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Presence of *Batrachochytrium dendrobatidis* at the Headwaters of the Mississippi River, Itasca State Park, Minnesota, USA

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The disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), chytridiomycosis, is one of several factors driving the global decline of amphibian populations (Blaustein and Kiesecker 2002; Lips et al.2006; Muths et al.2003). *Bd* has been found in amphibians at sites across North America including Minnesota, USA (Ouellet et al. 2005;Woodhams et al. 2008). The prevalence of *Bd* in wild anuran populations in Minnesota is unknown, and motivated the work described herein.

We investigated the occurrence of *Bd* at the University of Minnesota's Itasca Biological Station and Laboratories in Itasca State Park, site of the headwaters of the Mississippi River. Our research objectives were to: 1) verify if *Bd* is present in the park; 2) determine which anuran species are affected by the fungus; and 3) test if there are differences in infection rate among species.

Methods

We collected frogs in Itasca State park, Clearwater County, Minnesota in June and July 2008. We collected frogs by hand and with nets at night in breeding ponds and during the day near ponds and wetlands. We rinsed nets with 95% ethanol between outings and wore latex gloves in the field and the laboratory to prevent potential transfer of *Bd* among individuals. A subsample of frogs was toe-clipped and released in the field, but most were vouchered. Toe-clips were collected in individual plastic bags and we extracted genomic DNA immediately upon return to the laboratory. We kept frogs individually in plastic bags from time of capture until they were euthanised in the laboratory. Frogs were humanely euthanised with MS-222 (tricaine methanesulfonate) or topical application of benzocaine (Simmons 2002). MS-222 does not appear to inhibit growth or detection of *Bd* (Webb et al. 2005) and was the preferred method of euthanasia. We clipped one toe and a portion of

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adjacent webbing to obtain tissues. We stored tissues in 95% ethanol at 4°C until processing. All vouchers were deposited in the Bell Museum of Natural History, University of Minnesota (JFBM).

We extracted genomic DNA from tissues using the Qiagen extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. We used polymerase chain reaction (PCR) to amplify a 300-bp fragment consisting of part of internal transcribed spacer 1 (ITS1), ribosomal rRNA 5.8S, and part of internal transcribed spacer 2 (ITS2) using, *B. dendrobatidis* specific primers (Bd1a and Bd2a; Annis et al. 2004). PCR was performed in 12.5 μl reaction volumes under the following conditions: an initial denaturation of 94°C for 5 min; 30 cycles of denaturation (94°C for 45 sec), annealing (50°C for 45 sec) and extension (72°C for 1 min); followed by a final extension of 72°C for 5 min. All PCR reactions contained a negative control. PCR products were run on a 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Presence of a strong, defined band approximately 300 bp was considered a positive result.

We sequenced two positive samples to ensure that we had amplified *Bd* rather than non-target DNA. We purified PCR products using Exonuclease I and Shrimp Alkaline Phosphatase (Hanke and Wink 1994). Sequencing was performed using Big Dye (perkin Elmer, Boston, MA, USA) terminator cycle sequencing on an ABI 3730×1 at the Advanced Genetic Analysis Center, University of Minnesota. We used BLAST (Altschul et al. 1990) to verify that sequences were *Bd*.

We tested whether infection rates of *Bd* were significantly different among sampled species and among sampled families of frogs using a Chi-square contingency test. All statistical analyses were conducted using JMP 7.0 (sAS 2007).

Results

We collected tissues from 147 frogs and toads of three families from around Itasca State Park (Fig. 1), consisting of 133 vouchered specimens and 14 toe-clips of released frogs. Thirty-four of 147 (23%) individuals were *Bd*-positive. None of our negative controls produced bands on agarose gels. *Pseudacris maculata* had the highest percentage of positive individuals, 50% (Table 1), although it is difficult to interpret this given the small number of individuals captured. *Lithobates pipiens* had the second highest infection rate with 33.3% of samples being *Bd*-positive. *Hyla versicolor* and *Pseudacris crucifer* had no positive samples. We collected one dead *Lithobates sylvaticus* (JFBM 15957) at the Mississippi River headwaters on the North end of Lake Itasca that tested positive for *Bd*. No other dead animals were found, and no animals were observed with symptoms of illness.

Rates of *Bd* infection differed among species ($\chi^2 = 16.505$, $p < 0.0113$) and among families ($\chi^2 = 9.673$,$P < 0.0079$). Frogs in the family Ranidae had a higher infection rate than Bufonidae and Hylidae (Table 1).

We confirmed that DNA fragments amplified using PCR were *Bd* by sequencing two of our positive PCR products. Both sequenced samples, one from *Lithobates sylvaticus* (JFBM 15884, Genbank accession number FJ229469) and the other from *Lithobates pipiens* (JFBM 15918, Genbank accession number FJ229470) had an identical sequence. We compared our sequences to *Bd* sequences on Genbank using BLAST, confirming our samples as *Bd*. Our samples had 100% identity with the following *Bd* sequences from Genbank: EU779867, EU779864, EU779863, EU779862, EU779860, EU779859. Our samples had a 98% identity with *Bd* sequences AY997031 and EU779866.

Discussion

Rates of *Bd* infection in Itasca State Park varied among species and families. This variance is similar to observations from other North American sites for the same species (Longcore et aI.2007: Ouellet et al. 2005). North American hylid frogs, for example, typically have had low *Bd* infection rates (Longcore et al. 2007; Ouellet et al. 2005; Pearl et al. 2007) and we found no evidence of *Bd* in the hylid species *Hyla versicolor* and *Pseudacris crucifer*. *Pseudacris maculata* and *Acris blanchardi* appear to be exceptions to this rule (Ouellet et al. 2005; Steiner and Lehtinen 2008) and we found one infected *P.maculata* in the Park. We found the highest rates of infection in the three ranid species examined, *L. sylvaticus, L. pipiens and L. septentrionalis*. Several hypotheses have been proposed to explain the species-specific variance in *Bd* infection rates. Because *Bd* can persist in aquatic environments (Johnson and Speare 2003), species that spend more time in the water are thought to be at greater risk of infection than species spending less time in the water (Hero et al. 2005; Lips et al. 2003). *Bd* is prevalent in species breeding in permanent wetlands and streams (Kriger and Hero 2007) and in species that overwinter in aquatic environments (Longcore et al. 2007). There is also, potentially, a phylogenetic component to *Bd* (Corey and Waite 2008) with some amphibian lineages showing greater susceptibility to chytridiomycosis than others.

The presence of *Bd* in multiple frog species in Itasca State Park highlights the pathogen's pervasiveness in North America. North American amphibian populations have been exposed to *Bd* since the early 1960s and have been found across the continent (Ouellet et al. 2005). Presence of *Bd* does not always lead to mortality or population declines (Retallick and Miera 2007) but, given its potential as a pathogen, the presence of the fungus is cause for further monitoring of infected populations.

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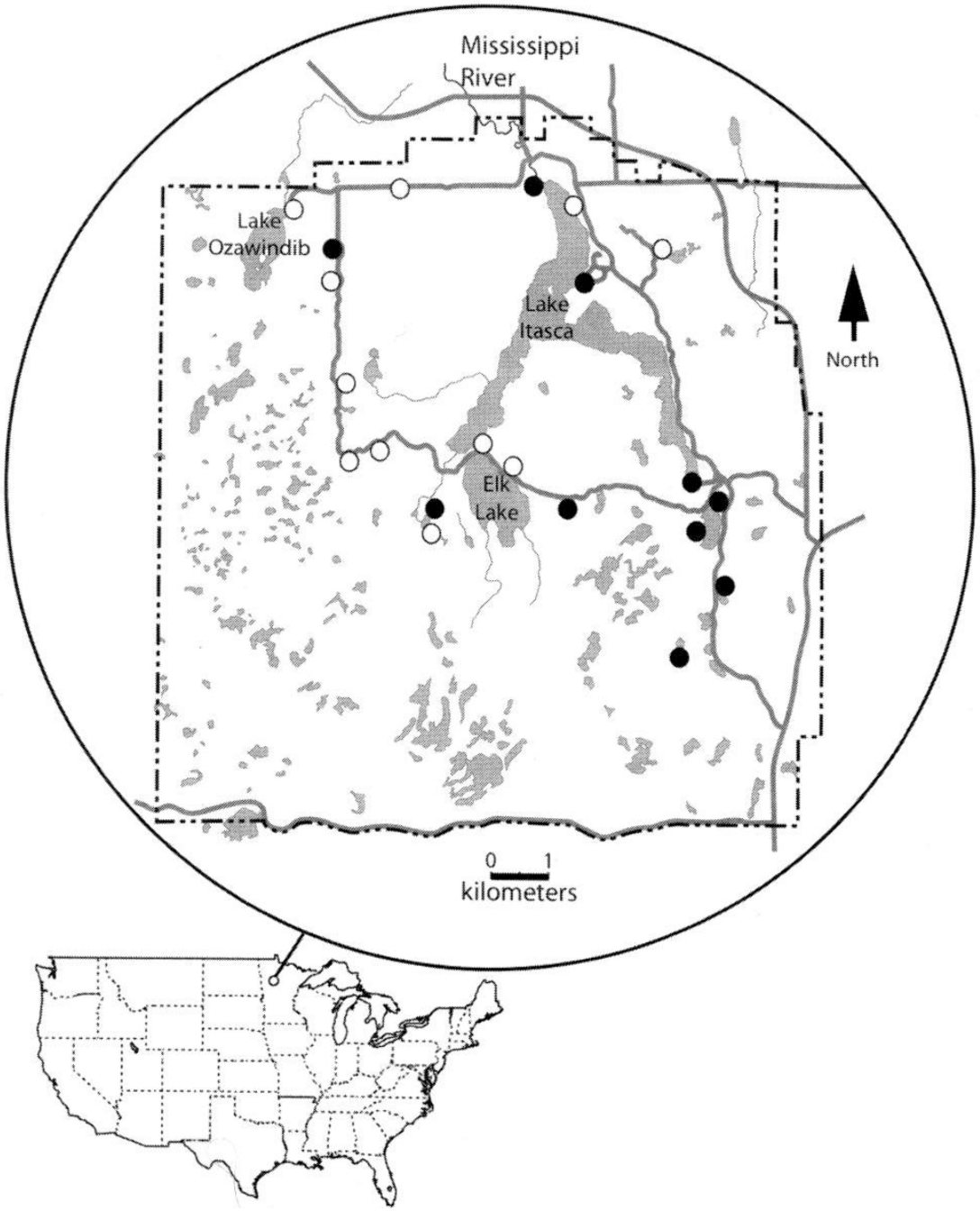

FIG. 1.

Collecting locations (circles) of anurans examined for *Batrachochytrium dendrobatitis* (*Bd*) at Itasca State park, Minnesota, USA. Localities with *Bd*-positive specimens indicated by filled cirlces, localities with no *Bd*-positive specimens indicated by open circles. Park boundaries shown by a dashed line. Light gray areas are water.

TABLE 1

Prevalence of *Batrachochytrium dendrobatidis* in seven frog species collected in Itasca State Park, Minnesota, USA, in 2008.

Species	Family	No. *Bd*-Negative Animals	No. *Bd*-Positive Animals	% Positive (95% C.I.)
Anaxyrus americanus	Bufonidae	20	1	4.8 (0–24.4)
Hyla versicolor	Hylidae	19	0	0 (0–19.8)
Pseudacris crucifer	Hylidae	2	0	0 (0–70.1)
Pseudacris maculata	Hylidae	1	1	50 (9.5–90.6)
Lithobates pipiens	Ranidae	14	7	33.3(17.1–54.8)
Lithobates septentrionalis	Ranidae	13	3	18.8(5.8–43.8)
Lithobates sylvaticus	Ranidae	78	22	22 (14.9–31.2)