**RESEARCH ARTICLE**

*Batrachochytrium dendrobatidis* infection in amphibians predates first known epizootic in Costa Rica

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**Abstract**

Emerging infectious diseases are a growing threat to biodiversity worldwide. Outbreaks of the infectious disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), are implicated in the decline and extinction of numerous amphibian species. In Costa Rica, a major decline event occurred in 1987, more than two decades before this pathogen was discovered. The loss of many species in Costa Rica is assumed to be due to *Bd*-epizootics, but there are few studies that provide data from amphibians in the time leading up to the proposed epizootics. In this study, we provide new data on *Bd* infection rates of amphibians collected throughout Costa Rica, in the decades prior to the epizootics. We used a quantitative PCR assay to test for *Bd* presence in 1016 anuran museum specimens collected throughout Costa Rica. The earliest specimen that tested positive for *Bd* was collected in 1964. Across all time periods, we found an overall infection rate (defined as the proportion of *Bd*-positive individuals) of 4%. The number of infected individuals remained relatively low across all species tested and the range of *Bd*-positive specimens was shown to be geographically constrained up until the 1980s; when epizootics are hypothesized to have occurred. After that time, infection rate increased three-fold, and the range of specimens tested positive for *Bd* increased, with *Bd*-positive specimens collected across the entire country. Our results suggest that *Bd* dynamics in Costa Rica are more complicated than previously thought. The discovery of *Bd*’s presence in the country preceding massive declines leads to a number of different hypotheses: 1) *Bd* invaded Costa Rica earlier than previously known, and spread more slowly than previously reported; 2) *Bd* invaded multiple times and faded out; 3) an endemic *Bd* lineage existed; 4) an earlier *Bd* lineage evolved into the current *Bd* lineage or hybridized with an invasive lineage; or 5) an earlier *Bd* lineage went extinct and a new invasion event occurred causing epizootics. To help...
visualize areas where future studies should take place, we provide a *Bd* habitat suitability model trained with local data. Studies that provide information on genetic lineages of *Bd* are needed to determine the most plausible spatial-temporal, host-pathogen dynamics that could best explain the epizootics resulting in amphibian declines in Costa Rica and throughout Central America.

Introduction

Amphibians are experiencing a global extinction event [1,2]. Though many factors contribute to population declines, the emergence of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is one of the most important [3]. The disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*), was first described in 1999 and has since been found all over the world [3–5]. *Bd* is composed of many genetic lineages that vary in virulence and affect host species differently. The panzootic disease is associated with *Bd*-GPL, a Global Panzootic Lineage of *Bd* associated with *Bd* epizootics and host population collapse [6]. Other lineages of *Bd* have been shown to be less virulent and have been identified in areas lacking epizootics [7]. *Bd* infects the skin of the amphibian and causes hyperkeratosis, the thickening of skin which disrupts the amphibian’s osmotic balance; leading to death by cardiac arrest in highly infected individuals [8,9].

The dynamics of *Bd* and its hosts, including pathogen invasion and the host-pathogen interactions that follow, are still not fully understood. For example, in some areas (e.g. South Korea, Brazil, and South Africa), *Bd* appears to be in an enzootic state with amphibian hosts [10–12], while in others (e.g. western North America [13], Central America and South America) there are repeated examples of epizootics and die offs of hosts. In these areas, *Bd*-GPL is associated with epizootics [14]. South Korea was recently proposed as a region of high *Bd* genetic diversity, with one of the *Bd* lineages identified as exhibiting genetic hallmarks that may be the source of the panzootic *Bd* (*Bd*-GPL) that emerged in the 20th century [15]. Many of the reported declines attributed to *Bd* in the New World such as in southern Mexico, Guatemala, and Costa Rica occurred decades before *Bd* was described, thus, retrospective studies can help create a timeline for *Bd* emergence and spread [16].

Causes of amphibian declines in Costa Rica, where some of the earliest reported declines of amphibians occurred, have been debated in the literature [17,18]. Some studies proposed *Bd* epizootics occurred when environmental factors weakened host immune systems making them more susceptible to endemic *Bd* [19]. Other studies refute this and show that an invasive *Bd* pathogen caused the epizootics [16,20]. Costa Rica had one of the earliest amphibian declines (1980s and 1990s) that was later associated with *Bd* epizootics [21–23]. These declines mostly affected stream-dwelling species at elevations between 1000 and 2500 meters and include sites such as Monteverde, where the amphibian community collapsed a decade before *Bd* was described [24]. At this site, around the year 1987, half of all amphibian species, along with the Costa Rican golden toad (*Incilius periglenes*), disappeared [22].

Like many other areas experiencing *Bd* epizootics, anuran (frogs and toads) species in Costa Rica experienced differential susceptibility to *Bd*. Whether this is due to different immune responses by hosts or possibly exposure to different lineages of *Bd* is not known [25]. For example, all nine frog species within the *Craugastor punctariolus* clade (robber frogs) [26,27] declined across all their elevational range, from 0 to 2300 meters a.s.l. [28], and yet decades later they appear to be slowly recovering from past *Bd* epizootics [25,29,30]. Similar
cases of catastrophic decline followed by apparent recovery have been observed in some high-
land populations of harlequin frogs, tree frogs and ranid frogs [31,32]. Population fluctuations
such as these elicit questions regarding the role of Bd transmission, virulence, and lineage in
this disease system. Recent studies have shown that Bd-GPL is unlikely to be endemic to Costa
Rica though it is possible that other endemic Bd lineages occur in Costa Rica and throughout
the Americas [33].

Retrospective studies analyzing the presence of Bd in specimens preserved in natural his-
tory collections have been useful to describe Bd invasions that may have led to amphibian declines [16,34] as well as situations where Bd has been present for a century [10,35,36].
Museum collection data has also contributed to tracking and identifying declined species [37–
39]. The utilization and analysis of accurate collections and databases is crucial to understand-
ing the historical context of population declines and can result in more applicable conservation
plans. We conducted a retrospective survey using a Bd qPCR assay effective on museum specimens [16] to describe the spatial and temporal patterns of Bd of anurans in Costa Rica from
1961–2011. We used logistic regression analysis to examine possible environmental factors correlated with Bd infections. Based on our data, we also constructed a habitat suitability
model for Bd in Costa Rica using a MaxEnt model in order to visualize Bd habitat suitability
for the region.

Materials and methods

Data collection

This study was approved by the San Francisco State University IACUC (Vredenburg #A16-
01). We sampled 1016 formalin-fixed, ethanol-preserved museum specimens including thirty-
four species of frogs from five taxonomic families. All specimens were collected in Costa Rica
between 1961 and 2011 and are housed in the Museum of Zoology (UC Berkeley) and at the
Universidad de Costa Rica (UCR). We focused our sampling efforts on anuran species that
were reported to have declined during the 1980s and 1990s [40]; however, museum specimens
collected in the past were not intended for disease studies therefore conclusions about the dis-
semination and presence of Bd are inherently limited. Most of the Craugastor species have not
recovered from population declines and are still classified as critically endangered or extinct
according the IUCN [41]. Craugastor frogs are direct developers with no aquatic tadpole
phase, therefore less dependent on water for reproduction. Because Bd is often considered a
water-borne pathogen, we questioned whether Craugastor declines were associated with Bd or
if something else was affecting survival. We also chose species with breeding behavior associ-
ated with water and which populations initially declined in the 1980s and 1990s and were
observed to be recovering by around 2010 or later (Agalychnis annae, Agalychnis lemur, Litho-
bates vibicarius and Lithobates warszewitschii). The data from our skin swabs, including qPCR
results from our survey and geographic coordinates for all samples used in this study can be
freely accessed on the amphibian disease portal (https://amphibiandisease.org), DOI: <https://
n2t.net/ark:/21547/Auf2>

Quantitative PCR assay

We collected skin swabs from formalin-fixed frogs following a standardized protocol that
reduces chances of cross-contamination between specimens and is described in Cheng et al.
(2011) [16]. Each museum specimen was removed from its jar using flame-sterilized forceps
and thoroughly rinsed with 70% ethanol to remove any surface contaminants. Flame-sterilized
forceps were used to hold the specimen while swabbing five times each of the following loca-
tions for a total of twenty-five strokes, using sterile fine rayon-tipped swabs (MW113, MWE,
United Kingdom); 1) ventral surface from mid abdomen to cloaca, 2) each inner thigh, and 3) the bottom side of the webbing between each digit. Swabs were stored in 1.5 mL tubes with tether cap (Fisher USA) and stored at -4˚C until processing. New latex or nitrile gloves were used at all times when handling tubes, jars, and specimens and were changed between handling each specimen. We followed the method detailed in Boyle et al. (2004) and Cheng et al. (2011) for our qPCR assay and we processed all of our samples in the Vredenburg Lab at San Francisco State University [16,43,44]. Before extraction, swabs were put in a spinvac to evaporate any ethanol which could inhibit qPCR. *Bd* was then extracted from swabs using the Prepman Ultra (Thermo Fisher Scientific) and diluted. For qPCR, positive and negative controls (water, TE buffer) were run in triplicate, while a PrepMan negative control were run in singlicate on every 96-well PCR plate to detect possible environmental contamination. Standard curves were calculated using positive controls with known 100, 10, 1, and 0.1 *B. dendrobatidis* zoospore genomic equivalents, provided by A. D. Hyatt. Samples were run on an Applied Biosystems 7300 Real-Time PCR thermocycler. All samples were tested in singlicate unless they showed exponential amplification before cycle 50 or amplification was observed in any of the negative control wells. Samples which amplified early or showed non-sigmoidal amplification curves were run two additional times. We determined those samples as positive if two of three runs showed exponential amplification before cycle 50 [16].

### Statistical analyses

We performed all statistical analyses using the software R (version 3.5.0). To characterize the temporal and spatial dynamics of *Bd* in Costa Rica, we calculated 95% confidence intervals for the proportion of *Bd*-positive individuals for each decade sampled based on a binomial probability distribution (Table 1). We quantified the presence of *Bd* as the number of *Bd*-positive individuals divided by the total number of sampled individuals within a taxonomic unit, geographic area, and/or time frame. We performed a linear regression for the likelihood of an individual to be *Bd*-positive, with *Bd* infection status as a response variable, assuming a binomial distribution as individuals are either infected or non-infected.

For the linear regression, we used the elevation and 19 bioclim variables available on WorldClim (http://www.worldclim.org) and reduced the number of variables by performing a Pearson-correlation test to eliminate highly correlated factors (>0.9 or < -0.9). The following variables were used: annual mean temperature, mean diurnal temperature range, day-to-night temperature oscillations relative to the annual oscillations (isothermality), temperature seasonality, annual precipitation, precipitation of the wettest month, precipitation of the warmest quarter, precipitation of the driest quarter, and precipitation of the coldest quarter. We then performed a stepwise regression to choose the best-fit model based on the AIC [45,46]. Fig 1 was generated using QGIS (http://qgis.osgeo.org), using elevation data from USGS (https://www.usgs.gov), and shapefiles from GADM (gadm.org).

#### Table 1. *Batrachochytrium dendrobatidis* (*Bd*) prevalence in museum specimens collected in Costa Rica. Pr (no *Bd*) is the probability of finding no *Bd*-positive samples in each time period if *Bd* were present with an enzootic prevalence of 11.0% [35].

| Decade   | Tested Negative | Tested Positive | Sample Size | Prevalence (%) | Pr (no Bd)  |
|----------|-----------------|----------------|-------------|----------------|-------------|
| 1960–1970| 333             | 15             | 348         | 4.31           | < 0.001     |
| 1971–1980| 355             | 17             | 372         | 4.57           | < 0.001     |
| 1981–1990| 151             | 20             | 171         | 11.70          | < 0.001     |
| 1991–2000| 72              | 9              | 81          | 11.11          | < 0.001     |
| 2001–2011| 37              | 7              | 44          | 15.91          | 0.006       |

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Lastly, we used MaxEnt to estimate *Bd* habitat suitability using the variables shown to be significant in predicting the possibility of finding *Bd*-positive individuals from the linear regression model [47].

**Results**

Our qPCR analysis of the 1016 museum specimens collected between 1961 and 2011 revealed sixty-eight *Bd*-positive anuran samples and 948 *Bd*-negative anurans for an overall *Bd* infection rate of *Bd* 6.7% (n = 1016). The earliest records were detected in four *Lithobates vibicarius* specimens collected in 1964 from the central volcanic mountain range, on the hillsides of Poas Volcano (Fig 1A, S1 Table). UCR museum records of the species included in this study begin in the 1960s, thus our retrospective survey begins with the oldest specimens collected in 1961 (n = 2). When grouped by decades, we find relatively low rate of *Bd* presence in the 1960s and 1970s (4.31% and 4.57%, n = 372 and n = 348; respectively), followed by an increase in the 1980s to 11.70% (n = 171). By the late '80s and '90s, *Bd* was detected in museum specimens collected throughout the entire country, whereas earlier positive samples were obtained only from frogs collected in the central regions of the country. Thus, *Bd* became more common throughout the country in the late 1980s and 1990s (Figs 1A and 2); however, conclusions derived from the presence of *Bd* based on museum specimens that were not collected for disease studies are limited. Overall, the majority of the *Bd* positive samples were found in mountains throughout Costa Rica at elevations ranging from 32–2550 meters a.s.l. We found that the rate of *Bd* detection between species ranged between 0.0% and 46.7%. The species with the highest percentage of *Bd* positive samples came from species that are highly dependent on water for reproduction or live in close proximity to water. For example, we found 46.7% *Bd* infection rate (n = 15 ) in the stream-breeding frog *Hyloscirtus palmeri*, and 45.9% prevalence (n = 37 ) in *Lithobates vibicarius*, a highland pond-breeding frog. The lowest level of *Bd* detection occurred in the Dendrobatidae and Bufonidae families, in species that spend much of their time on land rather than in water. However, we sampled only a small number of Dendrobatidae specimens (n = 7), and no samples were *Bd*-positive, whereas in Bufonidae we sampled...
171 specimens and found that 0.6% were Bd-positive (S1 Table). Overall, the Ranidae family showed the highest presence of Bd (22.4%; n = 300), followed by Craugastoridae (7.4%; n = 453). Most Craugastoridae samples were taken from direct developing streamside-breeding species of the Craugastor punctariolus clade, which are critically endangered across their entire distribution. Craugastor andi, however, a species not categorized within the punctariolus clade, is also found near streams [28]. In the Craugastoridae family, only four C. escoces individuals tested positive out of sixty-three specimens. All C. escoces samples were collected before the 1987 Costa Rican amphibian population decline epizootic [24]. The earliest Bd-positive C. escoces specimens were collected in 1975, and the last was collected in 1986.

Our power analysis showed that we had enough samples within each time period to have a robust statistical test (p < 0.01 across all time periods sampled; Table 1). In the model with the best AIC (AIC = -2853.44), we found that infection status has a positive relationship with elevation and annual mean temperature (p < 0.001 and p < 0.001; respectively). We also found that Bd infection status has a negative relationship with mean diurnal temperature range (p < 0.001). Infection status was not shown to have a significant relationship with the following factors: isothermality, precipitation of the warmest quarter, precipitation of the coldest quarter, precipitation of the wettest month, annual precipitation, temperature seasonality, and precipitation of the driest quarter (Table 2). Areas predicted by the Bd habitat suitability model to be suitable for Bd includes mid-elevation ranges across central Costa Rica (Fig 3). The areas predicted to be unsuitable include the lowland regions and the coasts.
Discussion

Our retrospective study using museum specimens revealed that *Bd* was present in Costa Rica at least two decades before declines were discovered at Monteverde [20], and four decades before *Bd* was described. It is possible that the DNA of old samples are more degraded than recent samples, which could lead to a spurious signal of emergence. However, several retrospective studies of *Bd* infections in amphibians have shown consistent and relatively high *Bd* detection rate (20–40%) for specimens over an entire 100-year period [35,48].

We found an increase in *Bd* detection throughout Costa Rica beginning in the 1970s (Fig 1A), with detection rate escalating during and after the decade of the first known epizootic (1980s), which supports the pattern expected with an invasive pathogen. Here, we show a spatial pattern where *Bd* appears to be present at a low detection rate across some of the region (Fig 1A), before purported enigmatic declines occurred. This could be indicative of an invasive pathogen that invades unsuccessfully for decades before epizootics develop, or it could be that the earlier *Bd* infections were the result of a non-virulent lineage of *Bd*, such as has been identified in other parts of the world [35,49,50]. Consistent with an invading pathogen, our limited data show a pattern of spread across the entire time of the study. We found *Bd*-positive specimens only in and around central Costa Rica in the earlier time period (1960s), but by the 1970s, we found *Bd*-positive individuals across a larger area, from northwestern areas to southern areas of Costa Rica. Our results from the 1980s show the greatest expansion of *Bd*, with *Bd*-positive individuals found on the eastern coast of Costa Rica and near the Panama-Costa Rica border to the southeast and all the way north close to Nicaragua. This pattern might reflect the *Bd*-epizootics that are proposed from that time period. In the more recent decades (e.g. 1990s), there were fewer specimens in museum collections that we could test. Thus, constructing a robust statement regarding the spatial distribution of *Bd* in the more recent time period is not possible given the available specimens. Our samples are not free from sampling biases, since museum specimens were collected for reasons unrelated to our study.

In some areas (e.g. South Korea, Brazil, and South Africa), where both the Global Panzootic Lineage (GPL) and an endemic strain of *Bd* occur in sympathy, direct competition between pathogen strains and potential cross immunity of hosts may explain the lack of epizootics [11,51]. Our study provides evidence that *Bd* was present in Costa Rica before the 1987 epizootic in Monteverde, but we acknowledge that there may have been previous undetected epizootics especially since the pathogen was yet to be described. The few specimens collected in the

| Variables                      | Model 1 | Model 2 | Model 3 | Model 4 |
|--------------------------------|---------|---------|---------|---------|
| Elevation (+)                  | X       | X       | X       | X       |
| Annual Mean Temperature (+)    | X       | X       | X       | X       |
| Mean Diurnal Temperature Range (-) | X     | X       | X       | X       |
| Isothermality                   | X       | X       | X       | X       |
| Precipitation of Wettest Month | X       | X       | X       | X       |
| Precipitation of Warmest Quarter | X     | X       | X       | X       |
| Precipitation of Coldest Quarter | X     | X       | X       | X       |
| Annual Precipitation            | X       | X       | X       | X       |
| Temperature Seasonality         | X       | X       |         |         |
| Precipitation of Driest Quarter |         |         |         |         |

|          | AIC      | AIC      | AIC      | AIC      |
|----------|----------|----------|----------|----------|
|          | -2853.44 | -2851.88 | -2849.93 | -2847.93 |
| Δ AIC    | 1.56     | 1.95     | 2.00     | N/A      |

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1970s showed relatively high levels of infection and, by the 1980s, both Bd infection rate and average zoospore equivalents increased (Figs 1A and 2). Our data do not refute the studies that show that epizootics in Central America are associated with Bd invasion, but they do help provide further data for interpretation. For example, identifying the Bd lineage of our earlier positive Bd samples would be extremely helpful, since having multiple pathogens that are closely related to each other in a population of hosts may help us understand why Bd has had such variable effects on hosts, even in areas with epizootics. Sub-lethal effects from fighting off the

Fig 3. Bd habitat suitability model. Areas in Costa Rica predicted to have Bd suitability. Increased intensity from white to green indicates increased suitability while blue dots are Bd-epizootic localities. (1) Monteverde, where the 1987 decline occurred and (2–4) indicates declines between 1993–1994.

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infection of one pathogen can suppress host immunity against other stressors, causing a larger effect [52–54]. However, populations can also benefit by being exposed to a lower virulence pathogen before being exposed to a similar yet more virulent pathogen. Direct competition between pathogens and/or cross immunity has been shown to assist the hosts in acquiring partial or total immunity to one pathogen from a previous infection by another closely related pathogen [55]. Additionally, climate change and the stress of an inconsistent environment may negatively affect amphibians and result in suppressed immune systems, which could make amphibians more vulnerable to chytridiomycosis [56–58]. Future studies involving \( \text{Bd} \) genotyping are required to determine whether \( \text{Bd} \) found in Costa Rica are of a single lineage and whether or not the \( \text{Bd} \) found in epizootics and enzootics are of the same lineage.

Consistent with other studies, our linear regression results found that \( \text{Bd} \) infection status has a positive relationship with elevation and mean temperature [59,60], but contrary to other studies, our best linear regression model (Table 2), did not show a relationship between precipitation and \( \text{Bd} \) detection rate [61–64]. This may be due to unintended sampling bias. For example, frogs in the genus \( \text{Craugastor} \) made up a large proportion of available specimens (434 of 1016) and yet most were negative. Frogs in the genus \( \text{Craugastor} \) develop directly from terrestrial eggs. This more terrestrial lifestyle may decrease exposure to the aquatic pathogen \( \text{Bd} \), although terrestrial life history alone is not associated with susceptibility to infection [65,66].

Our data shows a higher detection rate of \( \text{Bd} \) in mid to high elevation species, possibly due to a more suitable climate for \( \text{Bd} \) [67–69]. The \( \text{Bd} \) habitat suitability model (\( \text{Bd} \) HSM) we produced which is specific to Costa Rica, predicts high habitat suitability for \( \text{Bd} \) where epizootics occurred (Fig 3), and is similar to previous \( \text{Bd} \) HSM studies [64,65].

Our model also identifies high elevations along the central mountain range as having the highest \( \text{Bd} \) suitability and should be prioritized for further research and monitoring. The zoospore equivalents (i.e. the Zswab, a measure of infection intensity or host infection load) and detection of \( \text{Bd} \) observed in this study are typically consistent with enzootic dynamics [66], and unfortunately no samples were collected that would allow for a description of host/pathogen dynamics in populations of hosts [13]. Of the \( \text{Bd} \)-positive samples, none of the individuals showed observable symptoms of disease at the time of swabbing for \( \text{Bd} \). Many of these specimens were collected before \( \text{Bd} \) was described and were not collected for disease studies. Collectors do not typically select animals that look sick as representatives of their collections. Our study utilizes qPCR to detect \( \text{Bd} \), and we were able to detect specimens with low zoospore equivalents. Quantitative PCR was found to be more sensitive and less invasive than histological assays [Cheng et al. 2011]. Performing histology on otherwise healthy looking specimens would require destructive sampling of museum specimens and is beyond the scope of this study. Nonetheless, our results add important information that may help in our understanding of the mass die offs of amphibians that occurred in Costa Rica. Our results show that although \( \text{Bd} \) was present in the 1960s, the significant increase in \( \text{Bd} \)-positive individuals did not begin in the samples available until the 1980s (when the epizootics began). The data we provide in this study are not well-suited to test the novel vs endemic pathogen hypotheses for \( \text{Bd} \) (Fig 3) [18]; however, these samples could be used to test for \( \text{Bd} \) lineage in future studies that may clarify this question [49]. The steady increase in \( \text{Bd} \) presence throughout all elevations in Costa Rica after 1990 suggests that \( \text{Bd} \) has become more broadly established throughout the country [25,70] than it was previously. The recent rediscovery of some remnant populations of frogs once thought extinct provides new opportunities to assess the current impact of \( \text{Bd} \) in highly susceptible species [25,29,30,32]. Consistent with previous studies, we propose that \( \text{Bd} \) epizootics in amphibians may have begun in the central range of Costa Rica, affecting stream-breeding and pond-breeding species that inhabited this region (S1 Table and 2) such as \( \text{Lithobates vibicarius} \), \( \text{Isthmohyla angustilineata} \), \( \text{I. tica} \), \( \text{I. xanthosticta} \), \( \text{I. rivularis} \), Duellmanohyla
uranochroa Craugastor fleischmanni, C. ranoides, C. escoces, C. sp. (C. punctariolus clade), C. melanostictus, C. andi, Atelopus varius, A. senex (Harlequin frogs), and Incilus holdrigei.

Disease ecology research using museum specimens allows for a retrospective view. This can be extremely valuable as with the case of the global pandemic in amphibians caused by Bd [35,71,72]. Most of the declines attributed to Bd epizootics (including in Costa Rica) occurred before Bd was described (1999) [16,36,73]. In this study, we discovered Bd-infected frogs in Costa Rica twenty-three years before enigmatic amphibian declines occurred and 34 years before Bd was discovered [17]. The infected animals in this study could indicate the occurrence of any one or multiple epidemiological dynamics including: failed pathogen invasions (e.g. “pathogen fade out” theory [74,75]), slower than expected invasion dynamics resulting in epizootics, endemic lineages of Bd that may exhibit enzootic pathogen/host dynamics, an extinct endemic Bd lineage; or an earlier Bd lineage that evolved or hybridized into the current Bd lineage. Additional studies that sequence and identify Bd lineages from our data will help create a more complete understanding of Bd phylogenetics in Central America.

Supporting information
S1 Table. Bd observed in in museum specimens from Costa Rica. The table shows surveyed species, conservation status and proportion of samples with Bd including 95% binomial confidence intervals.

(DOCX)

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References

1. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, et al. Status and trends of amphibian declines and extinctions worldwide. Science (80- ). 2004; 306(5702):1783–6.

2. Wake DB, Vredenburg VT. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc Natl Acad Sci. 2008; 105(Supplement 1):11466–73.

3. Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Philott AD, et al. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. Ecohealth. 2007; 4(2):125–34.

4. Longcore JE, Pessier AP, Nichols DK. Batrachochytrium dendrobatidis gen. et sp. nov., a Chytrid Pathogenic to Amphibians. Mycologia. 1999 Mar; 91(2):219.

5. Berger L, Roberts AA, Voyles J, Longcore JE, Murray KA, Skerratt LF. History and recent progress on chytridiomycosis in amphibians. Fungal Ecol. 2016; 19:89–99.

6. Farrer RA, Weinert LA, Bielby J, Garner TWJ, Balloux F, Clare F, et al. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. Proc Natl Acad Sci. 2011; 108(46):18732–6. https://doi.org/10.1073/pnas.1119191108 PMID: 22065772

7. Becker CG, Greenspan SE, Tracy KE, Dash JA, Lambertini C, Jenkinson TS, et al. Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages. Fungal Ecol. 2017 Apr 1; 26:45–50.

8. Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, et al. Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines. Science (80- ). 2009 Oct 22; 326(5952):582 L–585.

9. Voyles J, Vredenburg VT, Tunstall TS, Park JM, Briggs CJ, Rosenblum EB. Pathophysiology in mountain yellow-legged frogs (Rana muscosa) during a chytridiomycosis outbreak. PLoS One. 2012; 7 (4).

10. Fong JJ, Cheng TL, Baille A, Pessier AP, Waldman B, Vredenburg VT. Early 1900s detection of Batrachochytrium dendrobatidis in Korean amphibians. PLoS One. 2015; 10(3).

11. Rodríguez D, Becker CG, Pupin NC, Haddad CFB, Zamudio KR. Long-term endemism of two highly divergent lineages of the amphibian-killing fungus in the Atlantic Forest of Brazil. Mol Ecol. 2014; 23 (4):774–87. https://doi.org/10.1111/mec.12615 PMID: 24471406

12. Tarrant J, Cilliers D, du Preez LH, Weldon C. Spatial Assessment of Amphibian Chytrid Fungus (Batrachochytrium dendrobatidis) in South Africa Confirms Endemic and Widespread Infection. PLoS One. 2013; 8(7).

13. Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ. Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proc Natl Acad Sci. 2010; 107(21):9689–94. https://doi.org/10.1073/pnas.0914111107 PMID: 20457913

14. Schloegel LM, Toledo LF, Longcore JE, Greenspan SE, Vieira CA, Lee M, et al. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Mol Ecol. 2012 Nov; 21(21):5162–77. https://doi.org/10.1111/j.1365-294X.2012.05710.x PMID: 22857789

15. O’Hanlon SJ, Rieux A, Farrer RA, Rosa GM. Recent Asian origin of chytrid fungi causing global amphibian declines. Science (80- ). 2018; 360(6389):621–7.

16. Cheng TL, Rovito SM, Wake DB, Vredenburg VT. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen Batrachochytrium dendrobatidis. Proc Natl Acad Sci. 2011; 108(23):9502–7. https://doi.org/10.1073/pnas.1105538108 PMID: 21543713

17. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci. 1998; 95(15):9031–6. https://doi.org/10.1073/pnas.95.15.9031 PMID: 9671799
18. Rachowicz LJ, Hero JM, Alford RA, Taylor JW, Morgan JAT, Vredenburg VT, et al. The novel and endemic pathogen hypotheses: Competing explanations for the origin of emerging infectious diseases of wildlife. Vol. 19, Conservation Biology. 2005. p. 1441–8.

19. Pounds JA, Bustamante MR, Coloma LA, Consuegra JA, Fogden MPL, Foster PN, et al. Widespread amphibian extinctions from epidemic disease driven by global warming. Vol. 439, Nature. 2006. p. 161–7. https://doi.org/10.1038/nature04246 PMID: 16407945

20. Lips KR, Brem F, Brénès R, Reeve JD, Alford RA, Voyles J, et al. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci. 2006; 103(9):3165–70. https://doi.org/10.1073/pnas.0506889103 PMID: 16481617

21. Lips KR. Decline of a Tropical Montane Amphibian Fauna. Vol. 12, Conservation Biology. 1998.

22. Lips KR, Green DE, Papendick R. Chytridiomycosis in Wild Frogs from Southern Costa Rica. J Herpetol. 2003; 37(1):215–8.

23. Puschendorf R, Bolaños F, Chaves G. The amphibian chytrid fungus along an altitudinal transect before the first reported declines in Costa Rica. Biol Conserv. 2006; 132(1):136–42.

24. Pounds JA, Crump ML. Amphibian Declines and Climate Disturbance: The Case of the Golden Toad and the Harlequin Frog. Soc Conserv Biol. 1994; 8(1):72–85.

25. Whitfield SM, Alvarado G, Abarca J, Zumbado H, Zuñiga I, Wainwright M, et al. Differential patterns of Batrachochytrium dendrobatidis infection in relict amphibian populations following severe disease-associated declines. Dis Aquat Organ. 2017; 126(1):33–41. https://doi.org/10.3354/dao03154 PMID: 28930083

26. Campbell JA, Savage JM. Taxonomic Reconsideration of Middle American Frogs of the Eleutherodactylus rugulosus Group (Anura: Leptodactylidae): A Reckonance of Subtle Nuances among Frogs. Herpetol Monogr. 2000; 14:186.

27. Hedges SB, Hedges SB, Duellman WE, Duellman WE, Heinicke MP, Heinicke MP. Zootaxa 1737. Vol. 1737, Zootaxa. 2008. 1–182 p.

28. Savage JM. The amphibians and reptiles of Costa Rica: a herpetofauna between two continents, between two seas. University of Chicago Press; 2002. 934 p.

29. Jiménez R, Alvarado G. Craugastor escoces (Anura: Craugastoridae) reappears after 30 years: Rediscovery of an “extinct” Neotropical frog. Amphib Reptil. 2017; 38(2):257–9.

30. Chaves G, Zumbado-Ulate H, García-Rodríguez A, Gómez E, Vredenburg VT, Ryan MJ. Rediscovery of the Critically Endangered Streamside Frog, Craugastor Taurus (Craugastoridae), in Costa Rica. Trop Conserv Sci. 2014; 7(4):628–38.

31. Nishida K. Encounter with Hyla angustilineata Taylor, 1952 (Anura: Hylidae) in a cloud forest of Costa Rica. Brenesia. 2006; 66Douglas(May 2005):78–81.

32. Gonzalez-Maya JF, Escobedo-Galvan AH, Wyatt SA, Schipper J, Belant JL, Fischer A, et al. Renewing hope: The rediscovery of Atelopus varius in Costa Rica. Amphib Reptil. 2013 Jan 1; 34(4):573–8.

33. Mutnale MC, Anand S, Eluvathingal LM, Roy JK, Reddy GS, Vasudevan K. Enzootic frog pathogen Batrachochytrium dendrobatidis in Asian tropics reveals high ITS haplotype diversity and low prevalence. Sci Rep. 2018; 8(1).

34. DeLeón ME, Vredenburg VT, Piovia-Scott J. Recent Emergence of a Chytrid Fungal Pathogen in California Cascades Frogs (Rana cascadae). Ecohealth. 2017; 14(1):155–61. https://doi.org/10.1007/s10393-016-1201-1 PMID: 27957606

35. Talley BL, Mulet CR, Vredenburg VT, Fleischer RC, Lips KR. A century of Batrachochytrium dendrobatidis in Illinois amphibians (1888–1989). Biol Conserv. 2015; 182:254–61.

36. Chaukulkar S, Sulaeman H, Zink AG, Vredenburg VT. Pathogen invasion and non-epizootic dynamics in Pacific newts in California over the last century. PLoS One. 2018; 13(7).

37. Soto-Azat C, Clarke BT, Fisher MC, Walker SF, Cunningham AA. Non-invasive sampling methods for the detection of Batrachochytrium dendrobatidis in archived amphibians. Dis Aquat Organ. 2009; 84(2):163–6. https://doi.org/10.3354/dao02029 PMID: 19476287

38. Ryan MJ, Bolanos F, Chaves G. Museums Help Prioritize Conservation Goals. Science (80- ). 2010; 1272:1273.

39. Garcia-Rodriguez A, Chaves G, Benavides-Varela C, Puschendorf R. Where are the survivors? Tracking relictual populations of endangered frogs in Costa Rica. Divers Distrib. 2012; 18(2):204–12.

40. Young BE, Lips KR, Reaser JK, Ibáñez R, Salas AW, Cedeño JR, et al. Population Declines and Priorities for Amphibian Conservation in Latin America. Conserv Biol. 2001 Jul 7; 15(5):1213–23.

41. IUCN 2018. The IUCN Red List of Threatened Species. Version 2018–2. http://www.iucnredlist.org. Downloaded on 09 October 2018. 2018.
Fungal pathogen predated first-known amphibian epizootic

42. AmphibiaWeb. 2018. University of California, Berkeley, CA, USA. Accessed 18 Oct 2018. [Internet]. 2018. Available from: https://amphibiandisease.org

43. Boyle DG, Boyle DB, Olsen V, Morgan JA T, Hyatt AD. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Organ. 2004; 60(2):141–8. https://doi.org/10.3354/dao060141 PMID: 15460858

44. Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, et al. Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis. Vol. 73, Diseases of Aquatic Organisms. 2007. p. 175–92. https://doi.org/10.3354/dao073175 PMID: 17330737

45. Schloegel LM, Picco AM, Kilpatrick AM, Davies AJ, Hyatt AD, Daszak P. Magnitude of the US trade in amphibians and presence of Batrachochytrium dendrobatidis and ranavirus infection in imported North American bullfrogs (Rana catesbeiana). Biol Conserv. 2009; 142(7):1420–6.

46. Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, et al. Mapping the Global Emergence of Batrachochytrium dendrobatidis, the Amphibian Chytrid Fungus. Stajich JE, editor. PLoS One. 2013 Feb 27; 8(2):e56802. https://doi.org/10.1371/journal.pone.0056802 PMID: 23463502

47. Phillips SJ, Dudik M, Schapire RE. A maximum entropy approach to species distribution modeling. In: Proceedings, Twenty-First Int Conf Mach Learn ICML 2004. 2004. p. 655–62.

48. Jenkinson TS, Betancourt Román CM, Lambertini C, Valencia-Aguilar A, Rodríguez D, Nunes-Del Almeida CHL, et al. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. Mol Ecol. 2016 Jul; 25(13):2978–96. https://doi.org/10.1111/mec.13599 PMID: 26939017

49. Rosenblum EB, James TY, Zamudio KR, Poorten TJ, Ilut D, Rodríguez D, et al. Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. Proc Natl Acad Sci. 2013; 110(23):9385–90. https://doi.org/10.1073/pnas.1300130110 PMID: 23650365

50. Burrowes PA, De la Riva I. Detection of the Amphibian Chytrid Fungus Batrachochytrium dendrobatidis in Museum Specimens of Andean Aquatic Birds: Implications for Pathogen Dispersal. J Wildl Dis. 2017 Apr; 53(2):349–55. https://doi.org/10.7589/2016-04-074 PMID: 28094607

51. Bataille A, Fong JJ, Cha M, Wogan GOU, Baek HJ, Lee H, et al. Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus Batrachochytrium dendrobatidis in wild Asian amphibians. Mol Ecol. 2013; 22(16):4196–209. https://doi.org/10.1111/mec.12385 PMID: 23802586

52. Parris MJ, Cornelius TO. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. Ecology. 2004 Dec; 85(12):3385–95.

53. Davidson C, Knapp RA. Multiple stressors and amphibian declines: Dual impacts of pesticides and fish on yellow-legged frogs. Ecol Appl. 2007 Mar; 17(2):587–97. https://doi.org/10.1890/06-0181 PMID: 17489262

54. Bliefy J, Fisher MC, Clare FC, Rosa GM, Garner TWJ. Host species vary in infection probability, sublethal effects, and costs of immune response when exposed to an amphibian parasite. Sci Rep. 2015; 5.

55. Daszak P, Berger L, Cunningham AA, Hyatt AD, Earl Green D, Speare R. Emerging infectious diseases and amphibian population declines. Emerg Infect Dis. 1998; 5(6):735–48.

56. Woodhams DC, Bosch J, Briggs CJ, Cashins S, Davis LR, Lauer A, et al. Mitigating amphibian disease: Strategies to maintain wild populations and control chytridiomycosis. Vol. 8, Frontiers in Zoology. 2011.

57. Voyles J, Rosenblum EB, Berger L. Interactions between Batrachochytrium dendrobatidis and its amphibian hosts: A review of pathogenesis and immunity. Vol. 13, Microbes and Infection. 2011. p. 25–32. https://doi.org/10.1016/j.micinf.2010.09.015 PMID: 20951224

58. Rollins-Smith LA. Amphibian immunity–stress, disease, and climate change. Dev Comp Immunol. 2017; 66:111–9. https://doi.org/10.1016/j.dci.2016.07.002 PMID: 27387153

59. Ron SR. Predicting the distribution of the amphibian pathogen Batrachochytrium dendrobatidis in the new world. Biotropica. 2005 Jun; 37(2):209–21.

60. Brem FMR, Lips KR. Batrachochytrium dendrobatidis infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. Dis Aquat Organ. 2008; 81 (3):189–202. https://doi.org/10.3354/dao08189 PMID: 18998584

61. Walls S, Barichivich W, Brown M. Drought, Deluge and Declines: The Impact of Precipitation Extremes on Amphibians in a Changing Climate. Biology (Basel). 2013; 2(1):399–418.

62. Cayuela H, Arsovski D, Bonnaire E, Duguet R, Joly P, Besnard A. The impact of severe drought on survival, fecundity, and population persistence in an endangered amphibian. Ecosphere. 2016; 7(2).

63. Scheele BC, Hunter DA, Banks SC, Pierson JC, Skerratt LF, Webb R, et al. High adult mortality in disease-challenged frog populations increases vulnerability to drought. J Anim Ecol. 2016; 85(6):1453–60. https://doi.org/10.1111/1365-2656.12569 PMID: 27380945
64. Adams AJ, Kupferberg SJ, Wilber MQ, Pessier AP, Grefsrud M, Bobzie S, et al. Extreme drought, host density, sex, and bullfrogs influence fungal pathogen infection in a declining lotic amphibian. Ecosphere. 2017; 8(3).

65. Catenazzi A, Lehr E, Rodríguez LO, Vredenburg VT. Batrachochytrium dendrobatidis and the collapse of anuran species richness and abundance in the Upper Manu National Park, southeastern Peru. Conserv Biol. 2011; 25(2):382–91. https://doi.org/10.1111/j.1523-1739.2010.01604.x PMID: 21054530

66. Langhammer PF, Burrowes PA, Lips KR, Bryant AB, Collins JP. Susceptibility to the amphibian chytrid fungus varies with ontogeny in the direct-developing frog, Eleutherodactylus coqui. J Wildl Dis. 2014; 50(3):438–46. https://doi.org/10.7589/2013-10-268 PMID: 24807186

67. Lips K, Reeve J, Witters L. Ecological traits predicting amphibian population declines in Central America. Conserv Biol. 2003; 17(4):1078–88.

68. La Marca E, Lips KR, Lötters S, Puschendorf R, Ibáñez R, Rueda-Almonacid JV, et al. Catastrophic population declines and extinctions in neotropical harlequin frogs (Bufo nidae: Atelopus). Vol. 37, Biotropica. 2005. p. 190–201.

69. Ryan MJ, Lips KR, Eichholz MW. Decline and extirpation of an endangered Panamanian stream frog population (Craugastor punctarius) due to an outbreak of chytridiomycosis. Biol Conserv. 2008; 141(6):1636–47.

70. Puschendorf R, Carnaval AC, Vanderwal J, Zumbado-Ulate H, Chaves G, Bolaños F, et al. Distribution models for the amphibian chytrid Batrachochytrium dendrobatidis in Costa Rica: Proposing climatic refuges as a conservation tool. Divers Distrib. 2009; 15(3):401–8.

71. Yap TA, Koo MS, Ambrose RF, Vredenburg VT. Introduced bullfrog facilitates pathogen invasion in the western United States. Fisher MC, editor. PLoS One. 2018 Apr 16; 13(4):e0188384. https://doi.org/10.1371/journal.pone.0188384 PMID: 29659568

72. Scheele BC, Pasmans F, Skerratt LF, Berger L, Martel A, Beukema W, et al. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science (80- ). 2019 Mar 29; 363(6434):1459–63.

73. Sette CM, Vredenburg VT, Zink AG. Reconstructing historical and contemporary disease dynamics: A case study using the California slender salamander. Biol Conserv. 2015; 192:20–9.

74. Anderson RM, May RM. The Population Dynamics of Microparasites and Their Invertebrate Hosts. Philos Trans R Soc B Biol Sci. 1981; 291(1054):451–524.

75. Swinton J, Woolhouse M, Begon M, Dobson A, Ferroglio E, Grenfell B, et al. “Microparasite transmission and persistence.” In: The ecology of wildlife diseases. 2002. p. 83–101.