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NOTE

Using environmental DNA for early detection of amphibian chytrid fungus *Batrachochytrium dendrobatidis* prior to a ranid die-off

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ABSTRACT: Amphibian chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) is an emerging infectious disease that has been associated with mass mortality and extinctions of amphibians worldwide. Environmental DNA (eDNA) techniques have been used to detect the presence of *Bd* in the environment, but not to detect *Bd* prior to an amphibian die-off. We collected eDNA using filtered water samples from 13 lakes across Sequoia Kings Canyon National Park. Seven of those sites had populations of mountain yellow-legged frogs, an amphibian highly susceptible to chytridiomycosis, and 3 of those populations experienced a *Bd* related die-off 1 mo post-eDNA sampling. We detected *Bd* in eDNA samples that were collected 1 mo prior to the observed *Bd*-caused die-off at all 3 sites affected by *Bd*, and we did not detect *Bd* at the other sites where no die-off was observed. Our study indicates the potential to use eDNA techniques for early detection of *Bd* in the environment.

KEY WORDS: *Batrachochytrium dendrobatidis* · Early detection · Environmental DNA · Infectious disease · Sierra Nevada

INTRODUCTION

Amphibian chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), is an emerging infectious disease that has been associated with mass mortality events and extinctions of amphibians worldwide (Berger et al. 1998, Stuart et al. 2004, Skerratt et al. 2007). Traditional techniques for detecting *Bd* include swabbing the keratinized portion of skin on frogs and tadpoles, and then analyzing the skin swabs for *Bd* zoospores using PCR techniques (Kriger et al. 2006). This method is dependent on finding and handling host individuals. Environmental DNA (eDNA) is a non-invasive alternative technique that has been used to accurately detect *Bd* through the collection and extraction of *Bd* DNA found in water samples (Kirshtein et al. 2007, Walker et al. 2007, Hyman & Collins 2012, Chestnut et al. 2014).

In the Sierra Nevada Mountain Range in California, USA, many persisting populations of amphibians are naïve to chytridiomycosis and can experience massive die-offs once *Bd* is introduced (Rachowicz et al. 2005, Vredenburg et al. 2010). Recent studies have shown that anti-fungal baths administered to symptomatic frogs can increase their chances of survival (Harris et al. 2009). Therefore, for these *Bd*-naïve populations, early detection of chytridiomycosis is a critical component in managing their recovery. In Sequoia Kings Canyon National Park, non-native fish are being removed to create habitat for the mountain yellow-legged frogs, *Rana muscosa*

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and *Rana sierrae*, 2 federally endangered amphibians highly susceptible to chytridiomycosis (Vredenburg et al. 2010). A few completed restoration sites (sites where non-native fish have been removed; hereafter referred to as restoration sites) have healthy populations of mountain yellow-legged frogs that are naïve to *Bd* (D. Bioano pers. comm.).

In this study, we collected eDNA samples from restoration sites across Sequoia Kings Canyon National Park. Our samples included 3 sites prior to a *Bd*-caused *R. muscosa* die-off. Our eDNA project presents a unique opportunity to determine if eDNA could provide an early signal of where die-offs may occur in these high alpine lakes.

MATERIALS AND METHODS

During June and July 2015, we collected water filter eDNA samples from 13 restoration sites in Sequoia Kings Canyon National Park (National Park Scientific Research and Collecting permit # SEKI-2015-SCI-0011). To accommodate variance in lake size, we collected a 50 ml water sample every 40 m along the shoreline. Every 5 water samples were combined into a 250 ml composite sample (see Table 1). Each 250 ml composite sample was filtered using 1.2 µm polycarbonate track-etched (PCTE) filter membrane (EMD Millipore) with a single-use 47 mm diameter filter funnel (Thermo Fisher Scientific). We used 1.2 µm PCTE filter membranes because our lake sampling effort was part of an eDNA sampling study where particle size distribution was being evaluated (Kamoroff 2016, C. Kamoroff & C. S. Goldberg unpubl. data), and PCTE filter membranes provide a fixed pore size (Turner et al. 2014). We filtered all water samples in the field using a polypropylene vacuum flask with a rubber stopper fixed to a hand pump. We used forceps soaked in a 50% bleach solution for at least 1 min and rinsed in distilled water to remove the filter membrane after filtration. All personnel wore single use disposable latex gloves during sample collection and changed gloves prior to handling filter membranes. All equipment used to collect water samples were single-use items and were properly disposed of after sample collection to prevent spread of *Bd*. No personal items (boots, clothing, camping equipment, etc.) were exposed to water prior to or during eDNA sampling. If any personal items were exposed to water after sampling, we cleaned or soaked them in quaternary ammonia to prevent the spread of disease (Johnson et al. 2003). During each sampling event, we filtered 250 ml of distilled water as a field negative control after all sampling was completed. Prior to DNA extraction, we stored filter membranes in 95% molecular-grade ethanol, at room temperature away from any light sources (Minamoto et al. 2016). We extracted samples within 6 mo of collection. Due to the nature of our eDNA collection methods in which the sampler was required to walk the perimeter of the lake collecting water samples, we opportunistically conducted a visual encounter survey at each site. We then corroborated yellow-legged frog sightings (or lack thereof) with biologists from Sierra Nevada Aquatic Research Lab (SNARL) and Sequoia Kings Canyon who regularly conduct amphibian surveys in the area.

In the laboratory, we cut each filter in half, used half the filter for DNA extraction, and stored the other half in 95% ethanol as a reserve. We used a QIAshredder/Qiagen DNeasy Blood and Tissue DNA extraction protocol (Goldberg et al. 2011) to extract DNA from filters in a limited-access clean room. The clean room is managed such that no high-quality DNA extraction or PCR product are handled in it, and personnel who have been exposed to PCR product or high-quality DNA are required to shower and change clothes before entering. We analyzed samples using the assay of Boyle et al. (2004). To quantify the initial DNA copy number for *Bd* eDNA samples, we created a standard curve by using a 4-point serial dilution (1 000 000 to 1000 copies) of a synthesized gene (gBlocks; Integrated DNA Technologies) designed using genomic sequences from *Bd*. We chose the range of the standard curve (1 000 000 to 1000 copies) because large quantities of *Bd* DNA generally persist during chytridiomycosis outbreaks. We can detect quantities of DNA above or below the range set by the standard curve, but exact quantification cannot be determined if the quantities are outside the range. We used 3 µl of DNA extract in each reaction and ran each quantitative PCR (qPCR) reaction in triplicate (3 technical replicates) using 1× QuantiTect Multiplex PCR Mix (Qiagen) with 0.2 µM of each primer and 0.2 µM of the probe. To run all reactions, we used a cycle of 15 min at 95°C followed by 50 cycles at 94°C for 60 s and 60°C for 60 s. Most eDNA studies use reactions with high PCR cycle numbers (50 to 55) to detect low quantities of DNA that may be present in the sample (Kirshtein et al. 2007, Goldberg et al. 2011). All wells included an exogenous internal positive control to insure no qPCR inhibition had occurred (IPC; Applied Biosystems). We created and analyzed negative extraction and qPCR controls with every extraction batch and plate.

RESULTS

During eDNA sampling (from June through July), we observed yellow-legged frogs at 7 of the 13 restoration sites (Table 1). None of the populations observed exhibited symptoms of illness common with *Bd* infections (e.g. dead frogs, lethargic frogs, or frogs with red legs or skin). Additionally, biologists from SNARL and Sequoia Kings Canyon regularly surveyed these restoration sites and did not observe sick frogs prior to August 2015. In August 2015 (1 mo post-eDNA sampling), a yellow-legged frog die-off caused by *Bd* was observed at 3 of the restoration sites (SEKI 1, 2, and 3) by a park wilderness ranger and verified by Dr. Roland Knapp and SNARL via physical diagnostics as well as laboratory analysis (R. Knapp unpubl. data). We analyzed samples from all 7 sites where yellow-legged frogs were present to see if we could detect *Bd* at the sites where the die-off occurred using eDNA methods.

We detected *Bd* using eDNA techniques at all 3 sites that experienced a *Bd* die-off (Table 1). Samples collected from SEKI-3 and SEKI-2 had at least 2 samples test positive for *Bd* (i.e. all technical replicates detected *Bd* DNA). We detected very low quantities of *Bd* DNA from all 3 samples collected from SEKI-1, and those samples yielded equivocal results because only 1 or 2 of the 3 technical replicates returned a positive detection of *Bd* DNA (see Hyatt et al. 2007) (Table 1). All technical replicates indicated a positive detection (e.g. beginning of an amplification curve) at or below a 40 cycle threshold. We analyzed all of our results using *Bd* DNA copy number per ml as recommended by Longo et al. (2013) (Table 1). We did not detect *Bd* at any of the 4 sites where yellow-legged frogs were present and no die-off was observed, as expected. All of the negative control samples, extraction negatives, and qPCR negatives tested negative. The standard curve for the *Bd* assay had an efficiency of 93.9% and $R^2 > 0.99$.

DISCUSSION

Environmental DNA is a promising, non-invasive alternative to skin swabs. Our sampling techniques accurately detected the presence of *Bd* at sites that experienced *Bd* related die-offs, and unlike swabs, did not require the capture or handling of target animals. Using non-invasive techniques such as eDNA is ideal when researching federally or state listed species because it may shorten or avoid a lengthy research permitting processes. Environmental DNA techniques are also useful when working with species or habitats sensitive to human impacts.

In addition to being non-invasive, our sampling techniques detected *Bd* at a critical time for management. Vredenburg et al. (2010) described the spread of *Bd* and subsequently the extinction or near extinction of 8 yellow-legged frog populations in the Sierra Nevada. For these 8 populations, *Bd* prevalence increased rapidly and infection intensity increased exponentially, with declines of frogs evident after average infection intensity of approximately 10 000 zoospores swab^{-1} (Vredenburg et al. 2010). Over the past 10 yr, low levels of *Bd* infection have been detected on yellow-legged frog skin swabs across our sample sites (R. Knapp unpubl. data). Although

Table 1. Meta-data for restoration sites with yellow-legged frogs present in Sequoia Kings Canyon National Park sampled using environmental DNA (eDNA) techniques. Each composite sample was composed of five 50 ml water samples collected at 40 m intervals around the lake. We analyzed each composite sample in triplicate (3 technical replicates) using quantitative PCR (qPCR) techniques. Parenthetical numbers represent number of technical replicates. Average DNA ml^{-1} is the averaged (±SE) copy number of DNA found in the qPCR replicates extrapolated to estimate the density of DNA ml^{-1} in the original sample. Negative samples were not included in calculating average or SE. NA: not available; dates are provided as mm/dd/yyyy

Site ID	Date sampled	Water filtered (ml)	Composite samples collected	% Positive technical replicates	Date die-off observed	Average DNA ml^{-1}
SEKI-1	7/15/2015	750	3 (9)	44	8/16/2015	< standard[a]
SEKI-2	7/15/2015	500	2 (6)	100	8/16/2015	775.1 ± 169.3
SEKI-3	7/15/2015	750	3 (9)	66.67	8/16/2015	1455.0 ± 56.8
SEKI-4	6/30/2015	500	2 (6)	0	None observed	NA
SEKI-5	7/01/2015	500	2 (6)	0	None observed	NA
SEKI-6	7/18/2015	500	2 (6)	0	None observed	NA
SEKI-7	7/20/2015	1500	6 (18)	0	None observed	NA

[a]Quantities of DNA detected from technical replicates were below lowest standard; precise DNA levels could not be calculated

background infection levels are common among persisting yellow-legged frog populations, these frogs appear healthy (R. Knapp unpubl. data). Detecting *Bd* when prevalence levels and infection intensities begins rapid/exponential growth but before lethal threshold levels (at ~10 000 zoospores) is the critical time for management to implement conservation strategies (i.e. anti-fungal baths). We did not observe any lethal effects of *Bd* infections at the time of eDNA sampling at Sites SEKI-1, 2, and 3 (sites that experienced a *Bd* related die-off), suggesting that we detected *Bd* below the ~10 000 zoospore threshold level. We also did not detect *Bd* at Sites SEKI-4, 5, 6 and 7 (sites with extant populations of yellow-legged frog that did not experience a die-off), suggesting that we did not detect low-level background infections. Our sampling techniques may indicate a critical time for yellow-legged frog conservation by detecting *Bd* before an epizootic outbreak.

Further research evaluating the relationship between *Bd* load on infected populations and amount of DNA detected in environmental samples could help determine when fatal effects are imminent and when salvage and treatment missions would be appropriate. The *Bd* infection intensity threshold of approximately 10 000 zoospores that is often associated with disease epidemics, mortality, and population extirpations (Vredenburg et al. 2010, Cheng et al. 2011, Kinney et al. 2011) was formulated from skin swab analysis and may be positively correlated but not directly applicable to filtered environmental samples. It is unclear at what zoospore or *Bd* DNA copy number detected in eDNA samples indicate background levels or potential epidemics because we did not couple our sampling with *Bd* swabbing of infected amphibians nor has this phenomena been examined by other eDNA studies. However, similar work from Hall et al. (2016) showed that quantities of ranavirus collected in eDNA samples were related to viral load detected in individuals, and viral load can predict the timing of a viral die-off. This is the first study that has used eDNA sampling to detect *Bd* in high alpine lakes in the Sierra Nevada, and our results indicate the potential for using eDNA techniques for early detection of *Bd* in the environment and for predicting epizootic outbreaks. With further research, eDNA could be used to predict *Bd* outbreaks for naïve amphibians at risk of extinction.

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