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Disease surveillance of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in Papua New Guinea

Deborah S. Bower[1,2] | **Charlotte K. Jennings**[3] | **Rebecca J. Webb**[4] | **Yolarnie Amepou**[5] | **Lin Schwarzkopf**[1] | **Lee Berger**[6] | **Ross A. Alford**[1] | **Arthur Georges**[5] | **Donald T. McKnight**[1,2] | **Leah Carr**[1] | **Dillian Nason**[7] | **Simon Clulow**[8,9]

[1]College of Science & Engineering, James Cook University, Townsville, Queensland, Australia

[2]School of Earth and Environmental Science, University of New England, Armidale, New South Wales, Australia

[3]Museum of Vertebrate Zoology, University of California, Berkeley, California

[4]College of Public Health, Medical and Veterinary Sciences, James Cook University

[5]Institute for Applied Ecology, University of Canberra, Canberra, Australian Capital Territory, Australia

[6]Melbourne Veterinary School, University of Melbourne, Werribee, Victoria, Australia

[7]Biological Science Department, University of Goroka, Goroka, Papua New Guinea

[8]School of Environmental and Life Science, University of Newcastle, Callaghan, New South Wales, Australia

[9]Department of Biological Sciences, Macquarie University, Sydney, New South Wales, Australia

Correspondence
Deborah S. Bower, University of New England, Armidale, NSW, 2350, Australia.
Email: deborah.bower@gmail.com

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Abstract

Emerging infectious diseases threaten the persistence of biodiversity globally. The amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, is one of the most widespread and damaging pathogens to biodiversity. New Guinea hosts 6% of the world's frogs and is the largest landmass where *B. dendrobatidis* remains undetected despite being largely climatically suitable for its persistence. We surveyed for *B. dendrobatidis* in Papua New Guinea, by swabbing live frogs in the Gulf Province and Eastern Highlands Province and by examining museum specimens from a range of sites and elevations. Here, we show that over a large geographical range, all 442 samples were negative for *B. dendrobatidis*. The spread of *B. dendrobatidis* to Papua New Guinea may have been thus far prevented by the remoteness of New Guinea and the hotter climate in its lowlands, which surrounds a more climatically suitable zone for *B. dendrobatidis* in the highlands. Alternatively, *B. dendrobatidis* may be present in isolated patches or at low levels and remain undetected, to date. Papua New Guinea remains at risk and would benefit from a national disease surveillance program for chytrid fungi and pre-emptive actions, designed to reduce the risk of pathogen transmission. Measures should include improved

Deborah S. Bower and Simon Clulow should be considered joint first authors.

biosecurity protocols for trade and travel and continued disease surveillance in areas of probable entry and spread.

KEYWORDS

biosecurity, disease, fungi, invasive, island, New Guinea, pathogen, policy

1 | INTRODUCTION

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*) has emerged throughout much of the world (Berger et al., 1998) and caused dramatic declines, even in the remote wilderness (O'Hanlon et al., 2018; Scheele et al., 2019). The most extensive declines and extinction events have occurred in amphibian populations in Australia and Central and South America, particularly in high elevation regions (Lips, Reeve, & Witters, 2003; Scheele et al., 2019). The origin of *Bd* is Asia (O'Hanlon et al., 2018) and its distribution has expanded to most areas that are climatically suitable. There are, however, still some areas where it has not been detected (Bower, Lips, Schwarzkopf, Georges, & Clulow, 2017; Hoskin, Hines, Webb, Skerratt, & Berger, 2019; Stockwell, Bower, Bainbridge, Clulow, & Mahony, 2015). One of these areas is New Guinea (Dahl et al., 2012), which is the largest climatically suitable landmass (Ron, 2005) where *Bd* remains undetected (Dahl et al., 2012; Swei et al., 2011). New Guinean frogs may be particularly susceptible to chytridiomycosis because they are closely related to Australian frogs, of which one fifth of species have declined, often owing to chytridiomycosis (Skerratt et al., 2016).

New Guinea is considered one of five global "High-Biodiversity Wilderness Areas," high in biodiversity and low in human disturbance (Mittermeier, Turner, Larsen, Brooks, & Gascon, 2011). Relatively low human population density, comparative lack of large-scale development, limited infrastructure for commerce, and importation of goods (Lenzen et al., 2012), and small scale of tourism contribute to low habitat disturbance across the island (Hannah, Carr, & Lankerani, 1995). Despite its disproportionate wealth of biological richness, New Guinea remains among the least studied places on earth in terms of conservation (Wilson et al., 2016). It harbors approximately 6% of global frog diversity in less than 1% of the world's terrestrial surface area and many species remain undescribed (Austin, Hayden, Bigilale, Dahl, & Anaminiato, 2008; Günther, 2006). New Guinea's high species richness makes it a priority refuge for global frog conservation.

Preemptive monitoring of emerging pathogens is key to minimizing biodiversity loss through preparation (identifying vulnerable species and possible actions) and early detection (Grant et al., 2017), yet surveillance for amphibian diseases in New Guinea has been limited to four geographically discrete surveys. Three of these surveys were opportunistic (Fu & Waldman, 2019; Ouellet, Mikaelian, Pauli, Rodrigue, & Green, 2005; Swei et al., 2011) and only one included intensive sampling, which occurred in the province of Madang in 2010 (Dahl et al., 2012). None of these detected *Bd*, however, and it is not known whether *Bd* continues to be absent from New Guinea, or if it is patchily distributed.

In this paper, we report results of the largest survey to date, based on a combination of specimens stored in museums and live frogs sampled in the field. We also did not detect *Bd* on the island of New Guinea.

2 | METHODS

2.1 | Sampling

We sampled for *Bd* by swabbing frogs from all available sources including live frogs during field surveys, and fixed museum specimens from previous collection trips (Richards-Hrdlicka, 2012). Sampling was opportunistic and included sites, species, and seasons that varied in their suitability as targets (e.g., their probability of being susceptible to infection) (Skerratt et al., 2008). Field collection sites ranged from highland towns and their surrounds (e.g., storm drains and fish ponds in Goroka), lowland villages (e.g., gardens throughout Kikori village) to remote tropical lowland rainforest (e.g., Wau Creek) and montane forest (e.g., Mt Otto, Mt Michael) (Figure 1; Table 1). We swabbed each specimen with an MV100-100 cotton swab for a total of 40 strokes, specifically: arm to hand (4 strokes), ventral body surface (8 strokes), dorsal body surface (8 stokes), ventral surface of left leg (4 strokes), dorsal surface of left leg (4 strokes), ventral surface of right leg (4 strokes), dorsal surface of right leg (4 strokes), and twice on each foot (4 strokes).

In November 2015, we swabbed 124 live frogs in the lowlands of the Gulf Province, and in 2017, we swabbed 108 live frogs in the Goroka region (Tables 1 and S1). Because we did not have access to refrigeration, we stored most swabs in 80% ethanol owing to the risk of

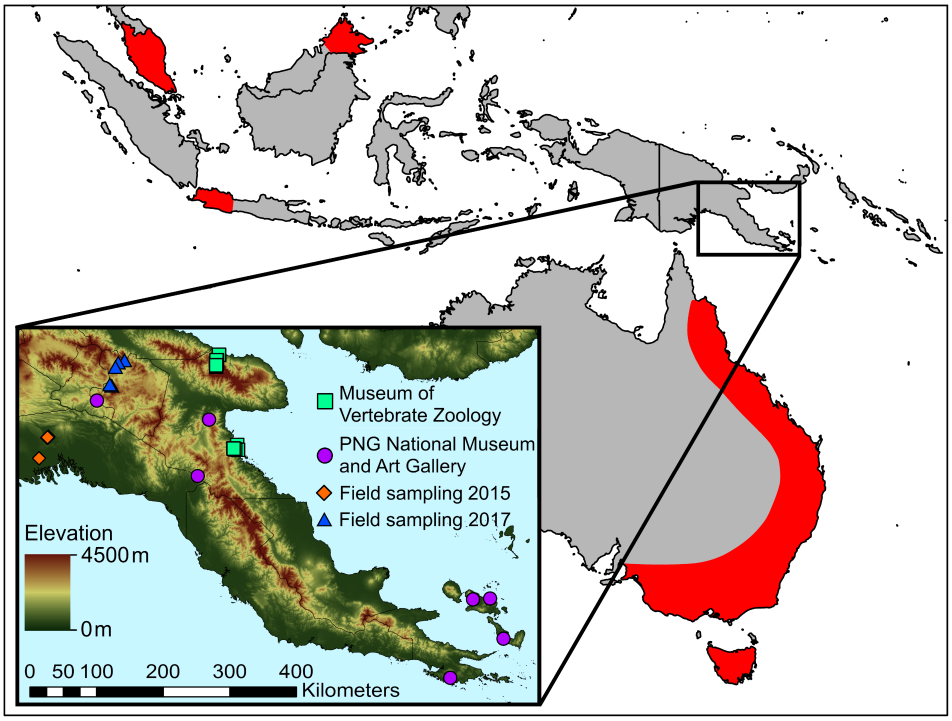

FIGURE 1 Map of the areas sampled in Papua New Guinea with surrounding landmasses (grey) and the approximate distribution of *Batrachochytrium dendrobatidis*

TABLE 1 Summary of sampling locations and swabs tested for *Batrachochytrium dendrobatidis* in Papua New Guinea

Sampling location	Province	Collection site	No. of samples
Field sampling	Gulf Province	Kikori (town)	7
Field sampling	Gulf Province	Wau Creek reserve	117
Field sampling	Goroka region	Goroka surrounds	37
Field sampling	Goroka region	Mt Otto	46
Field sampling	Goroka region	Mt Michael	25
Museum of vertebrate zoology, Berkeley	Morobe Province	Kamiali, Morobe Province, PNG	30
Museum of Vertebrate Zoology, Berkeley	Morobe Province	Huon peninsula	110
PNG National Museum and art gallery	Chimbu Province	Crater mountain	1
PNG National Museum and art gallery	Milne Bay Province	Awavula River, Fergusson Island	2
PNG National Museum and art gallery	Milne Bay Province	Normanby Island	1
PNG National Museum and art gallery	Milne Bay Province	Mt Kilkerran, Fergusson Island	14
PNG National Museum and art gallery	Milne Bay Province	Cloudy Mountains	25
PNG National Museum and art gallery	Morobe Province	Mt Shungol	6
PNG National Museum and art gallery	Morobe Province	Tekadu	7
PNG National Museum and art gallery	Unknown	Unknown location	14

Note: Further details are provided in Table S1.

mold. However, 63 swabs from Gulf Province were also stored dry (Table 2).

In October 2015, we swabbed 140 frog specimens that had been collected from a range of elevations from May through July 2014 in the Kamiali Wildlife Management Area and YUS Conservation Area, Morobe Province, PNG (Tables 1 and S1). These specimens had been fixed in formalin and stored in 70% ethanol at the Museum of Vertebrate Zoology, University of California, Berkeley, CA. These specimens had not previously been swabbed.

In November 2016, we swabbed 70 frog specimens that had been collected by a variety of researchers and in different locations over PNG (Tables 1 and S1). Most specimens had been fixed in formalin and stored in 70%

TABLE 2 Number of swabs analyzed for *Batrachochytrium dendrobatidis* in each taxonomic group

	Family	Genera	Species	Individuals
	Microhylidae	13	43	228
	Pelodryadidae	2	20	85
	Ranidae	1	13	90
	Limnodynastidae	1	1	11
	Bufonidae	1	1	3
	Ceratobatrachidae	2	2	25
Total		20	80	442

ethanol at the PNG National Museum and Art Gallery, PNG; however, we could not verify the storage history for some specimens and it is unknown whether these had previously been swabbed.

We complied with all ethical guidelines throughout our study (Animal ethics permit A2364) and approval was given by the Conservation and Environment Protection Authority in addition to landholders of each site we visited.

2.2 | DNA extraction and PCR assay

Swabs were analyzed by a commercial laboratory (Cesar, Australia). In all batches of swabs, a positive control swab was included, and in all cases, these were positive for *Bd*. We determined the number of *Bd* zoospore genome equivalents (ZGE) per swab with a real-time quantitative PCR protocol modified from Boyle, Boyle, Olsen, Morgan, and Hyatt (2004). Briefly, microcentrifuge tubes (1.5 ml) containing swabs in ethanol were centrifuged at 16,000*g* for 2 min, excess ethanol was removed by pipette, and swabs were left to air-dry in their original tubes for 30 min at room temp under a laminar flow. DNA was then extracted from swabs using either a Chelex® extraction protocol (Möhlenhoff, Müller, Gorbushina, & Petersen, 2001) or Qiagen DNeasy Blood and Tissue DNA extraction kit. Chelex® was used for all samples except the 108 samples collected in 2017 for which we used DNeasy. Although both protocols have previously been used for extracting DNA from fungal species (Chu et al., 2014), including *Bd*, DNeasy has a greater ability to detect low numbers of zoospores (Kosch & Summers, 2013), but it is more expensive. Therefore, it was used for samples from the most suitable *Bd* habitats to maximize the probability that *Bd* would be detected if it was currently present in PNG. The Chelex® protocol was modified by adding 3 μL of proteinase K and 200 μL of 5% Chelex® solution to the dried swabs. Samples were incubated at 55°C for 60 min then at 95°C for 15 min with periodic vortexing. The DNeasy protocol followed the animal tissues procedure outlined by Qiagen, and was modified by using an overnight digestion and eluting the DNA in two steps of 20 μL Qiagen buffer AE. Extracted DNA was stored at −20°C until required. Before PCR amplification, samples were centrifuged at 12,000*g* for 2 min and 2 μL of supernatant from just above the Chelex ® resin was used for PCR amplification.

Real-time Taqman PCR assays were conducted using a Roche LightCycler 480 system in a 384-well format. PCR reactions contained 5 μL of 2 × Qiagen multiplex PCR Master Mix (Qiagen), PCR primers at a concentration of 900 nM, the MGB probe at 250 nM and 2 μL of DNA brought to a final volume of 10 μL with nanopure water. PCR reaction mixes were prepared in triplicate. Included in each 96-well assay plate were control reactions containing DNA from 40, 4, 0.4, and 0.04 *Bd* zoospore genomic equivalents prepared and controls with no DNA template. The amplification conditions were 15 min at 95°C, followed by 15 s at 95°C and 1 min at 60°C for 50 cycles. The amplification profiles of each PCR were used to determine the crossing point (C_p) value using the Absolute Quantification module of the software package LightCycler® 480 (version 1.5, Roche Diagnostics Australia Pty Ltd., Castle Hill, New South Wales, Australia). A standard curve was constructed from the control reactions containing 40, 4, 0.4, and 0.04 *Bd* zoospores and the concentration determined for the test samples expressed as the number of zoospore genomic equivalents.

The standard for quantitation of *Bd* was provided by Australian Animal Health Laboratory, CSIRO. A 473 bp synthetic gene fragment of *Bd* ITS strain CW34 (gBlocks® Gene Fragments, Integrated DNA technologies, Baulkham Hills, New South Wales, Australia) was used to generate a 10-fold dilution series from 3,000,000 to 3 ITS copies. Included in triplicate on each 384-well assay plate was a 7-point ITS standard and/or a 4-point *Bd* spore standard and a no DNA template reaction (negative control). Quantitative PCR efficiencies were between 94 and 100% for all 384-well plates. The concentration of the test samples was determined using the Absolute Quantification module of the Roche LightCycler® 480 software package and expressed as the total copies of ITS or number of spores. Where absence is reported, it represents 0 ZGE in triplicate for each swab.

2.3 | Storage and detection experiment

To test whether our laboratory assay was capable of detecting *Bd* on frogs fixed in 10% buffered formalin

(which crosslinks DNA with associated proteins and renders it inaccessible to PCR; reviewed in Zimmermann et al., 2008), we experimentally infected 10 juvenile cane toads (*Rhinella marinus*) with *Bd*. Cultures of *Bd* were grown on agar plates made with Tryptone, Gelatin Hydrolysate Lactose (TGHL). We flooded plates with a dilute salt solution to obtain a solution of infective zoospores. The toads were inoculated by immersion in 5 ml of solution containing 1.6×10^6 zoospores for 8 hr. Toads were then housed at 18°C for 27 days to allow the infection to develop. Toads were euthanized with MS222 on day 27 and swabbed 10 times on their left ventral side. After swabbing, five toads were fixed in formalin for 48 hr and then stored in ethanol, whereas and five were stored in ethanol immediately. Toads were re-swabbed 7 days later and genomic zoospores equivalent (GZE) counts were compared to ensure *Bd* DNA was detectable.

3 | RESULTS

All toads infected with *Bd* in the laboratory tested positive for infection both with and without fixation in 10% formalin prior to storage in ethanol. Swabs taken after toads were euthanized, fixed, and stored had higher infection loads than live swabs: GZE counts from ethanol stored swabs were an average of 142,284 ± 113,212 (*SE*) higher after storage; GZE counts from swabs fixed in formalin were an average of 2,895 ± 1,927 (*SE*) higher after storage (Table S1).

In total, we swabbed 422 frogs from 80 species in 20 genera from six families. We did not detect *Bd* DNA on any skin swab taken from a frog swabbed in, or collected from PNG. If all species were equally vulnerable to infection, sites with more than 60 samples collected would have a 95% probability that the prevalence of *Bd* was less than 5% (Tables 1 and S2) (DiGiacomo & Koepsell, 1986). All positive templates detected *Bd* suggesting that the qPCR assay was successful, and that any negatives were true negatives.

4 | DISCUSSION

The absence of *Bd* in our samples from PNG, in addition to the negative detection results in four previous studies (Dahl et al., 2012; Fu & Waldman, 2019; Ouellet et al., 2005; Swei et al., 2011), suggests that this pathogen (a) has not yet invaded Papua New Guinea, (b) is not widely distributed in New Guinea, or (c) is present in low infection loads not detected by our sampling. We acknowledge that the apparent absence of *Bd* in our museum collected samples should be interpreted with caution. While the infection loads in the experimentally infected cane toads increased after 7 days, possibly because they became more easily dislodged from the skin after storage, this does not account for effects of longer storage times, to which the museum specimens were exposed. DNA can degrade on fixed museum specimens over time (Zimmermann et al., 2008) and the preservation history of our museum samples from PNG was unknown. In addition, lowland regions are not optimal climates for *Bd* growth (Bower et al., 2019), and many of the species we swabbed (e.g., terrestrial Microhylids) could have innate resistance, behavioral avoidance of *Bd* exposure or other factors that could reduce the probability of detecting *Bd*.

Further strategic sampling would strengthen existing data. A dataset that obtains a representative sample size from target species that are highly likely to become infected (e.g., stream dwelling *Litoria*), taken from sites optimal for *Bd* (e.g., highland areas in the coolest part of the year) would maximize the probability of detecting *Bd*. Histology of amphibian skin and eDNA in water samples would also be a useful additional sampling method. Places with maximal traffic, for example, ports, should be targeted as these areas are often infected first (Skerratt et al., 2008). Ongoing surveillance for *Bd* in frogs in PNG, as well as in the Indonesian province of Papua, is therefore critical to (a) create baseline data that can serve to quantify current frog diversity and assess any future changes in population status; (b) capture the arrival and spread of pathogens; and (c) direct management towards context-appropriate actions (Bower et al., 2017).

If *Bd* is absent from at least some regions of PNG, this presents an opportunity to collect frog population data prior to the invasion of the pathogen. Although the emergence of chytridiomycosis is regarded as a major contributor to amphibian declines globally (Scheele et al., 2019), the process of emergence is poorly understood and the cause and effect of proximate and ultimate processes is not always identifiable (Lambert et al., 2020). The declines and extinctions that occurred in northern Queensland have been attributed to the arrival of *Bd* and the consequent emergence of chytridiomycosis (Berger et al., 1998), but in this system and many others, data to explicitly test whether the arrival of *Bd* induced the declines is lacking (Lambert et al., 2020; Phillips, Puschendorf, VanDerWal, & Alford, 2012). Surveillance for *Bd* in New Guinea will provide the necessary data to test for causation in the event of frog declines. Furthermore, if chytridiomycosis eventually emerges in New Guinea, surveillance may make it possible to respond more effectively than has occurred in other areas. Published research in Australia suggests that frog populations have not declined in all areas where *Bd* occurs, but these

studies do not usually have pre-decline data to test this, which limits our capacity to draw conclusions (Riley, Berry, & Roberts, 2013). Similarly, studies of the impacts of emerging infectious diseases on host populations are often hindered by a lack of pre-decline tissue samples (McKnight, Schwarzkopf, Alford, Bower, & Zenger, 2017), and it would be useful to collect samples from New Guinea before an outbreak occurs.

It is unclear how the arrival of *Bd* in New Guinea will affect frog communities. While Asian species appear largely resistant and very tolerant to *Bd* (Swei et al., 2011), much of the frog fauna in PNG is closely affiliated with many susceptible Australian species. This includes more than 100 species of Pelodryadidae that occupy high elevation regions (Bower et al., 2019). These are high-risk taxa considering declines in closely related species (e.g., *Litoria* spp) elsewhere (Richards, McDonald, & Alford, 1993) and some species are susceptible to *Bd* under experimental exposure (Fu & Waldman, 2019). Nevertheless, diversification of amphibian fauna in New Guinea has predominantly occurred in the Microhylidae, and in Australia, the susceptibility of at least one Microhylid frog to chytridiomycosis appears to be very low (Hauselberger & Alford, 2012). Factors affecting the variation of susceptibility in closely related species and even populations, suggest that phylogeny alone cannot predict tolerance to *Bd* and suggest instead that physiological susceptibility is complex, involving skin morphology or innate immune defenses (such as MHC genes, AMPS and skin microflora) (Bataille et al., 2015). Microhylidae in New Guinea occupy a wider range of niches than those in Australia (including semi-aquatic species), and thus may be more susceptible to chytridiomycosis (Köhler & Günther, 2008). In addition, chytrid infection loads in South American species with direct developing embryos (a trait shared by all New Guinean microhylids) increase more rapidly and have higher mortality rates than species with aquatic larvae (Mesquita et al., 2017). Therefore, exposure trials would be useful to understand the susceptibility of New Guinea's frogs to *Bd*.

Collaborations between national communities, conservation biologists, disease ecologists and veterinarians can address these unknowns, quantify the biodiversity that is present and strengthen scientific partnerships and capacity within undeveloped countries. Efficient management of remaining refugia such as New Guinea requires not just scientific input, but integrated engagement of scientists, biosecurity departments, legislators, policymakers, on-ground managers, extractive industries and local landowners (Bower et al., 2017). We need to identify likely key points of entry to New Guinea and improve biosecurity to minimize the risk of entry (Bower et al., 2019). It will be imperative to identify avenues of potential spread and refugia from chytrid fungus should it arrive. Correspondingly, it is essential that we learn and record more about the frog biodiversity in areas most likely to be affected, and plan contingency responses. By investing the necessary funds now to coordinate pre-emptive efforts, many species and substantial funds can be saved in the long-term. Better to spend a penny now in prevention than a pound later on a cure.

Article impact statement: The amphibian chytrid fungus remains undetected in Papua New Guinea following wide geographical sampling of 442 frogs.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Data were collected by D. S. B., C. K. J., Y. A., D. N., A. G., and S. C. Laboratory work and analyses were completed by D. S. B., R. J. W., and L. C. Funding and resources were provided by L. S., A. G., R. A. A., and L. B. All authors contributed to concept, structure, writing, and revision of the manuscript.

DATA AVAILABILITY STATEMENT

Data are available in the supporting information.

ETHICS STATEMENT

The authors have followed the Publishing Ethics Guidelines.

ORCID

Deborah S. Bower https://orcid.org/0000-0003-0188-3290

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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