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Survey for *Batrachochytrium dendrobatidis* in the North Cascades National Park Service Complex, Washington, USA

We surveyed amphibians for the amphibian fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*; Longcore et al. 1999) as part of a larger study focused on the Coastal Tailed Frog, *Ascaphus truei*, in the North Cascades National Park Service Complex (which includes North Cascades National Park, Ross Lake National Recreation Area, and Lake Chelan National Recreation Area), Washington, USA (Fig. 1). This stream-associated frog is unique for a number of reasons, which provides rationale for assessing *Bd* presence among populations. Perhaps more so than other anurans, *A. truei* is tightly associated with aquatic habitats and is seldom found >100 m from its preferred habitat of high-gradient mountain streams (Wahbe et al. 2004). It is also unique among anurans in that its early-diverging phylogenetic placement is sister to all remaining frogs (along with the family Leiopalmatidae; Pyron and Wiens 2011), and a long generation time of approximately 2–8 years (Bury and Adams 1999; Nielson et al. 2001). Lastly, this species is endemic to mesic forests of the Pacific Northwest (e.g., northern California to southern British Columbia). To date, *Bd* has been detected in southern Washington state (http://www.bd-maps.net/; Fisher et al. 2009; Pearl et al. 2009), but it has not been detected in Olympic National Park (approximately 150–200 miles west of North Cascades National Park; Pearl et al. 2007). Although it is listed as a species of "least concern" across its range by the IUCN, *A. truei* is considered a species of concern in the state of Washington by the U.S. Fish and Wildlife Service.

We used hand-capturing and dipnetting to collect amphibians during diurnal surveys between June–October of 2012–2013 from streams that ranged 2–8 m wide in the North Cascades National Park Service Complex (Fig. 1). Because our focal species in the park was *A. truei*, our sampling was mainly limited to areas within ca. 10 km of the Skagit River, Gorge, Diablo, and Ross Lakes. Among all amphibian captures for our larger study, we opportunistically subsampled four species for *Bd*: *Ambystoma macrodactylum*, *Anaxyrus boreas*, *A. truei*, and *Dicamptodon tenebrosus*. Individuals were swabbed for >15 seconds using rayon-tipped swabs with plastic shafts (Medical Wire & Equipment, Wiltshire, England) following the protocol of Skerratt et al. (2008), focusing on dorsal regions in adults and oral regions in larvae. Swabs were then placed in 95% ethanol, then stored at 4°C for less than one month, at which point they were processed in the laboratory. All animals were alive when collected and processed for *Bd* sampling in accordance with University of Washington's Institutional Animal Care and Use Committee (PHS Animal Welfare Assurance number 4209-01) under scientific research and collecting permits issued by the United States Department of the Interior (permit nos. NOCA-2012-SCI-0044, NOCA-2013-SCI-0013) and the Washington Department of Fish and Wildlife (no. RCW 77-32-240, WAC 220-20-045).

Whole *Bd* genomic DNA was extracted from swabs (after allowing the ethanol to evaporate) using Life Technologies PrepMan® Ultra reagent (Carlsbad, California, USA), following manufacturer's recommendations, which was shown to perform better than alternative methods (Boyle et al. 2004). We also extracted one sample from a *Bd* stock (provided by the Kelly Zamudio Laboratory, Cornell University, New York, USA) and another using only PrepMan Ultra to serve as positive and negative controls, respectively. The TaqMan quantitative real-time qPCR (RT-qPCR) assay has been shown to have 100% diagnostic specificity, and is more sensitive to *Bd* detection than conventional histology methods (Hyatt et al. 2007). We therefore used a TaqMan RT-qPCR assay to survey for *Bd* in our samples.

We performed TaqMan real-time qPCR assays using an Applied Biosystems Step One Plus thermocycler (Applied Biosystems, Inc., Foster City, California, USA) with *Bd*-specific primers following the protocol of Boyle et al. (2004). Because we did not know the exact amount of zoospore equivalents (ZEs) in the positive control or standards processed in the Leaché laboratory, we were only able to perform presence/absence qPCR. Samples

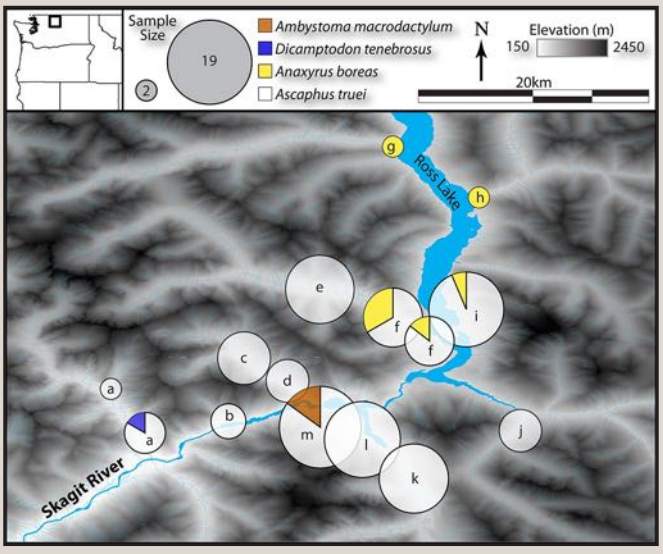

FIG. 1. Study area showing sampling sites and relevant geographic features in the North Cascades National Park Service Complex, Washington, USA. The bold square in top-left inset shows the location in Washington state of the enlarged study area. Letters correspond to the following streams, with sample sizes following stream name: a - Goodell (8), b - North Gorge (5), c - Stetattle (7), d - Sourdough (5), e - Big Beaver (19), f - Pierce (13), g - Little Beaver (2), h - Lightning (2), i - Roland (16), j – Panther (7), k - Thunder (15), l - Rhodes (16), m - Pyramid (19).

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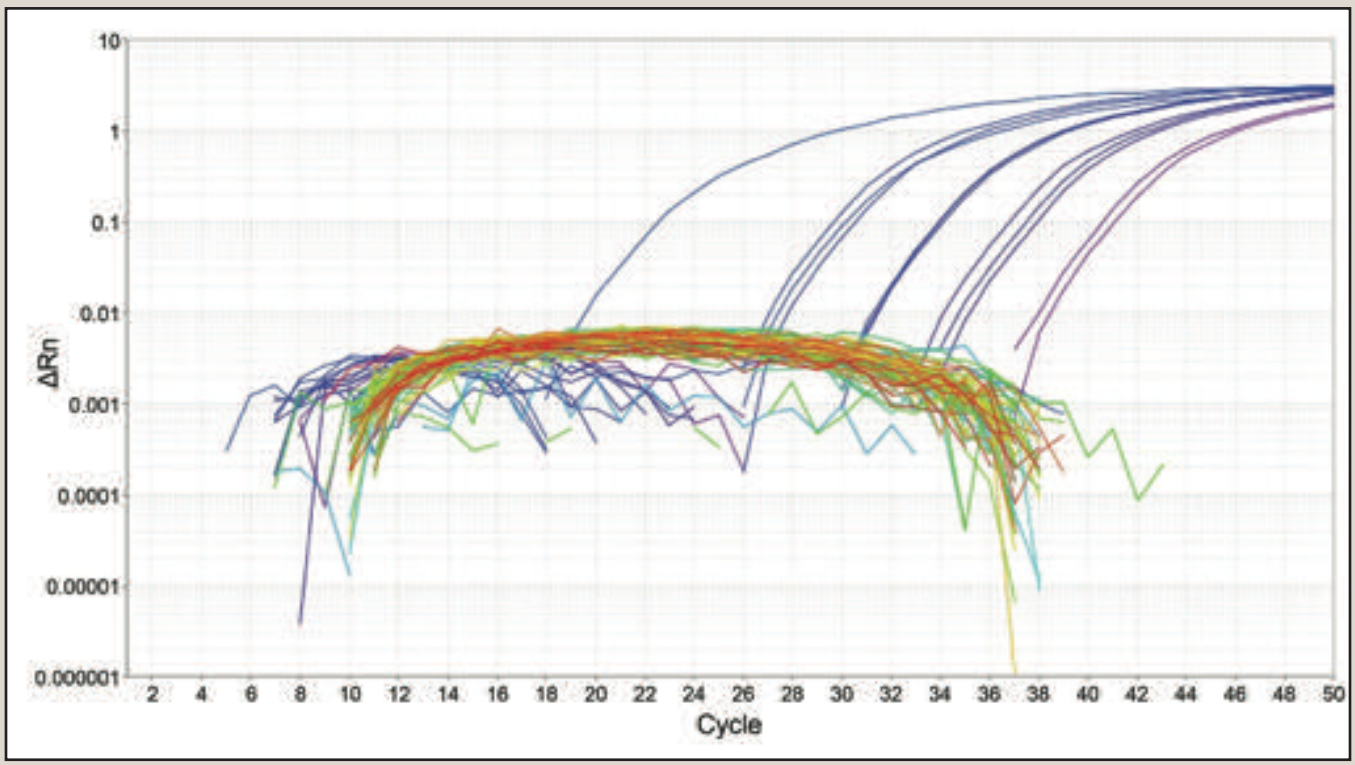

FIG. 2. Quantitative PCR (qPCR) amplification curves for the third run (performed in the Adam Leaché laboratory) that are representative of all qPCR reactions. Dark blue and purple samples represent standards, whereas all others are UWBM samples.

of extracted DNA were diluted 1:10 (5 µL sample + 45 µL TE buffer), then processed as in Boyle et al. (2004). qPCR standards were prepared by serially diluting positive control samples (5µL positive control per dilution) by four orders of magnitude, then combined with 20 µL qPCR master mix. The following thermocycler settings were used: 50°C for 2:00, 95°C for 10:00, then 50 cycles of (95°C for 0:15, 60°C for 1:00). We recorded the Ct value (cycle number in which the fluorescence signal of the probe during the qPCR reaction crosses a critical threshold) for all samples, and ran three separate qPCR runs (the first in the Vance Vredenburg Laboratory, San Francisco State University, California, USA, and the final two in the Adam Leaché Laboratory, University of Washington, Washington, USA). Only a single qPCR run was performed on each sample (e.g., not in triplicate).

We collected *Bd* samples from 134 specimens of the four amphibian species (47 during 2012 and 87 during 2013). Of our total sample, 119 (89%) were larvae (*A. macrodactylum* = 3, *A. truei* = 115, and *D. tenebrosus* = 1), 14 were adults (*A. boreas* = 9, *A. truei* = 5), and one *A. truei* was a metamorph. We did not detect *Bd* in any of the four amphibian species that we surveyed in the North Cascades National Park Service Complex (Fig. 2; Burke Museum of Natural History and Culture [Seattle, Washington] Herpetology Collection voucher nos. UWBM 7981–8301; Arctos Database information at http://arctos.database.museum/).

In Washington state to date, *Bd* has been detected in the Oregon Spotted Frog (*Rana pretiosa*; Pearl et al. 2009), however, those locations are in portions of the southern Washington Cascade Range near the Oregon border (e.g., nearly 200 miles away from our sites, as the spore flies); *Bd* has not yet been detected in the North Cascades of Washington. Also important to note is that Pearl et al. (2009) detected *Bd* in only ~3% (2/72) of the larval anurans that they sampled (genera *Rana* and *Anaxyrus*), whereas the detection rate in adults was much higher at 56% (133/238). Adult *A. truei* are seldom found during diurnal surveys (the technique that we employed), and we may have detected *Bd* had we surveyed a larger number of adults (Skerratt et al. 2008). Another possible methodological concern is that we did not run triplicates of our samples, nor did we test our samples at full (undiluted) concentrations. Both of these factors could result in false-negatives. However, we feel that it is unlikely that we are reporting any false negatives, as our qPCR standards gave results as expected, and our positive controls also reported low Ct values (e.g., high detectability). We encourage other amphibian researchers to continue surveying Washington's amphibian populations to document the temporal and spatial spread of this global pathogen.

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LITERATURE CITED

BOYLE, D. G., D. B. BOYLE, V. OLSEN, J. A. T. MORGAN, AND A. D. HYATT. 2004. Rapid quantitative detection of chytridiomycosis

(*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis. Aquat. Org. 60:141–148.

Bury, R. B., and M. J. Adams. 1999. Variation in age at metamorphosis across a latitudinal gradient for the tailed frog, *Ascaphus truei*. Herpetologica 55:283–291.

Fisher, M. C., T. T. Garner, and S. F. Walker. 2009. Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Ann. Rev. Microbiol. 63:291–310.

Hyatt, A., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines et al. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Dis. Aquat. Org. 73:175–192.

Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91(2):219–227.

Nielson, M., K. Lohman, and J. Sullivan. 2001. Phylogeography of the tailed frog (*Ascaphus truei*): implications for the biogeography of the Pacific Northwest. Evolution 55(1):147–160.

Pearl, C. A., J. Bowerman, M. J. Adams, and N. D. Chelgren. 2009. Widespread occurrence of the chytrid fungus *Batrachochytrium dendrobatidis* on Oregon spotted frogs (*Rana pretiosa*). EcoHealth 6(2):209–218.

———, E. L. Bull, D. E. Green, J. Bowerman, M. J. Adams, A. Hyatt and W. H. Wente. 2007. Occurrence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the Pacific Northwest. J. Herpetol. 41(1):145–149.

Pyron, R. A., and J. J. Wiens. 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol. Phylog. Evol. 61(2):543–583.

Skerratt, L. F., L. Berger, H. B. Hines, K. R. McDonald, D. Mendez, and R. Speare. 2008. Survey protocol for detecting chytridiomycosis in all Australian frog populations. Dis. Aquat. Org. 80:95–94.

Wilbur, T. R., F. L. Bunnell, and R. B. Bury. 2004. Terrestrial movements of juvenile and adult tailed frogs in relation to timber harvest in coastal British Columbia. Can. J. For. Res. 34(12):2455–2466.