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Using Environmental DNA Sampling for Simultaneous Detection of Hosts and Their Pathogens: A Case Study With the Critically Endangered Frog Genus *Atelopus*

Jamie Voyles[1] | Corinne L. Richards-Zawacki[2,3] | Allison Q. Byrne[4,5] | Angie Estrada[6] | Roberto Ibáñez[3] | Keely M. Rodriguez[1] | Caren S. Goldberg[7]

[1]Department of Biology, University of Nevada, Reno, Reno, Nevada, USA | [2]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA | [3]Smithsonian Tropical Research Institute, Panamá, Republic of Panama | [4]Department of Environmental Science, Policy and Management, University of California, Berkeley, Berkeley, California, USA | [5]Museum of Vertebrate Zoology, University of California, Berkeley, Berkeley, California, USA | [6]Summit Municipal Park, Panamá, Republic of Panama | [7]School of the Environment, Washington State University, Pullman, Washington, USA

Correspondence: Caren S. Goldberg (caren.goldberg@wsu.edu)

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ABSTRACT

Studying declining and rare species is inherently challenging, particularly when the cause of rarity is emerging infectious diseases (EIDs). Tracking changes in the distribution of pathogens that cause EIDs, and the species made scarce by them, is necessary for conservation efforts, but it is often a time and resource intensive task. Here, we demonstrate how using environmental DNA (eDNA) to detect rare species—and the pathogens that threaten them—can be a powerful tool to understand disease dynamics and develop effective conservation strategies. Amphibian populations around the world have undergone rapid declines and extinctions due to the emerging fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*). We developed and validated a qPCR assay using eDNA sampling methods for some of the most imperiled amphibian species, harlequin frogs (*Atelopus varius, Atelopus zeteki,* and *Atelopus chiriquiensis*), and applied this assay in concert with a standard qPCR assay for *Bd* in rainforest streams of Panamá. We confirmed the presence of *Atelopus* at sampling locations across three regions. In addition, we used genomic analysis of eDNA samples to show that *Bd* in Panamá falls within the Global Panzootic Lineage, a lineage associated with disease-induced declines. We detected *Bd* DNA in most of our historic sites, and its concentration in water samples correlated with stream characteristics and the pathogen load of the local amphibian community. These results suggest that some populations of *Atelopus* persist in their historic localities. They also show how eDNA analysis can be effectively used for monitoring species presence, pathogen concentrations, and the distribution and spread of pathogen lineages. EIDs are a growing threat to endangered species around the world. Simultaneous detection of rare and declining host species and their pathogens with eDNA will help to provide key insights for effective conservation management.

RESUMEN

Estudiar especies raras y en declive es intrínsecamente desafiante, particularmente cuando la causa de su rareza son las enfermedades infecciosas emergentes (EID). El seguimiento de los cambios en la distribución de los patógenos que causan las EID, junto con las especies que estas hacen escasas, es necesario para los esfuerzos de conservación, aunque a menudo es una tarea que requiere mucho tiempo y recursos. En este estudio, demostramos cómo el uso de ADN ambiental (eDNA, por sus siglas en Inglés) para detectar especies raras (y los patógenos que las amenazan) puede ser una herramienta poderosa para comprender la dinámica de las enfermedades y desarrollar estrategias de conservación efectivas. Las poblaciones de anfibios en todo el mundo han experimentado rápidas disminuciones y extinciones debido al hongo patógeno emergente *Batrachochytrium dendrobatidis* (*Bd*). Desarrollamos y validamos un ensayo de qPCR utilizando métodos de muestreo de eDNA para algunas de las especies de anfibios más amenazadas, las ranas arlequín (*Atelopus varius, A. zeteki y A. chiriquiensis*). Aplicamos este ensayo junto con un ensayo de qPCR estándar para *Bd* en Arroyos de la selva tropical de Panamá. Confirmamos la presencia de *Atelopus* en múltiples lugares de muestreo de varias regiones de Panamá. También utilizamos análisis genómicos de muestras de eDNA para mostrar que *Bd* en Panamá se encuentra dentro del Linaje Panzoótico Global, un linaje asociado con disminuciones inducidas por enfermedades. Detectamos ADN de *Bd* en la mayoría de nuestros sitios históricos, su concentración en muestras de agua se correlacionó con las características de los arroyos, y con la carga de patógenos de la comunidad de anfibios local. Estos resultados sugieren que algunas poblaciones de *Atelopus* persisten en sus localidades históricas. También muestran cómo el análisis de eDNA se puede utilizar eficazmente para monitorear la presencia de especies, las concentraciones de patógenos, y la distribución y propagación de linajes de patógenos. Las EID son una amenaza creciente para las especies en peligro de extinción en todo el mundo. El uso de eDNA para la detección simultánea de especies hospederas, raras, en declive y sus patógenos, ayudará a proporcionar información clave para una gestión eficaz de la conservación.

1 | Introduction

Monitoring and conserving the world's biodiversity hinges on our ability to detect and study shifts in populations, species, and community composition (Sewell et al. 2012). Yet, studying declining species—especially rare, cryptic, and endangered species—is inherently challenging (McDonald 2004; Schloegel et al. 2006; Pearl et al. 2009). Moreover, understanding the causes and consequences of species declines and extirpations becomes increasingly arduous as species become more difficult to detect (Schloegel et al. 2006; Ryan, Lips, and Eichholz 2008). While this is true for many of the diverse threats to biodiversity loss (e.g., invasive species, habitat modification, climate change), there are additional challenges to studying species declines due to emerging infectious disease because, as host species decline, frequently there is a concomitant loss of the pathogens that cause the disease (Figure 1a). Thus, the difficulties in detecting and studying both hosts and pathogens simultaneously exacerbate the problems associated with investigating, and potentially mitigating, disease-induced species loss.

In many lethal disease systems, outbreaks (i.e., epizootic events) are characterized by a rapid increase in pathogen prevalence and subsequent high mortality and declines in host populations (Briggs, Knapp, and Vredenburg 2010; Langwig et al. 2015). Once host species have declined and become rare, it can be extremely challenging to collect basic information, including data on host presence, health status, and recruitment, as well as pathogen presence, prevalence, and intensities of infection (Langwig et al. 2015). Moreover, it can be similarly difficult to track any shifts in disease dynamics (e.g., pathogen prevalence, attenuation, fadeout, and host species extinctions or recoveries) that may occur over an epizootic to enzootic transition (Figure 1b). As such, investigators can benefit from developing and optimizing tools that will allow for host and pathogen detection during and following epizootic events, when host detection probability is low and obtaining important information on disease dynamics is challenging (Figure 1a,b).

This pattern of disease-induced declines and the associated problems with studying post-epizootic dynamics are particularly evident for amphibian chytridiomycosis (Briggs, Knapp, and Vredenburg 2010; Crawford, Lips, and Bermingham 2010; Lips et al. 2006; Woodhams, Kilburn et al. 2008; Voyles et al. 2018). This disease is caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which colonizes the skin of amphibians, causing a range of pathophysiological effects and mortality (Berger et al. 1998; Longcore et al. 1999; Voyles et al. 2009). *Bd* emergence and spread has been linked to catastrophic declines, and even extinctions, that have occurred around the world (Berger et al. 1998; Longcore et al. 1999; Scheele et al. 2019). Some of the best-studied declines occurred in the mountains of Western Panamá, where chytridiomycosis spread through multiple amphibian assemblages between 1996 and 2007 (Lips et al. 2006; Woodhams, Kilburn et al. 2008; Crawford, Lips, and Bermingham 2010; Voyles et al. 2018). In this region, the disease-induced mortality events were especially severe in high-elevation rainforest streams and for stream-associated species (Lips et al. 2006; Crawford, Lips, and Bermingham 2010; Woodhams, Kilburn et al. 2008), which are notably difficult to study and for which we frequently lack basic natural history data of amphibian host species.

Panamá's harlequin frogs belong to the Neotropical genus *Atelopus*, which is arguably the most imperiled of all amphibian lineages (Lötters 2007; Lewis et al. 2019; Scheele et al. 2019). In western Panamá, three species of harlequin frogs (*Atelopus varius*, *Atelopus zeteki* and *Atelopus chiriquiensis*) are listed as critically endangered or extinct by the IUCN (IUCN SSC Amphibian Specialist Group). While *A. varius* was historically

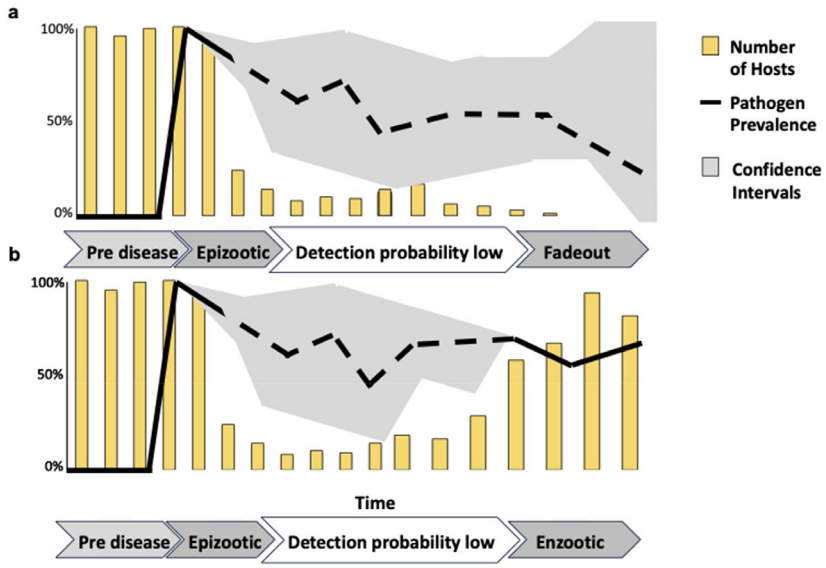

FIGURE 1 | Model of changes in number of hosts (yellow bars), pathogen prevalence (black lines), and confidence intervals (gray shading) over time following the emergence of a highly lethal disease through a transition to epidemic fadeout (a) or to an enzootic phase (b). Over time, detection hosts may initially decline following outbreak (epizootic) events due to the reduced host numbers, leading to uncertainty in pathogen prevalence.

found throughout much of montane Costa Rica and Western Panamá (Savage 1972), *A. varius* has disappeared from most of its range (Zippel et al. 2006; Richards and Knowles 2007; Richards-Zawacki 2009; Figure 2a). The historical range of *A. zeteki* was limited to the area near a volcanic crater at El Valle de Anton, Panamá (Zippel et al. 2006) and *A. chiriquiensis* was previously found only in the Talamanca mountains of western Panamá (Savage 1972).

Harlequin frogs are known to be highly susceptible to chytridiomycosis (Bustamante, Livo, and Carey 2010; Gass and Voyles 2022; Gass et al. 2024), were putatively driven to critically low levels by the emergence of *Bd* (Crawford, Lips, and Bermingham 2010; Lewis et al. 2019), and in some cases presumed to be extinct in the wild (Lewis et al. 2019). Yet, intensive visual surveys in the last decade have detected a small number of *A. varius* populations that appear to be persisting at very low densities despite *Bd* infection (Perez et al. 2014; Voyles et al. 2018; Byrne et al. 2021). These *A. varius* detections are exciting for multiple reasons. First, they raise the possibility that, with optimal tools, we will be able to find additional populations of amphibian hosts that have survived initial chytridiomycosis outbreaks (Byrne et al. 2021). Second, they suggest that we may be able to study the mechanism(s) that drive shifts in disease dynamics and lead to host recoveries (Langwig et al. 2015; Voyles et al. 2018). Third, they may allow us to understand what environmental characteristics allow for host persistence and recoveries (Scheele et al. 2017; Voyles et al. 2018). As such, detecting these now rare species, and gathering additional information on infection patterns, can help facilitate the development of effective, science-based conservation measures.

One tool that could facilitate the study of rare but persisting host populations, and the disease dynamics within their environments, is the analysis of environmental DNA (eDNA). The scientific community is increasingly turning to eDNA approaches to detect and monitor rare species (Zinger et al. 2020; Vörös et al. 2017; Kelly et al. 2023). eDNA detection in aquatic systems is highly sensitive and non-invasive, consisting of the collection and analysis of water samples (Rees et al. 2014). However, this method has been primarily used to detect single species or whole communities of a single taxon (e.g., fish). To date, it has not been used widely to detect hosts and pathogens simultaneously despite the insights such data could provide into host-pathogen dynamics.

Here, we demonstrate the potential for detection of rare hosts and their pathogens using eDNA techniques using the *Atelopus-Bd* system as a case study. We integrated field surveys with optimized techniques for using eDNA samples to (1) detect surviving harlequin frog populations across spatiotemporal gradients, (2) determine the presence and density of the pathogen in the streams where these frogs were historically found, and (3) obtain pathogen genomic information to identify pathogen lineages. This kind of integrated approach to understanding persistence and/or recovery of host populations may help to pinpoint locations and timeframes that are critical for effective conservation efforts. In addition, our approach may help advance our understanding of the distribution of different pathogen strains, pathogen transmission dynamics, and persistence in the environment. Such information is also critical for the disease ecology of EIDs, particularly in remote sites, and when attempting to detect rare and cryptic species.

2 | Materials and Methods

2.1 | Study Sites and Sampling Methods

We conducted field surveys within seven regions in Panamá (Figure 2b). These areas are tropical moist forests, which have two annual rainfall patterns: a dry season (which ranges from

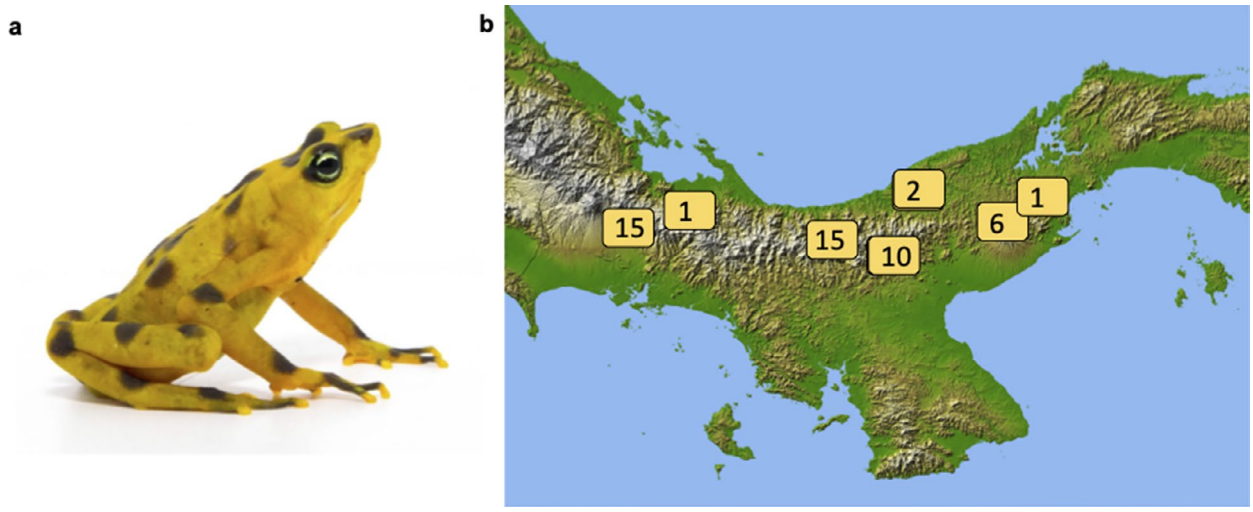

FIGURE 2 | Photo of and adult *Atelopus varius* detected by visual encounter survey (a) and (b) a map of Panama with seven regions and number of sites where we collected environmental DNA (eDNA) samples to detect three species in the amphibian genus *Atelopus* (*A. varius, A. zeteki, and A. chiriquensis*). source: photo credit for panel a to MD Basanta.

mid-December to mid-April) and a wet season (which ranges from mid-April to mid-December). To establish stream transects, we used GPS coordinates to identify stream sections that were either previously surveyed visually for amphibians (Lips et al. 2006; Richards and Knowles 2007; Woodhams, Kilburn et al. 2008) or that have been continuously visually sampled for amphibians since 2004. We used a measuring tape to determine 200 m and marked each transect every 10 m. During our surveys, 2–3 observers walked transects slowly, searching for amphibians according to established visual encounter survey protocols.

During visual encounter surveys, we temporarily captured amphibians using a fresh pair of gloves or an inverted plastic bag to minimize contamination and pathogen transmission. For each animal, we recorded the date, time, and location of capture, and we identified the species, sex, and age class, and measured snout-to-vent length (SVL) and body mass. We collected skin swab samples for all amphibians using standard swabbing techniques (Boyle et al. 2007). We froze the skin swab samples at −20°C and transported them to the laboratory to test for *Bd* infection using quantitative polymerase chain reaction (qPCR) according to established protocols (details below; Boyle et al. 2004, 2007).

2.2 | Environmental DNA Sample Collection

At each location, we collected two water samples, one sample from each edge of the stream, on the first visit to a site during each new season or year. We sampled facing upstream, at the most downstream point of the transect (meter 0). At the point where the water was sampled, we collected ecological data to get quantitative measures of stream characteristics, including stream width, depth, and flow rate. To measure water temperature and pH, we used a handheld calibrated water meter (Oakton, Vernon Hill, IL) at the start (meter 0, at the downstream end) of the transect. When possible, we also measured dissolved oxygen, total dissolved solids, and salinity. However, the water meter was less reliable for these water characteristics, and we subsequently omitted these parameters from our analyses. To measure stream width, we used a tape measure across the stream. We assessed stream depth using a dipstick at three locations across the stream. We took two measurements at 1 m in from the water's edges and one measurement was taken in the center of the stream. We also estimated stream velocity using a timed float for 1 m, canopy cover using a densiometer, and streambed substrate composition (e.g., percent boulders, rocks, pebbles, sand, silt) using visual observation.

When possible, we immediately preserved eDNA samples in the field using a hand-pump filter system. When on-site sampling was not possible, we kept samples cool or frozen until processing and filtered them using a vacuum pump. For on-site processing, we collected water samples using 1 L sterile Whirlpak bags. For off-site processing, we collected water samples using 1 L Nalgene bottles with screw top lids. Prior to using Nalgene bottles, we soaked bottles and lids in a 25% household bleach solution and subsequently rinsed both bottles and lids thoroughly to remove bleach residue before submerging them to collect stream water.

Before filtering, we homogenized the water by massaging the Whirlpak bag or gently agitating the Nalgene bottle. We used one filter per liter of stream water. We recorded the time to sample processing as well as the start and end time of each sample filtration. In addition, we recorded the volume of filtered samples, whether samples were cooled, and the duration of cooling. While processing water samples, we included one negative control for each site to monitor the quality of the protocol and possible contamination across sites. To collect a negative control sample, we filtered 1 L of store-bought bottled water following the same protocol described above.

In one location where we had visually detected *A. varius*, we collected additional eDNA samples ($N = 18$) to determine how far downstream from the detection we might detect *Atelopus*. We sampled at the site of the *Atelopus* detection, 200 m downstream from that location, and approximately 500 m downstream from that location once per week for four consecutive weeks.

2.3 | Diagnostics and *Batrachochyrium dendrobatidis* Prevalence in Swab Samples

For the *Bd* diagnostic assay of swabs, we analyzed all samples in triplicate with an internal positive control (IPC; Garland et al. 2010). We used a dilution set of plasmid standards (Pisces Molecular, Boulder, Colorado, USA) to quantify pathogen load. If 1 of 3 replicate wells turned up positive, we checked the cycle threshold (C_q) value to determine if non-amplification in 2 of 3 wells was caused by a low-level infection. We also verified that the qPCR was not inhibited (i.e., that the internal positive control amplified; Garland et al. 2010). In cases of inhibition or C_t values far from the detection threshold, we retested the sample and considered it positive if *Bd* was detected in any of the three retested wells (Boyle et al. 2007; Garland et al. 2010). We calculated *Bd* infection prevalence and 95% Clopper-Pearson binomial confidence intervals (CI) for sites and species.

2.4 | Detection of Host Species and *Batrachochytrium dendrobatidis* in eDNA Samples

For the eDNA samples, we used the same assay as described above to test for *Bd*, except with the substitution of environmental master mix, running for 50 cycles, and using a gBlock (IDT Inc) for the standard curve. For *Atelopus*, we developed a quantitative PCR assay for the three target *Atelopus* species using sequences from Richards and Knowles (2007) for *A. zeteki* and *A. varius* and including *A. chiriquiensis* (MVZ223270) in the validation (we were unable to produce a sequence from this sample, likely due to degradation). We designed an assay using Primer Express (ThermoFisher) software and validated this assay in silico using PrimerBlast (Ye et al. 2012). The assay consisted of AtCOIF: CGTTGAYACTCGAGCATATTTTAC, AtCOIR: GATTGTTCCT CCGTGYATTG, and 6FAM-CTACTATAATTATTGCCATTCCA-MGB at 0.2 μM each and 1X QuantiTect Multiplex PCR Mix (Qiagen Inc., Hilden, Germany) on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each qPCR included 3 μL of DNA extract in a total volume of 15 μL. We also included an internal positive control (IC; Qiagen) in each well to test for inhibition of the qPCR reaction. The cycling protocol started with 15 min at 95°C then ran for 50 cycles of 94°C for 60s followed by 60°C for 60s. We validated the sensitivity and specificity of the assay using tissue samples collected from *A. varius* ($N=7$), *A. zeteki* ($N=3$), *A. chiriquiensis* ($N=1$) (weak amplification consistent with a degraded sample), *Incilius coniferus* ($N=2$), *Rhaebo haematiticus* ($N=9$), *Rhinella horribilis* ($N=10$).

We extracted DNA from filter samples using the Qiashredder/DNeasy method described in Goldberg et al. (2011). Environmental DNA filter sample extraction and qPCR set up was carried out in a dedicated eDNA laboratory at Washington State University with limited access and clean protocols, following international eDNA guidelines (Goldberg et al. 2016). We included a negative extraction control with each set of extractions and included a negative qPCR control with each plate of samples. We ran all filter samples in triplicate, rerunning if the first set of results were inconsistent (1 or 2 wells positive). Each plate included a standard curve of a *A. varius* tissue sample in 4 ten-fold dilutions, run in duplicate. We considered samples inhibited if the *Cq* value was > 3 over that of the standard curve samples and cleaned samples testing as inhibited using a OneStepTM PCR Inhibitor Removal Kit (Zymo, Irvine, CA). We called a filter sample positive for detection if we saw exponential PCR amplification in all three of the original set run or at least 1 of each of the 2 triplicate runs.

2.5 | Analysis of Correlates of *Batrachochytrium dendrobatidis* Detection and Load in eDNA Samples

To test for correlations between eDNA detection (yes/no) and characteristics of the local environment, we used a generalized linear model (GLM) with a binomial distribution and a logit link. Predictor variables included stream characteristics (water temperature, wet width, mean depth, and pH) at the point of eDNA sampling and the mean load of *Bd* detected on amphibians sampled from the same transect and sampling period [in log (DNA copies +1)], as determined by qPCR on skin swab samples. For the subset of eDNA samples in which *Bd* was detected, we used a general linear model (LM) with the same predictors but *Bd* load per liter of water [in log (DNA copies)], as determined by qPCR, as the dependent variable. Both models were run in SPSS (version 26).

2.6 | Genomic Methods

We attempted to genotype 26 of the eDNA samples with the highest concentrations of *Bd* as determined by qPCR using a microfluidic multiplex PCR assay (Byrne et al. 2017). This assay uses 240 primer pairs designed to target phylogenetically informative regions of the *Bd* nuclear and mitochondrial genome (Byrne et al. 2017). We cleaned all eDNA extracts with an isopropanol precipitation (input volume between 25 and 40 uL) and DNA pellets were resuspended in 7 μL of low TE (10 mM Tris–HCl pH 8, 0.1 mM EDTA). Before sequencing, each cleaned sample was preamplified in two separate PCR reactions each with 120 primer pairs. After preamplification, each reaction was cleaned with 4 μL of ExoSAP-IT (Thermo Fisher Scientific) and diluted 1:5 with water. Finally, the cleaned and diluted products from each preamplification PCR were combined in equal proportions and sent to the University of Idaho IBEST Genomics Resources Core for amplification (using the Fluidigm Access Array platform) and sequenced on an Illumina MiSeq. After sequencing, we joined the raw reads using FLASH v.1.2.11 (Magoč and Salzberg 2011). We filtered the merged reads by selecting sequence variants represented by at least 5 reads and at least 5% of the total number of reads for each sample/locus combination. Next, we generated consensus sequences for each sample at each locus using the "reduced amplicons" R script (https://github.com/msettles/dbcAmplicons/blob/master/scripts/R/reduce_amplicons.R), which uses IUPAC ambiguity codes for multiple alleles.

For each sample, we counted the number of consensus sequences produced (out of a possible 240), and for samples with $N=5$ amplicon sequences, we created a *Bd* phylogeny using a gene-tree to species-tree with the program Astral (Zhang et al. 2018). To create each sample tree, we compared the amplicons sequenced in each of our samples to the same sequences from previously published *Bd* samples representing all major *Bd* lineages (Byrne et al. 2019). We first used MUSCLE (v.3.32.0, Edgar 2004) to align per-locus sequences and then used RAxML (v.8.2.11, Stamatakis 2014) with

rapid bootstrapping for 100 bootstraps and the GTR substitution model and searched for the best-scoring ML tree for each locus. We then used newick utils (v.1.6, Junier and Zdobnov 2010) to collapse all nodes in each locus tree with less than 10 bootstrap support. Finally, we used Astral (v.5.7.5) to create one tree from all input locus trees and collapsed all nodes in the astral tree with a posterior probability less than 0.5. We then determined the *Bd* genotype for each of our samples by comparing the position of our sample in the tree with the position of the reference sequences, considering a sample that forms a well-supported monophyletic grouping with all members of another major lineage as belonging to that *Bd* lineage.

3 | Results

3.1 | Surveys

Using eDNA methods, we detected *Atelopus* DNA at two of the four locations where we also detected *A. varius* individuals using VES (Table 1). However, on 15 of the 24 time points when we conducted both VES and eDNA surveys at four known *A. varius* sites, we failed to detect this species with both methods. On six occasions, we detected this species only with VES. On one occasion, we detected *Atelopus* with eDNA only. On two occasions, we had positive detections with both methods. We did not detect any *Atelopus* using eDNA or VES in the historic range of *A. chiriquiensis*. We discarded and re-collected a set of field samples that had been contaminated with eDNA from water obtained from the tap. All other negative controls tested negative.

When we repeatedly collected additional eDNA samples downstream of a known *Atelopus* location (2018 Dry Season, $N = 18$ samples), we only detected *Atelopus* in two samples collected nearest to the known *Atelopus* location. We did not detect *Atelopus* DNA from either of the additional sampling locations, 200–500 m away from the *Atelopus* location.

3.2 | Detection of *Batrachochytrium dendrobatidis* in *Atelopus* Sites

We detected *Bd* eDNA in at least one water sample at three of the four sites where *Atelopus* is known to persist. At the one site

TABLE 1 | Comparisons of *Atelopus varius* detection using visual encounter surveys (VES) versus environmental DNA surveys at sites where this species was detected and time points where both methods were used.

			Visual encounter surveys		eDNA surveys	
Site	**Year**	**Season**	**Survey days**	**Individuals seen**	**Samples taken**	**Samples (+) for *A. varius***
Cerro N.	2014	Wet	3	0	4	0
Cerro N.	2014	Dry	2	0	2	0
Cerro N.	2015	Wet	2	0	2	0
Cerro N.	2015	Dry	4	0	4	0
Cerro N.	2016	Wet	3	0	2	0
Rio B.	2014	Wet	3	0	2	0
Rio B.	2014	Dry	3	0	2	0
Rio B.	2015	Wet	3	1	2	0
Rio B.	2016	Wet	3	0	2	0
Rio B.	2016	Dry	2	0	5	0
Rio B.	2017	Dry	1	0	2	0
Rio B.	2018	Dry	4	0	2	0
Rio T.	2014	Wet	6	25	2	0
Rio T.	2014	Dry	2	4	6	5
Rio T.	2015	Wet	3	0	3	0
Rio T.	2015	Dry	3	2	2	2
Rio T.	2016	Dry	3	1	3	0
Rio T.	2017	Dry	1	0	2	0
Rio T.	2018	Dry	1	0	18	2
Site S.	2014	Wet	3	1	1	0
Site S.	2015	Wet	3	2	2	0
Site S.	2016	Wet	3	0	2	0
Site S.	2016	Dry	3	1	2	0
Site S.	2019	Dry	3	0	2	0

where *Bd* eDNA was never detected, we analyzed nine samples. At the site where *A. varius* has been seen most consistently since 2012, we detected *Bd* in only 2 of 36 water samples. The proportion of water samples that tested positive for *Bd* eDNA was much lower at the four sites where we detected *Atelopus* (12 of 92, or 13% of samples tested positive) than at sites where *Atelopus* had not been seen since before the epizootic event (90 of 238, or 38% of samples tested positive, $\chi^2_1 = 10.20$, $p = 0.001$). When considering only the samples in which *Bd* eDNA was detected, the estimated quantity of *Bd* eDNA copies per liter of water also showed some evidence of being lower at sites where *Atelopus* had been observed (mean ± SE: 632 ± 121) compared to sites where it had not (3143 ± 1395; $t_{90.29} = 1.79$, $p = 0.076$).

3.3 | Correlates of *Batrachochytrium dendrobatidis* Detection and Load in eDNA Samples

We detected *Bd* more often in water samples that were collected from shallow sections of stream and from sites where amphibians captured along the transect had greater loads of *Bd* on their skin (GLM: stream depth likelihood ratio $\chi^2_1 = 5.087$, $\beta = -0.085$, $p = 0.024$; skin swab *Bd* load likelihood ratio $\chi^2_1 = 6.720$, $\beta = -0.479$, $p = 0.010$; Figure 3a,b). Water temperature, pH, and stream width were not significant predictors of *Bd* detection in eDNA samples (GLM: all $\chi^2_1 \leq 3.748$, $p \geq 0.053$). For water samples from which *Bd* was detected, the concentration of *Bd* DNA detected was positively correlated with the mean load of *Bd* on the skin of the local amphibians (LM: $F_{1,13} = 10.874$, $\beta = 2.542$, $p = 0.006$; Figure 4a) and with water temperature (LM: $F_{1,13} = 7.613$, $\beta = 0.458$, $p = 0.016$; Figure 4b) and negatively correlated with stream depth (LM: $F_{1,13} = 10.647$, $\beta = -0.041$, $p = 0.006$, Figure 4c). Stream width and pH were not significant predictors of *Bd* concentration in water samples (LM: $F_{1,13} \leq 0.844$, $p \geq 0.375$).

3.4 | *Batrachochytrium dendrobatidis* Genomic Data From eDNA

We used 26 of the eDNA samples to assess our ability to collect *Bd* genomic data from stream samples (Table 2). Of the 26 samples, 18 did not return any amplicon sequences and 8 returned between 1 and 14 amplicon sequences. We were able to assign a *Bd*-lineage to four of the five samples that had at least five amplicon sequences (Figure 5). The phylogenetic trees indicate that these four samples belong to the *Bd*-GPL lineage (Figure 5).

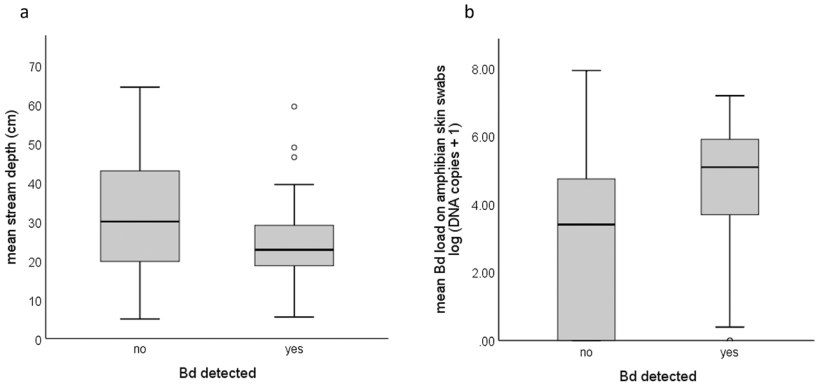

FIGURE 3 | Boxplots showing the distribution of stream depths (a) and mean loads of *Batrachochytrium dendrobatidis* (*Bd*) loads on swab samples from amphibian skin (b) for water samples in which *Bd* environmental DNA was (yes) and was not (no) detected.

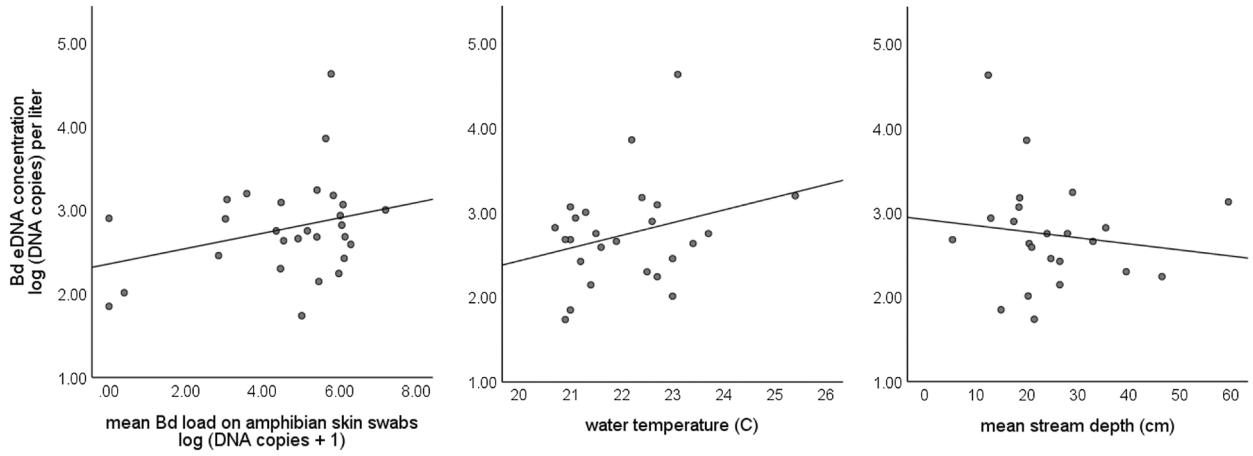

FIGURE 4 | Scatterplots and lines of best fit for the relationships between concentrations of *Batrachochytrium dendrobatidis* (*Bd*) and environmental DNA and *Bd* loads on amphibian skin (a), water temperature (b), and mean stream depth (c).

4 | Discussion

There are a wide range of challenges in studying rare, cryptic, and endangered species, especially in tropical ecosystems (McDonald 2004; Schloegel et al. 2006; Pearl et al. 2009). The use of eDNA has been transformative in these efforts because it allows scientists to collect critical information when species persist below visual detection thresholds (Lopes et al. 2021). While eDNA methods have previously been used in temperate riparian systems and employed to detect pathogens in aquatic environments (e.g., Amarasiri et al. 2021), it has been used far less in tropical rainforest streams and/or to detect cryptic host species and their pathogens. We developed eDNA methods for use in tropical stream systems to detect three species of amphibian hosts that recently declined due to the infectious disease chytridiomycosis. We also used our eDNA samples to detect *Bd*, the pathogen that causes the lethal disease, and understand how some ecological variables may influence pathogen detection in these stream systems.

We found that using an eDNA approach—notwithstanding some limitations which we detail below—was effective for detection of *Atelopus varius* in a tropical rainforest setting. This finding represents a great advantage for researchers studying disease-induced species declines during time periods when detection probability is low. Traditionally, this has involved labor- and time-intensive visual encounter surveys. Although we were not able to directly compare sampling effort between VES and eDNA, adding eDNA sampling may dramatically help in efforts to detect species that have declined to low levels due to disease. Yet, while using eDNA presents a variety of opportunities, it is critical to be aware of limitations, including challenges associated with collecting and preserving eDNA samples. For example, warm temperatures are associated with higher rates of DNA degradation (Strickler et al. 2015), both within the aquatic system and during transport if samples cannot be filtered on-site. Access to ice or refrigeration to keep samples cool prior to filtration can be limited in remote settings. Additionally,

TABLE 2 | Environmental DNA samples for a Fluidigm genotyping assay for *Batrachochytrium dendrobatidis* (*Bd*).

Sample	Region	*Bd* qPCR value (copies/μL)	*Bd* amplicons sequenced (out of 240)	*Bd* genotype
eDNA-SofiaR_P	El Cope	610.02	6	Bd-GPL
eDNA-CampanaR_z	Campana	1003.86	0	Undetermined
eDNA-SOFR-1z	El Cope	8.84	5	Bd-GPL
eDNA-MANL	El Cope	16.21	3	Undetermined
e-160624_RBR	El Cope	5.67	0	Undetermined
e-160603_Lz	Campana	340.28	0	Undetermined
e-SOF_R	El Cope	90.42	0	Undetermined
e-SOFIA_L	El Cope	34.70	0	Undetermined
e-MAN_L	El Cope	16.21	12	Bd-GPL
e-160608_AL	El Valle	13.51	0	Undetermined
e-CAM_R2z	Campana	11.96	0	Undetermined
e-150,608 _JL	El Valle	11.69	14	Bd-GPL
e-SOF_L-2z	El Cope	11.39	0	Undetermined
e-160625_SL	El Cope	9.83	0	Undetermined
e-MAN_R	El Cope	9.25	0	Undetermined
e-ADP_R	Santa Fe	8.56	0	Undetermined
e-SOF_R2z	El Cope	8.52	0	Undetermined
e-160607_L	El Valle	8.46	0	Undetermined
e-160608_AR	El Valle	8.10	5	Undetermined
e-MAR_AR	El Valle	7.94	0	Undetermined
e-160625_SR	El Cope	7.64	0	Undetermined
e-MAR_AL	El Valle	7.61	3	Undetermined
e-150628_G-Lz	El Cope	7.52	0	Undetermined
e-CAM_R	Campana	7.33	0	Undetermined
e-160725_CR	Campana	6.70	0	Undetermined
e-160714_L	Santa Fe	6.03	1	Undetermined

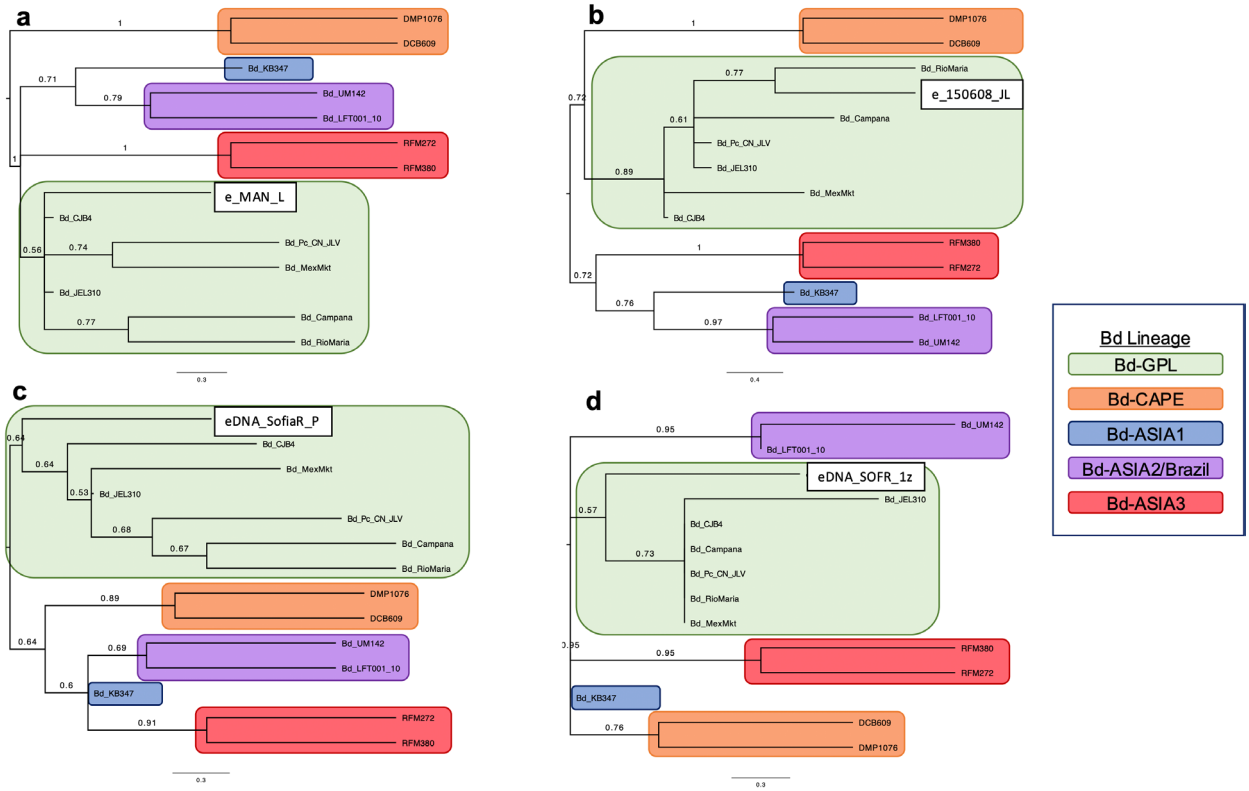

FIGURE 5 | Phylogenetic trees created using a gene-tree to species-tree approach for four eDNA samples and 13 previously published *Batrachochyrium dendrobatidis* (*Bd*) samples (Byrne et al. 2019). Trees were made from the following number of locus trees [12 (a), 14 (b), 6 (c), 5 (d)]. Nodes with less than 0.5 posterior probability are collapsed. The major *Bd* lineages (O'Hanlon et al. 2018; Byrne et al. 2019) are color-coded and labeled for the *Bd* lineage.

where field safety or permissions/access prevent filtering on-site (as they did at some sites in this study), sample volume will be limited. Whether sampled on-site or not, sample volume is constrained in high turbidity streams when filters clog. Much larger sample volumes (>100 L) have been collected from tropical streams using the VigiDNA filtering system (SPYGEN) (Lopes et al. 2017; Cantera et al. 2019). However, this filter is not yet widely available. Nevertheless, we expect that increasing sample volume (e.g., even from 1 to 2 L) is likely to have a large positive impact on detection probability (e.g., Bedwell and Goldberg 2020). Finally, lack of access to clean water can make decontamination problematic. During this study, we also experienced how imperative it is to collect field negatives in the same receptacles as those used for sample collection, use clean water to rinse after decontamination, and avoid reusing materials whenever possible. Overall, to effectively employ this method, it will be essential to carefully consider the limitations that might alter outcomes, such as those outlined here as well as the known distribution of the species, methodological/resource limitations, and dynamic environmental variables.

We did not detect other *Atelopus* species from Western Panamá, even with wide-ranging eDNA sampling in historic sites (Figure 2b). Despite extensive eDNA sampling for *A. chiriquiensis* at numerous historic localities and at multiple times of the year, we did not detect this *Atelopus* species, which was abundant until approximately 1996, near the town of Volcan. Our negative finding is also supported by recent visual survey efforts for this species in Western Panamá, which were also unsuccessful. The cause of decline of this species is thought to be chytridiomycosis (Lewis et al. 2019), and scientists have anecdotally commented that its survival and recovery may be unlikely because much of its previous habitat has been developed. As such, *A. chiriquiensis*'s IUCN status, which is currently listed as "Extinct" (IUCN SSC 2020), may be appropriate. Similarly, we did not detect the "critically endangered (possibly extinct in the wild)" *Atelopus zeteki* (IUCN SSC 2024), a species that was abundant at historical localities around El Valle de Anton, Panamá, until approximately 2006 (Zippel et al. 2006; Gagliardo et al. 2008). It is difficult to determine if this is due to true loss of this species or if more time and effort is needed to detect recoveries following epizootic events. Irrespective of the cause, it should also be noted that recent studies (Ramírez et al. 2020; Byrne et al. 2021) suggest that the status of *A. zeteki* as a distinct species from *A. varius* warrants further examination.

Environmental DNA analysis provided an opportunity to simultaneously investigate the presence of the amphibian hosts and *Bd* and to evaluate the factors influencing pathogen detection in tropical stream systems. We detected *Bd* in a high proportion of our water samples and uncovered some relationships with ecological variables that could be informative for future studies. First, both our ability to detect and determine the quantity of *Bd* DNA in eDNA samples were positively correlated with the infection intensity of local amphibians. Second, we found correlations between abiotic conditions at our sampling sites and our *Bd* eDNA results. For example, we

detected *Bd* more often and in greater quantities in shallower sections of the stream. This finding could reflect the fact that some amphibian species preferentially use shallower sections of streams more commonly than the deeper, faster flowing sections of the stream, but this possibility remains to be investigated. Nevertheless, these findings suggest that eDNA sampling may provide a fast and relatively cost-effective method for monitoring changes in pathogen dynamics in tropical amphibian assemblages.

An additional factor that may have influenced our detection of *Bd* in streams was water temperature. Specifically, we detected greater quantities of *Bd* DNA in areas of stream with slightly higher water temperatures (Figure 4b). In addition, all our positives for *Bd* eDNA were from the dry season when temperatures are generally slightly higher and stream velocity and flow are generally lower, compared to the wet season. The water temperatures were still within *Bd*'s thermal range (< 26°C, Voyles et al. 2017) but slightly higher than temperatures that are normally considered optimal for *Bd* (Piotrowski, Annis, and Longcore 2004, Woodhams, Alford et al. 2008; Voyles et al. 2017). The seasonal pattern that we observed (i.e., more positive *Bd* detections during the dry season) could be due to temperature effects on *Bd* (Voyles et al. 2012; Sonn, Berman, and Richards-Zawacki 2017), which has been suggested for temperate regions (Chestnut et al. 2014). However, we suggest that it is equally plausible that additional factors, such as seasonal fluctuations of host abundance (e.g., several frog species concentrate along the moist margins of streams during the dry season; Toft 1980; Ibáñez et al. 1995), host breeding phenology (e.g., some species congregate for breeding during the transition from wet to dry season; McCaffery, Richards-Zawacki, and Lips 2015), and other seasonal effects on host-pathogen dynamics, could be contributing to this pattern. Irrespective of the underlying determinants of this pattern, this finding underscores the importance of temporal sampling for eDNA.

In addition, we found that it is possible to genotype *Bd* using eDNA samples and a microfluidic multiplex PCR assay (Byrne et al. 2017). Our results corroborated other studies that show that *Bd* in Panamá is in the global panzootic lineage (BdGPL) (Voyles et al. 2018; Rothstein et al. 2021). We caution, however, that *Bd* genotyping using eDNA samples is difficult and somewhat unpredictable due to the presence of PCR inhibitors and low quality/quantity DNA in extracts. We suggest that further optimization of DNA cleaning (e.g., using a cleaning method that retains more DNA than isopropanol precipitation) should help to improve genotyping from eDNA samples in the future. Furthermore, more sensitive, or targeted approaches, such as lineage specific qPCR techniques may also be a useful tool for identifying *Bd* lineages from non-invasive eDNA samples. Overall, with improvements to these methods, we expect that eDNA samples may become a powerful tool for understanding pathogen genotypes within a given environment.

The most encouraging outcome of this research is that we can now leverage this tool to help direct science-based conservation efforts. Conservation initiatives are underway in Panamá for *Atelopus* and many other rare and endangered stream-breeding species (Lewis et al. 2019). Similar efforts are underway for other *Atelopus* species in this genus in multiple countries in Central and South American (Valencia and Fonte 2021). We suggest that these conservation initiatives can be considerably aided by a rapid and inexpensive eDNA testing to determine if rare species are present, if they may show signs of recovery, and to track the dynamics of any pathogens or parasites that may threaten them.

Ultimately, the ability to simultaneously detect the host species and the pathogen or parasite offers a wide range of options to understand changes in the health of species to ecosystems. For example, we could potentially use this approach in the context of eDNA metabarcoding to understand the species richness of an amphibian assemblage alongside the dynamics of the species they play host to (Lopes et al. 2021). We may also be able to better track host species phenological patterns as well as seasonal dynamics of disease (Rosa et al. 2022). These insights can help generate science-based conservation programs (e.g., head starting at an appropriate seasonal window where disease is less of a threat). Development underway for on-site eDNA testing using CRISPR (Baerwald et al. 2023) is also promising for quick-turnaround results at remote field sites (Sasso et al. 2017). Together with the eDNA methods we present here, scientists and conservationists can add to their tool kit for confronting species loss due to infectious disease.

By applying cutting-edge analysis techniques to detect rare species and their key pathogen, this research has the potential to discover populations that persist despite the persistence of *Bd*. These methods may also help identify characteristics that have given populations of three critically endangered *Atelopus* species a survival advantage. Understanding these characteristics will not only help understand the factors that contribute to variation in susceptibility to chytridiomycosis for other threatened taxa but will be important in devising informed conservation strategies for Panamanian *Atelopus*. In this way, our work contributes to understanding the biology of this globally important, emerging wildlife disease, and conserving some of Central America's most endangered species.

Author Contributions

J.V., C.R.Z., and C.G. conceived of the idea. All authors contributed to data collection. J.V., C.R.Z., C.G., and A.Q.B. analyzed the data. J.V. and C.R.Z. led writing with editorial assistance from the other authors.

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Ethics Statement

This research was approved under UNR IACUC protocol 20-08-1063 and the Smithsonian Tropical Research Institute IACUC protocol 2012-0901-2015.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in Dryad at https://datadryad.org/stash/share/vXjJcx9gXEDyNLG5oeNqLcwPNUpeDSnrtFv4xnAA8CQ.

References

Amarasiri, M., T. Furukawa, F. Nakajima, and K. Sei. 2021. "Pathogens and Disease Vectors/Hosts Monitoring in Aquatic Environments: Potential of Using eDNA/eRNA Based Approach." *Science of the Total Environment* 796: 148810.

Baerwald, M. R., E. C. Funk, A. M. Goodbla, et al. 2023. "Rapid CRISPR-Cas13a Genetic Identification Enables New Opportunities for Listed Chinook Salmon Management." *Molecular Ecology Resources* 23: 36847138.

Bedwell, M. E., and C. S. Goldberg. 2020. "Spatial and Temporal Patterns of Environmental DNA Detection to Inform Sampling Protocols in Lentic and Lotic Systems." *Ecology and Evolution* 10: 1602–1612.

Berger, L., R. Speare, P. Daszak, et al. 1998. "Chytridiomycosis Causes Amphibian Mortality Associated with Population Declines in the Rain Forests of Australia and Central America." *Proceedings of the National Academy of Sciences* 95, no. 15: 9031–9036.

Boyle, A. H. D., V. Olsen, D. B. Boyle, et al. 2007. "Diagnostic Assays and Sampling Protocols for the Detection of *Batrachochytrium dendrobatidis*." *Diseases of Aquatic Organisms* 73, no. 3: 175–192.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. Morgan, and A. D. Hyatt. 2004. "Rapid Quantitative Detection of Chytridiomycosis (*Batrachochytrium dendrobatidis*) in Amphibian Samples Using Real-Time Taqman PCR Assay." *Diseases of Aquatic Organisms* 60, no. 2: 141–148.

Briggs, C. J., R. A. Knapp, and V. T. Vredenburg. 2010. "Enzootic and Epizootic Dynamics of the Chytrid Fungal Pathogen of Amphibians." *Proceedings of the National Academy of Sciences of the United States of America* 107, no. 21: 9695–9700.

Bustamante, H. M., L. J. Livo, and C. Carey. 2010. "Effects of Temperature and Hydric Environment on Survival of the Panamá Nian Golden Frog Infected With a Pathogenic Chytrid Fungus." *Integrative Zoology* 5, no. 2: 143–153.

Byrne, A. Q., C. L. Richards-Zawacki, J. Voyles, K. Bi, R. Ibáñez, and E. B. Rosenblum. 2021. "Whole Exome Sequencing Identifies the Potential for Genetic Rescue in Iconic and Critically Endangered Panamánian Harlequin Frogs." *Global Change Biology* 27, no. 1: 50–70.

Byrne, A. Q., A. P. Rothstein, T. J. Poorten, J. Erens, M. L. Settles, and E. B. Rosenblum. 2017. "Unlocking the Story in the Swab: A New Genotyping Assay for the Amphibian Chytrid Fungus *Batrachochytrium dendrobatidis*." *Molecular Ecology Resources* 17, no. 6: 1283–1292.

Byrne, A. Q., V. T. Vredenburg, A. Martel, et al. 2019. "Cryptic Diversity of a Widespread Global Pathogen Reveals Expanded Threats to Amphibian Conservation." *Proceedings of the National Academy of Sciences of the United States of America* 116, no. 41: 20382–20387.

Cantera, I., K. Cilleros, A. Valentini, et al. 2019. "Optimizing Environmental DNA Sampling Effort for Fish Inventories in Tropical Streams and Rivers." *Scientific Reports* 9: 3085.

Chestnut, T., C. Anderson, R. Popa, et al. 2014. "Heterogeneous Occupancy and Density Estimates of the Pathogenic Fungus *Batrachochytrium dendrobatidis* in Waters of North America." *PLoS One* 9, no. 9: e106790.

Crawford, A. J., K. R. Lips, and E. Bermingham. 2010. "Epidemic Disease Decimates Amphibian Abundance, Species Diversity, and Evolutionary History in the Highlands of Central Panamá." *Proceedings of the National Academy of Sciences of the United States of America* 107, no. 31: 13777–13782.

Edgar, R. C. 2004. "MUSCLE: Multiple Sequence Alignment With High Accuracy and High Throughput." *Nucleic Acids Research* 32, no. 5: 1792–1797.

Gagliardo, R., P. Crump, E. Griffith, J. Mendelson, H. Ross, and K. Zippel. 2008. "The Principles of Rapid Response for Amphibian Conservation, Using the Programmes in Panamá as an Example." *International Zoo Yearbook* 42: 125–135.

Garland, S., A. Baker, A. D. Phillott, and L. F. Skerratt. 2010. "BSA Reduces Inhibition in a TaqMan® Assay for the Detection of *Batrachochytrium dendrobatidis*." *Diseases of Aquatic Organisms* 92, no. 2–3: 113–116.

Gass, J., A. J. Miller, C. Sheets, M. Long, and J. Voyles. 2024. "High Relative Humidity and Temperature Limit Disease Development and Mortality in Golden Frogs of Panamá, *Atelopus zeteki*, Infected With *Batrachochytrium dendrobatidis*." *Evolutionary Ecology* 38, no. 1: 141–156.

Gass, J., and J. Voyles. 2022. "When Defenses Fail: *Atelopus zeteki* Skin Secretions Increase Growth of the Pathogen *Batrachochytrium dendrobatidis*." *Integrative and Comparative Biology* 62, no. 6: 1595–1605.

Goldberg, C. S., D. S. Pilliod, R. S. Arkle, and L. P. Waits. 2011. "Molecular Detection of Vertebrates in Stream Water: A Demonstration Using Rocky Mountain Tailed Frogs and Idaho Giant Salamanders." *PLoS One* 6, no. 7: e22746.

Goldberg, C. S., C. R. Turner, K. Deiner, et al. 2016. "Critical Considerations for the Application of Environmental DNA Methods to Detect Aquatic Species." *Methods in ecology and evolution* 7, no. 11: 1299–1307.

Ibáñez, R., C. A. Jaramillo, M. Arrunátegui, Q. Fuenmayor, and F. A. Solís. 1995. "InventaRio Biológico del Canal de Panamá. Estudio herpetológico. Scientia (Panamá)." *Número Especial* 2: 111–159.

IUCN SSC Amphibian Specialist Group. 2020. "*Atelopus chiriquiensis*. The IUCN Red List of Threatened Species 2020: e.T54498A54340769." https://doi.org/10.2305/IUCN.UK.2020-3.RLTS.T54498A54340769.en.

IUCN. 2024. The IUCN Red List of Threatened Species. Version 2024-2. https://www.iucnredlist.org Accessed on January 1, 2025.

Junier, T., and E. M. Zdobnov. 2010. "The Newick Utilities: High-Throughput Phylogenetic Tree Processing in the UNIX Shell." *Bioinformatics* 26, no. 13: 1669–1670.

Kelly, R. P., D. M. Lodge, K. N. Lee, et al. 2023. "Toward a National eDNA Strategy for the United States." *Environmental DNA* 00: 1–10.

Langwig, K. E., J. Voyles, M. Q. Wilber, et al. 2015. "Context-Dependent Conservation Responses to Emerging Wildlife Diseases." *Frontiers in Ecology and the Environment* 13, no. 4: 195–202.

Lewis, C. H. R., C. L. Richards-Zawacki, R. Ibáñez, et al. 2019. "Conserving Panamá Nian Harlequin Frogs by Integrating Captive-Breeding and Research Programs." *Biological Conservation* 236: 180–187.

Lips, K. R., F. Brem, R. Brenes, et al. 2006. "Emerging Infectious Disease and the Loss of Biodiversity in a Neotropical Amphibian Community." *Proceedings of the National Academy of Sciences of the United States of America* 103, no. 9: 3165–3170.

Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. "Batrachochytrium dendrobatidis Gen. Et sp. nov., a Chytrid Pathogenic to Amphibians." *Mycologia* 91, no. 2: 219–227.

Lopes, C. M., D. Baêta, A. Valentini, et al. 2021. "Lost and Found: Frogs in a Biodiversity Hotspot Rediscovered With Environmental DNA." *Molecular Ecology* 30, no. 13: 3289–3298.

Lopes, C. M., T. Sasso, A. Valentini, et al. 2017. "eDNA Metabarcoding: A Promising Method for Anuran Surveys in Highly Diverse Tropical Forests." *Molecular Ecology Resources* 17: 904–914.

Lötters, S. 2007. "The Fate of the Harlequin Toads–Help Through a Synchronous Multi-Disciplinary Approach and the IUCN 'Amphibian Conservation Action Plan'?" *Zoosystematics and Evolution* 83, no. S1: 69–73.

Magoč, T., and S. L. Salzberg. 2011. "FLASH: Fast Length Adjustment of Short Reads to Improve Genome Assemblies." *Bioinformatics* 27, no. 21: 2957–2963.

McCaffery, R., C. L. Richards-Zawacki, and K. R. Lips. 2015. "The Demography of *Atelopus* Decline: Harlequin Frog Survival and Abundance in Central Panamá Prior to and During a Disease Outbreak." *Global Ecology and Conservation* 4: 232–242.

McDonald, L. L. 2004. "Sampling Rare Populations." In *Sampling Rare or Elusive Species: Concepts, Designs, and Techniques for Estimating Population Parameters*, edited by W. L. Thompson, 11–42. Washington, DC: Island Press.

O'Hanlon, S. J., A. Rieux, R. A. Farrer, et al. 2018. "Recent Asian Origin of Chytrid Fungi Causing Global Amphibian Declines." *Science* 360, no. 6389: 621–627.

Pearl, C. A., M. J. Adams, R. B. Bury, W. H. Wente, and B. McCreary. 2009. "Evaluating Amphibian Declines With Site Revisits and Occupancy Models: Status of Montane Anurans in the Pacific Northwest USA." *Diversity* 1, no. 2: 166–181.

Perez, R., C. Richards-Zawacki, A. R. Krohn, et al. 2014. "Field Surveys in Western Panamá Indicate Populations of *Atelopus varius* Frogs Are Persisting in Regions Where Batrachochytrium dendrobatidis Is Now Enzootic." *Amphibian & Reptile Conservation* 8, no. 2: 30–35.

Piotrowski, J. S., S. L. Annis, and J. E. Longcore. 2004. "Physiology of *Batrachochytrium dendrobatidis*, a Chytrid Pathogen of Amphibians." *Mycologia* 96, no. 1: 9–15.

Ramírez, J. P., C. A. Jaramillo, E. D. Lindquist, A. J. Crawford, and R. Ibáñez. 2020. "Recent and Rapid Radiation of the Highly Endangered Harlequin Frogs (*Atelopus*) Into Central America Inferred From Mitochondrial DNA Sequences." *Diversity* 12, no. 9: 360.

Rees, H. C., B. C. Maddison, D. J. Middleditch, J. R. Patmore, and K. C. Gough. 2014. "The Detection of Aquatic Animal Species Using Environmental DNA–a Review of eDNA as a Survey Tool in Ecology." *Journal of Applied Ecology* 51, no. 5: 1450–1459.

Richards, C. L., and L. L. Knowles. 2007. "Tests of Phenotypic and Genetic Concordance and Their Application to the Conservation of Panamá Nian Golden Frogs (Anura, Bufonidae)." *Molecular Ecology* 16: 3119–3133.

Richards-Zawacki, C. L. 2009. "Thermoregulatory Behaviour Affects Prevalence of Chytrid Fungal Infection in a Wild Population of Panamanian Golden Frogs." *Proceedings of the Royal Society of London, Series B* 277, no. 1681: 519–528.

Rothstein, A. P., A. Q. Byrne, R. A. Knapp, et al. 2021. "Divergent Regional Evolutionary Histories of a Devastating Global Amphibian Pathogen." *Proceedings of the Royal Society B* 288, no. 1953: 20210782.

Rosa, G. M., R. Perez, L. A. Richards, et al. 2022. "Seasonality of Host Immunity in a Tropical Disease System." *Ecosphere* 13, no. 7: e4158.

Ryan, M. J., K. R. Lips, and M. W. Eichholz. 2008. "Decline and Extirpation of an Endangered Panamá Nian Stream Frog Population (*Craugastor punctariolus*) due to an Outbreak of Chytridiomycosis." *Biological Conservation* 141, no. 6: 1636–1647.

Sasso, T., C. M. Lopes, A. Valentini, et al. 2017. "Environmental DNA Characterization of Amphibian Communities in the Brazilian Atlantic Forest: Potential Application for Conservation of a Rich and Threatened Fauna." *Biological Conservation* 215: 225–232.

Savage, J. M. 1972. "The Harlequin Frogs, Genus *Atelopus*, of Costa Rica and Western Panamá." *Herpetologica* 28, no. 2: 77–94.

Scheele, B. C., L. F. Skerratt, L. F. Grogan, et al. 2017. "After the Epidemic: Ongoing Declines, Stabilizations and Recoveries in Amphibians Afflicted by Chytridiomycosis." *Biological Conservation* 206: 37–46.

Scheele, B. C., F. Pasmans, L. F. Skerratt, et al. 2019. "Amphibian Fungal Panzootic Causes Catastrophic and Ongoing Loss of Biodiversity." *Science* 363, no. 6434: 1459–1463.

Schloegel, L. M., J. M. Hero, L. Berger, R. Speare, K. McDonald, and P. Daszak. 2006. "The Decline of the Sharp-Snouted Day Frog (*Taudactylus acutirostris*): The First Documented Case of Extinction by Infection in a Free-Ranging Wildlife Species?" *EcoHealth* 3: 35–40.

Sonn, J. M., S. Berman, and C. L. Richards-Zawacki. 2017. "The Influence of Temperature on Chytridiomycosis In Vivo." *EcoHealth* 14: 762–770.

Stamatakis, A. 2014. "RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies." *Bioinformatics* 30, no. 9: 1312–1313.

Strickler, K. M., A. K. Fremier, and C. S. Goldberg. 2015. "Quantifying Effects of UV-B, Temperature, and pH N eDNA in Aquatic Microcosms." *Biological Conservation* 183: 85–92. https://doi.org/10.1016/j.biocon.2014.11.038.

Sewell, D., G. Guillera-Arroita, R. A. Griffiths, and T. J. Beebee. 2012. When Is a Species Declining? Optimizing Survey Effort to Detect Population Changes in Reptiles.

Toft, C. A. 1980. "Seasonal Variation in Populations of Panamá Nian Litter Frogs and Their Prey: A Comparison of Wetter and Drier Sites." *Oecologia* 47, no. 1: 34–38.

Valencia, L. M., and L. F. M. Fonte. 2021. *Harlequin Toad (Atelopus) Conservation Action Plan (2021–2041)*. Atelopus Survival Initiative. https://www.iucn-amphibians.org/wp-content/uploads/sites/4/2021/08/HarleCAP-2021-English.pdf.

Vörös, J., O. Márton, B. R. Schmidt, J. T. Gál, and D. Jelić. 2017. "Surveying Europe's Only Cave-Dwelling Chordate Species (*Proteus anguinus*) Using Environmental DNA." *PLoS One* 12, no. 1: e0170945.

Voyles, J., L. R. Johnson, C. J. Briggs, et al. 2012. "Temperature Alters Reproductive Life History Patterns in *Batrachochytrium dendrobatidis*, a Lethal Pathogen Associated With the Global Loss of Amphibians." *Ecology and Evolution* 2, no. 9: 2241–2249.

Voyles, J., L. R. Johnson, J. Rohr, et al. 2017. "Diversity in Growth Patterns Among Strains of the Lethal Fungal Pathogen *Batrachochytrium dendrobatidis* Across Extended Thermal Optima." *Oecologia* 184: 363–373.

Voyles, J., D. C. Woodhams, V. Saenz, et al. 2018. "Shifts in Disease Dynamics in a Tropical Amphibian Assemblage Are Not due to Pathogen Attenuation." *Science* 359, no. 6383: 1517–1519.

Voyles, J., S. Young, L. Berger, et al. 2009. "Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines." *Science* 326, no. 5952: 582–585.

Woodhams, D. C., V. L. Kilburn, L. K. Reinert, et al. 2008. "Chytridiomycosis and Amphibian Population Declines Continue to Spread Eastward in Panamá." *EcoHealth* 5: 268–274.

Woodhams, D. C., R. A. Alford, C. J. Briggs, M. Johnson, and L. A. Rollins-Smith. 2008. "Life-History Trade-Offs Influence Disease in Changing Climates: Strategies of an Amphibian Pathogen." *Ecology* 89, no. 6: 1627–1639.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, and T. L. Madden. 2012. "Primer-BLAST: A Tool to Design Target-Specific Primers for Polymerase Chain Reaction." *BMC Bioinformatics* 13: 1–11.

Zhang, C., M. Rabiee, E. Sayyari, and S. Mirarab. 2018. "ASTRAL-III: Polynomial Time Species Tree Reconstruction From Partially Resolved Gene Trees." *BMC Bioinformatics* 19, no. 6: 15–30.

Zinger, L., J. Donald, S. Brosse, et al. 2020. "Advances and Prospects of Environmental DNA in Neotropical Rainforests." *Advances in Ecological Research* 62: 331–373.

Zippel, K. C., D. R. Ibáñez, E. D. Lindquist, C. L. Richards, A. C. A. Jaramillo, and E. J. Griffith. 2006. "Implicaciones en la Conservación de las Ranas Doradas de Panamá, Asociadas a su Revisión Taxonómica." *Herpetotropicos* 3, no. 1: 29–39.