Developing and Assessing an Environmental DNA Protocol for Detecting Amphibian Species in Lentic Systems in Alberta, Canada

by

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Abstract

Global declines of amphibian populations have resulted in increased interest in determining and monitoring the geographic ranges for both endangered and invasive amphibian species. Monitoring programs rely on detection techniques to amass data, techniques that must be dependable in order to create effective conservation plans. Using trace DNA found in the environment (environmental DNA or eDNA) to detect a species may provide better detection rates than conventional detection techniques, such as call and visual surveys, and trapping. A variety of methods have been developed, assessed and compared for detecting eDNA in environmental samples. Determining the most reliable, efficient and cost effective techniques should culminate in the creation of standardized protocols, allowing for more reliable comparison across projects and species. In this study, I designed and compared the efficiency and reliability of multiple methods for collecting eDNA samples and for detecting eDNA for the 10 amphibian species that occur in Alberta, Canada.

I used an Ion Torrent PGM™, a high through-put sequencing detection platform, to investigate the efficacy of three types of primer sets: universal primer set (targets all 10 species), genetically grouped species primer set (targets four groups of one to four species), and species-specific primer set. To amplify eDNA and label it with an identifying tag, I compared a single reaction PCR approach (one-step PCR) with a two reaction PCR approach (two-step PCR). I tested the primer sets on water samples collected from 39 wetlands known to contain one to four species of amphibians. The species-specific primer set combined with the two-step PCR was the most effective for detecting target species with eDNA and produced the lowest rate of false positive detections.

I identified three ponds in central Alberta that contained western toad (*Anaxyrus boreas*), wood frog (*Lithobates sylvaticus*) and boreal chorus frog (*Pseudacris maculata*). At each pond, 10 water and 10 sediment samples were collected and processed for eDNA detections for these three species on three different dates. I investigated: 1) how 10-fold dilutions to reduce potential PCR inhibition influence detection rates; 2) the

influence of processing samples with a high through-put sequencing and a quantitative PCR detection platforms on eDNA detection rates; 3) the influence of sample type, water versus sediment, on eDNA detection rates for each detection platform; and 4) how sampling at different times during the breeding and development season affects detection rates for both sampling methods and detection platforms. For each detection platform and sample collection method, detection rates for western toad and boreal chorus frog where low compared to wood frog. By comparing methods I determined that: 1) sample dilution to reduce potential PCR inhibition increased eDNA detections among samples that were negative prior to dilution and reduced eDNA detections among samples that were positive prior to dilution; 2) there was no significant difference in detection rates when samples were processed once with the qPCR and once with the ITPGM platform; however, analysis of samples processed in duplicate with the qPCR platform had a significantly higher detection rate than samples processed once with the ITPGM platform; 3) sediment samples provided higher detection rates than water samples for the ITPGM platform, but not for the qPCR platform; and 4) eDNA detection rates for both sampling methods and detection platforms changed seasonally, generally decreasing later in summer.

This study found that, regardless of the sample collection and processing method, eDNA detection rate varied across amphibian species that share a common habitat. Although I was able to show how detection rates differ across methodologies, I was unable to developing a reliable eDNA detection method for the 10 resident amphibians of Alberta.

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Chapter I: General Introduction

Amphibian populations have declined globally over the past three decades due to a combination of natural and anthropogenic etiologies such as the spread of chytridiomycosis and habitat loss through agricultural and urban development (Alford and Richards, 1999; Houlahan *et al*., 2000; Becker *et al*., 2007; Blaustein *et al*., 2011). As of 2008, it was estimated that 22.9% of all amphibian species were "Vulnerable", "Endangered" or "Critically Endangered", and 52.2% of species were considered "Data Deficient" for status assessment (International Union for Conservation of Nature, IUCN Red List 2008). Based on recent trends, many amphibian species are projected to deteriorate in future decades, moving them closer to extinction (Hoffman *et al*., 2010). Cataloguing regional amphibian distributions to create a large geographic database will facilitate the identification of areas that should be considered for conservation projects, as well as the creation of databases for contemporary amphibian ranges that can be used in the future to monitor the expansion and contraction of ranges. With such a large portion of amphibians listed as data deficient, and a rapid rate of species loss, it is critical that data, such as current distribution across large geographic ranges, are collected systematically. Determining if an amphibian is present in a potential habitat is the first phase of mapping species' distributions. For amphibians, conventional detection methods include auditory surveys, visual surveys, trapping and electrofishing. Conventional techniques require that targeted habitats are visited on multiple occasions (Gooch *et al*., 2006) to reduce detection biases created by environmental conditions (Dostine *et al*., 2013; Milne *et al*., 2013), observer misidentification (Genet and Sargent, 2003; Lotz and Allen, 2007; McClintock *et al*., 2010), and the secretive habits of many amphibian species. When mapping the geographic distribution of a species, multiple visitations to sites can become time consuming and increase research costs, factors that may limit the geographic scope of a project. Many conventional detection techniques also require the researcher to catch individuals either by hand, trapping or stunning them with electricity, causing stress and even mortality (Robertson *et al*., 1988; Portz *et al*., 2006; Donaldson *et al*,. 2011). Therefore, developing and testing detection

protocols that are efficient, cost effective and non-invasive are critical for creating regional distribution data for amphibian species while maintaining the integrity of their habitats.

Species that use freshwater habitats for foraging, reproduction, larval and juvenile development leave behind cellular debris from feces, urine, mucus and sloughed skin cells. Mitochondria and nuclei found in this cellular debris contain deoxyribonucleic acids (DNA) and through cellular disruption this DNA is released into the environment. These molecules contain nucleotide sequences that are specific to the species of origin and can serve as a species "fingerprint" in an environment. Detecting this trace DNA in environmental samples (environmental DNA or eDNA) can help catalogue the species using a habitat (Ficetola *et al*., 2008; Jerde *et al*., 2011; Goldberg *et al*., 2011; Dejean *et al*., 2012; Thomsen *et al*., 2012a,b; Spear *et al*., 2015; Fukumoto *et al*., 2015).

The practice of extracting and amplifying DNA in an environmental sample for species detection was developed when researchers found that the majority of naturally occurring soil microbes could not be cultured for identification using standard techniques (Brock 1987). To circumvent this issue, they began extracting DNA directly from soil, amplifying it by Polymerase Chain Reaction (PCR), and either sequencing the DNA or visualize it using gel electrophoresis (Steffen and Atlas 1988; Bruce *et al*., 1992). Currently, extracting DNA from sediment and soil samples coupled with more advanced DNA detection platforms, such as high through-put sequencing and quantitative PCR, have been used to identify a variety of species including plants (Lydolph *et al*., 2005; Haile *et al*., 2007; Willerslev *et al*., 2003; Willerslev *et al*., 2014), fungus (Epp *et al*., 2012; Lydolph *et al*., 2005), invertebrates (Thomsen *et al*., 2009; Bienert *et al*., 2012; Epp *et al*., 2012; Ficetola *et al*., 2015) and vertebrates (Willerslev *et al*., 2003; Hofreiter *et al*., 2003; Haile *et al*., 2009). DNA may persist in sediment and substrate samples for 100's to 1000's of years (Matisoo-Smith *et al*., 2008; Giguet-Covex *et al*., 2014) creating a large temporal window of detection, which may not benefit projects that seek to detect species currently using a site. Martellini *et al*. (2005) applied the eDNA technique to determine contamination from

human, bovine, ovine and porcine DNA in surface water. However, it was not until Ficetola *et al*. (2008) that the eDNA technique was used to detect a freshwater species in its habitat using water samples, which have a temporal eDNA detection window of ≤ 25 days (Dejean *et al*., 2011; Thomsen *et al*. 2012a,b; Barnes *et al*., 2014; Pilliod *et al*., 2014), gaining recognition as a tool for wild species monitoring and conservation. Initially, this technique was applied to invasive amphibian and fish species, specifically American bull frog (*Lithobates catesbeianus*; Ficetola *et al*., 2008; Dejean *et al*., 2012), brook trout (*Salvelinus fontinalis*; Wilcox *et al*., 2013) and carp species (*Hypophthalmichthys spp*.; Jerde *et al*., 2011). The eDNA technique crossed over to "at risk" amphibian species when Goldberg *et al*. (2011) used it to detect Rocky Mountain tailed-frogs (*Ascaphus montanus*) and Idaho giant salamanders (*Dicamptodon aterrimus*). Since then it has been used to detect rare, elusive or endangered amphibians such as eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*; Olson *et al*., 2012; Santas *et al*., 2013; Spear *et al*., 2015), common spadefoot toad (*Pelobates fuscus*), great crested newt (*Triturus cristatus*) (Thomsen *et al*., 2012a) and Japanese giant salamander (*Andrias japonicas*; Fukumoto *et al*., 2015).

Detection using eDNA has performed as well as or better than conventional detection methods for many amphibian species (Ficetola *et al*., 2008; Dejean *et al*., 2012; Thomsen *et al*., 2012a, Biggs *et al*., 2015) and has been promoted for its relatively greater detection sensitivity. However, like conventional methods, detection using eDNA is imperfect (i.e. may produce false negative detection) and detectability can vary across species. While some studies report eDNA detection rates of 79-100% at sites where a target species were detected using conventional methods (Ficetola *et al*., 2008; Dejean *et al*., 2012; Thomsen *et al*., 2012a), other studies have reported much lower detection rates. For example, Tréguier *et al*. (2014) detected red swamp crayfish (*Procambarus clarkia*) in 59% of sites where they were detected using trapping methods. Moyer *et al*. (2014) detected African jewelfish (*Hemichromis letourneuxi*) in 28 of the 324 samples collected from sites where the species was stocked and suggested that when abundances were moderate to low it may be necessary to collect

between 42-73 L of water to assure detection, which is unfeasible for most projects. Freshwater aquatic species that co-occur in a habitat may vary in abundances, size and DNA shed rate, factors that likely influence eDNA detections (Ficetola *et al*., 2008, Thomsen *et al*., 2012a). In this study I investigated how a variety field collection and laboratory techniques influence eDNA detections for amphibians that share common habitats. Understanding how these techniques influence eDNA detections may lead to the development of standardized eDNA detection methods that can be applied by biodiversity monitoring organizations, such as Alberta Conservation Association or Alberta Biodiversity Monitoring Institution, to establish and monitor species distributions across large geographic ranges.

In Chapter II, I describe the design of a panel of three primer sets to amplify eDNA from all 10 amphibian species that occur in Alberta, Canada. The three types of primer sets were: 1) a single primer set that targeted all 10 species (universal primer); 2) a primer set comprised of four primer pairs with each primer pair targeting a group of one to four species that shared genetic similarity in a ~550 bp region of the 16s mitochondrial region, but not necessarily taxonomically related (grouped species primer); and 3) primer pairs that were specific for a single target species (species-specific primer). To prepare samples for Ion Torrent PGM™ sequencing, eDNA amplicons are generated using primers that target eDNA, these amplicons are then labeled with a sample-specific identifying tag to facilitate the sequencing of multiple samples in parallel. I compared two approaches to amplifying and labeling eDNA: 1) a single-reaction PCR approach that joined the eDNA primers and sample identifier in a single reaction (one-step PCR) and 2) a two-reaction PCR where eDNA was amplified in the first PCR reaction and labeled with the sample identifier in the second PCR reaction (two-step PCR). I determined that the species-specific primer set in combination with the two-step PCR provided the highest detection rates. This protocol was used in Chapter III to compare detection rates between samples processed with the Ion Torrent PGM™ sequencing platform and a quantitative PCR platform.

In Chapter III, I targeted western toad (*Anaxyrus boreas*), wood frog (*Lithobates sylvaticus*) and boreal chorus frog (*Pseudacris maculata*), three amphibian species that co-occur in habitats in central Alberta; however, at different abundances. At 239 wetlands in / near my study site of Elk Island National Park, these species were captured at rates of 8 western toads, 25 wood frogs, and 17 boreal chorus frogs per 10 h of searching (Browne, 2010). The size at adulthood varies considerable among these species: western toad adults are 55-125 mm snout-vent length (SVL), wood frog adults are 30-60 mm SVL, and boreal chorus frog adults are 20-40 mm SVL (Russel and Bauer 2000). Although western toad is the largest of the three species, they typically occur in lower abundances than wood frog and boreal chorus frog in breeding sites in central Alberta and produce fewer larvae due to fewer females (Russel and Bauer 2000). Western toad is provincially listed as "Sensitive" in Alberta (Alberta Government, 2000), federally as "Species of Special Concern" in Canada (COSEWIC, 2012) and globally as "Near Threatened" (IUCN, 2004). Wood frog and boreal chorus frog are listed as "Secure" in Alberta (Alberta Government, 2005), federally as "Secure" in Canada (COSEWIC, 2012) and globally as "Least Concern" (IUCN, 2008 and 2014). Their conservation status, along with their relatively lower abundances per site, made western toad the focus of the project with data from wood frog and boreal chorus frog allowing for interspecific comparisons. For these three species I investigated: 1) how 10-fold dilutions to reduce the concentration PCR inhibitors influence detection rates for both sampling methods and detection platforms; 2) the influence of detection platform on eDNA detection rates; 3) the influence of environmental sampling method, water versus sediment, on eDNA detection rates for each detection platform; and 4) how sampling at different times during the breeding and active season for the target species affects detection rates for both sampling methods and detection platforms. Overall, I found detection rates varied across the three target species, 10-fold dilution increased detections, different detection platforms and sampling methods produced comparable results, and timing of collection influenced detection rate.

Overall, I found that eDNA detection rates were typically low for the amphibian species examined. I did not investigate what caused these low detection rates (i.e. lack of eDNA in the samples, low eDNA concentrations, or high concentrations of PCR inhibitors). Therefore, I recommend that future workers empirically determine the best method for detecting eDNA for species of interest on a case by case basis before the technique can be considered reliable.

Chapter II: Using Environmental DNA and High Through-Put Sequencing to Detect Amphibian Species in Alberta: Developing and Assessing Primer Sets for Multiple Species and Comparing One-step and Two-step PCR Protocols

2.1 Introduction

Globally declining populations and contracting species' distributions have brought amphibians to the forefront of ecological research and conservation projects. Traditional amphibian monitoring techniques focus on trapping, auditory and visual detection which are physically and temporally demanding and may be biased due to the secretive nature and small populations of many amphibian species (Jung *et* al., 2000, Tanadini and Schmidt, 2011; Dostine *et al*., 2013; Milne *et al*., 2013). Using conventional methods to map amphibian population occurrences across a large geographic area, such as a province or country, often requires multiple site visitations to obtain reliable detection data (Gooch *et al*., 2006) which is not feasible when resources are limited. This presents a need for innovative approaches to amphibian monitoring that are more sensitive, efficient and comprehensive.

When an amphibian species is present in an aquatic habitat, cellular debris accumulates from feces, urine, and epidermal sloughing. From such cellular debris, short deoxyribonucleic acid (DNA) fragments can be amplified, sequenced, and compared to catalogue organisms that inhabit freshwater ecosystems (Thomsen *et al*., 2012a,b; Kelly *et al*., 2014). Detection with environmental DNA (eDNA) may be more effective for some amphibian species than conventional monitoring techniques (Ficetola *et al*., 2008; Dejean *et al*., 2012; Thomsen *et al*., 2012a; Biggs *et al*., 2015).

Environmental samples often contain heterogeneous DNA from multiple source species. With high through-put sequencing (HTS) technology, this heterogeneous DNA can be sequenced in parallel allowing for DNA sequence data to be generated directly from an environmental sample (Sogin *et al*., 2006; Yergeau *et el*., 2012; Kelly *et al*., 2014). Processing eDNA samples with HTS has been used to assess microbial diversity (Sogin *et al*., 2006; Wegley *et al*., 2007; Yergeau *et el*., 2012; Navarrete *et al*., 2015), perform dietary analyses

(Bohmann *et* al., 2011; Shehzad *et* al., 2012; Deagle *et al*., 2013) and determine the vertebrate and invertebrate species present in various habitats (Hajibabaei *et al*., 2011; Thomsen *et* al., 2012b; Kelly *et al*., 2014). When determining the presence of a species in an environmental sample with HTS, eDNA must be extracted, then target loci are amplified with diagnostic primers and sequenced. The type of diagnostic primers used depends on the whether the goal is to detect one species or multiple species. When the goal is to detect multiple species, it is common to use universal primers or primers that amplify a large range of species typically belonging to the same class or order (Bell *et* al., 2011; Yergeau *et* al., 2012; Kelly *et al*., 2014).

For an eDNA sample, amplification rates for universal primers are influenced by how efficiently a primer amplifies DNA for the target species and by the amount of eDNA present per species; both factors may contribute to false negatives, i.e. lack of detection when a species is actually present (Kelly *et al*., 2014). For determining the presence of more restricted groups of more closely related species, such as members of the same family or genus, universal primers may be problematic. Universal primers are not specific to the species of interest, thus the presence of co-extracted non-targeted DNA may reduce amplification rates for target DNA by competing for PCR resources. Therefore, it may be more effective to use primers designed to be specific for targeted species within a group of interest (grouped primer set). Designing a grouped primer set that amplifies only target species and not co-extracted non-target DNA requires: 1) that the grouped species share sufficient genetic similarity so a single primer set will amplify all target species, 2) that the grouped primers do not amplify non-target species, and 3) that enough sequence divergence exists among the target species in the amplified loci to identify which species generated eDNA detected in a sample. These criteria make primer design challenging especially when accounting for annealing temperatures for the forward and reverse primers and reducing the potential for primer dimerization. This approach is also vulnerable to preferential amplification of DNA from species that display greater primer affinity. A species-specific primer pair may be used when the aforementioned criteria for primer design cannot be met for a group of target species. Species-specific primers

are designed, by definition, to detect only a single species, reducing the amount of competition with non-target eDNA during amplification via PCR and allowing for more flexibility during primer design.

To prepare a sample for high through-put sequencing, target eDNA is amplified with the appropriate primers. The resulting eDNA amplicons are labeled with a molecular identifying sequence (MID) tag specific to each sample; this facilitates the combination and sequencing of multiple samples in parallel. The two-step PCR method used in this study involves one PCR (PCR 1) reaction to amplify DNA and a second PCR (PCR 2) to MID label DNA. The sample preparation for PCR 2 creates a risk for sample cross-contamination, as PCR 1 products contain short genetic fragments that can be transferred to neighboring wells via splashing and aerosols. A single-reaction PCR approach (one-step PCR), similar to the protocol designed by Schuelke (2000), that includes the diagnostic primers and MID tag in the same PCR reaction, eliminates the intermediate step, potentially reducing the risk of sample cross-contamination.

In this study I developed and assessed three methods to investigate the application of universal, grouped and species-specific primer sets for amplification and sequencing of eDNA samples with the ITPGM. I tested the universal and grouped primer sets with one-step and two-step PCR amplification and MID labeling methods, whereas the species-specific primer set was processed with two-step PCR only. Each primer set was evaluated using a panel of positive control reactions containing tissue-extracted DNA from each of the 10 target amphibian species and using eDNA samples collected from two distinct geographic region where seven of the 10 target species are known to occur. The goal was to determine which combination of approaches was the most effective for detecting the 10 resident amphibian species found in Alberta, Canada.

2.2 Methods

2.2.1 Amphibian DNA tissue extraction and sequencing for diagnostic ITPGM primer design

I sequenced the 16S and CO1 mitochondrial regions for the 10 amphibian species found in Alberta: Canadian toad (*Anaxyrus hemiophrys*), Great Plains toad (*Anaxyrus cognatus*), western toad (*Anaxyrus boreas*),

wood frog (*Lithobates sylvaticus*), northern leopard frog (*Lithobates pipiens*), Columbia spotted frog (*Rana luteiventris*), boreal chorus frog (*Pseudacris maculata*), plains spadefoot toad (*Spea bombifrons*), western tiger salamander (*Ambystoma mavortium*) and long-toed salamander (*Ambystoma macrodactylum*). For each species I obtained a tissue sample from one individual at two or three geographic locations within Alberta separated by >150 km. Great Plains toad and plains spadefoot were the only exceptions. For Great Plains toad one tissue sample was collected from a Saskatchewan population < 50 km from the Alberta/Saskatchewan border. The second tissue sample was collected from an Alberta population geographically separated from the Saskatchewan population by >150 km. Both plains spadefoot tissue samples were collected from Saskatchewan populations < 50 km from the Alberta/Saskatchewan border and populations were < 150 km apart. I extracted DNA from all tissue samples using a DNeasy Blood and Tissue Extraction Kit (Qiagen) following the manufacturer's instructions; DNA extract was stored at -20°C until sequenced.

For each geographically isolated tissue sample from each species, I amplified and sequenced a ~550 bp segment of the 16S and ~ 600 bp segment of the cytochrome c oxidase subunit 1 (CO1) mitochondrial regions using three universal amphibian primer sets. For the 16S region, one universal primer set (16S) from Vence *et al*. (2005) was sufficient for all 10 species (Table 2.1). For the CO1 region, the first primer set (CO1) (Che *et al*., 2012) did not amplify boreal chorus frog, northern leopard frog, or plains spadefoot; therefore, a second primer set was required (Chm) (Che *et al*. (2012) (Table 2.1). Amplification for all primer sets were performed in 25 µL final volume reactions with 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.4 µM of forward and reverse primer each, and 0.75 units of Taq polymerase (proprietary) and 5 µL of template DNA (concentration between 2 and 15 ng/µL). Thermo-cycling conditions for the 16s primer set began with denaturing for 3 min at 95°C, 35 cycles of denaturing for 30 sec at 95°C, primer annealing for 30 sec at 52.5°C and elongation for 40 sec at 72°C with a final elongation step of 7 min at 72°C. Thermo-cycling conditions for both CO1 primer sets began with denaturing for 5 min at 95°C, 35 cycles of denaturing for 60 sec at 94°C, primer annealing for 60

sec at 44.5°C (CO1) or 43°C (Chm), elongation for 60 sec at 72°C and a final elongation step of 10 min at 72°C. Following the PCR reactions, primers and unincorporated dNTP's were removed from the PCR product using Illustra™ ExoStar™ (GE Healthcare Life Science; Buckinghamshire, UK) following the manufacturer's instructions. Cleaned PCR products were sequenced in both directions using a Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®; Foster City, CA) resolved on an ABI 3730 DNA Analyzer (Applied Biosystems®) following manufacturers' instructions.

2.2.2 ITPGM diagnostic primer design

Sequence data were visualized with SeqMan Pro (DNASTAR®; Madison, Wisconsin) and manually trimmed to remove primer sequences and low quality sequences from the terminal ends. Trimmed sequences were aligned in SeqMan Pro (DNASTAR®) for primer design. From preliminary ITPGM analyses, I found that diagnostic primers with specificity for human DNA can result in a large amount of sequenced human DNA fragments (or reads) which biased the ITPGM output. Therefore, all primer sets were assessed for similarity with human DNA using the nucleotide BLAST database (National Center for Biotechnological Information; Bethesda, Maryland) and primers were only selected if one primer (forward or reverse) had > 2 bp mismatches with *Homo sapiens* sequences from the BLAST database. Most primers had > 4 bp mismatches.

All diagnostic primers were designed as *forward*: 5'-16bp "Glenn" adaptor (CAGTCGGGCGTCATCA), the diagnostic forward sequence-3' (Table 2.2); *reverse*: 5'-truncated P1 adaptor (CCTCTCTATGGGCAGTCGGTGAT) provided by Ion Torrent PGM™ (trP1) and the diagnostic reverse sequence-3' (Table 2.2). Molecular identifying sequence (MID) tags for labeling eDNA amplicons were *forward*: 5'-30 bp A-adapter (Ion Torrent PGM™) (CCATCTCATCCCTGCGTGTCTCCGACTCAG), 10-11bp MID, and the 16 bp "Glenn" adaptor-3'; *reverse*: 5'-trP1 adapter provided by Ion Torrent PGM™ -3'.

2.2.3 Universal primer (Uni_P) design

I designed the Alberta amphibian universal primer set (Uni_P) as a single primer pair that amplified a ~60 bp locus in the 16S region for all 10 species. I aligned the 16S sequence data from all geographically distinct individuals for all 10 species in SeqMan (DNASTAR®) allowing for base-pair mismatches along the entire sequence to create a single alignment. The forward and reverse diagnostic primers were selected because they had < 1°C difference in annealing temperature, homopolymers were ≤ 4 bp long and each had ≤ 3 degenerate base pairs. The locus that was targeted had ≥1 bp pair-wise mismatches between all 10 species. Uni_P was tested against each species to ensure amplification using the PCR1 protocol (*see below*). PCR products were visualized using electrophoresis and a 1% agarose gel.

2.2.4 Species groups primer (Grp_P) design

I designed an Alberta amphibian grouped primer set (Grp_P) that included all 10 species by grouping genetically similar species, designing a primer pair for each group, and combining the primer pairs into a single primer cocktail for eDNA amplification. Four alignments were created based on species that shared genetic similarity for the 16S region (these alignments did not represent taxonomic relatedness) and I designed a single primer pair for each alignment that targeted the same ~115 bp locus for all species. These alignments consisted of: 1) Canadian toad, Great Plains toad, western toad and boreal chorus frog; 2) wood frog, northern leopard frog, and Columbia spotted frog; 3) plains spadefoot toad; 4) western tiger salamander and long-toed salamander. Among all primers, there were < 2.3°C difference in melting temperatures, homopolymers were ≤ 4 bp in length, and each primer had ≤ 3 degenerate base pairs. The locus that was targeted had ≥10 bp pair-wise mismatches between all species. All primer sets were tested against each Alberta amphibian species to ensure amplification success using the PCR 1 protocol (*see below*). PCR products were visualized using electrophoresis and a 1% agarose gel. The final primer cocktail was a single solution that contained all four primer pairs and is referred to as Grp_P from here on.

2.2.5 Species-specific primer (Ssp_P) design

Western toad, wood frog, boreal chorus frog and western tiger salamander were used for the development of the species-specific primer set (Ssp_P). These species were selected because they are common in the aquatic habitats I surveyed for eDNA in north-central Alberta (see below, also Chapter III). I used Primer3 v0.4.0 (Untergrasser *et al*., 2012) to generate multiple primer sets that targeted different ~115 bp loci in the CO1 region for each species. To maximize the primer base-pair mismatches between target species and the non-target amphibian species of Alberta, I aligned the CO1 region for all Alberta amphibian species in SeqMan (DNASTAR®) allowing for maximum base-pair mismatch to create a single alignment. I visually compared primer sequences generated with the Primer3 software (Untergrasser *et al*., 2012) to the alignment and selected primers with > 6 bp mismatches for all non-target Alberta amphibian species. The loci that were targeted had ≥10 bp pair-wise mismatches between all non-target species. The four primer pairs were tested against all 10 Alberta species to determine specificity using the PCR 1 protocol (*see below*) and PCR products were visualized using electrophoresis and a 1% agarose gel. The four species-specific primer pairs were not combined and each sample was exposed to each primer pair independently creating four eDNA amplification reactions per sample.

2.2.6 Field surveys, water sample collection and eDNA extraction

In July 2012, samples were collected from 25 sites located in central Alberta that consisted of: eight ponds (two storm-water ponds and six natural ponds) located within 20 km of Edmonton, Alberta, three natural ponds located in Elk Island National Park, two natural ponds located in Miquelon Lake Provincial Park, and 12 constructed "dugout" ponds located within 20 km of Redwater, Alberta. The amphibians that occur in this region are: Canadian toad, western toad, wood frog, boreal chorus frog and western tiger salamander. An additional 14 natural ponds were added in July of 2013 from Waterton Lakes National Park, Alberta. The amphibians that occur in this region are: western toad, Columbia spotted frog, boreal chorus frog, western tiger

salamander, and long-toed salamander. Northern leopard frog, Great Plains toad and plains spadefoot were not assessed for eDNA detections in environmental samples. Each site was monitored for amphibians by auditory and visual surveys conducted in mid-May and mid-June and all amphibian species identified were recorded, but individuals were not counted (Table A-2). Three or four water samples were collected at each site and preserved according to the protocol described by Ficetola *et al*. (2008). A 50 mL conical tube was submerged 5-10 cm below the surface of the pond where it was allowed to fill. Samples were decanted to 15 mL, then 1.5 mL of 3M sodium acetate (pH 5.2) and 33.5 mL of absolute ethanol were added. Samples were mixed by inversion and stored at -20°C until processed.

DNA and/or cellular remains were recovered from water samples by centrifugation of 6330 x g for 35 min at 4°C. Supernatant was decanted by gentle pouring; DNA/cellular debris pellets were air dried for a minimum of 10 min and DNA was extracted using a modified DNeasy Blood and Tissue Extraction Kit (Qiagen Hilden, Germany) protocol. I added 360 µL of ATL buffer and 40 µL of Proteinase K solution to the dried pellet and incubated for ~10 h at 56°C. Following incubation, samples were vortexed and 400 µL of AL buffer and 400 µL of absolute ethanol (pre-mixed) were added. From here on I followed the protocol according to the manufacturer's instructions and the final DNA was eluted into 150 µL of IDT nuclease free H_2O (nfH_2O) (Integrated DNA Technologies; Coralville, Iowa) and stored at -20°C until processed.

2.2.7 Ion Torrent™ PGM PCR protocols

To reduce the potential for PCR contamination, all micropipettes were cleaned with 20% sodium hypochlorite (bleach) solution. All one-step PCR reactions and the first PCR for the two-step reactions were prepared in a fume hood to reduce the transfer of aerosols between vessels, except for the first ITPGM sequencing round which was prepared on a laboratory bench top. The second PCR from the two-step PCR protocol was prepared in a room dedicated to handling of samples following PCR. Thus, all handling of samples

pre- and post- PCR was performed in separate rooms dedicated to each phase. eDNA sample preparation was performed on separate plates from positive PCR controls (*see below*).

2.2.8 One-step PCR protocol

The "Glenn" adaptor for the MID tag is located on the forward primer of each diagnostic primer set. The diagnostic forward primers are added at 1/4 final concentration of the MID tags. During the PCR reaction, the diagnostic forward primers are incorporated into the eDNA amplicons and are removed from the reaction. Once a majority of the forward primers are incorporated into the eDNA amplicons, the remaining MID tags can label these amplicons (Schuelke, 2000). One-step PCR reactions were performed in 15 µL final volume containing 1X High Fidelity PCR buffer (Life Technologies), 2 mM of $MgSO_4$ (Life Technologies), 0.16 mM dNTPs , 0.041 µM Glenn-F primer, 0.17 µM A-MID-Glenn-F and trP1-R each, 0.5 units of Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies) and 8.6 µL of eDNA extract. Thermo-cycling conditions for one-step PCR began with denaturing for 2 min at 94°C, 25 cycles of denaturing for 30 sec at 94°C, primer annealing for 30 sec at Tm (Table 2.2) and elongation for 60 sec at 68°C, then 15 cycles of denaturing for 30 sec at 94°C, primer annealing for 30 sec at Tm (Table 2.2) and elongation for 60 sec at 68°C with a final elongation step of 10 min at 68°C. The products from the one-step PCR were used to create ITPGM libraries (*see below*).

2.2.9 Two-step PCR protocol

For the two-step PCR protocol, PCR 1 (eDNA amplification) was performed once per sample for the Uni_P and Grp_P primer sets and four times per sample with the Spp_P, once per species primer set. Following PCR 1 all four Spp_P reaction were combined for each sample. PCR 1 was performed in 15 µL final volume reactions containing 1X High Fidelity PCR buffer (Life Technologies), 2 mM of $MgSO_4$, 0.2 mM dNTPs, 0.2 µM Glenn-F species-specific primers and trP1-R each, 0.5 units of Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies) and 5 µL of eDNA extraction. Thermo-cycling conditions for the PCR 1 began with denaturing for 2 min at 94°C, 35 cycles of denaturing for 30 sec at 94°C, primer annealing for 30 sec at

Tm (Table 2.2) and elongation for 60 sec at 68°C with a final elongation step of 10 min at 68°C. Uni_P and Grp_p PCR 1 products were diluted 50-fold with IDT nfH_2O to reduce the concentration of unincorporated primers and used as template for PCR 2. Following PCR 1 for the Spp_P set, the four individual reactions for each sample were combined then diluted to 10-fold with IDT nfH_2O. This served as the template DNA for PCR 2. PCR 2 (MID labeling PCR) was performed in a 15 µL final volume reaction containing 1X High Fidelity PCR buffer (Life Technologies), 2 mM $MgSO_4$, 0.2 mM dNTPs, 0.25 µM A-MID-Glenn-F and trP1-R primers each, 0.5 units of Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies) and 5 µL of diluted PCR 1 product. Thermo-cycling conditions for the PCR 2 began with denaturing for 2 min at 94°C, 25 cycles of denaturing for 30 sec at 94°C, primer annealing for 30 sec at 58°C and elongation for 60 sec at 68°C with a final elongation step of 10 min at 68°C. Post PCR 2 products were used to prepare ITPGM libraries (*see below*).

2.2.10 Positive and negative controls

eDNA extractions where performed in groups containing a negative extraction control as the last sample. Negative extraction controls were prepared in a sterile 50 mL conical tube using 15 mL of ddH_2O, 1.5 mL of 3M sodium acetate (pH 5.2) and 33.5 mL of absolute ethanol. Samples were mixed by inversion and stored at -20°C for at least 24 h. PCR negative controls were made with 5 µL of IDT nfH_2O substituted for the eDNA extract. PCR positive controls were prepared for the Uni_P and Grp_P runs with 5 µL of template from a 5 ng/µL stock of tissue-extracted DNA for each of the 10 Alberta amphibian species. For the Spp_P runs, positive controls were only made for western toad, wood frog, boreal chorus frog and western tiger salamander. All initial concentrations were measured with an Invitrogen™ Qubit® Fluorometer 1.0 (Life Technologies) and dilutions were made with IDT nfH_2O.

To assess the efficiency of the eDNA extraction method, I made two types of extraction positives: one that contained tissue-extracted DNA from five species that were likely to co-occur in central Alberta (Canadian toad, western toad, wood frog, boreal chorus frog and western tiger salamander); and one that contained tissue-

extracted DNA from all 10 species. A 10 ng/µL stock was made containing either five or 10 species at equal concentrations. From each stock 0.0067 ng/µL, 0.067 ng/µL and 0.14 ng/µL were made to 15 mL final volume with ddH_2O. These were treated with 1.5 mL of 3M sodium acetate (pH 5.2) and 33.5 mL of absolute ethanol and processed the same as field samples.

2.2.11 ITPGM Library preparation

Each round of sequencing contained 96 DNA amplification/MID labeling reactions comprised of eDNA samples, positive controls and their respective negative controls. I pooled the products for all eDNA samples and tissue-extracted DNA reactions separately. I concentrated 100 µL of the pooled eDNA solution with a 30 min centrifugation using a speed vacuum centrifuge; tissue-extracted DNA was not concentrated. For the pooled eDNA and tissue-extracted DNA, the DNA was purified based on size by electrophoresis and a 2% agarose gel. I extracted DNA between 90-120 bp (Uni_P), 130-180 bp (Grp_P) and 90-130 bp (Ssp_P), the length of the products following amplification with diagnostic primers and adaptors. This size-selective purification step excluded the majority of unincorporated primers, primer dimers, and non-target products from outside the size range as smaller molecular weight DNA can be preferentially amplified during emulsion PCR (emPCR). DNA was extracted from the 2% agarose using a QIAquick Gel Purification Kit following the manufacturer instructions and further purified using a QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions, and DNA was eluted into 30 µL IDT nfH_2O. For two sequencing rounds, samples processed with Uni_P and Grp_P (and positive control reactions for both) were combined at this step in equal volume. For the next two sequencing rounds, samples processed with Uni_P, Grp_P, and Spp_P were sequenced separately. I quantified eDNA and positive controls using a Invitrogen™ Qubit® Fluorometer 1.0 (Life Technologies) and diluted positive controls at a range of 1/10 to 1/1000 the concentration of eDNA depending on the ITPGM run (Appendix 1). This was done to prevent high quantities of positive control DNA from outcompeting eDNA for resources during emPCR. The eDNA and diluted positive

control DNA were combined at equal volumes to create ITPGM libraries. All libraries were re-quantified and diluted to a pM concentration specific to each sequencing round (Appendix 1). Libraries were amplified using an emulsion PCR Ion OneTouch™ 2 system and the Ion PGM™ Template OT2 200 Kit (Life Technologies) according to the manufacturer's instructions. Ion sphere particles (ISP) were enriched using Ion OneTouch™ Enrichment System. Enriched live ISPs were loaded and sequenced on an Ion PGM™ 314 chip v2 and an Ion PGM™ 200bp sequencing Kit v2 (Life Technologies) following the manufacturer's instructions.

2.2.12 Bioinformatics

Sequences were sorted according to their MID tags and adaptors were trimmed using the Torrent Suite™ and Torrent Server (Life Technologies). The output was a single ".fastq" file for each MID that represented an eDNA sample. Sequences were imported to CLC Genomic Workbench 7.0 (CLC bio, Aarhus, Denmark) where diagnostic primer sequences were trimmed. Trimmed sequences were filtered by length and by quality scores using a modified Mott-trimming algorithm (CLC Genomic Workbench 7.0). Reads were mapped to the 16S (Uni_P and Grp_P) or CO1 (Spp_P) reference sequences of all target amphibians using CLC Genomic Workbench 7.0. Reads were mapped to references with 100% read length match and 100% (Univ_P and Grp_P) or 98% (Spp_P) similarity in base pair compositions. Mismatch, insertion and deletion costs were set to 3, as stringent as CLC Genomic Workbench 7.0 allowed. For a sample to be considered positive for a species eDNA the number of aligned reads had to be ≥ 51 reads. This threshold was not determined from the data presented in this chapter due to the number of false positive detections. The threshold was determined using data from Chapter III where false positive were not possible and all negative controls had ≤ 50 aligned reads per species.

2.2.13 Analysis of Samples Collected During Field Surveys

When a species was encountered at a site, I expected a corresponding eDNA detection. For example, if three species were encountered at a site and four samples were collected from that site, then I expected 12 total detections for that site (3 species x 4 samples). When comparing the overall amount of eDNA detections for

each primer set, I treated a detection for a given species in a water sample as an independent data point (e.g. if a sample processed with the Uni_P set had three species detected, then there were three data points produced by this sample for that primer set). Positive DNA detections are defined as: "P_E", a species was encountered at a site and the corresponding water sample was positive for that species' eDNA; "P_N", a species was not encountered at a site but the corresponding water sample was positive for that species' eDNA and the site occurred within the species' known range; and "P_O", a species' DNA was detected in a water sample that corresponded to a site that was not located in the detected species' known range. Negative DNA detections are defined as: "N_E", a species was encountered at a site, but the corresponding water sample was negative for that species' eDNA; "$N_{N"}$, a species was not encountered at a site and the corresponding water sample was negative for that species' eDNA; and "N_O", a water sample was negative for a species' DNA and the sample was collected outside that species' known range. For each primer set, the detection rate [D_E] is defined as the ratio of positive detections to expected eDNA detections (i.e. the rate at which species were detected by eDNA at sites where they were encountered), the undocumented regional detection rate [D_N] is defined as the ratio of positive detections to undocumented regional eDNA detections (i.e. the rate at which species were detected by eDNA at sites where they were not encountered, but which occurred within the species' known ranges), and the false positive rate [D_O] is defined as the ratio of positive to unexpected, non-regional eDNA detections (i.e. the rate at which species' DNA was detected at sites outside species' known ranges). Detection rates were calculated using:

$$[D_E] = P_E / (P_E + N_E)$$

$$[D_N] = P_N / (P_N + N_N)$$

$$[D_O] = P_O / (P_O + N_O)$$

For each field sample sequencing round, the samples processed were collected within a ~100 km radius. Therefore, the samples for each round shared an expected suite of regional species. For each primer set and

sequencing round, the range of eDNA detections [D_E] is presented for each species that was encountered at a site.

2.3 Results

2.3.1 Diagnostic primer species specificity, sample extraction efficiency, and positive controls

All primers were tested against all 10 species using conventional PCR and visualized using an agarose gel and electrophoresis. The Uni_P set successfully amplified all 10 species. The Grp_P set was comprised of four primer pairs that were designed to target genetically similar groups. However, three of the four grouped primers amplified DNA from species outside the targeted group (Table 2.2). The Spp_P sets amplified their respective target species and did not amplify non-target amphibian species from Alberta.

The positive controls made with tissue-extracted DNA prepared for each ITPGM sequencing round were positive for DNA for their respective species. All the sample extraction positives controls detected DNA of the target species at starting concentrations of 0.0067 ng/µL, 0.067 ng/µL and 0.14 ng/µL. However, species detections varied across ITPGM preparation protocols (Appendix 1). Negative PCR and extraction controls produced false positive DNA detections for the universal and group primer sets (Appendix 1).

The following sections of the results are presented chronologically across ITPGM sequencing rounds to demonstrate the progression of the project and highlight issues with the sample preparation protocols. There were 96 MID tags for each sequencing round that were allocated to samples, negative and positive controls.

2.3.2 Sequencing round one – Uni P and Gp P sets and two-step PCR

For the first ITPGM sequencing round, samples collected from the 14 Waterton Lakes National Park sites were prepared using the Uni_P and Grp_P sets and 2-step PCR, and amplicons from both primer sets were sequenced simultaneously. The eDNA detection rates [D_E] were 0.11 for the Uni_P set and 0.01 for the Grp_P set (Table 2.3). The undocumented regional detection rates [D_N] was 0.075 for the Uni_P set; no such detections

were generated with the Grp_P set (Table 2.3). The false positive detection rates [D_O] were 0.032 for the Uni-P set and 0.042 for the Grp_P set (Table 2.3).

2.3.3 Sequencing round two- Uni P and Gp P sets and one-step PCR (simultaneous sequencing)

For the second ITPGM sequencing round, samples from the 14 Waterton Lakes National Park sites were prepared using the Uni-P and Grp_P sets and 1-step PCR, and the amplicons from both primer sets were sequenced simultaneously. The eDNA detection rates [D_E] were 0.21 for the Uni_P set and 0.019 for the Grp_P set (Table 2.3). The undocumented regional eDNA detection rates [D_N] were 0.052 for the Uni_P set and 0.014 for the Grp_P set (Table 2.3). The false positive detection rates [D_O] were 0.037 for the Uni-P set and 0.093 for the Grp_P set (Table 2.3).

2.3.4 Sequencing round three - Uni P and Gp P sets and one-step PCR (separate sequencing)

For the third ITPGM sequencing round, samples from the 25 central Alberta sites were prepared using the Uni-P and Grp_P sets and 1-step PCR, and the products from each primer set was sequenced separately. The eDNA detection rates [D_E] were 0.21 for the Uni_P set and 0.019 for the Grp_P set (Table 2.3). The undocumented detection rates [D_N] were 0.05 for the Uni_P set and 0.033 for the Grp_P set (Table 2.3).The false positive detection rates [D_O] were 0.041 for the Uni-P set and 0.031 for the Grp_P set (Table 2.3).

2.3.5 Sequencing round four - Spp_P set and two-step PCR

For the fourth ITPGM sequencing round, samples from a single site in central Alberta were prepared using the Spp_P set and 2-step PCR. There were 10 samples that were processed three times each (three replicates). If the species was detected in any of the replicates the sample was considered positive for that species. The 10 samples were collected from a single site where western toad, wood frog, boreal chorus frog, and tiger salamander were all encountered; therefore, there were 40 expected detections (10 samples x 4 species). There was an eDNA detection rate [D_E] of 0.25, all eDNA detections were for wood frog. The four species encountered at the sampling site represent all species known to occur in the region; therefore,

undocumented regional detections and false positives were not possible for this analysis. False positive detections occurred in the positive PCR control, potentially originating from the tissue DNA extraction. Once DNA was re-extracted, false positives were no longer observed.

2.3.6 Detection rates by primer set and species

For the Uni_P set, the expected detection rates [D_E] ranged from 0 to 0.13 for western toad, 0.048 to 0.05 for boreal chorus frog, 0.4 for wood frog, and 0.2 to 0.22 for Columbia spotted frog; Canadian toad, western tiger salamander and long-toed salamander were not detected. For the Grp_P set the expected detection rates [D_E] ranged from 0 to 0.042 for western toad, 0.36 for wood frog and 0.025 to 0.028 for Columbia spotted frog; Canadian toad, boreal chorus frog, western tiger salamander and long-toed salamander were not detected. For the Spp_P set the detection rate [D_E] was 1.0 for wood frog (Table 2.4); western toad, boreal chorus frog and western tiger salamander were not detected.

2.4 Discussion

From the DNA extraction and PCR positive controls I showed that the methods and primers used were successful in extracting and detecting DNA. The species-specific primer set produced better detection rates for eDNA samples than the universal and grouped primer sets, but only for wood frog. A different sample preparation protocol was used for the species-specific primer sequencing reaction making it difficult to directly compare detection rates across all three primer sets. To amplify and MID label eDNA from samples for Ion Torrent PGM™ sequencing, I found that the one-step and two-step PCR protocols produced similar detection rates for the universal and species grouped primer sets and both produced false positive detections. By combining the two-step PCR protocol with the species-specific primer set, detection rates for wood frog were substantially increased. The species-specific primer set combined with two-step PCR out-performed all other methodological combinations. However, this primer set was designed and tested for only four of the 10 target

amphibian species; thus it did not fulfill the original project goal of designing an all-purpose protocol for detecting eDNA for all 10 amphibian species that reside in Alberta.

The project began with creation of the universal and grouped primer sets and a two-step PCR protocol for amplifying eDNA from water samples (PCR 1) then labelling it with a MID tag (PCR 2). Samples for the first sequencing round were processed once with the universal primer set (Uni_P) and once with the grouped primer set (Grp_P). The products from both primer sets were combined to create a single eDNA library that was sequenced. Overall detection rates were higher for the universal primer set (0.11) compared to the grouped primer set (0.021); however, both detection rates were low. Both primer sets showed high rates of DNA detections for non-regional species (i.e. false positives; Table 2.3), contaminated extraction negative-controls, and contaminated PCR negative-controls (Table A-1). In two-step PCR, the 96-well plate containing the post-PCR 1 products is uncovered while samples are diluted and added to the PCR 2 reaction. During this step, every sample is potentially exposed to amplicons from adjacent samples via splashing and aerosols. A PCR reaction can generate up to 10^{12} amplicons per 0.1 µL of reaction solution (Kwok and Higuchi, 1989) and an aerosol droplet as small as 10^{-6} µL could potentially contain 10^{5} amplicons (Pershing, 1991). Therefore, there is great potential for sample "amplicon cross-contamination" during the post-PCR 1 / PCR 2 preparation phase. Post-PCR 1 "amplicon cross-contamination" is particularly problematic because these amplicons are not MID labeled, but they contain the MID adaptor sites making them ideal templates for the MID tags to amplify and label during PCR 2. Any DNA transferred between samples at this step will be incorrectly labeled with an MID tag potentially generating a false positive detection. To save project time and cost, Schuelke (2000) designed an effective protocol to fluorescently label microsatellite amplicons for fragment analysis that incorporates the microsatellite primers (forward and reverse) and the florescent label in a single nested PCR reaction. Applying this technique to the subsequent round of ITPGM sequencing, I chose to combine the diagnostic and MID tag

primers into a single PCR reaction, one-step PCR, to eliminate the intermediate step where sample cross-contamination was possible.

For sequencing round two, samples were processed with the universal and grouped primer sets and one-step PCR. Products for each primer set were combined to create a single ITPGM sequencing library. However, the rate of false positives was comparable to the previous sequencing round using two-step PCR for both primer sets (Table 2.3). Similar to round one, the universal primer set had a greater detection rate (0.12) than the grouped primer set (0.019), even though both primers were used on the same samples. During sequencing rounds one and two, the products for both primer sets were combined before the emPCR and ITPGM sequencing reaction to reduce project costs. However, the universal primer set amplified a ~60 bp segment of the 16S region which is much shorter than the ~115 bp 16S segment produced by the grouped primer. This can be problematic during the emPCR step as shorter DNA fragments may be preferentially amplified. I attempted to account for the fragment size discrepancies by averaging the fragment length produced by the two primer sets and using this value to calculate the DNA library concentration to be used in the emPCR step. However, I suspected the shorter amplicons generated by the universal primer set were out-competing the amplicons generated by the grouped primer set during emPCR resulting in the lower detection rates seen for the grouped primer set.

For sequencing round three, samples were processed with the universal primer set and the grouped primer set using one-step PCR and products from both primer sets were sequenced separately to reduce resource competition during emPCR. The universal primer set produced a detection rate (0.21) that was comparable to the grouped primer set (0.19) using this approach (Table 2.3). This suggests that the shorter amplicons from the universal primer had indeed out-competed the longer amplicons from the grouped primer set in the previous two rounds. Round three produced a large proportion of false positives for both primer sets (Table 2.3)

False positives are problematic for eDNA because there is growing interest in using this techniques to inform conservation and management decisions. When false positive detections occur at a substantial rate, such as in this study, it is difficult to determine which detections are legitimate and which ones result from "sample cross-contamination". Therefore, a species may be detected at a site when it is not actually present. I suspected that the false positives were a product of the lab techniques used to prepare samples; therefore, I had a second, highly-experienced, lab technician replicate the preparation protocol and sequencing reactions using the same set of samples. The technician experienced that same rates of false positive detection; however, they occurred for different samples and were positive for different species. After investigating the eDNA detections, extraction negatives, PCR negatives, and false positives for samples from sequencing rounds one, two and three, false positive detections appeared to occur randomly making it difficult to determine their origins. The PCR preparation for each sequencing round included many precautionary steps thought to reduce PCR cross-contamination, such as the use of rooms dedicated to working with samples pre and post-PCR, soaking pipettes in 20% bleach, using filtered pipette tips, keeping positive PCR reactions separate from sample PCR reactions, and changing reagents and primers for each sequencing round. The false positives generated during sequencing round one were originally attributed to sample cross-contamination during PCR 2 set-up resulting in the decision to use one-step PCR. However, one-step PCR produced a similar rate of false positive detections. I theorize that the false positives produced using the one-step PCR processes resulted from the method and not true cross-contaminations. It is possible that during the one-step PCR some diagnostic primers were not incorporated into eDNA amplicons. These primers could have become MID-labeled and bound to other amplicons within the target size-range. If they remained bound to the amplicons through the size-selective purification step and into the emPCR step, these primers could have bound to available DNA, ultimately producing new amplicons that were incorrectly MID-labeled. This outcome would be expressed in the sequencing output data as false positive detections. To my knowledge, this is the first time the one-step PCR

approach has been applied to amplification and MID labeling of DNA for high through-put sequencing and this technique requires further testing. If possible technical issues with one-step PCR could be remediated, this approach promises to be more cost effective and efficient than two-step PCR. However, the one-step PCR protocol used in this study was not reliable and is not recommended for future research without re-assessment.

Due to the high rates of false positives generated using the one-step PCR protocol, I determined that the two-step PCR protocol would be more effective for the next sequencing round using the species-specific primer set. This particular set was designed to reduce PCR competition between target and non-target eDNA in a sample. The species-specific primers were designed to target western toad, wood frog, boreal chorus frog and western tiger salamander. During sequencing round four, samples were amplified with the species-specific primer set using two-step PCR and each sample was processed three times per species. The samples tested with this primer set were collected from a site where all four species were directly observed. Wood frog was detected in all 10 samples, but western toad, boreal chorus frog and western tiger salamander were not detected in any sample. With no *a priori* knowledge about the relative population densities and shed rates of the four study species, it is difficult to infer why three were not detected. Studies have shown a positive correlation between a species' detectability using eDNA and its population density and biomass at a site (Ficetola *et* al., 2008; Dejean *et* al., 2011; Thomsen *et al*., 2012a; Takahara *et al*., 2012; Pilliod *et al*., 2013; Díaz-Ferguson *et al*., 2014; Pilliod *et al*., 2014). Pilliod *et al*. (2014) hypothesized that eDNA shed rates are influenced by metabolic processes such as urination and defecation, which typically varies across species. It is likely a combination of the aforementioned factors that resulted in lack of detection for three of the four species I investigated at field sites in central Alberta. However, detection rates are also influenced by protocol selection (Goldberg *et al*., 2011; Diener *et al*., 2014; Turner *et al*., 2014a,b), and when eDNA is in low abundance, increased sampling effort (Schmidt *et al*., 2013), collection of increased volumes of water (Díaz-Ferguson *et al*., 2014), and increased PCR replicates (Ficetola *et al*., 2014) can all increase detection rates. Therefore, detecting eDNA

from western toad, boreal chorus frog and western tiger salamander may require collection of larger volumes of water per sample more samples per site, and increased numbers of PCR replicates per sample.

Other studies that investigated amphibian detection using similar water collection and eDNA extraction protocols as described here, report detection rates between 0.76 and 1.0; however, these studies used different detection platforms, such as standard PCR accompanied by DNA visualization with gel electrophoresis (Ficetola *et al*., 2008; Dejean *et al*., 2012) and quantitative PCR (Thomsen *et al*., 2012a). In my study, wood frog had the highest detection rate per round of sequencing for the universal primer set (0.4), the grouped primer set (0.36) and the species-specific primer set (1.0). Wood frog detection rates for the universal and grouped primer set were substantially lower than detection rates reported by the aforementioned studies on amphibians. However, in sequencing round four, the species-specific primer set yielded comparable detection rates as reported in the literature (Table 2.4). Although it may have out-performed the universal and grouped primer sets, the lack of detection for western toad, boreal chorus frog and western tiger salamander using the species-specific primer set is troublesome, given that eDNA has been touted as a technique for monitoring species that are rare, in low abundances or difficult to detect with conventional methods. In fact, boreal chorus frog is a wide-spread, abundant species that is easy to detect via call surveys; however, eDNA detection rates for this species were low for each primer and methodological combination (Table 2.4). Conversely, there were a few occasions, based on a variety of protocols, when cryptic or low abundance species, such as the long-toed salamander, Canadian toad and western toad, were not encountered in the field, but were detected with eDNA and could have easily been present (Table 2.4). Detection rates were low for these species; however, in the case of the long-toed salamander, eDNA detection rates exceeded field encounter rates at sites in southwestern Alberta that represented good breeding habitat for the species. If a site is incorrectly accessed for the presence of a species "at risk" or an invasive species, the result may be inappropriate management actions. Thus the

occurrence of false negative or false positive detections, and generally low detection rates, represent equally troublesome problems requiring methodological solutions in the laboratory or the field.

The species-specific primer set, in combination with two-step PCR, was the most effective method, although only for wood frog. However, increase sampling effort and / PCR replications may increase detections for other species. Although, species-specific primers were designed for four of the 10 resident amphibian species of Alberta, designing primers for the remaining six species will require little effort now that 16S and CO1 sequence data are readily available. The eDNA amplification PCRs (PCR 1) for this primer set were performed independently for each species. With subsequent MID-labeling, emPCR, ITPGM sequencing, and the need to increase field sampling effort and PCR replicates, adding more species would substantially increase project cost. One solution may be to develop a panel of species-specific primer pairs with reduced pair-wise heterodimer potential. If the potential for primer dimerization is significantly reduced and primers are highly specific to their target species, the primer pairs for multiple species could be combined into a single primer cocktail. Samples could then be processed with multiple primer pairs in the same reaction, reducing cost and preparation time, especially if primer combinations are tailored to the target species expected to occur in the surveyed region.

Overall, I did not meet my original goal of developing a protocol to reliably detect all 10 resident amphibian species of Alberta. Although the universal and grouped primer sets had problems with false positives this may be attributed to the one-step PCR approach. The species-specific primer set provided a high detection rate for one of four target species, wood frog. I recommend that a species-specific primer set, in combination with two-step PCR, be used in future projects, although, further work is needed to refine techniques and to determine what biotic and abiotic factors influences detection rates.

Table 2.1. Primers used for sequencing the 16S and cytochrome c oxidase (CO1) mitochondrial regions for all 10 resident amphibian species of Alberta. The species amplified by each primer are listed as well as the publication source for the primer sequences.

Primer	Locus	Species	Forward	Source
16S	*16S*	Canadian toad western toad Great Plains toad wood frog northern leopard frog Columbia spotted frog boreal chorus frog plains spadefoot western tiger salamander long-toed salamander	f: 5'-CGCCTGTTTATCAAAAACAT-3' r: 5'-CCGGTCTGAACTCAGATCACGT-3'	Vence *et al* . (2005)
CO1	*CO1*	Canadian toad western toad Great Plains toad wood frog Columbia spotted frog western tiger salamander long-toed salamander	f: 5'-AYTCAACAAATCATAAAGATATTGG-3' r: 5'-ACYTCRGGRTGACCAAAAAATCA-3'	Che *et al* . (2012)
Chm	*CO1*	boreal chorus frog northern leopard frog plains spadefoot	f: 5'-TYTCWACWAAYCAYAAAGAYATCGG-3' r: 5'-ACYTCRGGRTGRCCRAARAATCA-3'	Che *et al* . (2012)

Table 2.2. Diagnostic primer sets used for the Ion Torrent PGM™ library preparation. The name of the diagnostic primer set, the primers pairs they contained, the species whose DNA primers were designed to amplify (target species), and the species whose DNA primers actually amplified (amplified species) are listed. Tm is the melting temperature for primer pairs.

Primer Set	Primer Pair	Tm	Sequence	Target Species	Amplified Species
Uni_P	Uni_P	50°C	f: 5'-CAGTGAAACTRATCTYCCCG-3' r: 5'-CATRGGGTCTTCTCGTCTTATR-3'	All 10 Species	All 10 Species
Grp_P	Grp_P1	52.8°C*	f: 5'-CGGTCACCCCAACCWAA-3' r: 5'-GAAGACCCTATGGAGCTTWAAAY-3'	Canadian toad western toad Great Plains toad boreal chorus frog	All 10 Species
	Grp_P2	52.6°C*	f: 5'-CCCCAACCYAAAACTARCAT-3' r: 5'-TAAGACGAGAAGACCCCATG-3'	wood frog columbia spotted frog northern leopard frog	wood frog northern leopard frog Canadian toad Great Plains toad boreal chorus frog western tiger salamander long-toed salamander
	Grp_P3	53.7°C*	f: 5'-CCCCAACCGAAAACATAAGC-3' r: 5'-TAAGACGAGAAGACCCCATG-3'	plains spadefoot	plains spadefoot
	Grp_P4	53.3°C*	f: 5'-TCGCCCCAACCYAAAATT-3' r: 5'-TATCATAAGACGAGAAGACCCTATG-3'	tiger salamander long-toed salamander	All 10 Species
Spp_P	WETO	56.3°C	f: 5'-TCTTCTCCTCTTAGCCTCTGC-3' r: 5'-AAGATAGTTAAGTCAACTGACGGC-3'	western toad	western toad
	WOFR	55.3°C	f: 5'-AGCTGGAGCTGGTACAGG-3' r: 5'-CCCCAAGATAGATGAAACACCAG-3'	wood frog	wood frog
	BCFR	54.5°C	f: 5'-TCCAAACCCTCCAATCAGG-3' r: 5'-ATTAAGTCAACCTGGCTCCC-3'	boreal chorus frog	boreal chorus frog
	TISA	52.7°C	f: 5'-TCGATCTGTCAGCAGTATTGTAATTC-3' r: 5'-CGCATCAATATCACAATATCAAACC-3'	western tiger salamander	western tiger salamander

*The group primer sets (Grp_P) were combined into a single primer cocktail and I used the Tm (52°C). This value was determined by taking the lowest Tm between all four primer sets and rounding down to account for base-pair degeneracies.

Table 2.3. The number of positive (+) and negative (-) eDNA detections for each ITPGM sequencing round compared to the number of samples that corresponded to a positive (+) or negative (-) species encounter (present/absence based on visual or auditory surveys) at the site where the sample was collected. Cells that correspond to eDNA (+) and field encounter (-) are referred to as "regional detections" and represent a sample with an eDNA detection for a species that could have occurred at the site (within its known range) but was not observed. The column labeled "non-regional" represent samples with eDNA detection for a species that does not occur in the region where the sample was collected.

Sequencing round		**Field encounter**		
Primer set		(+)	(-)	Non-regional
Round 1	eDNA (+)	11	15	9
Uni_p	eDNA (-)	92	184	276
2-step PCR	Total	103	199	285
Round 2	eDNA (+)	13	11	11
Uni_p	eDNA (-)	95	201	289
1-step PCR	Total	108	212	300
Round 3	eDNA (+)	18	18	16
Uni_p	eDNA (-)	66	345	374
1-step PCR	Total	84	363	390
Round 1	eDNA (+)	1	0	12
Grp_p	eDNA (-)	102	199	273
2-step PCR	Total	103	199	285
Round 2	eDNA (+)	2	3	28
Grp_p	eDNA (-)	106	209	272
1-step PCR	Total	108	212	300
Round 3	eDNA (+)	16	12	12
Grp_p	eDNA (-)	68	351	378
1-step PCR	Total	84	363	390
Round 4	eDNA (+)	10	n/a	n/a
Spp_p	eDNA (-)	30	0	60
2-step PCR	Total	40	0	60

n/a: not able to calculate these values because the sample site contained all target species and these species are the only species known to occur in the region.

Table 2.4 The total number of eDNA detections for each primer set and PCR protocol combination for each species that occurred in the region where eDNA samples were collected. eDNA detections are provided for all species that were encountered in the field: Canadian toad (CATO) western toad (WETO), wood frog (WOFR), Columbia spotted frog (CSFR), boreal chorus frog (BCFR), western tiger salamander (TISA), and long-toed salamander (LTSA).

Sequencing round											
Primer set		**Field Encounter**									
		WETO		BCFR		TISA		CSFR		LTSA	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Round 1	eDNA (+)	1	3	2	1	0	1	8	1	0	8
Uni_P	eDNA (-)	26	27	38	16	0	56	28	20	0	49
		WETO		BCFR		TISA		CSFR		LTSA	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Round 2	eDNA (+)	3	4	2	0	0	2	8	0	0	5
Uni_P	eDNA (-)	21	32	38	20	0	58	32	20	4	51
		WETO		BCFR		TISA		WOFR		CATO	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Round 3	eDNA (+)	0	4	1	3	0	1	17	6	0	1
Uni_P	eDNA (-)	9	65	20	54	6	71	25	30	6	71
		WETO		BCFR		TISA		CSFR		LTSA	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Round 1	eDNA (+)	0	0	0	0	0	0	1	0	0	0
Grp_P	eDNA (-)	27	30	40	17	0	57	35	21	0	57
		WETO		BCFR		TISA		CSFR		LTSA	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Round 2	eDNA (+)	1	1	0	0	0	0	1	1	0	1
Grp_P	eDNA (-)	23	35	40	20	0	60	39	19	4	55
		WETO		BCFR		TISA		WOFR		CATO	
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Round 3	eDNA (+)	0	2	0	0	0	0	15	5	1	5
Grp_P	eDNA (-)	9	67	21	57	6	72	27	31	5	67
		WETO		BCFR		TISA		WOFR			
		(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)		
Round 4	eDNA (+)	0	0	0	0	0	0	10	0		
Spp_P	eDNA (-)	10	0	10	0	10	0	0	0		

Chapter III: Environmental DNA: Assessing the Effect of Sampling Method, Sampling Date and Detection Platform on Species Detection Using eDNA for Western Toad (*Anaxyrus boreas*), Wood Frog (*Lithobates sylvaticus*) and Boreal Chorus Frog (*Pseudacris maculata*) in Central Alberta

3.1 Introduction

Detecting rare and invasive species to monitor their range expansions and contractions is critical to ecological conservation. Developing biodiversity catalogues by identifying habitats across large geographic ranges can facilitate efforts to monitor changes in species' distributions. Species' detection and identification are critical when building these catalogues; therefore, the methodologies employed must be comprehensive, reliable and efficient. Amphibians are often transient at aquatic habitats using them for different life stages and requirements such as foraging, breeding, larval development and overwintering. Traditional amphibian monitoring techniques focus on trapping, auditory and visual detection that may target one or all life phases. The techniques are physically demanding, time consuming and may be biased by species' abundances (Tanadini and Schmidt, 2011), environmental conditions (Dostine *et al*., 2013; Milne *et al*., 2013), sampling methods (Corn *et al*., 2000; Strain *et al*., 2009), and observer skills resulting in misidentification and omission of species (Genet and Sargent, 2003; Lotz and Allen, 2007; McClintock *et al*., 2010). A need exists for innovative approaches to amphibian monitoring that are more sensitive, efficient, and comprehensive. In aquatic habitats, the presence of cellular debris from feces, urine, epidermal sloughing, saliva, excretion from wounds, and dead organisms creates an accumulation of deoxyribonucleic acid (DNA). Amplifying, sequencing, and comparing these short DNA fragments from the environment (environmental DNA or eDNA) may be used to catalog the organisms that inhabit a freshwater ecosystem. To date, the use of eDNA to monitor freshwater biodiversity has been applied to rare and invasive amphibian and fish species (Ficetola *et al*., 2008; Jerde *et al*., 2011; Dejean *et al*., 2012; Thomsen *et al*., 2012a; Goldberg *et al*., 2013; Spear *et al*., 2014; Fukumoto *et al*., 2015).

Detecting vertebrates in freshwater ecosystems with eDNA techniques can be more effective than traditional methods (Ficetola *et al*., 2008; Jerde *et al*., 2011; Dejean *et al.*, 2012; Thomsen *et al.*, 2012a); however, species detection with eDNA is a relatively young and expanding technique and there are a variety of protocols to choose from when collecting and processing samples (Ficetola *et al.*, 2008; Jerde *et al.*, 2011; Goldberg *et al*., 2011; Deiner *et al*., 2014; Turner *et al.*, 2014a; Wilcox *et al*., 2014; Fukumoto *et al.*, 2015). Choice of protocol for sample collection (Deiner *et al*., 2014; Turner *et al.*, 2014a), eDNA extraction method (Goldberg *et al*., 2011; Deiner *et al*., 2014), and eDNA amplification (e.g. PCR conditions) (Goldberg *et al*. 2011) can influence the probability of detecting a species. Determining which methods provide the highest probability of detection is paramount to creating a protocol that is efficient and reliable.

PCR inhibitors such as humic and fulvic acids in environmental samples may bias detection of eDNA by reducing or blocking DNA amplification during PCR (Stevenson 1994; Albers *et al*., 2013; McKee *et al*., 2014; Jane *et al*., 2015). Diluting the DNA template (i.e. eDNA sample extract) may attenuate the effect of inhibitors during the PCR reaction (Hoshino and Inagaki, 2012; McKee *et al*., 2014). However, diluting a sample may also impede detection if the initial amount of eDNA is low to begin with (McKee *et al*., 2014). Understanding how different detection platforms are affected by PCR inhibitors and how samples dilution to reduce inhibition affects detection rates may influence platform selection.

A variety of platforms have been employed to detect eDNA, including high throughput sequencing (Thomsen *et al*., 2012a,b; Deiner *et al*., 2014; Kelly *et al*., 2014)and quantitative PCR (Goldberg *et al*., 2011; Wilcox *et al*., 2013; Spear *et al*., 2015; Jane *et al*., 2015). Platform selection is typically dictated by personal preference, cost, and laboratory equipment available to the researcher. Each platform may create biases due to the different methodologies involved for amplifying and detecting eDNA. Determining how eDNA detection rates compare across platforms will provide an understanding of the biases. Detectability should be taken into

consideration by researchers, not only when selecting a detection platform, but also when assessing the reliability of results when platform options are limited.

Environmental DNA in aquatic habitats originates from larger cellular debris suspended in the water column (Martellini *et al*., 2005; Caldwell *et al*., 2011; Turner *et al*., 2014a). While suspended, cellular debris has two fates: 1) it may rapidly breakdown, releasing eDNA into the water column where it remains detectable for 1-25 days following the absence of the source species (Dejean *et al*., 2011; Thomsen *et al*. 2012a,b; Barnes *et al*., 2014; Pilliod *et al*., 2014); or 2) it may settle from the water column into the aquatic habitat's sediment where it accumulates and its eDNA can potentially be detected for 100's to 1000's of years (Matisoo-Smith *et al*., 2008; Giguet-Covex *et al*., 2014). Turner *et al*. (2015) found that Asian carp (*Hypophthalmichthys spp*.) eDNA accumulates in pond bottom sediments at greater concentrations than in the water column. However, little is known about how detection rates from water samples compare to sediment samples and how detectability changes across life stages for amphibians.

My project objectives were to assess 1) how 10-fold dilutions to reduce potential PCR inhibition influence eDNA detection rates; 2) eDNA detection rates for two platforms: the Ion Torrent PGM™ (Life Technologies; Carlsbad, California USA) (high through-put sequencing) and the 7500 Fast Real-Time PCR Machine (Applied Biosystems; Foster City, CA, USA) (quantitative PCR); 3) the influence of sample type, water column versus sediment samples, on eDNA detection rates for each platform; and 4) how sampling across different life stages and seasons influence detection rates for both sampling methods and detection platforms. I used western toad (*Anaxyrus boreas*), wood frog (*Lithobates sylvaticus*) and boreal chorus frog (*Pseudacris maculata*) as the target species because they commonly co-occur in wetlands of central Alberta, Canada. At three water bodies, where all three species were encountered through visual and call surveys, I collected eDNA samples from the water column and the pond sediment on three different dates throughout the breeding and larval developmental periods. Samples were analyzed for eDNA using Ion Torrent PGM™ (ITGPM) and

quantitative PCR (qPCR) platforms. For each detection platform and sampling method, samples were processed at eDNA extraction concentration, then extractions were diluted and processed a second time to assess the influence of sample dilution on PCR inhibition.

3.2 Materials and Methods

3.2.1 Field collections

3.2.1.1 Study Area

The three study sites were natural wetlands located in Elk Island National Park (EINP), in the Aspen Parkland region of north-central Alberta, Canada (53°34'23.1"N, 112°50'30.4"W). The geographic landscape of EINP is upland boreal mixed-wood forest surrounded by grassland, wetlands, and shallow lakes. The forest is dominated by aspen (*Populus tremuloides*), balsam poplar (*Populus balsamifera*), white birch (*Betula papyrifera*), white spruce (*Picea glauca*), black spruce (*Picea mariana)* and the dominant shrubs are willow (*Salix spp*.), red osier dogwood (*Cornus sericea*) and beaked hazelnut (*Corylus cornuta*). Wetlands are dominated by sedges (*Carex spp.*), moss (*Sphagnum spp.*) and bulrush (*Typha spp*). In June the average daily temperature is 14.5°C with an average temperature range of 7.3 to 20.8°C and an average precipitation of 8.0 cm. In July the average daily temperature is 16.9°C with an average temperature range of 9.6 to 23.3°C and an average precipitation of 8.5 cm. In August the average daily temperature is 15.6°C with an average temperature range of 8.2 to 22.2°C and an average precipitation of 6.9 cm (Parks Canada, 2015).

3.2.1.2 Site description

Pond perimeter and surface area were calculated by walking the entire pond perimeter with a GPS unit to measure distance and calculate area on each collection date to create a seasonal mean for each site in 2014. Site 1 was an open water depression with a defined perimeter encompassed by mixed grass species merging into mixed-wood forest and contained open water with 2 to 3 m of emergent vegetation at the pond margin. The mean perimeter was 721±1 m and the mean surface area was 8826±596 m^2 (53°39'28.3"N, 112°50'38.7"W).

Site 2 was a shallow wetland habitat with a canopy of willow shrubs in the center and very little open water. It had a defined perimeter encompassed by mix-wood forest and contained 10 to 15 m of emergent vegetation at the pond margin. The mean perimeter was 749±2 m and the mean surface area was 16104±121 m^2 (53°40'37.7"N, 112°49'00.5"W). Site 3 was an open water pond with floating peat beds at the pond margin. It was surrounded by sprawling shallow wetlands with a poorly defined bank; I used the tree and shrub line to define the perimeter of Site 3. Therefore, the pond size was the same for each collection date; the perimeter was 850 m and the surface area was 18,016 m^2 (53°42'50.7"N, 112°50'22.8"W).

3.2.1.3 Call surveys and visual surveys

Western toad typically occur in lower abundances than wood frog and boreal chorus frog in central Alberta (Russel and Bauer 2000), thus due to fewer females they typically produce fewer larvae per breeding site. Western toad is provincially listed as "Sensitive" (Alberta Government, 2000), federally as "Species of Special Concern" in Canada (COSEWIC, 2012) and globally as "Near Threatened" (IUCN, 2004). Wood frog and boreal chorus frog are listed as "Secure" in Alberta (Alberta Government, 2005), federally as "Secure" in Canada (COSEWIC, 2012) and globally as "Least Concern" (IUCN, 2008 and 2014). Western toad was the main focus of this project due to is lower abundances per site and its conservation statuses. Field surveys and eDNA sample collection dates targeted three ontogenetic phases for western toad in EINP: early larval development, late larval development and post-metamorphosis. Although collection dates did not coincide with benchmark ontogenetic events (e.g. breeding and metamorphosis) for wood frog and boreal chorus frog, these species were included to assess eDNA detection rates because they typically occur in higher abundances than the western toad in central Alberta wetlands.

To establish the three study sites, surveys were conducted for the three species in EINP from May 1st through June 7th, 2014. Each site was assessed by two people walking the perimeter from 21:00 to 1:00 until all three species were heard and visually identified. Wood frog and boreal chorus frog reached peak breeding in

early and mid-May, respectively. Western toad began calling in late-May and early June and, within 2 weeks of encountering western toad, I began collecting water and sediment samples from the three sites.

3.2.2 eDNA sample collection and extraction

3.2.2.1 eDNA sampling methods

For each collection date, I established 10 evenly distributed sampling locations around the perimeter of each site. At each sampling location, I collected a 15 mL water sample and a >0.25 g sediment sample. A clean pair of gloves was used for each sample location. Sediment samples were taken after water samples to reduce sediment disturbance in the water column. I was careful not to step into the water unless necessary to obtain a clear water sample, in which case I took the samples quickly and as far away from my body as possible. Waders were cleaned using a spray bottle containing 50% bleach between each site.

Water samples were collected and preserved according to the protocol described by Ficetola *et al.* (2008) with slight modification. I submerged an inverted 50 mL conical tube to 10 to 20 cm from the pond bottom where it was allowed to fill. Samples were decanted to 15 mL and stored on ice for less than 1 h before adding 1.5 mL of 3M sodium acetate (pH 5.2) and 33.5 mL of absolute ethanol. Samples were mixed by inversion and placed on ice for < 2.5 h then stored at -20°C until processed. A field-based negative control for water sampling was taken at each site by transporting a clean 50 mL conical tube to the site, filling it with 15 mL of sterile de-ionized H_2O (ddH_2O), 1.5 mL of 3M sodium acetate (pH 5.2) and 33.5 mL of absolute ethanol following the collection of all other samples. It was stored and processed as a water sample thereafter.

Sediment samples were taken by submerging a gloved hand to the pond bottom and scraping the top 2-3 cm layer off the bottom and storing it in a 50 mL conical tube. Samples were placed on ice for < 2.5 h then stored at -20°C until processed. A field-based negative control for sediment sampling was taken at each site by transporting a clean 50 mL conical tube to the site, filling it with 15 mL of sterile ddH_2O following the collection of sediment samples. It was stored and processed as a sediment sample thereafter.

3.2.2.2 eDNA extractions

DNA and/or cellular remains were recovered from water samples by centrifugation of 6330 x g for 35 min at 4°C. Supernatant was decanted by gentle pouring, the pellet was air dried for a minimum of 10 min, and DNA was extracted using modified DNeasy Blood and Tissue Extraction Kit (Qiagen; Hilden, Germany) protocol. I added 360 µL of ATL buffer and 40 µL of Proteinase K solution to the 50 mL conical tube containing the pellet and incubated for ~10 h at 56°C. Following incubation, 400 µL of AL buffer and 400 µL of absolute ethanol (pre-mixed) were added and each sample was vortexed. From here on I followed the DNeasy Blood and Tissue Extraction Kit (Qiagen) protocol according to the manufacturer's instructions. Final DNA was eluted into 150 µL of IDT nuclease free H_2O (nfH_2O) (Integrated DNA Technologies; Coralville, Iowa) and stored at -20°C until processed. Samples from each site and collection date were processed as independent groups with their respective field-based negative as the last sample processed to control for eDNA cross-contamination.

Sediment samples were removed from -20°C and allowed to thaw at room temperature. Using clean forceps, I removed 250 µg of wet sediment from the top layer of the sample and extracted the DNA using a Power Soil® DNA Extraction Kit (MoBio Laboratories, Inc. Carlsbad, California USA) following the manufacturer's instructions. The final DNA was eluted into 150 µL IDT nfH_2O (Integrated DNA Technologies). The forceps were cleaned between samples by dipping in 10% bleach, soaking in 50% bleach ≥ 2 min, and rinsing them with ddH_2O. The same forceps were cleaned as above and dipped into the field-based negative sample; then 250 µL of the negative sample was processed the same as the sediment samples. Samples from each site and collection date were processed as independent groups with their respective field-based negative as the last sample processed to control for eDNA cross-contamination.

3.2.3 Ion Torrent PGM™

3.2.3.1 Primer design

To maximize the primer base pair mismatches between our target species and closely related amphibian species that may co-occur in targeted habitats in Alberta, I created an alignment of ~530 bp segment of the mitochondrial cytochrome c oxidase subunit one (CO1) region in SeqMan (DNASTAR®; Madison, Wisconsin USA) for western toad, Canadian toad (*Anaxyrus hemiophrys*), great plains toad (*Anaxyrus cognatus*), wood frog, northern leopard frog (*Lithobates pipiens*), Columbia spotted frog (*Rana luteiventris*), boreal chorus frog, plains spadefoot toad (*Spea bombifrons*), western tiger salamander (*Ambystoma mavortium*) and long-toed salamander (*Ambystoma macrodactylum*) (See Chapter II for DNA extraction, sequencing protocols and alignment). These are the known amphibian species resident in Alberta. All primer sets were tested against DNA from each species to determine specificity using the PCR 1 protocol (see below). PCR products were visualized using electrophoresis and a 1% agarose gel. All primers were positive for respective target species and negative for all other non-target amphibian species of Alberta.

I used a two-step PCR protocol to amplify eDNA from samples (PCR 1) and label the eDNA amplicons from each sample with a molecular identifier sequence (MID) tag (PCR 2). Two separate primer pairs were used: 1) PCR 1 had a primer pair for the species-specific locus amplification, the forward primer contained a "Glenn" adaptor on the 5' end followed by the species-specific forward primer sequence and the reverse primer contained the truncated P1 (trP1) sequence (Life Technologies) on the 5' end followed by the species-specific reverse primer sequence; and 2) PCR 2 had a primer pair containing a forward primer with a sample specific MID probe on the 5' end followed by the complementary "Glenn" sequence, and the reverse primer was the trP1 adaptor (Life Technologies). The "Glenn" adapter allowed us to leave the MID uncoupled from the species-specific forward primers, reducing the need for separate pre-MID labeled species-specific primers and the overall project cost. For each target amphibian the species-specific primer pairs for PCR 1 targeted a

different segment of the mitochondrial CO1 region that were 114 bp (western toad), 124 bp (wood frog) and 115 bp (boreal chorus frog) in length. Primers for PCR 1 were "Glenn"-species-specific forward: 5'-16bp "Glenn" adaptor (CAGTCGGGCGTCATCA) and species-specific forward primer (Table 3.1); trP1- species-specific reverse: 5'-trP1 adaptor (CCTCTCTATGGGCAGTCGGTGAT; Life Technologies) and species-specific reverse primer (Table 3.1). Primer pairs for PCR 2 were MID probe forward: 5'-30 bp A-adapter (CCATCTCATCCCTGCGTGTCTCCGACTCAG), 10-11bp MID sequence, and the 16 bp complementary "Glenn" adaptor; trP1 reverse: 5'-complementary trP1 adapter (Life Technologies).

3.2.3.2 Ion Torrent™ PGM PCR protocols

PCR 1 was performed three times per sample with each reaction containing one of the three species-specific primers. PCR 1 was performed in 15 µL final volume reactions of 1X High Fidelity PCR buffer (Life Technologies), 2 mM of $MgSO_4$, 0.2 mM dNTPs, 0.2 µM "Glenn"-species-specific forward and trP1-species-specific reverse primers each, 0.5 units of Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies) and 5 µL of eDNA extraction. Thermo-cycling conditions for the PCR 1 began with denaturing for 2 min at 94°C, 35 cycles of denaturing for 30 sec at 94°C, primer annealing for 30 sec at species' primer Tm (Table 3.1) and elongation for 60 sec at 68°C with a final elongation step of 10 min at 68°C. After PCR 1, the products from the three reactions per sample were combined then diluted 10 fold to reduce primer interactions during PCR 2. PCR 2 was performed in a 15 µL final volume reaction containing 1X High Fidelity PCR buffer (Life Technologies), 2 mM of $MgSO_4$, 0.2 mM dNTPs, 0.25 µM MID probe forward and trP1 reverse primers each, 0.5 units of Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies) and 5 µL of diluted PCR 1 product. Thermo-cycling conditions for the PCR 2 began with denaturing for 2 min at 94°C, 25 cycles of denaturing for 30 sec at 94°C, primer annealing for 30 sec at 58°C and elongation for 60 sec at 68°C with a final elongation step of 10 min at 68°C. All field-based negatives were prepared beside samples from their respective site and date. Each library included a PCR negative prepared using 5 µL of IDT nfH_2O (Integrated DNA

Technologies) following the two-step amplification and MID labeling protocol as above. I pooled the MID label reactions following PCR 2 to create the sample library. I concentrated 100 µL of the library with a 30 min centrifugation using a speed vacuum centrifuge. I size selectively purified the library with electrophoresis and a 2% agarose gel. I extracted DNA between 190-215 bps, the length of my products following amplification with adaptors. This purification step excludes the majority of primers, primer dimers, and non-target product as small molecular weight DNA may be preferentially amplified during emulsion PCR (emPCR). DNA was extracted from 2% agarose using a QIAquick Gel Purification Kit (Qiagen) following the manufacturer's instructions and further purified using a QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions; final DNA was eluted into 30 µL IDT nfH_2O.

For each sequencing library, I prepared three MID labeled PCR positives (one per target species) using 5 µL of template from a 5 ng/µL stock of DNA extracted from western toad, wood frog and boreal chorus frog tissue (see Chapter II for tissue extraction protocol). Initial template concentrations were measured with an Invitrogen™ Qubit® Fluorometer 1.0 (Life Technologies). PCR positives were amplified and labeled with a MID probe using the same two-step PCR protocol as above. Following PCR 2, the PCR positive products were combined to create a PCR positive control library. They were kept separate from the sample library through the gel extraction step. Following the gel extraction, both libraries were quantified using an Invitrogen™ Qubit® Fluorometer 1.0 (Life Technologies). Then the PCR positive library was added to the sample library at 1/100 concentration to reduce dominating amplification during emPCR. The combined library was re-quantified and diluted to 18 pM with IDT nfH_2O (Integrated DNA Technologies) to create the final Ion Torrent PGM™ library.

Libraries were amplified using an emulsion PCR Ion OneTouch™ 2 system (OT2) and the Ion PGM™ Template OT2 200 Kit (Life Technologies) according to the manufacturer's instructions. Ion sphere particles (ISP) were enriched using Ion OneTouch™ Enrichment System (Life Technologies) following the

manufacturer's instructions. Enriched live ISPs were loaded and sequenced on an Ion PGM™ 314 chip v2 using an Ion PGM™ 200bp sequencing Kit v2 (Life Technologies) following the manufacturer's instructions. All samples were processed at eDNA extraction concentration, then diluted 1/10 with IDT nfH_2O and processed a second time.

3.2.3.3 Bioinformatics

The sequences for each library were sorted according to their MID tag and the adaptor sequences were trimmed using the Torrent Suite™ and Torrent Server (Life Technologies). The output was a single ".fasta" file of sequences for each MID that represented an eDNA sample. Sequences were imported to CLC Genomic Workbench 7.0 (CLC bio, Aarhus, Denmark) where primers were trimmed. Trimmed sequences were filtered by length retaining reads between 50 and 150 bp and by quality scores using a modified Mott-trimming algorithm and the default settings (CLC Genomic Workbench 7.0). Reads were mapped to the CO1 reference regions of the three species using CLC Genomic Workbench 7.0 with 100% read length match to reference and 98% similarity in base pairs to reference. Mismatch, insertion and deletion cost were set to 3 which is as stringent as the program allows. A large proportion of samples produced ≤ 50 aligned reads. However, all negative controls had ≤ 50 aligned reads for each species; therefore, I used a threshold of ≥ 51 reads per species as a minimum read count for a sample to be considered positive for eDNA for that species.

3.2.4 Quantitative PCR

3.2.4.1 Probe Design

One qPCR PrimeTime® Standard Assay primers and fluorescent probe set was designed per species to target a different ~ 150 bp segment of the CO1 region (Table 3.1). These segments did not coincide with the DNA segments targeted using the ITPGM platform. Primers were designed using Primer Express® Software v3.0.1 (Applied Biosystems). Using SeqMan (DNASTAR®) I created an alignment of ~530bp segment of the CO1 region for the four amphibian species known to occur in EINP: western toad, wood frog, boreal chorus

frog and western tiger salamander. To increase primer specific to target species, I compared the selected primer sets to this alignment to ensure all forward and reverse primers contained at least 3 bp mismatches with non-target species with at least one of these mismatches occurring in the last 7 bp on the 3' end. All primer and probe sets were tested against their target species and three other amphibian species that co-occur in EINP using 2.5 µL of 5 ng/µL stock of tissue-extracted DNA following and the 7500 Fast Real-Time PCR Machine (Applied Biosystems) (see below for protocol). All primers and probes tested positive for respective target species and negative for all three non-target species.

3.2.4.2 qPCR Protocol

qPCR reactions were analyzed on a 7500 Fast Real-Time PCR Machine (Applied Biosystems) using 1.5µL IDT nfH_2O (Integrated DNA technologies), 5µL of proprietary 2x Master Mix (Appendix 2), 1 µL of 10x custom PrimeTime® Standard qPCR assay containing primers and probes (Integrated DNA Technologies) and 2.5 µL of extracted eDNA sample. The qPCR thermal cycling conditions were one cycle of 95°C for 2 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. All samples were processed in duplicate to determine how replication influences detection rates. I chose two replicates per sample to keep project costs low; however, more than two replicates may be preferred if detection probabilities are low (Ficetola *et al*., 2015). Field-based negative controls were processed beside samples from their respective ponds. A qPCR reaction was considered positive for eDNA when the quantity of amplified PCR product exceeded the Ct (the cycle at which PCR product exceeds background level). Ct threshold was automatically set by the 7500 Fast Real-Time PCR Machine software (Applied Biosystems). An eDNA sample was considered positive for a species if at least one reaction crossed the Ct threshold. All samples were processed at eDNA extraction concentration, then diluted 1/10 with IDT nfH_2O (Integrated DNA Technologies) and processed in duplicate a second time.

3.2.5 Data Analysis

Mean detection probabilities were calculated for each species, sampling method and detection platform for the June, July and August sampling periods by averaging the detection probabilities between all three sites. Each sample was analyzed for eDNA from each target species once with the ITPGM and twice with qPCR detection platforms. To make a direct comparison between platforms, I conducted a refraction analysis on the qPCR data. For each sample, using a random number generator, I randomly selected results from one of the two PCR reactions to compare with ITPGM results (i.e. if reaction 1 was positive, reaction 2 was negative and reaction 2 was randomly selected, then the samples was considered negative for the qPCR platform). In a less rigorous evaluation of qPCR results, I conducted a second analysis that "combined" both qPCR reactions to compare detection rates with ITPGM rates (i.e. if one of the two reactions was positive for eDNA, a sample was considered positive for the qPCR platform). To compare the detection rates for each species by platform, I combined the results for the water and sediment samples across all three sites and collection dates for both platforms (n=360) and performed a McNemar's test for paired dichotomous data. McNemar's test determines if there is a significantly greater number of discordant pairs than would occur by chance alone (McNemar, 1947). For this analysis, a discordant pair would be a sample that was positive for the eDNA of a target species using the ITPGM platform and negative for that species' eDNA using the qPCR platform, and vice versa. For each species, I also used a McNemar's test to compare total detection rates between water and sediment sampling. To determine if either sampling method had greater detection rates during a single collection period (month), I used a McNemar's comparison between sampling methods for each collection period and detection platform.

To determine the influence of sampling date on detectability, I conducted a Cochran's Q test for multiple comparisons across the three sampling periods (months) for each species (Cochran, 1950). If a species' detection rates differed significantly among months, I performed a post-hoc pair-wise McNemar's comparison between the three sampling periods to identify which period(s) had significantly greater detection rates

(McNemar, 1947). All statistical analyses were performed in R version 3.2.0 (R Core Team, 2015) and the R packages 'stats' (R Core Team, 2015), 'binom' version 1.1-1 (Doria-Raj, 2015) and 'RVAidMemoire' version 0.9-50 (Hervé, 2015) with an alpha level of 0.05.

3.3 Results

3.3.1 PCR inhibitors

The number of positive samples for each site, period and species can be categorized into two groups: eDNA detection at standard concentration and eDNA detection after dilution to reduce potential PCR inhibition (Table 3.2). For samples processed with the ITPGM platform there were a total of 110 eDNA detections (of a potential 540 detections) across all three species, collection dates and sampling methods combined. Of these 110 detection, 61 occurred before inhibitor dilution (55.4%) and 49 occurred after inhibitor dilution (44.5%). Of the 61 pre-diluted detections, 44 were not detected post-dilution (40% loss of eDNA detections). Processing a sample in duplicate with the qPCR platform was more cost effective than processing a sample once with the ITPMG platform (See Discussion 3.4.2 for cost analysis). Therefore, unless stated otherwise, all qPCR detection data is derived from samples processed in duplicate. For the qPCR platform at least one of the two replicates was positive for eDNA on 133 occasions (of a potential 540 detections) across all three species for both sampling methods combined. Of these 133 detection, 105 occurred before inhibitor dilution (78.9%) and 28 came after inhibitor dilution (21.1%). Of the 105 pre-diluted detections, 55 were not detected post-dilution (41.3% loss of eDNA detections). For the remaining analysis, the subsequent total detection rates represent the detections of eDNA at standard concentration plus the additional detections that occurred only after inhibitor dilutions. All diluted sediment samples from Site 2 collected on July 15th were removed due to a field-based negative control that was positive for wood frog eDNA. All other field and lab negatives showed no contamination.

3.3.2 Field surveys and eDNA detection per site

Western toad young-of-the-year (YOY) were visually encountered in July at Site 1 and Site 2, verifying that these were successful breeding locations. At Site 3 western toads were heard calling and adults were seen but YOY were not observed and it is unknown if this site produced any metamorphs. Wood frog and boreal chorus frog young-of-the-year were seen at all three sites during the July sampling period.

Western toad eDNA was detected at Site 1 using both sampling methods and detection platforms. Western toad eDNA was not detected at Site 2 with either sampling methods or detection platforms. Western toad eDNA was detected at Site 3 when water samples where processed on the ITPGM and qPCR platforms. Wood frog eDNA was detected at every site for both sampling methods and detection platforms. Boreal chorus frog eDNA was detected at Site 1 using both sampling methods and detection platforms. Boreal chorus frog eDNA was detected at Site 2 when sediment samples were processed with the ITPGM and qPCR platforms. Boreal chorus frog eDNA was not detected at Site 3 with either sampling method or either detection platform (Table 3.2).

3.3.3 Comparing detection platforms: Ion Torrent PGM™ and qPCR with and without rarefaction analysis

After combining sediment and water samples from all three sites and dates, there were 180 samples processed with the ITPGM and with the qPCR platforms. With the ITPGM platform: western toad was detected in 18 samples (10% detection probability), wood frog was detecting in 81 samples (45% probability), and boreal chorus frog was detected in 11 samples (6.1% probability). For the qPCR platform including rarefaction analyses: western toad was detected in 10 samples (5.6% detection probability), and there was no significant difference between platform performance (χ^2=0.53, df=1, p=0.47). Wood frog was detected in 93 samples (57% detection probability), and there was no significant difference between platform performance (χ^2=3.13, df=1, p=0.077). Boreal chorus frog was detected in 10 samples (5.6% detection probability), and there was no significant difference between platform performance (χ^2=0.09, df=1, p=0.76). With qPCR and samples

processed in duplicate for the 180 samples (representing 360 qPCR runs): western toad was detected in 20 samples (11.1% detection probability), and there was no significant difference between platforms (χ^2=0.29, df=1, p=0.59). Wood frog was detected in 108 samples (60% detection probability), and detection rates were significantly greater for the qPCR platform compared to the ITPGM platform (χ^2=13.76, df=1, p=2.4 x 10^{-4}). Boreal chorus frog was detected in 13 samples (7.2% detection probability), and there was no significant difference between platforms (χ^2=0.33, df=1, p=0.56).

3.3.4 The effect of sample collection method on eDNA detections

Detection probabilities for water and sediment sampling methods were compared for each target species and detection platform. After combining all three sites and dates there were 90 water samples and 90 sediment samples. For the Ion Torrent PGM™ western toad was detected in 10 of the water samples (11.1% detection probability) and 8 of the sediment samples (8.9% detection probability). There was no significant difference in detection probabilities between sampling methods (χ^2=2.78, df=1, p=0.095). Wood frog was detected in 35 of the water samples (38.9% detection probability) and 46 of the sediment samples (51.1% detection probability). Detection probability for wood frog was significantly higher for sediment samples (χ^2=3.9, df=1, p=0.048). Boreal chorus frog was detected in 8 of the water samples (8.9% detection probability) and 3 of the sediment samples (3.3% detection probability). There was no significant difference in detection probability between sampling methods (χ^2=0.11, df=1, p=0.74).

For qPCR samples run in duplicate, western toad was detected in 11 of the water samples (12.2% detection probability) and 9 of the sediment samples (10% detection probability). There was no significant difference in detection probability for sampling method (χ^2=0.33, df=1, p=0.56). Wood frog was detected in 54 of the water samples (60% detection probability) and 54 of the sediment samples (60% detection probability). There was no significant difference in detection probability between sampling methods (χ^2=0, df=1, p=1). Boreal chorus frog was detected in 6 of the water samples (6.7% detection probability) and 7 of the sediment

samples (7.7% detection probability). There was no a significant difference in detection probability between methods (χ^2=0.11, df=1, p=0.74).

3.3.5 Assessing the effect of sampling periods on the eDNA detections

Detection rates were compared between monthly sampling periods for each sample collection method using combined data from all three sites. There was no significant differences among months for water samples analyzed with the Ion Torrent PGM™ platform for western toad (χ^2=8, df=2, p=0.37) and boreal chorus frog (χ^2=4.33, df=2, p=0.11). There was a significant difference in detection rates across the sampling periods for wood frog (χ^2=16.38, df=2, p=0.0002). Wood frog detection rates did not differ significantly between June and July (χ^2=0.4, df=1, p=0.53); however, both June and July had significantly higher detection rates than August (χ^2=10.29, df=1, p=0.0013; χ^2=10.89, df=1, p=0.00097, respectively). Detection rates for sediment samples processed with the Ion Torrent PGM™ platform did not differ significantly across sampling periods for western toad (χ^2=3.17, df=2, p=0.16), wood frog (χ^2=3.2, df=2, p=0.2) or boreal chorus frog (χ^2=6, df=2, p=0.05).

For water samples analyzed in duplicate with the qPCR platform, detection rates did not differ significantly across the three sampling periods for western toad (χ^2=3.2, df=2, p=0.2) and boreal chorus frog (χ^2=4, df=2, p=0.14). There was a significant difference in detection rates across the sampling periods for wood frog (χ^2=13.06, df=2, p=0.0015). Wood frog detection rates were not significantly different between June and July (χ^2=0.2, df=1, p=0.65); however, both June and July had significantly greater detection rates than August (χ^2=8.07, df=1, p=0.0045; χ^2=7.14, df=1, p=0.0075, respectively). Monthly detection rates for sediment samples processed with the qPCR platform differed significantly for western toad (χ^2=9, df=2, p=0.011) and wood frog (χ^2=15, df=2, p=0.00067). For western toad, July had a significantly greater detection rate than June (χ^2=6, df=1, p=0.014) or August (χ^2=8.07, df=1, p=0.0045). Detection rate for June and August did not differ significantly (χ^2=0, df=1, p=1). For wood frog, June and July detection rates did not differ significantly (χ^2=1.8, df=1, p=0.17). However, June and July detection rates were significantly greater than August (χ^2=6.2, df=1,

p=0.012; χ^2=6.2, df=1, p=0.0013, respectively). Detection rates for sediment samples were not significantly different across the three sampling periods for boreal chorus frog (χ^2=3.5, df=2, p=0.17).

3.3.6 Comparing detection rates between sampling methods for each sample collection period

Detection rates for both sampling methods, water versus sediment, processed with the ITPGM were compared for each month. For western toad, detection rates did not differ between sampling methods for June (χ^2=0, df=1, p=1), July (χ^2=0.2, df=1, p=0.65) or August (χ^2=3, df=1, p=0.083). For wood frog detection rates did not differ significantly between sampling methods for June (χ^2=1.14, df=1, p=0.29) or July (χ^2=0, df=1, p=1). Methods differed significantly for August (χ^2=5.33, df=1, p=0.021) with a greater detection rate for sediment samples. For boreal chorus frog detection rates did not differ significantly between sampling methods for June (χ^2=0.33, df=1, p=0.56), July (χ^2=0.66, df=1, p=0.41) or August (χ^2=1, df=1, p=0.32).

For samples analyzed in duplicate with the qPCR platform, there was no significant difference between sampling methods for western toad in June (χ^2=0.33, df=1, p=0.56), July (χ^2=0.2, df=1, p=0.65) or August (χ^2=1.8, df=1, p=0.18); wood frog in June (χ^2=0.09, df=1, p=0.76), July (χ^2=0.2, df=1, p=0.65) or August (χ^2=0.11, df=1, p=0.74); or for boreal chorus frog in June (χ^2=1, df=1, p=0.32), July (χ^2=0.2, df=1, p=0.65) or August (χ^2=0.2, df=1, p=0.65).

3.4 Discussion

In this study, I determined that 1) sample dilution to reduce potential PCR inhibition influenced eDNA detection for each platform; 2) if samples were processed in duplicate for the qPCR platform, there was a significant difference between the number of positive eDNA samples between detection platforms, with detection via qPCR being greater than ITPGM for wood frog; 3) the number of positive detections for water versus sediment samples when analyzed with the ITPGM were significantly different for eDNA of one species, wood frog, with a higher detection probability for sediment samples; 4) there was no significant difference in detection between sampling methods for any species using the qPCR platform; 5) eDNA detection rates for both

sampling methods changed seasonally, for two species, western toad and wood frog, generally decreasing later in summer; and 6) the number of positive wood frog detections in August were significantly greater for sediment samples versus water samples when processed with the ITPGM platform.

3.4.1 PCR inhibitors

Detection rates for both detection platforms where influenced by 10-fold sample dilution to attenuate the potential influence of PCR inhibitors. For the ITPGM platform, 44.5% of the total detections were obtained after sample dilution. For the qPCR platform 21.1% of the total detections came after sample dilution. The abundance of eDNA in a sample may be low when a species is present in low abundances or if animals produce low levels of eDNA. If a sample requires a 10- fold dilution to attenuate potential PCR inhibition, the starting abundance of template eDNA is reduced by 90% which may cause eDNA to reach levels that are undetectable (McKee *et al*., 2014). In this study, of the samples that were positive prior to dilution, 40% for ITPGM and 41.3% for qPCR were negative post-dilution. This indicates that these samples likely had low starting concentration of eDNA, the 10-fold dilution may have reduced the eDNA to undetectable levels. Therefore, diluting samples 10-fold may create false negative detections (type II error), which is problematic if the eDNA technique is being used to influence habitat management decisions or to detect the presence of an invasive species. Column purified PCR inhibitor removal kits reduce starting eDNA by only 25% (McKee *et al*. 2014) and may be more useful than a 10-fold dilution when working on systems where eDNA concentrations are low.

3.4.2 Detection platforms

I compared the detection rates between the Ion Torrent PGM™ and qPCR detection platforms. Using rarefaction analysis, I found that western toad, wood frog and boreal chorus frog detection rates were comparable between platforms. Overall, detection rates for western toad and boreal chorus frog were low which may bias the platform comparison. For wood frog, which had higher overall detection rates, I found that samples processed in duplicate on the qPCR platform had significantly greater detection rates than the ITPGM

platform. Due to the cost of processing samples with the ITPGM, samples were only processed once using this platform. Ficetola *et al*. (2015) showed that detection probabilities increase with the number of PCR replicates; therefore, it was not surprising that replication increased detection rate for the qPCR platform.

Both detection platforms used in this study have become increasingly common in eDNA analysis; however, they are typically used for different applications. High throughput sequencing (HTS) has been used predominately in metagenomic applications when the goal is to assemble a community of co-occurring organisms based on an environmental sample. Using universal primers that target large taxonomic groups, such as a class or order, eDNA is amplified from a sample and sequenced to generate data for wide range of species. These sequences are compared to a large sequence database to determine which species generated the DNA (i.e. what species are associated with the site where the samples was collected) (Hajibabaei *et al*., 2011; Thomsen *et al*. 2012b; Kelly *et al*., 2014). This approach has been effective in detecting multiple species simultaneously in an environment where little is known *a priori* about community composition. With this approach, false negatives are likely to occur when eDNA from a species is in low abundance or if the universal primers have reduced specificity for particular individual species (Kelly *et al*., 2014). Using species-specific primers may aid in detecting low concentrations of eDNA when universal primers fail (Kelly *et al*., 2014); however, beyond the present project few studies have compared detection rates for HTS species-specific primer approach with other reliable platforms (although see Murray *et al*., 2011). Quantitative PCR is used in eDNA studies that target one to three species using species-specific primers and this platform has become the norm in eDNA analysis due to relatively simple laboratory preparation, cost, and reliability. In this study, for wood frog, I found that sample replication on the qPCR platform gave significantly greater detection rates than single reactions on the ITPGM detection platform.

Using the qPCR protocol from this study, the cost to process a sample for one species is $1.50 CAD for each reaction and an additional $1.50 CAD for each additional species or replicated reaction. Using the ITPGM

protocol from this study, the cost to process a sample for one species is $6.15 CAD per reaction and $1.17 for each additional species. However, to process a sample in duplicate is an additional $6.15 CAD per reaction. The cost of processing a sample once with the qPCR platform does not exceed the cost of processing a sample once with the ITPGM platform until the number of target species is 15. When starting DNA concentrations are low, which is often the case with eDNA samples, samples processing replication is recommended to provide the highest likelihood of detection (Ficetola *et al*., 2008; Dejean *et al*., 2012; Ficetola *et al*., 2015). Therefore, when the project goal is to detect 14 or fewer target species it is more cost effective and more reliable to use the qPCR detection platform and replicate the detection process.

3.4.3 Sampling method

Comparing water samples and sediment samples, I found a significant difference for detection rates for only one case, the ITPGM platform for wood frog eDNA, where sediment samples yielded greater detection rates. Detection rates for water and sediment samples were not significantly different for any species when samples were processed with the qPCR platform. Previous studies show that fish and amphibian aqueous eDNA degrades to undetectable levels between 0-25 days (Dejean *et al*., 2011; Thomsen *et al*., 2012a; Barnes *et al*., 2014; Pilliod *et al*., 2014). In surficial sediment samples, bigheaded Asian carp (*Hypophthalmichthys spp*.) eDNA persisted for >132 days (Turner *et al*. 2014), while in unfrozen lake core sediment samples, fish eDNA was detectable for 3600 yr (Matisoo-Smith *et al*. 2008) and mammalian eDNA was detectable for up to 4800 yr (Giguet-Covex *et al*., 2014). This discrepancy between the lengths of time that eDNA persist in each sampling medium suggests that aqueous and sediment sampling examine different temporal detection ranges. In this study neither sampling method produced significantly greater detection rates for the qPCR platform; therefore, when using the qPCR detection platform sampling method selection should depend on the aim of the project. Turner *et al*. (2015) recommend that water column sampling provides better temporal resolution and should be used to determine current or recent occupancy. Sediment samples, which have lower temporal resolution, may

be used to determine historical presence of a species (Matisoo-Smith *et al*., 2008; Giguet-Covex *et al*., 2014) or when the target species has no history of prior occupancy in a habitat of interest (Treguier *et al*., 2014). However, when using the ITPGM detection platform, it may be beneficial to use sediment sampling if temporal resolution is not a concern.

3.4.4 Species detection patterns across sampling periods

I selected sample collection periods based on the ontology of the western toad. Western toads in Alberta typically have synchronized egg-laying at breeding sites, within a 1 week period (Russel and Bauer 2000). I started collections within 1 to 2 weeks of hearing western toads calling at each site to target early larval development in June. To target later larval development and metamorphosis for this species, I re-sampled each site 3 - 4 weeks later in July. To target post-metamorphosis, I re-sampled another 4 weeks later in August. In June, I encountered western toad adults. In July, I encountered western toad adults and young-of-the-year emerging from Sites 1 and 2; I never encountered young-of-the-year at Site 3. In August I did not encounter any life stages for western toad. Environmental DNA detection rates for western toad changed significantly through the season for sediment samples analyzed with the qPCR platform. However, detection rate were negligible for Sites 2 and 3 for all months, making it difficult to infer which collection period yields the best detection rate for this species. While the source species is present in an aquatic habitat, the rate of eDNA accumulation should exceed the rate of degradation (Thomsen *et al*., 2012a). Prior to the July collection period, western toad larvae had occupied the sites for approximately 3 or more weeks. If population abundance and shed rates were sufficient for eDNA to reach a detectable level, three weeks should have been ample time for it to accumulate at each site; however, for two of the three sites western toad was not detected with either sampling methods or detection platforms. It is difficult to attribute these false negative detections to a lack of western toad eDNA without further investigation into the role of co-extracted DNA (e.g. the use of blocking primers for wood frog and boreal chorus frog DNA) and PCR inhibition (e.g. the application of PCR inhibitor removal kits).

Identifying the cause of false negatives would be valuable for determining whether western toad can be reliably detecting using eDNA.

In Alberta, wood frog typically reaches peak breeding in May, approximately 3 to 4 weeks prior to the June collection period (Russel and Bauer 2000). Wood frog larvae typically develop to metamorphosis in 6 to 12 weeks (Russel and Bauer 2000). For this project, I did not collect on dates that tracked life-history patterns of this species, in part because I was focused on the western toad, which breeds almost 1 month later. At all three sites in June, I encountered larvae and adults; in July and August I encountered young-of-the-year and adults. Through the season, detection rates for water samples processed with the ITPGM platform for wood frog eDNA changed significantly. I found that June and July had similar detection rates with a significant decline in detection rates for August (Figure 1). The lack of difference between June and July may be attributed to the presence of wood frog larvae and adults for multiple weeks prior to the June collection and their continued occupation of ponds through July. Once the wood frog larvae vacated the ponds, between July and August, terrestrial juveniles and adults that visited the ponds for foraging and refuge would have been the only source of eDNA. During this period eDNA accumulation rates most likely decreased and were exceeded by the rate of eDNA degradation, resulting in undetectable levels of eDNA in August using the water sampling method and the ITPGM detection platform. Therefore, when using the ITPGM platform to process water samples for wood frog, DNA collections should be obtained in mid-June or mid-July and not in mid-August. Wood frog eDNA detection rates for sediment samples processed with the ITPGM platform did not decline during the August sampling period and detection rates were significantly greater for sediment samples compared to water samples. I expected to see this disparity between water and sediment detection rates in August due to the persistence of eDNA (Matisoo-Smith *et al*., 2008; Giguet-Covex *et al*., 2014; Turner *et al*., 2015), high concentration of eDNA in sediment (Honjo *et al*. 2012; Turner *et al*., 2015) and rapid eDNA degradation rate in the water column (Dejean *et al*., 2011; Thomsen *et al*. 2012a; Pilliod *et al*. 2014). Therefore, when using the ITPGM

platform to process sediment samples for wood frog, DNA collections could be obtained in mid-June, mid-July or mid-August.

For the qPCR platform, wood frog detection rates were significantly different across the three sampling periods for both water and sediment samples. For water samples this was expected due to the ≤ 25 days of eDNA detection before degradation in the water column reported by other studies (Dejean *et al*., 2011; Thomsen *et al*. 2012a; Pilliod *et al*. 2014). However, sediment eDNA is expected to persist much longer (Turner *et al*., 2015) and I expected detection rates would not decrease in August for this sampling method, similar to the trends observed with the ITPGM platform. However, the qPCR platform produced significantly lower detection rates for August for sediment samples, as well as water samples when compared to June and July. When sampling for wood frog eDNA using the qPCR platform, it is recommended that samples are collected in mid-June and mid-July for both methods.

In Alberta, boreal chorus frog reaches peak breeding in mid-May and continues to breed into early June (Russel and Bauer 2000). For all three sites I encountered adults in June, I encountered young-of-the-year in July, but I did not encounter any boreal chorus frogs in August. There was no significant change in detection across months for any processing combinations. Although not significantly different from other periods, the highest detection rates for this species were in July. Boreal chorus frog was very common at all three sites; however, for both platforms and collection methods this species consistently had low detection rates. Whiting (2010) surveyed ponds in Elk Island National Park and found that boreal chorus frog had greater mean tadpole densities ($\bar{x}$ = 0.085, from 0.001 to 0.51 tadpoles/L, n = 40 ponds) than wood frog ($\bar{x}$ = 0.059, from 0 to 0.31 tadpoles/L, n = 40 ponds). However, boreal chorus frog adults are typically smaller in snout to vent length (20-40 mm) compared to wood frog (30-60 mm) (Russel and Bauer 2000), and their young-of-the-year are smaller at metamorphosis (12.5 to 14.5 mm) compared to wood frog (17.5 to 20.5 mm) (Whiting 2010). Body size has been shown to influences eDNA shed rates in mesocosms containing Idaho giant salamander (*Dicamptodon*

aterrimus; Pilliod *et al*., 2014) and may play a critical role in the detectability of boreal chorus frog. Investigating how each of these factors influence eDNA detection between these amphibians may aid in future protocol design, e.g. if a species occurs in high abundance but is relatively small and known to have a low eDNA shed rate, perhaps collection effort should be increased. However, the methods used in this project to detect boreal chorus frog eDNA were not reliable.

Recent studies that investigate using eDNA to detect of amphibians species in lentic systems report detection rates (i.e. proportion of positive samples to total samples collected) between 0.76 - 1.0 in sites where the target amphibian species were known or expected to occur (Ficetola *et al*., 2008; Dejean *et al*., 2012; Thomsen *et al*., 2012a). In each study, the researcher collected three triangulated perimeter samples per site to increase site coverage (Ficetola *et al*., 2008; Dejean *et al*., 2012; Thomsen *et al*., 2012a). In this study I collected 10 samples, evenly spaced along the perimeter for each site. Wood frog was the only species that I targeted that had comparable detection rates to other studies (Table 3.3). Western toad and boreal chorus frog detection rates were considerably lower for all three collection periods for both sampling methods and detection platforms (Table 3.3). In July, western toad produced false negative detection at Sites 2 and 3 for both detection platforms and sampling method, while boreal chorus frog detections were consistently negligible (Table 3.2; Figure 3.1 and 3.2). The failure to detect a species' eDNA can be caused by insufficient DNA in the environmental sample, eDNA extraction method selection (Goldberg *et al*., 2011; Deiner *et al*., 2014), low number of PCR replicates (Ficetola *et al*., 2008; Dejean *et al*., 2012; Ficetola *et al*., 2015), lack of primer to species specificity (Wilcox *et al*., 2013) and PCR inhibition from humic substances (Stevenson 1994; Albers *et al*., 2013; McKee *et al*., 2014; Jane *et al*., 2015) and / or co-extracted DNA (Alvarez *et al*., 1995).

I was able to detect eDNA for all three species using my water and sediment DNA extraction protocols, indicating that these protocols can collect and recover eDNA; however, without an internal positive control (e.g. known concentration of DNA from an exogenous source), I could not estimate the extraction efficiencies. Not

obtaining a complete extraction from each sample could lead to non-detection if the concentration of a species' eDNA is initially low (Deiner *et al*., 2014). When there is a low probability of detecting a species' eDNA, increasing the number of PCR replicates can reduce the potential for false negatives (Ficetola *et al*., 2008; Ficetola *et al*., 2015). Ficetola *et al*. (2014) showed that when a species' eDNA detection probability is <0.5 is may be necessary to run ≥ 8 PCR replicates per sample. To reduce costs each sample was analyzed as a single reaction for the ITPGM platform and in duplicates for the qPCR platform. In this study, I found that duplicating the detection process with the qPCR platform can significantly increase detection rates for wood frog. I also found that positive detections are not ubiquitous across samples collected at a site where a species is known to occur. This indicates that eDNA detection is spatially variable and may be influenced by the distribution of eDNA within a habitat. Therefore, increasing dispersed sampling effort beyond 10 samples may provide better likelihood of detection.

Although sampling effort and PCR replication may influence detection rates, the variable habits and phenology of target species should be considered. None of the three species in my study are highly aquatic and all forage and hibernate on land. They vary in tadpole and adult body size, inhabit aquatic sites at different abundances, and may have different eDNA shed rates; bufonids, for example, have relatively dry, keratinized skin. All these factors contribute to the presence and persistence of eDNA and they typically vary among amphibians. Therefore, certain species may require a tailored eDNA protocol for detection (e.g. increase sampling effort and / or PCR replications) and some species may not be reliably detected using eDNA, especially if natural PCR inhibitors are present in the environment.

The primary goal of this project was to compare eDNA detection rates of western toad, a species that generally occurs in low abundances, to wood frog and boreal chorus, species that are generally more abundant in Alberta ponds. I found that the methods used in this study would not provide reliable detection rates for the western toad. The low detection rates for boreal chorus frog, an abundant species but with a smaller body size

than wood frog and western toad, was unexpected and suggests that there are many unrecognized variables that may influence detectability of a species using eDNA. I found that eDNA detection rates did not vary between detection platforms unless samples were processed in duplicate using the qPCR platform. I found that collection method did not influence detection rates; however, the timing of collections through the breeding season was influential on detection rates. For all three target species, sampling during the late larval/metamorphosis stage gave the highest detection rates. However, western toad and boreal chorus frog detection rate were low for two of the three sites, suggesting that techniques used in this study for eDNA detection are not dependable enough to be included as a major element in programs designed to survey or monitor these amphibians in Alberta.

Table 3.1. Species-specific primers and probes used for Ion Torrent PGM™ (ITPGM) analysis and quantitative PCR (qPCR) analysis for western toad (WETO), wood frog (WOFR), and boreal chorus frog (BCFR).

Platform	Species	Tm	Forward	Reverse	Probe
ITPGM	WETO	56.3°C	5'-CTCCCTTCTAGGCGATGACC-3'	5'-ACAAGCCAGTTTCCAAAGCC-3'	
	WOFR	55.3°C	5'-GTACAGGTTGAACAGTTTATCCACCC-3'	5'-CCCAAGATAGATGAAACACCAGC-3'	
	BCFR	54.5°C	5'-TCCAAACCCTCCAATCAGG-3'	5'-ATTAAGTCAACCTGGCTCCC-3'	
qPCR	WETO	60°C	5'-ATAAAATTAATCGCCCCCAGAATA-3'	5'-ACGCAGGGCCGTCAGTT-3'	5'-TCTTCTCTC/ZEN/TTCATCTGGCGGGTGTTT C-3'
	WOFR	60°C	5'-GAGTATGGTGATTCCAGCAGCTAA-3'	5'-CACCTCTTTTTGTTTGATCCGTACTA-3'	5'-ACTGCAGTC/ZEN/CTGCTTCTCTTATCACTCCCA-3'
	BCFR	60°C	5'-TTGTTTATTCGTGGGAAGGCTATAT-3'	5'-TGGAGGGTTTGGAAACTGACTT-3'	5'-CCCTCTGAT/ZEN/GATCGGAGCCCCG-3'

Table 3.2. Number of samples positive for eDNA for each species at each site across the three collection dates. On each date water samples (n=10) and pond bottom sediment samples (n=10) were taken and each sample was analyzed once for eDNA using Ion Torrent PGM™ (IT) and in duplicate for quantitative PCR (qPCR). Samples negative for eDNA at eDNA extraction concentration were diluted 1/10 to reduce PCR inhibition and were re-analyzed.

Species	**June**				**July**				**August**			
Site	**Water**		**Sediment**		**Water**		**Sediment**		**Water**		**Sediment**	
	IT	**qPCR**	**IT**	**qPCR**	**IT**	**qPCR**	**IT**	**qPCR**	**IT**	**qPCR**	**IT**	**qPCR**
	(e/d)	**(e/d)**	**(e/d)**	**(e/d)**	**(e/d)**	**(e/d)**	**(e/d)**	**(e/d)**	**(e/d)**	**(e/d)**	**(e/d)**	**(e/d)**
Western toad												
Site 1	0/1	3/0	1/1	1/0	3/2	4/1	3/2	4/3	2/0	2/0	1/0	1/0
Site 2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Site 3	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/0	0/0	0/0
Wood frog												
Site 1	1/1	1/0	3/0	3/1	1/0	1/1	2/2	2/3	1/0	2/0	1/0	0/0
Site 2	1/5	6/0	4/2	6/0	0/8	6/4	7/0	8/0	0/0	0/6	4/0	4/1
Site 3	3/6	7/2	6/4	10/0	4/4	7/3	8/0	10/0	1/1	3/0	7/0	6/0
Boreal chorus frog												
Site 1	0/1	1/0	0/0	0/0	3/2	4/0	1/1	1/2	1/1	0/0	0/0	1/0
Site 2	0/0	0/0	0/0	1/0	0/0	0/0	1/0	2/0	0/0	0/0	0/0	1/0
Site 3	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0

e = Number of eDNA detection at eDNA extraction concentration.

d = Number of additional eDNA detections after 1/10 dilution to reduce PCR inhibition.

Table 3.3. Mean detection probabilities and standard error from the three sites for two sampling methods (water and sediment) for each species during three sampling periods using the Ion Torrent PGM™ (ITPGM), one run per sample, and quantitative PCR (qPCR) detection platforms, duplicate runs per sample.

		Water			Sediment		
Platform	**Species**	**June**	**July**	**August**	**June**	**July**	**August**
ITPGM	western toad	0.067±0.033	0.17±0.17	0.067±0.07	0.067±0.067	0.17±0.17	0.033±0.033
	wood frog	0.57±0.2	0.57±0.23	0.1±0.058	0.63±0.20	0.63±0.12	0.4±0.17
	boreal chorus frog	0.033±0.033	0.17±0.17	0.067±0.067	0	0.1±0.58	0
qPCR	western toad	0.1±0.1	0.17±0.17	0.1±0.58	0.033±0.033	0.23±0.23	0.033±0.033
	wood frog	0.53±0.23	0.7±0.3	0.37±0.12	0.6±0.18	0.77±0.15	0.37±0.19
	boreal chorus frog	0.033±0.033	0.13±0.13	0	0.033±0.033	0.17±0.088	0.067±0.033

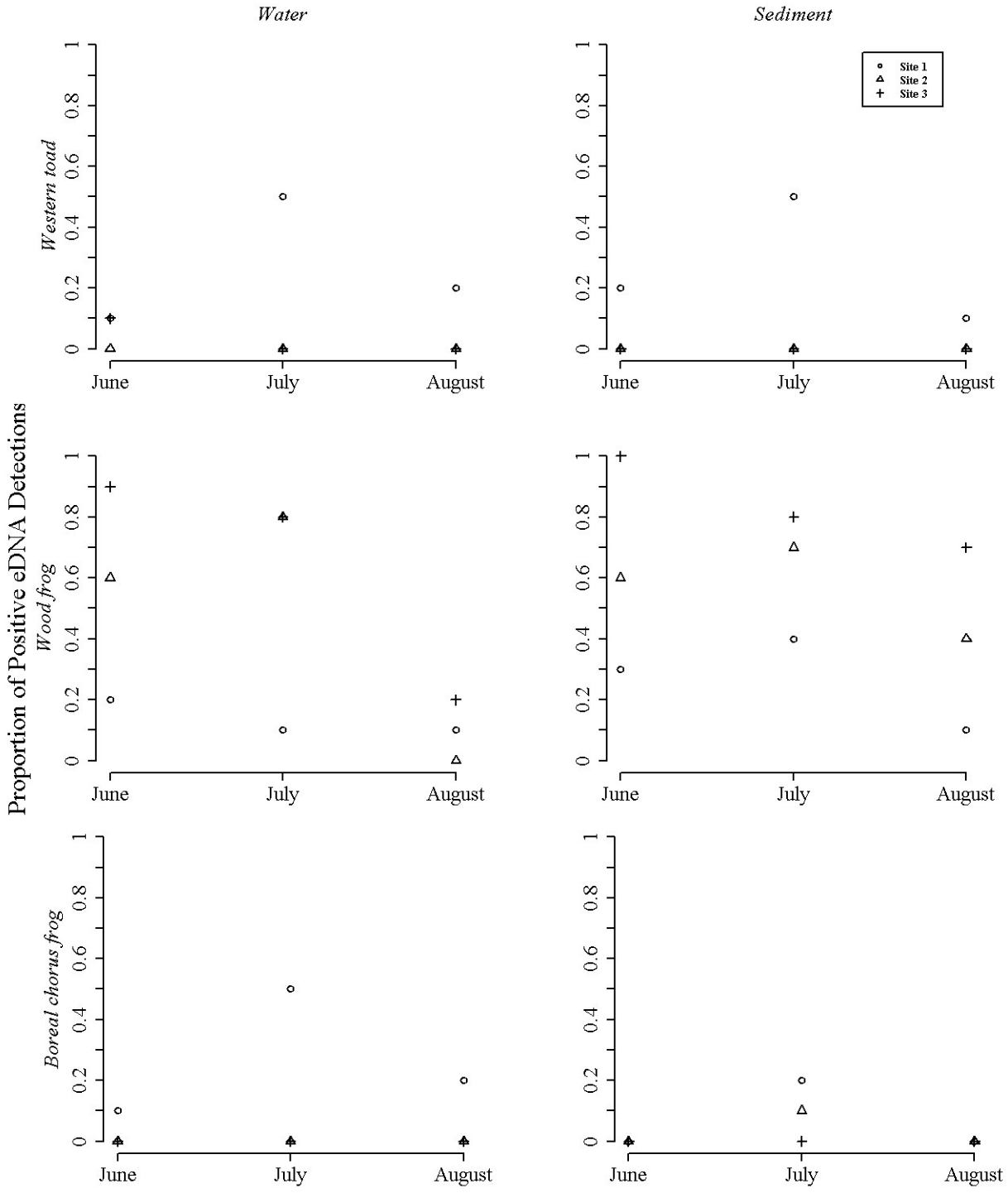

Figure 3.1. The detection rates for each site (n=10) for each sampling period for water samples (*left column*) and sediment samples (*right column*) for western toad (*top row*), wood frog (*middle row*) and boreal chorus frog (*bottom row*) for samples processed with Ion Torrent PGM™.

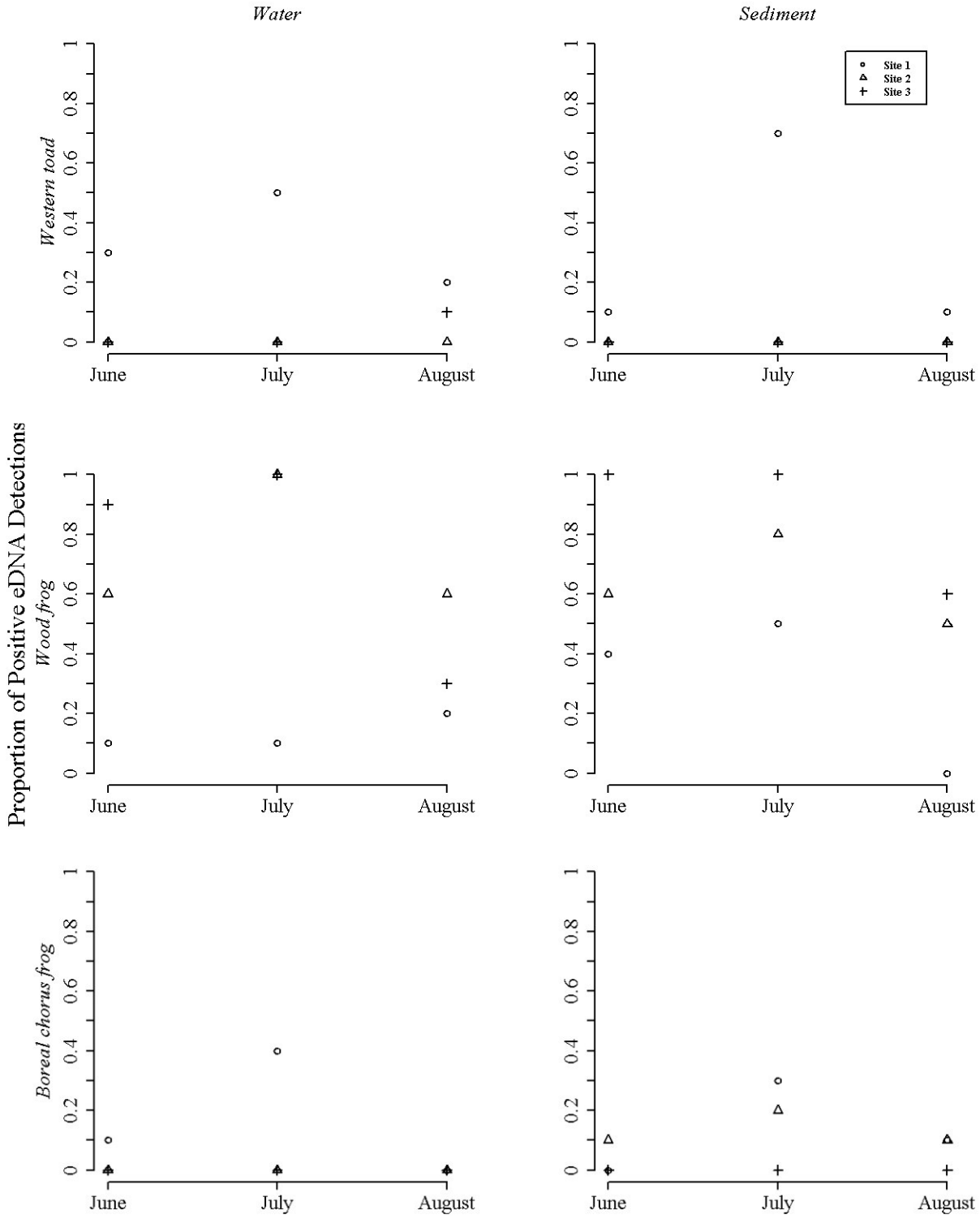

Figure 3.2. The detection rates for each site (n=10) for each sampling period for water samples (*left column*) and sediment samples (*right column*) for western toad (*top row*), wood frog (*middle row*) and boreal chorus frog (*bottom row*) for samples processed with qPCR.

Chapter IV: Conclusion

In a time when habitat loss, climate change, the spread of disease and invasive species are impacting ecosystems around the globe, it is critical to catalogue the distribution of species across large geographic ranges for monitoring and conservation programs. The global rate at which amphibian species have recently declined and their projected continued decline in future decades (Hoffman *et al*., 2010) necessitates that regional distribution datasets are developed as soon as possible for all species. The eDNA detection technique has proven to be as reliable as conventional detection techniques for certain amphibians (Ficetola *et al*., 2008; Dejean *et al*., 2012; Thomsen *et al*., 2012a; Biggs *et al*., 2015) and detection data are accumulated at a much faster rate. Therefore, this technique promises to expedite the development of regional distribution atlases for amphibians. The aim of this study was to develop an eDNA protocol that would facilitate the detection of the 10 amphibian species that reside in Alberta, Canada. This protocol could then be used to document their distributions across the province in a standardized fashion in conjunction with major monitoring programs, e.g, Alberta Biodiversity Monitoring Institute. The eDNA detection technique is still in its infancy and development of standardized protocols will facilitate more accurate comparisons of results across projects and species. Comparing protocols to determine which are the most efficient and effective is critical for determining which methods should be set as standards. In this study, I found that the methods used to sample eDNA from aquatic habitats and to process them in the laboratory have significant impacts on detection rates. Furthermore, I showed that detection rates are highly variable across amphibians that co-occur at the same location and these rates change through the phenology of the reproductive season. Lastly, I found that diluting eDNA samples to reduce potential PCR inhibition influences detection rates, both positively and negatively.

When targeting the 10 amphibian species of Alberta, I found that a species-specific primer set outperformed a universal primer set that consisted of a single primer pair that targeted all 10 species, and a grouped primer set, comprised of four primer pairs that targeted species that were grouped based on genetic

similarity in the target DNA region. Because the universal and grouped primer sets target multiple species, they are subject to detection biases created by variable eDNA abundances and / or differing primer affinity across species (Pompanon *et al*., 2012; Deagle *et al*., 2013; Kelly *et al*., 2014). The species-specific primer set was the only set that produced detection rates comparable to rates reported by other amphibian eDNA studies (Ficetola *et al*., 2008; Dejean *et al*., 2012; Thomsen *et al*., 2012a), albeit the comparable rate was only for one of the four species examined, wood frog. Although western toad, boreal chorus frog and western tiger salamander were encountered during field surveys they were not detected in my initial analysis of survey data using the species-specific primer set. To a lesser degree the species-specific primers may incur the same biases caused by DNA abundance and / or primer affinity, particularly during the second PCR phase in the two-step PCR protocol when PCR 1 products are multiplexed and MID labeled (Deagle *et al*., 2013). Without further investigation into biological factors, such as eDNA shed rates and relative abundances of these species, it is difficult to determine what caused a lack of detections.

I found that the two-step PCR protocol outperformed the one-step PCR protocol when preparing the eDNA samples for HTS sequencing. The one-step PCR protocol was tested because I believed it would reduce sample cross-contamination and false positive detections; however, this technique produced false positive detections at a rate that was too high to allow data to be considered reliable. The two-step PCR approach is widely used among other HTS studies and my findings suggest it should not be replaced by the one-step PCR protocol. Therefore, the species-specific primer set in combination with the two-step PCR protocol was the most effective and reliable detection approach. This protocol combination was used to compare analytical platforms in Chapter III.

In Chapter III, I compared detection rates between water and sediment eDNA samples for a HTS and a qPCR detection platform. I targeted three species that co-occur in central Alberta: western toad, wood frog and boreal chorus frog. Western toad and boreal chorus frog had very low detection rates for both sampling methods

and detection platforms. This may be attributed to low eDNA shed rates (Thomsen *et al*., 2012a), eDNA extraction method selection (Goldberg *et al*., 2012; Deiner *et al*., 2014), low number of PCR replicates (Ficetola *et al*., 2008; Dejean *et al*., 2012; Ficetola *et al*., 2015), lack of primer specificity to the target species (Wilcox *et al*., 2013) and / or PCR inhibition from co-extracted DNA and water chemistry (Stevenson 1994; Alvarez *et al*., 1995; Albers *et* al., 2013). Due to these low detection rates, it was difficult to compare methods using these species; significantly different detection rates were documented mostly for wood frog. Similar to Murray *et al*. (2011) for fecal analysis, I found that sample replication with the qPCR platform outperformed single reactions using HTS platform for eDNA, producing significantly higher detection rates for wood frog. Even when processing samples in duplicate, the qPCR method is more cost effective and less labour intensive than the HTS platform. Therefore, qPCR with replicated samples is recommended for studies designed to detect amphibian eDNA.

Diluting samples 10-fold to reduce potential PCR inhibition affected each detection platform differently. Of the total positive species detection, 44.5% were from samples that were negative prior to dilution for the HTS platform and 21.1% for the qPCR platform. This suggests that the HTS platform was more sensitive to PCR inhibition. For the samples that were positive prior to dilution there was a 40% loss of detection for the HTS platform and a 41.3% loss in detection for the qPCR platform. Even though the 10-fold dilution increased detection rates for some samples, there were likely samples that had low starting eDNA concentrations and suffered from PCR inhibition. Following the 10-fold dilution, the eDNA would have reached undetectable levels resulting in false negative results even though the PCR inhibitors were diluted. McKee *et al*. (2014) had similar results in samples collected from lentic systems and they recommend the use of PCR inhibitor removal kits over a 10-fold dilution to attenuate PCR inhibition. However, these kit will increase the overall project cost and when funding is limited the 10-fold dilution approach may be acceptable if detection biases are acknowledged.

Turner *et al*. (2015) found that eDNA concentration for Asian carp (*Hypophthalmichthys spp*.) were higher in sediment samples than in water column samples and speculated that this would increase detection rates for sediment samples. I found that detection rates for sediment samples were significantly greater than water samples for the HTS platform; however, they were not significantly different for the qPCR platform. It is important to note the each sampling method examines a different temporal detection window as eDNA can persist in sediment for months to years (Matisoo-Smith *et al*., 2008; Giguet-Covex *et al*., 2014, Turner *et al*., 2015) and typically persists in the water column for ≤ 25 days (Dejean *et al*., 2011; Thomsen *et al*. 2012a,b; Barnes *et al*., 2014; Pilliod *et al*. 2014). Therefore, the sampling method used should depend on a project's goal. If the goal is to detect a species' historical presence at a site or to detect contemporary presence, when historic status is not a concern, then sediment sampling would suffice. If the goal is to detect a species that was present at a site within the last 25 days, and prior historical occurrences are a concern, then water sampling should be used.

Lastly, I found the disparity in the temporal detection windows between sampling methods was illustrated when samples collected in June, July and August were processed with the HTS platform. Wood frog detection rates in water samples were comparable in June and July and were significantly reduced for August, following larval metamorphosis. For the sediment samples, the detections rates were not significantly different among months which may indicate a persistence of eDNA in the sediment samples after larvae underwent metamorphosis. However, the same trends were not observed with the qPCR platform when detection rates significantly decreased in August for both sediment and water samples.

When setting the foundation of a large scale project, such as mapping geographic ranges of amphibian species across a province, intrinsic methodological challenges will occur and need to be overcome. Although I was not able to develop a reliable protocol for detecting the 10 amphibian species that occur in Alberta, I was able to further elucidate some of the methodological challenges that are inherent in using eDNA for conducting

surveys, such as variability across species and sites. I was able to compare methods and to identify advantages associated with some approaches. Detection of species with eDNA is an emerging technique that requires rigorous testing to build understanding of its applicability and its limitations. I believe this project facilitates the growth of this body of knowledge and will aid future researchers in protocol selection and development.

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Appendix 1

Table A-1: The read counts for each sample, primer and PCR protocol combination for Chapter II. Sampling locations are given in UTM's.

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_1	Uni_p	2-step	303525	5436411	11U										
WLNP_2	Uni_p	2-step	303525	5436477	11U					2404					
WLNP_3	Uni_p	2-step	303542	5436475	11U	75						57			
WLNP_4	Uni_p	2-step	303525	5436477	11U										
Lab (-ve)	Uni_p	2-step													
WLNP_5	Uni_p	2-step	293544	5443978	11U										
WLNP_6	Uni_p	2-step	293544	5443978	11U				318						
WLNP_7	Uni_p	2-step	293544	5443978	11U										427
WLNP_8	Uni_p	2-step	293544	5443978	11U		233								312
WLNP_9	Uni_p	2-step	288914	5439090	11U										
WLNP_10	Uni_p	2-step	288914	5439090	11U										
WLNP_11	Uni_p	2-step	288914	5439090	11U										658
WLNP_12	Uni_p	2-step	288914	5439090	11U	532						573			544
WLNP_13	Uni_p	2-step	291545	5440529	11U										
WLNP_14	Uni_p	2-step	291545	5440529	11U										
WLNP_15	Uni_p	2-step	291545	5440529	11U										
WLNP_16	Uni_p	2-step	291545	5440529	11U										
Lab (-ve)	Uni_p	2-step				832	97		765			801		593	
WLNP_17	Uni_p	2-step	289006	541185	11U										
WLNP_18	Uni_p	2-step	289006	541185	11U										
WLNP_19	Uni_p	2-step	289006	541185	11U		124		1600						
WLNP_20	Uni_p	2-step	289006	541185	11U										
WLNP_21	Uni_p	2-step	292497	5445475	11U										
WLNP_22	Uni_p	2-step	292497	5445475	11U										
WLNP_23	Uni_p	2-step	292497	5445475	11U										138
WLNP_24	Uni_p	2-step	292497	5445475	11U										
WLNP_25	Uni_p	2-step	295763	5439416	11U					102					
WLNP_26	Uni_p	2-step	295763	5439416	11U				491						
WLNP_27	Uni_p	2-step	295763	5439416	11U										
WLNP_28	Uni_p	2-step	295763	5439416	11U	144						161			
WLNP_29	Uni_p	2-step	297116	5438234	11U										
WLNP_30	Uni_p	2-step	297116	5438234	11U					300					
WLNP_31	Uni_p	2-step	297116	5438234	11U									887	
WLNP_32	Uni_p	2-step	297116	5438234	11U										829
WLNP_33	Uni_p	2-step	299469	5438654	11U				96	3121					
WLNP_34	Uni_p	2-step	299469	5438654	11U					1288					
WLNP_35	Uni_p	2-step	299469	5438654	11U					2491					

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_36	Uni_p	2-step	299469	5438654	11U					4461					
WLNP_37	Uni_p	2-step	299743	5438674	11U										
WLNP_38	Uni_p	2-step	299743	5438674	11U		138		2033						
WLNP_39	Uni_p	2-step	299743	5438674	11U										
WLNP_40	Uni_p	2-step	299743	5438674	11U										
Lab (-ve)	Uni_p	2-step					958							1343	
WLNP_41	Uni_p	2-step	304000	5435067	11U		69								
WLNP_42	Uni_p	2-step	304000	5435067	11U										
WLNP_43	Uni_p	2-step	304000	5435067	11U					367					2374
WLNP_44	Uni_p	2-step	304000	5435067	11U										
WLNP_45	Uni_p	2-step	289277	5441865	11U										60
WLNP_46	Uni_p	2-step	289277	5441865	11U					3562					
WLNP_47	Uni_p	2-step	289277	5441865	11U										
WLNP_48	Uni_p	2-step	289277	5441865	11U										
Lab (-ve)	Uni_p	2-step													
PCR (-VE)	Uni_p	2-step													
Ext(5)_1	Uni_p	2-step				117	284		167			118		516	
Ext(5)_2	Uni_p	2-step				93	398		228			97		662	
Ext(5)_3	Uni_p	2-step				178	390		300			166		640	
Lab (-ve)	Uni_p	2-step					65	486	362	664			209		
Ext(10)_1	Uni_p	2-step				149	149	237	446		101	143	571	360	182
Ext(10)_2	Uni_p	2-step				159	125	219	232		85	159	622	346	164
Ext(10)_3	Uni_p	2-step				165	128	215	285		78	167	883	367	211
Lab (-ve)	Uni_p	2-step					1388						1619		
PCR (-ve)	Uni_p	2-step											664		
CATO(+ve)	Uni_p	2-step													
WETO(+ve)	Uni_p	2-step					367								
GPTO(+ve)	Uni_p	2-step						549	56						
WOFR(+ve)	Uni_p	2-step							580						
CSFR(+ve)	Uni_p	2-step								564					
NLFR(+ve)	Uni_p	2-step									296				
BCFR(+ve)	Uni_p	2-step				151						182			
PLSF(+ve)	Uni_p	2-step											470		
TISA(+ve)	Uni_p	2-step												459	
LTSA(+ve)	Uni_p	2-step													1450
10 spp(+ve)	Uni_p	2-step							106	108					
Lab (-ve)	Uni_p	2-step													
PCR (-ve)	Uni_p	2-step													
WLNP_5	Uni_p	1-step	293544	5443978	11U										
WLNP_6	Uni_p	1-step	293544	5443978	11U										
WLNP_7	Uni_p	1-step	293544	5443978	11U										
WLNP_8	Uni_p	1-step	293544	5443978	11U										

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_9	Uni_p	1-step	288914	5439090	11U										
WLNP_10	Uni_p	1-step	288914	5439090	11U										
WLNP_11	Uni_p	1-step	288914	5439090	11U										
WLNP_12	Uni_p	1-step	288914	5439090	11U	145						126			1229
WLNP_13	Uni_p	1-step	291545	5440529	11U										
WLNP_14	Uni_p	1-step	291545	5440529	11U										
WLNP_15	Uni_p	1-step	291545	5440529	11U										
WLNP_16	Uni_p	1-step	291545	5440529	11U					166					
Lab (-ve)	Uni_p	1-step													
WLNP_17	Uni_p	1-step	289006	541185	11U										
WLNP_18	Uni_p	1-step	289006	541185	11U										
WLNP_19	Uni_p	1-step	289006	541185	11U										
WLNP_20	Uni_p	1-step	289006	541185	11U										
WLNP_21	Uni_p	1-step	292497	5445475	11U										
WLNP_22	Uni_p	1-step	292497	5445475	11U										
WLNP_23	Uni_p	1-step	292497	5445475	11U		373		466						
WLNP_24	Uni_p	1-step	292497	5445475	11U								334	78	
WLNP_25	Uni_p	1-step	295763	5439416	11U		357								
WLNP_26	Uni_p	1-step	295763	5439416	11U		115								
WLNP_27	Uni_p	1-step	295763	5439416	11U						1066				
WLNP_28	Uni_p	1-step	295763	5439416	11U										
WLNP_29	Uni_p	1-step	297116	5438234	11U					1370					
WLNP_30	Uni_p	1-step	297116	5438234	11U										
WLNP_31	Uni_p	1-step	297116	5438234	11U										
WLNP_32	Uni_p	1-step	297116	5438234	11U				529						571
WLNP_33	Uni_p	1-step	299469	5438654	11U				609	803					
WLNP_34	Uni_p	1-step	299469	5438654	11U					1524					326
WLNP_35	Uni_p	1-step	299469	5438654	11U					2355					
WLNP_36	Uni_p	1-step	299469	5438654	11U	118				152		111			
WLNP_37	Uni_p	1-step	299743	5438674	11U										
WLNP_38	Uni_p	1-step	299743	5438674	11U										
WLNP_39	Uni_p	1-step	299743	5438674	11U								1592		
WLNP_40	Uni_p	1-step	299743	5438674	11U										
Lab (-ve)	Uni_p	1-step													
WLNP_41	Uni_p	1-step	304000	5435067	11U		252								
WLNP_42	Uni_p	1-step	304000	5435067	11U										1851
WLNP_43	Uni_p	1-step	304000	5435067	11U										
WLNP_44	Uni_p	1-step	304000	5435067	11U										
WLNP_45	Uni_p	1-step	289277	5441865	11U		1210								
WLNP_46	Uni_p	1-step	289277	5441865	11U										

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_47	Uni_p	1-step	289277	5441865	11U										
WLNP_48	Uni_p	1-step	289277	5441865	11U										
WLNP_49	Uni_p	1-step	288863	5442151	11U										
WLNP_50	Uni_p	1-step	288863	5442151	11U										
WLNP_51	Uni_p	1-step	288863	5442151	11U										
WLNP_52	Uni_p	1-step	288863	5442151	11U		1690								
WLNP_53	Uni_p	1-step	291891	5445240	11U					893					
WLNP_54	Uni_p	1-step	291891	5445240	11U			242	153					118	
WLNP_55	Uni_p	1-step	291891	5445240	11U				169						
WLNP_56	Uni_p	1-step	291891	5445240	11U					3001					
WLNP_57	Uni_p	1-step	282474	5443659	11U		131								
WLNP_58	Uni_p	1-step	282474	5443659	11U										
WLNP_59	Uni_p	1-step	282474	5443659	11U										
WLNP_60	Uni_p	1-step	282474	5443659	11U										358
Lab (-ve)	Uni_p	1-step					684								
PCR (-ve)	Uni_p	1-step													
Ext(5)_1	Uni_p	1-step				58	184		112			67		226	
Ext(5)_2	Uni_p	1-step				72	327		139			74		294	
Ext(5)_3	Uni_p	1-step				134	288		157			141		275	
Lab (-ve)	Uni_p	1-step									564				
Ext(10)_1	Uni_p	1-step				162	80	164	226			163	416	226	154
Ext(10)_2	Uni_p	1-step				129	86	187	127		70	129	346	159	145
Ext(10)_3	Uni_p	1-step				76	61	140	138		54	98	465	128	125
Lab (-ve)	Uni_p	1-step													
CATO(+ve)	Uni_p	1-step													
WETO(+ve)	Uni_p	1-step					110								
GPTO(+ve)	Uni_p	1-step						166							
WOFR(+ve)	Uni_p	1-step							125						
CSFR(+ve)	Uni_p	1-step				58				179					
NLFR(+ve)	Uni_p	1-step									73				
BCFR(+ve)	Uni_p	1-step													
PLSF(+ve)	Uni_p	1-step											120		
TISA(+ve)	Uni_p	1-step												62	
LTSA(+ve)	Uni_p	1-step													226
10 spp(+ve)	Uni_p	1-step													
Lab (-ve)	Uni_p	1-step													
PCR (-ve)	Uni_p	1-step													
NCAB_1	Uni_p	1-step	327325	5925304	12U										
NCAB_2	Uni_p	1-step	327343	5925332	12U										
NCAB_3	Uni_p	1-step	327378	5925304	12U				423						

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NCAB_4	Uni_p	1-step	378254	5939594	12U		579		2554						
NCAB_5	Uni_p	1-step	378259	5939582	12U										
NCAB_6	Uni_p	1-step	378262	5939585	12U		214		88						
NCAB_7	Uni_p	1-step	378017	5940669	12U										
NCAB_8	Uni_p	1-step	378025	5940671	12U										
NCAB_9	Uni_p	1-step	378039	5940668	12U										
NCAB_10	Uni_p	1-step	377187	5945134	12U										
NCAB_11	Uni_p	1-step	377190	5945142	12U										
NCAB_12	Uni_p	1-step	377196	5945124	12U										
NCAB_13	Uni_p	1-step	373192	5900423	12U										
NCAB_14	Uni_p	1-step	373197	5900431	12U				2528						
NCAB_15	Uni_p	1-step	373198	5900438	12U				859						
NCAB_16	Uni_p	1-step	374294	5901396	12U			150					810		
NCAB_17	Uni_p	1-step	374295	5901385	12U										
NCAB_18	Uni_p	1-step	374296	5901376	12U			695					60		109
NCAB_19	Uni_p	1-step	324277	5943860	12U										
NCAB_20	Uni_p	1-step	324280	5943840	12U				1114						
NCAB_21	Uni_p	1-step	324302	5943865	12U										
NCAB_22	Uni_p	1-step	694127	5944779	11U								441		
NCAB_23	Uni_p	1-step	694156	5944760	11U										
NCAB_24	Uni_p	1-step	694179	5944733	11U		181		907	665					1959
NCAB_25	Uni_p	1-step	694209	5945747	11U				2396						
NCAB_26	Uni_p	1-step	694209	5945759	11U										
NCAB_27	Uni_p	1-step	694232	5945748	11U										
NCAB_28	Uni_p	1-step	693908	5945977	11U				253						
NCAB_29	Uni_p	1-step	693914	5945970	11U										
NCAB_30	Uni_p	1-step	693921	5945986	11U				1746						
NCAB_31	Uni_p	1-step	314509	5922008	12U										
NCAB_32	Uni_p	1-step	314540	5921918	12U					62					
NCAB_33	Uni_p	1-step	314577	5921932	12U										
NCAB_34	Uni_p	1-step	326471	5927024	12U										
NCAB_35	Uni_p	1-step	326465	5927045	12U										
NCAB_36	Uni_p	1-step	326494	5927033	12U										
NCAB_37	Uni_p	1-step	326500	5926987	12U										
NCAB_38	Uni_p	1-step	326505	5926974	12U										
NCAB_39	Uni_p	1-step	326522	5926975	12U										
Lab (-ve)	Uni_p	1-step					228		4164						985
NCAB_40	Uni_p	1-step	354216	5994841	12U										
NCAB_41	Uni_p	1-step	354240	5994809	12U										
NCAB_42	Uni_p	1-step	354236	5994787	12U	597	360	619	361	360	321	173	930	174	664

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NCAB_43	Uni_p	1-step	354282	5994755	12U				4831						
NCAB_44	Uni_p	1-step	354290	5994758	12U										
NCAB_45	Uni_p	1-step	354297	5994760	12U										
NCAB_46	Uni_p	1-step	352392	5996476	12U										
NCAB_47	Uni_p	1-step	352383	5996463	12U										
NCAB_48	Uni_p	1-step	354209	5996456	12U				326		438				
NCAB_49	Uni_p	1-step	352483	5996464	12U				3383			88			
NCAB_50	Uni_p	1-step	352485	5996461	12U										
NCAB_51	Uni_p	1-step	352484	5996465	12U				2588			125			
NCAB_52	Uni_p	1-step	343213	5993520	12U										
NCAB_53	Uni_p	1-step	343211	5993535	12U										
NCAB_54	Uni_p	1-step	343211	5993537	12U										
NCAB_55	Uni_p	1-step	347616	5993635	12U				2087						
NCAB_56	Uni_p	1-step	347613	5993596	12U				1959						
NCAB_57	Uni_p	1-step	347673	5993583	12U				4659						
NCAB_58	Uni_p	1-step	347716	5993597	12U										
NCAB_59	Uni_p	1-step	347696	5993602	12U				1026						
NCAB_60	Uni_p	1-step	347677	5993612	12U										
NCAB_61	Uni_p	1-step	347697	5993508	12U				2143			166			
NCAB_62	Uni_p	1-step	347689	5993500	12U										
NCAB_63	Uni_p	1-step	347700	5993500	12U										
NCAB_64	Uni_p	1-step	351141	5993194	12U				2942						
NCAB_65	Uni_p	1-step	351144	5993205	12U				54						
NCAB_66	Uni_p	1-step	351143	5993217	12U				3485						
NCAB_67	Uni_p	1-step	352133	5993089	12U										
NCAB_68	Uni_p	1-step	352120	5993069	12U										
NCAB_69	Uni_p	1-step	352141	5993043	12U										
NCAB_70	Uni_p	1-step	352169	5989802	12U			88							
NCAB_71	Uni_p	1-step	352153	5989797	12U										
NCAB_72	Uni_p	1-step	352155	5989778	12U										
NCAB_73	Uni_p	1-step	352188	5989671	12U										
NCAB_74	Uni_p	1-step	352193	5989671	12U										
NCAB_75	Uni_p	1-step	352183	5989674	12U										
Lab (-ve)	Uni_p	1-step				214	267	127	573	715	174	64	288		1468
PCR(-ve)	Uni_p	1-step													
CATO(+ve)	Uni_p	1-step				6282									
WETO (+ve)	Uni_p	1-step					4731								
GPTO (+ve)	Uni_p	1-step						6573							
WOFR (+ve)	Uni_p	1-step							6936						
CSFR (+ve)	Uni_p	1-step								7079					

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NLFR (+ve)	Uni_p	1-step									4047				
BCFR (+ve)	Uni_p	1-step										5997			
PLSF (+ve)	Uni_p	1-step											3587		
TISA (+ve)	Uni_p	1-step												4082	
LTSA (+ve)	Uni_p	1-step													9179
10 spp (+ve)	Uni_p	1-step				425	1141	715	1340	447	1630	956	359	493	215
Lab (-ve)	Uni_p	1-step													
PCR (-ve)	Uni_p	1-step													
WLNP_1	Grp_p	2-step	303525	5436411	11U										
WLNP_2	Grp_p	2-step	303525	5436477	11U								714		
WLNP_3	Grp_p	2-step	303542	5436475	11U										
WLNP_4	Grp_p	2-step	303525	5436477	11U										
Lab (-ve)	Grp_p	2-step													
WLNP_5	Grp_p	2-step	293544	5443978	11U				277						
WLNP_6	Grp_p	2-step	293544	5443978	11U										
WLNP_7	Grp_p	2-step	293544	5443978	11U										
WLNP_8	Grp_p	2-step	293544	5443978	11U										
WLNP_9	Grp_p	2-step	288914	5439090	11U										
WLNP_10	Grp_p	2-step	288914	5439090	11U										
WLNP_11	Grp_p	2-step	288914	5439090	11U										
WLNP_12	Grp_p	2-step	288914	5439090	11U										
WLNP_13	Grp_p	2-step	291545	5440529	11U										
WLNP_14	Grp_p	2-step	291545	5440529	11U				501						
WLNP_15	Grp_p	2-step	291545	5440529	11U										
WLNP_16	Grp_p	2-step	291545	5440529	11U										
Lab (-ve)	Grp_p	2-step													
WLNP_17	Grp_p	2-step	289006	541185	11U										
WLNP_18	Grp_p	2-step	289006	541185	11U				338						
WLNP_19	Grp_p	2-step	289006	541185	11U										
WLNP_20	Grp_p	2-step	289006	541185	11U										
WLNP_21	Grp_p	2-step	292497	5445475	11U										
WLNP_22	Grp_p	2-step	292497	5445475	11U										
WLNP_23	Grp_p	2-step	292497	5445475	11U										
WLNP_24	Grp_p	2-step	292497	5445475	11U										
WLNP_25	Grp_p	2-step	295763	5439416	11U										
WLNP_26	Grp_p	2-step	295763	5439416	11U				202						
WLNP_27	Grp_p	2-step	295763	5439416	11U										
WLNP_28	Grp_p	2-step	295763	5439416	11U				62						
WLNP_29	Grp_p	2-step	297116	5438234	11U										
WLNP_30	Grp_p	2-step	297116	5438234	11U										

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_31	Grp_p	2-step	297116	5438234	11U										
WLNP_32	Grp_p	2-step	297116	5438234	11U										
WLNP_33	Grp_p	2-step	299469	5438654	11U										
WLNP_34	Grp_p	2-step	299469	5438654	11U										
WLNP_35	Grp_p	2-step	299469	5438654	11U										
WLNP_36	Grp_p	2-step	299469	5438654	11U				59						
WLNP_37	Grp_p	2-step	299743	5438674	11U										
WLNP_38	Grp_p	2-step	299743	5438674	11U								60		
WLNP_39	Grp_p	2-step	299743	5438674	11U										
WLNP_40	Grp_p	2-step	299743	5438674	11U				63						
Lab (-ve)	Grp_p	2-step													
WLNP_41	Grp_p	2-step	304000	5435067	11U										
WLNP_42	Grp_p	2-step	304000	5435067	11U										
WLNP_43	Grp_p	2-step	304000	5435067	11U				335	69					
WLNP_44	Grp_p	2-step	304000	5435067	11U								56		
WLNP_45	Grp_p	2-step	289277	5441865	11U										
WLNP_46	Grp_p	2-step	289277	5441865	11U										
WLNP_47	Grp_p	2-step	289277	5441865	11U										
WLNP_48	Grp_p	2-step	289277	5441865	11U						85				
Lab (-ve)	Grp_p	2-step							309						
PCR (-VE)	Grp_p	2-step													
Ext(5)_1	Grp_p	2-step													
Ext(5)_2	Grp_p	2-step							81						
Ext(5)_3	Grp_p	2-step							58						
Lab (-ve)	Grp_p	2-step													
Ext(10)_1	Grp_p	2-step											58		
Ext(10)_2	Grp_p	2-step							65				288		
Ext(10)_3	Grp_p	2-step							84				378		
Lab (-ve)	Grp_p	2-step													
PCR (-ve)	Grp_p	2-step											76		
CATO (+ve)	Grp_p	2-step				68									
WETO (+ve)	Grp_p	2-step													
GPTO (+ve)	Grp_p	2-step													
WOFR (+ve)	Grp_p	2-step													
CSFR (+ve)	Grp_p	2-step													
NLFR (+ve)	Grp_p	2-step													
BCFR (+ve)	Grp_p	2-step													
PLSF (+ve)	Grp_p	2-step													
TISA (+ve)	Grp_p	2-step													
LTSA (+ve)	Grp_p	2-step													

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
10 spp(+ve)	Grp_p	2-step													
Lab (-ve)	Grp_p	2-step													
PCR (-VE)	Grp_p	2-step													
WLNP_5	Grp_p	1-step	293544	5443978	11U		465								
WLNP_6	Grp_p	1-step	293544	5443978	11U										
WLNP_7	Grp_p	1-step	293544	5443978	11U										
WLNP_8	Grp_p	1-step	293544	5443978	11U										
WLNP_9	Grp_p	1-step	288914	5439090	11U										
WLNP_10	Grp_p	1-step	288914	5439090	11U										
WLNP_11	Grp_p	1-step	288914	5439090	11U										
WLNP_12	Grp_p	1-step	288914	5439090	11U				227						
WLNP_13	Grp_p	1-step	291545	5440529	11U										
WLNP_14	Grp_p	1-step	291545	5440529	11U						104				
WLNP_15	Grp_p	1-step	291545	5440529	11U										
WLNP_16	Grp_p	1-step	291545	5440529	11U	81			147						
Lab (-ve)	Grp_p	1-step													
WLNP_17	Grp_p	1-step	289006	541185	11U										
WLNP_18	Grp_p	1-step	289006	541185	11U				391						
WLNP_19	Grp_p	1-step	289006	541185	11U				172						
WLNP_20	Grp_p	1-step	289006	541185	11U										
WLNP_21	Grp_p	1-step	292497	5445475	11U										
WLNP_22	Grp_p	1-step	292497	5445475	11U										
WLNP_23	Grp_p	1-step	292497	5445475	11U										
WLNP_24	Grp_p	1-step	292497	5445475	11U										
WLNP_25	Grp_p	1-step	295763	5439416	11U				80						
WLNP_26	Grp_p	1-step	295763	5439416	11U										
WLNP_27	Grp_p	1-step	295763	5439416	11U						145				
WLNP_28	Grp_p	1-step	295763	5439416	11U								2873		
WLNP_29	Grp_p	1-step	297116	5438234	11U										
WLNP_30	Grp_p	1-step	297116	5438234	11U				233						
WLNP_31	Grp_p	1-step	297116	5438234	11U	196									
WLNP_32	Grp_p	1-step	297116	5438234	11U										
WLNP_33	Grp_p	1-step	299469	5438654	11U	80									
WLNP_34	Grp_p	1-step	299469	5438654	11U										
WLNP_35	Grp_p	1-step	299469	5438654	11U										
WLNP_36	Grp_p	1-step	299469	5438654	11U	137									
WLNP_37	Grp_p	1-step	299743	5438674	11U										
WLNP_38	Grp_p	1-step	299743	5438674	11U										
WLNP_39	Grp_p	1-step	299743	5438674	11U										
WLNP_40	Grp_p	1-step	299743	5438674	11U								265		

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
Lab (-ve)	Grp_p	1-step													
WLNP_41	Grp_p	1-step	304000	5435067	11U										
WLNP_42	Grp_p	1-step	304000	5435067	11U				341						
WLNP_43	Grp_p	1-step	304000	5435067	11U										
WLNP_44	Grp_p	1-step	304000	5435067	11U								385		
WLNP_45	Grp_p	1-step	289277	5441865	11U	338									93
WLNP_46	Grp_p	1-step	289277	5441865	11U	872									
WLNP_47	Grp_p	1-step	289277	5441865	11U										
WLNP_48	Grp_p	1-step	289277	5441865	11U						106				
WLNP_49	Grp_p	1-step	288863	5442151	11U										
WLNP_50	Grp_p	1-step	288863	5442151	11U										
WLNP_51	Grp_p	1-step	288863	5442151	11U		98		2323		429				
WLNP_52	Grp_p	1-step	288863	5442151	11U										
WLNP_53	Grp_p	1-step	291891	5445240	11U				249	314			332		
WLNP_54	Grp_p	1-step	291891	5445240	11U										
WLNP_55	Grp_p	1-step	291891	5445240	11U										
WLNP_56	Grp_p	1-step	291891	5445240	11U				292						
WLNP_57	Grp_p	1-step	282474	5443659	11U										
WLNP_58	Grp_p	1-step	282474	5443659	11U			113	239	55			82		
WLNP_59	Grp_p	1-step	282474	5443659	11U						99				
WLNP_60	Grp_p	1-step	282474	5443659	11U										
Lab (-ve)	Grp_p	1-step													
PCR (-ve)	Grp_p	1-step													
Ext(5)_1	Grp_p	1-step													
Ext(5)_2	Grp_p	1-step				1017	941		1547					63	
Ext(5)_3	Grp_p	1-step				687	664		1045						
Lab (-ve)	Grp_p	1-step													
Ext(10)_1	Grp_p	1-step				53		61	571		113		1268		
Ext(10)_2	Grp_p	1-step				66	53	88	706		346		2410		
Ext(10)_3	Grp_p	1-step				55	51	126	809		327		4334		
Lab (-ve)	Grp_p	1-step													
CATO(+ve)	Grp_p	1-step													
WETO(+ve)	Grp_p	1-step					80								
GPTO(+ve)	Grp_p	1-step						87							
WOFR(+ve)	Grp_p	1-step							151						
CSFR(+ve)	Grp_p	1-step								127					
NLFR(+ve)	Grp_p	1-step									70				
BCFR(+ve)	Grp_p	1-step													
PLSF(+ve)	Grp_p	1-step											81		
TISA(+ve)	Grp_p	1-step													

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
LTSA(+ve)	Grp_p	1-step													
10 spp(+ve)	Grp_p	1-step													
Lab (-ve)	Grp_p	1-step													
PCR (-ve)	Grp_p	1-step													
NCAB_1	Grp_p	1-step	327325	5925304	12U								88		
NCAB_2	Grp_p	1-step	327343	5925332	12U				179						
NCAB_3	Grp_p	1-step	327378	5925304	12U				81						
NCAB_4	Grp_p	1-step	378254	5939594	12U										
NCAB_5	Grp_p	1-step	378259	5939582	12U										
NCAB_6	Grp_p	1-step	378262	5939585	12U										
NCAB_7	Grp_p	1-step	378017	5940669	12U										
NCAB_8	Grp_p	1-step	378025	5940671	12U	192			1093						
NCAB_9	Grp_p	1-step	378039	5940668	12U				384						
NCAB_10	Grp_p	1-step	377187	5945134	12U										
NCAB_11	Grp_p	1-step	377190	5945142	12U										
NCAB_12	Grp_p	1-step	377196	5945124	12U										
NCAB_13	Grp_p	1-step	373192	5900423	12U										
NCAB_14	Grp_p	1-step	373197	5900431	12U										
NCAB_15	Grp_p	1-step	373198	5900438	12U										
NCAB_16	Grp_p	1-step	374294	5901396	12U				523	302					
NCAB_17	Grp_p	1-step	374295	5901385	12U				784						
NCAB_18	Grp_p	1-step	374296	5901376	12U										
NCAB_19	Grp_p	1-step	324277	5943860	12U	685							85		
NCAB_20	Grp_p	1-step	324280	5943840	12U	1996	125								
NCAB_21	Grp_p	1-step	324302	5943865	12U	466			1073						
NCAB_22	Grp_p	1-step	694127	5944779	11U				611	905					
NCAB_23	Grp_p	1-step	694156	5944760	11U										
NCAB_24	Grp_p	1-step	694179	5944733	11U										
NCAB_25	Grp_p	1-step	694209	5945747	11U										
NCAB_26	Grp_p	1-step	694209	5945759	11U										
NCAB_27	Grp_p	1-step	694232	5945748	11U										
NCAB_28	Grp_p	1-step	693908	5945977	11U										
NCAB_29	Grp_p	1-step	693914	5945970	11U										
NCAB_30	Grp_p	1-step	693921	5945986	11U	1015									
NCAB_31	Grp_p	1-step	314509	5922008	12U										
NCAB_32	Grp_p	1-step	314540	5921918	12U						1273				
NCAB_33	Grp_p	1-step	314577	5921932	12U					748			686		
NCAB_34	Grp_p	1-step	326471	5927024	12U										
NCAB_35	Grp_p	1-step	326465	5927045	12U										
NCAB_36	Grp_p	1-step	326494	5927033	12U										

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NCAB_37	Grp_p	1-step	326500	5926987	12U										
NCAB_38	Grp_p	1-step	326505	5926974	12U										
NCAB_39	Grp_p	1-step	326522	5926975	12U										
Lab (-ve)	Grp_p	1-step						127	442						
NCAB_40	Grp_p	1-step	354216	5994841	12U										
NCAB_41	Grp_p	1-step	354240	5994809	12U				98						
NCAB_42	Grp_p	1-step	354236	5994787	12U										
NCAB_43	Grp_p	1-step	354282	5994755	12U										
NCAB_44	Grp_p	1-step	354290	5994758	12U										
NCAB_45	Grp_p	1-step	354297	5994760	12U										
NCAB_46	Grp_p	1-step	352392	5996476	12U										
NCAB_47	Grp_p	1-step	352383	5996463	12U										
NCAB_48	Grp_p	1-step	354209	5996456	12U				336						
NCAB_49	Grp_p	1-step	352483	5996464	12U				871						
NCAB_50	Grp_p	1-step	352485	5996461	12U										
NCAB_51	Grp_p	1-step	352484	5996465	12U				744						
NCAB_52	Grp_p	1-step	343213	5993520	12U										
NCAB_53	Grp_p	1-step	343211	5993535	12U										
NCAB_54	Grp_p	1-step	343211	5993537	12U		327								
NCAB_55	Grp_p	1-step	347616	5993635	12U				2099	791			76		
NCAB_56	Grp_p	1-step	347613	5993596	12U				3989	1878					
NCAB_57	Grp_p	1-step	347673	5993583	12U				3082						
NCAB_58	Grp_p	1-step	347716	5993597	12U										
NCAB_59	Grp_p	1-step	347696	5993602	12U										
NCAB_60	Grp_p	1-step	347677	5993612	12U										
NCAB_61	Grp_p	1-step	347697	5993508	12U				459						
NCAB_62	Grp_p	1-step	347689	5993500	12U				136						
NCAB_63	Grp_p	1-step	347700	5993500	12U										
NCAB_64	Grp_p	1-step	351141	5993194	12U	96			1825						
NCAB_65	Grp_p	1-step	351144	5993205	12U				2121						
NCAB_66	Grp_p	1-step	351143	5993217	12U				4047	143					
NCAB_67	Grp_p	1-step	352133	5993089	12U										
NCAB_68	Grp_p	1-step	352120	5993069	12U								268		
NCAB_69	Grp_p	1-step	352141	5993043	12U										
NCAB_70	Grp_p	1-step	352169	5989802	12U										
NCAB_71	Grp_p	1-step	352153	5989797	12U										
NCAB_72	Grp_p	1-step	352155	5989778	12U										
NCAB_73	Grp_p	1-step	352188	5989671	12U										
NCAB_74	Grp_p	1-step	352193	5989671	12U										
NCAB_75	Grp_p	1-step	352183	5989674	12U										

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
Lab (-ve)	Grp_p	1-step													
PCR(-ve)	Grp_p	1-step													
CATO (+ve)	Grp_p	1-step				6428									
WETO (+ve)	Grp_p	1-step					4065								
GPTO (+ve)	Grp_p	1-step						5139							
WOFR (+ve)	Grp_p	1-step							8461						
CSFR (+ve)	Grp_p	1-step								8065					
NLFR (+ve)	Grp_p	1-step									7347				
BCFR (+ve)	Grp_p	1-step										2723			
PLSF (+ve)	Grp_p	1-step											4357		
TISA (+ve)	Grp_p	1-step												902	
LTSA (+ve)	Grp_p	1-step													3088
10spp(+ve)	Grp_p	1-step					80	590	187			750	477	313	
Lab (-ve)	Grp_p	1-step													
PCR (-ve)	Grp_p	1-step													
NCAB_76	Ssp_p	2-step	372580	5952185	12U				3933						
NCAB_77	Ssp_p	2-step	372580	5952185	12U										
NCAB_78	Ssp_p	2-step	372580	5952185	12U				3895						
NCAB_79	Ssp_p	2-step	372580	5952185	12U				5155						
NCAB_80	Ssp_p	2-step	372580	5952185	12U				2359						
NCAB_81	Ssp_p	2-step	372580	5952185	12U				6128						
NCAB_82	Ssp_p	2-step	372580	5952185	12U				3993						
NCAB_83	Ssp_p	2-step	372580	5952185	12U				3992						
NCAB_84	Ssp_p	2-step	372580	5952185	12U				3908						
NCAB_85	Ssp_p	2-step	372580	5952185	12U										
Lab (-ve)	Ssp_p	2-step													
PCR(-ve)	Ssp_p	2-step													
NCAB_76	Ssp_p	2-step	372580	5952185	12U				3900						
NCAB_77	Ssp_p	2-step	372580	5952185	12U				2635						
NCAB_78	Ssp_p	2-step	372580	5952185	12U				6828						
NCAB_79	Ssp_p	2-step	372580	5952185	12U				3945						
NCAB_80	Ssp_p	2-step	372580	5952185	12U				4182						
NCAB_81	Ssp_p	2-step	372580	5952185	12U				5924						
NCAB_82	Ssp_p	2-step	372580	5952185	12U				4272						
NCAB_83	Ssp_p	2-step	372580	5952185	12U				327						
NCAB_84	Ssp_p	2-step	372580	5952185	12U				631						
NCAB_85	Ssp_p	2-step	372580	5952185	12U				6346						
Lab (-ve)	Ssp_p	2-step													
PCR(-ve)	Ssp_p	2-step													

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NCAB_76	Ssp_p	2-step	372580	5952185	12U				2924						
NCAB_77	Ssp_p	2-step	372580	5952185	12U				224						
NCAB_78	Ssp_p	2-step	372580	5952185	12U				3533						
NCAB_79	Ssp_p	2-step	372580	5952185	12U				5502						
NCAB_80	Ssp_p	2-step	372580	5952185	12U				4578						
NCAB_81	Ssp_p	2-step	372580	5952185	12U				5903						
NCAB_82	Ssp_p	2-step	372580	5952185	12U				4253						
NCAB_83	Ssp_p	2-step	372580	5952185	12U				4415						
NCAB_84	Ssp_p	2-step	372580	5952185	12U				5897						
NCAB_85	Ssp_p	2-step	372580	5952185	12U				6382						
Lab (-ve)	Ssp_p	2-step													
PCR(-ve)	Ssp_p	2-step													
CATO (+ve)	Ssp_p	2-step													
WETO (+ve)	Ssp_p	2-step					4946								
GPTO (+ve)	Ssp_p	2-step					324								
WOFR (+ve)	Ssp_p	2-step							9965						
CSFR (+ve)	Ssp_p	2-step							1021						
NLFR (+ve)	Ssp_p	2-step													
BCFR (+ve)	Ssp_p	2-step							152			8467			
PLSF (+ve)	Ssp_p	2-step										1466			
TISA (+ve)	Ssp_p	2-step												10099	
LTSA (+ve)	Ssp_p	2-step													
10spp(+ve)	Ssp_p	2-step					1088		4800			3247		4954	
Lab (-ve)	Ssp_p	2-step													
PCR (-ve)	Ssp_p	2-step													

Table A-2: The expected amphibian detections for each sample, i.e. the species that were encountered during visual and call surveys for each site for Chapter II. Sampling locations are given in UTM's.

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_1	Uni_p	2-step	303525	5436411	11U		•								
WLNP_2	Uni_p	2-step	303525	5436477	11U		•								
WLNP_3	Uni_p	2-step	303542	5436475	11U		•								
WLNP_4	Uni_p	2-step	303525	5436477	11U		•								
Lab (-ve)	Uni_p	2-step													
WLNP_5	Uni_p	2-step	293544	5443978	11U							•			
WLNP_6	Uni_p	2-step	293544	5443978	11U							•			
WLNP_7	Uni_p	2-step	293544	5443978	11U							•			
WLNP_8	Uni_p	2-step	293544	5443978	11U							•			
WLNP_9	Uni_p	2-step	288914	5439090	11U		•					•			
WLNP_10	Uni_p	2-step	288914	5439090	11U		•					•			
WLNP_11	Uni_p	2-step	288914	5439090	11U		•					•			
WLNP_12	Uni_p	2-step	288914	5439090	11U		•					•			
WLNP_13	Uni_p	2-step	291545	5440529	11U		•			•		•			
WLNP_14	Uni_p	2-step	291545	5440529	11U		•			•		•			
WLNP_15	Uni_p	2-step	291545	5440529	11U		•			•		•			
WLNP_16	Uni_p	2-step	291545	5440529	11U		•			•		•			
Lab (-ve)	Uni_p	2-step													
WLNP_17	Uni_p	2-step	289006	541185	11U					•		•			
WLNP_18	Uni_p	2-step	289006	541185	11U					•		•			
WLNP_19	Uni_p	2-step	289006	541185	11U					•		•			
WLNP_20	Uni_p	2-step	289006	541185	11U					•		•			
WLNP_21	Uni_p	2-step	292497	5445475	11U					•		•			
WLNP_22	Uni_p	2-step	292497	5445475	11U					•		•			
WLNP_23	Uni_p	2-step	292497	5445475	11U					•		•			
WLNP_24	Uni_p	2-step	292497	5445475	11U					•		•			
WLNP_25	Uni_p	2-step	295763	5439416	11U		•			•		•			
WLNP_26	Uni_p	2-step	295763	5439416	11U		•			•		•			
WLNP_27	Uni_p	2-step	295763	5439416	11U		•			•		•			
WLNP_28	Uni_p	2-step	295763	5439416	11U		•			•		•			
WLNP_29	Uni_p	2-step	297116	5438234	11U		•			•		•			
WLNP_30	Uni_p	2-step	297116	5438234	11U		•			•		•			
WLNP_31	Uni_p	2-step	297116	5438234	11U		•			•		•			
WLNP_32	Uni_p	2-step	297116	5438234	11U		•			•		•			
WLNP_33	Uni_p	2-step	299469	5438654	11U					•		•			
WLNP_34	Uni_p	2-step	299469	5438654	11U					•		•			
WLNP_35	Uni_p	2-step	299469	5438654	11U					•		•			

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_36	Uni_p	2-step	299469	5438654	11U					•		•			
WLNP_37	Uni_p	2-step	299743	5438674	11U		•			•					
WLNP_38	Uni_p	2-step	299743	5438674	11U		•			•					
WLNP_39	Uni_p	2-step	299743	5438674	11U		•			•					
WLNP_40	Uni_p	2-step	299743	5438674	11U		•			•					
Lab (-ve)	Uni_p	2-step													
WLNP_41	Uni_p	2-step	304000	5435067	11U					•		•			
WLNP_42	Uni_p	2-step	304000	5435067	11U					•		•			
WLNP_43	Uni_p	2-step	304000	5435067	11U					•		•			
WLNP_44	Uni_p	2-step	304000	5435067	11U					•		•			
WLNP_45	Uni_p	2-step	289277	5441865	11U					•		•			
WLNP_46	Uni_p	2-step	289277	5441865	11U					•		•			
WLNP_47	Uni_p	2-step	289277	5441865	11U					•		•			
WLNP_48	Uni_p	2-step	289277	5441865	11U					•		•			
Lab (-ve)	Uni_p	2-step													
PCR (-VE)	Uni_p	2-step													
Ext(5)_1	Uni_p	2-step				•	•		•			•		•	
Ext(5)_2	Uni_p	2-step				•	•		•			•		•	
Ext(5)_3	Uni_p	2-step				•	•		•			•		•	
Lab (-ve)	Uni_p	2-step													
Ext(10)_1	Uni_p	2-step				•	•	•	•	•	•	•	•	•	•
Ext(10)_2	Uni_p	2-step				•	•	•	•	•	•	•	•	•	•
Ext(10)_3	Uni_p	2-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Uni_p	2-step													
PCR (-ve)	Uni_p	2-step													
CATO(+ve)	Uni_p	2-step				•									
WETO(+ve)	Uni_p	2-step					•								
GPTO(+ve)	Uni_p	2-step						•							
WOFR(+ve)	Uni_p	2-step							•						
CSFR(+ve)	Uni_p	2-step								•					
NLFR(+ve)	Uni_p	2-step									•				
BCFR(+ve)	Uni_p	2-step										•			
PLSF(+ve)	Uni_p	2-step											•		
TISA(+ve)	Uni_p	2-step												•	
LTSA(+ve)	Uni_p	2-step													•
10 spp(+ve)	Uni_p	2-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Uni_p	2-step													
PCR (-ve)	Uni_p	2-step													
WLNP_5	Uni_p	1-step	293544	5443978	11U							•			
WLNP_6	Uni_p	1-step	293544	5443978	11U							•			
WLNP_7	Uni_p	1-step	293544	5443978	11U							•			
WLNP_8	Uni_p	1-step	293544	5443978	11U							•			

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_9	Uni_p	1-step	288914	5439090	11U		•					•			
WLNP_10	Uni_p	1-step	288914	5439090	11U		•					•			
WLNP_11	Uni_p	1-step	288914	5439090	11U		•					•			
WLNP_12	Uni_p	1-step	288914	5439090	11U		•					•			
WLNP_13	Uni_p	1-step	291545	5440529	11U		•			•		•			
WLNP_14	Uni_p	1-step	291545	5440529	11U		•			•		•			
WLNP_15	Uni_p	1-step	291545	5440529	11U		•			•		•			
WLNP_16	Uni_p	1-step	291545	5440529	11U		•			•		•			
Lab (-ve)	Uni_p	1-step													
WLNP_17	Uni_p	1-step	289006	541185	11U					•		•			
WLNP_18	Uni_p	1-step	289006	541185	11U					•		•			
WLNP_19	Uni_p	1-step	289006	541185	11U					•		•			
WLNP_20	Uni_p	1-step	289006	541185	11U					•		•			
WLNP_21	Uni_p	1-step	292497	5445475	11U					•		•			
WLNP_22	Uni_p	1-step	292497	5445475	11U					•		•			
WLNP_23	Uni_p	1-step	292497	5445475	11U					•		•			
WLNP_24	Uni_p	1-step	292497	5445475	11U					•		•			
WLNP_25	Uni_p	1-step	295763	5439416	11U		•			•		•			
WLNP_26	Uni_p	1-step	295763	5439416	11U		•			•		•			
WLNP_27	Uni_p	1-step	295763	5439416	11U		•			•		•			
WLNP_28	Uni_p	1-step	295763	5439416	11U		•			•		•			
WLNP_29	Uni_p	1-step	297116	5438234	11U		•			•		•			
WLNP_30	Uni_p	1-step	297116	5438234	11U		•			•		•			
WLNP_31	Uni_p	1-step	297116	5438234	11U		•			•		•			
WLNP_32	Uni_p	1-step	297116	5438234	11U		•			•		•			
WLNP_33	Uni_p	1-step	299469	5438654	11U					•		•			
WLNP_34	Uni_p	1-step	299469	5438654	11U					•		•			
WLNP_35	Uni_p	1-step	299469	5438654	11U					•		•			
WLNP_36	Uni_p	1-step	299469	5438654	11U					•		•			
WLNP_37	Uni_p	1-step	299743	5438674	11U		•			•					
WLNP_38	Uni_p	1-step	299743	5438674	11U		•			•					
WLNP_39	Uni_p	1-step	299743	5438674	11U		•			•					
WLNP_40	Uni_p	1-step	299743	5438674	11U		•			•					
Lab (-ve)	Uni_p	1-step													
WLNP_41	Uni_p	1-step	304000	5435067	11U					•		•			
WLNP_42	Uni_p	1-step	304000	5435067	11U					•		•			
WLNP_43	Uni_p	1-step	304000	5435067	11U					•		•			
WLNP_44	Uni_p	1-step	304000	5435067	11U					•		•			
WLNP_45	Uni_p	1-step	289277	5441865	11U					•		•			
WLNP_46	Uni_p	1-step	289277	5441865	11U					•		•			

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_47	Uni_p	1-step	289277	5441865	11U					•		•			
WLNP_48	Uni_p	1-step	289277	5441865	11U					•		•			
WLNP_49	Uni_p	1-step	288863	5442151	11U		•								
WLNP_50	Uni_p	1-step	288863	5442151	11U		•								
WLNP_51	Uni_p	1-step	288863	5442151	11U		•								
WLNP_52	Uni_p	1-step	288863	5442151	11U		•								
WLNP_53	Uni_p	1-step	291891	5445240	11U					•					•
WLNP_54	Uni_p	1-step	291891	5445240	11U					•					•
WLNP_55	Uni_p	1-step	291891	5445240	11U					•					•
WLNP_56	Uni_p	1-step	291891	5445240	11U					•					•
WLNP_57	Uni_p	1-step	282474	5443659	11U										
WLNP_58	Uni_p	1-step	282474	5443659	11U										
WLNP_59	Uni_p	1-step	282474	5443659	11U										
WLNP_60	Uni_p	1-step	282474	5443659	11U										
Lab (-ve)	Uni_p	1-step													
PCR (-ve)	Uni_p	1-step													
Ext(5)_1	Uni_p	1-step				•	•		•			•		•	
Ext(5)_2	Uni_p	1-step				•	•		•			•		•	
Ext(5)_3	Uni_p	1-step				•	•		•			•		•	
Lab (-ve)	Uni_p	1-step													
Ext(10)_1	Uni_p	1-step				•	•	•	•	•	•	•	•	•	•
Ext(10)_2	Uni_p	1-step				•	•	•	•	•	•	•	•	•	•
Ext(10)_3	Uni_p	1-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Uni_p	1-step													
CATO(+ve)	Uni_p	1-step				•									
WETO(+ve)	Uni_p	1-step					•								
GPTO(+ve)	Uni_p	1-step						•							
WOFR(+ve)	Uni_p	1-step							•						
CSFR(+ve)	Uni_p	1-step								•					
NLFR(+ve)	Uni_p	1-step									•				
BCFR(+ve)	Uni_p	1-step										•			
PLSF(+ve)	Uni_p	1-step											•		
TISA(+ve)	Uni_p	1-step												•	
LTSA(+ve)	Uni_p	1-step													•
10 spp(+ve)	Uni_p	1-step													
Lab (-ve)	Uni_p	1-step													
PCR (-ve)	Uni_p	1-step													
NCAB_1	Uni_p	1-step	327325	5925304	12U										
NCAB_2	Uni_p	1-step	327343	5925332	12U										
NCAB_3	Uni_p	1-step	327378	5925304	12U										

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NCAB_4	Uni_p	1-step	378254	5939594	12U				•			•			
NCAB_5	Uni_p	1-step	378259	5939582	12U				•			•			
NCAB_6	Uni_p	1-step	378262	5939585	12U				•			•			
NCAB_7	Uni_p	1-step	378017	5940669	12U				•			•			
NCAB_8	Uni_p	1-step	378025	5940671	12U				•			•			
NCAB_9	Uni_p	1-step	378039	5940668	12U				•			•			
NCAB_10	Uni_p	1-step	377187	5945134	12U				•			•			
NCAB_11	Uni_p	1-step	377190	5945142	12U				•			•			
NCAB_12	Uni_p	1-step	377196	5945124	12U				•			•			
NCAB_13	Uni_p	1-step	373192	5900423	12U				•						
NCAB_14	Uni_p	1-step	373197	5900431	12U				•						
NCAB_15	Uni_p	1-step	373198	5900438	12U				•						
NCAB_16	Uni_p	1-step	374294	5901396	12U				•						
NCAB_17	Uni_p	1-step	374295	5901385	12U				•						
NCAB_18	Uni_p	1-step	374296	5901376	12U				•						
NCAB_19	Uni_p	1-step	324277	5943860	12U										
NCAB_20	Uni_p	1-step	324280	5943840	12U										
NCAB_21	Uni_p	1-step	324302	5943865	12U										
NCAB_22	Uni_p	1-step	694127	5944779	11U										
NCAB_23	Uni_p	1-step	694156	5944760	11U										
NCAB_24	Uni_p	1-step	694179	5944733	11U										
NCAB_25	Uni_p	1-step	694209	5945747	11U										
NCAB_26	Uni_p	1-step	694209	5945759	11U										
NCAB_27	Uni_p	1-step	694232	5945748	11U										
NCAB_28	Uni_p	1-step	693908	5945977	11U				•						
NCAB_29	Uni_p	1-step	693914	5945970	11U				•						
NCAB_30	Uni_p	1-step	693921	5945986	11U				•						
NCAB_31	Uni_p	1-step	314509	5922008	12U										
NCAB_32	Uni_p	1-step	314540	5921918	12U										
NCAB_33	Uni_p	1-step	314577	5921932	12U										
NCAB_34	Uni_p	1-step	326471	5927024	12U										
NCAB_35	Uni_p	1-step	326465	5927045	12U										
NCAB_36	Uni_p	1-step	326494	5927033	12U										
NCAB_37	Uni_p	1-step	326500	5926987	12U									•	
NCAB_38	Uni_p	1-step	326505	5926974	12U									•	
NCAB_39	Uni_p	1-step	326522	5926975	12U									•	
Lab (-ve)	Uni_p	1-step													
NCAB_40	Uni_p	1-step	354216	5994841	12U										
NCAB_41	Uni_p	1-step	354240	5994809	12U										
NCAB_42	Uni_p	1-step	354236	5994787	12U										

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NCAB_43	Uni_p	1-step	354282	5994755	12U							•			
NCAB_44	Uni_p	1-step	354290	5994758	12U							•			
NCAB_45	Uni_p	1-step	354297	5994760	12U							•			
NCAB_46	Uni_p	1-step	352392	5996476	12U				•						
NCAB_47	Uni_p	1-step	352383	5996463	12U				•						
NCAB_48	Uni_p	1-step	354209	5996456	12U				•						
NCAB_49	Uni_p	1-step	352483	5996464	12U	•			•						
NCAB_50	Uni_p	1-step	352485	5996461	12U	•			•						
NCAB_51	Uni_p	1-step	352484	5996465	12U	•			•						
NCAB_52	Uni_p	1-step	343213	5993520	12U									•	
NCAB_53	Uni_p	1-step	343211	5993535	12U									•	
NCAB_54	Uni_p	1-step	343211	5993537	12U									•	
NCAB_55	Uni_p	1-step	347616	5993635	12U		•		•			•			
NCAB_56	Uni_p	1-step	347613	5993596	12U		•		•			•			
NCAB_57	Uni_p	1-step	347673	5993583	12U		•		•			•			
NCAB_58	Uni_p	1-step	347716	5993597	12U		•		•			•			
NCAB_59	Uni_p	1-step	347696	5993602	12U		•		•			•			
NCAB_60	Uni_p	1-step	347677	5993612	12U		•		•			•			
NCAB_61	Uni_p	1-step	347697	5993508	12U		•		•			•			
NCAB_62	Uni_p	1-step	347689	5993500	12U		•		•			•			
NCAB_63	Uni_p	1-step	347700	5993500	12U		•		•			•			
NCAB_64	Uni_p	1-step	351141	5993194	12U	•			•						
NCAB_65	Uni_p	1-step	351144	5993205	12U	•			•						
NCAB_66	Uni_p	1-step	351143	5993217	12U	•			•						
NCAB_67	Uni_p	1-step	352133	5993089	12U										
NCAB_68	Uni_p	1-step	352120	5993069	12U										
NCAB_69	Uni_p	1-step	352141	5993043	12U										
NCAB_70	Uni_p	1-step	352169	5989802	12U				•						
NCAB_71	Uni_p	1-step	352153	5989797	12U				•						
NCAB_72	Uni_p	1-step	352155	5989778	12U				•						
NCAB_73	Uni_p	1-step	352188	5989671	12U				•						
NCAB_74	Uni_p	1-step	352193	5989671	12U				•						
NCAB_75	Uni_p	1-step	352183	5989674	12U				•						
Lab (-ve)	Uni_p	1-step													
PCR(-ve)	Uni_p	1-step													
CATO(+ve)	Uni_p	1-step				•									
WETO (+ve)	Uni_p	1-step					•								
GPTO (+ve)	Uni_p	1-step						•							
WOFR (+ve)	Uni_p	1-step							•						
CSFR (+ve)	Uni_p	1-step								•					

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NLFR (+ve)	Uni_p	1-step									•				
BCFR (+ve)	Uni_p	1-step										•			
PLSF (+ve)	Uni_p	1-step											•		
TISA (+ve)	Uni_p	1-step												•	
LTSA (+ve)	Uni_p	1-step													•
10 spp (+ve)	Uni_p	1-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Uni_p	1-step													
PCR (-ve)	Uni_p	1-step													
WLNP_1	Grp_p	2-step	303525	5436411	11U		•								
WLNP_2	Grp_p	2-step	303525	5436477	11U		•								
WLNP_3	Grp_p	2-step	303542	5436475	11U		•								
WLNP_4	Grp_p	2-step	303525	5436477	11U		•								
Lab (-ve)	Grp_p	2-step													
WLNP_5	Grp_p	2-step	293544	5443978	11U							•			
WLNP_6	Grp_p	2-step	293544	5443978	11U							•			
WLNP_7	Grp_p	2-step	293544	5443978	11U							•			
WLNP_8	Grp_p	2-step	293544	5443978	11U							•			
WLNP_9	Grp_p	2-step	288914	5439090	11U		•					•			
WLNP_10	Grp_p	2-step	288914	5439090	11U		•					•			
WLNP_11	Grp_p	2-step	288914	5439090	11U		•					•			
WLNP_12	Grp_p	2-step	288914	5439090	11U		•					•			
WLNP_13	Grp_p	2-step	291545	5440529	11U		•			•		•			
WLNP_14	Grp_p	2-step	291545	5440529	11U		•			•		•			
WLNP_15	Grp_p	2-step	291545	5440529	11U		•			•		•			
WLNP_16	Grp_p	2-step	291545	5440529	11U		•			•		•			
Lab (-ve)	Grp_p	2-step													
WLNP_17	Grp_p	2-step	289006	541185	11U					•		•			
WLNP_18	Grp_p	2-step	289006	541185	11U					•		•			
WLNP_19	Grp_p	2-step	289006	541185	11U					•		•			
WLNP_20	Grp_p	2-step	289006	541185	11U					•		•			
WLNP_21	Grp_p	2-step	292497	5445475	11U					•		•			
WLNP_22	Grp_p	2-step	292497	5445475	11U					•		•			
WLNP_23	Grp_p	2-step	292497	5445475	11U					•		•			
WLNP_24	Grp_p	2-step	292497	5445475	11U					•		•			
WLNP_25	Grp_p	2-step	295763	5439416	11U		•			•		•			
WLNP_26	Grp_p	2-step	295763	5439416	11U		•			•		•			
WLNP_27	Grp_p	2-step	295763	5439416	11U		•			•		•			
WLNP_28	Grp_p	2-step	295763	5439416	11U		•			•		•			
WLNP_29	Grp_p	2-step	297116	5438234	11U		•			•		•			
WLNP_30	Grp_p	2-step	297116	5438234	11U		•			•		•			

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
WLNP_31	Grp_p	2-step	297116	5438234	11U		•			•		•			
WLNP_32	Grp_p	2-step	297116	5438234	11U		•			•		•			
WLNP_33	Grp_p	2-step	299469	5438654	11U					•		•			
WLNP_34	Grp_p	2-step	299469	5438654	11U					•		•			
WLNP_35	Grp_p	2-step	299469	5438654	11U					•		•			
WLNP_36	Grp_p	2-step	299469	5438654	11U					•		•			
WLNP_37	Grp_p	2-step	299743	5438674	11U		•			•					
WLNP_38	Grp_p	2-step	299743	5438674	11U		•			•					
WLNP_39	Grp_p	2-step	299743	5438674	11U		•			•					
WLNP_40	Grp_p	2-step	299743	5438674	11U		•			•					
Lab (-ve)	Grp_p	2-step													
WLNP_41	Grp_p	2-step	304000	5435067	11U					•		•			
WLNP_42	Grp_p	2-step	304000	5435067	11U					•		•			
WLNP_43	Grp_p	2-step	304000	5435067	11U					•		•			
WLNP_44	Grp_p	2-step	304000	5435067	11U					•		•			
WLNP_45	Grp_p	2-step	289277	5441865	11U					•		•			
WLNP_46	Grp_p	2-step	289277	5441865	11U					•		•			
WLNP_47	Grp_p	2-step	289277	5441865	11U					•		•			
WLNP_48	Grp_p	2-step	289277	5441865	11U					•		•			
Lab (-ve)	Grp_p	2-step													
PCR (-VE)	Grp_p	2-step													
Ext(5)_1	Grp_p	2-step				•	•		•			•		•	
Ext(5)_2	Grp_p	2-step				•	•		•			•		•	
Ext(5)_3	Grp_p	2-step				•	•		•			•		•	
Lab (-ve)	Grp_p	2-step													
Ext(10)_1	Grp_p	2-step				•	•	•	•	•	•	•	•	•	•
Ext(10)_2	Grp_p	2-step				•	•	•	•	•	•	•	•	•	•
Ext(10)_3	Grp_p	2-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Grp_p	2-step													
PCR (-ve)	Grp_p	2-step													
CATO (+ve)	Grp_p	2-step				•									
WETO (+ve)	Grp_p	2-step					•								
GPTO (+ve)	Grp_p	2-step						•							
WOFR (+ve)	Grp_p	2-step							•						
CSFR (+ve)	Grp_p	2-step								•					
NLFR (+ve)	Grp_p	2-step									•				
BCFR (+ve)	Grp_p	2-step										•			
PLSF (+ve)	Grp_p	2-step											•		
TISA (+ve)	Grp_p	2-step												•	
LTSA (+ve)	Grp_p	2-step													•

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
10 spp(+ve)	Grp_p	2-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Grp_p	2-step													
PCR (-VE)	Grp_p	2-step													
WLNP_5	Grp_p	1-step	293544	5443978	11U							•			
WLNP_6	Grp_p	1-step	293544	5443978	11U							•			
WLNP_7	Grp_p	1-step	293544	5443978	11U							•			
WLNP_8	Grp_p	1-step	293544	5443978	11U							•			
WLNP_9	Grp_p	1-step	288914	5439090	11U		•					•			
WLNP_10	Grp_p	1-step	288914	5439090	11U		•					•			
WLNP_11	Grp_p	1-step	288914	5439090	11U		•					•			
WLNP_12	Grp_p	1-step	288914	5439090	11U		•					•			
WLNP_13	Grp_p	1-step	291545	5440529	11U		•			•		•			
WLNP_14	Grp_p	1-step	291545	5440529	11U		•			•		•			
WLNP_15	Grp_p	1-step	291545	5440529	11U		•			•		•			
WLNP_16	Grp_p	1-step	291545	5440529	11U		•			•		•			
Lab (-ve)	Grp_p	1-step													
WLNP_17	Grp_p	1-step	289006	541185	11U					•		•			
WLNP_18	Grp_p	1-step	289006	541185	11U					•		•			
WLNP_19	Grp_p	1-step	289006	541185	11U					•		•			
WLNP_20	Grp_p	1-step	289006	541185	11U					•		•			
WLNP_21	Grp_p	1-step	292497	5445475	11U					•		•			
WLNP_22	Grp_p	1-step	292497	5445475	11U					•		•			
WLNP_23	Grp_p	1-step	292497	5445475	11U					•		•			
WLNP_24	Grp_p	1-step	292497	5445475	11U					•		•			
WLNP_25	Grp_p	1-step	295763	5439416	11U		•			•		•			
WLNP_26	Grp_p	1-step	295763	5439416	11U		•			•		•			
WLNP_27	Grp_p	1-step	295763	5439416	11U		•			•		•			
WLNP_28	Grp_p	1-step	295763	5439416	11U		•			•		•			
WLNP_29	Grp_p	1-step	297116	5438234	11U		•			•		•			
WLNP_30	Grp_p	1-step	297116	5438234	11U		•			•		•			
WLNP_31	Grp_p	1-step	297116	5438234	11U		•			•		•			
WLNP_32	Grp_p	1-step	297116	5438234	11U		•			•		•			
WLNP_33	Grp_p	1-step	299469	5438654	11U					•		•			
WLNP_34	Grp_p	1-step	299469	5438654	11U					•		•			
WLNP_35	Grp_p	1-step	299469	5438654	11U					•		•			
WLNP_36	Grp_p	1-step	299469	5438654	11U					•		•			
WLNP_37	Grp_p	1-step	299743	5438674	11U		•			•					
WLNP_38	Grp_p	1-step	299743	5438674	11U		•			•					
WLNP_39	Grp_p	1-step	299743	5438674	11U		•			•					
WLNP_40	Grp_p	1-step	299743	5438674	11U		•			•					

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
Lab (-ve)	Grp_p	1-step													
WLNP_41	Grp_p	1-step	304000	5435067	11U					•		•			
WLNP_42	Grp_p	1-step	304000	5435067	11U					•		•			
WLNP_43	Grp_p	1-step	304000	5435067	11U					•		•			
WLNP_44	Grp_p	1-step	304000	5435067	11U					•		•			
WLNP_45	Grp_p	1-step	289277	5441865	11U					•		•			
WLNP_46	Grp_p	1-step	289277	5441865	11U					•		•			
WLNP_47	Grp_p	1-step	289277	5441865	11U					•		•			
WLNP_48	Grp_p	1-step	289277	5441865	11U					•		•			
WLNP_49	Grp_p	1-step	288863	5442151	11U		•								
WLNP_50	Grp_p	1-step	288863	5442151	11U		•								
WLNP_51	Grp_p	1-step	288863	5442151	11U		•								
WLNP_52	Grp_p	1-step	288863	5442151	11U		•								
WLNP_53	Grp_p	1-step	291891	5445240	11U					•					•
WLNP_54	Grp_p	1-step	291891	5445240	11U					•					•
WLNP_55	Grp_p	1-step	291891	5445240	11U					•					•
WLNP_56	Grp_p	1-step	291891	5445240	11U					•					•
WLNP_57	Grp_p	1-step	282474	5443659	11U										
WLNP_58	Grp_p	1-step	282474	5443659	11U										
WLNP_59	Grp_p	1-step	282474	5443659	11U										
WLNP_60	Grp_p	1-step	282474	5443659	11U										
Lab (-ve)	Grp_p	1-step													
PCR (-ve)	Grp_p	1-step													
Ext(5)_1	Grp_p	1-step				•	•		•			•		•	
Ext(5)_2	Grp_p	1-step				•	•		•			•		•	
Ext(5)_3	Grp_p	1-step				•	•		•			•		•	
Lab (-ve)	Grp_p	1-step													
Ext(10)_1	Grp_p	1-step				•	•	•	•	•	•	•	•	•	•
Ext(10)_2	Grp_p	1-step				•	•	•	•	•	•	•	•	•	•
Ext(10)_3	Grp_p	1-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Grp_p	1-step													
CATO(+ve)	Grp_p	1-step				•									
WETO(+ve)	Grp_p	1-step					•								
GPTO(+ve)	Grp_p	1-step						•							
WOFR(+ve)	Grp_p	1-step							•						
CSFR(+ve)	Grp_p	1-step								•					
NLFR(+ve)	Grp_p	1-step									•				
BCFR(+ve)	Grp_p	1-step										•			
PLSF(+ve)	Grp_p	1-step											•		
TISA(+ve)	Grp_p	1-step												•	

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
LTSA(+ve)	Grp_p	1-step													•
10 spp(+ve)	Grp_p	1-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Grp_p	1-step													
PCR (-ve)	Grp_p	1-step													
NCAB_1	Grp_p	1-step	327325	5925304	12U										
NCAB_2	Grp_p	1-step	327343	5925332	12U										
NCAB_3	Grp_p	1-step	327378	5925304	12U										
NCAB_4	Grp_p	1-step	378254	5939594	12U				•			•			
NCAB_5	Grp_p	1-step	378259	5939582	12U				•			•			
NCAB_6	Grp_p	1-step	378262	5939585	12U				•			•			
NCAB_7	Grp_p	1-step	378017	5940669	12U				•			•			
NCAB_8	Grp_p	1-step	378025	5940671	12U				•			•			
NCAB_9	Grp_p	1-step	378039	5940668	12U				•			•			
NCAB_10	Grp_p	1-step	377187	5945134	12U				•			•			
NCAB_11	Grp_p	1-step	377190	5945142	12U				•			•			
NCAB_12	Grp_p	1-step	377196	5945124	12U				•			•			
NCAB_13	Grp_p	1-step	373192	5900423	12U				•						
NCAB_14	Grp_p	1-step	373197	5900431	12U				•						
NCAB_15	Grp_p	1-step	373198	5900438	12U				•						
NCAB_16	Grp_p	1-step	374294	5901396	12U				•						
NCAB_17	Grp_p	1-step	374295	5901385	12U				•						
NCAB_18	Grp_p	1-step	374296	5901376	12U				•						
NCAB_19	Grp_p	1-step	324277	5943860	12U										
NCAB_20	Grp_p	1-step	324280	5943840	12U										
NCAB_21	Grp_p	1-step	324302	5943865	12U										
NCAB_22	Grp_p	1-step	694127	5944779	11U										
NCAB_23	Grp_p	1-step	694156	5944760	11U										
NCAB_24	Grp_p	1-step	694179	5944733	11U										
NCAB_25	Grp_p	1-step	694209	5945747	11U										
NCAB_26	Grp_p	1-step	694209	5945759	11U										
NCAB_27	Grp_p	1-step	694232	5945748	11U										
NCAB_28	Grp_p	1-step	693908	5945977	11U				•						
NCAB_29	Grp_p	1-step	693914	5945970	11U				•						
NCAB_30	Grp_p	1-step	693921	5945986	11U				•						
NCAB_31	Grp_p	1-step	314509	5922008	12U										
NCAB_32	Grp_p	1-step	314540	5921918	12U										
NCAB_33	Grp_p	1-step	314577	5921932	12U										
NCAB_34	Grp_p	1-step	326471	5927024	12U										
NCAB_35	Grp_p	1-step	326465	5927045	12U										
NCAB_36	Grp_p	1-step	326494	5927033	12U										

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NCAB_37	Grp_p	1-step	326500	5926987	12U									•	
NCAB_38	Grp_p	1-step	326505	5926974	12U									•	
NCAB_39	Grp_p	1-step	326522	5926975	12U									•	
Lab (-ve)	Grp_p	1-step													
NCAB_40	Grp_p	1-step	354216	5994841	12U										
NCAB_41	Grp_p	1-step	354240	5994809	12U										
NCAB_42	Grp_p	1-step	354236	5994787	12U										
NCAB_43	Grp_p	1-step	354282	5994755	12U							•			
NCAB_44	Grp_p	1-step	354290	5994758	12U							•			
NCAB_45	Grp_p	1-step	354297	5994760	12U							•			
NCAB_46	Grp_p	1-step	352392	5996476	12U				•						
NCAB_47	Grp_p	1-step	352383	5996463	12U				•						
NCAB_48	Grp_p	1-step	354209	5996456	12U				•						
NCAB_49	Grp_p	1-step	352483	5996464	12U	•			•						
NCAB_50	Grp_p	1-step	352485	5996461	12U	•			•						
NCAB_51	Grp_p	1-step	352484	5996465	12U	•			•						
NCAB_52	Grp_p	1-step	343213	5993520	12U									•	
NCAB_53	Grp_p	1-step	343211	5993535	12U									•	
NCAB_54	Grp_p	1-step	343211	5993537	12U									•	
NCAB_55	Grp_p	1-step	347616	5993635	12U		•		•			•			
NCAB_56	Grp_p	1-step	347613	5993596	12U		•		•			•			
NCAB_57	Grp_p	1-step	347673	5993583	12U		•		•			•			
NCAB_58	Grp_p	1-step	347716	5993597	12U		•		•			•			
NCAB_59	Grp_p	1-step	347696	5993602	12U		•		•			•			
NCAB_60	Grp_p	1-step	347677	5993612	12U		•		•			•			
NCAB_61	Grp_p	1-step	347697	5993508	12U		•		•			•			
NCAB_62	Grp_p	1-step	347689	5993500	12U		•		•			•			
NCAB_63	Grp_p	1-step	347700	5993500	12U		•		•			•			
NCAB_64	Grp_p	1-step	351141	5993194	12U	•			•						
NCAB_65	Grp_p	1-step	351144	5993205	12U	•			•						
NCAB_66	Grp_p	1-step	351143	5993217	12U	•			•						
NCAB_67	Grp_p	1-step	352133	5993089	12U										
NCAB_68	Grp_p	1-step	352120	5993069	12U										
NCAB_69	Grp_p	1-step	352141	5993043	12U										
NCAB_70	Grp_p	1-step	352169	5989802	12U				•						
NCAB_71	Grp_p	1-step	352153	5989797	12U				•						
NCAB_72	Grp_p	1-step	352155	5989778	12U				•						
NCAB_73	Grp_p	1-step	352188	5989671	12U				•						
NCAB_74	Grp_p	1-step	352193	5989671	12U				•						
NCAB_75	Grp_p	1-step	352183	5989674	12U				•						

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
Lab (-ve)	Grp_p	1-step													
PCR(-ve)	Grp_p	1-step													
CATO (+ve)	Grp_p	1-step				•									
WETO (+ve)	Grp_p	1-step					•								
GPTO (+ve)	Grp_p	1-step						•							
WOFR (+ve)	Grp_p	1-step							•						
CSFR (+ve)	Grp_p	1-step								•					
NLFR (+ve)	Grp_p	1-step									•				
BCFR (+ve)	Grp_p	1-step										•			
PLSF (+ve)	Grp_p	1-step											•		
TISA (+ve)	Grp_p	1-step												•	
LTSA (+ve)	Grp_p	1-step													•
10spp(+ve)	Grp_p	1-step				•	•	•	•	•	•	•	•	•	•
Lab (-ve)	Grp_p	1-step													
PCR (-ve)	Grp_p	1-step													
NCAB_76	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_77	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_78	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_79	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_80	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_81	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_82	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_83	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_84	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_85	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
Lab (-ve)	Ssp_p	2-step													
PCR(-ve)	Ssp_p	2-step													
NCAB_76	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_77	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_78	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_79	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_80	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_81	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_82	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_83	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_84	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_85	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
Lab (-ve)	Ssp_p	2-step													
PCR(-ve)	Ssp_p	2-step													

Sample	Primer	PCR	Easting	Northing	Zone	CATO	WETO	GPTO	WOFR	CSFR	NLFR	BCFR	PLSF	TISA	LTSA
NCAB_76	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_77	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_78	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_79	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_80	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_81	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_82	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_83	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_84	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
NCAB_85	Ssp_p	2-step	372580	5952185	12U		•		•			•		•	
Lab (-ve)	Ssp_p	2-step													
PCR(-ve)	Ssp_p	2-step													
CATO (+ve)	Ssp_p	2-step													
WETO (+ve)	Ssp_p	2-step					•								
GPTO (+ve)	Ssp_p	2-step													
WOFR (+ve)	Ssp_p	2-step							•						
CSFR (+ve)	Ssp_p	2-step													
NLFR (+ve)	Ssp_p	2-step													
BCFR (+ve)	Ssp_p	2-step										•			
PLSF (+ve)	Ssp_p	2-step													
TISA (+ve)	Ssp_p	2-step												•	
LTSA (+ve)	Ssp_p	2-step													
5spp(+ve)	Ssp_p	2-step					•		•			•		•	
Lab (-ve)	Ssp_p	2-step													
PCR (-ve)	Ssp_p	2-step													

Table A-3: Index of abbreviations and terminology for Table A-1 and A-2.

Sample ID	Sample Description
WLPN	Waterton Lakes Nation Park sample
NCAB	north central Alberta sample
CATO(+ve)	Canadian toad
WETO(+ve)	wetern toad
GPTO(+ve)	Great Plains toad
WOFR(+ve)	wood frog
CSFR(+ve)	Columbia spotted frog
NLFR(+ve)	northern leopard frog
BCFR(+ve)	boreal chorus frog
PLSF(+ve)	plains spadefoot
TISA (+ve)	wetstern tiger salamander
LTSA(+ve)	long-toed salamander
5spp(+ve)	WETO, WOFR, BCFR, TISA
10spp(+ve)	10 spp listed above combined
Lab(-ve)	Lab-based extraction negative control
PCR(-ve)	PCR negative control
Ext(5)_1	0.0067 ng/uL extraction control using 5 spp DNA
Ext(5)_2	0.067 ng/uL extraction control using 5 spp DNA
Ext(5)_3	0.14 ng/uL extraction control using 5 spp DNA
Ext(10)_1	0.0067 ng/uL extraction control using 10 spp DNA
Ext(10)_2	0.067 ng/uL extraction control using 10 spp DNA
Ext(10)_3	0.14 ng/uL extraction control using 10 spp DNA

Appendix 2

qPCR Mastermix for Chapter 3:

The 2X QPCR Mastermix (*Dynamite*) used in this study is a proprietary mix developed, and distributed by the Molecular Biology Service Unit (MBSU), in the department of Biological Science at the University of Alberta, Edmonton, Alberta, Canada. It contains Tris (pH 8.3), KCl, $MgCl_2$, Glycerol, Tween 20, DMSO, dNTPs, ROX as a normalizing dye, and an antibody inhibited Taq polymerase.