# SURVEY FOR BATRACHOCHYTRIUM DENDROBATIDIS IN HELLBENDERS AND MUDPUPPIES IN PA WATER WAYS

Rachel Love Hazlewood Lewis

# Survey for *Batrachochytrium dendrobatidis* in Hellbenders and Mudpuppies in PA water ways

A Thesis

Presented to the College of Graduate Studies

In Partial Fulfillment of the Requirements for

Master's Degree

Rachel Love Hazlewood Lewis

To the Graduate Council:

I am submitting herewith a thesis written by Rachel Love Hazlewood Lewis entitled "Survey of *Batrachochytrium dendrobatidis* in Hellbenders and Mudpuppies in PA water ways". I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

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## DEDICATION

I dedicate this thesis to my husband, Clarke Lewis. He helped motivate me to start this journey and has been a source of constant support and encouragement. He has always been understanding though all of my late nights, days without sleep and emotional outburst due to stress or lack of sleep. Life would not be complete without you.

## LIST OF FIGURES

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## ABSTRACT

*Batrachochytrium dendrobatidis* (*Bd*) is the known fungal agent responsible for Chytridiomycosis. Chytridiomycosis is a fatal disease that affects over 200 species of amphibians on a global scale. Chytridiomycosis invades the epidermis of amphibians where it degrades the keratin typically leading to death. Limited research has been conducted in Pennsylvania to determine the geographical extent and severity of Chytridiomycosis in local amphibian populations. Skin swab samples were collected thought out water ways in Pennsylvania from 2009-2011 from *Cryptobranchus alleganiensis*, the Eastern Hellbender, and *Necturus maculosus*, the Common Mudpuppy. Traditional and nested PCR were used to determine presence or absence of *Bd* from these samples. The data reveals that *Bd* is present in the Eastern Hellbender and the Common Mudpuppy throughout water ways in Pennsylvanin. Nested PCR was found to be $1x10^{13}$ times more sensitive than traditional PCR. This increase in sensitivity revealed an increase of infected sampled amphibians to be >40%.

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# CHAPTER I

# INTRODUCTION

## Amphibian Importance

Amphibians are located on every continent with the exception of Antarctica and Greenland (4) (Fig. 1). Most amphibians have a complex life cycle consisting of an aquatic and terrestrial stage of development (11,12). Due to this, amphibians play a pivotal role in the hierarchy of aquatic and terrestrial food webs (27). Adult amphibians are secondary consumers aiding in insect pest control and provide a significant food source for tertiary consumers in both aquatic and terrestrial environments. Larvae fill primary and secondary consumer positions depending on the stage of development. Amphibians make excellent bioindicators for environmental changes and water quality (11,12,27,30). Permeable skin as well as soft unprotected egg casings make amphibians more susceptible to environmental toxins, temperature changes, ultraviolet radiation, and infectious diseases during all stages of development (12,13,17,26).

## Amphibian Decline

In 1989, the First World Congress of Herpetology officially recognized that amphibian populations around the globe were declining (42). During the late 1980's, approximately 31%, or 1,856, of amphibian species were listed as globally threatened and only 4% were critically endangered (42). Since the late 1980's over 400 species of amphibians have experienced a population decline of which half cannot be explained (42). Of the 34 known extinct species of amphibians, 9 of those extinctions have

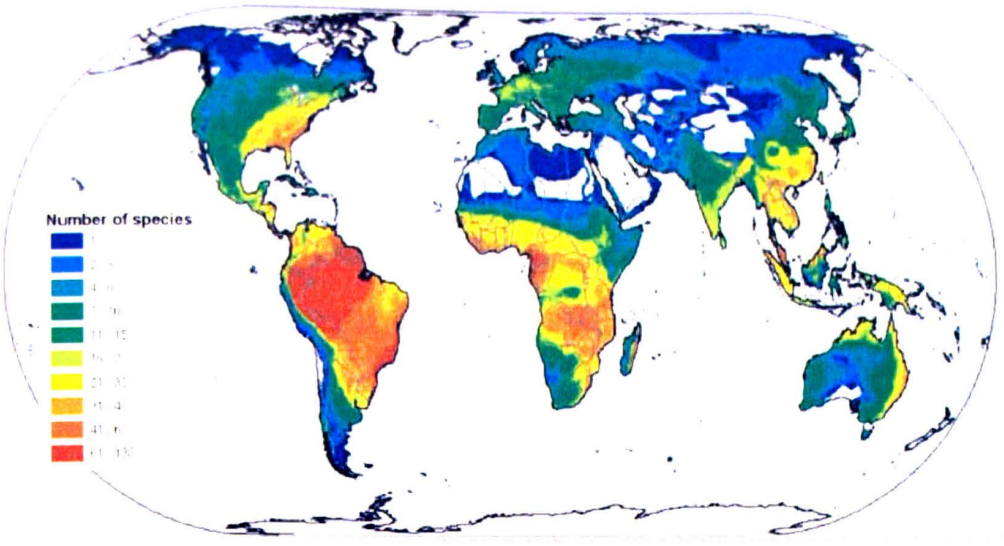


Figure 1. Global Distribution of amphibian species (4).

occurred since the late 1980's (39,42). One hundred and thirteen more species have not been reported in the last three decades and are feared to be extinct (42).

In 2004, the Global Amphibian Assessment (GAA) reported that worldwide, approximately 33% of amphibian species were threatened (2,3,42) (Fig 2.). This is a 2% increase of over 300 species from the late 1980's. Currently, it is estimated that 43% of amphibian species are experiencing some form of population decline with only 0.5% increasing in population size (2,3). According to the International Union of Conservation of Nature (IUCN) 7.2% of amphibian species are currently critically endangered (2,3,42) (Table 1). This threat is undoubtedly underestimated due to the fact that approximately 22.5% of the amphibian species are too poorly known to assess (2,3,42). Extinction of amphibian species and populations could have severe repercussion on the ecosystem and wreck havoc on the delicate food web all organisms are dependent upon.

The amphibian population decline has been attributed to several factors such as habitat destruction and fragmentation, overexploitation, pesticide use, introduction of non-native species, and disease caused by fungal or viral infections (13,21,39,42). Chytridiomycosis, a disease caused by a fungal infection of an amphibian's epidermis, has been found at numerous sites in sync with population declines due to unknown causes (9,15,17,27,39). Although the reasons are unclear, many have interpreted the wide scale amphibian population decline as a warning sign of increasing environmental decline (30). Many speculate and believe that Chytridiomycosis is one of the leading causes for these unexplained declines (39).

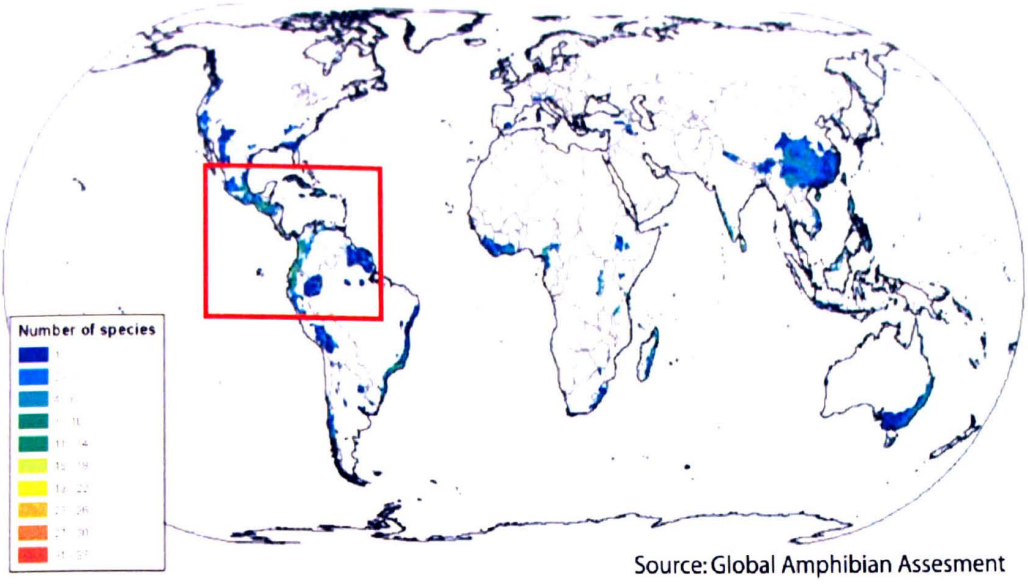


Figure 2. Global distribution of threatened amphibians (4).

Table 1. Criteria for qualifying an amphibian as critically endangered outlined by the International Union of Conservation of Nature (1).

**CRITICALLY ENDANGERED (CR)**

A taxon is Critically Endangered when the best available evidence indicates that it meets any of the following criteria (A to E), and it is therefore considered to be facing an extremely high risk of extinction in the wild:

A. Reduction in population size based on any of the following:

1. An observed, estimated, inferred or suspected population size reduction of ≥90% over the last 10 years or three generations, whichever is the longer, where the causes of the reduction are clearly reversible AND understood AND ceased, based on (and specifying) any of the following:

(a) direct observation

(b) an index of abundance appropriate to the taxon

(c) a decline in area of occupancy, extent of occurrence and/or quality of habitat

(d) actual or potential levels of exploitation

(e) the effects of introduced taxa, hybridization, pathogens, pollutants, competitors or parasites.

2. An observed, estimated, inferred or suspected population size reduction of ≥80% over the last 10 years or three generations, whichever is the longer, where the reduction or its causes may not have ceased OR may not be understood OR may not be reversible, based on (and specifying) any of the following:

(a) direct observation

(b) an index of abundance appropriate to the taxon

(c) a decline in area of occupancy, extent of occurrence and/or quality of habitat

(d) actual or potential levels of exploitation

(e) the effects of introduced taxa, hybridization, pathogens, pollutants, competitors or parasites.

3. A population size reduction of ≥80%, projected or suspected to be met within the next 10 years or three generations, whichever is the longer (up to a maximum of 100 years), based on (and specifying) any of the following:

(b) an index of abundance appropriate to the taxon

(c) a decline in area of occupancy, extent of occurrence and/or quality of habitat

(d) actual or potential levels of exploitation

(e) the effects of introduced taxa, hybridization, pathogens, pollutants, competitors or parasites.

4. An observed, estimated, inferred, projected or suspected population size reduction of ≥80% over any 10 year or three generation period, whichever is longer (up to a maximum of 100 years in the future), where the time period must include both the past and the future, and where the reduction or its causes may not have ceased OR may not be understood OR may not be reversible, based on (and specifying) any of the following:

(a) direct observation

(b) an index of abundance appropriate to the taxon

(c) a decline in area of occupancy, extent of occurrence and/or quality of habitat

(d) actual or potential levels of exploitation

(e) the effects of introduced taxa, hybridization, pathogens, pollutants, competitors or parasites

Table 1. Criteria for qualifying an amphibian as critically endangered outlined by the International Union of Conservation of Nature Continued (1)

| CRITICALLY ENDANGERED (CR) |
|---|
| B. Geographic range in the form of either B1 (extent of occurrence) OR B2 (area of occupancy) OR both: |
| 1. Extent of occurrence estimated to be less than 100 $km^2$, and estimates indicating at least two of a-c: |
| a. Severely fragmented or known to exist at only a single location. |
| b. Continuing decline, observed, inferred or projected, in any of the following: |
| (i) extent of occurrence |
| (ii) area of occupancy |
| (iii) area, extent and/or quality of habitat |
| (iv) number of locations or subpopulations |
| (v) number of mature individuals. |
| c. Extreme fluctuations in any of the following: |
| (i) extent of occurrence |
| (ii) area of occupancy |
| (iii) number of locations or subpopulations |
| (iv) number of mature individuals. |
| 2. Area of occupancy estimated to be less than 10 $km^2$, and estimates indicating at least two of a-c: |
| a. Severely fragmented or known to exist at only a single location. |
| b. Continuing decline, observed, inferred or projected, in any of the following: |
| (i) extent of occurrence |
| (ii) area of occupancy |
| (iii) area, extent and/or quality of habitat |
| (iv) number of locations or subpopulations |
| (v) number of mature individuals. |
| c. Extreme fluctuations in any of the following: |
| (i) extent of occurrence |
| (ii) area of occupancy |
| (iii) number of locations or subpopulations |
| (iv) number of mature individuals. |
| C. Population size estimated to number fewer than 250 mature individuals and either: |
| 1. An estimated continuing decline of at least 25% within three years or one generation, whichever is longer, (up to a maximum of 100 years in the future) OR |
| 2. A continuing decline, observed, projected, or inferred, in numbers of mature individuals AND at least one of the following (a-b): |
| (a) Population structure in the form of one of the following: |
| (i) no subpopulation estimated to contain more than 50 mature individuals, OR |
| (ii) at least 90% of mature individuals in one subpopulation. |
| (b) Extreme fluctuations in number of mature individuals. |
| D. Population size estimated to number fewer than 50 mature individuals. |
| E. Quantitative analysis showing the probability of extinction in the wild is at least 50% within 10 years or three generations, whichever is the longer (up to a maximum of 100 years). |

## Chytrids

Chytridiomycosis is a fatal disease caused by the fungus, Batrachochytrium dendrobatidis (Bd) that affects amphibians worldwide (7,15,17,22,27,28,35,38,43,43) see Figure 3. Chytridiomycosis was fully described and associated with amphibian population declines in 1998 (8,17,43). *Batrachochytrium dendrobatidis* originates from the Phylum Chytridiomycota, Class Chytridiomycetes, Order Chytridiales, and is the only member of Chytridiomycota that infects vertebrates (8,22). Members of Chytridiomycota are typically referred to as chytrids and reside in aquatic habitats and moist soils where they degrade the cellulose, chitin, and keratin typically found in plants, algae, protists, and invertebrates (8,34). Chytridiomycosis, unlike any other chytrid, invades the epidermis of amphibians and degrades the keratin that resides in the stratum corneum (27,34,38). Destruction of the epidermis leads to lethargy, abnormal posture, loss of limb function, lesions, abnormal sloughing of the epidermis, ulcerations, hemorrhages in the eye and skin; reddening of the digits and ventral surface as well as congestion of internal organs (7,9,15,17).

There are at least 80 isolates of Bd that have been identified from Australia and North America (15). The fungus is known to affect at least 200 species of amphibians around the world and has been specifically linked to at least one species extinction (22). The earliest known cases of Chytridiomycosis were discovered in archived *Xenopus laevis,* the African clawed frog, specimens which had been collected in South Africa from 1879 to 1999 (43). Less than 3% of specimens in this collection were determined to be infected with Bd. Chytridiomycosis was first seen outside of Africa in 1970 in the United States in *Bufo canorus* and *Rana pipien,* Yosemite toad and Northern Leopard

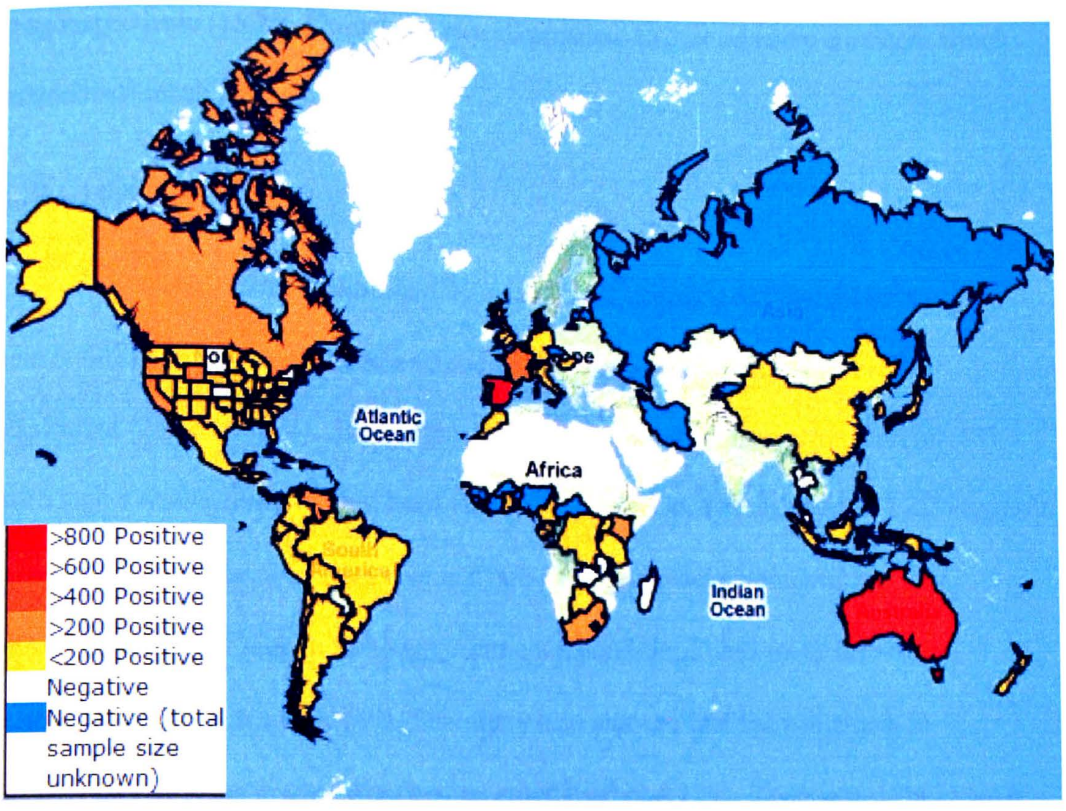


Figure 3. Distribution of samples taken for Chytridiomycosis reported by the World Conservation Unit, Global Amphibian Assessment, eBurts, and EDGE Amphibians (4).

frog respectively (16,24). Currently Chytridiomycosis occurs on every continent which amphibians inhabit (43,44).

**Life Cycle:**

*Batrachochytrium dendrobatidis,* has two life stages, a uniflagellated zoospore that is substrate independent and a reproductive zoosporangium that is substrate dependent (24,38) (Fig. 4). Both stages require a moist environment for survival (31). Although a resting stage has not been identified (6,38) a study published in 2003 suggests zoosporangia may be able to survive in a non-discernible developmental state for extended periods of time in harsh environmental conditions until more favorable conditions become available (24). This study also showed that Bd will attach to arthropods and algae in the absence of an amphibian host (24). Zoospores (Fig 5) have been shown to re-infect the same substrate or use the aquatic environment to locate a new substrate. Location to a new substrate may occur through chemotaxis toward the cysteine component of keratin found in amphibian skin (29). This includes the oral disk of larvae and the epidermis of adults (29,36). Once a zoospore has located and infected its substrate it develops into a zoosporangium for reproduction. Zoospores will develop within the zoosporangium and be released into the environment via discharge tubes on the surface of the epidermis (29,38) (Fig. 6 & 7). It is important to note that Bd cannot survive desiccation in any life stage (26,27). As a zoospore Bd is lacking a cell wall and as a zoosporangium the cell wall is thin and easily damaged (31). The pH of water plays a role in longevity of zoospores and zoosporangium with an optimal growth range between pH 6-7 (25).

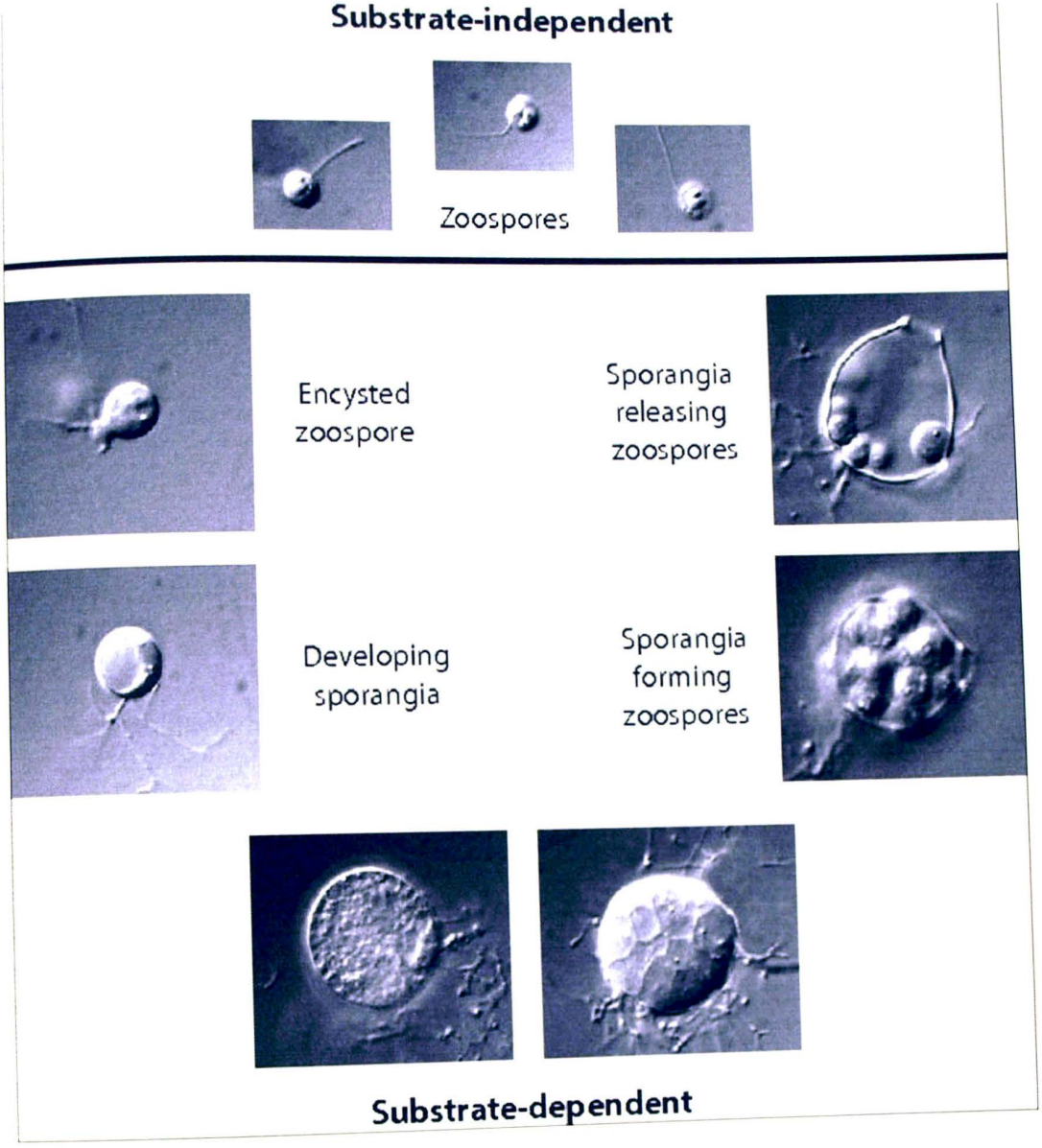


Figure 4. Life cycle of *Batrachochytrium dendrobatidis* (38).

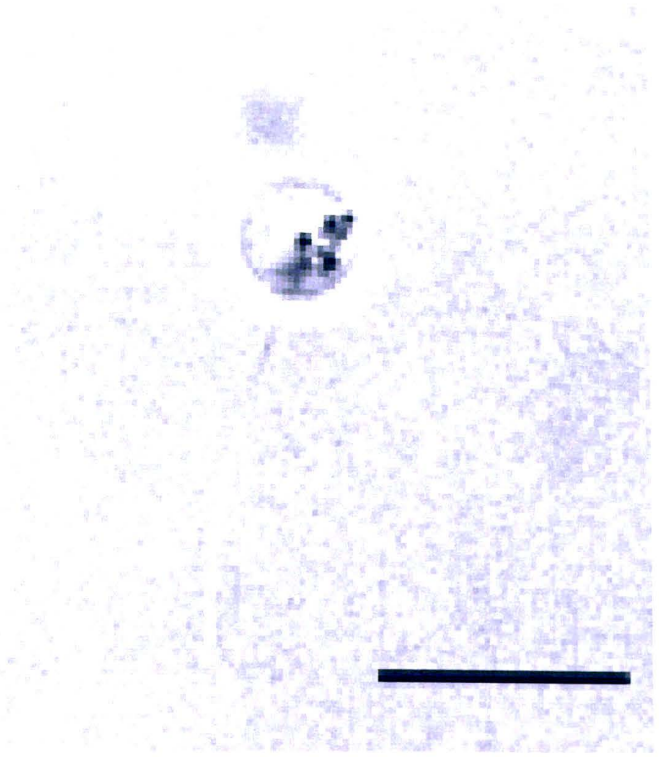

Figure 5. Light micrograph of live cultured zoospore of *Batrachochytrium dendrobatidis* (6).

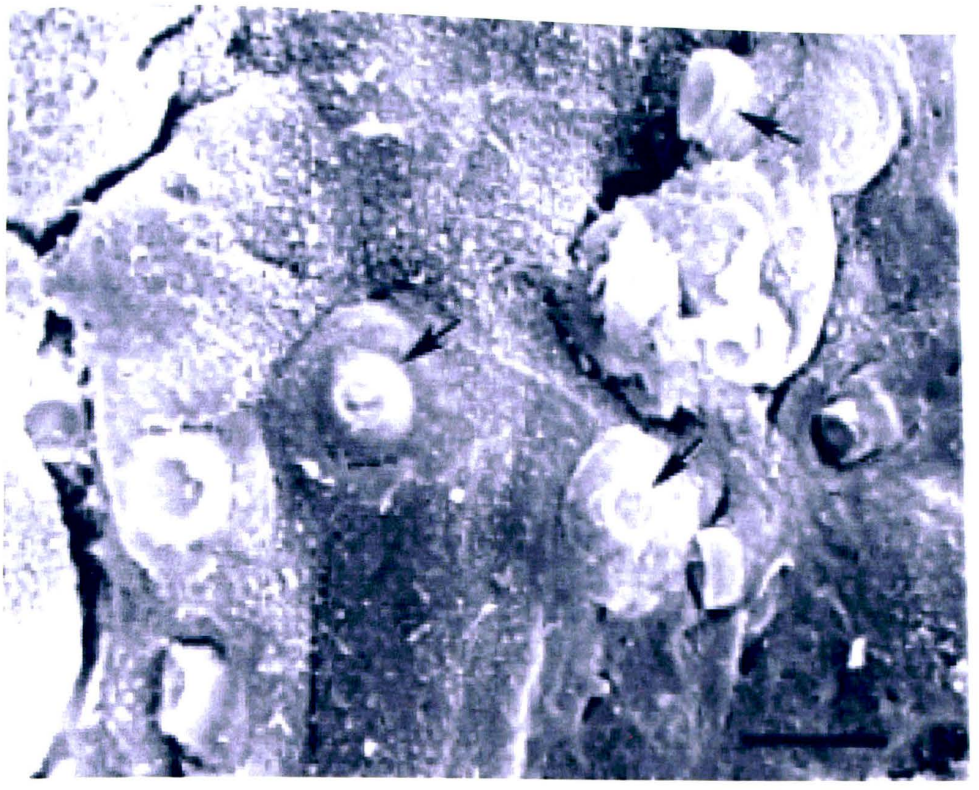

Figure 6. Scanning electron micrographs of positively infected Bd frog skin (17) Arrows indicate mature unopened sporangia discharge tubes that contain zoospores. Bar=35µm.

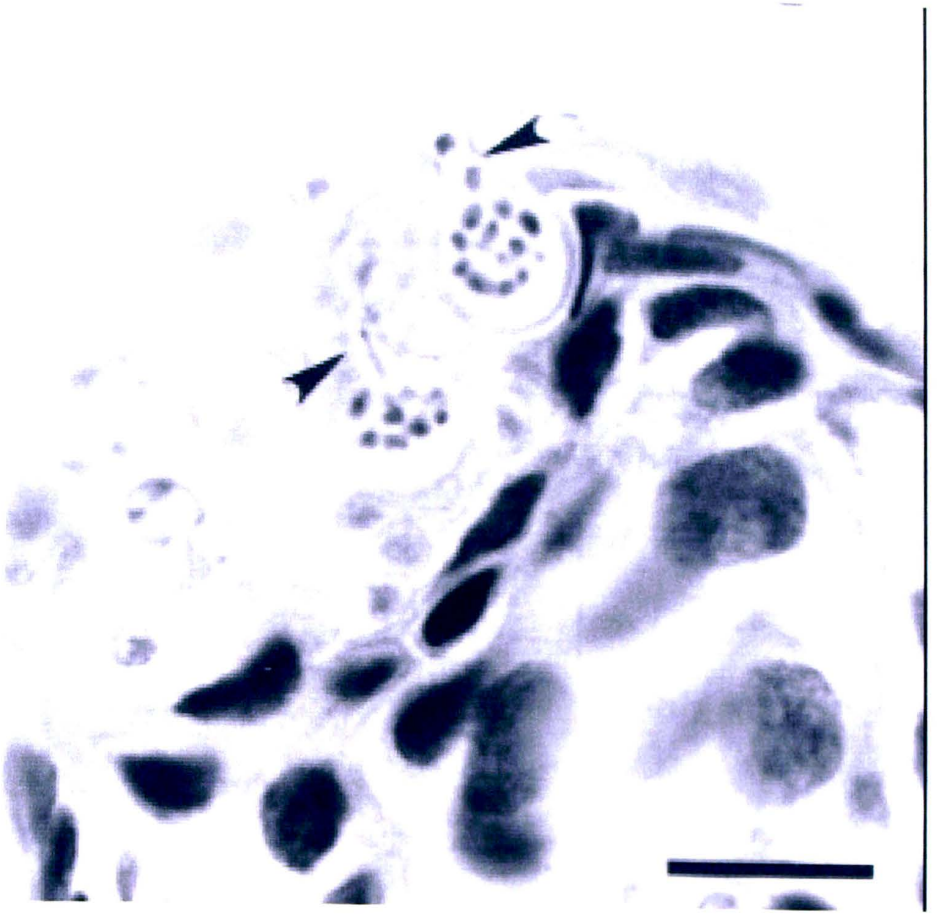

Figure 7 Histological section of positively Bd infected frog skin (17).

Arrows indicate mature sporangia full of zoospores ready to be released. Bar=35μm.

**Spread/Transmission:**

Studies have shown transmission of Chytridiomycosis can occur via shared bodies of water, physical interactions, between life stages of amphibians, and postmortem to healthy amphibians (24,27,36). Laboratory studies have shown that zoospores can even remain infectious in sterile water lacking a host for up to 6 weeks (24,36). Zoospores have also been shown to be intolerant of warm water and dry conditions, however a laboratory study found that birds may transport Chytrid from one body of water to another (25). Human interaction has also been suggested as a mode of transportation for Bd from one site to the next (25,31,33). Handling, disinfecting, and equipment storage protocols have been established to reduce possible spread of Bd (41).

The international trade of *Xenopus Laevis*, African clawed frog, that began in the 1930's has been proposed to be the original dissemination of Bd across the globe (19,43). The global amphibian trade is a substantial industry involving every country where Bd-infected amphibians reside (19). Many amphibians involved in the global trade are collected from the wild and introduced into new habitats (19) such as *Xenopus laevis* and *Rana catesbeiana*, the African clawed frog and the North American Bullfrog respectively. Xenopus species are known to harbour Bd (19,31). The North American bullfrog are known asymptomatic carriers of Bd (19). Many other species involved in the global trade are known to be susceptible to Bd infection even at low rates. This implies they could be vectors of Bd into new habitats and populations (19). Screenings of amphibian species involved in global trade have shown 28 species to be carriers of Bd and some are experiencing die-offs associated with Chytridiomycosis (19). Amphibian

trade was once unregulated and even today smugglers still transport amphibians across the globe undetected (33,43).

## Detection

Infection of Bd can lead to Chytridiomycosis in amphibian. Chytridiomycosis is a disease that destroys the keratin within the epidermis of amphibians. This destruction can cause many clinical symptoms such as ulcerations, thickening of the epidermis, lesions, swelling and loss of limb functions, reddening of the ventral surface and digits, abnormal posture and lethargy in adult amphibians (7,9,15,17). In larval stages such as tadpoles Chytridiomycosis infects the oral disc and causes loss of pigmentation of the jawsheeth (36). Although many of these symptoms can be observed without invasive diagnostic testing, not all amphibians infected with Bd exhibit these symptoms thus ruling out visual inspection as a primary survey method.

Culturing and histological examination of amphibian skin has also been utilized as detection methods for Bd (36). These methods require euthanization of amphibians. Culturing can take weeks to confirm infection and histology procedures are extremely labor intensive. Detection can easily be missed if the infection rate is low (14). Cytology has also been implemented as a detection method by scraping epithelial cells from adult amphibians and the jaw sheath of tadpoles to be examined under a microscope for sporangia (36). This is less invasive and is a more amphibian friendly method of detection.

Polymerase chain reaction (PCR) has also been utilized in detection of Bd DNA present in samples of amphibian skin, swabs of skin, toe clips, water samples and soil

samples (19,22,40). PCR is the most sensitive technique and least invasive method of detection for Bd and real-time PCR (qRT-PCR) quantifies the number of Bd zoospores present in a sample (40). With the use of experimentally infected amphibians, detection has been demonstrated two weeks prior to histological and cytological methods and is less time and labor intensive (14).

# CHAPTER II

# MATERIALS AND METHODS

## Animal collection

All samples in this study were collected by Dr. Kurt Regester and his team of undergraduate and graduate students from Clarion University in Clarion Pennsylvania. All samples used in this study were collected from water ways in Pennsylvanina. (Fig. 8). Species collected include *Cryptobranchus a. alleganienis*, *Necturus m. maculosus*, the Eastern Hellbender and common Mudpuppy respectively. To minimize contamination each salamander was placed in a plastic tote or mesh bag during sampling. New latex gloves and sterile cotton tipped swabs were used to collect samples from each salamander. Each salamander was swabbed on the ventral surface of the feet, dorsolateral folds, and the cloaca five times in each location. Salamanders were then released at the respective capture location. Swabs were stored in individual 2ml screw cap vials with 1ml of 70% ethanol (EtOH) labeled with a species code, location code, and date. Plastic totes and mesh bags were reused. However, to prevent cross contamination, each bag/tote was treated with a 10% bleach solution and allowed to dry for 24 hours before reuse (37). A total of 229 individuals were swabbed for Chytridiomycosis between 2009 and 2011. Collections were made from June through September of each year.

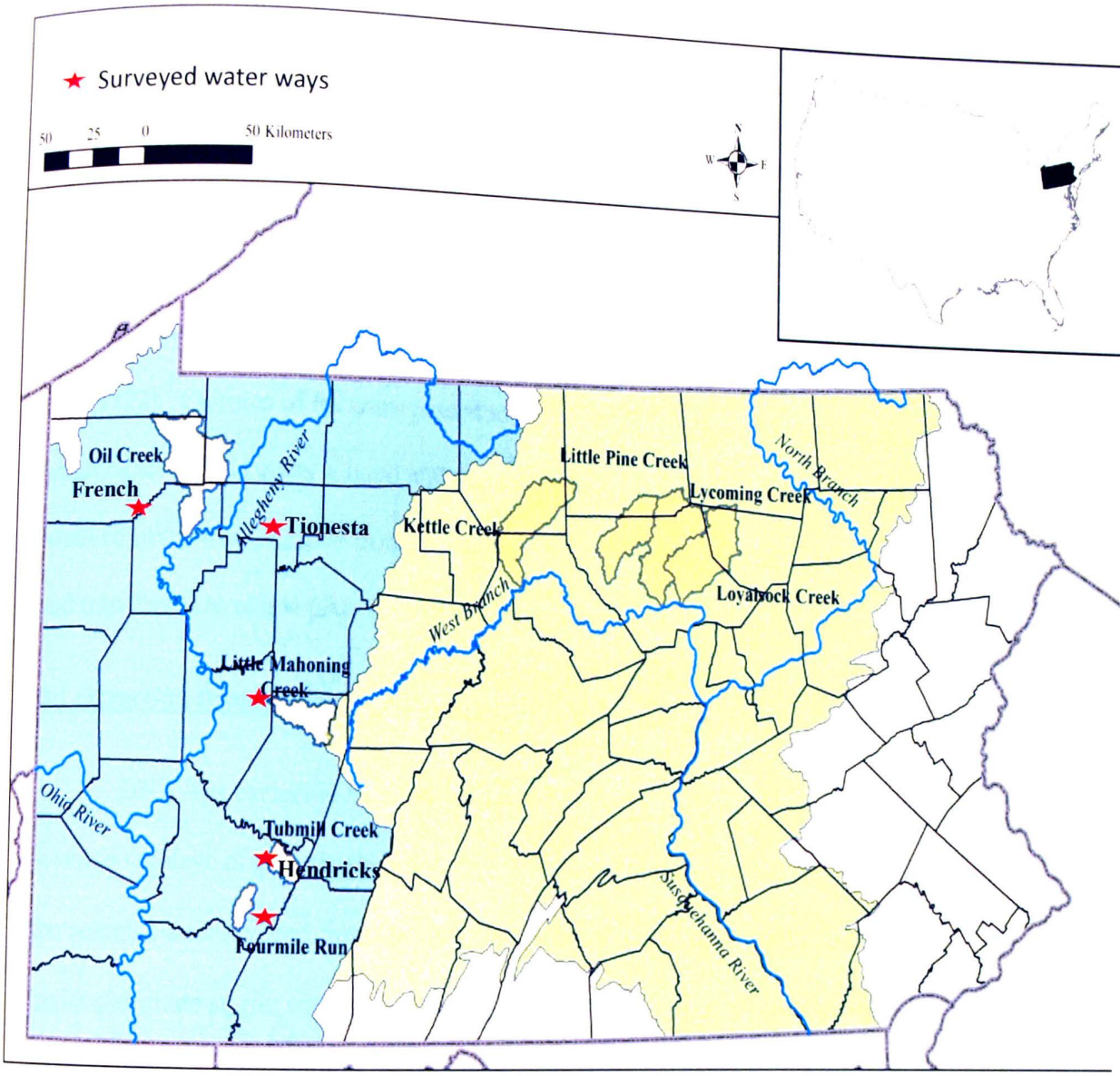


Figure 8. Water ways surveyed in Pennsylvania. (20)

## Batrachochytrium dendrobatidis cultivation

*Batrachochytrium dendrobatidis* (Bd) was cultivated from a stock solution obtained from J.E. Longcore at the School of Biology and Ecology in Orono Maine. Tryptone agar plates were made using 16g Tryptone (Sigma), 2g hydrolysed gelatine (Acros), 4g lactose (Sigma), 10g bacteriological agar (Sigma) and 1000ml of deionized water. The mixture was autoclaved at 121°C for 15 minutes and allowed to cool before pouring into culture dishes (23). Cultures of Bd were passed every seven to ten days and stored at room temperature (22°C) under a hood and monitored daily for growth. When passing Bd, two small colonies were scraped from the surface of the plate using a sterile inoculating loop and transferred to a new plate using aseptic technique.

## Bd extraction from Bd for positive control

DNA was extracted from Bd cultures for later use as positive controls. After seven to ten days of growth, the Bd was passed onto another plate and the remainder of the colonies were scraped from the plate and added to a 1.5ml tube with 1000µl of molecular grade sterile water (Mediatech, Inc). A sterile disposable pestle was used for homogenization of the solution as well as vortexing for 30 secconds. The sample was then boiled for 10 minutes.

## Comparison of field sample DNA extraction techniques:

To determine the best DNA extraction method of the field samples, DNA was extracted from Bd cultures in the lab using sterile cotton swabs to mimic field sampling techniques. A cotton swab was used to remove five colonies of Bd from Bd cultures in

the lab. The swab was then cut and stored in 1ml of 70% EtOH for 24 hours in a micro centrifuge tube at room temperature. The swab was partially removed from the tube using sterile forceps. The swab was then rolled against the inside of the storage tube to remove excess EtOH. Next, another set of sterile forceps were used to squeeze the swab and "press" any residual EtOH from the cotton swab. All initial and recovered EtOH from the swab and storage tube was centrifuged at max speed (14000rpms) for 10 minutes to pellet DNA. After centrifugation the supernatant was removed and discarded. All excess EtOH was removed pelleted DNA derived from the alcohol in a Thermo Fisher Speedvac and resuspended in 100µl molecular grade water.

To determine if DNA remained on the swab, the tip of the cotton was also processed by cutting the end of the cotton swab using sterile scissors and placing it in a micro centrifuge tube. All excess EtOH was removed from the cotton swab in a Thermo Fisher Speedvac and resuspended in 100µl molecular grade water.

The samples were then boiled for 10 minutes and centrifuged to pellet any cell debris. Serial dilutions of DNA isolated from the swab and DNA isolated from the EtOH was PCR amplified to determine the most sensitive technique for processing field samples expected to yield low amounts of DNA.

**Field sample DNA extraction**

Field samples were mailed to Austin Peay State University to be processed. Upon arrival at Austin Peay State University, the field collected samples were stored at 4°C until DNA extraction could be performed. Excess fluid was removed from the swab using the same protocol above; all fluid was transferred from the 2ml vial to a 1.5ml tube

and centrifuged at max speed for 10 minutes to pellet the DNA. After centrifugation, the supernatant was removed and transferred back to the original field sample vial and stored with the swab at 4°C. Excess EtOH was removed from the pelleted DNA (Thermo Fisher Speedvac) and each pellet resuspended in 50µl molecular grade water (Mediatech, Inc) then boiled for 10 minutes. Sample DNA was stored at -20°C. Samples received from Pisces Molecular, a commercial company, were already processed with a Qiagen DNeasy 96 kit and stored in AE Buffer (Qiagen).

**Primer design for Traditional PCR**

The primer set for PCR amplification was designed to hybridize to the ITS1 and ITS2 regions in the Bd genome (5,18). The forward and reverse primers 5' CAG TGT GCC ATA TGT CACG 3' and 3' CAT GGT TCA TAT CTG TCC AG 5' respectively were obtained through Integrated DNA technologies. Each primer was centrifuged at 14000rpms for 10min to pellet the primers and then resuspended to 100mM stock volume with molecular grade water (Mediatech, Inc). Next the forward and reverse primers were diluted to a 1:10 ratio to make a primer mix for downstream reactions. Each PCR reaction required 2µL of primer solution at 10µM concentration.

**Identification of Bd in swab samples**

Polymerase chain reaction was utilized to detect and amplify Bd genomic DNA. Polymerase chain reactions were set up in 20µl reactions consisting of 10µl 2XGoTaq polymerase, 2µl primer solution, 3µl molecular grade sterile water (Mediatech, Inc), and 5µl of sample. Positive and negative controls (Fig. 9) were used for each set of reactions

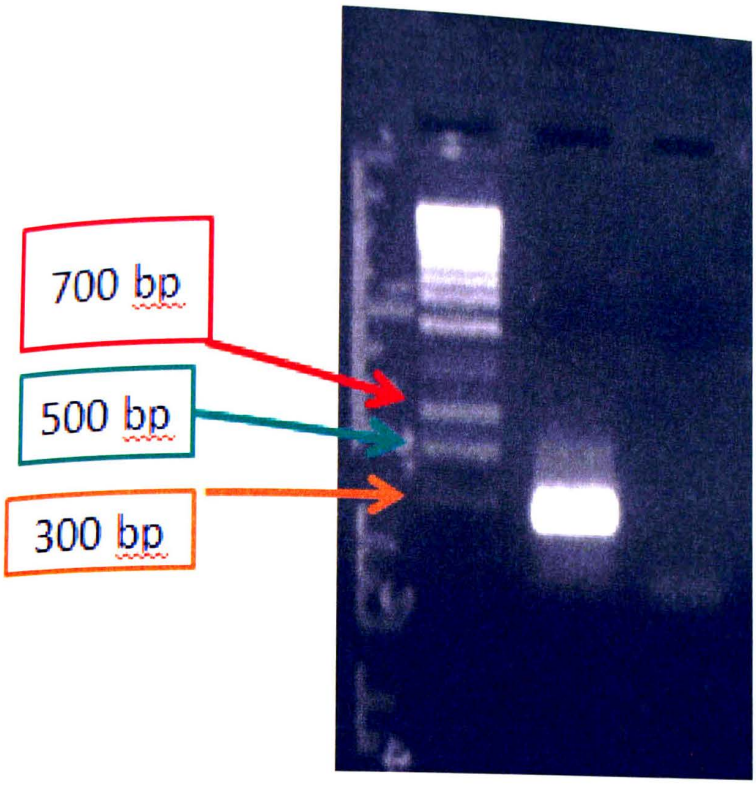


Figure 9. Molecular ladder alongside a positive and negative control for Bd.

to ensure proper amplification was occurring and each reaction was contamination free. Positive controls used 5µl of Bd DNA extracted in the lab as indicated above. Negative controls contained 5µl of molecular grade sterile water (Mediatech, Inc) instead of sample or Bd DNA from the lab. Amplification was performed with a thermal cycler (Applied Biosystems Veriti) and consisted of 40 cycles at 95°C for 30 seconds to denature the DNA, 55°C for 30 seconds for annealing and 72°C for 60 seconds for elongation. Products were visualized using electrophoresis with a 1% agarose gel with ethidium bromide. Images were recorded and saved using a Kodak 1D 3.6 camera. All samples were completed in triplicate. Each sample that tested positive for Bd at least once during the triplicate run was considered positive for Bd. All remaining samples were tested for inhibition with the use of spike controls. Twenty micro liter spike controls contained 1µl of isolated Bd DNA.

All positive spiked samples were considered negative for Bd. All negative spiked samples indicated inhibition within the sample and were diluted at a 1:5 ratio. All negative samples were spiked again at the 1:5 dilution following the same protocol as before for PCR. Of the diluted spiked samples all negative samples were determined to be inhibited beyond detection capabilities. Of the diluted spiked samples, all positive samples were considered inhibited at the original sample size. Polymerase chain reaction was then completed in quintuplicate, without spiking, on these samples to equal a single run at the original sample concentration. All samples that tested positive at least once were considered positive with PCR inhibitors within the sample. All samples that tested negative for Bd were considered negative for Bd with PCR inhibitors within the sample.

## Nested primer and biotinylated oligonucleotide design

Two sets of forward and reverse primers were designed nested PCR and a biotinylated probe for Bd DNA capture was used in this study (Table 2). Nested primers were designed to hybridize inside of the original primer set to increase specificity and detection. Biotinylated probes were selected based on prior unpublished research at Austin Peay State University, David Henley a previous undergraduate, each specific for single stranded Bd DNA. Primers and biotinylated probes were resuspended in molecular grade water to make a 100mM stock solution.

## Nested PCR

Four sets of nesting primers were tested to determine the most accurate results (Table 2). Each primer set was determined to be accurate. From these results, the Bd3fwd and Bd4rev were used in all nesting PCR reactions. Polymerase chian reactions were set up in 20µl volume using 10µl 2xGoTaq (Promega), 7µl molecular grade water, 2µl forward and reverse nesting primer, and 1µl of previously amplified sample DNA.

## Preparation of Streptavidin beads:

Magnabind™ Streptavidin beads (Thermoscientific) were utilized to capture biotinylated Bd primer. Streptavidin beads in the original vial were mixed by rotation and the calculated number of beads necessary for an experiment were removed and added to a PCR tube. Beads were removed from suspension, for 1-2 minutes, with a magnet. The supernatant was removed by aspiration and the remaining pellet was resuspended in

Table 2. Primers used for PCR analysis and Bioltinylated oligonucleotides used for *Batracochcytrium dendrobatidis* capture.

| Primer | Primer Type | Primer/oligonucleotide Sequence |
|---|---|---|
| Bd1a | Forward | 5'- CAGTGTGCCATATGTCACG -3' |
| Bd2a | Reverse | 5'- CATGGTTCATATCTGTCCAG -3' |
| Bd3fwd | Forward | 5'- TGTCACGACGTCGAACAAAATTTAT -3' |
| Bd3rev | Reverse | 5'- CTGTCCAGTCAATTCGGAGAAT -3' |
| Bd4fwd | Forward | 5'- AGTCGAACAAAATTTATTATTTTTTC -3' |
| Bd4rev | Reverse | 5'- TCAATTCGGACAATGTATTTTTATAA -3' |
| Biotin-Bd1a | Biotinylated | 5'- CAGTGTGCCATATGTCACG -3' |

wash binding buffer (WBB) (2M NaCl, 1mM EDTA, 10mM Tris-HCl, ph 7.5) in a volume equal to the original volume removed from the stock solution. The wash was repeated three times and the beads were re-suspended in WBB for storage and use.

**Binding biotinylated probes to streptavidin magnetic beads**

Biotinylated probes were prepared with molecular water at a calculated concentration not to exceed the carrying capacity of the Streptavidin beads. This solution was added to prepared beads and incubated at 28°C in a Techne, HB-1D Hybridiser with agitation for two hours. After incubation, the beads were pelleted with a magnet for 1-2 minutes and the supernatant was removed. The probe-bound beads were then washed a total of five times with molecular grade water and resuspended for experimentation. Washes were completed to insure removal of all unbound probes from solution.

**Capture of *Batrachochytrium dendrobatidis* by biotinylated probe-bound beads**

A serial dilution of Bd genomic DNA was prepared at a 1:10 dilution. One hundred microliters of each dilution was denatured at 95°C for 10 minutes on the thermal cycler and immediately placed on ice to prevent annealing. Probe-bound bead suspension was then added to each 0.2 ml PCR tube and placed in a hybridizer at 55°C for 3 hours to allowing annealing of biotinylated probe-bound beads to single stranded Bd genomic DNA. After hybridization, the Bd DNA captured probe-bound beads were mixed using trituration. Following trituration, 5µl was removed and used for PCR. The remaining suspension was pelleted using a magnet for 2-3 minutes. The supernatant was removed and the beads were washed with molecular grade water three times. The DNA

containing pellet was then re-suspended in molecular water at a concentration not to exceed 4µg/µl and 2µl of suspension was used for PCR.

# CHAPTER III

## RESULTS

Amplification of DNA processed from the alcohol yielded a positive result at a 1:100,000 dilution. Amplification of DNA processed from the swab yielded a positive result at a 1:100 dilution (Fig. 10). Amplification of traditional PCR serial dilution yielded a positive result at a 1:100,000 dilution. Amplification of nested PCR serial dilution yielded a positive result at a $1:10^{18}$ dilution (Fig. 11). Amplification of DNA processed from alcohol utilizing Streptavidin beads as a DNA capture mechanism yielded a positive results at a 1:100 dilution (Fig. 12).

Of the 11 samples dating June-September 2009, 4 were positive for Bd, 1 negative, 1 positive with inhibition and 5 inhibited beyond our means (Fig. 13). A total of 19 samples dating June-September 2010 revealed 4 positive, 6 negative, 5 positive with inhibition, and 5 unknown due to PCR inhibition (Fig. 14). Samples dating June-September 2011 revealed 126 negative and 17 unknown due to PCR inhibition for a total of 143 samples using traditional PCR (Fig. 15). Nested PCR of these samples revealed 38 positive and only 104 to be negative (Fig. 16). This is an increase of 26.8% positive results over the traditional PCR.

One hundred and nineteen samples dating June-September 2010 previously analyzed by Pisces Molecular, a commercial company, revealed multiple banding patterns (Fig. 17) during the initial PCR amplification to which only 20 samples were determined to be positive. Nested PCR resulted in better visual results as well as

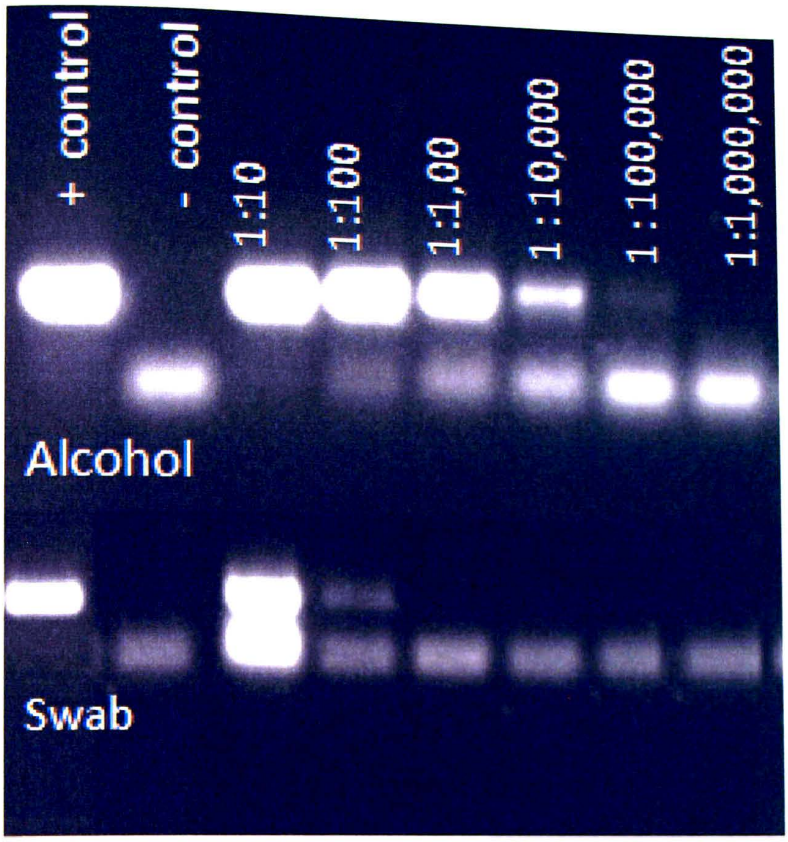


Figure 10. PCR amplification comparison of field sample DNA extraction technique.

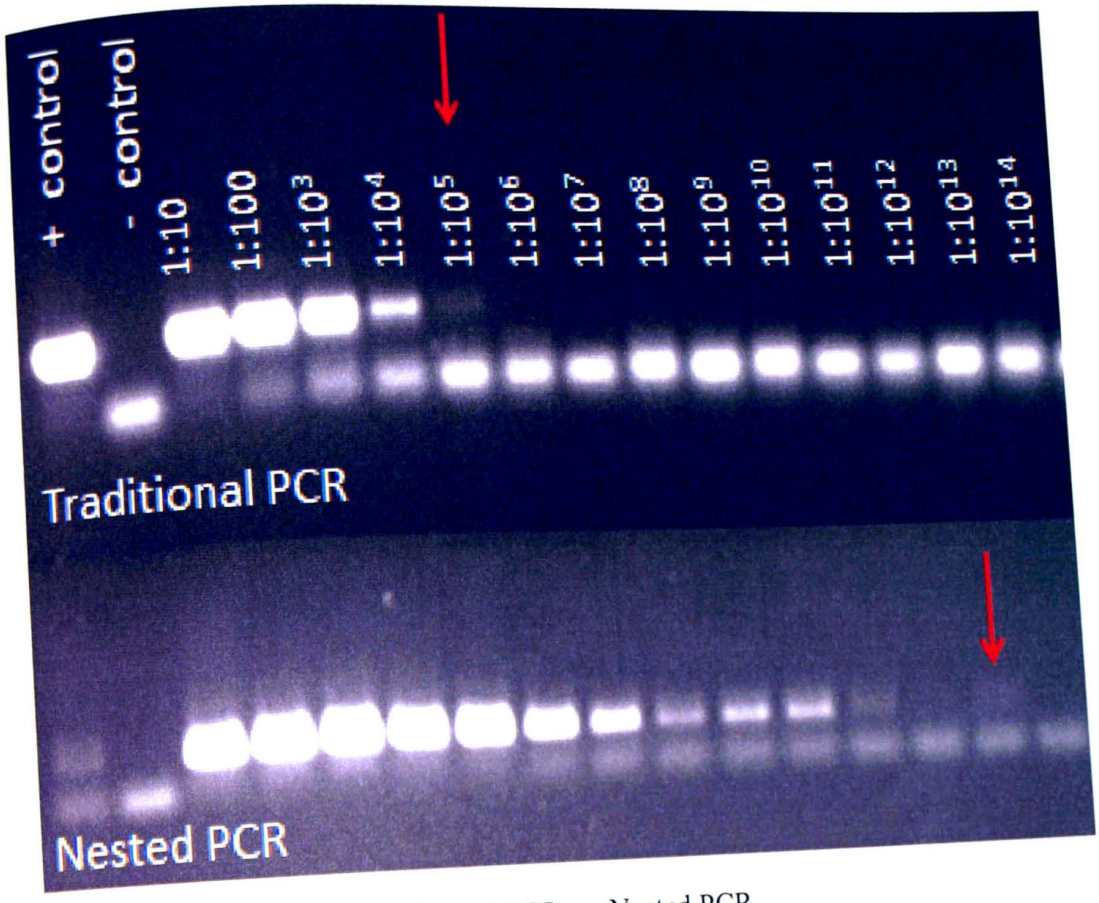


Figure 11. Comparison of Traditional PCR vs. Nested PCR.

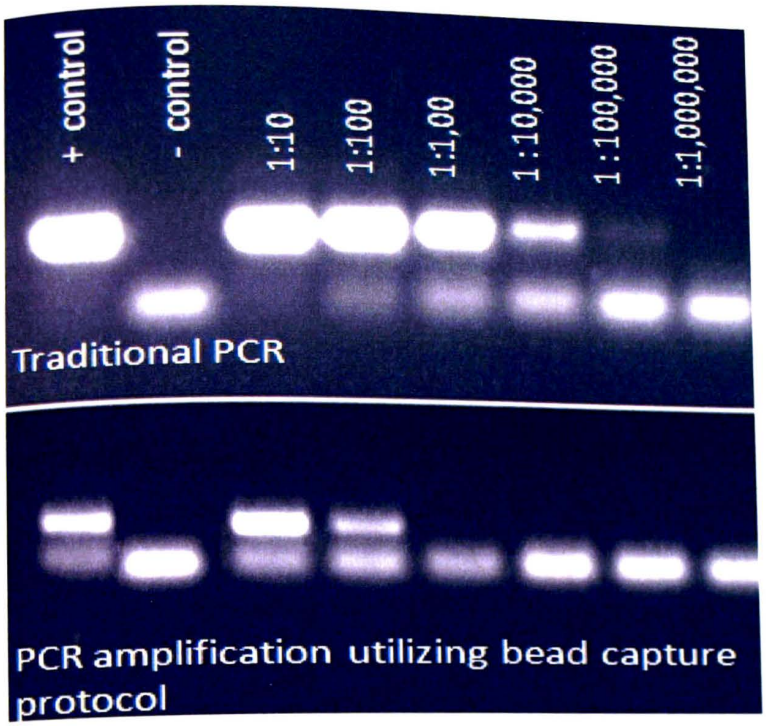


Figure 12. PCR amplification comparison of traditional PCR and PCR utilizing DNA bead capture protocol.

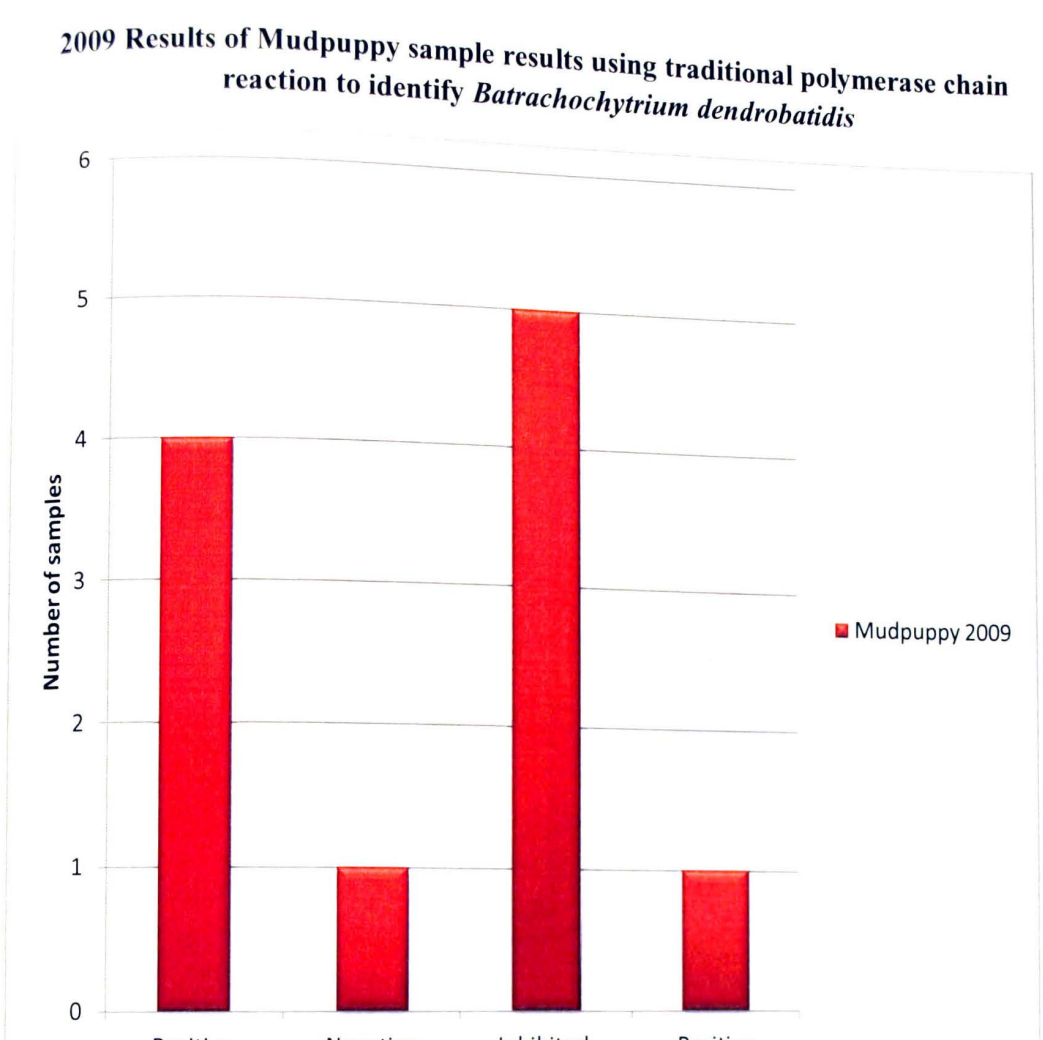


Figure 13. Summary of all results for presence/absence of Bd from 2009 field samples assessed in this study.

**2010 results of Hellbender and Mudpuppy samples processed in full at Austin Peay State University using traditional polymerase chain reaction to identify *Batrachochytrium dendrobatidis*.(not including samples from Pisces Molecular).**

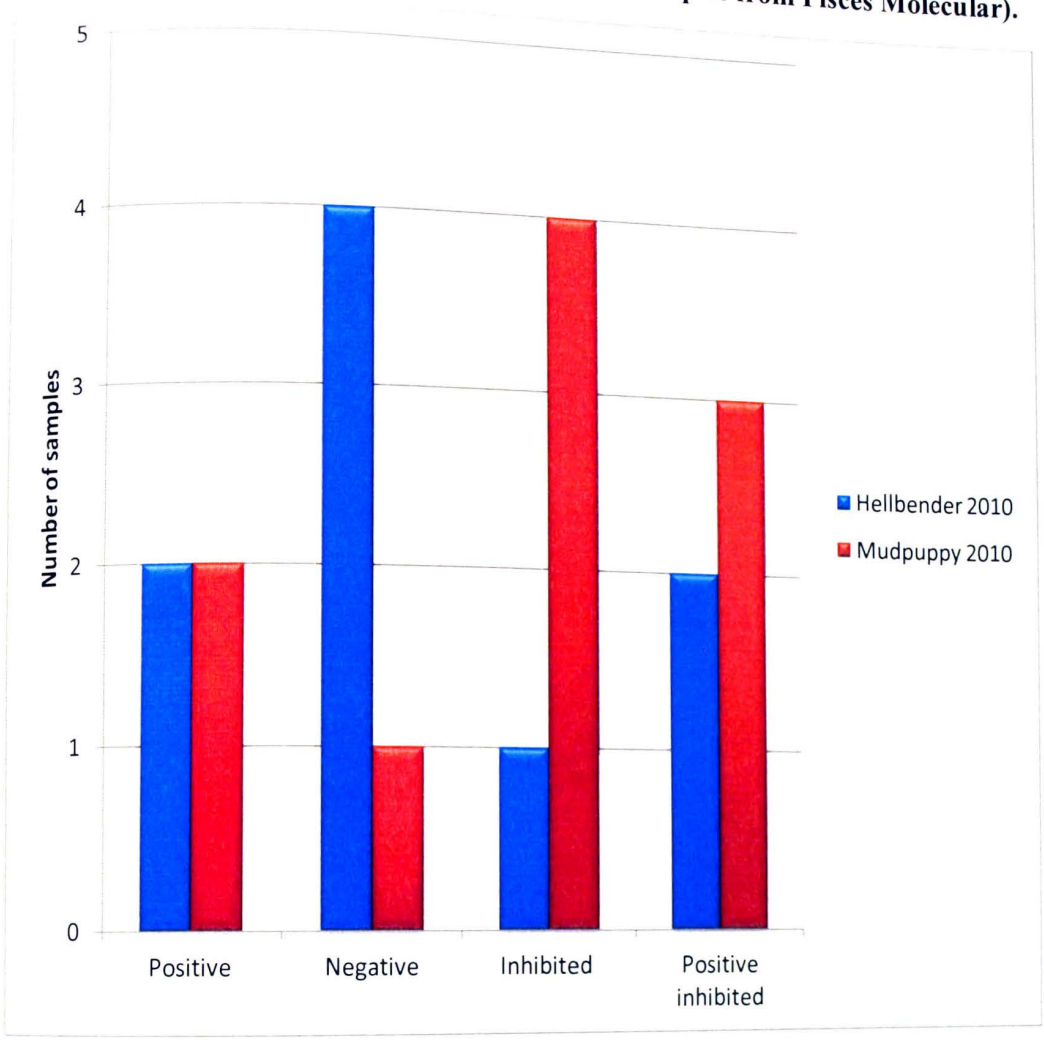


Figure 14. Summary of initial results for presence/absence of Bd from 2010 field samples processed in full at APSU assessed for this study.

**2011 Hellbender and Mudpuppy sample results using traditional polymerase chain reaction to identify *Batrachochytrium dendrobatidis*.**

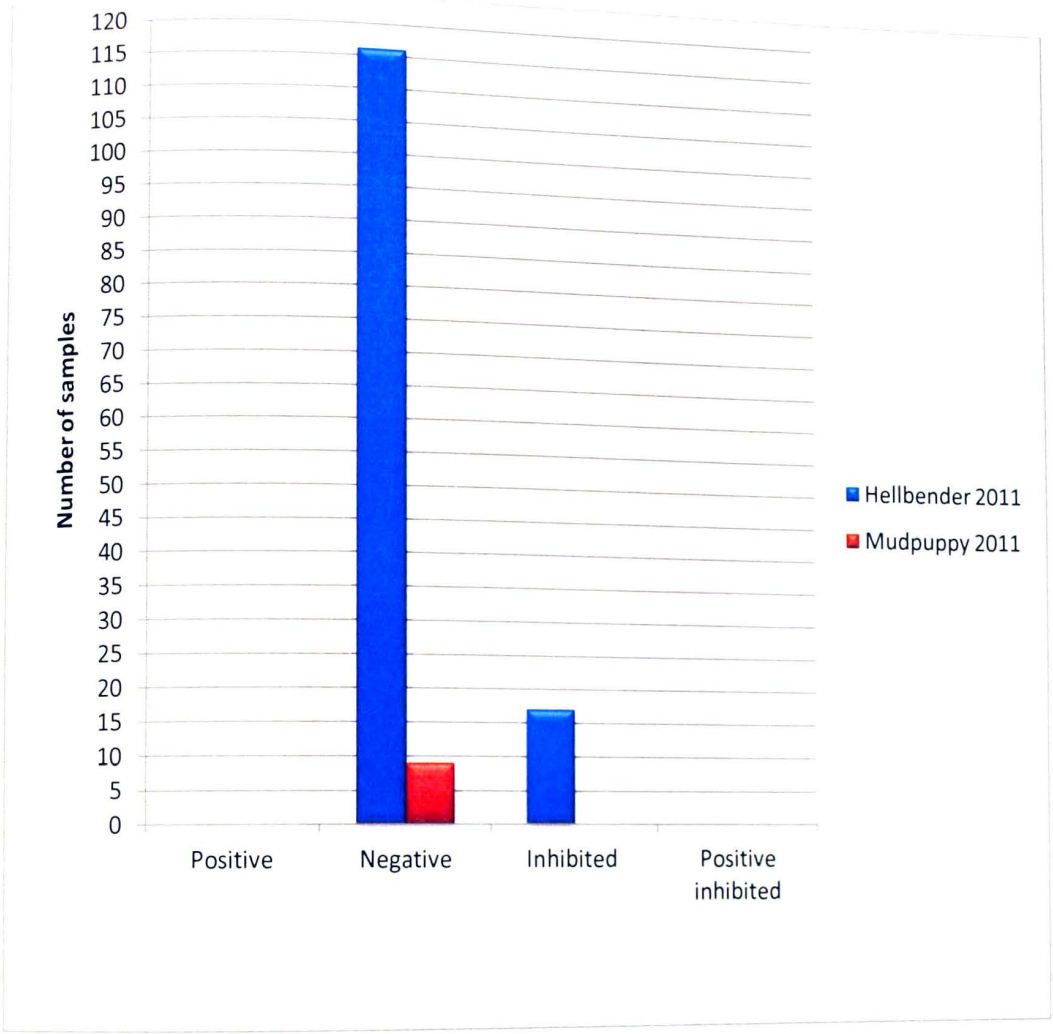


Figure 15. Summary of all results for presence/absence of Bd from 2011 field samples assessed in this study.

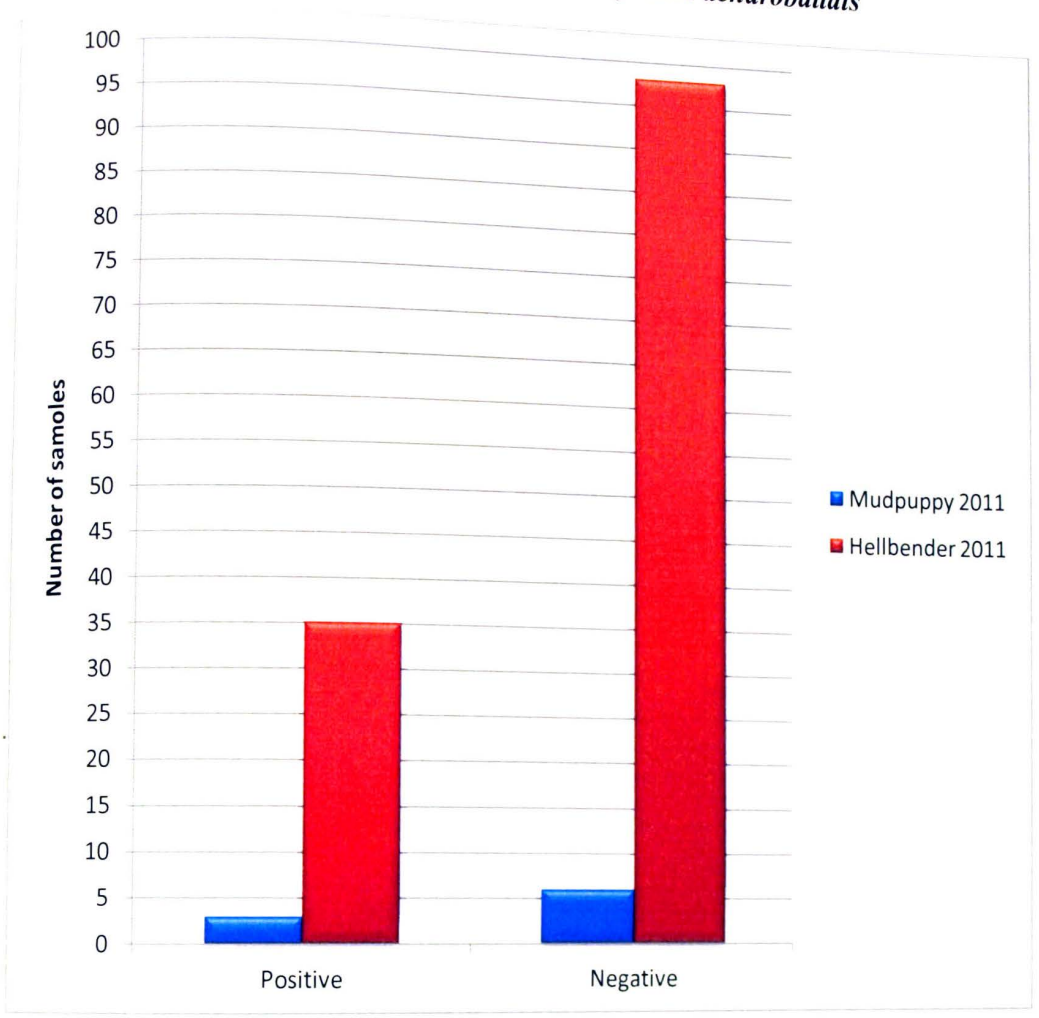


Figure 16. Summary of all results for presence/absence of Bd from 2011 field samples assessed in this study using nested PCR.

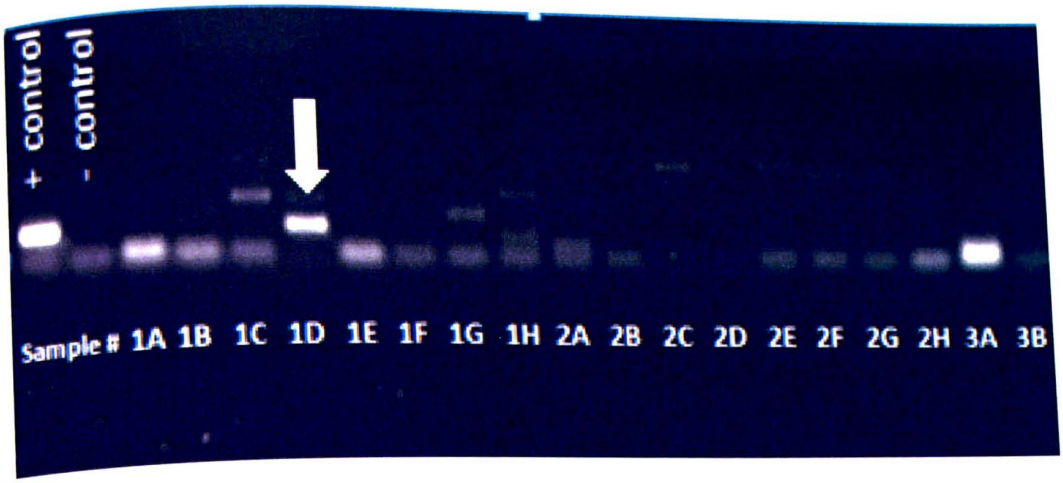


Figure 17. Example of traditional PCR gel of samples from Pisces Molecular.

Arrow indicates the only positive sample for this set of samples.

increased sensitivity (Fig. 18). Nested PCR revealed 68 positive samples for Bd (Fig.19). Initial PCR reactions yielded a 16.8% positive infection for Bd whereas nested PCR revealed 57.4% positive infection for Bd.

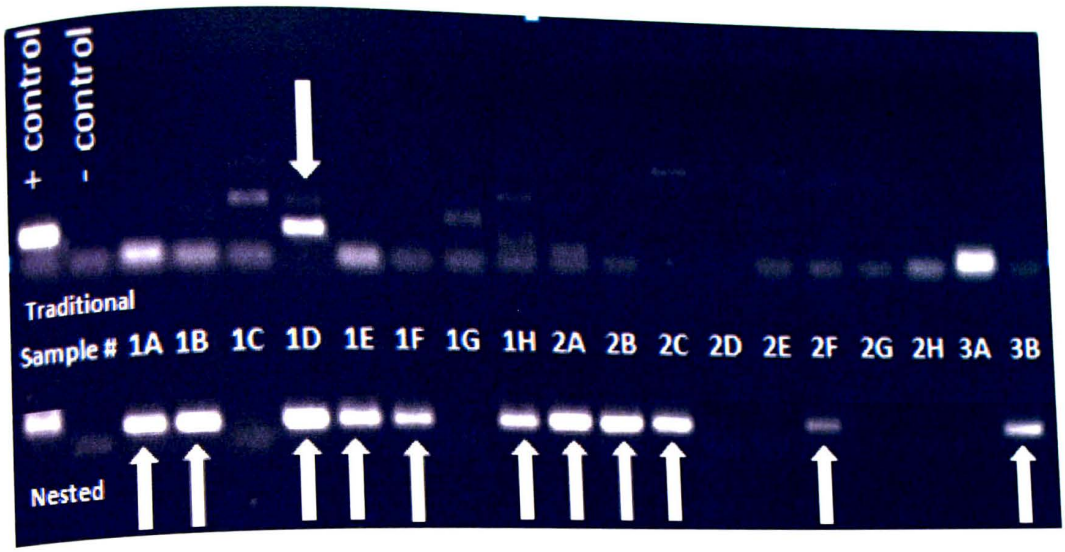


Figure 18. Example of Traditional PCR and nested PCR results for samples 1A-3B from 2010 samples originally processed by Pisces Molecular.

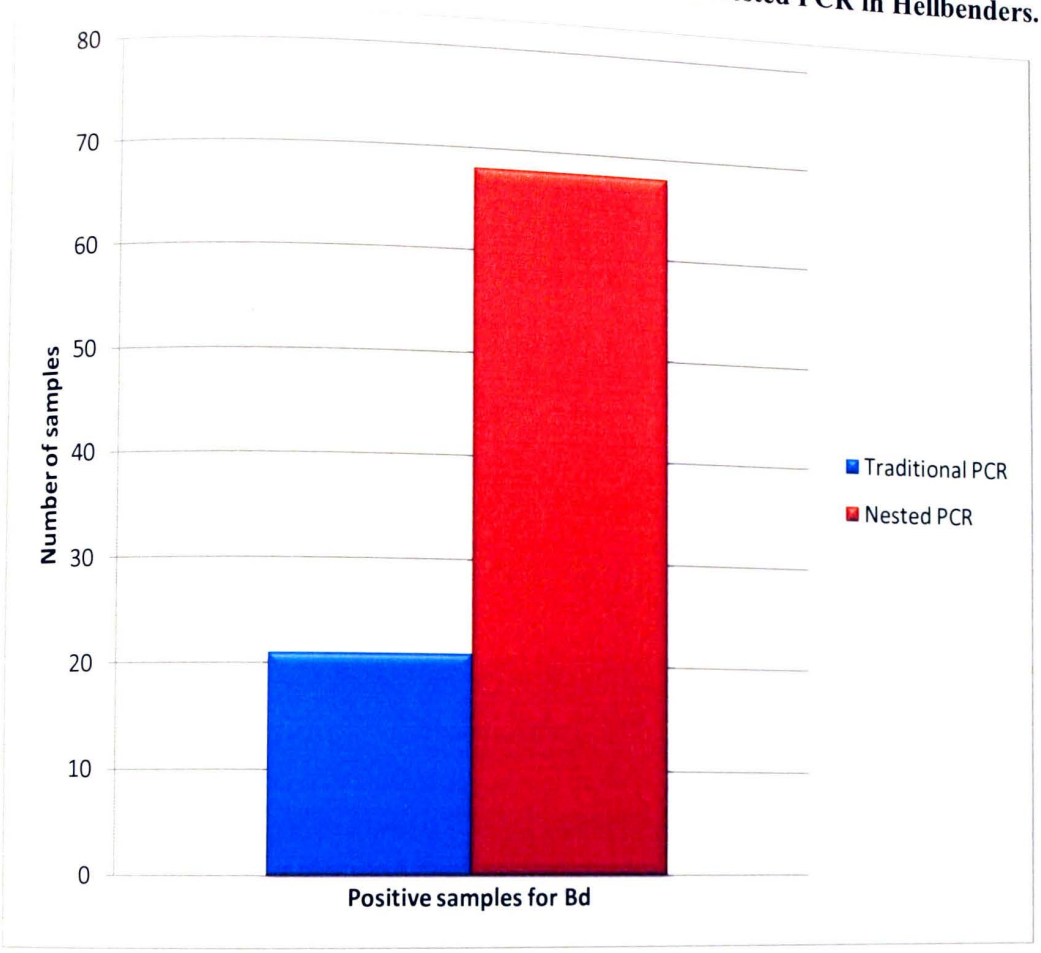


Figure 19. Traditional vs. Nested PCR results for samples received from Pisces Molecular.

# CHAPTER IV

## DISCUSSION

Comparison of field sample DNA extraction techniques using traditional PCR revealed processing of alcohol provided 1,000 times greater sensitivity than swab DNA extraction. Comparison of traditional PCR and nested PCR revealed that nested PCR was 10,000,000,000,000 times more sensitive than traditional PCR. Traditional PCR was also compared to traditional PCR utilizing Streptavidin beads to capture DNA prior to amplification. Results showed a decrease in sensitivity when beads were present during amplification. Beads caused a decrease in sensitivity of 1,000 times.

A positive result is derived when a sample returns a positive result at least once during the initial triplicate run of PCR for those samples of DNA extracted using the boiling method. A negative result is derived after the use of spike controls to determine if the sample is truly negative or simply inhibited by something in the PCR reaction. If the spike controls came back positive then the samples were determined to be truly negative and not inhibited. If the sample came back negative with spike controls then the sample was determined to be inhibited and further analysis was needed. All samples that came back negative during the spike control were diluted at a 1:5 ratio and re-spiked to try and dilute out any inhibitors in the sample DNA. All dilution spikes that came back positive were determined to be inhibition free at the dilution factor and were processed again 5 times without spike controls to determine positive and negative results. Samples that were positive at least once during the quintuplet run without spike controls were

determined to be positive with inhibition. Samples that came back negative at the 1:5 dilution with spike controls were determined to be inhibited beyond our means. These samples could have been diluted again, however, with every dilution in attempts to decrease the concentration of inhibitors, sample DNA was also being diluted, decreasing the possibility of yielding positive results. It is also important to note that with a dilution factor of five, five PCR reactions would only equivalate to one of the original PCR reactions. Dilutions not only decrease the likelihood of picking up inhibitors from the samples, but also any possible DNA in the sample.

There are several known inhibitors for PCR reactions derived from laboratory protocols for extracting DNA or storage of field samples such as phenols, salts, and alcohols (10). For this study we chose to extract DNA by boiling the samples to reduce possible inhibition and loss of DNA during the cleaning process. Although samples were stored in 70% EtOH until processing could be completed, extra precaution was taken to remove all EtOH from sample DNA to insure no inhibition due to processing. As a control, DNA extracted from cultures in the laboratory were also stored in 70% EtOH over a 24 hour period and then extracted to ensure removal of all alcohol in the extraction process.

There are several naturally occurring inhibitors for PCR reactions such as complex polysaccharides, humic acid from plant materials, bile salts and urea (10). Humic acid, which occurs naturally in soil, has been shown to yield false negatives in PCR samples due to soil being present in collected field samples (10,32). Pessier also reports that wooden handled swabs have shown to cause inhibition in PCR reactions (32). All of the samples processed at APSU were from wooden handled swabs.

A total of 28 samples, 16.3%, that were processed in full at APSU were determined to be PCR inhibited, 19 of which were hellbenders and 9 mudpuppies. When we compared the number of inhibited hellbenders to the overall total of sampled hellbenders, we find that 13.4% of sampled hellbenders were PCR inhibitory. When we compare the number of inhibited mudpuppy samples to the overall total of sampled mudpuppy, we find 30% were PCR inhibitory. Further analysis of samples could be done to try and decipher the cause of PCR inhibition; however, this study did not investigate inhibitors, it simply identified them as a problem in field sampling.

In this study traditional PCR results for samples originally processed by Pisces Molecular have been reported as well as nested PCR results for 2011 samples. Due to Pisces DNA extraction method, Qiagen DNeasy kit, field inhibitors should not be of concern thus samples were ran two fold and then nested. The use of nested PCR clearly shows an increase in sensitivity and yields higher results with an increase >40% of positive infection for Bd. This is a large increase from the traditional PCR results of only 16.8% positively infected with Bd. To further support nested PCR, 2011 samples initially revealed 0% infection of Bd even at the dilution factor. When 2011 samples were nested, a 26.8% of samples were identified as positive for Bd infection. These results show that nested PCR should be considered as part of the normal necessary protocol for testing field samples for Bd.

## APPENDIX

Table 3. Results of all data collected for DNA extracted at Austin Peay State University.

| APSU ID # | Source ID | Source Information | Traditional PCR | Dilution PCR | Nested PCR |
|---|---|---|---|---|---|
| 1 | LM103 NMAC | LM103 07-06-2010 NMAC | - | x | n/a |
| 2 | LM102 NMAC | LM102 07-06-2010 NMAC | - | Positive | n/a |
| 3 | LM009 NMAC | LM009 07-21-2009 NMAC | - | Positive | n/a |
| 4 | LM004 NMAC | LM004 07-16-2009 NMAC | POSITIVE | x | n/a |
| 5 | LM006 NMAC | LM006 07-21-2009 NMAC | - | x | n/a |
| 6 | LM015 CALL | LM015 07-06-2010 CALL | - | Positive | n/a |
| 7 | LM007 NMAC | LM007 07-21-2009 NMAC | - | (inhibited) | n/a |
| 8 | FR002 NMAC | FR002 09-18-2010 NMAC | - | Positive | n/a |
| 9 | LM002 NMAC | LM002 07-16-2009 NMAC | - | (inhibited) | n/a |
| 10 | FR001 NMAC | FR001 06-30-2010 NMAC | - | (inhibited) | n/a |
| 11 | FMR001 NMAC | FMR001 07-28-2010 NMAC | - | Positive | n/a |
| 12 | LM101 NMAC | LM101 07-06-2010 NMAC | - | (inhibited) | n/a |
| 13 | LM106 NMAC | LM106 07-27-2010 NMAC | - | (inhibited) | n/a |
| 14 | LM003 NMAC | LM003 07-16-2009 NMAC | - | (inhibited) | n/a |
| 15 | LM105 NMAC | LM105 07-27-2010 NMAC | - | (inhibited) | n/a |
| 16 | LM004 CALL | LM004 07-01-2010 CALL | POSITIVE | x | n/a |
| 17 | LM011 NMAC | LM011 07-21-2009 NMAC | POSITIVE | x | n/a |
| 18 | LM001 NMAC | LM001 07-16-2009 NMAC | POSITIVE | x | n/a |
| 19 | TBLV001 RCAT | TBLV001 07-08-2010 RCAT | POSITIVE | x | n/a |
| 20 | LM104 NMAC | LM104 07-06-2010 NMAC | POSITIVE | x | n/a |
| 21 | LM018 CALL | LM018 07-06-2010 CALL | POSITIVE | x | n/a |
| 22 | LM010 NMAC | LM010 07-21-2009 NMAC | - | (inhibited) | n/a |
| 23 | LM002 CALL | LM002 07-01-2010 CALL | - | (inhibited) | n/a |

*Table 3. Results of all data collected for DNA extracted at Austin Peay State University Continued.*

| APSU ID # | Source ID | Source Information | Traditional PCR | Dilution PCR | Nested PCR |
|---|---|---|---|---|---|
| 24 | LM005 NMAC | LM005 07-21-2009 NMAC | - | (inhibited) | n/a |
| 25 | LM008 NMAC | LM008 07-21-2009 NMAC | POSITIVE | x | n/a |
| 26 | LM029 CALL | LM029 07-06-2010 CALL | | Positive | n/a |
| 27 | LM034 CALL | LM034 07-06-2010 CALL | - | x | n/a |
| 28 | LM035 CALL | LM035 07-06-2010 CALL | - | x | n/a |
| 29 | LM042 CALL | LM042 07-27-2010 CALL | - | x | n/a |
| 30 | LM024 CALL | LM024 07-06-2010 CALL | - | x | n/a |
| 31 | LM001 NMAC | LM001 07-12-2011 NMAC | - | - | |
| 32 | LM002 NMAC | LM002 07-12-2011 NMAC | - | - | - |
| 33 | LM003 NMAC | LM003 07-12-2011 NMAC | - | - | - |
| 34 | LM004 NMAC | LM004 07-12-2011 NMAC | - | - | - |
| 35 | LM005 NMAC | LM005 07-21-2011 NMAC | - | - | - |
| 36 | LM006 NMAC | LM006 07-21-2011 NMAC | - | - | Positive |
| 37 | LM007 NMAC | LM007 07-231-2011 NMAC | - | - | - |
| 38 | TION-B-001 NMAC | TION-B-001 07-18-2011 NMAC | - | - | Positive |
| 39 | TION-B-002 NMAC | TION-B-002 07-18-2011 NMAC | - | - | Positive |
| 40 | SALM001 CALL | SALM001 07-19-2011 CALL | - | - | - |
| 41 | SALM002 CALL | SALM002 07-19-2011 CALL | - | - | Positive |
| 42 | TB001 CALL | TB001 07-15-2011 CALL | - | - | - |
| 43 | TB002 CALL | TB002 7-15-2011 CALL | - | (inhibited) | - |
| 44 | TB003 CALL | TB003 7-15-2011 CALL | - | - | - |
| 45 | TB004 CALL | TB004 7-15-2011 CALL | - | - | - |
| 46 | TB005 CALL | TB005 7-15-2011 CALL | - | - | - |

*Table 3. Results of all data collected for DNA extracted at Austin Peay State University Continued.*

| APSU ID # | Source ID | Source Information | Traditional PCR | Dilution PCR | Nested PCR |
|---|---|---|---|---|---|
| 47 | TB006 CALL | TB006 7-15-2011 CALL | - | - | - |
| 48 | TB007 CALL | TB007 7-15-2011 CALL | - | - | - |
| 49 | TB008 CALL | TB008 8-2-2011 CALL | - | - | - |
| 50 | TB009 CALL | TB009 8-2-2011 CALL | - | - | Positive |
| 51 | TB010 CALL | TB010 8-2-2011 CALL | - | - | Positive |
| 52 | TB011 CALL | TB011 8-2-2011 CALL | - | - | - |
| 53 | TB012 CALL | TB012 8-2-2011 CALL | - | - | - |
| 54 | LM001 CALL | LM001 7-12-2011 CALL | - | - | - |
| 55 | LM002 CALL | LM002 7-12-2011 CALL | - | - | - |
| 56 | LM003 CALL | LM003 7-12-2011 CALL | - | - | Positive |
| 57 | LM004 CALL | LM004 7-12-2011 CALL | - | - | - |
| 58 | LM005 CALL | LM005 7-12-2011 CALL | - | - | - |
| 59 | LM006 CALL | LM006 7-21-2011 CALL | - | - | - |
| 60 | LM007 CALL | LM007 7-12-2011 CALL | - | - | Positive |
| 61 | LM008 CALL | LM008 7-12-2011 CALL | - | - | - |
| 62 | LM009 CALL | LM009 7-12-2011 CALL | - | - | Positive |
| 63 | LM010 CALL | LM010 7-12-2011 CALL | - | - | Positive |
| 64 | LM011 CALL | LM011 7-12-2011 CALL | - | - | Positive |
| 65 | LM012 CALL | LM012 7-12-2011 CALL | - | (inhibited) | - |
| 66 | LM013 CALL | LM013 7-12-2011 CALL | - | - | - |
| 67 | LM014 CALL LF #3 CLIPPED | LM014 7-21-2011 CALL | - | - | Positive |
| 68 | LM015 CALL | LM015 7-21-2011 CALL | - | (inhibited) | - |
| 69 | LM016 CALL | LM016 7-21-2011 CALL | - | - | - |

*Table 3. Results of all data collected for DNA extracted at Austin Peay State University Continued.*

| APSU ID # | Source ID | Source Information | Traditional PCR | Dilution PCR | Nested PCR |
|---|---|---|---|---|---|
| 70 | LM017 CALL | LM017 7-21-2011 CALL | - | - | - |
| 71 | LM018 CALL | LM018 7-21-2011 CALL | - | - | - |
| 72 | LM019 CALL | LM019 7-21-2011 CALL | - | - | - |
| 73 | LM020 CALL | LM020 7-21-2011 CALL | - | - | - |
| 74 | LM021 CALL | LM021 8-2-2011 CALL | - | - | - |
| 75 | TION001 CALL | TION001 7-26-2011 CALL | - | - | - |
| 76 | TION002 CALL | TION002 7-26-2011 CALL | - | - | - |
| 77 | TION003 CALL | TION003 7-26-2011 CALL | - | - | - |
| 78 | TION004 CALL | TION004 7-26-2011 CALL | - | - | Positive |
| 79 | TION005 CALL | TION005 7-26-2011 CALL | - | - | - |
| 80 | TION006 CALL | TION006 7-26-2011 CALL | - | - | - |
| 81 | TION007 CALL | TION007 7-26-2011 CALL | - | - | - |
| 82 | TION008 CALL | TION008 7-26-2011 CALL | - | - | - |
| 83 | TION009 CALL | TION009 7-26-2011 CALL | - | - | - |
| 84 | TION010 CALL | TION010 7-26-2011 CALL | - | - | - |
| 85 | TION011 CALL | TION011 08-04-2011 CALL | - | - | - |
| 86 | TION012 CALL | TION012 08-04-2011 CALL | - | - | - |
| 87 | TION013 CALL | TION013 08-04-2011 CALL | - | - | - |
| 88 | TION014 CALL | TION014 08-04-2011 CALL | - | - | Positive |
| 89 | TION015 CALL | TION015 08-04-2011 CALL | - | - | - |
| 90 | TION016 CALL | TION016 08-04-2011 CALL | - | - | Positive |
| 91 | TION017 CALL | TION017 08-04-2011 CALL | - | - | - |
| 92 | TION018 CALL | TION018 08-04-2011 CALL | - | - | - |
| 93 | TION019 CALL | TION019 08-04-2011 CALL | - | - | - |

*Table 3. Results of all data collected for DNA extracted at Austin Peay State University Continued.*

| APSU ID # | Source ID | Source Information | Traditional PCR | Dilution PCR | Nested PCR |
|---|---|---|---|---|---|
| 94 | TION020 CALL | TION020 08-04-2011 CALL | - | - | - |
| 95 | TION021 CALL | TION021 08-04-2011 CALL | - | - | - |
| 96 | TION022 CALL | TION022 08-04-2011 CALL | - | - | - |
| 97 | TION023 CALL | TION023 08-06-2011 CALL | - | - | - |
| 98 | TION024 CALL | TION024 08-06-2011 CALL | - | - | - |
| 99 | TION025 CALL | TION025 08-08-2011 CALL | - | - | - |
| 100 | TION026 CALL | TION026 08-08-2011 CALL | - | - | - |
| 101 | TION027 CALL | TION027 08-08-2011 CALL | - | - | - |
| 102 | TION028 CALL | TION028 08-08-2011 CALL | - | - | - |
| 103 | TION029 CALL | TION029 08-09-2011 CALL | - | (inhibited) | - |
| 104 | TION030 CALL | TION030 08-09-2011 CALL | - | - | Positive |
| 105 | TION031 CALL | TION031 08-09-2011 CALL | - | - | Positive |
| 106 | TION032 CALL | TION032 08-13-2011 CALL | - | - | - |
| 107 | TION033 CALL | TION033 08-13-2011 CALL | - | - | - |
| 108 | TION034 CALL | TION034 08-13-2011 CALL | - | (inhibited) | Positive |
| 109 | TION035 CALL | TION035 08-13-2011 CALL | - | - | Positive |
| 110 | TION036 CALL | TION036 08-13-2011 CALL | - | - | Positive |
| 111 | TION037 CALL | TION037 08-13-2011 CALL | - | - | Positive |
| 112 | TION038 CALL | TION038 08-13-2011 CALL | - | - | Positive |
| 113 | TION039 CALL | TION039 08-13-2011 CALL | - | - | Positive |
| 114 | TION040 CALL | TION040 08-13-2011 CALL | - | - | Positive |
| 115 | TION041 CALL | TION041 08-13-2011 CALL | - | (inhibited) | Positive |
| 116 | TION042 CALL | TION042 08-13-2011 CALL | - | - | Positive |

*Table 3. Results of all data collected for DNA extracted at Austin Peay State University Continued.*

| APSU ID # | Source ID | Source Information | Traditional PCR | Dilution PCR | Nested PCR |
|---|---|---|---|---|---|
| 117 | TION-B-001 CALL | TION-B-001 07-18-2011 CALL | - | (inhibited) | Positive |
| 118 | TION-C-001 CALL | TION-C-001 07-19-2011 CALL | - | - | Positive |
| 119 | TION-C-002 CALL | TION-c-002 07-19-2011 CALL | - | - | Positive |
| 120 | TION-C-003 CALL | TION-C-003 07-19-2011 CALL | - | - | Positive |
| 121 | TION-C-004 CALL | TION-C-004 07-19-2011 CALL | - | - | - |
| 122 | TION-C-005 CALL | TION-C-005 07-19-2011 CALL | - | - | Positive |
| 123 | FR001 CALL | FR001 07-14-2011 CALL | - | (inhibited) | - |
| 124 | FR002 CALL | FR002 07-14-2011 CALL | - | - | - |
| 125 | FR003 CALL | FR003 07-14-2011 CALL | - | - | Positive |
| 126 | FR004 CALL | FR004 07-14-2011 CALL | - | - | - |
| 127 | FR005 CALL | FR005 07-14-2011 CALL | - | - | - |
| 128 | FR006 CALL | FR006 07-14-2011 CALL | - | - | - |
| 129 | FR007 CALL | FR007 07-14-2011 CALL | - | (inhibited) | Positive |
| 130 | FR008 CALL | FR008 08-11-2011 CALL | - | - | - |
| 131 | FR009 CALL | FR009 08-11-2011 CALL | - | - | - |
| 132 | FR010 CALL | FR010 08-11-2011 CALL | - | - | - |
| 133 | FR011 CALL | FR011 08-11-2011 CALL | - | - | - |
| 134 | FR012 CALL | FR012 08-11-2011 CALL | - | - | - |
| 135 | FR013 CALL | FR013 08-11-2011 CALL | - | - | Positive |
| 136 | FR014 CALL | FR014 08-11-2011 CALL | - | - | - |
| 137 | FR015 CALL | FR015 08-11-2011 CALL | - | (inhibited) | Positive |
| 138 | FR016 CALL | FR016 08-11-2011 CALL | - | - | - |
| 139 | FR017 CALL | FR017 08-11-2011 CALL | - | - | - |

*Table 3. Results of all data collected for DAN extracted at Austin Peay State University Continued.*

| APSU ID # | Source ID | Source Information | Traditional PCR | Dilution PCR | Nested PCR |
|---|---|---|---|---|---|
| 140 | FR018 CALL | FR018 08-11-2011 CALL | - | (inhibited) | - |
| 141 | FR019 CALL | FR019 08-11-2011 CALL | - | - | - |
| 142 | FR020 CALL | FR020 08-11-2011 CALL | - | (inhibited) | - |
| 143 | FR021 CALL | FR021 08-11-2011 CALL | - | - | - |
| 144 | FR022 CALL | FR022 08-11-2011 CALL | - | - | Positive |
| 145 | FR023 CALL | FR023 08-11-2011 CALL | - | - | - |
| 146 | FR024 CALL | FR024 08-11-2011 CALL | - | - | - |
| 147 | FR025 CALL | FR025 08-11-2011 CALL | - | (inhibited) | - |
| 148 | FR026 CALL | FR026 08-11-2011 CALL | - | - | - |
| 149 | FR027 CALL | FR027 08-11-2011 CALL | - | (inhibited) | - |
| 150 | FR028 CALL | FR028 08-11-2011 CALL | - | (inhibited) | - |
| 151 | FR029 CALL | FR029 08-11-2011 CALL | - | - | - |
| 152 | FR030 CALL | FR030 08-11-2011 CALL | - | - | Positive |
| 153 | FR031 CALL | FR031 08-12-2011 CALL | - | - | - |
| 154 | FR032 CALL | FR032 08-12-2011 CALL | - | - | - |
| 155 | FR033 CALL | FR033 08-12-2011 CALL | - | - | - |
| 156 | FR034 CALL | FR034 08-12-2011 CALL | - | - | - |
| 157 | FR035 CALL | FR035 08-12-2011 CALL | - | - | - |
| 158 | FR036 CALL | FR036 08-12-2011 CALL | - | (inhibited) | - |
| 159 | FR037 CALL | FR037 08-12-2011 CALL | - | - | - |
| 160 | FR038 CALL | FR038 08-12-2011 CALL | - | - | - |
| 161 | FR039 CALL | FR039 08-12-2011 CALL | - | - | - |
| 162 | FR040 CALL | FR040 08-12-2011 CALL | - | - | - |

*Table 3. Results of all data collected for DNA extracted at Austin Peay State University Continued.*

| APSU ID # | Source ID | Source Information | Traditional PCR | Dilution PCR | Nested PCR |
|---|---|---|---|---|---|
| 163 | FR041 CALL | FR041 08-12-2011 CALL | - | - | - |
| 164 | FR042 CALL | FR042 08-12-2011 CALL | - | - | - |
| 165 | FR043 CALL | FR043 08-12-2011 CALL | - | - | - |
| 166 | FR044 CALL | FR044 08-12-2011 CALL | - | (inhibited) | - |
| 167 | FR045 CALL | FR045 08-12-2011 CALL | - | - | - |
| 168 | FR046 CALL | FR046 08-12-2011 CALL | - | - | Positive |
| 169 | FR047 CALL | FR047 08-12-2011 CALL | - | - | - |
| 170 | FR048 CALL | FR048 08-12-2011 CALL | - | - | - |
| 171 | FR049 CALL | FR049 08-12-2011 CALL | - | - | - |
| 172 | FR050 CALL | FR050 08-12-2011 CALL | - | - | - |

Table 4. Results of data collected from samples originally processed by Pisces Molecular and re-tested at Austin Peay State University.

| Plate # | Well # | Pisces # | Source ID | Source Information | Traditional PCR | Nested PCR |
|---|---|---|---|---|---|---|
| Plate 1 | 1A | 104790 | FR006 | 7-22-10 CALL (on tube)French Creek (on bag) | - | POSITIVE |
| Plate 1 | 1B | 104791 | FC012 | 7-22-10 CALL (on tube)French Creek (on bag) | - | POSITIVE |
| Plate 1 | 1C | 104792 | FC013 | 7-22-10 CALL (on tube)French Creek (on bag) | - | - |
| Plate 1 | 1D | 104793 | FC014 | 7-22-10 CALL (on tube)French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 1E | 104794 | FC015 | 7-22-10 CALL (on tube)French Creek (on bag) | - | POSITIVE |
| Plate 1 | 1F | 104795 | FC016 | 7-22-10 CALL (on tube)French Creek (on bag) | - | POSITIVE |
| Plate 1 | 1G | 104796 | FC017 | 7-22-10 CALL (on tube)French Creek (on bag) | - | - |
| Plate 1 | 1H | 104797 | FR012 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 2A | 104798 | FR013 | 8-25-10 CALL (on tube) French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 2B | 104799 | FR014 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 2C | 104800 | FR015 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 2D | 104801 | FR016 | 8-25-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 2E | 104802 | FR017 | 8-25-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 2F | 104803 | FR018 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 2G | 104804 | FR019 | 8-25-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 2H | 104805 | FR020 | 8-25-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 3A | 104806 | FR021 | 9-18-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 3B | 104807 | FR022 | 9-18-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 3C | 104808 | FR023 | 9-18-10 CALL (on tube) French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 3D | 104809 | FR024 | 9-18-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 3E | 104810 | FR025 | 9-18-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 3F | 104811 | FR027 | 9-18-10 CALL (on tube) French Creek (on bag) | - | - |

*Table 4. Results of data collected for samples originally processed by Pisces Molecular and re-tested at Austin Peay State University Continued.*

| Plate # | Well # | Pisces # | Source ID | Source Information | Traditional PCR | Nested PCR |
|---|---|---|---|---|---|---|
| Plate 1 | 3G | 104812 | FR028 | 9-18-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 3H | 104813 | FR029 | 9-18-10 CALL (on tube) French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 4A | 104814 | FR030 | 9-18-10 CALL (on tube) French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 4B | 104815 | FR031 | 9-18-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 4C | 104816 | FR032 | 9-18-10 CALL (on tube) French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 4D | 104817 | LM 002 | 2 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 4E | 104818 | LMAH 008 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 4F | 104819 | LMAH 009 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 4G | 104820 | LMAH 010 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 4H | 104821 | LM 008 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 5A | 104822 | LM 009 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 5B | 104823 | LM 010 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 5C | 104824 | LM 011 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 5D | 104825 | LM 012 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 5E | 104826 | LM 013 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 5F | 104827 | LM 014 | 2 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 5G | 104828 | LM 017 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 5H | 104829 | LM 019 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 6A | 104830 | LM 020 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 6B | 104831 | LM 021 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 6C | 104832 | LM 022 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 6D | 104833 | LM 023 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 6E | 104834 | LM 025 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |

*Table 4. Results of data collected for samples originally processed by Pisces Molecular and re-tested at Austin Peay State University Continued.*

| Plate # | Well # | Pisces # | Source ID | Source Information | Traditional PCR | Nested PCR |
|---|---|---|---|---|---|---|
| Plate 1 | 6F | 104835 | LM 026 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 6G | 104836 | LM 028 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 6H | 104837 | LM 030 | 07-13-10 CALL (on tube) Tubmil/Hendricks (on bag) | - | POSITIVE |
| Plate 1 | 7A | 104721 | FC002 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 7B | 104722 | FR003 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 7C | 104723 | FC004 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 7D | 104724 | FR005 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 7E | 104725 | FC006 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 7F | 104726 | FC007 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 7G | 104727 | FC008 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 7H | 104728 | FC009 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 8A | 104729 | FC010 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 8B | 104730 | FC011 | 7-22-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 8C | 104731 | FR012 | 8-25-10 CALL (on tube) French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 8D | 104732 | FR013 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 8E | 104733 | FR014 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 8F | 104734 | FR015 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 8G | 104735 | FR016 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 8H | 104736 | FT017 | 8-25-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 9A | 104737 | FR018 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 9B | 104738 | FR019 | 8-25-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 9C | 104739 | FR020 | 8-25-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 9D | 104740 | FR021 | 9-28-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |

*Table 4. Results of data collected for samples originally processed by Pisces Molecular and re-tested at Austin Peay State University Continued.*

| Plate # | Well # | Pisces # | Source ID | Source Information | Traditional PCR | Nested PCR |
|---|---|---|---|---|---|---|
| Plate 1 | 9E | 104741 | FR022 | 9-28-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 9F | 104742 | FR023 | 9-28-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 9G | 104743 | FR024 | 9-28-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 9H | 104744 | FR025 | 9-28-10 CALL (on tube) French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 10A | 104745 | FR027 | 9-28-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 10B | 104746 | FR028 | 9-28-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 10C | 104747 | FR029 | 9-28-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 10D | 104748 | FR030 | 9-28-10 CALL (on tube) French Creek (on bag) | - | POSITIVE |
| Plate 1 | 10E | 104749 | FR031 | 9-28-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 10F | 104750 | FR032 | 9-28-10 CALL (on tube) French Creek (on bag) | - | - |
| Plate 1 | 10G | 104751 | LM 001 | 1 July 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 10H | 104752 | LMAH 005 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 11A | 104753 | LMAH 006 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 11B | 104754 | LMAH 007 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 11C | 104755 | LM 008 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 11D | 104756 | LM 009 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 11E | 104757 | LM 010 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 11F | 104758 | LM 011 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 11G | 104759 | LM 012 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 11H | 104760 | LM 013 | 1 Jul 10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 1 | 12A | 104761 | LM 014 | 1 July 10 CALL (on tube) Little Mahoning (on bag) | 0 | - |
| Plate 1 | 12B | 104762 | LM 017 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |

*Table 4. Results of data collected for samples originally processed by Pisces Molecular and re-tested at Austin Peay State University Continued.*

| Plate # | Well # | Pisces # | Source ID | Source Information | Traditional PCR | Nested PCR |
|---|---|---|---|---|---|---|
| Plate 1 | 12C | 104763 | LM 019 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 12D | 104764 | LM 020 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 12E | 104765 | LM 021 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 12F | 104766 | LM 022 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 1 | 12G | 104767 | LM 023 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 1 | 12H | 104768 | LM 025 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | POSITIVE |
| Plate 2 | 1A | 104769 | LM 026 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 2 | 1B | 104770 | LM 028 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 2 | 1C | 104771 | LM 030 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 2 | 1D | 104772 | LM 031 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 2 | 1E | 104773 | LM 032 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 2 | 1F | 104774 | LM 033 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 2 | 1G | 104775 | LM 036 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 2 | 1H | 104776 | LM 037 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 2 | 2A | 104777 | LM 038 | 07-6-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 2 | 2B | 104778 | LM 039 | 7-27-10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 2 | 2C | 104779 | LM 040 | 7-27-10 CALL (on tube) Little Mahoning (on bag) | POSITIVE | POSITIVE |
| Plate 2 | 2D | 104780 | LM 044 | 7-27-10 CALL (on tube) Little Mahoning (on bag) | - | - |
| Plate 2 | 2E | 104781 | TION 002 | 7-7-10 CALL (on tube) Tionesta (on bag) | - | - |
| Plate 2 | 2F | 104782 | Tion 003 | 7-7-10 CALL (on tube) Tionesta (on bag) | - | - |
| Plate 2 | 2G | 104783 | Tion 004 | 7-7-10 CALL (on tube) Tionesta (on bag) | - | POSITIVE |
| Plate 2 | 2H | 104784 | TION 005 | 7-7-10 CALL (on tube) Tionesta (on bag) | - | POSITIVE |

Table 4. Results of data collected for samples originally processed by Pisces Molecular and re-tested at Austin Peay State University Continued.

| Plate # | Well # | Pisces # | Source ID | Source Information | Traditional PCR | Nested PCR |
|---|---|---|---|---|---|---|
| Plate 2 | 3A | 104785 | TION 006 | 7-7-10 CALL (on tube) Tionesta (on bag) | - | - |
| Plate 2 | 3B | 104786 | TION 007 | 7-7-10 CALL (on tube) Tionesta (on bag) | - | - |
| Plate 2 | 3C | 104787 | FC003 | 7-22-10 CALL (on tube)French Creek (on bag) | - | POSITIVE |
| Plate 2 | 3D | 104788 | FR004 | 7-22-10 CALL (on tube)French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 2 | 3E | 104789 | FC005 | 7-22-10 CALL (on tube)French Creek (on bag) | POSITIVE | POSITIVE |
| Plate 2 | 3F | 104838 | LM 031 | 7-22-10 CALL (on tube)French Creek (on bag) | - | - |
| Plate 2 | 3G | 104839 | HC 007 | 7-22-10 CALL (on tube)French Creek (on bag) | - | - |

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