Prevalence of *Batrachochytrium dendrobatidis* in *Xenopus* Collected in Africa (1871–2000) and in California (2001–2010)

Vance T. Vredenburg¹,²,³*, Stephen A. Felt⁴, Erica C. Morgan⁴, Samuel V. G. McNally¹, Sabrina Wilson⁴, Sherril L. Green⁴

¹ Department of Biology, San Francisco State University, San Francisco, California, United States of America, ² California Academy of Sciences, San Francisco, California, United States of America, ³ Museum of Vertebrate Zoology Berkeley, Berkeley, California, United States of America, ⁴ Department of Comparative Medicine, Stanford University, Stanford, California, United States of America

Abstract

International trade of the invasive South African clawed frog (*Xenopus laevis*), a subclinical carrier of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been proposed as a major means of introduction of *Bd* into naïve, susceptible amphibian populations. The historical presence of *Bd* in the indigenous African population of *Xenopus* is well documented. However, there are no reports documenting the presence of *Bd* in wild *Xenopus* populations in the US, particularly in California where introduced populations are well-established after intentional or accidental release. In this report, a survey was conducted on 178 archived specimens of 6 species of *Xenopus* collected in Africa from 1871–2000 and on 23 archived specimens (all wild-caught *Xenopus laevis*) collected in California, USA between 2001 and 2010. The overall prevalence rate of *Bd* in the tested Xenopus was 2.8%. The earliest positive specimen was *X. borealis* collected in Kenya in 1934. The overall prevalence of *Bd* in the *X. laevis* collected in California was 13% with 2 positive specimens from 2001 and one positive specimen from 2003. The positive *Xenopus* (3/23) collected in California were collected in 2001 (2/3) and 2003 (1/3). These data document the presence of *Bd*-infected wild *Xenopus laevis* in California. The findings reported here support the prevailing hypothesis that *Bd* was present as a stable, endemic infection in *Xenopus* populations in Africa prior to their worldwide distribution likely via international live-amphibian trade.

Introduction

The appearance of the amphibian chytrid *Batrachochytrium dendrobatidis* (*Bd*), a fungal pathogen and the causative agent of chytridiomycosis has caused amphibian deaths and population declines worldwide [1–3]. This highly transmissible fungus has led to the recent decline or extinction of approximately 200 species of frogs [4]. A retrospective study of museum specimens performed by Weldon and colleagues [5] identified *Xenopus laevis*, the South African clawed frog, as an asymptomatic but *Bd*-transmitting species responsible for the pathogen’s global spread. In that study, infected *Xenopus* were identified in specimens collected as early as 1938 and another study found an infected *Xenopus* collected from Cameroon from 1953 [6]. Global distribution of *Xenopus* via the pet and zoo exhibit trade was well-established by the late 1900’s and by 1940, with the discovery that injection of urine from a pregnant woman caused egg-laying in *Xenopus*, the frogs were widely used in hospital laboratories world-wide as a means of detecting human pregnancy [7]. This practice persisted through the 1970’s. Though no longer used for pregnancy testing, *Xenopus* have since become a major animal model in biomedical and basic science research [8]. Significant populations of *Xenopus* (largely *X. laevis* and to a lesser extent, *X. tropicalis*) are currently housed in research laboratories.

Global trade of *Bd* infected *Xenopus* and their release into the wild (either accidentally or intentionally) may have contributed to the present *Bd* epidemic, and may be one of the major means by which the pathogen was introduced into the United States. There is currently a US-based movement to limit the importation, trade and transportation of this species [9]. However, to date there is no report documenting *Bd* infection in wild caught *X. laevis* collected in the US, or from California in particular, a geographic location well-known to be relatively recently invaded [1–3],[10].

In this report, we determined the prevalence of *Bd* in archival *Xenopus* specimens from the herpetological collection at the California Academy of Sciences (CAS), one of the oldest and largest collections in North America. This collection includes specimens from 6 different *Xenopus* species (including *X. laevis*). The samples were collected in the wild from southern, eastern, and western Africa, as well as from established introduced populations in California.
**Materials and Methods**

A retrospective study was conducted on the entire collection of 201 archived specimens (collected between 1901 and 2001) of the genus *Xenopus* housed in the California Academy of Sciences. Specimens archived in the CAS were collected by several groups and were not collected for the purpose of disease surveillance. The specimens examined from this archive were collected mainly from Kenya, Uganda, and a variety of other African nations and from Los Angeles, San Diego, and San Francisco County in California, USA. The frog specimens, most preserved in ethanol, were sampled using swabbing techniques and PCR as previously described [11]. Briefly, the skin of each of the 201 preserved amphibians was swabbed with a MW100 sterile cotton-tipped swab. Each specimen was swabbed a total of 30 times: 10 strokes each along each side of the ventral surfaces (including abdomen, pelvis, and thighs), and 5 strokes on each hind foot webbing. To prevent possible cross contamination from multiple specimens cohabiting in a single jar, each specimen was rinsed with 70% ethanol prior to swabbing, and a new pair of disposable gloves was used for each specimen. Swabs were stored in 1.5 mL microcentrifuge vials and refrigerated at 4°C until extraction. Prior to extraction, swab vials were placed in a SpinVac for 15–20 min or under a fume hood for ~1 hr to evaporate residual ethanol. Swabs were extracted with PrepMan Ultra, and extractions diluted 1:10 in 0.25xTE Buffer. Presence of *Bd* was determined using a real-time PCR (ABI 7300) assay for *Bd* according to methods described by Boyle et al [12], and revised for museum specimens as specified in Cheng et al [11]. Samples were run in triplicate along with negative controls (H2O, TE Buffer) and positive standards at dilutions of 100, 10, 1.0, and 0.1.

**Results**

Overall, prevalence of *Bd* in the CAS archived *Xenopus* specimens (African and Californian) was 4.0% (8 positives out of 201 specimens) (Table 1). The 8 positive samples were positive in all three of the triplicate runs, but the genomic equivalents were 201 specimens) (Table 1). The 8 positive samples were positive in all three of the triplicate runs, but the genomic equivalents were

| Species     | No. examined | No. positive | % positive (95% CI) |
|-------------|--------------|--------------|---------------------|
| *K. laevis* | 145          | 6            | 4.1 (1.5,8.8)       |
| *K. borealis* | 3          | 2            | 66.7 (9.4, 99.2)    |
| *K. fraseri* | 2           | 0            | 0 (0, 84.2)         |
| *K. muelleri* | 11         | 0            | 0 (0, 28.5)         |
| *K. tropicalis* | 2          | 0            | 0 (0, 84.2)         |
| *K. wittei* | 38           | 0            | 0 (0, 9.3)          |
| Total       | 201          | 8            | 4.0 (1.7, 7.7)      |

Table 1. Prevalence of *Batrachochytrium dendrobatidis* in all *Xenopus* specimens tested in this study and archived at the California Academy of Sciences.

The overall prevalence of *Bd* in archived African *Xenopus* was 2.8%, remarkably close to the overall prevalence previously reported by Weldon (2.7%) [5]. Notably, though the sample size is small, the overall prevalence of *Bd* in *Xenopus* collected from California was relatively high: 15% (time interval 2001–2010) but is consistent with the prevalence rate reported in by Ouellet et al [10], who surveyed archival amphibian specimens collected in Canada and the United States between 1895–2001. *Xenopus* were not amongst the specimens surveyed from that collection, but the earliest positive infections dated from the 1960’s and were found in a native North American anuran species, *Rana clamitans*. The presence of wild *Xenopus* populations in California was first documented the 1970’s; these populations are thought to have been established via release or escape from pet shops and hobbyists, from museum and zoo exhibits, or from hospital or research laboratories [13–16]. The findings reported here provide temporal evidence that *Bd*-infected *Xenopus* were present in California at least as far back as 2001. This supports the epidemic pathogen hypothesis in California, i.e. that *Bd*-positive *Xenopus*, a non-native, invasive species imported from Africa and released in California are one possible means of spreading *Bd* to naive amphibian hosts.

Eleven states (AZ, CA, HI, MN, NV, NJ, NC, OR, UT, VA and WA) in the U.S. currently restrict the importation of *Xenopus*, either by necessitating special permit requirements for research laboratories and exhibitors, and/or by not allowing this species to be sold as pets [9]. The U.S. Department of Interior’s Fish and Wildlife Service Agency is currently reviewing a petition to list all live amphibians and their eggs under the Lacy Act (thus restricting their importation and intra- and interstate transport) and determining this species as “injurious” unless certified as free of *Bd*, the amphibian chytrid fungus [9]. Although *Xenopus* are no longer used for pregnancy testing, the impact of additional regulations restricting the trade of *Xenopus* would be significant, particularly to basic and biomedical research. While the exact numbers of *Xenopus laevis* used in research world-wide are difficult to determine, since 1970 to the present, there have been >25,000 research reports using *Xenopus* [17].

Quellet et al [10], reported *Bd* positive anurans in Quebec, Canada in 1961. It is not known if *Bd* was present in California before *Xenopus* arrived, however, based on the results reported here, it does appear that wild *Bd*-infected *Xenopus* were present in California at least 12 years ago and that *Xenopus* may be, in part a
means of spread of this fungus to susceptible native amphibians. Another potential source of the spread of \textit{Bd} in California is the American Bullfrog (\textit{Rana catesbeiana}) which was introduced to the US West Coast as a food source in the 1920's. This species has been widely implicated in the spread of \textit{Bd} and studies are currently underway to determine if archival specimens from this species are \textit{Bd} positive.

Our results indicate that \textit{Bd}-infected \textit{Xenopus} were present in California; however, no attempt was made to sequence the material from these archival specimens. While the PCR amplification curves were normal, the genomic equivalent values were very low (0.0075, 0.01, 0.001), which is typical of results using this \textit{Bd} PCR assay on museum specimens [11]. Thus we cannot at this time ascertain if the \textit{Bd} material identified in these specimens could be an endemic \textit{Bd} strain, or a more virulent introduced pathogenic strain or even a new sexual hybrid [18], as has been proposed to cause the recent massive amphibian die offs. Evidence was recently reported for a hypervirulent strain of \textit{Bd} on introduced \textit{Rana catesbeiana} [18] alongside other less virulent strains on native fauna, and this makes it harder to interpret our historical findings. The question remains: Which \textit{Bd} strain(s) did we discover on the historical specimens reported here and did it differ in Africa from those we discovered in California? Our study cannot address this issue. Studies that use the \textit{Bd} qPCR assay [12] should be aware that different strains of \textit{Bd} may yield different infection intensities based on variable ITS copy number by strain [19]. Studies that employ the \textit{Bd} assay for testing museum specimens are less likely to be affected by this, however, because there is less emphasis on infection intensities and more on simple positive negative results [11]. Additionally, there are potential sources of contamination that we could not control. For example, contemporary field measures to prevent cross contamination between specimens cannot be applied retrospectively to specimens collected in the past. When working with specimens, it is not possible to know the exact history of how each specimen was stored since the day it was collected. Many specimens, for example, do not have accompanying field notes and the only data available are those attached on the tags attached to the specimens. Future studies may be able to use new technologies to recover sequence data from museum swab PCR assays with very little recoverable \textit{Bd} DNA, however we believe that retrospective \textit{Bd}-assays on amphibian specimens provide an important basis for our understanding of the epidemiology of this disease. If wild \textit{Xenopus} appeared in California decades ago, during the 1970s as first reported [14],[15] then by strong inference and supported by the findings reported here, introduction of \textit{Bd} into this geographical area could have occurred in part via \textit{Bd} infected \textit{Xenopus laevis}.

### Table 2. Prevalence of \textit{Batrachochytrium dendrobatidis} in archived \textit{Xenopus}, by location.

| Location               | No. examined | No. positive | % positive (95%CI) |
|------------------------|--------------|--------------|--------------------|
| Botswana               | 1            | 0            | 0 (0, 97.5)        |
| Ghana                  | 2            | 0            | 0 (0, 84.2)        |
| Kenya                  | 42           | 3            | 7.1 (1.5, 19.5)    |
| Namibia                | 4            | 0            | 0 (0, 60.2)        |
| Nigeria                | 1            | 0            | 0 (0, 97.5)        |
| Rwanda                 | 4            | 0            | 0 (0, 60.2)        |
| South Africa           | 4            | 0            | 0 (0, 60.2)        |
| Tanzania               | 10           | 0            | 0 (0, 30.9)        |
| Uganda                 | 88           | 2            | 2.3 (0.3, 8.0)     |
| Zaire                  | 9            | 0            | 0 (0, 33.6)        |
| Zambia                 | 9            | 0            | 0 (0, 33.6)        |
| Unspecified, Africa    | 4            | 0            | 0 (0, 60.2)        |
| USA, San Francisco, California | 3      | 1            | 33.3 (0.8, 90.6)   |
| USA, San Diego, California | 8   | 2            | 25 (3.2, 65.1)    |
| USA, Los Angeles, California | 12 | 0            | 0 (0, 26.5)        |
| Total                  | 201          | 8            | 4.0 (1.7, 7.7)     |

*CICl = Bayesian Credible Interval.

doi:10.1371/journal.pone.0063791.t002

### Table 3. Prevalence of \textit{Batrachochytrium dendrobatidis} in archived \textit{Xenopus} by time intervals, specimens collected in various countries in Africa and North America.

| Time interval   | No. examined | No. positive | % positive (95%CI) |
|-----------------|--------------|--------------|--------------------|
| 1871–1940b      | 18           | 2            | 11 (1.9, 30)       |
| 1941–1950       | 4            | 0            | 0 (0, 45)          |
| 1951–1960       | 28           | 0            | 0 (0, 9.8)         |
| 1961–1970       | 4            | 0            | 0 (0, 45)          |
| 1971–1980       | 40           | 1            | 2.5 (0.11, 1)      |
| 1981–1990       | 17           | 0            | 0 (0, 15.3)        |
| 1991–2000       | 65           | 2            | 3 (0.4, 9.4)       |
| 1991–2000*      | 12           | 0            | 0 (0, 20.5)        |
| 2001–2010       | 2            | 0            | 0 (0, 63.1)        |
| 2001–2010*      | 11           | 3            | 27.2 (8.2, 54.8)   |
| Total           | 201          | 8            | 4.0 (1.7, 7.7)     |

*CICl = Bayesian Credible Interval;
bearliest positive specimen collected in 1934, \textit{X. Borealis}, in Kenya.

*samples collected in California, USA.

doi:10.1371/journal.pone.0063791.t003
Acknowledgments

We wish to thank J. Vindum, the Senior Collections Manager of the California Academy of Sciences Herpetology Department, who granted permission to access and sample the *Xenopus* collection.

Author Contributions

Conceived and designed the experiments: VTV SAF. Performed the experiments: VTV ECM SVM SSW SLG. Analyzed the data: VTV SAF ECM SVM SSW SLG. Contributed reagents/materials/analysis tools: VTV SAF. Wrote the paper: VTV SAF.

References

1. Rachowicz LJ, Knapp RA, Morgan JAT, Stice MJ, Vredenburg VT, et al. (2006) Emerging infectious disease as a proximate cause of amphibian mass mortality. Ecology 87(7): 1671–1683.
2. Wake DB, Vredenburg VT (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc Natl Acad Sci U S A 105: 11466–11473.
3. Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large-scale amphibian population extinctions. Proc Natl Acad Sci U S A 107: 9689–9694.
4. Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. Ecohealth 4: 125–134.
5. Weldon C, du Preez LH, Hyatt AD, Muller R, Spears R (2004) Origin of the amphibian chytrid fungus. Emerg Infect Dis 10: 2100–2105.
6. Soto-Azat C, Clark BT, Poynton JC, Cunningham A (2010) Widespread historical presence of *Batrachochytrium dendrobatidis* in African pipid frogs. Diversity Distrib. 16: 126–131.
7. Green SL (2009) Preface. In: Suckow MA, editors. The Laboratory *Xenopus* sp. Boca Raton: CRC Press. pp. xi–xii.
8. Deuchar EM (1972) *Xenopus laevis* and developmental biology. Biol Rev 47: 37–112.
9. United States Fish and Wildlife Service website. Available http://www.fws.gov/policy/library/2010/2010-23039.html. Accessed 2013 April 12.
10. Oudelet M, Mikaelian I, Pauli BD, Rodrigue J, Green DM (2005) Historical evidence of widespread chytrid infection in North American amphibian populations. Conservation Biology · Boston MA 19: 1431–1440.
11. Cheng TL, Rovito SM, Wake DB, Vredenburg VT (2011) Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proc Natl Acad Sci U S A 108: 9302–9307.
12. Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Die Aquatik Org 60, 141–148.
13. St.Amant JA, Hoover EJ (1973) African clawed frog, *Xenopus laevis laevis* (Daudin), established in California. Calif Fish Game 39: 151–153.
14. Green SL (2009) Husbandry. In: Suckow MA, editors. The Laboratory *Xenopus* sp. Boca Raton: CRC Press. 19–62.
15. McCoid MJ (1980) Observations of feral populations of *Xenopus laevis* (Pipidae) in Southern California. Bulletin of the Southern California Academy of Science 79: 82–86.
16. Bury RB, Luckenback RA (1976) Introduced amphibians and reptiles in California. Biol Cons 10: 1–14.
17. PubMed website. http://www.ncbi.nlm.nih.gov/pubmed. Accessed 2013 April 12.
18. Schloegel LM, Toledo LF, Longcore JE, Greenspan SE, Vleira CA, et al. (2012) Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade Mol Ecol 21: 5162–77.
19. Longo AV, Rodriguez D, da Silva Leite D, Toledo LF, Mendoza Almeralla C, et al. (2013) ITS1 Copy Number Varies among *Batrachochytrium dendrobatidis* Strains: Implications for qPCR Estimates of Infection Intensity from Field-Collected Amphibian Skin Swabs. PLoS ONE 8(3): e59499. doi:10.1371/journal.pone.0059499.