The ecology of chytrid lineages in southern Africa

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Abstract

The inter- and intraspecific diversity of microbial communities is known to be an important, but difficult to disentangle, factor in pathogen ecology. Conspecific or interspecific microbial interactions may result in competitive suppression, the evolution of pathogens to greater levels of virulence, environmental niche separation and coexistence or even result in the generation of novel recombinant pathogen genotypes. *Batrachochytrium dendrobatidis* (*Bd*), the causative agent of chytridiomycosis, is a uniquely destructive pathogen – it is the proximate driver behind the population declines of an unprecedented number of amphibian species and has undergone a global dispersal. It has also become clear that within *Bd* are multiple phylogenetically deeply diverged lineages. There is evidence that these lineages vary in ecology and virulence, but diagnostic limitations have hampered research assessing the importance of lineage and lineage interactions on *Bd* epidemiology. I have developed a novel qPCR-based diagnostic to type the *Bd* lineage present in amphibian skin swabs, museum specimens and experimental animals quickly and economically, to facilitate the collection of baseline data on chytrid lineage distributions globally and to enable experimental work on lineage interactions and ecology. Using this novel diagnostic assay I have delineated *Bd* lineage distributions over one of the widest areas to date in South Africa and the Lesotho highlands, where both *Bd*GPL and *Bd*CAPE are shown to coexist, but are associated with different environmental conditions and exhibit distinctly different population structures. The data collected from this fieldwork were used to inform experimental work investigating whether the distributions observed in reality may be due to the lineages exhibiting divergent thermal optima. Finally, I considered the role that the wider fungal community may play in modulating pathogen dynamics by investigating whether a novel Malagasy chytrid may be preventing *Bd* from establishing on Madagascar, a biodiversity hotspot with a diverse endemic amphibian community.

Key words

Batrachochytrium dendrobatidis, chytrid, diagnostics, qPCR, Africa, mycobiome

Statement of Originality

I confirm that the contents of this thesis are original and any work carried out or devised by contributors is acknowledged in the relevant chapters.

Copyright declaration

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Acronyms and abbreviations

Bd	*Batrachochytrium dendrobatidis*
Bsal	*Batrachochytrium salamandrivorans*
EID	Emerging infectious disease
EFP	Emerging fungal pathogen
FAO	Food and Agricultural Organisation
IBD	Isolation by distance
ICL	Imperial College London
IoZ	Institute of Zoology, London
NWU	North-West University
OD	Optical density
OIE	World Organisation for Animal Health
RACE	Risk Assessment of Chytridiomycosis to European Amphibians
SDM	Species distribution model
SNP	Single Nucleotide Polymorphism
TGhL	Tryptone, Gelatin hydrosolate, Lactose culture broth
WGS	Whole genome sequencing
WHO	World Health Organisation

Chapter 1

General Introduction

1.1 Emerging infectious diseases (EIDs) and the "One Health" approach

Only recently, health professionals had begun to consider that the tide had turned in the war on infectious disease and non-communicable diseases became the greatest threat to human life in upper middle-income countries (Wishnow & Steinfeld, 1976). However, although deaths due to infectious diseases are continuing to fall worldwide, in 2016 lower respiratory tract infections, diarrhoeal disease and tuberculosis occupied three of the top 10 global causes of deaths, and infectious diseases continue to be five of the 10 leading causes of death in lower-income countries (World Health Organisation, 2018). Lower respiratory tract infections are still the leading cause of death in lower-income countries and killed 3 million people in 2016 (World Health Organisation, 2018) and globally, infectious diseases cause 15 million deaths per year (Morens & Fauci, 2012); clearly, the battle to mitigate the threat posed by pathogens is not over and epidemiological research is still essential to improve the health and socioeconomic stability of populations worldwide. Recent outbreaks of EIDs in humans have focussed attention on the threat pathogens continue to pose to human health and prosperity, from the AIDs crisis of the 1980s to the more recent Ebola and Zika outbreaks (Gibbs, 2005; Bloom, Black & Rappuoli, 2017; Cunningham, Daszak & Wood, 2017).

The threat of emerging and re-emerging infectious diseases – diseases that are either rapidly increasing in incidence, virulence or range, or have recently appeared in a population (Morse, 1995; Farrer & Fisher, 2017) – from all classes of microbial pathogens is severe and growing globally (Jones *et al*., 2008; Smith *et al.*, 2014; Allen *et al*., 2017). A 2008 study found that EID events have steadily risen among humans since 1940 and reached a peak in the 1980s, probably due to the AIDs pandemic (Jones *et al*., 2008). Smith *et al.*'s updated work from 2014 has subsequently found that since the 1980s this trend has not abated and, even though *per capita* the rate of emerging

infectious disease events is decreasing, the total number of infectious disease outbreaks continues to rise (Smith *et al*., 2014). Although the impact that EIDs have on humans and our food resources is reasonably well documented, the impact they have on biodiversity and wildlife has historically received much less attention (Jenkins *et al*., 2015; Cunningham, Daszak & Wood, 2017). At the beginning of the 21st century, concern over zoonotic disease outbreaks was growing and it was formally recognised that human, crop, wildlife and ecosystem health were essentially interlinked and should be addressed contemporaneously and holistically (Alder & Easton, 2005; Anonymous, 2005; Gibbs, 2014). This recognition led to the "One Health" approach which has been adopted by, amongst others, the World Organisation for Animal Health (OIE), the Food and Agricultural Organisation (FAO) and the World Health Organisation (WHO) (Gibbs, 2014). A One Health approach, while difficult to define precisely, broadly aims to foster a collaborative and cross-disciplinary approach to infectious disease management and research (Gibbs, 2014), recognising that in terms of pathogens, humans, wildlife and ecosystems do not exist in isolation. As we begin to ask why we are seeing, in our age of ever-improving healthcare and sanitation, a steady increase in EID events, the One Health approach provides a holistic framework within which to work.

1.2 Emerging fungal pathogens and a globalised world

The world is increasingly connected politically, socially and economically through trade and human migration. Globalisation carries unquestionable economic and social benefits but also risks, including the risk of the unintended transportation of pathogens to new areas, or "pathogen pollution" (Mömer *et al*., 2002; Cunningham, Daszak & Rodríguez, 2003; Patil, Kumar & Bagvandas, 2017). It is now estimated that 25% of total global produce is exported, a 4,000-fold increase since 1913 (Ortiz-Ospina, Beltekian & Roser, 2018). As well as goods, wildlife and people are also moving around the world more and at a faster rate than ever before. In 2015, over 1 billion people crossed international borders for the purposes of tourism, compared with 25 million in 1950 (Glaesser *et al*., 2017). This trend shows no sign of abating, with international tourism arrivals

predicted to reach 1.8 billion by 2030 (Glaesser *et al*., 2017). Animals and plants, as livestock, pets and wildlife, also make up a sizeable and a growing portion of this global movement; in 2012, nations imported approximately US$187.3 billion of wildlife products legally (Chan *et al*., 2015). This is particularly pertinent given that an estimated 43.3% of emerging infectious diseases have a zoonotic origin, emerging in human populations after crossing over from wildlife reservoirs (Allen *et al*., 2017).

Concurrent with this rise in globalisation and tourism, the risk of pathogen pollution also rises (Jones *et al*., 2008; Smith *et al*., 2014; Semenza *et al*., 2016; Findlater & Bogoch, 2018) as microbes, pathogens and parasites are transported along with goods, wildlife and people. One gram of soil, perhaps carried on a tourist's shoe, could contain up to 5×10^6 bacteria, 5×10^5 fungi, three plant seeds, 40 nematodes and even mites (Hulme, 2015). Drivers of individual EID events are difficult to define, but an analysis of 116 EID events in Europe that occurred between 2008 and 2013 found that the most frequent and robust driver of EID outbreaks were globalisation and the environment, specifically travel and tourism (Semenza *et al*., 2016). Rising EID events, as well as being a concern for the human population, have an impact on biodiversity and conservation (Cunningham, Daszak & Rodríguez, 2003; Cunningham, 2005; Patil, Kumar & Bagvandas, 2017), and disproportionately affect wildlife species that are already threatened with extinction (Biodiversa, 2013; Heard *et al*., 2013).

Although they receive comparatively little attention in research effort, funding and general awareness, emerging fungal pathogens (EFPs) in particular appear to be capitalising on our increasingly globalised world (Fisher *et al*., 2012; Ghosh, Fisher & Bates, 2018). Fungal pathogens (mycoses) do not contribute the highest proportion of morbidity or mortality caused by EIDs, but, despite poor levels of surveillance and research investment making their true significance difficult to assess, it is clear that their impact on human health, ecosystems, crops and wildlife is far from negligible (Brown *et al*., 2012; Fisher *et al*., 2012; Bongomin *et al*., 2017, Konopka *et al.*, 2019). In

the agricultural sphere it was estimated that at the beginning of the 21st century, microbes caused a 16% reduction in global crop yields. The vast majority of that loss – 70% - 80% – was due to fungi (Oerke, 2006; Moore, Robson & Trinci, 2011). EFPs have caused extreme human suffering, both directly and indirectly, throughout history and continue to do so to this day (Konopka *et al*., 2019). For example, in East Bengal (now part of Bangladesh) unusually heavy rainfall late in 1942 led to a dramatic increase in incidence of leaf blight on rice crops, now thought to have been caused by the fungal pathogen *Cochliobolus miyabeanus*. The resultant epiphytotic led to a drop in rice yield of between 40% and 90% in the region, and the deaths by starvation of around 2 million people (Scheffer, 1997; Vurro, Bonciani & Vannacci, 2010; Manamgoda *et al*., 2011).

In the present day, antimicrobial drug resistance has led to the emergence or re-emergence of fungal pathogens that are increasingly difficult to treat, such as *Candida auris*. *C. auris* is a nosocomial fungal pathogen that was only described in 2009 (Satoh *et al*., 2009). Of the isolates that have been recovered, 50% are multi-drug resistant (while some are pan-drug resistant), and the fungus has now been detected in at least 19 countries (Clancy & Nguyen, 2017; Rhodes *et al*., 2018). *C. auris* inflicts a 70% mortality rate and predominantly infects patients in intensive care units; the rapid spread and extreme virulence of this fungus in the most vulnerable patients demonstrates the extreme difficulty of treating some of the newly emerging infections of the present day (Clancy & Nguyen, 2017; Chowdhary, Sharma & Meis, 2017).

Mycoses are known to cause a serious or life-threatening infection in 150 million people annually and over a million deaths (Gow & Netea, 2016; Bongomin *et al*., 2017). In total, mycoses directly affect over a billion people each year (Bongomin *et al*., 2017), but these figures are not reflected in the resources allocated to researching, monitoring and tackling fungal pathogens. Mycoses command just under 2% of UK philanthropic and public funding invested in infectious disease research and are subject of a mere 3% of funded research studies (Head *et al*., 2014). Despite this lack of surveillance and research investment, it has become clear that the share of infectious disease

morbidity attributable to fungal pathogens is increasing globally, with the number of EID event alerts to ProMED (the Program for Monitoring Emerging Diseases) attributable to fungi rising from 1% to 7% of the total number of alerts between 1995 and 2010 (Fisher *et al*., 2012; Brandt & Park, 2013; Vallabhaneni *et al*., 2016; Benedict *et al*., 2017). When EFPs occur, they have the potential to be extremely serious (Table 1.1). Fungi are capable of extreme virulence and in recent notable outbreaks have inflicted very high mortality rates of more than 50% and occasionally nearly 100% on the host population (Wilder *et al*., 2011; Brown *et al*., 2012; Stegen *et al*., 2017). The extreme virulence that fungal pathogens are capable of displaying is reflected in that they are more likely than any other class of microbial pathogen to cause a population decline in their host species (Fisher *et al*., 2012). Furthermore, from an anthropocentric perspective, the biochemical similarity of fungal cells to our own means that a much smaller toolkit of drugs is available to tackle these infections when they do occur, compared with those available for bacterial infections, making multi-drug resistance in fungi a serious and growing problem (Roemer & Krysan, 2014).

Table 1.1 Examples of serious emerging and re-emerging fungal pathogens.

Pathogen (phylum) & disease	Host	Emergence context & impact
Batrachochytrium dendrobatidis (*Bd*) (Chytridiomycota) Chytridiomycosis	Amphibians	Described and isolated from an infected amphibian in 1999, since when *Bd* has been detected on over 700 amphibian species and implicated as a proximate driver of declines in over 500 species (Longcore & Pessier, 1999; Skerratt *et al*., 2007; Olson *et al.*, 2013; Berger *et al*., 2016; Scheele *et al*., 2019).
Psdueogymnoascus destructans (Ascomycota) White Nose Syndrome (WNS)	Bats	WNS was first reported in New York State, USA in 2006 and *P. destructans* was described in 2009. In North America, WNS is spreading at 200 - 900km per year and triggered a 75% population crash in bats at affected sites in two years following discovery. *P. destructans* has been identified throughout the Americas and Eurasia, but appears to be tolerated in Eurasian bats. The fungus is predicted to extirpate regionally the previously common *Myotis lucifugus* (Blehert *et al.*, 2009; Gargas *et al*., 2009; Frick *et al.,* 2010; Langwig *et al*., 2012; Lorch *et al.,* 2016; Zukal *et al.*, 2016; Campana *et al*., 2017)
Fusarium graminearum (Ascomycota) Fusarium Head Blight	Cereals	First described in England in 1884 and caused significant losses in the early 20th century (Wegulo *et al*., 2015). Head Blight re-emerged in the 1980s and 1990s and continues to exert a significant impact today, which may increase under changing climactic conditions (Madgwick *et al*., 2011;West *et al*., 2012).
Phakopsora pachyrhizi (Basidiomycota) Soybean Rust	Legumes	*P. pachyrhizi* originates in Asia-Australia and was first reported outside that region when it was detected in Hawaii in 1994, following which it was detected in South America in 2001 and continental USA in 2004 (Schneider *et al*., 2005;Goellner *et al*., 2010).
Candida auris (Ascomycota) Candidiasis	Humans	*C. auris* was isolated from the external ear canal of a Japanese patient in 2009 (Satoh *et al*., 2009). It has since emerged as a nosocomial pathogen globally, predominantly isolated from patients in intensive care units and present in at least 19 countries (Chowdhary, Sharma & Meis, 2017; Rhodes *et al*., 2018).
Aspergillus fumigatus (Ascomycota) Azole-resistant aspergillosis	Humans	*A. fumigatus* is an ubiquitous fungus and also the primary causative agent of aspergillosis worldwide (Chowdhary *et al*., 2013). The earliest known case of azole-resistant aspergillosis is from a man suffering with AIDs in San Francisco, United States in 1988 (Denning *et al*., 1997). Multiple European countries have since reported cases (Vermeulen, Lagrou & Verweij, 2013) and in the UK and the Netherlands, incidence is significantly increasing (Wiederhold & Patterson, 2015).

1.3 Drivers leading to EFPs

Several particular characteristics of fungal pathogens may make them a) predisposed to be capable of capitalising on our increasingly globalised world and b) tend towards extreme virulence. Firstly, many fungal pathogens have a sporulation stage which is often capable of environmental persistence outside their host. This confers a two-fold benefit for fungal pathogens. Production of environmentally resistant spores allows fungi to disseminate over large distances (Fisher *et al*., 2012). Sporulation also allows fungal pathogens to persist in the environment when there is no host available for direct transmission. Pathogenic spores have been found persisting environmentally for long periods of time whilst remaining infective, sometimes up to months (Kramer, Schwebke & Kampf, 2006; Mitchell *et al*., 2008; Lindner *et al*., 2011; Lorch *et al*., 2013; Al-Shorbaji *et al*., 2015). Both of these factors reduce the reliance on host species maintaining high population densities for pathogen survival, allowing many mycoses to operate outside of the classical theory of density dependent host-pathogen co-evolution (Fisher *et al*., 2012).

Many fungal pathogens are also extreme generalists, capable of infecting and causing pathogenesis in a staggeringly high number of species. For example *Bd*, the amphibian pathogen, has been identified infecting nearly 700 species from all three amphibian orders (Olson & Ronnenberg, 2014); *Sclerotinia sclerotiorum* (Lib.) de Bary is a necrotrophic pathogen of over 400 species of plant, threatening crops ranging from sunflowers to peas to tulips (Bolton, Thomma & Nelson, 2006); *Botrytis cinerea*, another necrotrophic mycosis, infects over 200 plant species, fuelling a €540 million botrycide-specific sector of the fungicide market (Dean *et al*., 2012).

Crucially, not all species that are susceptible to fungal infection will be equally so. Some may be tolerant (where the host does not prevent pathogen colonisation but is able to persist due to less severe symptoms following infection) and some may be resistant (where the host is able to minimise pathogen burden by reducing the ability of the pathogen to colonise in the first place) (Råberg, Graham & Read, 2009). Variation may even occur within the same species, as is seen

in the toleration of *Bd* infection by many tadpoles, which only have colonisable keratin-rich mouthparts, compared with juvenile and adult frogs (Langhammer *et al*., 2014; McMahon & Rohr, 2015). The variation in susceptibility means that populations, species, or even specific host life stages that are less vulnerable can act as a reservoir, enabling the continual reinfection of those that are most vulnerable even when the population falls to densities that would otherwise render pathogen transmission unviable (Fisher *et al*., 2012).

Fungal genomics also play a role in "pre-adapting" fungal pathogens for the modern world. Fungi are extremely flexible in their ability to recombine, hybridise and undergo horizontal gene transfer (Fraser *et al*., 2005; Mallet, 2007; Inderbitzin *et al*., 2011; Mehrabi *et al*., 2011; Croll & McDonald, 2012), allowing rapid generation of genetic diversity. As we move fungi around the world, not only may this genomic plasticity allow fungal pathogens to adapt quickly to new hosts and environments but also, as fungal lineages come into anthropogenically-mediated contact, hybridisation and recombination may occur (Brasier, 2000; Slippers, Stenlid & Wingfield, 2005).

There is precedent for hybrid and recombinant fungal pathogens to be more virulent than either of the parent lineages, or to result in expanded host ranges – this is particularly pertinent given the discovery of recombinant *Bd* isolates (Schloegel *et al*., 2012; Jenkinson *et al*., 2016; O'Hanlon *et al*., 2018). In the USA, two commercially grown sections of poplar, *Populus* sect. *Aigeiros* and *Populus* sect. *Tacamahaca* are sympatrically isolated. No species from *P.* sect. *Aigeiros* grow in the Pacific Northwest, where *P.* sect. *Tacamahaca* species are found; likewise no *P.* sect. *Tacamahaca* species can be found east of the Appalachian Mountains, where *P.* sect *Aigeiros* grows. Similarly, each section is affected by a specific species of the *Melamspora* leaf rust pathogen. *Melamspora medusae* is typically found on *P.* sect. *Tacamahaca* in the west of the USA and *Melamspora occidentalis* infects *P*. sect. *Aigeiros* species to the east. In the 1980s, a commercial hybrid of two species of *Populus* (produced from *P. deltoides* of *P*. sect. *Aigeiros* and *P. trichocarpa* from *P*. sect. *Tacamahaca*) was bred to be resistant to *M. occidentalis* and widely planted in the Pacific

Northwest. These clones remained free of leaf rust until 1991, when it became apparent that a hybrid pathogen, the result of a cross between *M. medusae* and *M. occidentalis*, was attacking the new Poplar clones. Furthermore, it emerged that the new leaf rust hybrid pathogen was able to attack both parent Poplar species, as well as the commercial hybrid clone (Brasier, 2000; Newcombe *et al*., 2000).

Similarly, triticale is a hybrid multipurpose crop first developed in 1875 by crossing wheat and rye. Triticale is now widely grown for animal feed and as a cover crop thanks to its hardiness and plasticity, which enable growth in a wide range of environments combined with a high grain yield, rapid growth and nutritional content. In 2014, 17 million tons of triticale grain was produced, at an increase of 8% in acreage and 17% in yield compared with 2017 (Ayalew *et al*., 2018). An additional benefit of triticale as a crop was that until recently it was resistant to *Blumeria graminis*, the powdery mildew pathogen of grasses which in extreme circumstances is capable of causing yield reductions of up to 60% (Singh *et al*., 2016). Subspecies of *B. graminis* affect both the wheat and rye parent species of triticale. However, in 2001 powdery mildew was reported on triticale crops in France, and subsequently across Europe (Walker *et al*., 2011) with yield losses approaching 20% in some cases, thus seriously undermining economic incentives to cultivate the crop (Walker *et al*., 2011). In 2016, it was discovered that the triticale-specialising powdery mildew pathogen is a hybrid of *B. graminis* f. sp. *tritici*, a wheat specialist, and *B. graminis* f. sp. *secalis*, a rye specialist. The new hybrid, *B. graminis* f. sp. *dicocci*, is a triticale specialist and an emerging pathogen in multiple countries (Menardo *et al*., 2016).

Finally, climate change, along with the multitude of other global challenges it presents, may lead to an increase in the incidence and range of fungal pathogens. Currently, although fungi are capable of causing serious morbidity and mortality in commercially important crops, some animals and immunocompromised humans, it is unusual for them to cause severe infections in immunocompetent mammals. One reason for this is that mammals are endothermic, maintaining

an internal environment that is substantially warmer than the ambient temperature. To take advantage of pathogen protection via endothermy, mammals must maintain a substantial temperature differential compared with the surrounding environment, one that will be reduced as more of the world becomes warmer under a changing climate. As environmental conditions warm, thermotolerant fungi will be selected for. Circumstantial evidence shows the potential consequence of this – rates of *Cryptococcus* infection in immunocompromised patients in Africa are up to 25% higher than in immunocompromised patients in temperate regions. As well as direct selection for pathogenic fungi "pre-adapted" to be able to grow at mammalian body temperatures, as the climate warms more heat-tolerant fungi will be able to expand their ranges, potentially introducing EFPs into wider areas (Garcia-Solache & Casadevall, 2010). It is possible that we are already witnessing the effects of this, with the emergence of *C. auris*. Phylogenetic analyses of temperature susceptibility of *C. auris* and close relatives shows that *C. auris* is more thermotolerant than most of its close, non-pathogenic relatives, raising the possibility that if the fungus recently acquired thermotolerance, this ability will have enabled it to colonise the human body where it now causes severe disease (Casadevall, Kontoyiannis & Robert, 2019).

1.4 *Bd* biology and chytridiomycosis

Bd is a fungal pathogen of amphibians, nested within the phylum Chytridiomycota. Chytridiomycetes are an early diverging fungal lineage, characterised by their motile zoospores and the majority are obligate parasites of vascular plants (James *et al*., 2006). *Bd* and the recently described *Batrachochytrium salamandrivorans* (*Bsal*) (Martel *et al*., 2013) are the only chytrid species known to infect vertebrates and both are capable of causing catastrophic disease in susceptible populations of amphibians. The impact of *Bd* on global amphibian populations has been so extreme that the fungus has been described as having caused "the most spectacular loss of vertebrate biodiversity due to disease in recorded history" (Skerratt *et al.*, 2007). *Bd* was first isolated and described from a captive *Dendrobatidis tinctorius azureus* (blue poison dart frog)

(Longcore & Pessier, 1999). The fungus has two distinct life stages; the zoosporangium (the reproductive stage) and the motile zoospore (the infective stage). Zoospores are small, only 3–5µm in diameter, with a long flagellum up to 10µm in length and are free living (Berger *et al*., 2005; Institute of Medicine, Board on Global Health & Forum on Microbial Threats, 2011). Amphibians may become infected either by direct transmission from another amphibian or from contacting zoospores present in the environment (Institute of Medicine, Board on Global Health & Forum on Microbial Threats, 2011). Experimental work has shown that the majority (>95%) of zoospores are only active for a short period of time, less than 24 hours, and swim less than 2cm before encysting (Piotrowski, Annis & Longcore, 2004). This suggests that many zoospores encyst close to where they are released, building up infection burdens on the same individual host animal (Briggs, Knapp & Vredenburg, 2010). *Bd* zoospores are chemotactically attracted to, among other molecules, keratin, which is a major component of the adult amphibian epidermis and the mouthparts of many larval amphibians (Moss *et al*., 2008). When *Bd* infects larval mouthparts, it does not cause significant morbidity or mortality, the most obvious symptom being depigmentation of the keratinised regions of the mouth (Figure 1.1) (Rachowicz & Vredenburg, 2004; Rooij *et al*., 2015).

Figure 1.1. A *Bd* infected *Hadramophryne natalensis* tadpole, Royal Natal National Park, South Africa. Brown colouration around the black keratinised mouthparts indicates presence of *Bd*.

In post-metamorphic amphibians, zoospores encyst deep in the amphibian skin epidermis and develop into immature zoosporangia (Berger *et al*., 2005). As the epidermal cells differentiate, the developing zoosporangia are carried up the skin layers, with most reaching the *stratum corneum* upon maturity (Berger *et al*., 2005).

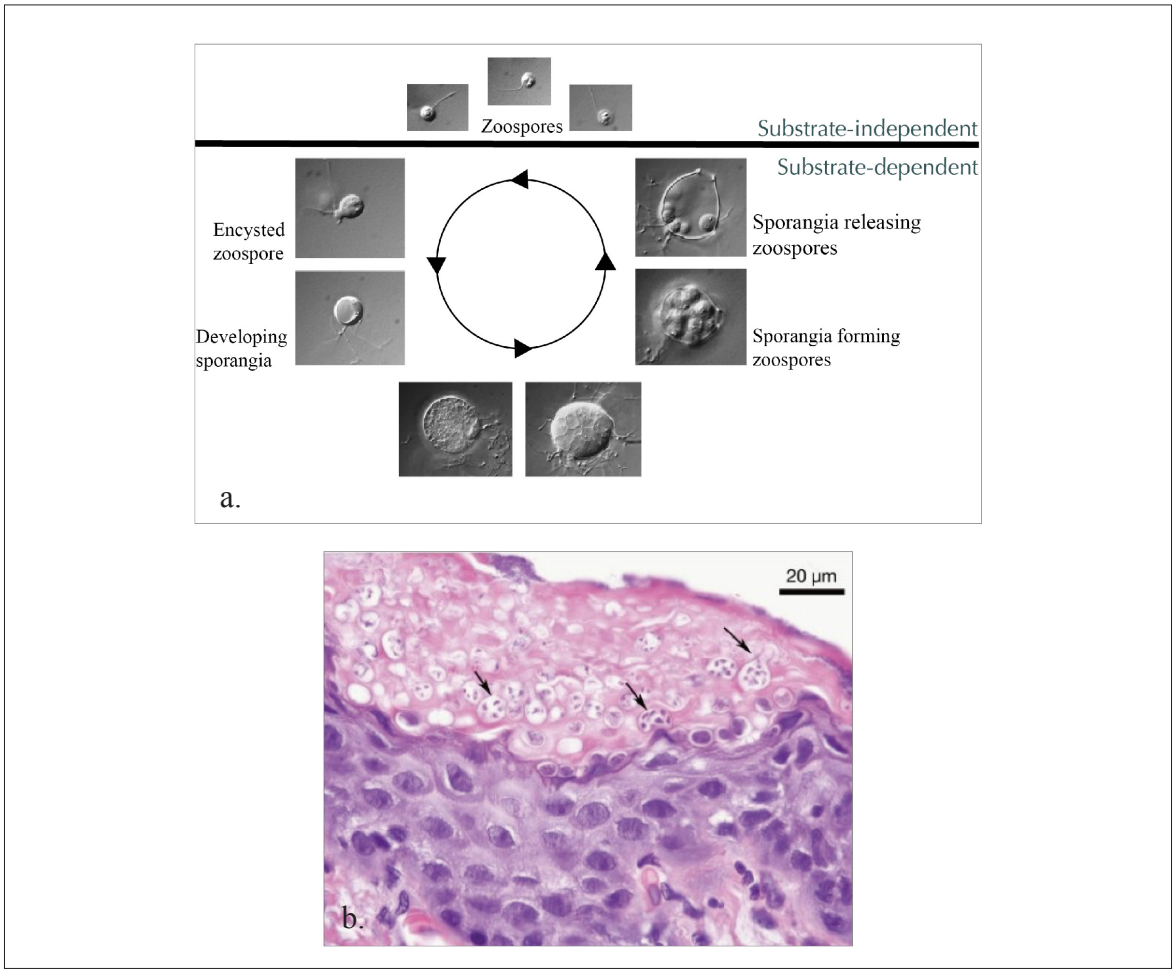


Figure 1.2. Life cycle and histology of *Bd*. (a) Life cycle of *Bd*, image taken from Rosenblum *et al*., 2008 (b) Histological section of amphibian skin infected with *Bd* and displaying epidermal hyperplasia (arrows indicate *Bd* sporangia, image taken from Jones *et al*., 2012).

Discharge tubules are produced by zoosporangia which orientate towards the surface of the skin layer and protrude to the external environment through a gap in the amphibian epidermal cell membrane. As this process progresses, the epidermal cells surrounding zoosporangia undergo hyperkeratosis (Figure 1.2). Clinical signs of infection include excessive skin sloughing, anorexia, a

loss of the righting reflex and abnormal positioning of the hind legs (Berger *et al*., 2005).

Amphibian skin is a hugely complex organ, performing multiple critical functions such as being a component of the immune system (functioning both as a physical barrier and harbouring protective microbes) (Becker *et al*., 2015; Varga, Bui-Marinos & Katzenback, 2018) and allowing gaseous, ion and liquid exchange between the amphibian and the environment (Vanburen, Norman & Fröbisch, 2018). The disruption to the amphibian skin system caused by the hyperkeratosis, hyperplasia and tissue erosion directly impairs amphibian skin functions such as osmoregulation. Critically, the host's ionic regulatory and osmoregulatory abilities may be severely damaged (Berger *et al*., 2005; Voyles *et al*., 2009). Severely infected amphibians may experience a drop in plasma electrolytes of between 25% and 70% (Berger *et al*., 2016). It seems likely that this in turn causes abnormal cardiac electrical activity, resulting in death by cardiac arrest (Berger *et al*., 2005; Voyles *et al*., 2009).

1.5 The global amphibian extinction crisis and the emergence of *Bd*

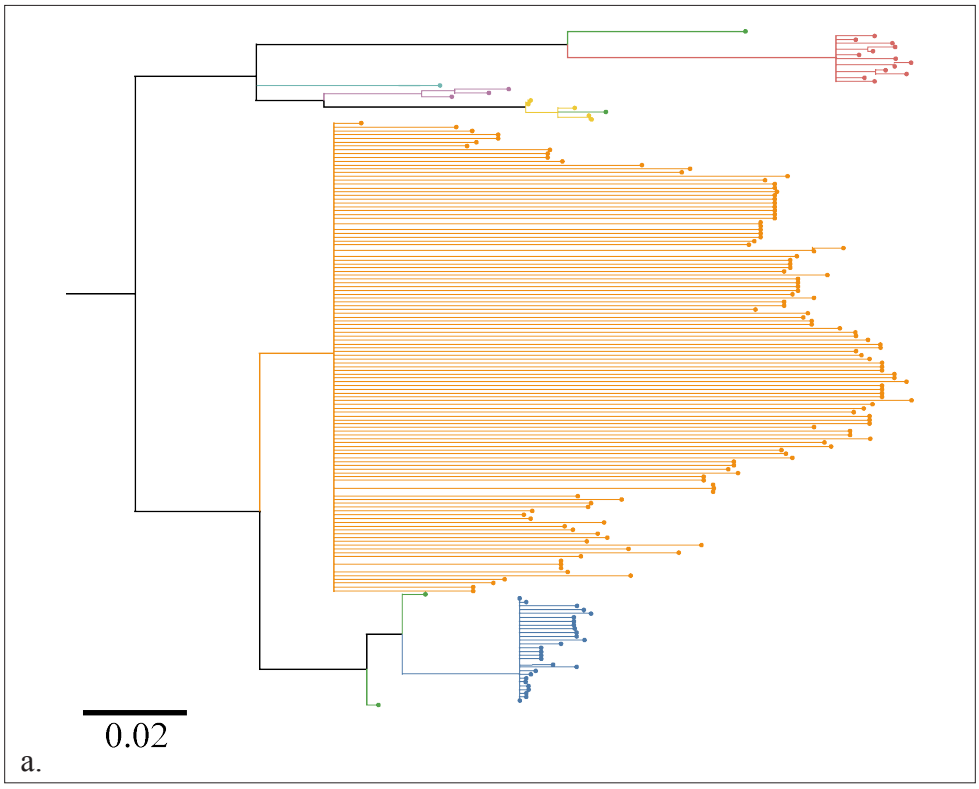
The emergence of *Bd* is a contributing factor in an ongoing extinction crisis among the world's amphibians. The amphibians (the Anura, Caudata and Gymnophonia) are a highly diverse and ancient group of vertebrates of approximately 7,000 species (Catenazzi, 2015). Amphibians are uniquely threatened among vertebrate classes, with 40% of amphibians assessed by the IUCN Red List falling into a threatened category; the only group more highly threatened is the cycads. Among vertebrates, amphibians are the most highly threatened group; in comparison 25% of mammal species and 14% of bird species are classified as threatened. Furthermore, this is likely to be an underestimate as amphibians remain a poorly described class. At the moment, 24% of amphibian species are classified as Data Deficient, the category species fall into when there is insufficient information known about the species or threats (or lack thereof) it faces to assign it an IUCN category (Nori, Villalobos & Loyola, 2018) and many of these are likely to be small, at-risk populations – species on the IUCN list classified as Data Deficient tend to have an elevated risk

of extinction (Heard *et al*., 2013; Bland *et al.*, 2015). Current estimates put the rate of amphibian species extinctions at about 2,000 times faster than the historical average (Catenazzi, 2015; Alroy, 2015). The rate of amphibian declines is so extreme that it has been suggested that the phenomenon qualifies as a sixth mass extinction (Wake & Vredenburg, 2008). Amphibians are faced with multiple threats as well as EIDs, such as climate change, habitat loss, over harvesting and pollution. Concerningly, areas that harbour the greatest levels of amphibian biodiversity are also experiencing the greatest number of threats (Hof *et al*., 2011). Despite the critical and uniquely threatened status of the world's amphibians, the most threatened species of amphibian are the least likely among vertebrates to have any range overlap with the global protected area network (Venter *et al*., 2014; Nori *et al*., 2015).

Amphibian declines appeared to begin accelerating in the 1980s and 1990s and it was noted that many of these declines were enigmatic, unattributable to the usual suspects such as habitat loss, and occurring in non-threatened areas such as in nature reserves among previously common species (Alroy, 2015). Many of these declines have now been attributed to the emergence of *Bd* and chytridiomycosis (Alroy, 2015). *Bd*-driven declines are thought to have begun in the late 1970s in Australia and the Americas, long before the fungus was finally described in 1999 (Berger *et al*., 1998; Longcore & Pessier, 1999; Berger *et al*., 2016). In Australia, the realisation that more than 14 endemic species had disappeared or declined by over 90% within 15 years, even in pristine habitats, triggered investigations that ultimately concluded that a deadly and novel pathogen was spreading through the country at a rate of about 100km per year (Laurance, McDonald & Speare, 1996; Berger *et al*., 2016), although it is worth noting that this conclusion was not without controversy (McCallum, 2005; Phillips *et al*., 2012). Concurrently, a similar pattern was observed in Central America, with mass mortalities of adult amphibians appearing to progress in the wave-like pattern characteristic of EIDs through the region (Lips *et al*., 2006). In affected areas, amphibian populations crashed within a few months of *Bd* arriving in a naïve area, resulting in over half amphibian species being extirpated and those that persisted only did so at about 20% of their

prezootic abundance (Berger *et al*., 1998; Lips *et al*., 2006).

Genomic analysis has recently revealed that *Bd* originates in South East Asia, and this is where the majority of genetic diversity is seen today (O'Hanlon *et al*., 2018). However, it is now found on every continent except Antarctica (where no amphibian hosts exist) and has been detected on ~50% of amphibian species sampled, totalling infection of nearly 700 species (Olson & Ronnenberg, 2014). Recent analyses indicate that *Bd* has caused 90 presumed extinctions and has been a factor in the decline of at least 500 amphibian species (Scheele *et al.*, 2017). Over 100 countries have sampled for *Bd*, albeit patchily, and 71 have identified the fungus on their amphibians (Olson *et al*., 2013; Olson & Ronnenberg, 2014). It is notable that the Amazon basin and Central Asia are chronically under-sampled, and surveillance in these areas would greatly inform *Bd* epidemiological understanding. A further gap in *Bd* surveillance work lies in terms of the taxonomic level at which sampling is taking place. Within the species *Bd* are harboured at least six phylogenetically deeply diverged lineages: *Bd*GPL is a panzootic lineage with a global distribution; *Bd*CAPE is predominantly found in Africa and was thought to be restricted to Africa and a single known introduction in Mallorca until recently when it was detected in Honduras and more widely in Europe as well; *Bd*ASIA-1 is a highly diverse lineage so far only identified on amphibians native to the Korean peninsula and the only lineage which shows the characteristics of pathogen endemism in its genome; *Bd*ASIA-2/BRAZIL is associated with invasive frogs in the Korean Peninsula and also the Brazilian Atlantic Forest; *Bd*ASIA-3, which was described in 2019 and is widespread in South East Asia; and *Bd*CH, which has only been isolated once from Switzerland (Walker *et al*., 2008; Goka *et al*., 2009; Farrer *et al*., 2011; Jenkinson *et al*., 2016; O'Hanlon *et al*., 2018; Byrne *et al*., 2019) (Figure 1.3).



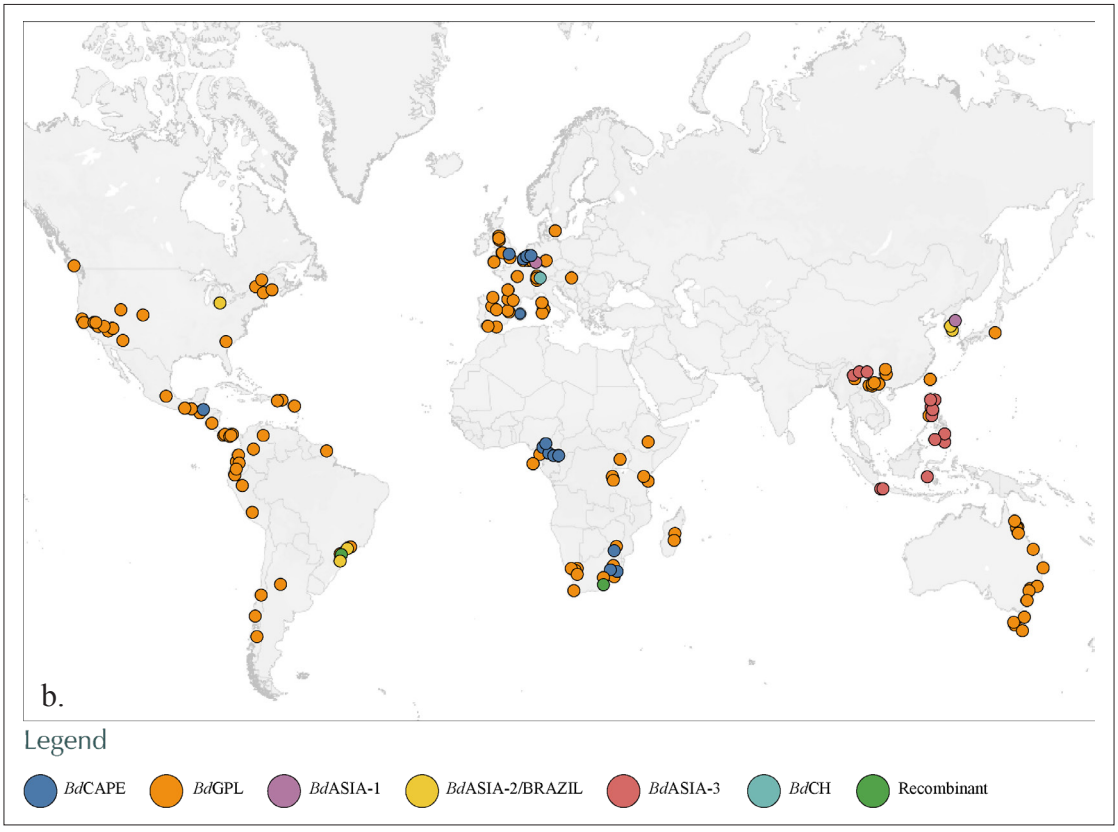


Figure 1.3. Global *Bd* lineage isolations: (a) *Bd* lineage phylogeny, image taken from Byrne *et al.*, 2019; (b) map showing locations of *Bd* lineage-typed samples. Compiled from data taken from O'Hanlon *et al.*, 2018 & Byrne *et al.*, 2019). Map generated in Tableau™.

Bd lineage is now known to be an important epidemiological variable. To date, only *Bd*GPL and, in a single case on Mallorca, *Bd*CAPE have confirmed associations with amphibian population declines (O'Hanlon *et al*., 2018). Multiple studies have consistently shown that virulence and traits associated with virulence, such as zoosporangia size and zoospore production rate, vary with lineage (Fisher *et al*., 2009; Farrer *et al*., 2011; Lambertini *et al*., 2016; Voyles *et al*., 2017; Becker *et al*., 2017; O'Hanlon *et al*., 2018). *Bd*GPL is the only lineage with a global distribution that consistently demonstrates hypervirulence with respect to other lineages both experimentally and in the wild, and likely emerged in the early 20th century, although precisely from where it emerged remains unknown. *In vivo* experiments have shown that infection with *Bd*GPL is significantly more likely to result in host death than infection with *Bd*CAPE, *Bd*ASIA-1 or *Bd*CH in model host species. Analysis of field isolates has shown that both *Bd*GPL and *Bd*CAPE are significantly more likely to be associated with symptoms of chytridiomycosis in nature than the other lineages (O'Hanlon *et al.*, 2018).

Recently, recombinant lineages in both South Africa and Brazil have been identified by whole genome sequencing (WGS) (Jenkinson *et al*., 2016; O'Hanlon *et al.*, 2018). Not only does this show that *Bd* lineages are capable of undergoing either sexual reproduction or parasexual recombination (neither of which have been observed under laboratory conditions to date), but also that *Bd* lineages that are historically isolated from each other may interact and recombine given anthropogenically-mediated re-contacting. Even more concerningly, it seems that *Bd* may follow the precedence of other fungal pathogens which have hybridised or recombined to produced hyper-virulent offspring: isolates that are recombinants of *Bd*ASIA-2/BRAZIL and *Bd*GPL have been shown to be more virulent than either parent lineage from the same region *in vivo* (Greenspan *et al*., 2018).

It is worth noting here that there is no accepted terminology for describing *Bd* isolates produced by the sexual reproduction of two distinct *Bd* lineages. Most research to date has described these isolates as "hybrids" (Schloegel *et al*., 2012; Ghosh & Fisher, 2016; Jenkinson *et al*., 2016;

O'Hanlon *et al*., 2018), but this term leads to the implication that *Bd* lineages are in fact distinct species. Defining species is notoriously complex, particularly among the fungi (Taylor *et al*., 2000; Lobuglio & Taylor, 2002; Matute *et al.*, 2006; Milgroom *et al*., 2014). However, the identification of *Bd* isolates with dual-lineage parentage multiple times from two continents (Schloegel *et al*., 2012; Jenkinson *et al*., 2016; O'Hanlon *et al*., 2018), and evidence that there is a routinely recombining, highly genetically diverse population in South East Asia (O'Hanlon *et al*., 2018), strengthens the argument that the lineages should not be treated as distinct species, and thus the offspring of different lineages are in fact recombinants, rather than hybrids. For this reason, "recombinant" will be used throughout this thesis in lieu of "hybrid", in accordance with the Genealogical Concordance Phylogenetic Species Recognition Concept (Taylor *et al*., 2000).

1.6 *Bd* in Africa

Before genomic analysis revealed the true origin of *Bd* to be in South East Asia, Africa was considered a candidate source region for the pathogen (Weldon *et al*., 2004; Doherty-Bone *et al.*, 2019). Unlike in the Americas, Europe and Australasia, until recently there had been no reports of chytridiomycosis-driven amphibian population declines (Hirschfeld *et al*., 2016; Lips, 2016; Weldon *et al*., 2019), and until 2014 the oldest known detection of *Bd* was from a *Xenopus fraseri* specimen collected from Cameroon in 1933 and now held at the Natural History Museum in London (Soto-Azat *et al*., 2010). A subsequent survey of museum specimens collected from the Atlantic Forest in Brazil reported detection of *Bd* on a *Hypsiboas pulchellus* collected in 1894, the lineage of which was deduced via analysis of the Internal Transcribed Spacer (ITS) region to be *Bd*GPL. This was followed by the 2015 discovery of *Bd* (lineage untyped) on a *Rana* (L.) *sphenocephala* collected in Illinois, USA in 1888 (Talley *et al*., 2015).

However, the ITS region is not reliable at discriminating phylogenetic relationships beyond species level, particularly among early diverging fungi such as the Chytridiomycota (Schoch *et al*., 2012, O'Hanlon *et al.,* 2018), calling the lineage typing of the *H. pulchellus* specimen into question.

Brazil currently houses two lineages in its Atlantic Forest, *Bd*GPL and *Bd*ASIA-2/BRAZIL. If the *Bd* present on the *H. pulchellus* specimen has been incorrectly typed to *Bd*GPL instead of *Bd*ASIA-2/BRAZIL, then this result would not conflict with the emergence of *Bd*GPL in the 20th century, but also leaves Africa as a candidate source continent for *Bd*GPL. Additionally, two lineages also occur in Africa, *Bd*CAPE and *Bd*GPL, and although this continent is severely under-surveyed at the lineage level, it is clear that they both occur over a much wider area than the two lineages that occur in Brazil (Rodriguez *et al.*, 2014). The circumstantial evidence suggests that *Bd*GPL may have been in Africa for longer than in much of the rest of the world, and crucially it is clear that two lineages have been present on the continent since at least 2008 with few clinical signs of chytridiomycosis, making this a key continent to explore for insights into the evolutionary history and the long term ecology of *Bd*.

1.7 Pathogen competition and the importance of niche

Many studies of hosts and their diseases focus on single host-pathogen systems. This approach generally cannot account for the fact that both within the host and at the landscape scale, many pathogen strains and species may be interacting and affecting the disease outcome for the host (Balmer *et al*., 2009; Abdullah *et al*., 2017). Where interacting pathogens are closely related, such as when they are strains or genotypes of the same species, competition is theoretically even more likely as their resource and environmental requirements are more likely to overlap substantially (Godoy, Kraft & Levine, 2014; Venail *et al*., 2014). According to Gause's principle, competitive exclusion will occur provided the following two conditions are met: a) the resource requirements overlap beyond a certain critical point and b) one of the strains or species is a superior competitor for these common resource requirements (Hardin, 1960; Aarssen, 1983). Being a superior competitor does not necessarily mean an ability to monopolise a contested resource; it could mean a better ability to tolerate a reduction in the contested resource. Thus, competitive microbes or conspecific pathogen strains have the potential to impact pathogen distributions and densities

strongly. Competitors in this way contribute to defining the realised niche of a pathogen (where the realised niche is the distribution which is observed in reality, constrained by biotic and abiotic pressures including competitors and in contrast to the fundamental niche, which is the full range of the organism in the absence of any suppressing pressures) (Hutchinson, 1959; Connell, 1961; Vanhove *et al*., 2017). Understanding how pathogens interact with conspecific strains and other competing microbes can therefore provide critical insights into how pathogens and the diseases they cause may spread across a landscape and progress within a host population.

Multiple-strain infections in a host lead to intraspecific interactions and have been shown to have important ecological and evolutionary effects on both the host and the parasites. For example, experimental *in vivo* coinfection of mice with two *Trypanosoma brucei* strains (the causal pathogen of trypanosomiasis, or sleeping sickness), causes mutual competitive suppression, suppressing the population of the more virulent strain and thus increasing host survival probability by up to 15% (Balmer *et al.*, 2009). The dengue virus displays similar behaviour — when two dengue strains are cultured simultaneously, or together with a time lag between each strain being introduced to the culture environment — both strains exhibit significantly reduced rates of replication, and consequently lower overall virus titres, which could have major implications for viral transmission, should this be replicated in the mosquito vector. Further research has shown that it is likely that within-host (in this case, the mosquito vector) competition limits the ability of sylvatic dengue to infect human populations, thus preventing regular zoonotic outbreaks (Pepin & Hanley, 2008; Pepin, Lambeth & Hanley, 2008).

Conversely, under other conditions coinfection with multiple pathogen strains could be predicted to result in the evolution of increased virulence, with each strain driven to greater virulence in order to outcompete other strains (May & Nowak, 1995). Competition for resources should theoretically be greatest and strongest among organisms that a) have the highest overlap in resource requirements and b) come into contact frequently resulting in population mixing (Bauer *et al*., 2018). Conspecific

pathogen strains are likely to meet both these conditions due to their phylogenetic similarity, making them prime candidates for observing strong competition. It is clear that to appreciate fully pathogen and therefore disease dynamics, the genetic diversity of infections must be considered.

Similarly, the range of environmental factors leading to disease emergence, and their interactions with pathogen genetic diversity and microbial communities, may be large-scale and multi-faceted, both temporally and spatially, making causal inference and prediction of pathogen dynamics extremely difficult (Plowright *et al*., 2008). Where this is the case, and no one strategy to identify the drivers of pathogen dynamics can be relied upon, it is necessary to triangulate towards important factors by employing a suite of approaches, from traditional hypothesis testing, experimental work, observational studies and epidemiologic causal criteria, and predictive modelling (Plowright *et al*., 2008). Taking such a holistic view enables the clarification of what the key factors are in determining disease emergence and outcome.

1.8 Project overview, aims and objectives

In this project, I aim to investigate the ecology of chytrid strains in southern Africa, where multiple strains of the amphibian-killing chytrid fungus *Bd* are known to exist. Several factors necessitate taking advantage of a broad suite of approaches to elucidate the distributions and behaviours of these strains. Firstly, the distribution of chytrid strains in Africa has been poorly characterised, hampered by an inability to diagnose the lineage of *Bd* infecting an amphibian from a skin swab, resulting in a severe lack of baseline data. Secondly, the two known chytrid lineages in the region, *Bd*GPL and *Bd*CAPE, where they have been identified, appear to occur over an extremely wide area, being found both close to the northern border of South Africa, and at the southern tip of the country in the Western Cape. Thirdly, previous work which has attempted to identify traits that may impact disease distribution and virulence, such as thermal envelopes, have been confounded by strain and hampered by few isolates available for testing. Finally, the diversity of the fungal mycobiome and whether it impacts *Bd* survival and colonisation ability has been largely

unexplored. The discovery of a novel amphibian skin-associated chytrid in Madagascar presents the first opportunity to investigate the interactions of *Bd* with the wider chytridiomycete community.

I first developed qPCR-based diagnostics for *Bd*GPL and *Bd*CAPE which were applicable for use in the field setting (Chapter 3). The distributions of the two lineages in South Africa were identified across a near complete east to west transect of the country, following the course of the Orange River from its mouth on the western coast to its source in the Maloti-Drakensburg Mountains of Lesotho (Chapter 4). Observational data from this fieldwork informed experimental work to identify whether the lineages were constrained into different regions by environmental conditions (Chapter 4). The genomes of isolates collected were interrogated for insights into the lineages' population structure (Chapter 4) and finally, I investigated whether multi-parasite dynamics could, theoretically, provide an explanation for inconclusive infection data emerging from Madagascar, a critically important biodiversity hotspot for amphibians (Chapter 5) (Table 1.2).

Table 1.2. Project aims and objectives.

Aim	Objectives	Relevant chapter
Develop a diagnostic capable of identifying *Bd*GPL and *Bd*CAPE from amphibian skin swabs, including for sub clinical infections.	Design a candidate qPCR diagnostic using TaqMan Minor Groove Binder (MGB) probes and primers and confirm specificity and sensititvity using *Bd* isolates typed by WGS. Confirm the applicability of the assay in a field and experimental context, as well as for preserved archive specimens.	Chapter 3
Investigate *Bd* lineage distributions in a region known to harbour multiple lineages.	Carry out field transects across South Africa and Lesotho highlands using novel lineage-specific qPCR assay. Search for environmental correlates of *Bd* lineage distribution. Test the impact of environmental correlates observed in a field setting on *in vitro Bd* isolate survival.	Chapter 4
Interrogate population structure of *Bd*GPL and *Bd*CAPE in southern Africa.	Compare genetic diversity of African *Bd*GPL and African *Bd*CAPE. Investigate whether African *Bd*GPL and African *Bd*CAPE display different population structures.	Chapter 4
Assess optical density as a tool for measuring *Bd* growth.	Use standard curve analysis to confirm whether a microplate reader can accurately carry out *Bd* quantification.	Chapter 5
Investigate the phylogenetic context and distribution of a novel Malagasy chytrid.	Use the ITS region and WGS to resolve the position of a novel Malagasy chytrid within the Chytridiomycota. Develop a novel qPCR diagnostic for the Malagasy chytrid based on the ITS region and screen stored amphibian skin swab samples for the presence of the Malagasy chytrid.	Chapter 5
Investigate potential for competitive exclusion between chytrids *in vitro*.	Co-culture the Malagasy chytrid and *Bd*GPL *in vitro* and compare chytrid growth to that seen in pure culture.	Chapter 5

Chapter 2

General Methods

2.1 Chytrid culturing conditions

Unless otherwise specified, all chytrid isolates are held in Fisher Lab's culture collection at Imperial College London (ICL), in a 4°C active collection stored in 25cm^2 vent/close angled neck EasYFlasks™, Nunclon™Δ cell culture flasks (Nunclon™ culture flasks) (ThermoFisher Scientific, Massachusetts, USA). All isolates in the collection are re-passaged every three to four months, and are also held in a cryopreserved state at -80°C. All chytrids are maintained in a Tryptone-based (TGhL) broth (see Appendix 1 for recipe). Newly inoculated or re-passaged cultures are incubated at 18°C for approximately a week, until healthy growth has established. Isolates may also be cultured on TGhL 1% agar plates (Appendix 1). All work with live cultures, aside from initial tissue collection and cleaning, is carried out in a BioSafety ACDP Class 2 cabinet or equivalent to prevent contamination of isolates with environmental fungi and bacteria.

2.2 Swabbing amphibians for *Bd*

Adult and juvenile amphibians were swabbed for *Bd* using tubed, fine-tipped MW100 swabs (MWE, UK). Amphibians were held using gloves changed between individuals or the plastic bag the individual had been placed in was manipulated to allow swabbing without direct contact with the animal. The swabbing process, illustrated in Figure 2.1, was to hold the animal by its back legs (Figure 2.1a) and firmly run the swab over the back and legs on the dorsal side (Figure 2.1b), being particularly careful to ensure the back legs and toes were well swabbed (Figure 2.1c). The animal was then flipped over and swabbed on the ventral side, particularly on the pelvic patch and back legs (Figure 2.1d). *Bd* is most likely to be found on the highly keratinised areas of the back legs, toes and pelvic patch, so these areas were focussed on for swabbing (Hyatt *et al*., 2007).

The animals were then released at the site at which they had been collected as soon as possible. Swabs were labelled with as specific a location as possible (ideally GPS coordinates), the species of amphibian if known, the date and any additional notes such as sex, developmental stage or condition of the individual where relevant. Swabs were stored at 4°C, or simply kept as cool as possible in instances where refrigeration was not available (Hyatt *et al*., 2007).

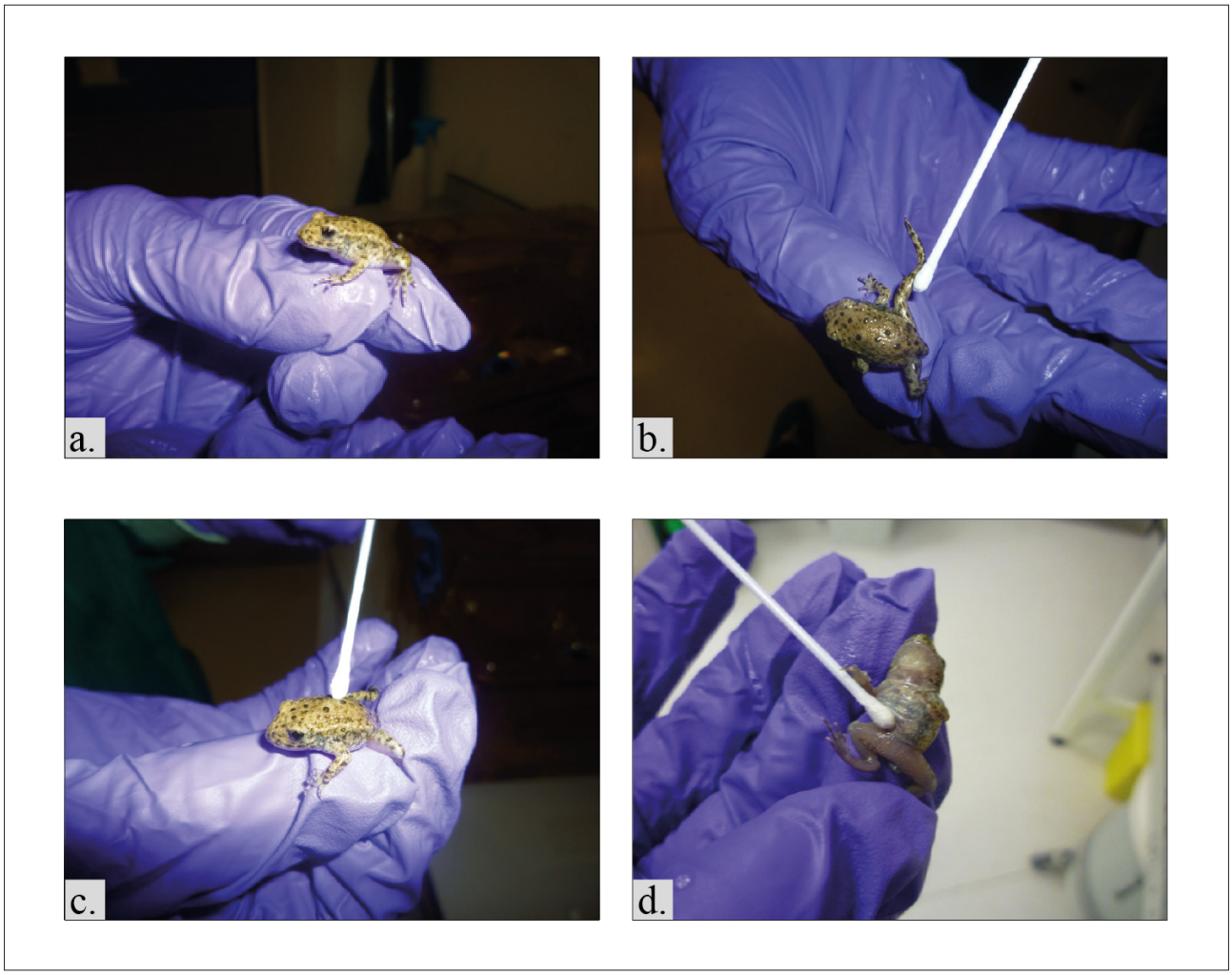


Figure 2.1. Swabbing protocol for *Bd*.

2.3 *Bd* isolation, toe clipping, tadpole euthanasia and toe cleaning

Non-lethal isolation protocols for chytrids have been well established and are widely used in the field (Fisher *et al*., 2018). Adult and juvenile amphibians with fully developed back legs were toe-clipped for *Bd* isolation. All surgical tools were thoroughly disinfected with ethanol of at least 70%

proof in between processing individuals. Where possible, gloves were used to handle amphibians and changed in between individuals, or the animals were manipulated in the plastic bag in which they had been caught to allow toe clipping without direct contact with the individual. Animals were held firmly at the top of the back legs, as in Figure 2.1a. Counting from inside to outside, the fourth toe was amputated at the distal interphalangeal joint on the left back leg. The toe was then buried in a TGhL 1% agar plate infused with antibiotics (Appendix 1) for transportation to a site where further processing could take place. Once at a fieldwork base, the toes were put onto a fresh antibiotic-infused TGhL 1% agar plate and thoroughly cleaned of superficial bacteria, which could prevent the growth of *Bd in vitro*. A sterile needle was used to drag the toe through the agar, moving the toe up and down through the body of the agar. Up to four toes were cleaned per plate, ensuring no toe passed over any agar that had previously been disrupted. Once clean, toes were placed in a sterile tube containing antibiotic-infused TGhL broth and stored in a cool place or refrigerated until they could be processed further under sterile conditions in a laminar flow hood.

In a laminar flow hood, tissue samples were decanted into 24- or 48-well sterile culture plates, and the antibiotic-infused TGhL broth was replenished. Samples were observed daily for up to two weeks under a light microscope for either moving zoospores or developing sporangia and incubated at 18^{o}C. Samples that showed contamination with fungal or bacterial blooms were disposed of. Once zoospores or sporangia had been observed, samples were divided into further wells to reduce the risk of losing the isolate to contamination. When a culture began to show growth (zoospore production), it was re-passaged into a well containing no antibiotics and eventually, when a pure culture was observed under the microscope, into a 25cm^2 Nunclon^{TM} culture flask for storage at 4^{o}C. All isolates were also cryopreserved for long-term storage.

Bd in tadpoles colonises the keratinised mouthparts and so euthanasia was necessary for *Bd* isolation. At all field sites, we aimed to collect 10 tadpoles (ideally of the same species) and no more than 30 individuals in order to minimise the number of lethal samples. In some species, such

as *Hadramophryne natalensis*, it is possible to observe *Bd* on the tadpole mouthparts using a hand lens due to the size of the tadpole (Figure 1.1). Where this was the case, tadpoles that looked as though they were positive for *Bd* by this method were preferentially selected for the purpose of isolation. Tadpoles were collected in plastic bags containing water from the site at which they were collected and kept cool during transportation. Only tadpoles of the same species from the same site were kept in the same bag and only for as long as was necessary to transport them to a site suitable for processing. Tadpoles were immersed in a buffered solution of Tricane methanesulfonate (MS-222) for at least five minutes and until their righting and tail reflexes were no longer functional. Death was confirmed by cutting the spinal cord at the base of the head. The tadpole mouthparts were excised and the bodies preserved in 70% etOH and stored at North-West University (NWU) in case they could be used for future analyses. The mouthparts were then cleaned and processed in the same way as adult amphibian toe clips. Large mouthparts were divided up into smaller pieces of tissue for processing.

2.4 Chytrid cryopreservation and thawing

Chytrid cultures were cryopreserved as soon as possible for long-term storage. 500µl of refrigerated culture in TGhL broth was added to each of two 175cm^2 Nunclon^{TM} culture flasks containing 40ml of fresh TGhL broth. Flasks were incubated at 18^{o}C for six days or until a dense culture was observed. The sides and bases of the flasks were aseptically scraped and the entire contents transferred into two 50ml sterile Falcon tubes which were spun at 17,000g for 10 minutes. The supernatant was then gently poured off, taking care to follow the slope of the pellet and avoid disturbing it. In a BioSafety ACDP Class 2 cabinet or equivalent, the pellet was re-suspended in 3 to 4.5ml of prepared 10% dimethyl sulfoxide (DMSO), 10% Fetal Calf Serum (FCS) in TGhL broth and transferred to six 1.8ml cryotubes. Cryotubes were then placed in an isopropanol-containing plastic cryocontainer and stored overnight at -80^{o}C. After overnight incubation, they were removed from the cryocontainer for long-term storage at -80^{o}C.

To thaw the cultures, cryotubes were incubated at 43°C until the culture was just beginning to liquefy (approximately 30 seconds to 1 minute of incubation). The cryotubes were then incubated at room temperature until thawing was complete. The thawed chytrid was divided either between two TGhL 1% agar plates or two 25cm^2 Nunclon™ culture flasks containing 10ml of TGhL broth. Agar plates were allowed to dry, then parafilmed and incubated inverted at 18°C. Culture flasks were transferred directly to incubation at 18°C.

2.5 Chytrid zoospore harvesting and quantification for preparation of DNA standards for qPCR and experimental inoculates

To harvest chytrid zoospores, 500µl of liquid culture was transferred to a TGhL 1% agar plate, allowed to dry and then parafilmed and incubated inverted at 18°C. Plates were observed regularly under a light microscope for high levels of zoospore production. When this was observed, the plates were washed with 1ml of TGhL broth and incubated for 10 minutes at room temperature. The TGhL broth was then pipetted off the plate and this process was repeated three times in total. The concentration of live zoospores in the collected supernatant was counted using a Neubauer haemocytometer. Live zoospores were identified by whether they were moving or had a "halo" effect around them, as many zoospores stop moving soon after transfer to the Neubauer slide (personal observation). Once the concentration of zoospores in the original supernatant had been identified, a solution of the desired concentration of zoospores could be obtained by dilution with TGhL broth. DNA quantitation standards of 10,000 genomic equivalents (GE) were prepared by making a zoospore solution of 10,000 zoospores per ml, which was then divided between 2ml Eppendorf tubes that had been prepared with 0.03 to 0.04g of silica homogenisation beads (Thistle Scientific, Glasgow, UK). DNA extraction following the Risk Asessement of Chytridiomycosis to European Biodiversity (*RACE*) protocol (Section 2.6) was then carried out, before dilution of the resultant DNA extract in a 1 in 10 dilution series using filtered water to make quantitation standards.

2.6 *Bd* DNA extraction and pan-lineage qPCR

Two methods were used for DNA extraction from tissue samples, swabs and live chytrid cultures, based either on the protocol developed under the *RACE* project (ERA LEARN, 2008) or an adapted version of Qiagen's DNeasy™ Blood and Tissue Kit (Qiagen, Venlo, Netherlands).

For the *RACE* protocol, tissue samples larger than 1cm^2 were diced with a sterile scalpel blade before being placed into a 1.5ml Safe-Lock Eppendorf tube (Eppendorf, Hamburg, Germany) containing 0.03 to 0.04g of 0.5mm silica homogenisation beads and 60µl of Prepman Ultra (ThermoFisher Scientific, Massachusetts, USA). Tissue samples that were smaller than or approximately equal to 1cm^2 were added to the tube intact. For swab samples, the tips of swabs were snapped off directly into Safe-Lock Eppendorf tubes prepared in the same way as above. For live chytrid samples, 100µl of culture was pipetted into Safe-Lock Eppendorf tubes containing 0.03 to 0.04g of 0.5mm silica homogenisation beads before being spun for 10 minutes at a minimum of 7,000rpm. The supernatant was gently removed by pipetting, taking care not to disturb the pellet before 60µl of Prepman Ultra was added to the tube. All sample types from this point onward were treated the same. Samples were bead-beated for 45 seconds at 30Hz in a Qiagen TissueLyser II (Qiagen, Venlo, Netherlands) before being centrifuged at 14,500rpm for 30 seconds, a process that was repeated twice in total. Samples were then incubated at 100^{o}C for 10 minutes, cooled for two minutes, then centrifuged again at 14,500rpm for three minutes. The supernatant was collected and transferred to a fresh 0.5ml or 1.5ml Safe-Lock Eppendorf tube.

Cultures to be extracted using Qiagen's DNeasy™ Blood and Tissue kit were grown under the conditions described in Section 2.1 in 175cm^2 Nunclon™ culture flasks. Once healthy growth was observed, the base and sides of the flask were aseptically scraped before the entire contents of the flask were poured into a 50ml Falcon centrifuge tube and spun for five minutes at 4,000rpm. The culture medium was gently poured away and discarded, taking care not to disturb the pellet. The pellet was then resuspended by pipetting in 180µl of ATL buffer and transferred to

a Safe-Lock Eppendorf tube containing 0.03 to 0.04g of 0.5mm silica homogenisation beads. Samples were transferred to a Qiagen TissueLyser II and bead-beated for 45 seconds at 30Hz twice, with one minute's rest in between. The samples were then centrifuged at 4,000rpm for two minutes to eliminate bubbles. 20µl of Proteinase K was added before vortexing thoroughly and incubation at 56°C overnight. From this point on, the Qiagen DNeasy Blood and Tissue Kit protocol for purification of Total DNA from Animal Tissues (Spin-Column Protocol) was followed (commencing from step 3). Tissue samples were diced finely before being added to Safe-Lock Eppendorf tubes containing 0.03g to 0.04g of 0.5mm silica homogenisation beads and 180µl ATL buffer, and then treated in the same way as cultures. A one in 10 dilution with filtered water was made from field sample DNA extracts for onward analyses. Sample dilutions that were to be used for onward analyses within seven days were stored at 4°C, otherwise sample dilutions and DNA extracts were stored at -20°C.

Pan-lineage *Bd* detection via qPCR followed the protocol described by Boyle *et al*. (Boyle *et al*., 2004). The reaction volume was a total of 25µl made up of 20µl master mix (12.5µl TaqMan Universal PCR Master Mix (ThermoFisher Scientific, Massachusetts, USA); 1.25µl ITS1 primer; 1.25µl 5.8s primer; 0.0625µl TaqMan MGB probe (ThermoFisher Scientific, Massachusetts, USA); 6.49735µl of filtered water). For field samples, 0.2µl of Bovine Serum Albumin (BSA) was added, with a corresponding quantity of water removed in order to combat PCR inhibition that may be introduced via dirty swabs. Primers all had an initial starting concentration of 10µM, and probe of 100µM. The reaction profile was as follows: two minutes at 50°C followed by 10 minutes at 95°C, then 50 cycles of 15 seconds at 95°C and one minute at 60°C. All qPCR assays were run on either a 7300 Real Time PCR System (Applied Biosystems, Life Technologies, USA) or a QuantStudio 7 Flex System (Applied Biosystems, Life Technologies, USA). All samples were run in duplicate with two no DNA template negative controls and quantified using standard curve analysis with DNA quantitation standards of 100GE, 10GE, 1GE and 0.1GE of isolate IA042.

2.7 Multi Locus Sequence Typing (MLST) DNA Sanger sequencing (for a 50µl PCR reaction)

MLST DNA Sanger sequencing was carried out on PCR products obtained from chytrid cultures using primers ITS4 and ITS1F and is outlined here in four stages.

Stage 1: PCR clean-up reaction

60µl of PEG/2.5 NaCl was added to each PCR reaction well in a 96-well PCR plate, which were then resealed and vortexed, before being spun at 1,000rpm for 10 seconds. Samples were then incubated at room temperature for 30 minutes or 4^{o}C overnight. To pellet the PCR product, the samples were centrifuged at 3,200rpm at 4^{o}C for one hour, following which the supernatant was removed by unsealing the plate and inverting it onto a folded paper towel. The plate and towel were wrapped in clingfilm and placed into the centrifuge bucket, still inverted and spun at 500rpm for 20 seconds. The towel and clingfilm were discarded, and then the plate was inverted again onto a fresh paper towel, wrapped in clingfilm again and centrifuged inverted at 500rpm for a further one minute. The DNA pellet was then washed by adding 150µl of 70% etOH. The plate was sealed and centrifuged at 3,200rpm for 20 minutes. The seal was removed again, and the plate inverted on to fresh paper towel, then immediately moved onto a second fresh paper towel, wrapped in clingfilm and centrifuged inverted at 500rpm for one minute. The above centrifugation steps were repeated once. To dry the DNA pellet, the plate was placed on a thermocycler at 37^{o}C for two minutes without a seal or lid. The pellet was then resuspended in 10µl of nuclease-free water, sealed and vortexed before being centrifuged at 1,000rpm for 10 seconds. The plate was then incubated at 4^{o}C for 10 minutes to allow the pellet to resuspend and then vortexed and centrifuged at 1,000rpm for 10 seconds again to ensure the clean PCR product was at the bottom of the well.

Stage 2: Measuring DNA using a NanoDrop 2000

The standard protocol for measuring DNA content using a NanoDrop 2000 machine (ThermoFisher

Scientific, Massachusetts, USA) was followed. Ideally, the DNA concentration of PCR products should be ≥50µg/ml. In a fresh plate, 2µl of each DNA sample was added to sufficient nuclease-free water to achieve a final concentration of 2 to 6.5ng/ml of DNA.

Stage 3: Setting up the sequencing reaction

Primers were used at 1pM/µl, and the forward and reverse reactions were carried out separately, each in half of a fresh plate. Two master mix reactions were made up on ice (one for the forward reaction and one for the reverse reaction), in sufficient quantity for all samples, multiplied by the number of replicates plus 5%.

Table 2.1. Sanger sequencing reaction master mix.

Reagent	Volume per reaction (µl)
Forward or reverse primer	4
TaqFS (BigDye v1.1)	0.5
Sequencing buffer	1.75
Nuclease-free H_2O	1.75

8µl of master mix (Table 2.1) was added to each well, followed by 2µl of diluted PCR product. The plate was sealed, vortexed and centrifuged at 1,000rpm for 10 seconds. The sealed plate was placed on a thermocycler and incubated at 96°C for 10 seconds, followed by cycling between 50°C for five seconds and 60°C for two minutes, repeated 25 times. The temperature was then lowered by 0.1°C per second until reaching 4°C.

Stage 4: Second PCR clean-up reaction

To each well, 9µl of 3M sodium acetate (pH 5.2 in a 4:11 ratio) was added followed by 51µl of 95% etOH. The plate was then resealed, vortexed and centrifuged at 1,000rpm for 10 seconds, then incubated at -20°C for one hour. After incubation, the plate was centrifuged at 3,500rpm at 4°C for one hour and as soon as centrifugation was complete, the seal was removed and the plate inverted onto fresh paper towel to remove the supernatant. The inverted plate was placed onto fresh paper

towel and spun inverted at 500rpm for one minute to remove any residual ethanol. The DNA pellet was then washed again by adding a further 150µl of 70% etOH, the plate resealed, and centrifuged at 3,500rpm for 30 minutes. Immediately upon completion of centrifugation, the supernatant was again discarded by inverting the plate onto fresh paper towel and spinning the plate inverted on paper towel at 800rpm for one minute to dry the pellet. The plate was then resealed and stored at -20°C for sequencing. The sequencing reaction plates were sent to Imperial College London's Medical Research Council Core Sequencing Centre (MRC CSC) Genomics Core Laboratory for sequencing to be carried out. CodonCode v4.2.5 (CodonCode Corporation, Massachusetts, USA) was used to trim low quality ends and Phred-base calling was used to assign consensus sequences.

2.8 *Bd* lineage identification using WGS

Many of the isolates collected as part of this project were whole genome sequenced as part of a large body of work to describe the global diversity and origin of *Bd* (O'Hanlon *et al*., 2018).

Isolates were grown in 175cm^2 Nunclon™ cell culture flasks for one to two weeks, depending on speed of growth, at 18°C. The DNA was extracted using Qiagen Genomic Tips 20/G (Qiagen, Venlo, Netherlands) and DNeasy™ Blood and Tissue Kits before a Tapestation™ 2200 (Agilent Technologies, California, United States) and Qubit™ fluorimeter (ThermoFisher Scientific, Massachusetts, USA) were used to quantify the DNA present. The DNA was then prepared for sequencing on an Illumina HiSeq™ platform (Illumina, California, USA) which was carried out at the Natural Environmental Research Council (NERC) Biomolecular Analysis Facility (NBAF) GenePool genomics facility at the University of Edinburgh, generating 125 + 125 base pair (bp) paired-end sequencing using Illumina HiSeq™ high output V4 chemistry (Illumina, California USA). Cutadapt v1.10 (Martin, 2011) was used to quality trim and clean the raw sequencing reads of adapter sequences before mapping the sequences to isolate JEL 423, the *Bd* reference genome, using Burrows-Wheeler Aligner (BWA) (v0.7.8) (Li & Durbin, 2010) , producing sequence alignment/map (SAM) files. SAMtools (v1.3.1) (Li *et al*., 2009) was used to prepare the files for

variant searching and variant discovery was carried out using freebayes vdbb6160 (Garrison & Marth, 2012), assuming diploidy. All variant positions across all samples were then merged into a single variant call format (VCF), which was then quality filtered using *bcftools* (Phred quality score of greater than or equal to five where any reads cover that position; the alternate allele(s) are in the called genotypes but without supporting reads; reads supporting alternate alleles are of low quality; quality scaled by depth of supporting reads is less than a threshold; called allele is not present on forward and reverse strands; alleles are only supported by reads entirely left or right of the query variant). The individual filtered VCF files were merged into a single VCF using *vcfstreamsort*. Phylogenetic analyses were carried out in RAxML v8.2.9 (Stamatakis, 2014), using a generalised time reversible (GTRCAT) model bootstrapped 500 times in rapid bootstrapping mode (O'Hanlon *et al*., 2018).

2.9 Ethics Statement

All South African fieldwork sampling was carried out with permission from landowners or, in National Parks (Ezemvelu KZN Wildlife; Department of Economic Development, Tourism and Environmental Affairs for Free State Province), under a collection licence. Sampling methods were approved by the NWU Research Ethics committee (permit no. NWU-00015-16S5). Research and export permits were supplied by the Direction des Eaux et Forêts, Ministry of the Environment, and Madagascar National Parks for fieldwork sampling in Madagascar.

Chapter 3

Lineage-Specific Diagnostic Development for *Bd*

3.1 Abstract

The ability to detect and monitor infectious diseases at an appropriate cost and scale is critical to our ability to manage them. Without this information, it is impossible to gather the baseline data which enables the past and future movements of pathogens to be investigated; research into pathogen ecology is hampered; and it is difficult to detect rapid changes in pathogen distribution or frequency to implement timely control measures. Developing pathogen surveillance tools for monitoring wild populations presents a further challenge as diagnostics must be sensitive enough to detect the pathogen in asymptomatic animals; minimise stress to animals while sampling is being carried out; and be stable under field conditions. It has become increasingly clear that the lineage of *Bd*, a fungal pathogen of amphibians, is an important epidemiological factor determining infection outcome but research into the extent to which this is the case has been hindered by an inability to type lineage quickly and easily. Here, a novel lineage-specific TaqMan qPCR assay is presented to detect the two lineages of *Bd* most commonly associated with chytridiomycosis symptoms in nature (*Bd*GPL and *Bd*CAPE). This assay is applicable for surveillance of natural populations using swab and tissue samples, isolate cultures and museum specimens.

3.2 Introduction

3.2.1 Infectious disease diagnostics for wildlife and pathogen surveillance

The first line of defence against invasive parasites is effective monitoring and surveillance, so that prompt action can be taken to contain EID events and routes and so that mechanisms of transmission can be clarified (Voyles *et al*., 2014; Langwig *et al*., 2015). This, however, requires substantial resources in terms of skills, funds, time and political engagement. Monitoring diseases among wildlife populations is inherently even more difficult and is hindered further by fewer resources, but is crucial to protect global biodiversity and understand the ecology of wild populations (Mömer *et al*., 2002). To date, the global approach for monitoring and surveillance of wildlife pathogen movement remains inadequate for the scale of the task, reflected in the slow response to multiple devastating wildlife epidemics, such as the emergence of chytridiomycosis in amphibians and WNS in bats (Grogan *et al*., 2014; Martel *et al*., 2014; Voyles *et al*., 2014).

Molecular diagnostics for surveillance of pathogens hold several advantages over traditional typing methods such as histological identification or identification via culture and isolation. Many pathogens cannot be cultured under laboratory conditions and, even where culturing is possible, this can be time consuming and may require substantial technical skill (Irinyi *et al*., 2016; Ghosh, Fisher & Bates, 2018). Molecular diagnostics also allow cryptic pathogen diversity to be identified, such as pathogen genotype (which may not be distinguishable by traditional phenotyping) and have been repeatedly demonstrated to be sensitive, fast and quantitative (Girones *et al*., 2010; Byrne *et al.*, 2019).

Ideally, a diagnostic for wildlife pathogens should be designed with a limited resource setting in mind. Funding is often limited for wildlife conservation projects, so it should be cheap enough to be viable and rely on samples that can be collected easily with minimal training while also minimising invasiveness and distress to the animal. Furthermore, as wildlife diagnostics will often be required

in a fieldwork setting, it is best if they require samples that are relatively stable. For example, for shipping and storage they would ideally have minimal refrigeration requirements in case they cannot be processed immediately and would be easy and cheap to transport (Ryser-Degiorgis, 2013).

Additionally, sensitivity (even in animals that are not displaying clinical symptoms) is important as it may not be obvious when an animal is infected. For example, a study testing apparently healthy captive amphibians in the Netherlands for *Bd* found 3% overall prevalence and 13.6% of collections held at least one *Bd*-positive animal (Martel *et al*., 2011). Finally, it is important that the diagnostic functions at a useful taxonomic scale. Many pathogen diagnostics for wildlife and livestock do not distinguish between strains (Ryser-Degiorgis, 2013; Vidic *et al*., 2017) and an inability to understand the phylogenetic diversity and interactions of pathogen genotypes impedes our ability to control the diseases they cause (Cooper, 2001; Metcalf *et al*., 2015).

The OIE has produced a framework on which to base the validation of a wildlife pathogen diagnostic, structured into three stages (Diaz, 2016). Stage 1 assesses whether the diagnostic is actually able to detect the target under laboratory conditions. Stage 2 assesses whether the diagnostic is useful under more realistic conditions and is able to detect the target in infected animals and how it compares to any existing assay. Stage 3 assesses whether the assay is reproducible across different laboratories and testing centres, indicating how robust the diagnostic is.

3.2.2 *Bd* diagnostics to date

There have been three primary ways in which *Bd* has been diagnosed from live amphibians and museum specimens to date: histological examination of toe clips and skin scrapings; examination showing oral disc abnormalities in tadpoles; and PCR or quantitative PCR (qPCR) of skin swabs or tissue samples (Berger, Speare & Kent, 2000; Boyle *et al*., 2004; Weldon & Du Preez, 2006; Hyatt *et al*., 2007; Smith & Weldon, 2007). A pan-lineage qPCR assay for *Bd* targeting the ITS region of

the genome has become the favoured method for diagnosis in most cases (Boyle *et al*., 2004). This is because the assay has been shown to be extremely sensitive, detecting sub-clinical infections, and specific (Kriger *et al*., 2006; Garland, Wood & Skerratt, 2011; Skerratt *et al*., 2011). As the assay works with skin swabs taken from adult amphibians, this method also has the significant advantage of being minimally invasive and sampling can be carried out with minimal training (Boyle *et al*., 2004; Hyatt *et al*., 2007). Skin swabs are light, easy to carry in difficult field settings and easy to store – although refrigeration is ideal, they can be stored at temperatures up to 23^{o}C if necessary (Hyatt *et al*., 2007; Van Sluys *et al*., 2008)

As with the Boyle *et al.*'s pan-lineage qPCR assay, most molecular based diagnostics of *Bd* have generally relied on the ITS region of the nuclear genome. The ITS region is made up of three rRNA genes: the 18S, the 5.8S and the 28S. These three genes are split following transcription, excising two internal transcribed spacers and the 5.8S gene. The two internal transcribed spacers and the 5.8S gene make up the ITS region (Schoch *et al*., 2012). The ITS region is the fungal universal DNA barcode. The region accumulates diversity due to the excision of the two internal transcribed spacers relaxing selection pressure within the spacers and it is well characterised in databases with over 170,000 sequences on Genbank. It is used as a standalone for fungal identification in 35% of studies and is generally accepted as being reliable at resolving phylogenies at the species level (Nilsson *et al*., 2008; Schoch *et al*., 2012; Badotti *et al*., 2018).

However, this region is not without its problems. The Chytridiomycota are an early diverging branch of fungi, even considered by some to be a transitional group between the protists and the fungi (James *et al*., 2006b, 2006a). The ITS region has lower powers of discrimination when applied to basal branches of the fungal kingdom such as the Chytridiomycota, an issue acknowledged when it was initially proposed as the universal fungal DNA barcode (Schoch *et al*., 2012). In multiple fungal phyla, the ITS region does not contain sufficient levels of variation to delineate sub species and lineages (Nilsson *et al*., 2008), which is pertinent in the case of *Bd* where lineage is known to

be epidemiologically relevant (Farrer *et al.*, 2011; Becker *et al*., 2017; O'Hanlon *et al*., 2018).

Bd isolates and lineages are hugely variable in the ploidy and content of their nuclear DNA. For example, JEL423, a *Bd*GPL isolate collected in Panama, contains double the DNA of the *Bd*ASIA-2/Brazil isolates JEL468 and UM142 (Schloegel *et al*., 2012), and this is reflected in how variable ITS copy number can be between different *Bd* isolates (Longo *et al*., 2013). ITS copy number variation confounds quantification attempts and although this problem can be somewhat mitigated by the inclusion of DNA standards, it would be unwise to view the quantification of the *Bd* pan-lineage qPCR as an absolute measure for field samples. Rather, it should be considered a semi-quantitative indication of the relative infection burden (Clare, 2014). However, many surveillance studies report extremely precise infection burdens based on the pan-lineage qPCR (Longo, Burrowes & Joglar, 2010; Kielgast *et al*., 2010; Kolby, 2014; An & Waldman, 2016). Finally, as well as being of variable copy number, the ITS region can be very diverse within individual strains of *Bd*. A single *Bd* isolate may harbour up to 22 identifiable ITS alleles within the array, rendering any sub-specific phylogenetic analysis based on this region impossible (Schloegel *et al*., 2012).

The inability of the currently widely used ITS region-based qPCR assay for *Bd* to indicate the *Bd* lineage present is a major drawback in its utility. Although the reasons are not clear why some amphibian populations suffer catastrophic declines following *Bd* introduction and some are able to persist, sometimes even asymptomatically, the lineage of *Bd* present has been shown to be an important epidemiological factor (Farrer *et al*., 2011; Becker *et al*., 2017; Greenspan *et al.*, 2018; O'Hanlon *et al*., 2018). All known chytrid-driven mass amphibian population declines to date, have occurred following infection with *Bd*GPL and *Bd*GPL shows elevated virulence with respect to the other lineages in a laboratory setting (Farrer *et al*., 2011; O'Hanlon *et al*., 2018). Many important epidemiological factors may contribute to the infection outcome in a population, but an inability to identify lineage quickly, easily and cheaply from routine *Bd* surveillance samples has hindered

progress in understanding how *Bd* lineages are distributed and therefore understanding their ecology and epidemiological importance.

To date, the most reliable way of identifying chytrid lineage has been to carry out WGS. Although comparative genomic analyses have been extremely valuable in the chytrid research field, this approach is impractical on the large scale required for pathogen surveillance. Firstly, it requires a pure culture of the *Bd* isolate, which is difficult and time-consuming to obtain; it requires a skin biopsy to be taken which is particularly undesirable when working with threatened species and requires access to sterile lab conditions. Secondly, although costs are decreasing rapidly, it is expensive at approximately £200 per isolate (based on 2016 costs to ICL). Finally, it requires substantial technical knowledge, training, bioinformatics skills and time to carry out the necessary analyses. An alternative approach taken in many studies has been to carry out MLST either on the ITS region (which is unreliable) or on other regions of the genome. This method can be carried out from a swab (Byrne *et al*., 2017) and can, if using appropriate polymorphisms, accurately recover the *Bd* phylogeny as determined by WGS typing. A substantial drawback, however, is that it requires reasonably high infection burdens and so is not a suitable approach for all *Bd*-positive regions (Byrne *et al*., 2017) and is also very labour intensive as it requires manual PCR and sequencing of all MLST loci independently.

An alternative candidate region for *Bd* lineage diagnostics is the mitochondrial DNA (mtDNA). The *Bd* mitochondrial genome is 178,280kb long and has recently been fully sequenced (O'Hanlon *et al*., 2018). This molecule is present at high copy number and thus is both amenable to PCR techniques and is likely to be detectable at low infection burdens. Additionally, the rate of recombination relative to the nuclear genome is low, making it a stable region on which to base a molecular diagnostic. Phylogenetic analyses based on *Bd* mtDNA recover the same lineage groups as those recovered from whole genome sequence analysis (O'Hanlon *et al*., 2018) and there is a precedent for using mtDNA for phylogenetic studies across a wide range of taxa (Barr *et al.*, 2011;

Li *et al*., 2016; Schreeg *et al*., 2016; Song *et al*., 2016; Penry *et al*., 2018). Before the ITS region was widely adopted as the universal fungal DNA barcode, the cytochrome c oxidase subunit 1 (CO1) region of the mitochondrial genome was used by the Consortium for the Barcode of Life as the default DNA barcode for all relevant organisms, including fungi that had mitochondria (Schoch *et al*., 2012).

3.2.4 Taqman MGB probes and qPCR

qPCR is a highly sensitive and specific method of molecular identification. The fluorescent probe, following attachment of a primer pair, binds to a specific diagnostic region of DNA and on binding emits a fluorescent reporter dye. The level of fluorescence increases in proportion to the quantity of target DNA in a sample. Samples with a higher starting quantity of target DNA will therefore reach a detectable level of fluorescence, the threshold cycle (Ct), faster than samples with a lower starting quantity of DNA. TaqMan MGB probes are ideal for work on closely related organisms as they have further enhanced specificity due to the inclusion of the MGB at the 3' end of the probe. The MGB makes the probe-DNA complex more stable than regular qPCR probes, making it possible to design much shorter probes, which are more sensitive. The more stable complex and shorter probes also allow the annealing temperature to be raised, which further increases specificity (Kutyavin *et al*., 2000).

In this chapter, novel mtDNA-based diagnostics are presented to identify *Bd*CAPE and *Bd*GPL, the only two *Bd* lineages shown to have been associated with amphibian population declines in the wild (O'Hanlon *et al.*, 2018, Doddington *et al*., 2013), using TaqMan MGB probes in a qPCR assay. Samples may be from adult amphibian skin swabs, pure chytrid culture or tissue samples from adult or larval amphibians. The assay utilises the same technology as the ITS region-based pan-lineage *Bd* diagnostic and thus can be easily incorporated into the repertoire of any diagnostic laboratory already working with *Bd* surveillance samples. Finally, this novel diagnostic was used to identify the lineage of *Bd* infecting known *Bd*-positive animals from museum archives, which was then

confirmed with shotgun sequencing. Retrospective surveys of *Bd* lineages in museum archives is proposed as a particular utility of this very sensitive tool.

3.2.5 Southern Africa as a testing system

Southern Africa is one of only two regions in the world known to harbour more than one *Bd* lineage over a large area and apparently over the long term (Jenkinson *et al*., 2016; O'Hanlon *et al*., 2018), the other being the Brazilian Atlantic Forest where *Bd*ASIA-2/BRAZIL and *Bd*GPL can be found. Both *Bd*GPL and *Bd*CAPE were isolated from South Africa between 2008 and 2015 and were lineage-typed by WGS, but further baseline data on lineage distributions in the country are severely lacking due to an inability to diagnose quickly and economically the type of *Bd* present in a sample from amphibian skin swabs (O'Hanlon *et al*., 2018). South Africa is also home to over 100 species of amphibian (Measey, 2011), several of which are known to build up substantial *Bd* infection burdens (such as *Hadramophryne natalensis*, the Natal ghost frog) (Weldon & Du Preez, 2006). This makes it an ideal country to test the utility of this novel lineage-specific diagnostic in the field.

Although there have been few reports of chytridiomycosis in the region, there has been one putative chytridiomycosis-driven extirpation in nearby Tanzania, of the Kihansi spray toad (*Nectophrynoides asperginis*) (Weldon *et al*., 2019). Histological examination of toads collected around the time of the population crash show the arrival of *Bd* immediately prior to the population crash. *N. asperginis* was subsequently declared extinct in the wild in 2005 (IUCN SSC Amphibian Specialist Group, 2015). However, the lineage of *Bd* associated with this outbreak has never been identified.

The ability to type lineage from museum specimens, such as the Kihansi spray toads that were preserved for histological examination, would enable the generation of hugely valuable baseline data on a global scale. Currently, this potential data source is largely untapped due to the difficulty and expense involved in obtaining sufficient quantity of high-quality DNA for sequencing. Although the point-of-origin of *Bd* as a whole has now been identified as South East Asia (O'Hanlon *et al.*, 2018), the origin and patterns of expansion around the world have yet to be resolved. qPCR's highly

sensitive nature means the quantity and quality of DNA required compared with that for WGS is greatly reduced, making the method an excellent candidate tool for unlocking the information hidden in museum archives around the world, and for the generation of more detailed baseline data from which to gauge past and future *Bd* movement.

3.3 Methods

3.3.1 Assay design and optimization

A 175,295kb long mtDNA sequence alignment (O'Hanlon *et al*., 2018) for 145 isolates of *Bd* representing all known lineages at the time of testing (*Bd*CH, *Bd*CAPE, *Bd*ASIA-1, *Bd*ASIA-2/BRAZIL, *Bd*GPL) was manually screened for lineage-specific single nucleotide polymorphisms (SNPs). SNPs that consistently appeared in one lineage and that were not shared with any of the other lineages were considered candidate diagnostic SNPs. 200bp sequences surrounding candidate SNPs were imported into Primer Express Software v3.0 (ThermoFisher Scientific, Massachusetts, USA) for probe and primer design. Probes were then designed around candidate SNPs following Applied Biosystem guidelines in the Primer Express v3.0 user manual (Applied Biosystems, 2004). Following identification of an acceptable probe, the software was directed to search for suitable primer pairs. A candidate primer pair that contained an additional lineage-specific SNP or, where this was not possible, the primer pair with the lowest penalty score was selected and ordered along with the corresponding TaqMan MGB probe (ThermoFisher Scientific, Massachusetts, USA).

Primer concentrations were optimized in singleplex using a checkerboard system, and DNA quantitation standards for the DNA template. The same process was carried out to assess optimal probe concentration. For each combination of primer or probe combinations, the R^2 values and reaction efficiency for each test was calculated by plotting the log DNA concentration against the mean Ct value (from quantitation standards run in duplicate) and fitting a regression line. The reaction efficiency could then be calculated from the slope (m) of the regression line using the following equation:

$$\% \text{ Efficiency} = (10^{-1/m}-1) \times 100$$

100% efficiency would mean that the reaction is doubling the quantity of product with every PCR cycle. The R^2 value is the coefficient of correlation for the standard curve and is a measure

of how reliable the assay quantification is across a plate. A high R^2 value would indicate that the quantity of template in unknown samples can be reliably predicted from the DNA standards. Final primer and probe concentrations were selected based on a combination of maximizing reaction efficiencies compared to oligonucleotide concentration, while ensuring that the mean Ct values for a given quantity of DNA remained as similar as possible between the assays, and high R^2 values were produced. Consistency between the assays meant that it was possible to multiplex the two diagnostics to make a single assay diagnosing both *Bd*GPL and *Bd*CAPE. All optimization and validation reactions were carried out on both a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and a QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

The cycling conditions were based on the protocol for the pan-lineage *Bd* qPCR diagnostic (Boyle *et al*., 2004), but the number of cycles was reduced and the annealing temperature was raised in order to increase specificity. The final reaction profile was two minutes at 50°C followed by 10 minutes at 95°C, then 15 seconds at 95°C and one minute at 62°C, cycled 40 times. Following Boyle's protocol, the Ct threshold was set at $\Delta Rn = 0.1$ for all reactions, and all reactions were 25µl total volume (20µl master mix and 5µl DNA template). The optimised multiplexed reaction mix for a single 25µl reaction was as follows: 12.5µl TaqMan Fast Advanced MasterMix (ThermoFisher Scientific, Massachusetts, USA); 0.125µl each of *Bd*GPL-specific forward and reverse primers; 0.225µl each of *Bd*CAPE-specific forward and reverse primers; 0.03125µl of *Bd*GPL-specific TaqMan MGB probe; 0.09375µl of *Bd*CAPE-specific TaqMan MGB probe; 0.2µl BSA; 6.475µl filtered water. The starting concentrations of all primers and probes was 100µM. When the assays were run un-multiplexed, the same quantities of all reagents were used except for an increase in the volume of filtered water commensurate with the quantity of primer and probe removed. Unless otherwise stated, all reactions were run in duplicate and two negative controls, where the DNA template was replaced with filtered water, were included on every plate. The starting DNA present in a reaction was quantified via standard curve analysis using DNA standards made from isolate

IA042 (*Bd*GPL) and isolate TF5a1 (*Bd*CAPE). Further details of the isolates used to make the DNA quantitation standards are supplied in Appendix 2. Both isolates were previously lineage-typed by WGS as part of a larger project to investigate global diversity of *Bd* using the protocols described in the General Methods (Section 2.8) (O'Hanlon *et al*., 2018), and are well established with vigorous

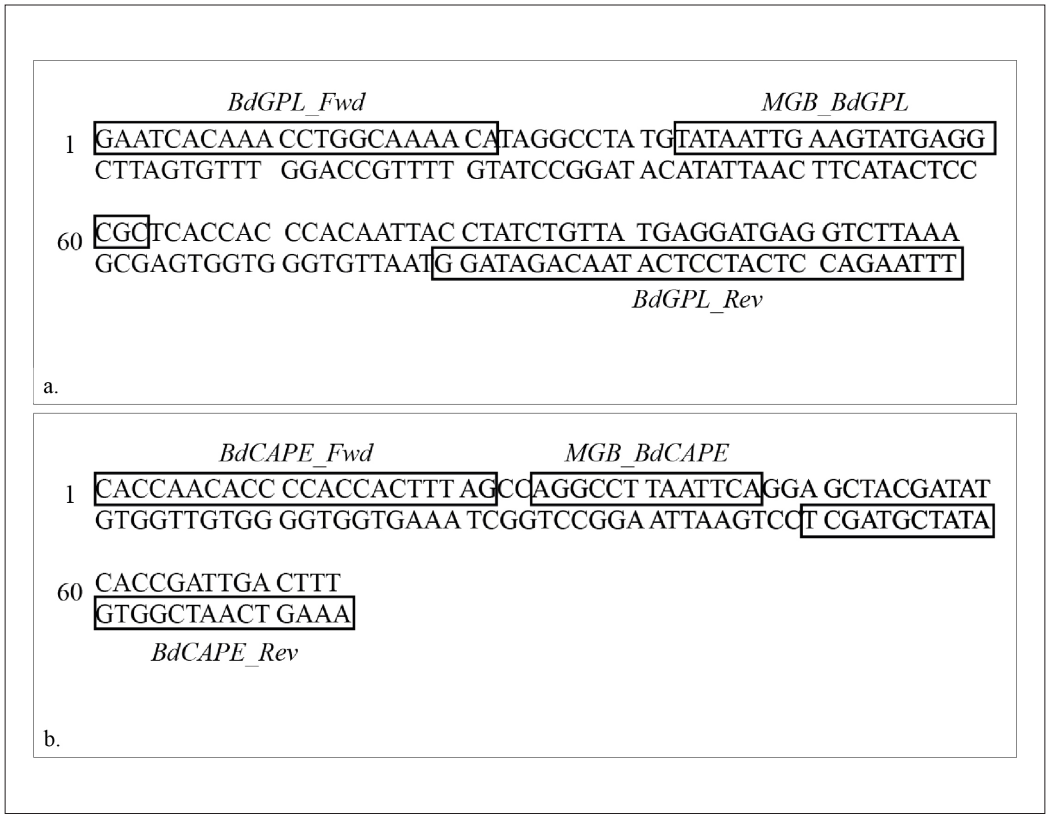


Figure 3.1. Primer and TaqMan MGB probe sequences for lineage-specific qPCR showing (a) *Bd*GPL-specific qPCR assay and (b) *Bd*CAPE-specific qPCR assay.

growth displayed *in vitro*, making them amenable to zoospore harvesting. The final primer and TaqMan MGB probe sequences are shown in Figure 3.1.

3.3.2 Sample preparation

Live cultures, field swabs and tissue samples were prepared, and DNA extracted as described in the General Methods (Section 2.6). Museum specimens from the Natural History Museum,

London were sampled by taking skin scrapings using an Oral-B interdental toothbrush (Soto-Azat *et al.*, 2009). The toothbrush was used to scrape the inner thighs, back feet and ventral side of the amphibian, with particular focus on the pelvic patch. The interdental toothbrushes were then processed in the same way as swabs.

3.3.3 OIE wildlife diagnostic validation structure

The flow chart in Figure 3.2 shows the structure used to guide validation of the lineage specific qPCR assay described here, and the tests selected for each stage of validation.

Stage 1: Assay characteristics

A broad-range specificity panel was assembled by carrying out DNA extractions using the *RACE* protocol (Section 2.6) on 54 cultures held by Prof. Matthew Fisher at Imperial College, London. All isolates had previously been lineage typed using WGS as described in general methods (Section 2.8) (O'Hanlon *et al*., 2018). Isolates were selected which represented all five of the known lineages of *Bd* at the time of testing (40 *Bd*GPL, six *Bd*CAPE, four *Bd*ASIA-1, three *Bd*ASIA-2/BRAZIL and one *Bd*CH) and from 17 countries as well as the global amphibian trade (Figure 3.3)(Appendix 3). The bias towards *Bd*GPL isolates reflects the over-representation of this lineage in isolate collections due to its high global prevalence. DNA extracts for isolates UM142 and CLFT-065 were provided by Prof. Tim James, University of Michigan. DNA from all 54 isolates was put through qPCR using the new assays to check for cross-reactivity in singleplex. All assays after this stage were run in multiplex. Analytical sensitivity was tested by creating an extended 10-fold dilution series of each set of DNA quantitation standards in duplicate to assess at what point quantification was no longer reliable for each assay individually. Once a recommended standard curve range had been identified, repeatability of the assay within and between plates was assessed by running the multiplexed assay in duplicate on three plates to compare reaction efficiency, R^2 values and Ct values across and within plates.

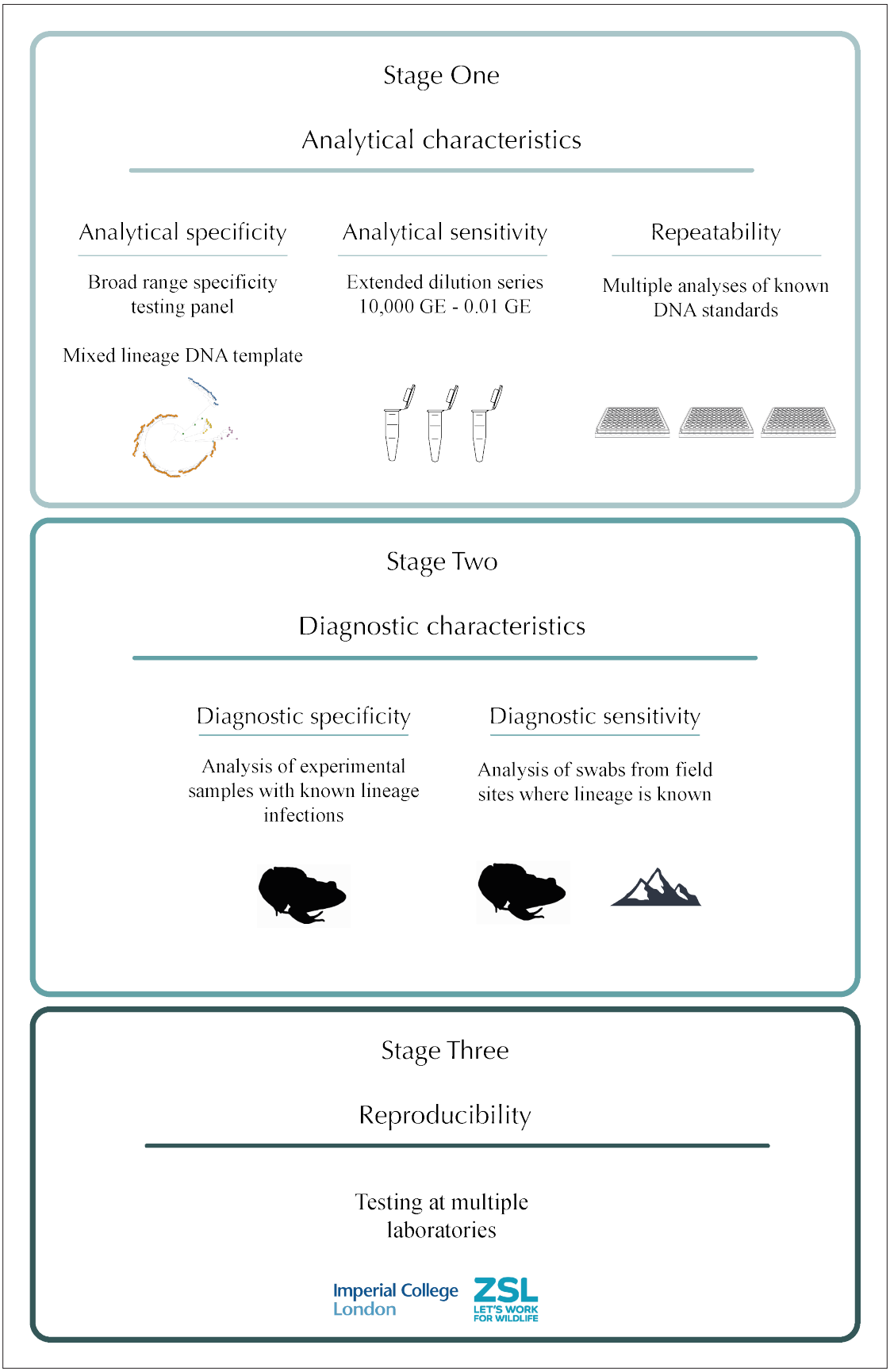


Figure 3.2. Workflow for OIE-based wildlife diagnostic validation structure.

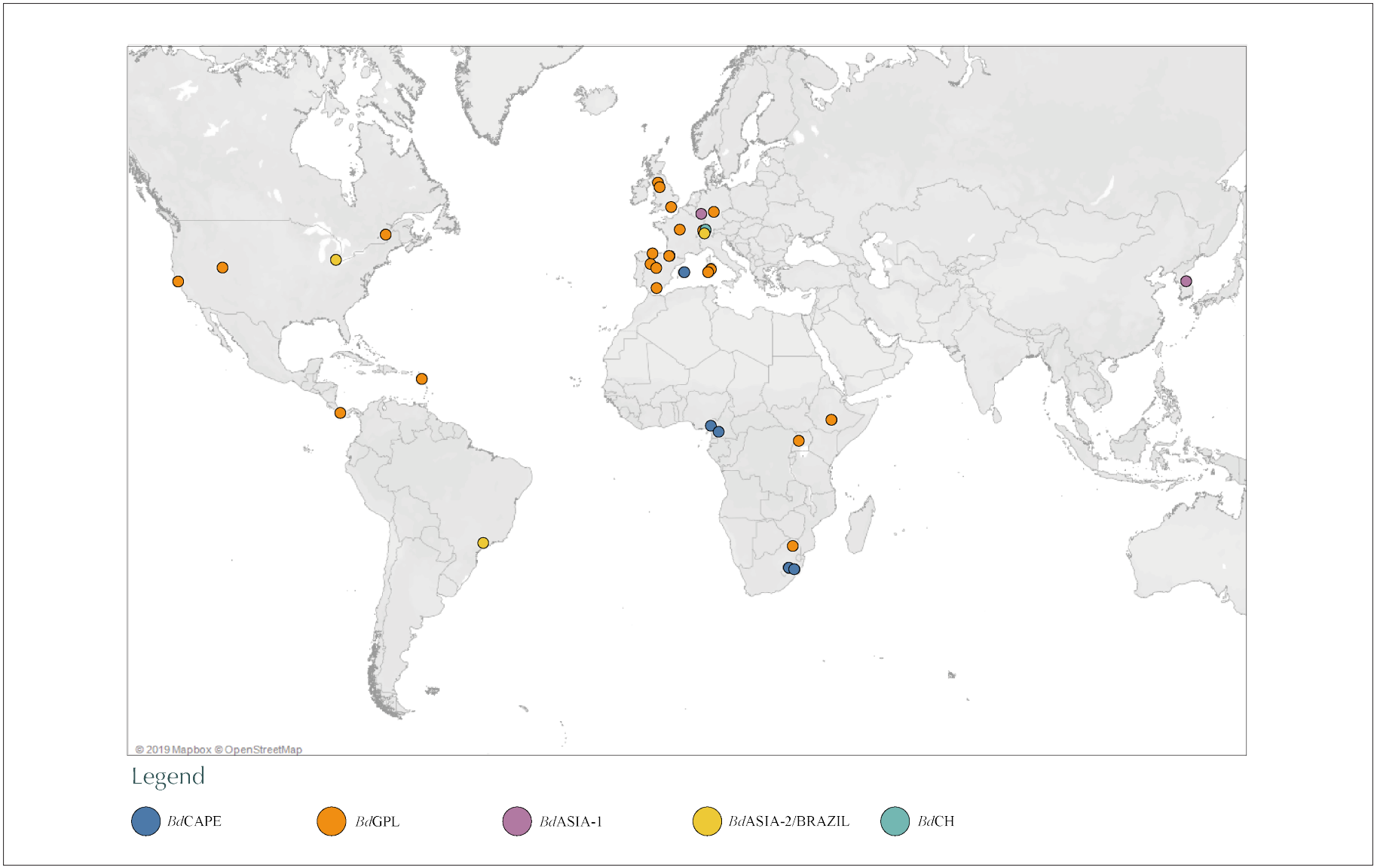


Figure 3.3. Map showing source locations of *Bd* isolates used for qPCR assay specificity testing. Map generated in Tableau.

Stage 2: Diagnostic characteristics

Diagnostic specificity and sensitivity were tested by an *in vivo* infection experiment carried out by collaborators at NWU. In the interests of applying the 3Rs (**R**eplace use of animals were possible, **R**educe the number of animals used for experimentation and **R**efine methods to minimise animal suffering) to animal research, the assay was tested on an experiment planned for another research project but which fit with the aims of investigating the performance of the assay on experimental animals. The primary purpose of the experiment was to assess rates of transmission between animals exposed to *Bd* and naïve animals, or animals previously exposed to a different lineage. African common toad egg strings (*Sclerophrys guttaralis*) were collected from the wild (approximately 50% of eggs per clutch harvested) and raised in a group as metamorphs in a sterile environment at NWU. Upon metamorphosis, the animals were individually housed in 500ml clear plastic boxes with damp tissue paper substrate and a sterilised PVC half pipe as a cover object. Throughout the experiment, toads were fed seven pinhead crickets every day and sprayed daily with aged filtered water to maintain humidity. The paper substrate was replaced and any debris removed every four days and the boxes were rotated daily. Gloves were changed between handling animals from different treatments and all laboratory surfaces were disinfected with 70% EtOH between husbandry and processing of different treatments. These husbandry procedures were kept constant throughout the experiment.

For the purposes of this work, the treatments can be grouped into four: control animals; animals exposed only to *Bd*CAPE; animals exposed only to *Bd*GPL; and animals exposed to both *Bd*CAPE and *Bd*GPL. Animals were randomly assigned into one of six treatment groups (17 to 20 animals) or a control group (36 animals) (Table 3.1). The variation in subject number in the treatment groups was due to unrelated deaths occurring prior to dosing in the metamorph population.

Table 3.1. Treatment groups for *in vivo* lineage exposure experiment.

Treatment group	Initial exposure	Paired treatment	Lineages exposed to (infection + housing)
Control	Sham	None	None
N1	Sham	C1	*Bd*CAPE
C1	*Bd*CAPE	N1	*Bd*CAPE
N2	Sham	G2	*Bd*GPL
G2	*Bd*GPL	N2	*Bd*GPL
C3	*Bd*CAPE	G3	*Bd*CAPE + *Bd*GPL
G3	*Bd*GPL	C3	*Bd*CAPE + *Bd*GPL

Animals were exposed by immersion for three hours in an individual petri dish containing 20ml of aged borehole water. On the day of dosing, zoospores were harvested directly from culture flasks by pipetting from the body of the culture medium. The number of live zoospores were counted using a haemocytometer (Section 2.5) and the final dose of both isolates was obtained by diluting the number of zoospores in the more highly concentrated isolate down to the same quantity as that of the less concentrated isolate, using TGhL broth so that all animals received the same quantity of zoospores on the dose day in question. On each dose day, all individuals received doses of between 110,000 to 145,000 zoospores per ml by pipetting 500µl of the relevant lineage of *Bd* directly onto the animal in the petri dish. Animals in the control group received a sham dose containing only TGhL broth. Four doses were carried out over five days (animals dosed on days one and two, followed by a rest day on day three, and then two more doses on days four and five). After all doses had been carried out, the animals were left undisturbed for two days, following which they were housed in a $500ml^3$ plastic tub, identical to before, with an individual from a paired treatment for 14 more days. At this point the experiment was ended and all subjects were euthanised by immersion in MS222. Any animals that displayed symptoms of chytridiomycosis (loss of righting reflex, inability to feed) were euthanised before the experimental endpoint and the bodies were preserved individually in 70% etOH in 2ml Eppendorf tubes for further analysis. At the experimental endpoint, all toads were individually placed in 2ml Eppendorf tubes containing 70% etOH following euthanasia for preservation before being transported to the Institute of Zoology,

London (IoZ) for further analysis.

At the IoZ, the left hind limb from each toad was removed using a sterile scalpel blade and DNA was extracted using the *RACE* protocol for tissue samples (Section 2.6). DNA extracts were diluted one in 10 and then all treatment and control samples underwent lineage-specific qPCR in multiplex. Four DNA quantitation standards of 1,000GE to 1GE were used for quantification and all samples and quantitation standards were carried out in duplicate with two negative controls per plate. Reactions were carried out on an Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies) and samples that produced amplification equating to more than 1GE, after accounting for dilution, in duplicate were considered positive for the lineage indicated.

The diagnostic specificity and sensitivity are much harder to assess in a field setting. To address this, the results from amphibian adult skin swabs and tissue biopsies tested with lineage-specific qPCR from South African field sites were compared with the lineage of isolates collected from the same sites, as assigned by WGS (O'Hanlon *et al*., 2018) (Section 2.8). A recombinant isolate collected from South Africa (SA-EC5, putative F1 recombinant between *Bd*CAPE and *Bd*GPL) was also tested with the multiplexed assay to see if recombinants could be indicated by the new diagnostic.

Stage 3: Reproducibility

Finally, the reproducibility of the assay between laboratories was tested by sending reagents and quantitation standards to another laboratory (the IoZ) where plates identical to the repeatability tests from Stage 1 were run, three times on an Applied Biosystems StepOnePlus™ RealTime PCR System (Applied Biosystems, Life Technologies). Reagents, DNA quantitation standards and a protocol were provided to the testing laboratory which routinely carries out pan-lineage *Bd* diagnostics, but where the investigator had not previously carried out this lineage-specific assay.

3.3.4 Archive specimen testing

Museum specimens from the Natural History Museum, London and preserved Kihansi spray toads collected and stored by Prof. Ché Weldon, NWU were sampled to assess the applicability of the lineage-specific qPCR to archival specimens. From the Natural History Museum, London, five *Xenopus spp*. specimens from Africa (two from Uganda, two from South Africa and one from Cameroon) were sampled that had previously been tested for *Bd* and identified as positive, but no lineage had been typed (Soto-Azat *et al*., 2010). From Tanzania, four *N. asperginis* (Kihansi spray toads), collected in 2003 were sampled. Accession numbers for all archive specimens tested are provided in Appendix 4.

The Natural History Museum, London specimens underwent skin scraping with an Oral-B interdental toothbrush and DNA extraction following the *RACE* protocol (Section 2.6). One foot from each Kihansi spray toad was removed for analysis and DNA extracted using Qiagen DNEasy Blood and Tissue Kit (Section 2.6), with a change to the two-step final wash at the end of the protocol. The supernatant from each of the two washes was collected in separate 1.5ml Eppendorf Safe-Lock tubes, resulting in two DNA extracts per foot. Samples from the Natural History Museum, London were initially tested using Boyle *et al*.'s pan-lineage *Bd* diagnostic in duplicate. Samples that showed amplification were then tested a second time using the pan-lineage *Bd* diagnostic in triplicate to confirm the result, before any samples which were positive in triplicate (GE > 1) were tested with the novel lineage-specific qPCR assay in multiplex. It was not possible to obtain tissue samples from the Natural History Museum samples, so no further analysis on these specimens could be carried out.

Due to a need to conserve DNA available for shotgun sequencing, Kihansi spray toad DNA extracts were not tested with the pan-lineage *Bd* diagnostic but moved straight onto the lineage-specific qPCR assay. As two extractions were available per animal, and each extraction was tested in duplicate, each animal was effectively replicated in quadruplicate. The samples that showed the

highest number of *Bd* GEs (AC040803_A and AC290703_A2) were then shotgun-sequenced to confirm the lineage diagnosis.

3.3.5 Shotgun sequencing and analysis methods

The Kihansi spray toad DNA extracts underwent library preparation at the University of Copenhagen (Carøe *et al*., 2018). Libraries were built using the Nebnext Ultra II FS library kit (Illumina, California, USA), following the Chapter 1 protocol (protocol for use with inputs ≤ 100ng), with a fragmentation time of 10 minutes to obtain an average target length of 300bp to 700bp fragments. Adaptor-ligated libraries were purified with 1x SPRI beads (SPRIselect® Reagent Kit, Beckman Coulter, Inc.). qPCR was carried out to estimate amplification cycle numbers for the indexing PCR with primers randomly chosen from the BEST protocol list of index primers (Caroe, 2018, unpubl.). The 10µl qPCR reaction mix was as follows: 5µl Q5 Polymerase mastermix; 0.5µl SYBR GREEN/ROX solution; 0.25µl each of forward and reverse primer (both at a 10µM starting concentration); 3µl filtered water; 1µl of DNA template, diluted 10 times. The qPCR was run on an Agilent MX3005 instrument with a reaction profile of 90^{o}C for 30 seconds, followed by 40 cycles of 98^{o}C for 10 minutes, 62^{o}C for 30 seconds and 65^{o}C for 45 seconds. Primer sequences are provided in Appendix 5.

The Index PCR reactions were set up as 50µl reaction mixes using 10µl of DNA template (25µl Q5 polymerase MasterMix; 1µl each of forward and reverse index primers (both at a 10µM starting concentration); 13µl filtered water), and 10 cycles in the reaction profile. One blank reaction was also run. Reactions were carried out on a 2720 thermocycler and purified with 1x SPRI beads. The purified libraries were analysed on an Agilent 2100 Bioanalyzer in the 150 to 1,000bp range, following which the two samples were pooled giving a total concentration of 2nM. The pooled samples were sequenced at the Danish National High Throughput Sequencing Centre in Copenhagen, Denmark. Sequencing was done using a HiSeq 4000 instrument in single read mode for 80 cycles.

DNA sequences generated from the Kihansi spray toad specimens were trimmed to remove adapter sequences and low-quality ends (Phred score <20) using cutadapt v1.9.1. Following this, any reads that were less than 25bp were discarded. BWA v0.7.8 was used to align the trimmed reads to the mitochondrial assembly of the *Bd* isolate JEL423 (the *Bd* reference genome isolate). Of the two Kihansi spray toad samples, AC040803A was found to have the greatest coverage of the mtDNA (85% coverage at a depth of two reads; 61% coverage at a depth of five reads), and therefore only this sample was taken onwards for downstream analyses. PCR duplicates were marked using MarkDuplicates from the GATK toolkit v3.5, following which variant detection was performed using HaplotypeCaller (GATK) with ploidy set to one. SelectVariants (GATK) was used to generate an SNP-only VCF file, and bcftools v1.3.1 was then used to filter for high-quality variants (GQ > 20; Depth > 5, QUAL > 5). The Kihansi spray toad *Bd* sequence was then merged with previously generated VCF files of *Bd* mtDNA using CombineVariants (GATK). Phylogenetic analysis was performed in RAxML v8.2.9, using a generalised time reversible model (GTRCAT) and bootstrapped 500 times in rapid bootstrapping mode. The phylogeny displaying the most reliable topology was imported into RStudio (v1.2.1335) (Rstudio Team, 2015) and visualised using ggtree (v1.16.6) (Yu *et al*., 2017).

3.4 Results

3.4.1 Stage 1: Analytical characteristics

Both diagnostic assays were able to identify all isolates assigned to the relevant lineage by WGS. The *Bd*GPL assay also detected one isolate from the *Bd*ASIA-1 lineage, UM142, albeit at a low level for an extract taken from pure culture (mean Ct = 31.762, mean GE = 39.06). Additionally, the amplification plot for UM142 showed that the threshold was not crossed during the exponential phase as would be expected in a true positive result, as can be seen when compared with true amplification of DNA quantitation standards (Figure 3.4). Crucially, all isolates originating in Africa, the study region for this research, were correctly typed by both lineage specific assays.

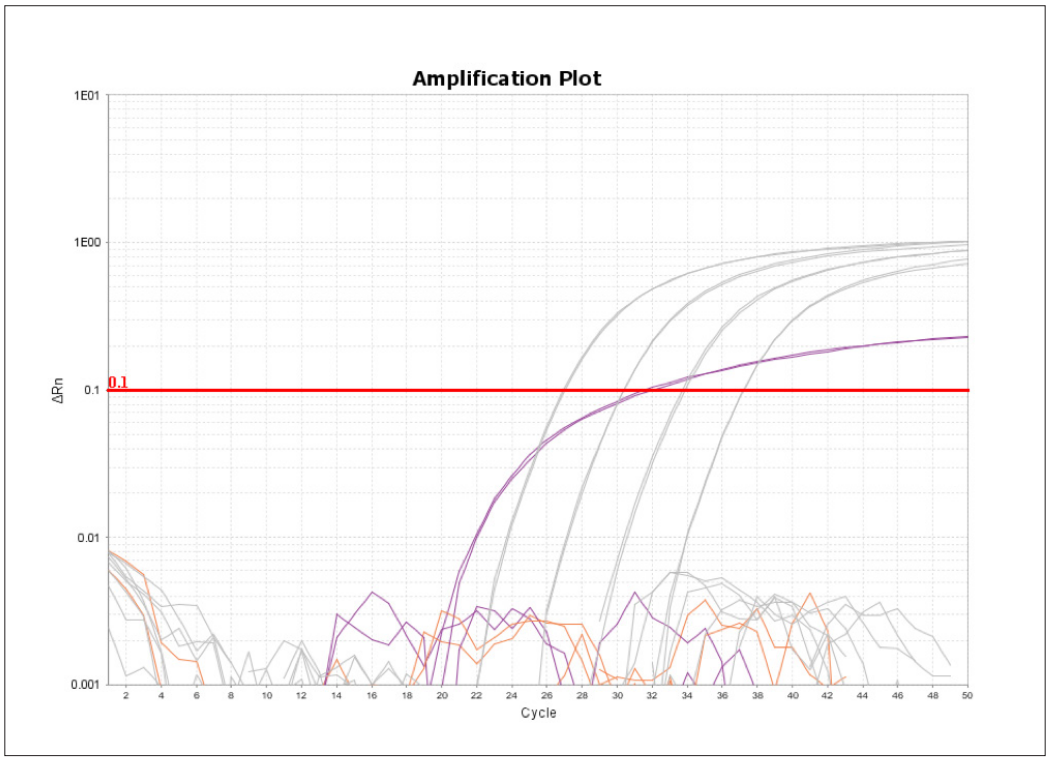


Figure 3.4. qPCR trace for isolate UM142 (purple) with *Bd*GPL-specific assay and showing *Bd*GPL DNA quantitation standards from 1,000GE to 1GE (grey).

Both lineage-specific assays had a limit of detection of 1GE (Figures 3.5a and b). It can be seen from the *Bd*CAPE-specific assay plot that the 10,000GE quantitation standard fluorescent plot

plateaus earlier than would be expected given the plots of the other quantitation standards. When there is too much target DNA present in a sample, it can actually inhibit the PCR reaction, as reagents are used up at such a fast rate that the reaction begins to slow prematurely, which is likely what is happening in this case. Based on these results, the recommended quantitation standard range for both assays is 1,000GE, 100GE, 10GE, and 1GE.

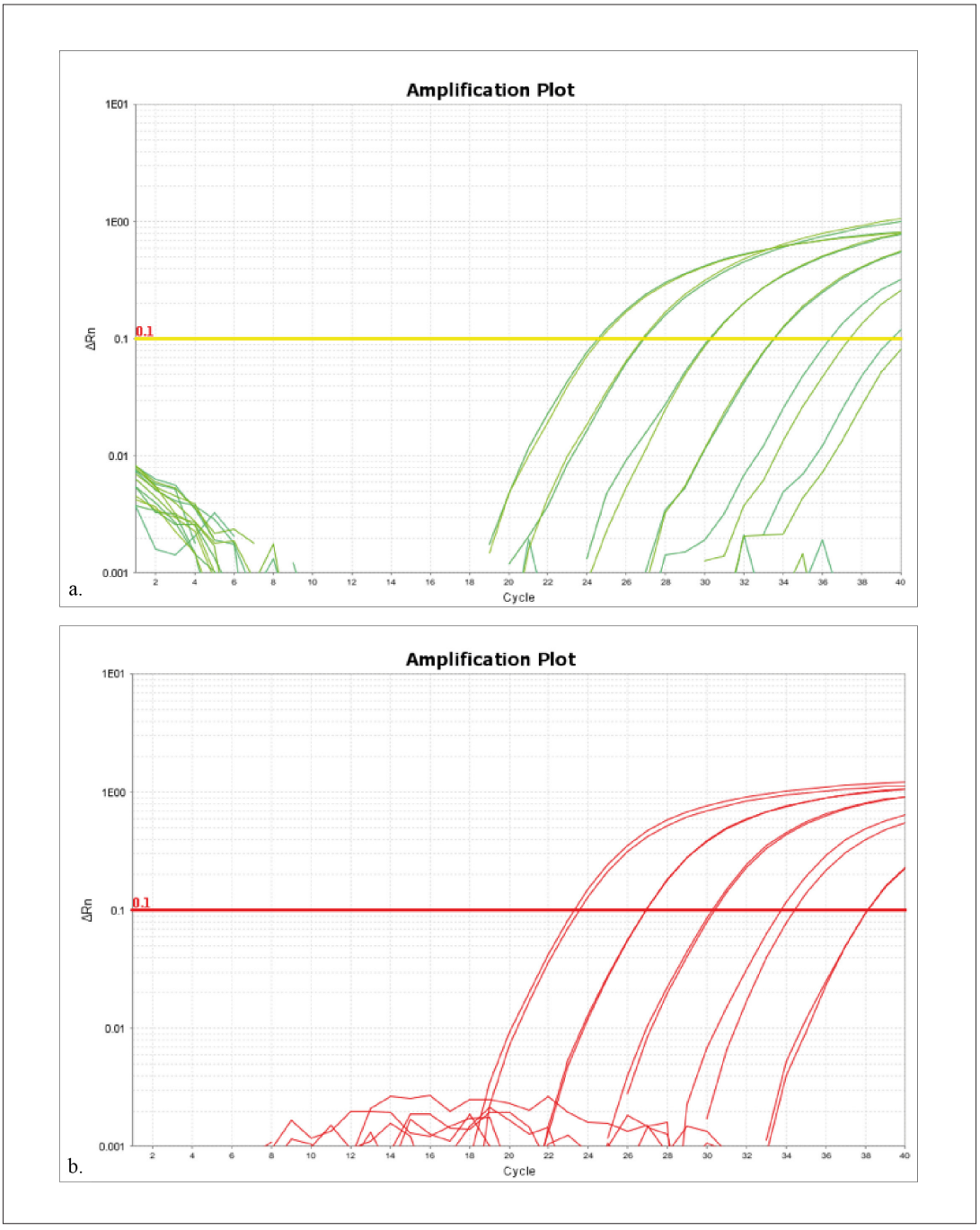


Figure 3.5. qPCR traces of extended dilution series with (a) *Bd*CAPE-specific assay and (b) *Bd*GPL-specific assay. Dilution series consists of 10,000GE, 1,000GE, 100GE, 10GE, 1GE and 0.1GE.

Repeatability testing showed both assays performed well over three separate plates. The overall mean percentage efficiency for the *Bd*CAPE-specific assay was 108.87% (range from 99.35% to 126.32%), and the overall mean percentage efficiency for the *Bd*GPL-specific assay was 97.35% (range from 92.93% to 104.21%). All R^2 values, whether averaged across and within plates or run on individual Ct values, were within and across plates were >0.95 (range from 0.98 to 0.99) and the Ct values obtained for the DNA quantitation standards were also consistent, with all crossing the Ct threshold within one cycle of each other.

3.4.2 Stage 2: Diagnostic characteristics

No assay returned a positive result for any animals in the control group which received a sham dose of TGhL broth only. Additionally, all positive results for a particular lineage were obtained from an animal that had received a dose of that lineage (*i.e.* the qPCR did not detect any lineage in an animal that had not been exposed to that lineage). Some coinfections were detected in animals exposed to both lineages, with the lowest number of GEs detected in a coinfected animal being 28.6GE, indicating the assay is able to identify coinfection under experimental conditions. However, only three out of 38 animals that were exposed to both lineages returned a positive result for both lineages (Table 3.2, Figure 3.6). This low detection rate may be due to the second lineage that subjects were exposed to failing to colonise hosts, or due to an inability of the diagnostic to detect low positives when an animal is coinfected.

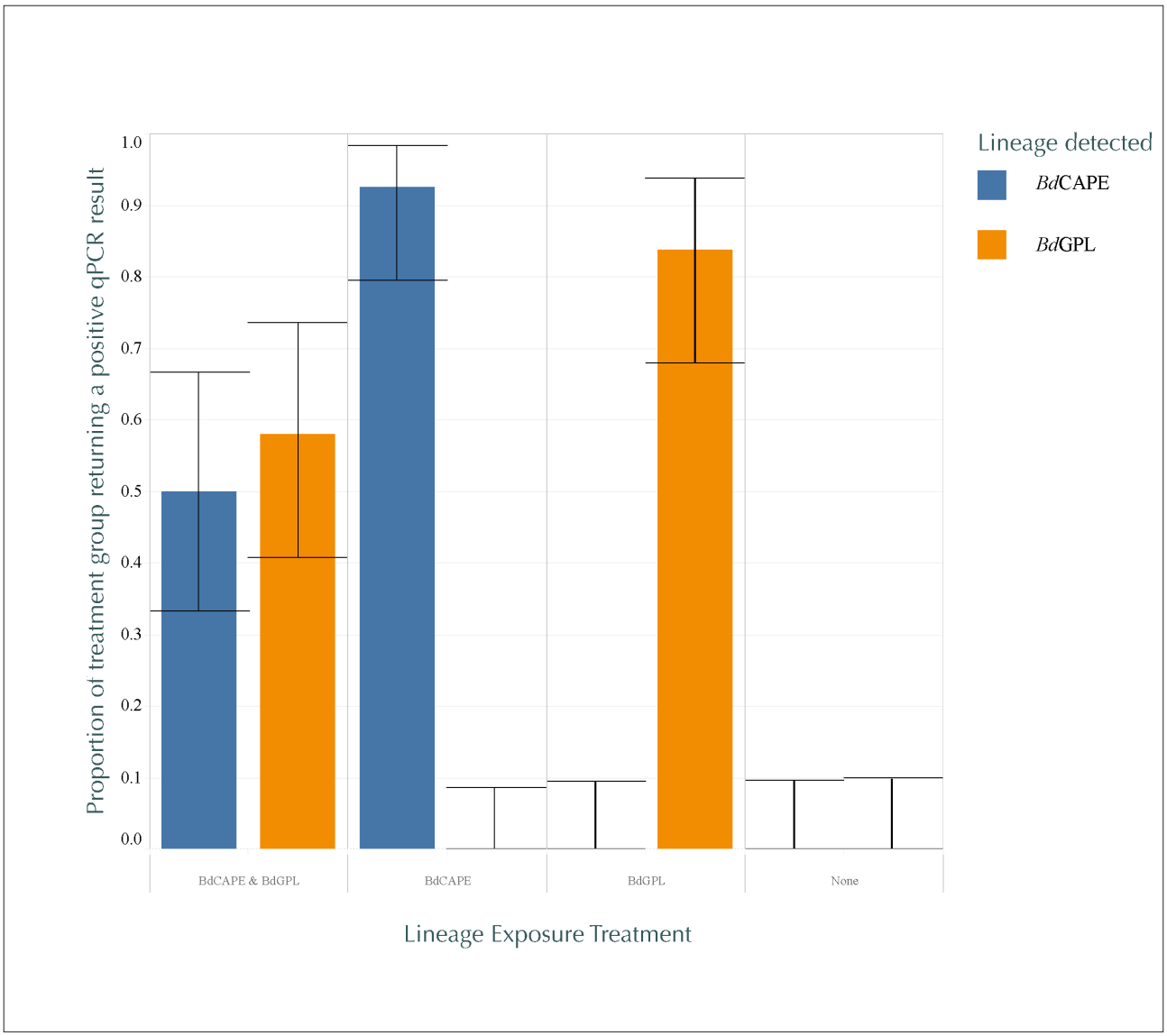


Figure 3.6. Bar plot showing results of *in vivo* lineage exposure experiment. Bars indicate proportion of animals returning a positive result for *Bd*CAPE or *Bd*GPL with lineage-specific qPCR by experimental treatment. Error bars represent 95% binomial confidence intervals.

Table 3.2. Lineage detection results for *in vivo* lineage exposure experiment, using novel lineage-specific qPCR assay.

Lineage Exposure Treatment Group	Proportion of treatment group in which lineage was detected (95% CI)			
	*Bd*CAPE only	*Bd*GPL only	*Bd*CAPE & *Bd*GPL	Negative
*Bd*CAPE	0.925 (0.80-0.98)	0 (0-0.09)	0 (0-0.09)	0.075 (0.02-0.2)
*Bd*GPL	0 (0-0.1)	0.84 (0.68-0.94)	0 (0-0.10)	0.16 (0.06-0.32)
*Bd*CAPE & *Bd*GPL	0.42 (0.26-0.58)	0.5 (0.33-0.67)	0.08 (0.02 - 0.21)	0 (0-0.09)
None	0 (0-0.10)	0 (0-0.10)	0 (0-0.10)	1 (0.90-1.00)

Over the course of this project, *Bd* isolates were collected from 10 sites that were also typed for lineage by qPCR from adult skin swab samples. In all cases except Aliwal North, where the lineage via qPCR was unidentifiable and which yielded a recombinant *Bd* isolate (SA-EC5), the lineage typing of the site by qPCR and by isolate WGS was in agreement (Table 3.3, Figure 3.7). The results from Douglas, a site in the Northern Cape, were noteworthy: both *Bd*CAPE and *Bd*GPL were detected from the same pond, and in one case on a single frog, via lineage-specific qPCR in 2016. In 2017, subsequent sampling recovered isolates of both *Bd*GPL and *Bd*CAPE from the same site, confirming the presence of both lineages in a single pond over at least a two-year period. SA-EC5 returned a positive result for *Bd*CAPE only via lineage-specific qPCR.

Table 3.3. Comparison of lineages detected at sites by lineage-specific qPCR and Isolation with WGS showing prevalence estimates with 95% binomial confidence intervals.

Site	qPCR Diagnosis	qPCR Prevalence (95% Confidence Intervals)	WGS Diagnosis	WGS prevalence (95% Confidence Intervals)
Lotheni	*Bd*CAPE	0.05 (0.00-0.25)	*Bd*CAPE	0.2 (0.06-0.44)
Cobham	*Bd*CAPE	0.20 (0.04-0.8)	*Bd*CAPE	0.02 (0.00-0.12)
Sani Pass	*Bd*CAPE	0.64 (0.31-0.89)	*Bd*CAPE	0.04 (0.00-0.19)
Mont Aux Sources	*Bd*CAPE	0.43 (0.3-0.58)	*Bd*CAPE	0.09 (0.02-0.25)
Royal Natal National Park	*Bd*CAPE	0.60 (0.31-0.89)	*Bd*CAPE	0.10 (0.03-0.23)
Garden Castle	*Bd*CAPE	0.64 (0.31-0.89)	*Bd*CAPE	0.11 (0.03-0.26)
Douglas	*Bd*CAPE & *Bd*GPL	0.13 (0.08-0.20)	*Bd*CAPE & *Bd*GPL	0.04 (0.01-0.11)
Onseepkans	*Bd*GPL	0.86 (0.42-1.00)	*Bd*GPL	0.29 (0.04-0.71)
Pella	*Bd*GPL	1.0 (0.78-1.00)	*Bd*GPL	0.40 (0.16-0.68)
Aliwal North	Unidentifiable	0.03 (0.00-0.38)	*Bd*GPL & recombinant	0.25 (0.05-0.57)

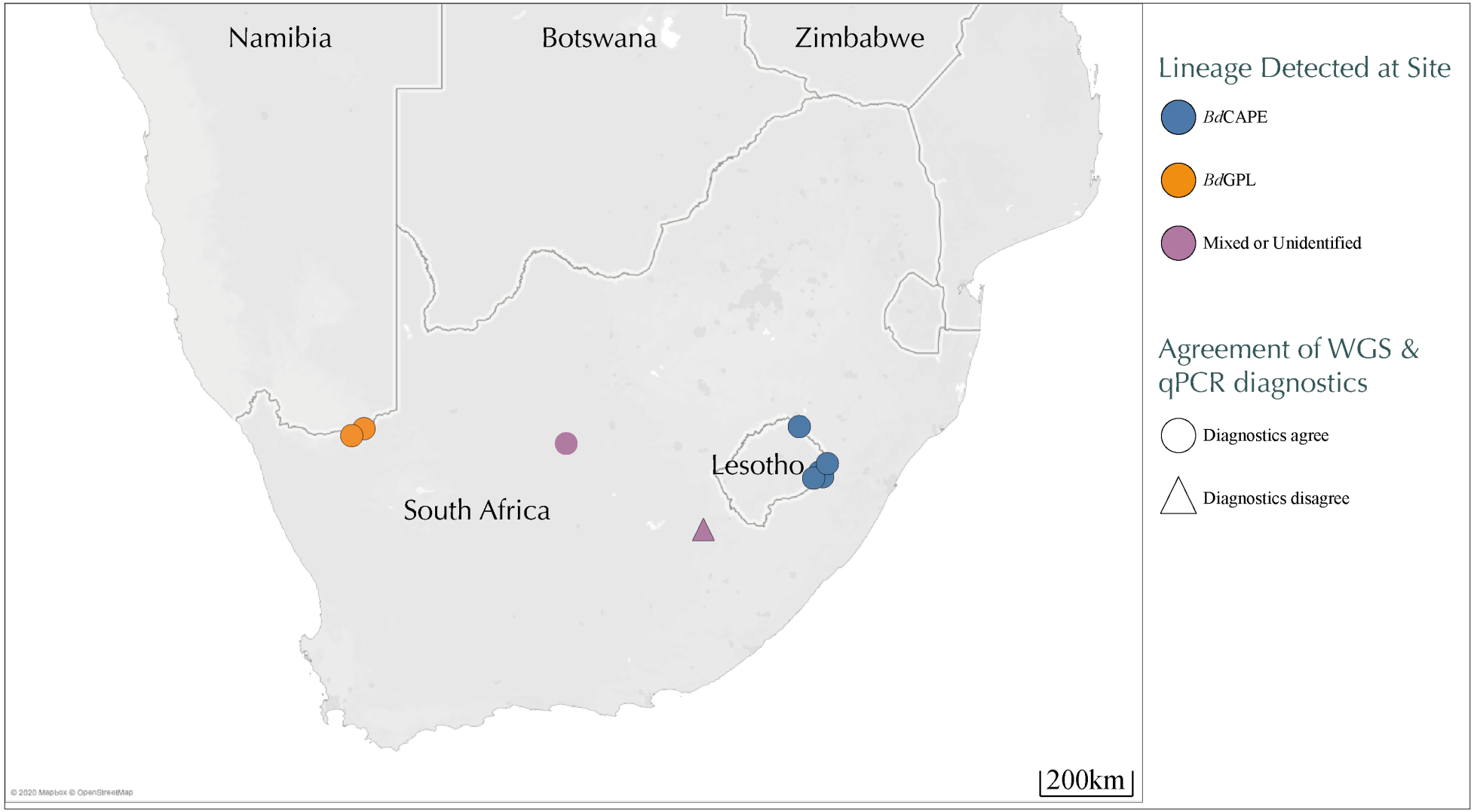


Figure 3.7. Comparison of *Bd* lineage typing for South African fieldsites using lineage-specific qPCR and WGS. The diagnostics agreed at all sites except for Aliwal North, where WGS diagnosed isolates as *Bd*GPL or recombinant, but lineage-specific qPCR was unable to assign any lineage.

3.4.3 Stage 3: Reproducibility

Results produced by laboratories at the IoZ and ICL were quantitatively very similar. The mean percentage efficiency across three independent plates was lower at the IoZ than at ICL, but all remained within an acceptable window (90% to 110% (Taylor *et al*., 2010)). R^2 values in all cases were >0.95, indicating quantification remained reliable across plates. All quantification standards were detected on plates run at ICL, while at the IoZ on two plates a single well of *Bd*CAPE quantitation standard = 1GE failed to amplify, and on one plate, a single well of *Bd*GPL quantitation standard = 1GE also failed to amplify. There was some variation in the Ct value for the quantitation standards, particularly for *Bd*CAPE which on average amplified approximately three cycles later at the IoZ. Conversely, on one plate at the IoZ, the *Bd*GPL quantitation standards amplified approximately three cycles earlier than all other plates. The percentage efficiency of one *Bd*CAPE assay at ICL was slightly higher than is ideal, at 126.3%. However, on average both assays performed consistently both within and between institutions (Table 3.4).

Table 3.4. Comparison of reproducibility measures obtained for novel lineage-specific qPCR between ICL and the IoZ.

Measure	ICL (mean of 3 plates)	IoZ (mean of 3 plates)	Overall mean
*Bd*CAPE % efficiency	108.87%	96.93%	102.9%
*Bd*GPL % efficiency	97.35%	91.51%	94.43%
*Bd*CAPE R2	>0.99	>0.99	>0.99
*Bd*GPL R2	>0.99	>0.99	>0.99
*Bd*CAPE Ct for 1000GE	26.23	29.54	27.89
*Bd*GPL Ct for 1000GE	26.64	26.62	26.63

3.4.4 Archive specimen testing

One of the five specimens tested from the Natural History Museum, London (1948.1.8.74, a *Xenopus fraseri* collected from Cameroon in 1933) returned a positive result (duplicate amplification, GE > 0.1) with the pan-lineage *Bd* diagnostic. A second specimen (1935.10.10.295, a *Xenopus laevis bunyoniensis* collected from Uganda in 1934) showed amplification of GE > 0.1

in one out of the two reaction wells. When these two specimens were then tested in triplicate with the pan-lineage *Bd* diagnostic, specimen 1948.1.8.74 showed amplification of GE > 0.1 in all three reaction wells, and specimen 1935.10.10.295 showed amplification of GE > 0.1 in a single reaction well. As only 1948.1.8.74 returned a definitively positive result, only this specimen was tested for lineage with qPCR. Lineage testing returned a stronger positive result than with the pan-lineage *Bd* diagnostic. The specimen was positive for *Bd*GPL (mean GE = 5.7 after accounting for dilution) in duplicate and negative for *Bd*CAPE.

All Kihansi spray toads showed amplification for *Bd*CAPE; four animals showed amplification in all four reaction wells, and one in three reaction wells. None showed any amplification for *Bd*GPL. The mean GE per animal ranged from 5.25GE up to 147GE. Shotgun sequencing confirmed the identity of the *Bd* isolated from *N. asperginis* in 2003, two years before the species was declared extinct in the wild, to be *Bd*CAPE.

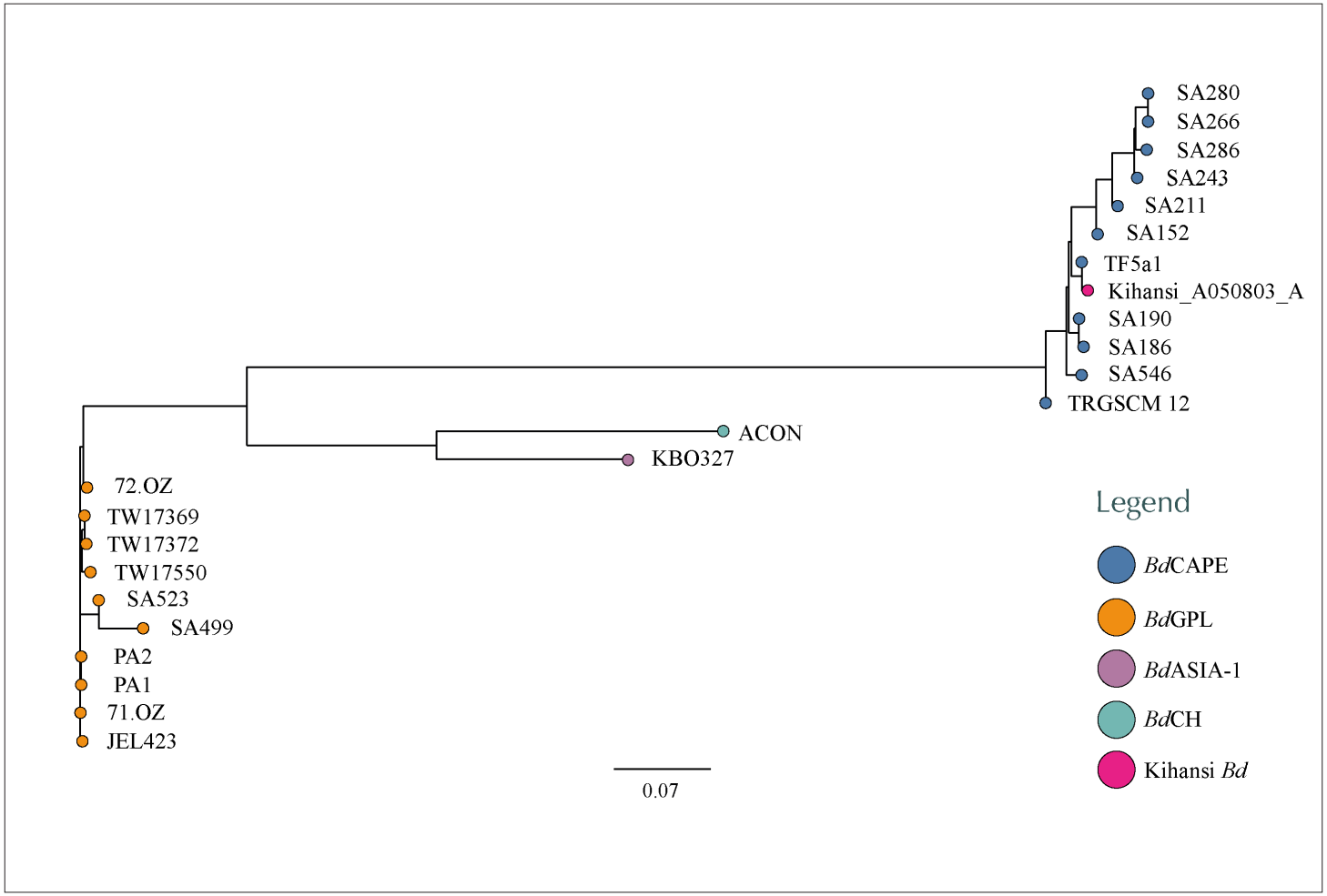


Figure 3.8. Unrooted phylogeny of *Bd* based on mtDNA showing placement of *Bd* DNA extracted from a Kihansi spray toad within the *Bd*CAPE lineage.

3.5 Discussion

The increased incidence of emerging disease events in our globalised world is an issue that cannot be managed or understood without the appropriate diagnostic tools: tools that are at an appropriate economic price point; that can be widely operated; that are straightforward to interpret; and that report information that is relevant to the epidemiology of the pathogen in question. For many years, it has been generally accepted that the pan-lineage diagnostic for *Bd* met these criteria. However, as the epidemiological importance of lineage becomes increasingly clear, the inability of this gold-standard test to diagnose lineage has become a major drawback in a) gathering baseline data at an epidemiologically useful scale and b) conducting further research into precisely how lineage interactions may have impacted the global emergence of *Bd*. Here, this research gap is addressed by the development of a novel, rapid and economical lineage-specific qPCR assay applicable to archival, field and experimental samples.

3.5.1 Assay performance

Collectively, the two novel lineage-specific qPCR assays for *Bd* described here were able to type correctly 53 out of 54 isolates. The *Bd*GPL-specific assay showed some cross-reactivity with a *Bd*ASIA-2/BRAZIL isolate, UM142. The sequencing reads for this isolate were screened for the presence of the *Bd*GPL-specific probe sequence, which was not found. Therefore, the most likely explanation for the cross-reactivity is that the DNA extract for this isolate was contaminated with *Bd*GPL DNA at some point after WGS. This result highlights that it is always necessary to examine the amplification plots manually to assess whether any look atypical, such as the trace seen for UM142, and to confirm results via multiple methods if feasible. Importantly, the assays were able to type presence or absence of the target lineage correctly in over 98% of cases and in all cases for Africa, the study system used for this research. The assay was highly sensitive, able to detect one genomic equivalent of *Bd*, compared with the 150GE limit of detection reported for alternative rapid *Bd* genotyping assays (Byrne *et al*., 2017). Furthermore, using standard curve analysis this

assay is able to give an indication of infection burden, with the important caveat that mtDNA copy number has not been quantified for any *Bd* isolate. With this in mind, we recommend that in order to remain consistent with the literature, the pan-lineage *Bd* diagnostic continues to be utilised for reporting *Bd* quantification (again, however, bearing in mind that ITS copy number may vary, so reported genomic equivalents should be viewed as semi-quantitative).

Initial testing at ICL showed both assays had good reaction percentage efficiencies and consistently produced R^2 values of over 0.95, indicating minimal within-plate variation. Ct values for standards were also extremely consistent across plates, showing good reproducibility between assays. A major limitation in exploring the epidemiological importance of lineage to date has been an inability to recover the lineages coinfecting amphibians under experimental conditions. Here we show that this assay is able to identify correctly experimentally coinfected animals and does not show cross-reactivity under experimental conditions. However, further work will be necessary to calculate the sensitivity and specificity of this assay in an *in vivo* context, particularly for coinfected animals, where it is possible that a very high concentration of one lineage on an animal may confound the ability of the assay to detect a lower infection intensity from a second lineage. This is a difficult paradox to untangle, but carrying out an *in vivo* experiment where single animals are exposed simultaneously to both *Bd*GPL and *Bd*CAPE in equal quantities would be an extremely valuable first step.

Diagnostic characteristics are inherently challenging to test in a field setting, due to an inability to have *a priori* knowledge of target pathogen locations. However, agreement of qPCR lineage typing of sites and WGS lineage typing of isolates from the same sites strongly supports the use of this assay under field conditions. Crucially, the results from Douglas (Section 3.4.2) indicate that the assay is able to identify sites where mixed-lineage infections are occurring and even single amphibians which are coinfected with two lineages of *Bd*, making the assay appropriate for use in putative contact zones between lineages where key interactions are most likely to be taking place.

The results of a mixed-lineage site can also be compared with sites known to harbour recombinant isolates of *Bd*. Testing of a site that returned a recombinant isolate, Aliwal North (SA-EC5), and the pure culture of another recombinant isolate collected from another site nearby (Hogsback), suggested that the assay is unable to indicate the presence of a recombinant isolate. In the case of the recombinant SA-EC5, which returned a positive result for *Bd*CAPE only, this suggests uniparental inheritance of mitochondria, a new insight into *Bd* biology.

The consistency of any diagnostic assay across multiple laboratories is essential to its utility, as is its ease of use. The sensitivity, speed and specificity of Boyle's (Boyle *et al*., 2004) pan-lineage *Bd* diagnostic means that the vast majority of *Bd* monitoring projects use this method to analyse their samples (Briggs *et al*., 2005; Kriger, Pereoglou & Hero, 2007; Cheng *et al*., 2011; Peralta-García *et al*., 2018). As a result, any laboratory working on *Bd* is likely to have access to the reagents and facilities required to perform the lineage-specific assay described here already. Comparison of results between the two institutions showed that the new assay performed well and reasonably consistently. For logistical reasons, qPCR reagents and quantitation standards were transported from ICL to the IoZ for this work and the slightly lower reaction percentage efficiency and Ct values for *Bd*CAPE quantitation standards may be due to this. qPCR is extremely sensitive in terms of target DNA detection, but also in terms of any reagent variation or degradation. This variation highlights the importance, for all qPCR-based diagnostics, of ensuring all reagents and quantitation standards are carefully maintained and results continue to be monitored for signs of degradation.

3.5.2 Lineage typing of archival specimens

We have shown here that the lineage present in specimens collected over 80 years ago can be typed using a non-invasive sampling method via this qPCR assay. In doing so, we have shown that *Bd*GPL, the globally invasive lineage, has been present in Africa since at least 1933, the oldest record of *Bd* on the continent. Although we now know that *Bd* as a species arose in South East Asia at the beginning of the 20th century (O'Hanlon *et al*., 2018), it is not clear when individual lineages

emerged or how and when they migrated out of South East Asia. The point-of-origin of *Bd*GPL, the lineage of greatest conservation concern, is therefore of significant interest. In 2014, Rodriguez *et al* reported the presence of *Bd*GPL in the Brazilian Atlantic Forest since at least 1894 (Rodriguez *et al*., 2014), slightly before the recently calculated estimated emergence of *Bd*GPL in the 20th century (O'Hanlon *et al*., 2018). However, the specimen from 1894 was lineage typed using the ITS1 region of the genome, which is unsuitable for sub-specific phylogenetic analyses in the case of members of the Chytridiomycota, such as *Bd* (Rodriguez *et al*., 2014; O'Hanlon *et al*., 2018). It would be pertinent to re-examine this result to confirm the lineage identification reported using the more reliable mtDNA, as if this specimen has been mistyped to *Bd*GPL, there would be no conflict with an emergence of *Bd*GPL in the 20th century. As it stands, the results presented here contribute to a body of evidence (lack of amphibian population declines; widespread yet disjunct distribution; apparently stable prevalence on the continent both temporally and spatially (Weldon *et al*., 2004)) that *Bd*, and crucially *Bd*GPL, has existed in Africa for longer than in other affected regions and therefore may represent a post-epidemic system.

Retrospective lineage typing of the *Bd* present on preserved Kihansi spray toads has provided evidence of only the second known population decline and first population extirpation attributable to *Bd*CAPE. Previous work has shown that *Bd*CAPE is more likely than *Bd*ASIA-1 and *Bd*ASIA-2/BRAZIL to be associated with symptoms of chytridiomycosis *in situ*, but except in a single case of *Bd*CAPE introduction into Mallorcan midwife toads (*Alytes muletensis*), it has not been observed to be associated with population declines (Walker *et al*., 2008; Doddington *et al.*, 2013, O'Hanlon *et al*., 2018). It is possible that if *Bd*CAPE, as is hypothesised, has been present in Africa for an extremely long period of time, amphibian populations may have developed tolerance or resistance to the fungus. It is likewise possible that a population of amphibians existing in a habitat as remote and difficult to access as that of the Kihansi spray toads would have been protected from *Bd* simply by nature of their location. Retrospective histological surveys first detected *Bd* in Kihansi spray toads three years following initial work to construct the Kihansi hydropower project in

the gorge that the toads lived in (IUCN SSC Amphibian Specialist Group, 2015; Weldon *et al*., 2019). This construction work would have greatly increased traffic to a previously extremely isolated area. *Bd*, while easily killed by commercial fungicides and disinfectants (Johnson *et al*., 2003) can be extremely persistent outside the host even in nutrient-poor environments, such as on moist substrates like sand or in tap water or even on feathers (Johnson & Speare, 2003, 2005). It is possible to speculate that construction on the dam and corresponding increase in movement of material and people into the area introduced the fungus and thus triggered the population extirpation of *N. asperginis*.

Although historically it has been considered unusual for a disease to be a proximate driver of species extinctions (Gulland, 1995; Smith, Acevedo-Whitehouse & Pedersen, 2009; McCallum, 2012), small isolated populations, such as *N. asperginis* in the early 2000s are particularly vulnerable to population stochasticity, such as that which could be induced by the arrival of a novel infectious agent. Typically, it would be expected that R_0 (the expected number of secondary infections resulting from a typical index case in a completely susceptible population (van den Driessche & Watmough, 2008)) would be pushed below one due to the low density of the host, thus ending the disease outbreak. However, instead of recovery, an extremely small and isolated population will then be highly vulnerable to extinction due to secondary factors, simply by virtue of its limited size (Smith, Acevedo-Whitehouse & Pedersen, 2009; McCallum, 2012). Interestingly, the phylogeny produced here shows the Kihansi spray toad *Bd* isolate to be most closely related to TF5a1, the isolate that has caused severe population declines in Mallorcan midwife toads, and the only other *Bd*CAPE isolate known to have been the proximate driver of population declines in wild populations. This raises the question of whether a subset of the *Bd*CAPE lineage has evolved elevated virulence. Further work is needed to interrogate the genomes of these two isolates for signatures of elevated pathogenicity and to compare their virulence levels under experimental conditions with other *Bd*CAPE isolates. Greater confidence in the results produced here could be gained by resequencing the Kihansi spray toad DNA samples to increase the mtDNA genome

coverage.

3.5.3 Future directions and utility

The novel *Bd* lineage-specific qPCR assay presented here has been shown to type *Bd* lineage reliably from adult amphibian skin swabs taken in the field, to recover lineages infecting experimental animals accurately, to be capable of identifying co-infection both in the wild and under experimental conditions and to be suitable for typing *Bd* lineage in archived specimens. It is worth noting that caution should be applied to interpreting results in the case of abnormal qPCR traces, that quantification is only considered to be semi-quantitative, and very low infection intensities may not be detectable. I have shown using this novel diagnostic that *Bd*GPL has been present in Africa since at least 1933 and evidence is presented of the first *Bd*CAPE-associated population extirpation.

For the purposes of remaining consistent with the literature, it is recommended that practitioners wishing to quantify *Bd* infection intensity first carry out standard pan-lineage diagnostics, followed by lineage-specific qPCR on positive samples to type the *Bd* present. However, wherever the copy number of the target region has not been quantified, as is the case for most *Bd* isolates, caution must still be applied when interpreting these results. Under experimental conditions, this problem could be resolved by first quantifying the copy number of the region targeted by the chosen molecular diagnostic for the experimental isolates before carrying out any experimental work. In the case of field and archival specimens, where there is no corresponding isolate and the effects of storage further impact quantification, caution should always be taken not to view quantification of *Bd* infection intensities as absolute.

The TaqMan MGB assay is easily adoptable by any laboratory already carrying out *Bd* research, since it utilises the same technology as the ubiquitous pan-lineage *Bd* diagnostic. The results are produced quickly and are simple to interpret for anyone familiar with traditional *Bd* qPCR protocols. A further advantage of this assay is its low cost compared to WGS. Again, as the same

technology is utilised as for the pan-lineage diagnostic, the cost per lineage identification will be comparable for laboratories (£3.47 per sample, using prices quoted to ICL, correct September 2019 and based on running every sample in duplicate with two no template controls and four duplicate DNA quantitation standards per lineage). This represents a substantially reduced cost both financially and in terms of time compared with WGS.

The process of developing this *Bd*GPL- and *Bd*CAPE-specific assay should be replicated as a priority for the other lineages of *Bd*. This would allow the rapid and widespread generation of baseline data from field samples and archived specimens, allowing much finer delineation of both contemporary and historical *Bd* lineage distributions globally. Lineage diagnostics also allow *in vivo* and *in vitro* experimental manipulation of *Bd* lineages, thus providing further insight into *Bd* population dynamics and structure.

3.6 Ethics statement

Permits for collection of *S. guttaralis* eggs were granted by North West Province, South Africa (permit number HQ18/05/16-088NW, issued by Dept. of Rural, Environmental and Agricultural Development). The *in vivo* experiment was approved by NWU's Animal Research Ethics Committee (AREC-130913-015, ethics number NWU-00015-16S5).

3.7 Acknowledgments and contributions

mtDNA sequences for designing lineage-specific primers and TaqMan MGB probes were compiled and aligned by Adrien Rieux, University College London. The alignment was searched for lineage-specific SNPs and primers and probes were designed by Pria Ghosh. DNA extracts from UM142 and CLFT065 for specificity testing were provided by Prof. Tim James, University of Michigan. All other isolates used for specificity tested were sourced from Prof. Matthew Fisher's live *Bd* collection, held at ICL, and were DNA extracted and prepared for testing by Pria Ghosh. Jean Ruhan Verster, NWU, designed and carried out the *in vivo* lineage infection experiment and conducted the lineage-specific diagnostic tests for that experiment. Lineage-specific diagnostic validation tests conducted at ZSL were carried by Claudia Wierzbicki. Execution of all other tests, and data cleaning, analysis and evaluation of all tests were carried out by Pria Ghosh. The Kihansi spray toad specimens were collected in 2003 by Ché Weldon, NWU. DNA was extracted from the specimens by Pria Ghosh. Shotgun sequencing library preparation was carried out by Claudia Wierzbicki, ICL, and Christian Carøe, University of Copenhagen, at the University of Copenhagen. Analysis of phylogenetic data was planned by Pria Ghosh with help from Thomas Sewell, ICL; the shotgun sequencing data was cleaned and analysis was carried out by Thomas Sewell. Access to the Natural History Museum archives for specimen sampling was facilitated by Jeff Streicher (Senior Curator, Amphibians and Reptiles, Natural History Museum, London). All qPCR tests on NHM specimens were carried out and analysed by Pria Ghosh.

Chapter 4

Bd Lineage Ecology in South Africa and the Lesotho Highlands

4.1 Abstract

Bd lineage is now known to be an epidemiologically important variable. While much progress has been made in understanding the differences between lineages via a comparative genomics approach, an inability to diagnose lineage reliably from field samples has, until now, hindered investigations into their distributions, interactions and ecologies. Addressing this research gap may shed light on the evolutionary pathway of *Bd* and resolve inconsistencies currently seen in the *Bd* literature regarding pathogen distribution and disease emergence. Southern Africa is known to harbour the two lineages of *Bd* that have been associated with chytridiomycosis-driven amphibian population declines, *Bd*GPL and *Bd*CAPE. In this chapter, a traditional comparative genomics via isolation and WGS lineage-typing approach is combined with the use of a novel lineage-specific qPCR assay to delineate *Bd* lineage distributions over one of the largest areas to date, in South Africa and the Lesotho highlands. Genomic analyses of collected isolates revealed that *Bd*GPL and *Bd*CAPE have fundamentally different population structures in Southern Africa; *Bd*GPL exhibits a strong signature of isolation by distance while *Bd*CAPE clusters into two distinct populations. However, this study revealed that while in nature *Bd*GPL and *Bd*CAPE had significantly different environmental profiles and evidence of divergent population structures, there was no lineage effect to isolates' growth responses to a heat shock treatment under experimental conditions. This work raises important questions regarding relative importance of environmental conditions versus *Bd* lineage interactions, and how they are affected by each other, in determining lineage distribution and pathogen dispersion in Southern Africa as well as shedding light on the lineages' evolutionary past in the region.

4.2 Introduction

4.2.1 The disease triangle and intra-pathogen variation

The disease triangle (Figure 4.1) is a conceptual model that was originally developed in the field of plant pathology and illustrates the interacting role of the environment, the host and the pathogen on the emergence and severity of disease (Scholthof, 2007; Gurr, Samalova & Fisher, 2011). Critically, the model varies from the commonly used single host-pathogen model approach by encouraging a consideration of much greater complexity that more accurately reflects natural disease systems (Dobson, 2004; Abdullah *et al*., 2017; Fountain-Jones *et al*., 2018). The disease triangle illustrates how a disease outbreak is not a certainty when a novel pathogen arrives in a naïve area, but is modulated by variations in the environment, pathogen biology and inter-microbial interactions, as well as host factors (James *et al*., 2015).

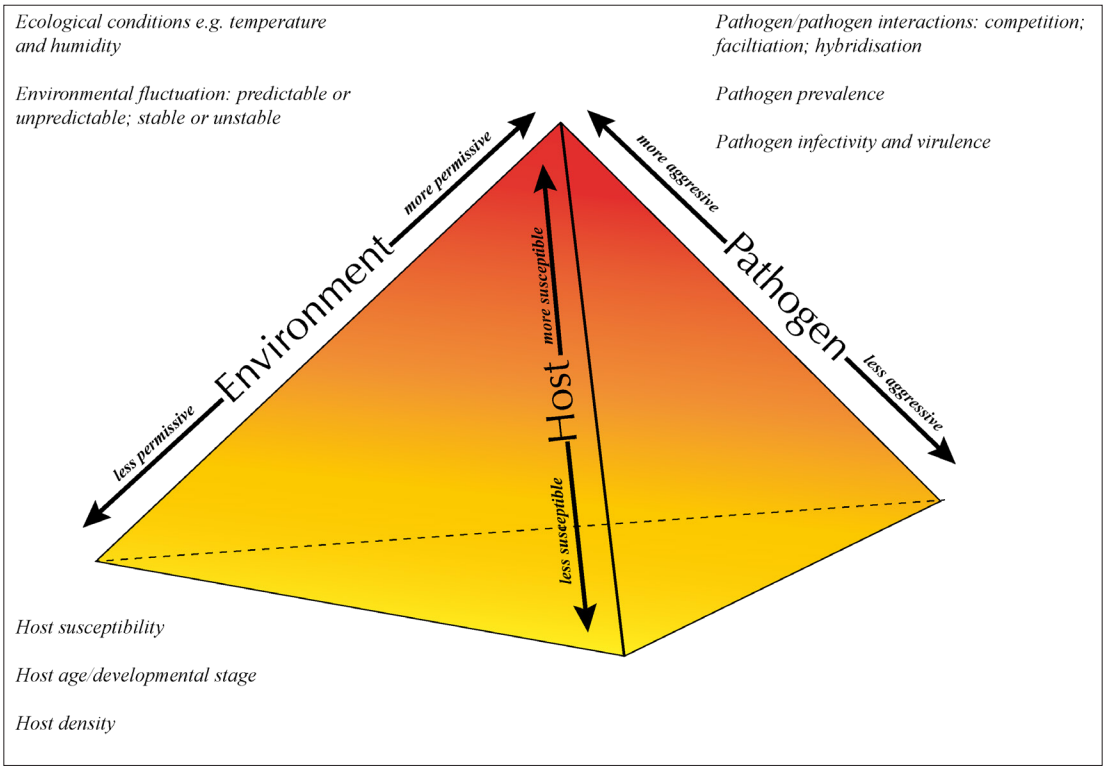


Figure 4.1. Diagram of the disease triangle. Adapted from Barrett *et al*., (2009). Progression in colour from yellow to red indicates increasing disease intensity or propensity towards an outbreak.

Understanding under what conditions pathogens invade, spread or cause elevated harm to their hosts is vital to understanding the level of threat diseases pose to biodiversity (Barrett *et al*., 2009). The model emphasises that considering the natural complexity inherent in many disease systems is essential to understanding disease dynamics and managing pathogens (Barrett *et al*., 2009). Within *Bd* research, much of the literature investigates the host/environment axes and their interface (Kriger & Hero, 2007; Longo, Burrowes & Joglar, 2010; Cohen *et al*., 2017; Spitzen-Van Der Sluijs *et al*., 2017; Sonn, Berman & Richards-Zawacki, 2017; Campbell *et al*., 2019). However, a layer of complexity that has been neglected until very recently is the genetic variation within the pathogen itself and how *Bd* lineage interacts with the environment to influence pathogen distribution and so disease patterns.

Comparative genomics of the lineages has revealed differences in genetic diversity and population structure at a global scale. While unsuitable for large-scale surveillance due to the cost and skill involved, a comparative genomics approach continues to be extremely valuable in revealing the contrasting evolutionary pathways of *Bd* lineages. It is now clear that the *Bd* lineages are deeply phylogenetically diverged and harbour variable levels of genetic diversity: *Bd*ASIA-1 has the greatest level of diversity, and signatures of pathogen endemicity within its genome; in contrast *Bd*GPL, despite its global distribution, has extremely low levels of genetic diversity (Farrer *et al*., 2011; O'Hanlon *et al*., 2018).

We now know that *Bd* lineage is an epidemiologically important variable; that *Bd* lineages vary in their distributions globally; that *Bd*GPL can outcompete enzootic lineages under experimental conditions; and that *Bd* lineages can recombine (Goka *et al*., 2009; Farrer *et al*., 2011; Rodriguez *et al*., 2014; Jenkinson *et al*., 2016; O'Hanlon *et al*., 2018). Furthermore, the discovery of *Bd* lineage recombinants in Brazil and South Africa have revealed that lineages are to some extent already interacting and recombining, adding an additional layer to the *Bd* lineage story (Schloegel *et al*., 2012; Ghosh & Fisher, 2016; Jenkinson *et al*., 2016; O'Hanlon *et al*., 2018).

4.2.2 Resolving environmental effects and lineage effects on pathogen distribution

Species distribution models (SDMs), and analysis of *Bd* environmental correlates, have rarely taken into account intra-pathogen variation or biotic interactions (Puschendorf *et al*., 2009; Rödder *et al*., 2009; Wisz *et al*., 2013; Penner *et al.*, 2013, Seimon *et al*., 2015; Xie, Olson & Blaustein, 2016; Miller *et al*., 2018), the extent of which has only recently been recognised (Becker *et al*., 2017; Kearns *et al*., 2017; Greenspan *et al.*, 2018) and due to the difficulty in typing *Bd* lineages, has not been practically resolvable using field samples until recently (Byrne *et al*., 2016). While data limitations have largely precluded adequately accounting for *Bd* lineage in investigations into the environmental preferences of *Bd*, several studies have shown that different isolates of *Bd* can vary in their growth and other life history traits under experimental environmental manipulation (Stevenson *et al.*, 2013; Voyles *et al*., 2017) and different *Bd* genotypes have been found to be associated with different environments in nature (Kaiser & Pollinger, 2012; Muletz-Wolz *et al*., 2019).

To date, the environmental envelope for *Bd* is still not satisfactorily resolved, and there have been several seemingly conflicting studies on the environmental correlates of *Bd* epizootics (Venesky *et al*., 2014). Some of this variation could be partly explained if different *Bd* lineages exhibited different environmental niche characteristics, possibly reflecting their varying evolutionary trajectories since emerging from South East Asia and being largely allopatrically separated from each other since then (O'Hanlon *et al.*, 2018). As it is now known that in the Americas and in Africa two *Bd* lineages, presumed to have been isolated in their recent evolutionary past, are re-contacting (Jenkinson *et al*., 2016; O'Hanlon *et al*., 2018), the question is raised to what extent *Bd* lineage distributions and disease emergence will be modulated by the presence of interspecific diversity.

The inability to take account of pathogen and microbial level interactions may be affecting the results of the many SDMs and range estimates for *Bd*. Pathogen strain interactions have been

demonstrated to impact disease outcome in other diseases through competitive interactions (May & Nowak, 1995; Grenfell & Gog, 2001; Gower & Webster, 2005; Pepin, Lambeth & Hanley, 2008; Pepin & Hanley, 2008; Balmer *et al*., 2009), and sometimes by leading to hybrid or recombinant offspring which may display elevated virulence or an expanded host range compared to the parental strains (Brasier, Cooke & Duncan, 1999; Newcombe *et al*., 2000; Brasier & Buck, 2001; Stukenbrock, 2016; Menardo *et al*., 2016; Greenspan *et al*., 2018). The variance in virulence displayed by *Bd* lineages also suggests an alternative hypothesis for the determinants of lineage distributions. Recent work focussing on environmental fungi has found that the moisture and thermal optima for most fungi are remarkably similar across taxa. This research has suggested that rather than varying in their optimum environmental niche, the structure of fungal communities instead depends on a trade-off between niche width, or the tolerance of fungi to abiotic environmental stressors, and competitive ability (measured as a fungus's ability to displace another fungus in co-culture under laboratory conditions) (Maynard *et al*., 2019). It is well established that biotic interactions can influence species distributions, and that the nature of the biotic interactions may be influenced by changing the environmental conditions (Wisz *et al*., 2013). A dominance/ environmental trade-off would allow fungi with very similar optimal niches to coexist and would also mean that the outcome of lineage interactions would vary depending upon the environment in which they take place.

Identifying areas where lineages are in direct contact would enable better parameterisation of the lineage's fundamental and realised niches. Identification of lineage contact points, 'hot spots' and 'cold spots' would enable targeted research on what factors limit lineage proliferation in certain areas. A cold spot may be the result of lineage interactions, via mutual competitive suppression, or it may be that the lineages having divergent environmental niches and the cold spot represents a region where neither is able to dominate due to environmental constraints. It is also possible that cold spots only appear to be so due to lineage distributions being in disequilibrium – it is possible that a cold spot is simply in the process of being colonised.

The complexity of potential interacting factors in research such as this makes it necessary to adopt a multi-disciplinary approach, combining phylogenetics and genomics with traditional spatial epidemiology, and observation studies with experimental manipulation in order to triangulate towards the key factors determining disease dynamics (Grenfell & Gog, 2001; Grenfell *et al*., 2004; Ostfeld, Glass & Keesing, 2005; Plowright *et al*., 2008; Johnson, Roode & Fenton, 2015; Fountain-Jones *et al*., 2018).

To begin investigating whether environmental conditions determine *Bd* lineage distribution and the extent to which *Bd* lineage interactions are epidemiologically relevant, it is first necessary to gather baseline data on lineage distributions in areas of lineage co-existence (Bataille *et al.*, 2013; James *et al*., 2015; Ghosh & Fisher, 2016). Some work has been carried out in the Brazilian Atlantic Forest (Schloegel *et al*., 2012; Rodriguez *et al*., 2014; Jenkinson *et al*., 2016; Greenspan *et al*., 2018), where recombinant lineages have been found, but nothing is known about fine-scale lineage distribution outside of Brazil.

4.2.3 *Bd* in Africa

South Africa is already known to harbour two lineages of *Bd*, *Bd*GPL and *Bd*CAPE (Farrer *et al*., 2011; O'Hanlon *et al*., 2018), and has done so since at least 2008 (Figure 4.2). The earliest record of *Bd* in Africa comes from a *Xenopus fraseri* specimen collected from Cameroon in 1933 and now held at London's Natural History Museum (Soto-Azat *et al.*, 2010). *Bd* appears to be widely spread in Africa, with a disjunct distribution, however sampling is distinctly patchy (Weldon *et al*., 2004; Olson & Ronnenberg, 2014, Doherty-Bone *et al*, 2019). The distribution of *Bd* lineages, however, in Africa is poorly resolved.

For some time Africa was considered to be a candidate continent for the origin of *Bd* itself, due to the continent being the source of, at the time, the oldest record of *Bd*; the apparent lack of impact of the fungus on wild amphibian populations in Africa; its widespread distribution on the continent; and apparent stability of prevalence of the fungus at just under 3% in South Africa, based on

surveys of archived amphibian specimens (Weldon *et al*., 2004). However, the picture has changed in recent years.

It is now known that that the origin of *Bd* as a species is in South East Asia (O'Hanlon *et al*., 2018), and the oldest record of *Bd* infection is now from the USA (a *Rana* (L.) *sphenocephala* specimen collected in Illinois in 1888) (Talley *et al.*, 2015). There have also now been reports of amphibian population declines or extirpations attributable to *Bd* infection, such as the 2003 collapse of the Kihansi spray toads (*N. asperginis*) in Tanzania (Krajick, 2006; Lips, 2016; Weldon *et al*., 2019) and the population decline of numerous amphibian species in Cameroon contemporaneously with increasing prevalence of *Bd* in the relevant areas (Hirschfeld *et al*., 2016).

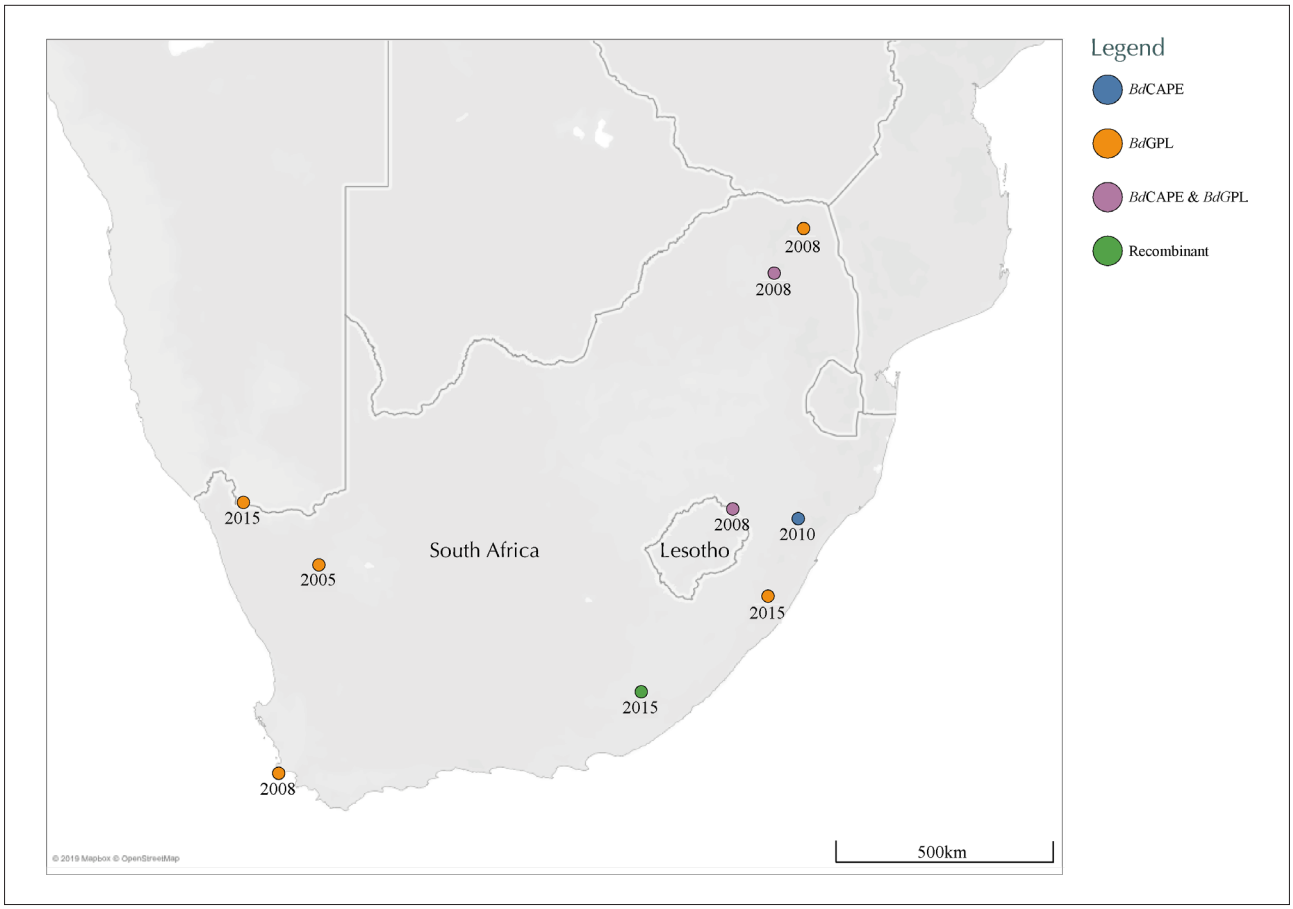


Figure 4.2. Map of known distribution of *Bd*GPL and *Bd*CAPE in South Africa and Lesotho, correct as of October 2015 (O'Hanlon *et al*., 2018). Map generated in Tableau.

4.2.4 *Bd* in South Africa and the Drakensberg – Orange/Gariep River study system

South Africa is one of the few countries on the African continent that has been reasonably well surveyed for *Bd* infection (Tarrant *et al*., 2013). The amphibian community of 124 species is well characterised and studied, with none being classified as Data Deficient by the IUCN (Measey, 2011; Hughes, 2019; UNESCO World Heritage, n.d.). While wide areas of the country are considered environmentally suitable for *Bd* occurrence on the basis of correlative SDMs, and *Bd* has been found widely distributed across the country (Weldon *et al*., 2004; Tarrant *et al*., 2013), there have been no confirmed reports of chytridiomycosis outbreaks driving population declines (Weldon *et al*., 2004; Lips, 2016).

The Orange River (referred to by the Khoi people as the Gariep or Great River and as the Senqu River in Lesotho) is the seventh longest in Africa, with a catchment area of approximately 900,000km^2, encompassing four countries (Botswana, Namibia, Lesotho and South Africa) (Mungatana & Hassan, 2007). The Orange travels more than 2,300km from the cool, wet highlands of Lesotho in the Drakensberg Mountains to the arid and hot Alexander Bay in the Northern Cape Province of South Africa, where it spills into the Atlantic Ocean (Cambray *et al*., 1986; Mungatana & Hassan, 2007) (Figure 4.3). Consequently, the range of environmental conditions through which this river passes is enormous: the highlands of Lesotho and the Drakensberg may receive over 1,000mm of precipitation per year (Nel, 2009) and temperatures can drop as low as -10^{o}C in winter (Cambray *et al*., 1986; Conley & Van Niekerk, 2000), while at the river mouth precipitation levels may be as low as 50mm of rain per year with temperature highs of up to 50^{o}C (Conley & Van Niekerk, 2000).

Parts of the Drakensberg mountain range have been intensively surveyed for *Bd*, and prevalence is relatively high (Smith *et al*., 2007; Meyer, 2009; Pretorius, 2016). The majority of the range is covered by the 2,500km^2 Maloti-Drakensberg National Park, a transboundary UNESCO world

heritage site that encompasses the Sehlathebe National Park of Lesotho and the uKhahlamba Drakensberg National Park of South Africa. The uKhahlamba Drakensberg National Park is itself formed from 12 smaller protected areas including a RAMSAR site, Royal Natal National Park. The rivers of the southern Maloti-Drakensberg National Park drain into the Orange River, while the Tugela River, which rises on the Lesotho highland plateau of Mont-aux-Sources with the Orange River, drains eastwards towards the Indian Ocean. The combination of a range of environmental conditions, the widespread presence of *Bd* and the existence of two *Bd* lineages in relatively close proximity makes this region an ideal study system for investigating the ecology and population dynamics of *Bd* lineages in nature.

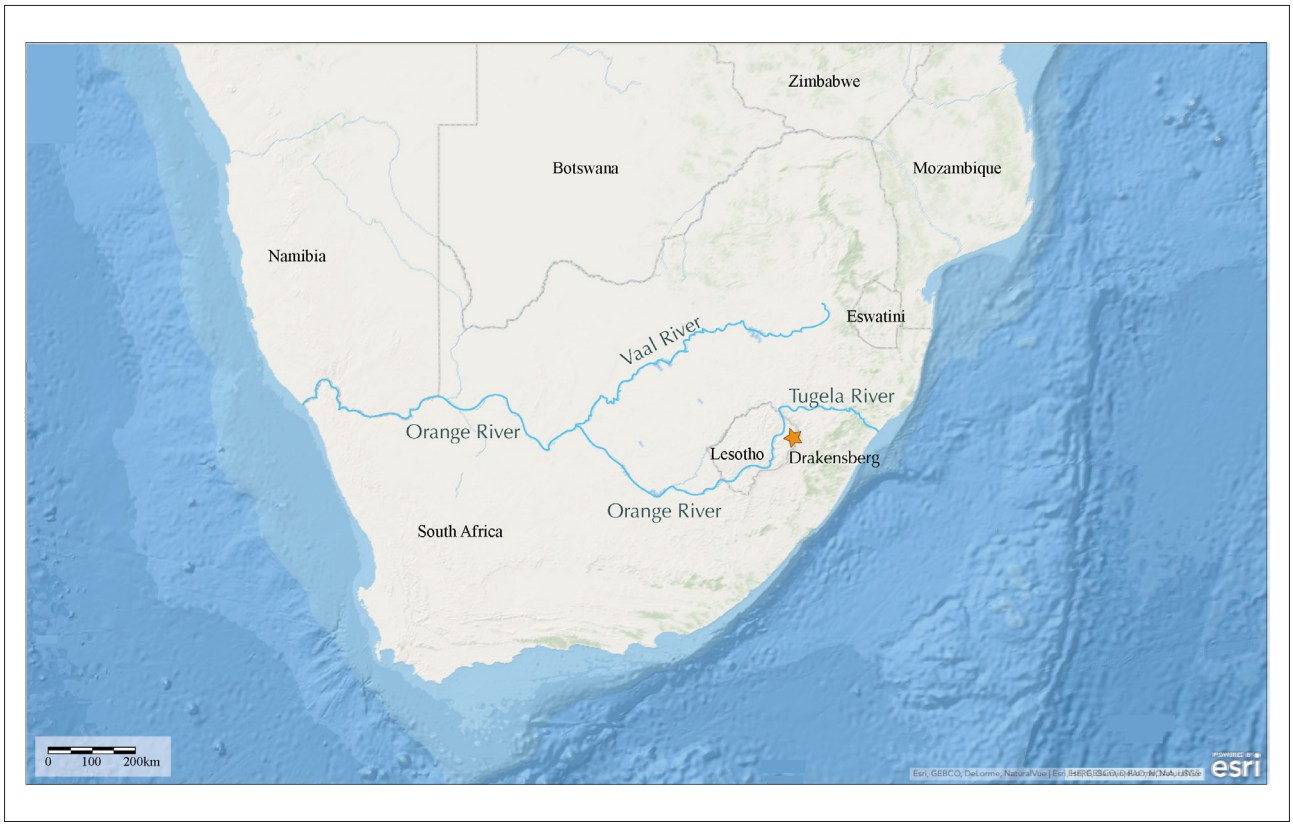


Figure 4.3. Map of the rivers of South Africa and Lesotho. Major rivers, the Vaal, the Orange and the Tugela are highlighted. Map generated in ArcGIS using Esri Ocean Basemap, with annotations added in Adobe Illustrator®.

In this chapter, a novel lineage-specific diagnostic qPCR is combined with traditional isolation and lineage typing by WGS to establish baseline data on the distributions of *Bd*GPL and *Bd*CAPE in South Africa and the Lesotho highlands, focussing mainly on the course of the Orange River. This data is then interrogated for lineage associations with environmental variables that could impact fungus growth. Informed by the field data, an experimental hypothesis-testing approach is taken to see whether associations identified in the field are reflected in *in vitro* experimental results. I hypothesised that, based on its distribution in nature, *Bd*GPL would be able to grow better following subjection to heat shock treatment than *Bd*CAPE. Finally, comparative genomic analyses are used to reveal whether *Bd*GPL and *Bd*CAPE exhibit signs of different population structure that may shed light on their recent evolutionary past in Africa.

4.3 Methods

4.3.1 Field sample collection and processing

Tissue and swab samples were collected between 2016 and 2018. Samples were mostly collected between January and March (during the rain season), when amphibians are easiest to find in this region and *Bd* prevalence is highest (C. Weldon pers. comm.). Sites were initially selected by targeting regions known to be positive for *Bd*, specifically sites between areas known to harbour different *Bd* lineages, in order to identify zones of potential lineage transition or co-occurrence. Ultimately, following reassessment with each field season, the main focus of the study became the Orange River transect, tracing the course of the Orange River over 1,500km from Vioolsdrift Settlement near the river estuary in the Northern Cape Province of South Africa (~155m asl), to the Drakensburg Mountains (the most easterly site of which was Lotheni camp in the uKhahlamba Drakensberg National Park (~1,600m asl)). Smaller transects and opportunistic sampling where possible were also carried out in an attempt to identify other zones of interest regarding *Bd* lineage distribution (Figure 4.4). Wherever possible, sites were not more than 100km apart and sites closer than approximately 10km from each other along the same watercourse or within the same river system were treated as a single site in analyses. Site selection was dependent on a) existence of a water body or wetland area with amphibians, either as larvae or adults, b) the ability to gain permission of the landowner to sample the land, and c) maximising the safety of the transect route.

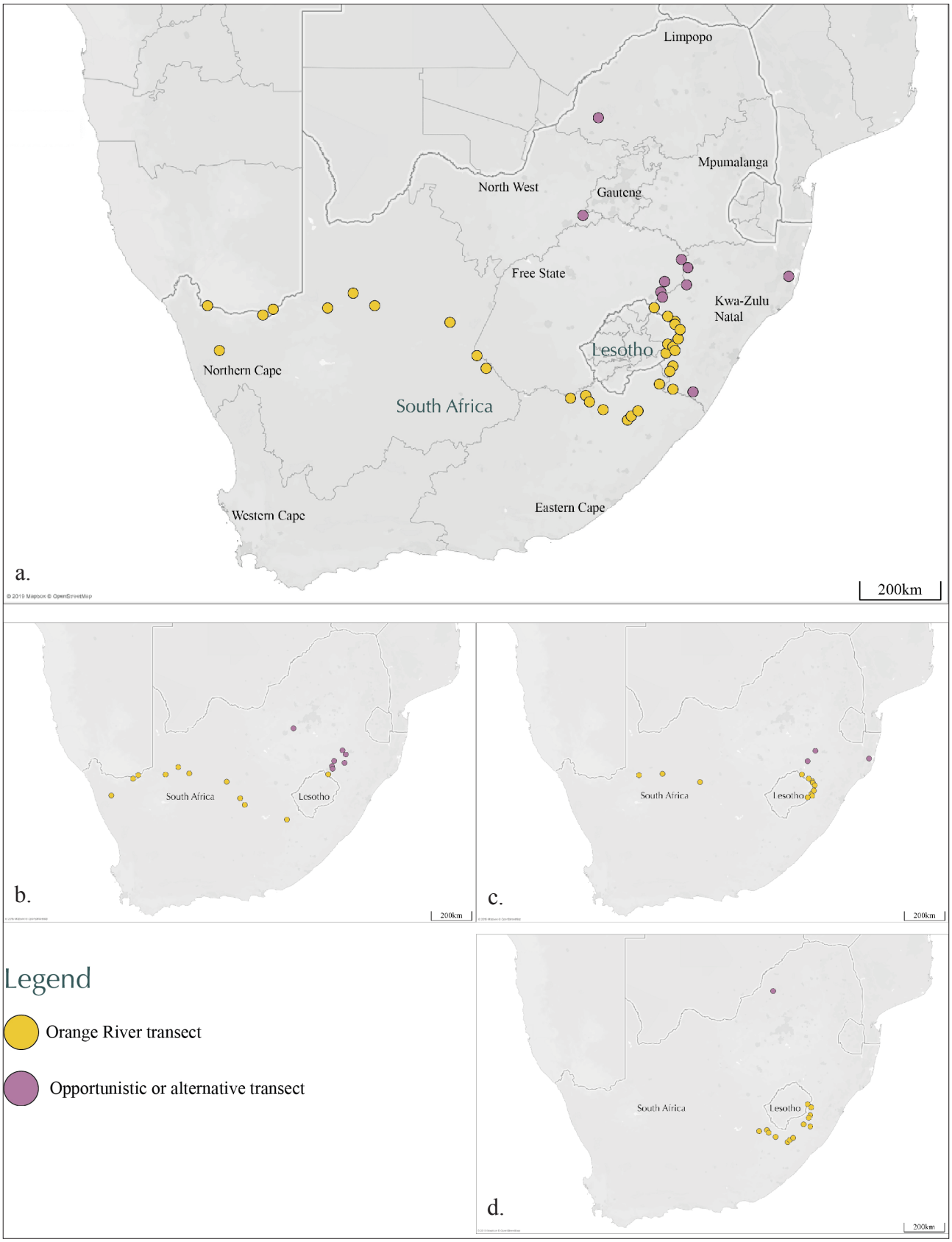


Figure 4.4. Maps of sampling sites across South Africa and Lesotho showing sites sampled during (a) all years 2016 - 2018, (b) during 2016, (c) during 2017, and (d) during 2018 only. Maps generated in Tableau.

For work carried out in 2016, all amphibians were sampled opportunistically as limited information was available on the detectability of *Bd* in natural populations of African species. The Maloti-Drakensberg Park harbours 26 amphibian species, several of which are in IUCN 'threatened' classes (IUCN & UN Environment World Conservation Monitoring Centre, 2013). Host effects, including variation within the same genus and even different populations of the same species, have a strong influence on *Bd* susceptibility (Briggs, Knapp & Vredenburg, 2010; Searle *et al*., 2011; Rooij *et al*., 2015; Bacigalupe *et al*., 2017; Cohen *et al*., 2017; Brannelly *et al*., 2018). This makes eliminating the impact of unknown host effects on results almost impossible for any field study on *Bd*. However, the park is also home to several species that are members of the *Amietia*, a large genus (which is frequently the subject of taxonomic reassessment) of around 16 cryptic species (https://amphibiaweb.org/lists/Pyxicephalidae.shtml). The *Amietia* are distributed throughout sub-Saharan Africa, including along the entire length of the Orange River and on top of the Lesotho plateau. Additionally, members of the *Amietia* genus are readily infected with *Bd* (C. Weldon, pers. comm.).

Following analysis of 2016 samples, it was decided to not sample any toad species, as no toads were found infected with *Bd* during sampling. It was also decided to particularly target members of the *Amietia* genus wherever possible, as both tadpoles and adults regularly carried *Bd* infection in collected samples, although no animals displayed symptoms of chytridiomycosis throughout the study. Since this work was undertaken over too large an area to focus on a single host species to control for inter-specific variation in susceptibility, we considered a focus on a single genus a step towards mitigating the effect of taxonomic variation in infection susceptibility observed in many *Bd* studies (Woodhams *et al*., 2007; Searle *et al*., 2011; Gahl, Longcore & Houlahan, 2012).

If insufficient *Amietia* were found at a site, other frog species as either larvae or adults were caught instead until the required sample size was met. As prior to initiating this study minimum expected prevalence for an infected population of amphibians in the study sites was not known, sample sizes were largely dictated by permit restrictions and ability to find target amphibians. Due to weather

conditions (a severe drought in 2016 and flooding in 2017), it was not always possible to catch the target number of animals but wherever possible a minimum of 10 individuals were sampled per site. If tadpoles were collected, a maximum of 30 individuals were collected as lethal sampling was necessary for amphibian larvae. All sampling followed the methods described in Section 2.2 and Section 2.3. Adult amphibians were swabbed for *Bd* lineage identification via qPCR and toe-clipped to attempt *Bd* isolation for WGS. Tadpole mouthparts were excised for Bd isolation following euthanasia. Due to the difficulty in acquiring sufficient adult samples for *Bd* lineage surveillance in 2016 and 2017 (as a result of the extreme weather), it was decided that in 2018, the final year of sampling, 50% of each excised tadpole mouthpart would be preserved in 70% etOH and processed for qPCR testing as described in Section 2.6, with the remaining 50% reserved for attempting *Bd* isolation.

For all samples collected, the site name and coordinates at which the amphibian was caught, species, life stage, date of collection and the type of sample taken (swab or tissue sample, or both) was recorded. The location of sites was recorded using a handheld GPS device. Elevation data for sites visited in 2016 was downloaded from Google Earth Pro (v 7.3.2.5776) and for sites visited in 2017 and 2018, this information was collected at the time of the site sampling using a handheld GPS device. For all sites at which *Bd* lineage had been typed, either by WGS or lineage-specific qPCR, several environmental covariates were compiled: mean temperature (earth skin temperature in ^{o}C, averaged between 1981 and 2018, recorded daily), mean daily precipitation (mm per day, averaged between 1981 and 2018) and mean annual dew/frost point at 2m above ground (^{o}C averaged between 1981 and 2018, recorded daily) were downloaded from the NASA POWER website (https://power.larc.nasa.gov). These variables were selected as previous work has suggested that they may be key determinants of *Bd* distribution (Puschendorf, Bolaños & Chaves, 2006; Puschendorf *et al*., 2009; Murray *et al*., 2011). Sites from which recombinant isolates were collected and those that were positive for both *Bd*CAPE and *Bd*GPL by lineage-specific qPCR, or returned isolates of both lineages from the same water body, were grouped together.

For *Bd* lineage testing via qPCR, DNA extracts from field samples first underwent pan-lineage *Bd* qPCR (Section 2.6). Samples were considered positive if they showed amplification in duplicate with a GE > 0.1 after accounting for dilution. Samples that showed amplification in duplicate with a GE > 1 then underwent lineage specific-*Bd* qPCR as described in Chapter 3. Lineage distribution maps were produced in Tableau Desktop (v2019.3).

4.3.2 WGS lineage typing of collected isolates

Isolates collected prior to this PhD research had previously been whole genome sequenced and lineage typed and the majority of isolates collected during this study were processed and reported as part of the same work, as described in Section 2.8 (O'Hanlon *et al*., 2018). Isolates were cryopreserved as soon as possible following isolation (Section 2.4). Metadata for all isolates used or collected during this research can be found in Appendix 6. A phylogeny of Southern African isolates only (subsetted from O'Hanlon *et al.*, 2018, Appendix 6) was generated from a multi-sample VCF file consisting of all 46 genomes available for South African or Lesotho isolates, using the methods described in Section 2.8 (also those used by O'Hanlon *et al*., 2018). The combined VCF file was inputted into RStudio (v1.0.143) and converted into a genlight object using vcfr (v1.8.0). vcfr found 14,179 loci with more than two alleles. As objects of class genlight only support loci with two alleles, these loci were omitted from the genlight object, reducing overall diversity. A midpoint rooted maximum likelihood phylogeny of all 46 Southern African *Bd* genomes was generated using SNP positions only to maintain alignment between sites (*i.e.* insertions and deletions were removed). The phylogeny was generated over all non-ambiguous sites (279,600 variant sites, or 1.15% of the total genome). A FASTA file was generated with SNP positions only (loci where at least one isolate varied from the reference genome) and the tree was generated using RAxML, a generalised time reversible model (GTRCAT) and bootstrapped 500 times in rapid bootstrapping mode.

4.3.3 Field data analysis

I analysed the data to determine whether there were any differences in the environmental characteristics exhibited at sites at which each of the *Bd* lineage groups were detected. All analyses were conducted in RStudio v1.0.143. Box and whisker plots of the sites' lineage identification were used to visualise variation in mean temperature and mean precipitation across sites grouped by *Bd* lineage. Kruskal Wallis tests (which show if there are significant differences between groups for a given variable, but not which specific groups are different from each other) were carried out to see if sites of different lineage identities showed significant differences in terms of a) mean temperature (^{o}C), b) mean precipitation (mm day^{-1}), c) mean annual dew point (^{o}C) and d) elevation. It was also tested whether the prevalence of *Bd* was significantly different at sites of different lineage identity. Kruskal Wallis tests were selected as the data was non-normal. Elevation was predicted to correlate heavily with the other variables. A Pairwise Wilcox test was then carried out for any of those variables that returned a significant difference for the Kruskal Wallis test to identify which groups differed from each other, with Holm used as the p-value adjustment method.

A principal component analysis (PCA) was also conducted on lineage-typed samples in RStudio v1.2.1335 using packages FactoMineR v1.42 and factoextra v1.0.5 and visualised using ggplot2 v3.2.1. Mean annual temperature, mean precipitation, elevation and mean annual dew point were used as explanatory variables. A PCA is a dimension reduction technique that works by identifying axes along which the variation in data is at maximal, and using those to create fewer new variables (the principal components) from the ones provided in the data set that still capture the important variation in the data (Ringnér, 2008). This simplifies the data interpretation and plotting by allowing each sample to be represented by the few principal components that account for the greatest amount of variation in the data set. A PCA was used here to identify the relative importance of different environmental variables as predictors of the lineage present at a site and to see if sites at which different lineages were found clustered together according to their environmental conditions.

4.3.4 *In vitro* experimental work to determine response of *Bd* lineages to heat shock

A particularly notable feature of the *Bd*GPL 'hot spot' revealed in the field data is the extreme temperatures reached in summer months (>50°C at the mouth of the Orange River). I therefore hypothesised that ability to withstand periodic exposure to thermal stress could be a determining factor in lineage distributions. Five lineages of *Bd*CAPE, five lineages of *Bd*GPL and one recombinant isolate, all collected from South Africa between 2015 and 2017 (Appendix 7), were thawed from cryopreservation (Section 2.4) and once healthy growth had established, 500µl of each was transferred to a 175cm^2 Nunclon™ cell culture flask containing 40ml of TGhL broth. The cultures were incubated at 18°C for seven days before each isolate was divided into 18 25cm^2 Nunclon™ cell culture flasks. Each new culture flask contained 9ml of TGhL broth and 1ml of *Bd* culture. All new culture flasks were then incubated at 18°C for 48 hours. After 48 hours, 100µl of culture was removed from each flask and placed into a 1.5ml Safe-Lock Eppendorf tube prepared with 0.03 – 0.04g of 0.5mm silica homogenisation beads, and immediately frozen at -20°C. At this stage all small flasks were randomly assigned to one of four treatment temperatures (28°C, 32°C, 36°C, 40°C) or one of two control temperatures (18°C positive control and 70°C negative control), resulting in a sample size of three flasks per temperature treatment per isolate. 28°C is generally accepted as the thermal maximum for *Bd*, although some isolates have been observed to survive temporary incubation at this temperature or higher (Piotrowski, Annis & Longcore, 2004; Stevenson *et al*., 2013; Voyles *et al*., 2017).

As I wanted to explore the thermal limits of the fungus, 28°C was selected as the lowest temperature treatment. Each flask represented a single replicate and each temperature had three replicates per isolate. Due to limitations in time and resources, not all treatments could be carried out in the same model of incubator. However, incubators were independently monitored using an iButton data logger for a week before the experiment took place and throughout incubation, as well as traditional thermometers being placed in each incubator where they could be checked without opening the

incubator door to ensure temperatures remained stable. The 70°C negative control flasks were incubated in a hot water bath and independently monitored with a thermometer. Incubation time was two hours measured from when the incubators reached target temperature (as this dropped when the doors were opened to put the flasks inside). Following two hours incubation, flasks were left at room temperature for two hours and then returned to the 18°C incubator for seven days. After seven days, another 100µl of culture was removed from each flask and transferred to a Safe-Lock Eppendorf tube prepared as before, and also immediately frozen at -20°C until further processing could take place. Figure 4.5 shows the experimental process for a single isolate at one treatment temperature. In total, the isolates were allowed to grow for nine days as pilot trials measuring optical density with a microplate reader showed that peak density was achieved at around 10 days of incubation at 18°C (Appendix 8).

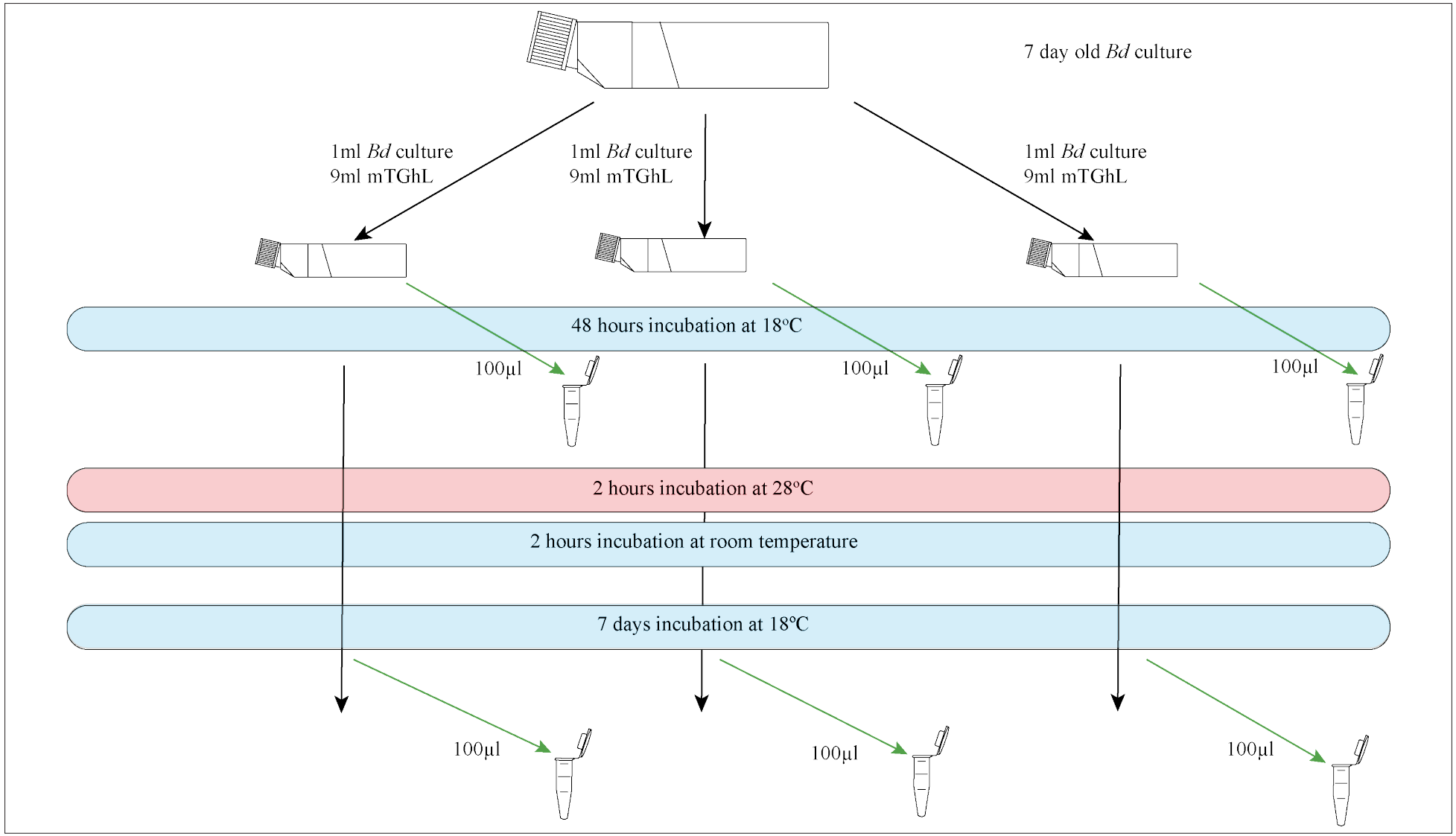


Figure 4.5. Diagram of *in vitro* heat-shock experimental process for one isolate in the 28°C treatment group.

To process samples, each 1.5ml Safe-Lock Eppendorf tube was thawed at room temperature and then centrifuged at 7,000rpm for 10 minutes. Following centrifugation, the supernatant was pipetted off, taking care not to disturb the DNA pellet. 60µl of Prepman Ultra was added to each tube and then the DNA was extracted following the *RACE* protocol (Section 2.6). DNA extracts were diluted 1 in 10 for qPCR analysis with the *Bd* pan-lineage diagnostic following the standard protocol (Boyle *et al*., 2004) and with DNA quantitation standards from isolate IA042 to quantify the mean concentration of zoospores at the beginning and end of the experiment for each replicate of each isolate at every temperature.

All analysis for the *in vitro* experiment was conducted using RStudio v1.2.14335. *Bd* isolates which showed zero growth in the positive control treatment of 18^{o}C (two *Bd*CAPE, SA546 and SA280, and one *Bd*GPL, NC82.1) were removed from the analyses. In addition, two isolates showed growth in a single replicate at 70^{o}C. These were assumed to be due to contamination and removed as well. This left four *Bd*GPL isolates, three *Bd*CAPE isolates and one hybrid isolate. The relative growth (final GE – baseline GE) was calculated for each isolate at each temperature. Growth data was visualised using box and whisker plots and bar plots. A linear mixed effects model (R package lme4 v1.1.21 (Bates *et al*., 2015)) was used to examine *Bd* growth as a function of temperature, lineage and their interaction, with *Bd* isolate included as a random effect. *Bd* growth data was log transformed to improve model fit. Quantile-quantile plots and histograms were used to check normality of model residuals. P-values for fixed effects were approximated using the Kenward Rogers method using the mixed package (Singmann *et al*., 2017). Tukey HSD was used to carry out post-hoc pairwise comparison tests (R package multcomp v1.4.10 (Hothorn, Bretz & Westfall, 2008)) (Appendix 10).

4.3.5 Genomic analyses of *Bd*CAPE and *Bd*GPL isolates

The combined VCF file described in Section 4.3.2 was used to generate a Minimum Spanning Network (MSN) (showing the genetic, nei, distance between isolates), a Principal Coordinate

Analysis (showing separation of lineages) and isolation by distance analyses for each lineage. The MSN was created using the poppr package (v2.8.1), the Principal Coordinate Analysis was generated using the adegent package (v2.1.1) and visualised using ggplot2 (v3.1.0). The isolation by distance analyses show the correlation between geographic (Euclidian) distance and genetic distance (Nei) for each lineage group. Euclidian distances were calculated using the R function dist in the R stats package (v3.5.1) and the genetic distance Nei, which assumes that genetic distance arises due to mutation and genetic drift, was calculated using the StAMPP package (v1.5.1). A Mantel test was carried out to assess if correlations between the genetic and geographic distances were statistically significant. An isolation by distance (IBD) model assumes genetic differentiation as a function of distance (Jenkins *et al*., 2010) with a high R^2 value indicating that genetic distance and geographic distance are correlated. IBD does not take into account any geographic complexity that may predict population structure and is used here as an exploratory baseline analysis to investigate whether either lineage deviates from a simple genetic distance/geographical distance correlation.

4.4 Results

4.4.1 Field data collection

1,477 individual amphibians were sampled over the three years of fieldwork; 40 new isolates of *Bd* were collected of which 22 have been whole genome sequenced (O'Hanlon *et al*., 2018). 32 species of amphibian were sampled, as well as some individuals that were either unidentifiable or could only be typed to Genus level (8.17% of total samples). The *Amietia* dominated sampling, representing over 60% of all samples collected (Figure 4.6). In total, seven *Amietia* species were collected throughout the three years, the vast majority of which were *Amietia delalandii* (26.25% of total samples) and *Amietia quecketti* (21.06% of total samples).

Lineage-specific qPCR was successfully used to delineate *Bd* lineage distributions in South Africa and the Lesotho highlands over one of the largest contiguous areas to date, including the identification of a zone of regular lineage co-occurrence in the Northern Cape Province (Figure 4.7). A further potential recombinant zone was identified close to the Drakensberg Mountains, between Royal Natal National Park and Memel. Unfortunately, weather conditions throughout the study meant that this zone could not be further investigated, and no isolates were obtained from the intermediary area after 2016. The highest number of samples for qPCR was collected in 2016 (466 individuals) from 18 sites as in this year two field trips were run, one led by collaborators at NWU, to attempt to develop baseline data on which to base future work rapidly. In 2017, 310 samples were collected for qPCR from 15 sites and in 2018, 327 samples were collected from 14 sites.

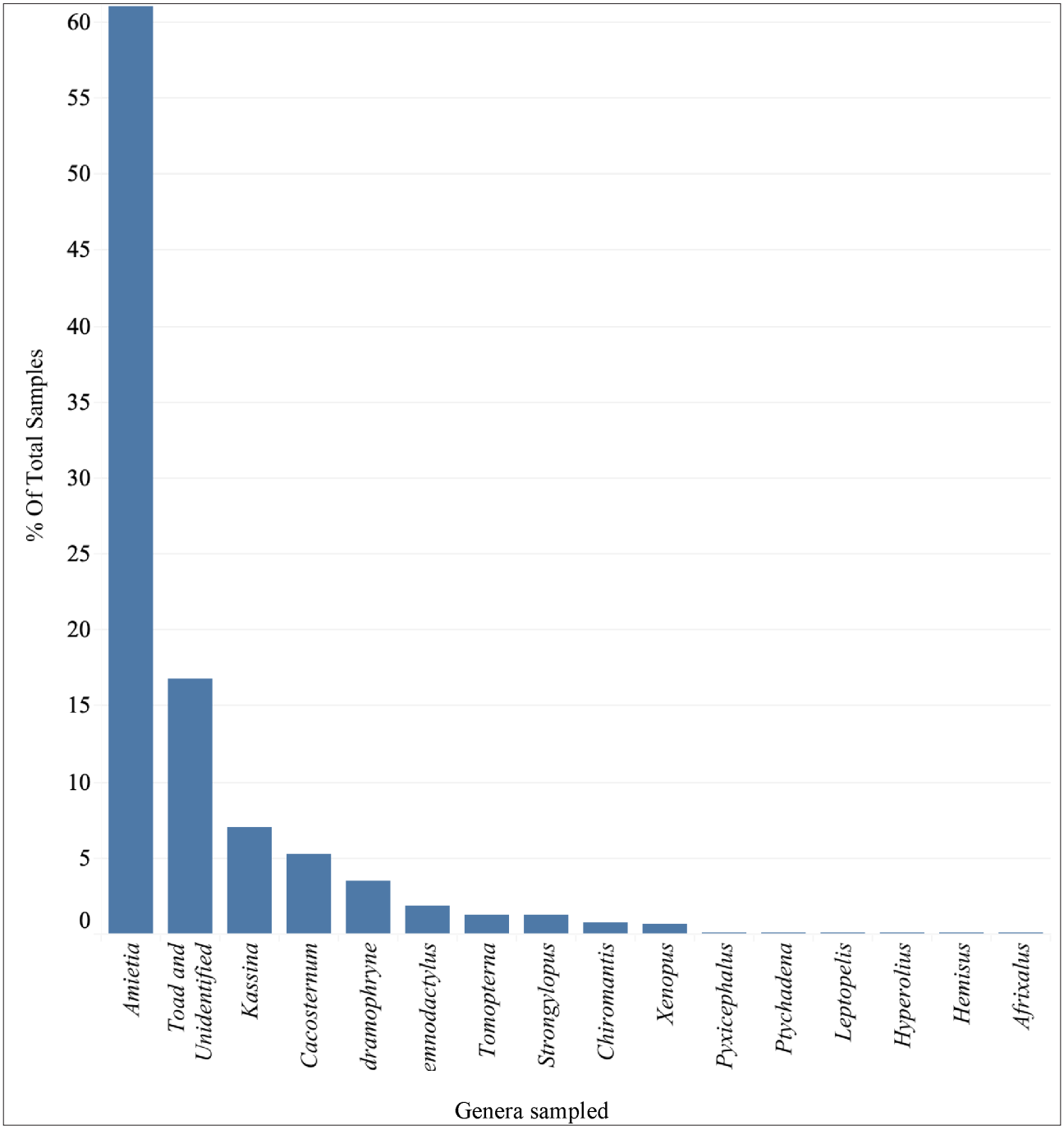


Figure 4.6. Genera collected throughout fieldwork, as a percentage of the total number of samples collected.

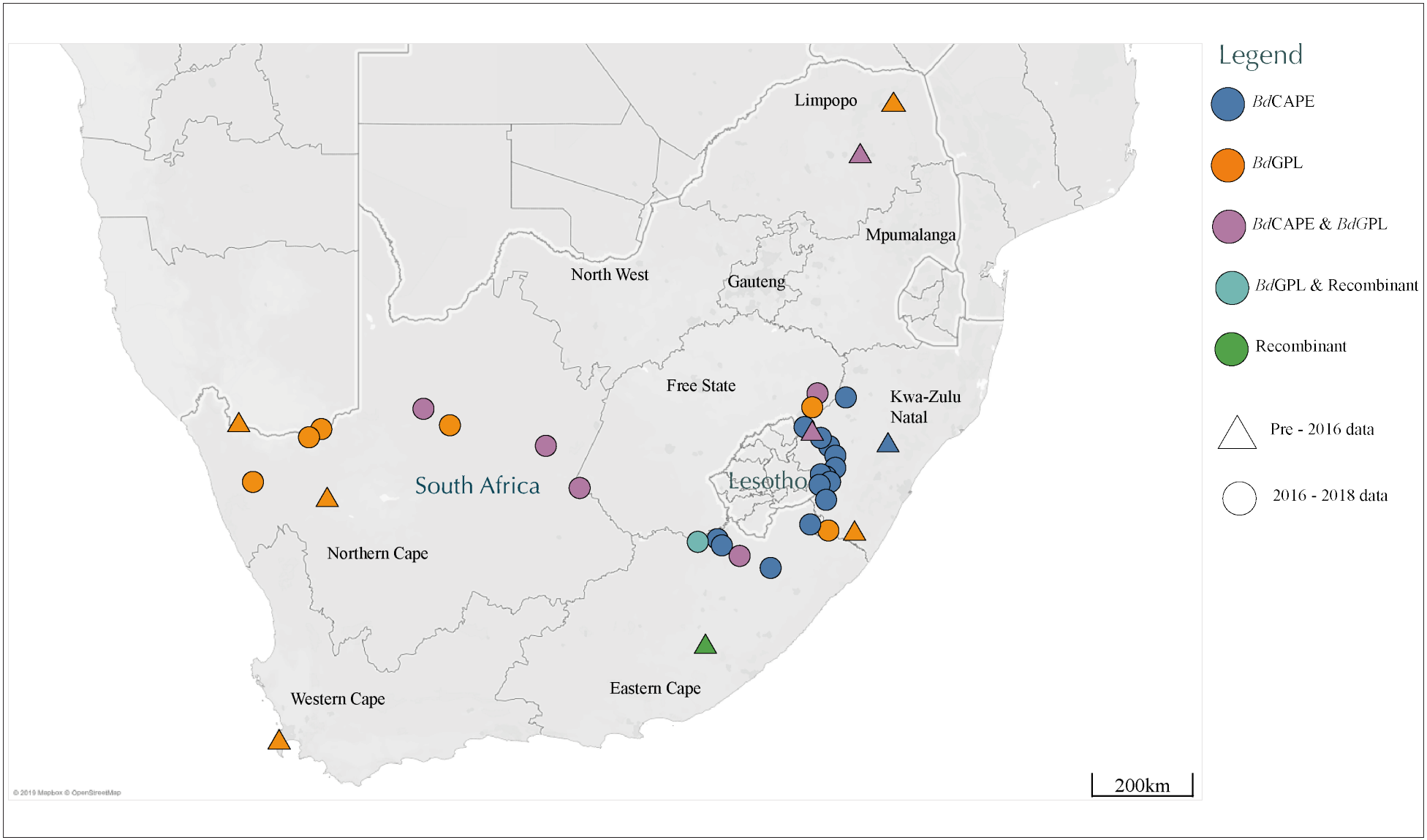


Figure 4.7. Updated map of *Bd* lineage distributions in South Africa and Lesotho. Map generated in Tableau.

Mean *Bd* prevalence was highest in 2016, at 28.97% (95% CI = 24.9% to 33.3%) reflecting the fact that sampling in this year was carried out in what turned out to be hot spots for *Bd*GPL and *Bd*CAPE, in the Northern Cape and in the Drakensberg, respectively. Mean *Bd* prevalence in 2017 and 2018 were extremely similar, at 10.97% (95% CI = 7.7% to 15.0%) and 10.7% (95% CI = 7.6% to 14.6%) respectively. It is worth noting that the small sample sizes collected mean that these prevalence estimates are associated with a high variance and therefore a change in prevalence between years may simply be undetected rather than absent (Skerratt *et al.*, 2008). In 2017 and 2018, some *Bd* cultures were isolated from sites that did not return a positive result by pan-lineage qPCR, highlighting the importance of a large sample size and multi-year sampling to combat the risk of false negatives, fluctuations of detectability and natural variation in prevalence.

In total, 16 *Bd*CAPE-positive sites and six *Bd*GPL-positive sites were identified as part of this study. Additionally, six sites returned isolates of both lineage or a lineage and a recombinant (n = 2), or tested positive for both lineages via lineage-specific qPCR (n = 4). Combined with sites previously typed for lineage using WGS, in total 17 *Bd*CAPE-positive sites, 11 *Bd*GPL-positive sites and nine mixed-lineage or recombinant sites have now been identified across South Africa and Lesotho. As expected, all isolates collected during this work were either *Bd*GPL or *Bd*CAPE or a recombinant lineage (Figure 4.8).

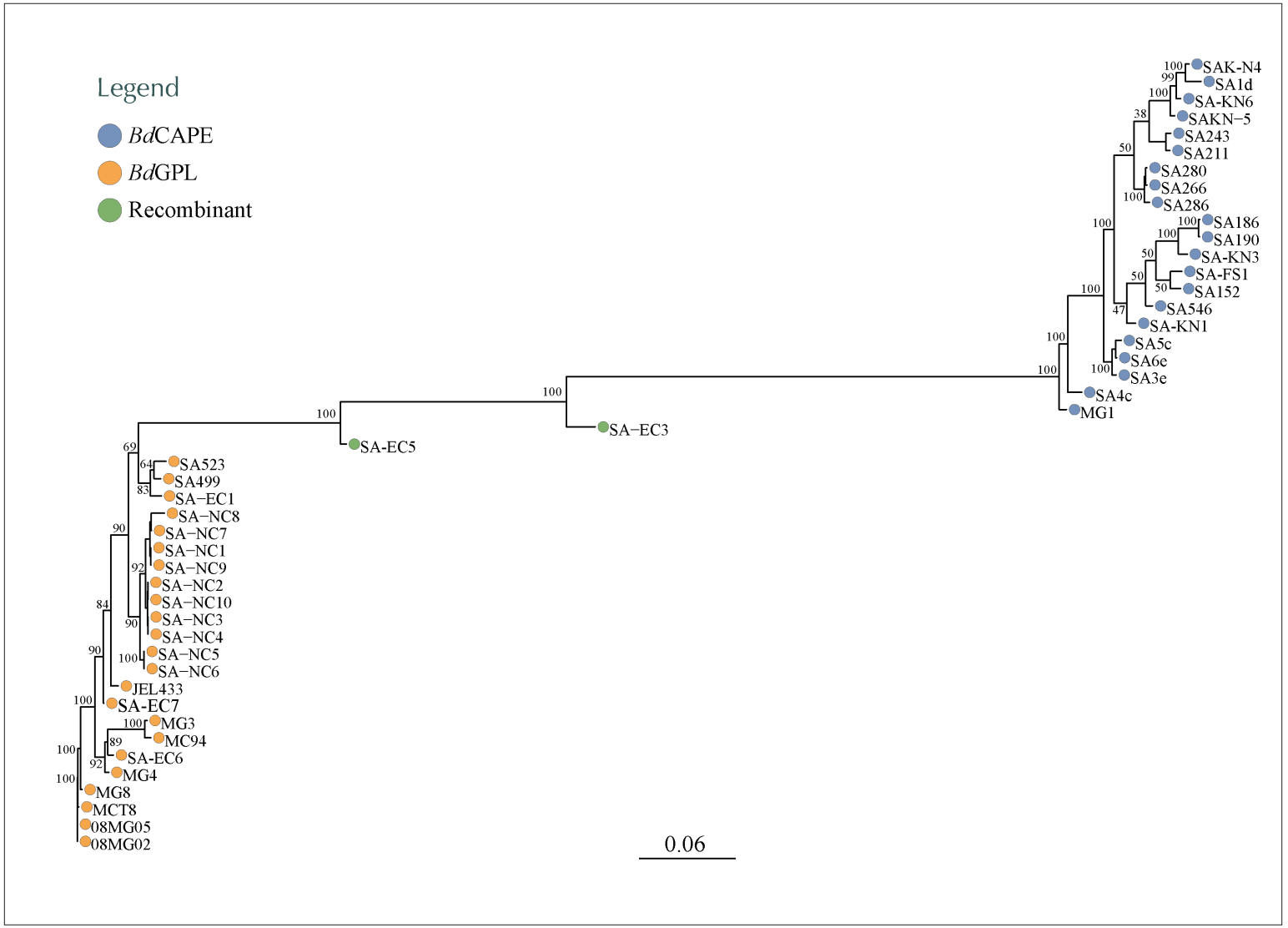


Figure 4.8. Unrooted phylogeny of South African and Lesothoan *Bd* isolates.

4.4.2 Site lineage identification correlates with environmental parameters

Bd was detected across the entire range of environments sampled, from the border of the Karoo in the Northern Cape to the top of the Drakensberg (Figure 4.7). When the proportion of infected animals at each site is taken into account (according to lineage-specific qPCR with data from WGS isolates removed from the map), it is clear that *Bd*GPL and *Bd*CAPE have very different areas in which they are most likely to be found. Overlaying the data on a satellite image highlights the different environments of the two lineage hot spots (Figure 4.9).

The Northern Cape on the western coast of South Africa is extremely hot and dry, bordering the Karoo semi-desert, and *Bd*GPL has highest prevalence in this region. In contrast, *Bd*CAPE has highest prevalence in the Drakensberg Mountains, where temperatures are cooler and precipitation and elevation are higher. No *Bd*CAPE isolates were found in the *Bd*GPL hotspot, and aside from a single isolation of *Bd*GPL in the Drakensberg in 2008, there are no records of *Bd*GPL in the *Bd*CAPE hotspot. Furthermore, the prevalence-scaled map shows that the centre of South Africa is a cold spot for both lineages.

Box and whisker plots of environmental variables plotted against the site lineage identification (Figure 4.10) show that sites positive for *Bd*GPL are drier and hotter than sites that are positive for *Bd*CAPE, and also that *Bd*GPL-positive sites have a higher variance in environmental conditions than *Bd*CAPE-positive sites. A scatter plot comparing the mean daily precipitation and mean annual temperature of different sites further illustrate the correlation between the environmental variables and the lineage identification of a site (Figure 4.11). *Bd*CAPE-positive sites are clustered at the low temperature, high precipitation end, while the *Bd*GPL-sites tend towards the high temperature, low precipitation side of the plot, but are scattered much more widely than *Bd*CAPE-sites. Mixed-lineage sites are distributed throughout the range. A notable outlier for *Bd*GPL is the isolate collected at Harding, which falls to the extreme end even of the *Bd*CAPE sites in terms of both environmental variables, but particularly precipitation (Figure 4.11). The lineage separation is even

more apparent when the data (with WGS-typed only sites removed, due to an inability to compare prevalence across methods) is scaled by prevalence of *Bd* (Figure 4.12). There was no significant difference in prevalence between *Bd*GPL, *Bd*CAPE or mixed and qPCR-typed sites, but it is possible that the sample sizes were too low for a difference to be detected.

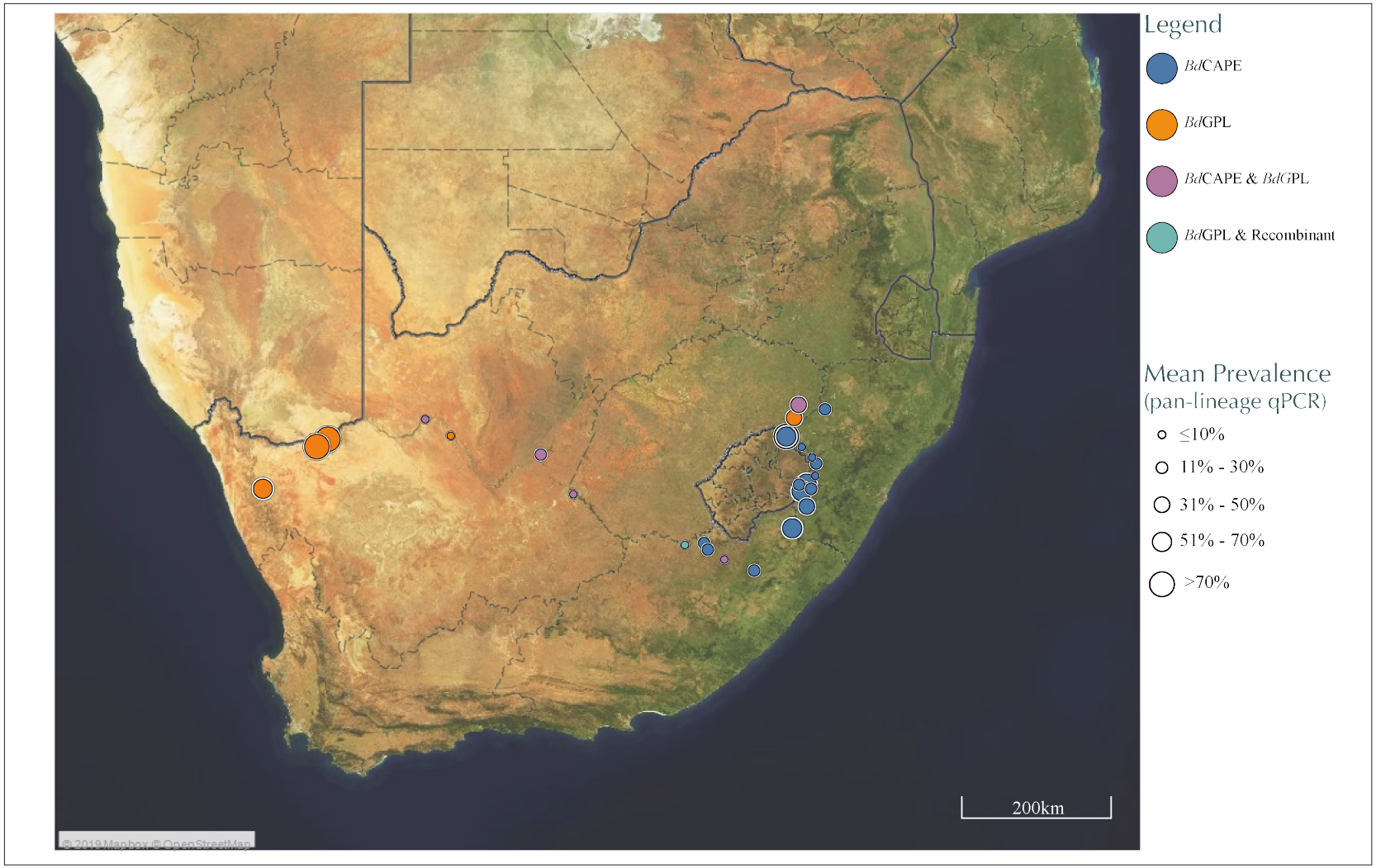


Figure 4.9. Satellite map of *Bd* lineage distributions in South Africa and Lesotho, scaled by prevalence of *Bd* at each site. Map generated in Tableau using the Satellite base map sourced from ©Mapbox ©OpenStreetMap.

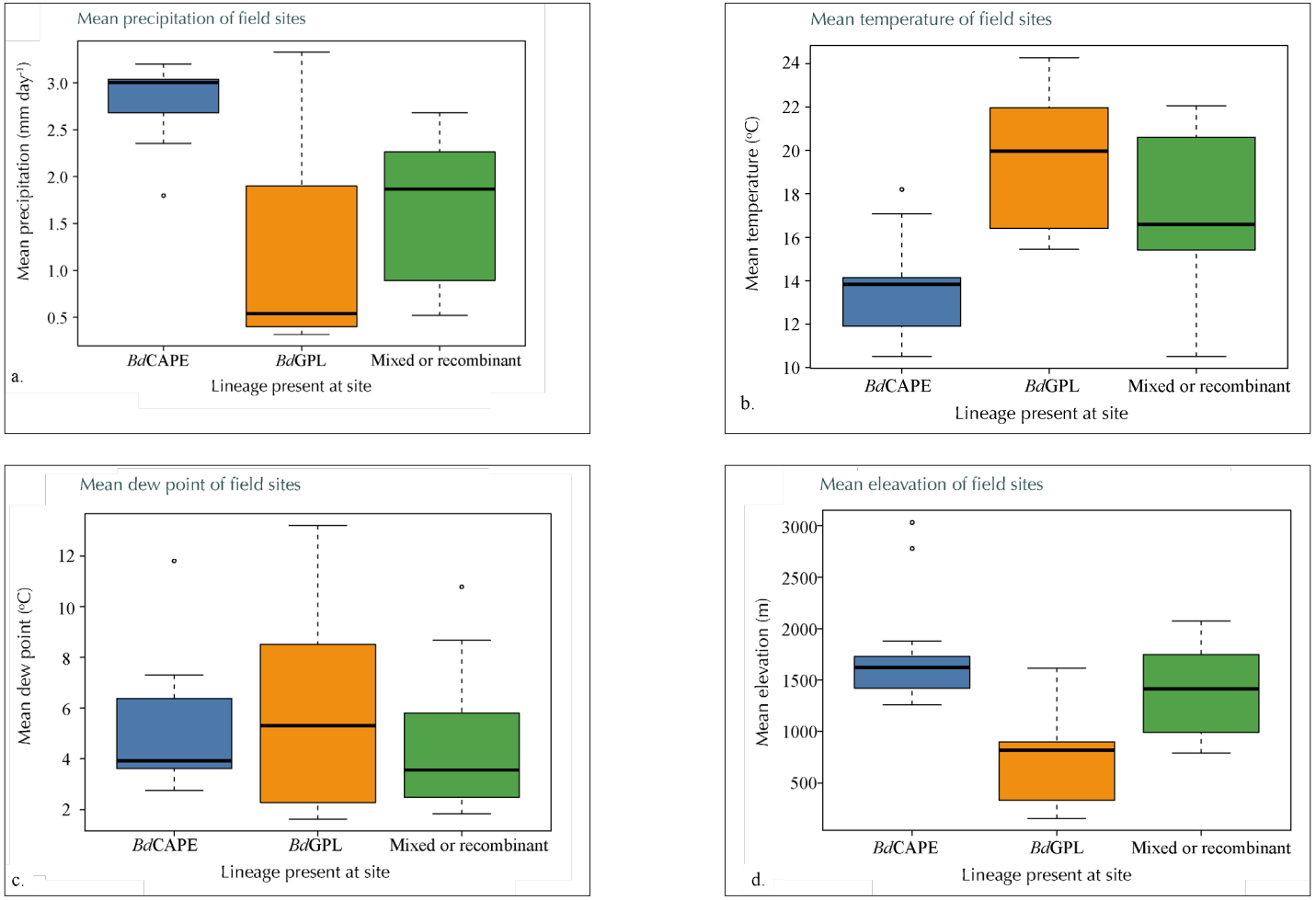


Figure 4.10. Box and whisker plots of environmental site variables against lineage detected showing (a) mean precipitation, (b) mean temperature, (c) mean dew point and (d) mean elevation. Boxes indicate the interquartile range (IQR), the horizontal black line indictes the median, the upper whisker limit indicates the 75th percentile + (1.5 x IQR), the lower whisker limit indicates the 25th percentile - (1.5 x IQR).

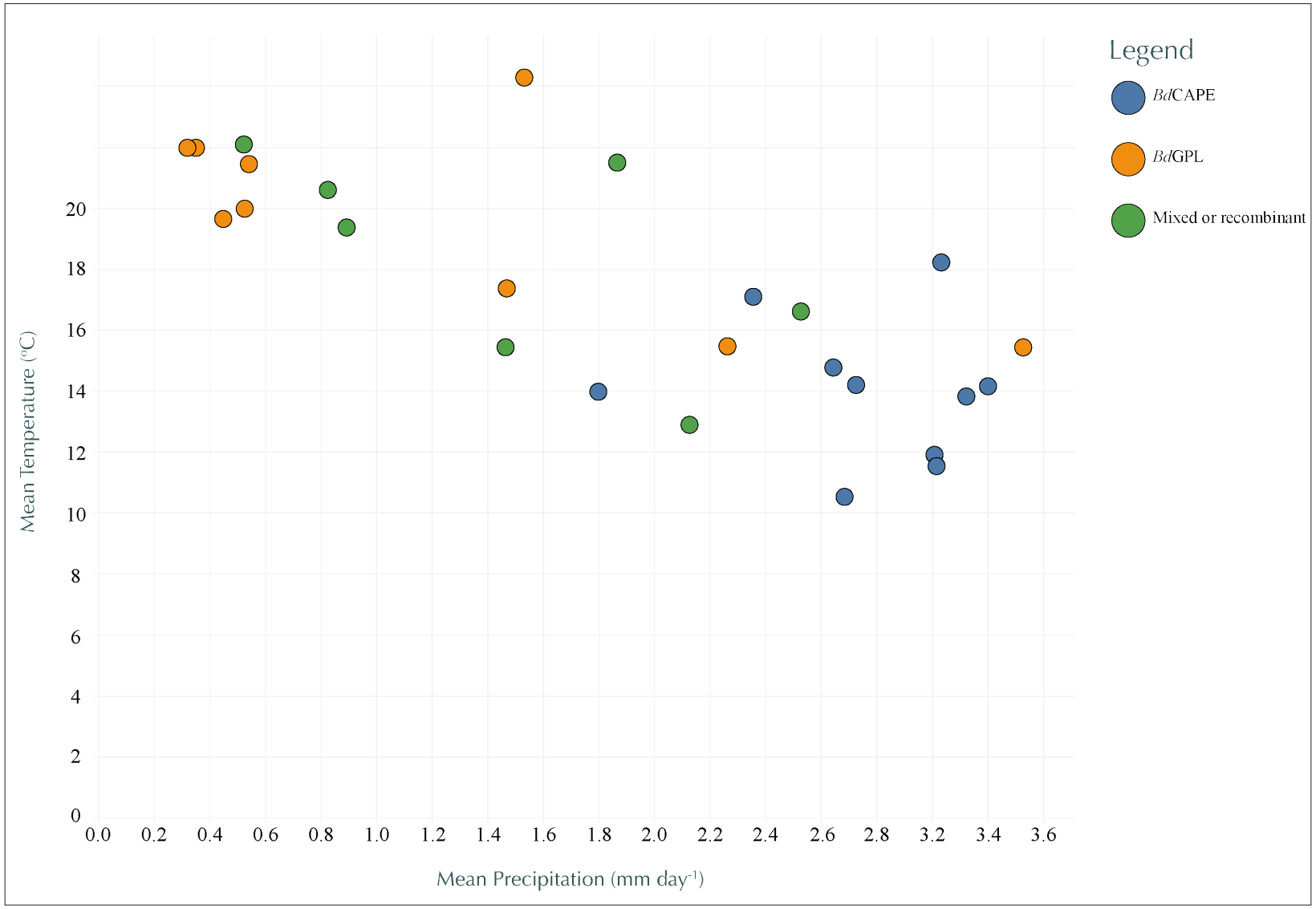


Figure 4.11. Scatter plot showing mean temperature and mean precipitation at sites where lineage was typed.

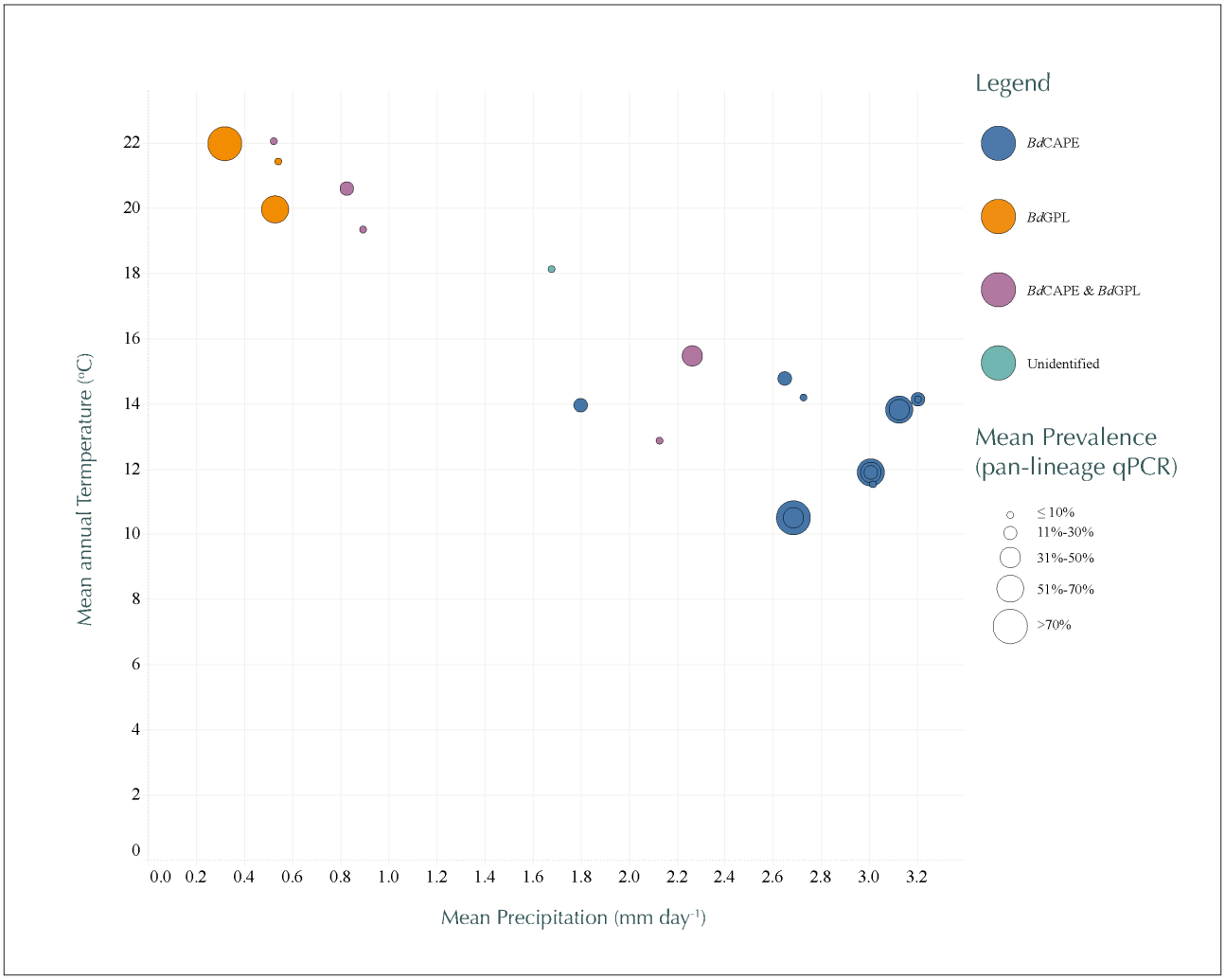
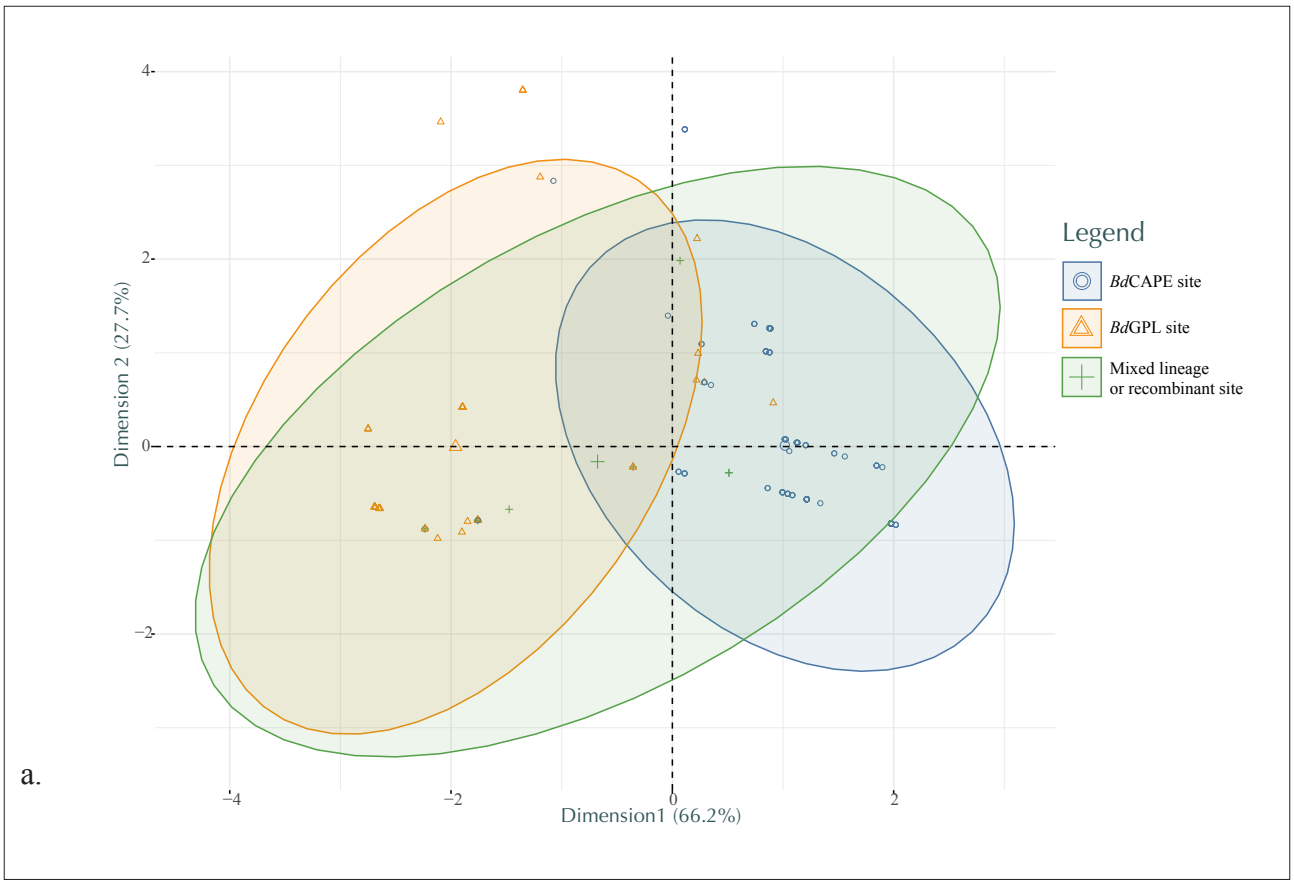
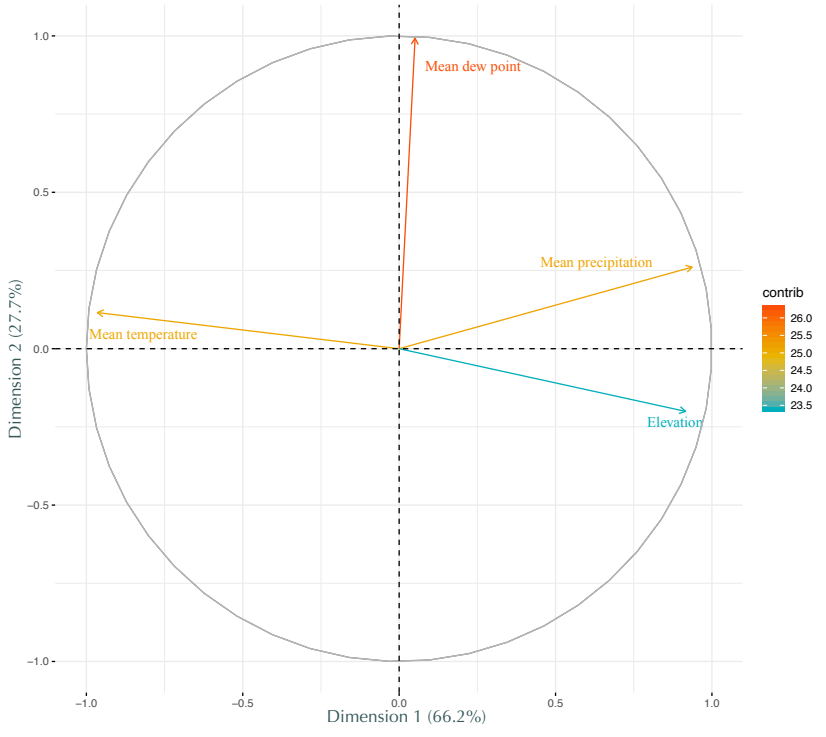


Figure 4.12. Scatter plot showing qPCR lineage-typed sites only, scaled by prevalence of *Bd* at each site.

The Kruskal-Wallis test showed there was a significant difference between groups for all variables tested except for the mean annual dew point (mean annual temperature; mean daily precipitation and elevation $p<0.05$). The Wilcoxon Rank Sum tests showed that the mean annual temperature and mean annual precipitation of *Bd*CAPE-positive sites were significantly different from both *Bd*GPL and mixed-lineage sites ($p<0.05$), but that *Bd*GPL and mixed-lineage sites did not significantly differ from each other. *Bd*CAPE and *Bd*GPL sites were also significantly different in terms of elevation ($p<0.05$), which is likely highly correlated with the environmental variables. Mixed-lineage sites, in contrast to the result seen for mean annual precipitation and temperature, were significantly different from *Bd*GPL sites in terms of elevation ($p<0.05$), but not *Bd*CAPE sites. No lineage groups were significantly different from each other in terms of the mean annual dew point.

Results from the PCA also showed that *Bd*CAPE and *Bd*GPL were associated with different environmental conditions, with recombinants and mixed-lineage samples being distributed across most of the range of both lineages, but not at the extreme of either single lineage distribution (Figure 4.13a). Dimensions 1 and 2 accounted together for over 90% of the variance in the data set (Figure 4.13b). Dimension 1 closely corresponded to an interaction between mean annual temperature, mean daily precipitation and elevation, and Dimension 2 corresponded to the mean annual dew point (Figure 4.13b). Eigen values and additional plots relating to the PCA are provided in Appendix 9.





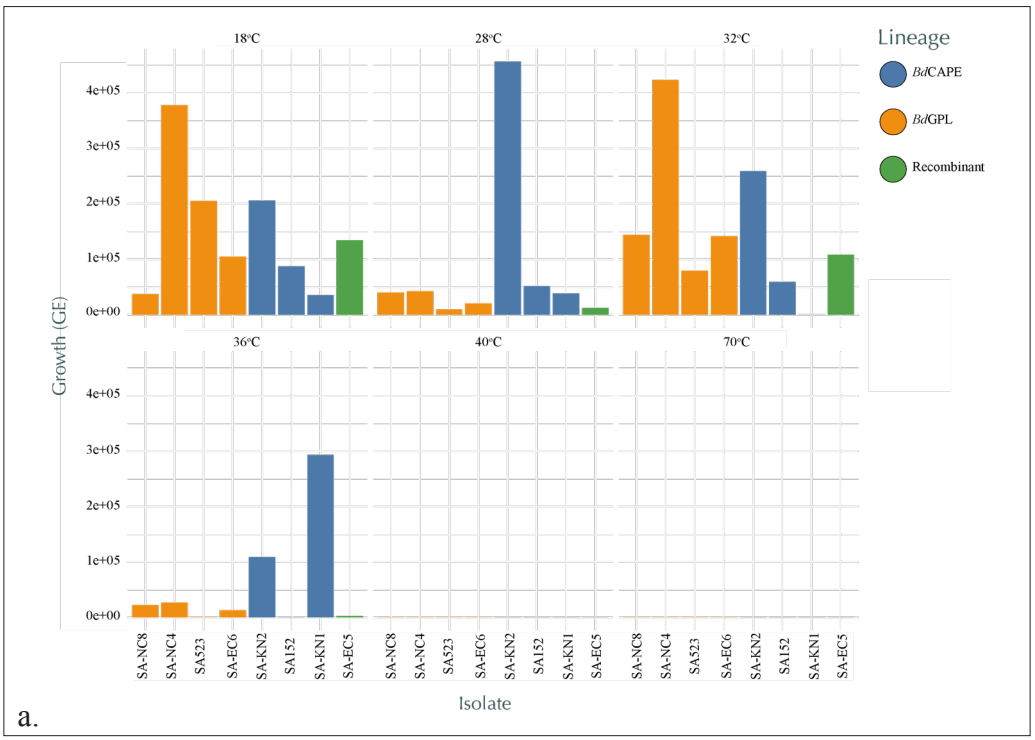
b.

Figure 4.13 Principal component analysis visualisations showing environmental variables accounting for majority of variation between lineages identfied at field sites in South Africa. (a) PCA plot showing separation of lineage-typed sites along environmental variables and (b) correlation of variables to Dimensions 1 and 2.

4.4.3 Lineage does not affect heat shock response

All analysed isolates grew following incubation at 28°C, which is generally accepted as approximately *Bd*'s thermal maximum (Piotrowski, Annis & Longcore, 2004; Stevenson *et al*., 2013; Sonn, Berman & Richards-Zawacki, 2017) (Figure 4.14). Surprisingly, all but one isolate also grew following incubation at 32°C, and two *Bd*CAPE isolates grew following incubation at 36°C. Curiously, an isolate that did not grow following incubation at 32°C did grow following incubation at 36°C.

The mixed effect model showed that only temperature had a significant effect on relative growth rate ($F=43.48$, $p<0.05$). An effect of lineage, or an effect of any interaction between temperature and lineage, on *Bd* growth was not detected. Plots of residuals and results of post-hoc multiple comparisons using Tukey HSD are provided in Appendix 10.



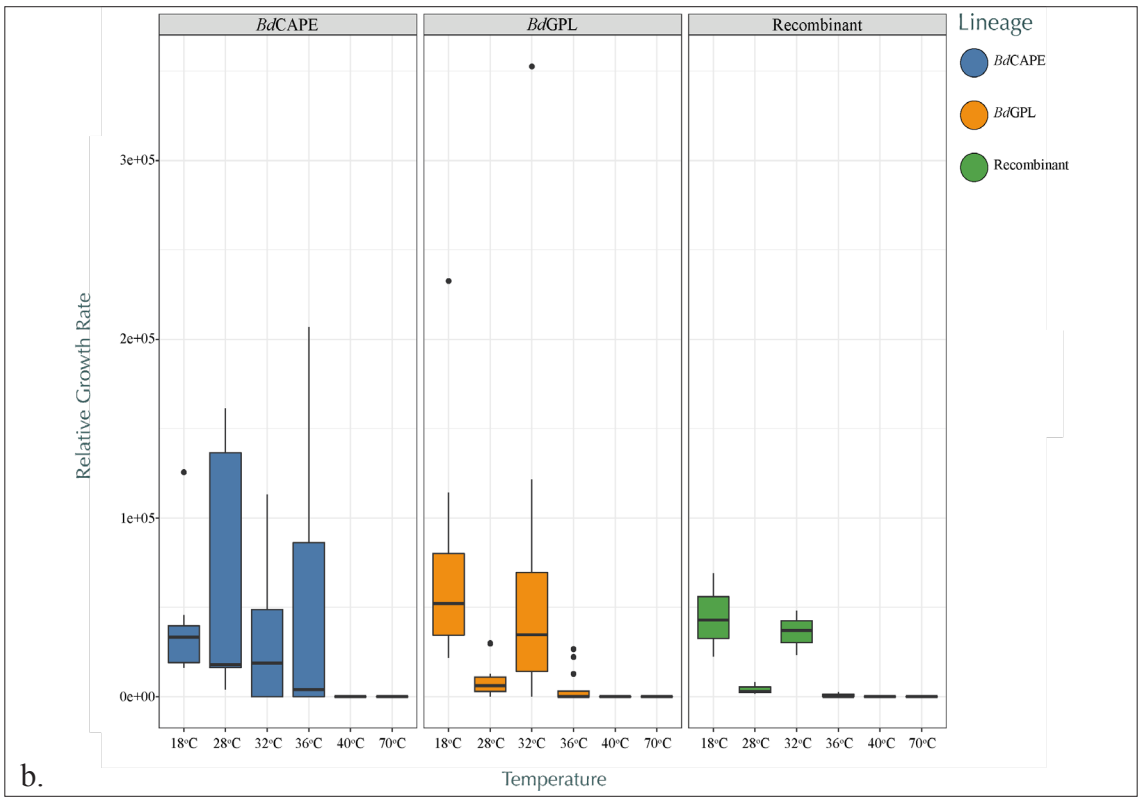
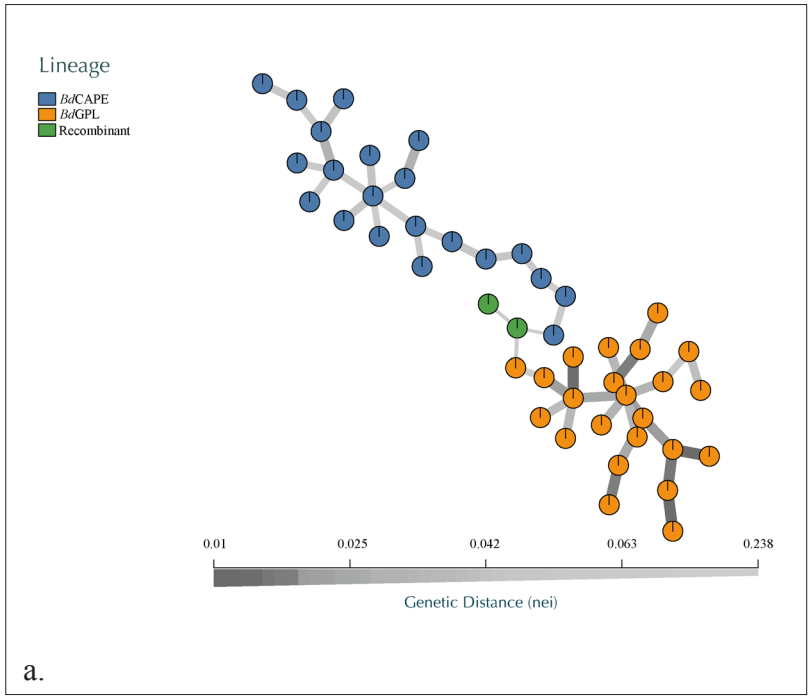
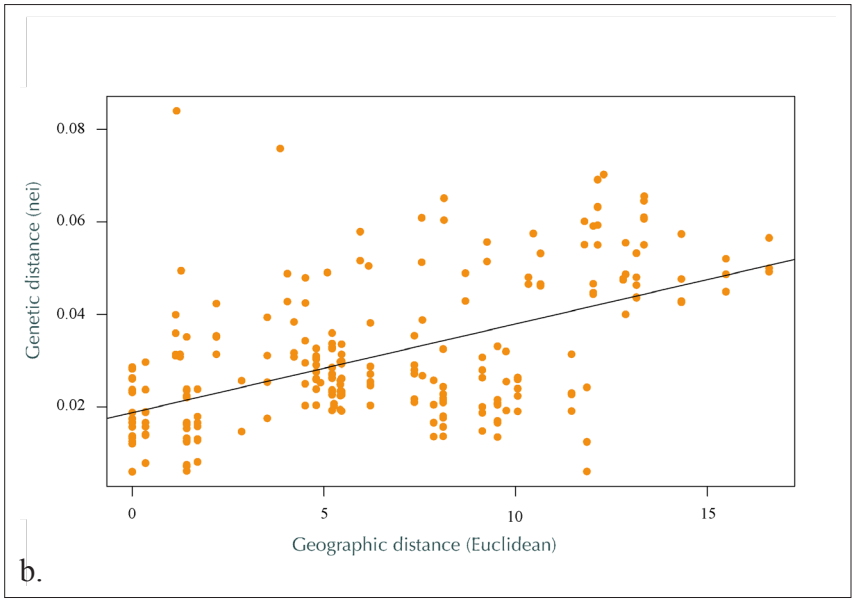


Figure 4.14. Bar and box and whisker plots for *in vitro* heat shock experiment. (a) Bar plot of isolate growth at each temperature treatment. (b) Box and whisker plot of mean growth of lineages at each temperature treatment. The boxes represent the IQR, the median is indicated by the horizontal black line, the upper whisker limit indicates the 75th percentile + (1.5 x IQR), the lower whisker limit indicates the 25th percentile - (1.5 x IQR).

4.4.5 *Bd*CAPE shows evidence of stronger population structure, and higher genetic diversity, than *Bd*GPL

The minimum spanning network confirms the lineage identifications resolved in the phylogeny, but illustrates the greater clonality among *Bd*GPL isolates compared with *Bd*CAPE isolates (Figure 4.15a). Closer genetic relatedness is illustrated by darker and thicker connecting lines between nodes, which represent individual isolates. IBD analyses were carried out on both lineages to see if there was evidence of any non-random population structure. As can be seen from Figure 4.15b, *Bd*GPL shows strong isolation by distance, with an R^2 value of 0.31 ($p<0.05$). Conversely, *Bd*CAPE (Figure 4.15c) shows evidence of a more complex population structure, with the isolates falling into two clusters and no significant correlation between genetic and geographic distance ($R^2 <0.05$, $p >0.05$). The presence of two *Bd*CAPE clusters was also seen in the Principal Coordinate Analysis (Figure 4.16a). The first two principal components here account for 94.89% of the total genetic variation. Further investigation reveals the two *Bd*CAPE clusters to be both temporally and spatially clustered (Figure 4.16b). All isolates in Cluster 1 were collected in 2017, whereas isolates in Cluster 2 were collected during a range of years from 2008 to 2016, indicating that these data are also temporally confounded. Therefore, it is not possible to identify whether the clustering is due to a temporal effect (and therefore, possibly an artefact of sampling) or due to reproductive barriers.





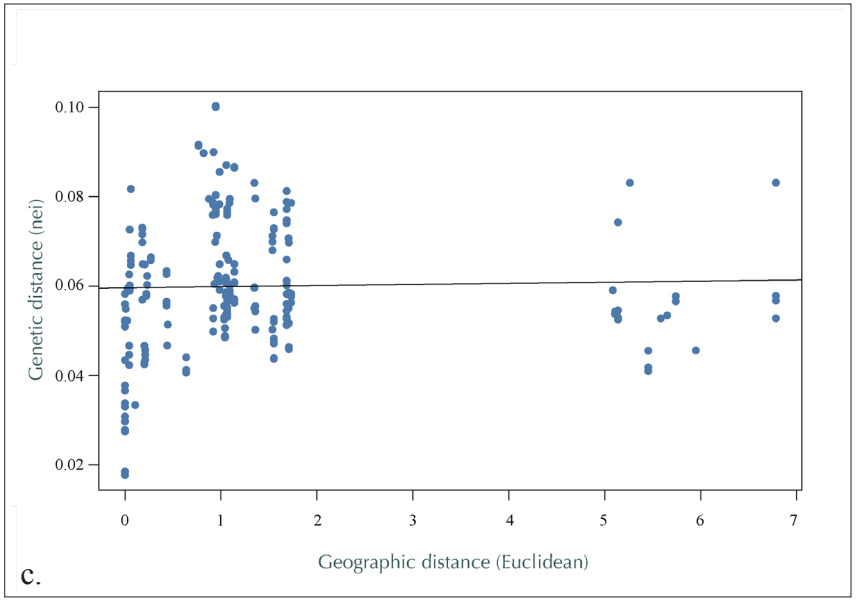


Figure 4.15. Comparative genomic analyses of *Bd* lineages (a) Minimum Spanning Network showing genetic distance between isolates, (b) IBD plot for *Bd*GPL and (c) IBD plot for *Bd*CAPE.

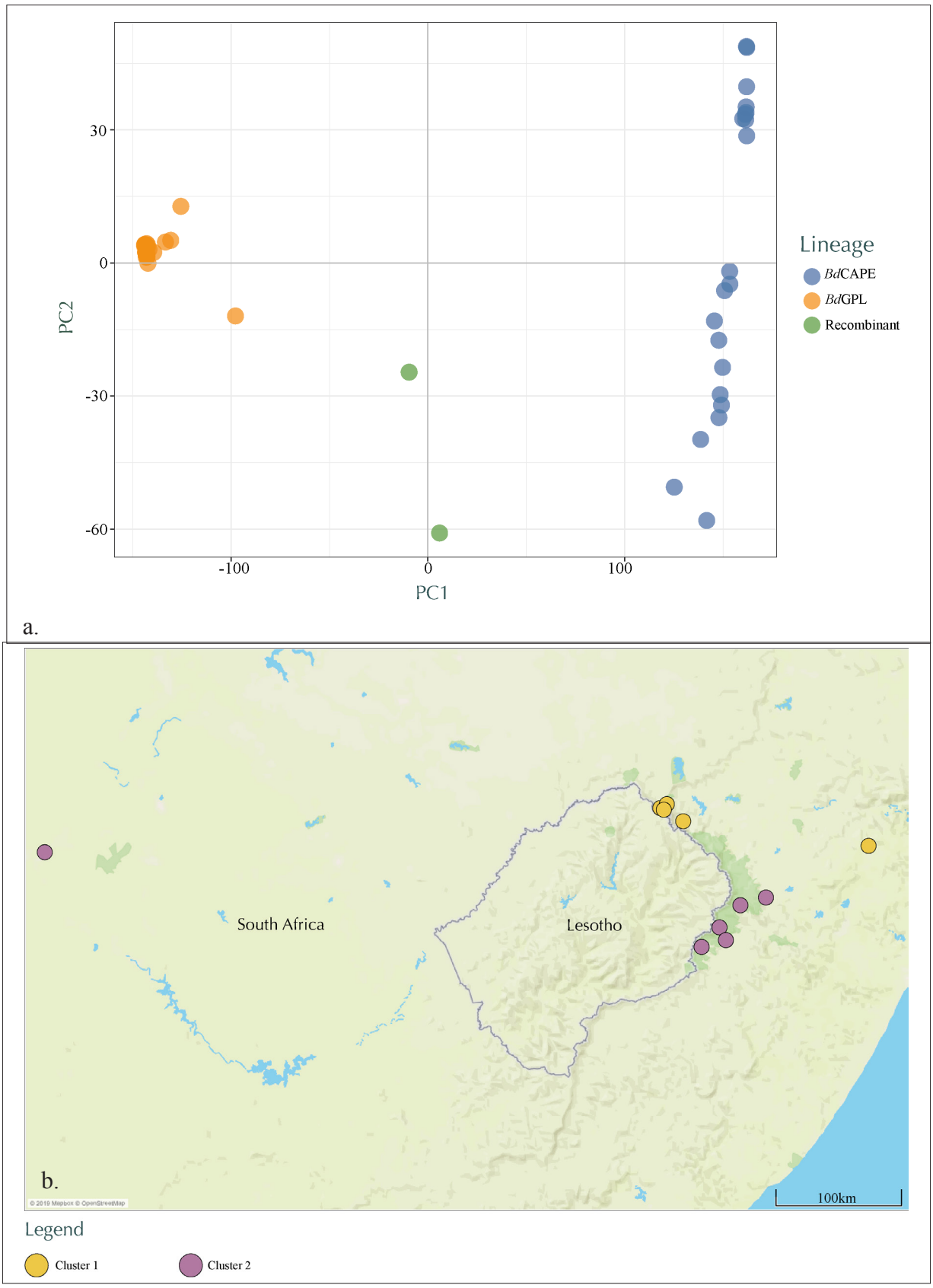


Figure 4.16. *Bd*CAPE population clusters shown in (a) a Principal Coordinate Analysis of *Bd*GPL, *Bd*CAPE and recombinant genomes and (b) on a terrain map of South Africa and Lesotho showing location around the Drakensberg Mountains. Map generated in Tableau using the Outdoors base map sourced from Mapbox OpenStreetMap.

4.5 Discussion

In this chapter, the distributions of two lineages of *Bd* across one of the largest areas to date is described. Different environmental profiles for *Bd*GPL and *Bd*CAPE were observed and the results show that *Bd*GPL and *Bd*CAPE have fundamentally different population structures in South Africa. The isolates collected as part of this work have, in line with previous research, shown that *Bd*CAPE harbours more genetic diversity than *Bd*GPL, and also that lineage recombination is taking place in Southern Africa. As well as the recombination sites, one area of lineage co-existence has been identified as well as another putative contact zone. Furthermore, although experimental work to investigate the lineage response to heat shock revealed no lineage effect, it was shown that some *Bd* isolates are able to survive two hours incubation at 8^{o}C above the published thermal maximum.

4.5.1 Distribution of lineages along the Orange River

The data gathered here show that *Bd*GPL and *Bd*CAPE are strongly associated with different environmental conditions in the wild in Southern Africa. *Bd*GPL is generally found at sites that have a higher mean temperature and lower mean daily precipitation level, while *Bd*CAPE is found in cooler, wetter sites. Each lineage appears to have a particular 'hot spot' where they are found at high prevalence; *Bd*GPL has highest prevalence on the western coast, near the mouth of the Orange River, while *Bd*CAPE is most likely to be found in the Drakensberg mountain range. Where the prevalence of one lineage was particularly high, no evidence of the other lineage was found. As well as the identification of lineage hot spots, a lineage cold spot could be seen in the middle of the Orange River course. This cold spot was a region where neither lineage had high prevalence, but both could be found regularly. The results of the Orange River transect could be explained in two ways; the lineage distributions seen here are the result of lineages assorting along environmental lines due to niche separation. In this case, the distributions would be expected to stay stable over time, with lineage contact and potentially recombination, or lineage competition, where the ranges overlap at the environmental niche margins. In this case, the low prevalence of lineages in the

central region of the Orange River could be due to each lineage being at the edge of its optimal thermal envelope. Alternatively, one or both lineages encompass the entire range within their fundamental niche, and one or both are migrating towards each others' hotspots. In the latter case, it is possible that lineage contact could result in lineage replacement through competition, and under this scenario it would be expected that if the same transect is monitored over multiple years, the lineage distributions will shift. The low prevalence at the centre of the transect could in this case be due to mutual suppression through competitive interactions. The identification of this cold spot presents a target for future research; by identifying factors that are limiting lineage proliferation in this area, there is huge opportunity to gain insight into the drivers of *Bd* elsewhere (James *et al*., 2015).

4.5.2 Exploration of mixed-lineage and recombinant sites

The large environmental space occupied by the mixed and recombinant group in the PCA is curious. It seems surprising that if such a large environmental range could be associated with this group, that only two recombinant isolates have been successfully identified and isolated in culture. It is possible that this means even if *Bd*GPL and *Bd*CAPE encounter each other, that recombination occurs rarely, due to some kind of reproductive barrier between the lineages. Alternatively, recombination may occur frequently, but the offspring isolates are less fit than either parent lineage and therefore do not exist at a high enough frequency to be regularly isolated. This finding links to the field site at Douglas, where both *Bd*GPL and *Bd*CAPE exist in a single pond, but from where no recombinant isolates have been identified. Long-term monitoring of this site and the surrounding area presents an opportunity to shed light on lineage interactions in nature.

The huge environmental envelope indicated by the mixed and recombinant sites however, also raises the possibility that the *Bd*GPL and *Bd*CAPE fundamental niche envelopes may be wider than that which is currently observed in nature. If this is the case, either mutual competitive suppression may be resulting in the appearance of stronger clustering than would occur otherwise, or the

lineages are spreading outwards from separate introduction points and simply have not colonised the intermediary environmental regions yet.

4.5.3 Lack of evidence for a lineage effect on isolates' response to heat shock under experimental conditions

Results from *in vitro* experimentation to determine the lineages' response to heat shock showed a significant effect of temperature on *Bd* growth, but no significant difference in growth of lineages at different temperatures, nor was there a signficant effect of any interaction between lineage and temperature on growth. However, the small number of replicates and the diversity of isolates used in this study may mean that the experiment was too underpowered to detect small but significant effects. Furthermore, it is also possible that a lineage effect was not detected due to the high level of variability between replicates combined with a small sample size. Unfortunately, the experiment could not be expanded to include more replicates as it was planned at the maximum that was logistically possible in this instance. The fact that three isolates failed to grow at 18^{o}C is another reason to treat the results presented here with caution, as it suggests experimental error may have affected the experiment. It would be beneficial to re-approach this experiment in the future with increased capacity to attempt to untangle the extent of any inter-lineage or inter-isolate variation and resolve whether there is a mechanistic explanation for the different environmental profiles of *Bd*GPL and *Bd*CAPE seen in the field.

4.5.4 Implications of *Bd*GPL's and *Bd*CAPE's divergent population structures

Analysis of isolates collected during this work have concurred with previous studies showing that *Bd*GPL is less genetically diverse than *Bd*CAPE, and that *Bd*CAPE and *Bd*GPL have recombined in South Africa to produce recombinant isolates. The apparent presence of two clusters within the *Bd*CAPE population suggests that there is some as yet unidentified barrier preventing equal mixing of the two clusters. The two clusters are separated by multiple valleys and river courses, which may be functioning as reproductive barriers. However, Cluster 1 is composed of isolates from the

bottom and the top of the Drakensberg escarpment, a short lateral distance but a vertical distance of approximately 1,000m. There is therefore no obvious reason why the geography of the Drakensberg should represent such a reproductive barrier. To address this question, sampling in between the two clusters would be beneficial to investigate the geography of the intervening area more completely and see which clusters isolates from in between fall into. It would also be beneficial to take on the previous recommendation to sample these sites in multiple years to rule out the possibility that the clusters are due to a temporal effect, since all the isolates in Cluster 2 were collected in a single year. Alternatively, the two clusters could result from two independent introductions. In contrast, the isolation by distance plot for *Bd*GPL suggests that no such reproductive barriers exist in this population. The strong isolation by distance displayed by *Bd*GPL suggests the population has relatively low rates of gene flow, and progressive stepping-stone dispersal from a single point-of-origin, unimpeded by geographical or environmental barriers. Further work should concentrate on identifying point-of-origin locations for both lineages and estimating the rate of spread to establish whether the populations are still on the move, and where they might reach, in order to carry out appropriate risk assessments.

4.5.5 Study limitations and future directions

A limitation of this data set, due to the focus on gathering data from as wide an area as possible, is that the majority of sites were not visited in multiple years. As a result, the data are temporally confounded. A priority for future research should therefore be to revisit as many sites as possible to confirm whether the lineage identification of the site is consistent over multiple years. It is possible that the lineage distributions do not represent a preference of either lineage for particular environmental conditions, but their occurrence is a result of spatial autocorrelation. This could reflect the location of historical introductions and given time or translocation to a new area, existence in different environmental conditions would be perfectly possible. It is not possible to differentiate between these two possibilities based on this data set alone, as it is effectively a single

replicate of a dry and hot to wet and cold environmental gradient. This question could be resolved firstly by confirming the environmental envelope of the lineages under experimental conditions, and secondly the environmental niche separation hypothesis would be greatly strengthened if a similar pattern was found along multiple environmental gradients. Due to the nature of the fieldwork, this was beyond the scope of this research, but should be addressed in future work, with areas of environmental transition a target for future sampling. It would also be helpful in future to return to sites that have only been lineage-typed by isolation and WGS, to carry out sampling for qPCR and obtain consistent prevalence estimates across the entire transect and therefore reveal whether prevalence is also associated with environmental conditions or the lineage at a site. Unfortunately, sampling along the other transect through a putative lineage contact zone, from Royal Natal National Park to Memel in Free State Province was hindered by a severe drought in 2016 and flooding in 2017. This region should be a priority area for future sampling.

The *in vitro* experiment presented here was conceived in recognition of the fact that previous attempts to delineate the environmental envelope of *Bd* have been confounded by lineage. In many cases either all isolates have been from a single lineage (Stevenson *et al*., 2013); the lineage has been unknown or unreported (Seimon *et al*., 2015); the lineage has been typed using the ITS region which is unreliable (Miller *et al*., 2018); or there have been insufficient replicates of isolates for each lineage to unpick inter-lineage variation versus inter-isolate variation (Muletz-Wolz *et al*., 2019). This is a key research gap. Multiple studies have indicated that temperature may be an important epidemiological indicator for *Bd*, so generation of thermal performance curves and determination of the thermal maximum and minima for all lineages is an important next step to confirm the result here that lineage does not determine growth rates at different temperatures. If this is the case, combined with the contrasting level of variance seen in the environmental data for the two lineages, and the wide environmental space occupied by the recombinant and mixed-lineage group in the PCA, this suggests that the theory of niche width/competitive ability trade-offs should be investigated as a potential determinant of lineage distributions (Maynard *et al*., 2019). What

was clear was that several *Bd* isolates were able to persist following incubation for two hours at temperatures well above the published thermal maximum for *Bd*. It would be interesting to identify whether these isolates are outliers next, further strengthening the argument for an expanded niche width being critical in determining lineage interactions, or if the accepted thermal envelope for *Bd* as a whole should be expanded. This result also indicates that *Bd* may be preadapted to climate change and could threaten areas that are currently considered low risk for *Bd*.

In vitro and *in vivo* work to establish the extent of any competition between lineages is a crucial next step in this research, as is accurately defining lineage environmental niche spaces. It may have appeared an obvious step to develop SDMs for *Bd*GPL and *Bd*CAPE based on the presence data collected here. However, SDMs are based on two fundamental assumptions – firstly that species are at equilibrium in the environment and secondly that the data used to develop the SDM represent the full environmental niche of the organism (Puschendorf *et al*., 2013). The points discussed here demonstrate why neither of these assumptions are met, and in fact are not definitively met in many studies where SDMs have been produced for *Bd* since it is generally acknowledged the species environmental optimum has not been satisfactorily resolved (Ron, 2005; Rödder *et al*., 2009; Rödder, Kielgast & Lötters, 2010; Venesky *et al*., 2014). It will be necessary to establish greater understanding of the impact of environment on *Bd* lineages and of lineage interactions, and whether they are in fact determinants of distribution or rather just correlates, before generating further SDMs is useful for predicting *Bd* distributions.

4.6 Acknowledgements and contributions

2016 sample collection of the Orange River transect was carried out by Jean Ruhan Verster, with field assistance from Estée Matthews. All other fieldwork was planned and conducted by Pria Ghosh with assistance in various years from Jean Ruhan Verster, Abigail Pretorius, Nadine Lepart, Allécia Van Dyk, Ryno Van Dyk, Maryke Gericke, Ché Weldon, Trent Garner, Claudio Soto-Azat, Jos Kielgast, Maroni Campos Cerquiera, Xavier Harrison, Thomas Smallwood, Jamie Bosch and Kieran Bates. All data entry, cleaning and analysis was carried out by Pria Ghosh, except where specified in the following cases. Kieran Bates conducted the linear mixed effects model for the *in vitro* lineage thermal maxima experiment; Thomas Sewell carried out whole genome sequence analyses, based on a pipeline developed by Simon O'Hanlon (O'Hanlon *et al*., 2018); the principal component analysis was conducted by Pria Ghosh with assistance from Sonia Tiedt. Particular thanks are due to Ché Weldon, Trent Garner and Jean Ruhan Verster for helpful discussions regarding site selection and the study as a whole.

Chapter 5

Chytrids in Madagascar

5.1 Abstract

Madagascar is a global hotspot for biodiversity and the island contains a unique endemic amphibian assemblage. For this reason, there has been great concern over the impact the arrival of *Bd* could have on this megadiverse amphibian assembly. Although *Bd* has now been detected on the island, whether the pathogen has established has been widely debated and there is speculation that amphibian skin-associated microbes of Malagasy amphibians could aid resistance of native amphibians to invasion by fungus. In this, however, fungal diversity has largely been overlooked. Our discovery of a novel amphibian-associated chytrid on wild Malagasy amphibians has raised the possibility that the mycobiome of the frogs may be equally, if not more, important than the microbiome in protecting amphibians against this deadly pathogen. Here, the position of the novel Malagasy chytrid within the Chytridiomycota and an associated co-occurring basidiomycete within the Tremellaceae, is resolved. *In vitro* experiments reveal that the fungi isolated from Malagasy frogs are capable of suppressing the growth of *Bd* under experimental conditions, highlighting promising future research avenues to protect Malagasy amphibian diversity and possibly explaining the confusing status of *Bd* on Madagascar.

5.2 Introduction

5.2.1 Madagascar

Madagascar is the world's fourth largest island, sitting off the southeast coast of Africa in the Indian Ocean. The formation of the island began approximately 160 million years ago in the mid-Jurassic when, with the break-up of Gondwana, it separated from the African continent along with the rest of what is now the Indian subcontinent, East Antarctica, Australia and the Seychelles (Gibbons, Whittaker & Müller, 2013; Halliday, Prasad & Goswami, 2017). Madagascar subsequently broke off from India between 84 and 96 million years ago (Halliday, Prasad & Goswami, 2017).

Today, Madagascar is home to over 25 million people and is one of the poorest countries in the world, ranking 155 out of 187 countries on the Human Development Index and with an average life expectancy of just under 66 years. The majority of Madagascar's population live below the poverty line, on less than US$1.90 per day (Marks *et al*., 2016; Oxford Poverty and Human Development Initiative, 2018), with economic growth not expected to rise in the period up to 2021 (World Bank, 2018). Healthcare provision in the country is often hard to access and inadequate, reflected in periodic outbreaks of diseases that much of the world consider to be historical problems, such as plague which killed 40 people in 2014 (Marks *et al*., 2016). Up to 40% of the population have no access to primary healthcare and those that do have access, routinely need to travel extensive distances to reach the facilities available (Marks *et al*., 2016).

Although Madagascar experiences such an extreme level of human poverty, geographic isolation for such an extended period of time has resulted in an exceptional level of endemic biodiversity even at high taxonomic levels, which is particularly unusual (Ganzhorn *et al*., 2001). In 2000, the concept of a "biodiversity hot spot" was formalised, which aimed to aid the prioritisation of global conservation funding. There are just 25 global biodiversity hot spots which are defined by extraordinary endemism combined with high rates of habitat loss. Madagascar falls within

the top five of these prioritised global hotspots owing to its vast level of endemism compared to surface area: the top five hotspots (Madagascar, the Brazilian Atlantic Forest, the Tropical Andes, Sundaland and the Caribbean) comprise 45% of the total of all the hotspots' endemic plants and vertebrates on just 0.4% of the earth's surface (Myers *et al.*, 2000). Over 90% of vascular plants, 50% of birds and more than 98% of mammals, reptiles and amphibians are endemic to the island (Ganzhorn *et al*., 2001) and as a result Madagascar is recognised as being of particular importance for global conservation efforts (Brooks *et al*., 2006). It is estimated that less than 10% of Madagascar's original land cover remains (Buerki *et al.*, 2015) and what does remain continues to be at risk in large part due to the high proportion of the population dependent on natural resources for survival and income (Gardner *et al.*, 2018). Madagascar's human population has doubled since 1985 while rainforest cover has decreased by 66% in the same period, indicating the extreme pressures the natural resources of the island are now under (Alroy, 2015). External pressure from high-income foreign countries for Madagascar's rare species, such as rosewood and tortoises, also plays a large role in risking the island's natural capital for the future (Gardner *et al*., 2018).

Another ongoing challenge to socio-economic development and biodiversity conservation in Madagascar is the high level of political instability experienced by the country. Madagascar, formerly a French colony, gained independence in 1960 and has experienced multiple coups since then. Most recently, in 2009 violent protests resulted in the deaths of multiple civilians, the closure of independent media organisations, the flight of then President Marc Ravalomanana to South Africa and the dissolution of the government. The Senate was not re-elected until six years later in 2015, since when an uneasy peace has been maintained (https://www.bbc.co.uk/news/world-africa-13861843, BBC News, 2018). The instability and lack of strong governance has resulted in reduced investments from donors in Malagasy biodiversity conservation and poor implementation of laws surrounding environmental exploitation (Andreone *et al*., 2012; Rakotomanana, Jenkins & Ratsimbazafy, 2013).

5.2.2 Malagasy amphibian diversity

Madagascar is home to over 280 species of amphibian, only two of which are introduced and non-native (Bletz *et al*., 2015b), and with many more waiting to be described it is estimated that the true number is likely to be over 500 species (Andreone *et al*., 2012; Perl *et al*., 2014). The level of endemism is astonishingly high – 88% of genera and 100% of native species are endemic to the island, falling into just five evolutionary radiations (Andreone *et al*., 2008; Vences *et al*., 2009; Vieites *et al*., 2009). Until recently, although many of Madagascar's amphibians were highly threatened, none were thought to have become extinct in recent times (Andreone *et al*., 2008). However, a comprehensive analysis estimating extinction rates of reptiles and amphibians globally has suggested that in fact up to 187 frog species may have disappeared from the island (Alroy, 2015). As with much of the rest of Madagascar's biodiversity, Malagasy amphibians are threatened by deforestation, habitat change and overharvesting to satisfy the demands of the global pet market (Andreone & Luiselli, 2003; Andreone, Mercurio & Mattioli, 2006; Rabemananjara *et al.*, 2008; Bletz *et al.*, 2015a).

5.2.3 *Bd* in Madagascar

The IUCN SSC Amphibian Specialist Group in Madagascar held a workshop in 2010 to address the potential threat of *Bd* to the island's amphibian megadiversity (Andreone *et al.*, 2012). The *Bd* status of Madagascar at the time was unknown, but no chytrid-related deaths had been documented. The result of the workshop was that a three-year, biannual monitoring programme was instigated at eight particularly sensitive sites across Madagascar, following a standardised protocol, with the aim of clarifying the *Bd* infection status of Malagasy frogs (Andreone *et al*., 2012). At the culmination of the three-year program, an international consortium of stakeholders established the Chytridiomycosis Working Group (CWG) in Madagascar, to act as a coordination centre for *Bd* research and mitigation across the island (Weldon *et al*., 2013). The CWG developed a pre-emptive National Monitoring Plan (NMP) for *Bd* in Madagascar, following the same framework as the

previous three-year program, to detect *Bd* early enough that, should it arrive, mitigation measures against the fungus could be enacted.

The *Bd* infection status of Madagascar remains unclear, despite this extensive monitoring. The fungus was first detected in 2010, on the Makay Massif in the southwest of the country (Rabemananjara, Andreone & Rabibisoa, 2011), and *Bd* has been detected on amphibians originating from Madagascar in global trade networks (Kolby & Skerratt, 2015). Since that first report of *Bd*, the pathogen has been detected at multiple sites over several years (Bletz *et al*., 2015b). However, it has been debated whether these positive test results represent true colonisation of the island by *Bd*, or instead are the product of multiple introductions which fail to establish, or even are the result of laboratory contamination from the DNA of amplified PCR products (Kolby & Skerratt, 2015; Bletz *et al*., 2015a; Kolby *et al*., 2015). Furthermore, to this date no amphibians have been found displaying clinical symptoms of chytridiomycosis in the wild in Madagascar, despite *ex situ* testing showing that multiple Malagasy species are susceptible to the fungus under laboratory conditions (Weldon *et al.*, 2013), and *Bd* has never been isolated from the island.

5.2.4 A Malagasy chytrid?

In September 2015, as part of an international research project attempting to isolate *Bd* from Madagascar, a putatively new species of the Chytridiomycota was isolated from frogs at two sites in Madagascar, but no *Bd* was found. As *Bd* and *Bsal* are to date the only two members of the Chytridiomycota known to parasitise vertebrates, the isolation of the new chytrid from amphibian skin raises the possibility that a third member of the group may also have adopted vertebrate parasitism. The possible presence of an additional chytrid on Malagasy frogs raises the possibility that interspecific competition between chytrids may have a role to play in *Bd* colonisation and transmission on the island.

Interspecific competition at the microbial level can impact host resistance to pathogens (Belden & Harris, 2007). It has already been shown that some amphibians harbour microbial defences to

Bd. For example *Janthinobacterium lividum*, a bacteria that naturally occurs on the skin of *Bd*-resistant populations of *Plethodon cinereus* (Red-backed salamander), is now known to produce an antifungal metabolite, violacein, in quantities capable of repelling or, in some cases, killing *Bd* zoospores (Brucker *et al*., 2008; Harris *et al*., 2009). However, avirulent microbes may also aid hosts in resisting pathogens indirectly. It is possible that highly diverse microbial communities may protect a host by making them less susceptible to invasion by pathogens due to competitive microbial interactions. Studies across multiple systems and taxa have shown that changing a host's microbial composition can result in an increase in disease, from humans to fish to insects (Harris *et al*., 2009). This phenomenon has been particularly well established in plant pathology. In the case of wheat, experimental evidence has shown that individuals harbouring high levels of microbial diversity in their rhizosome were better able to resist invasion by the opportunistically pathogenic fungus *Pseudomonas aeruginosa* (Matos, Kerkhof & Garland, 2005). A long-standing hypothesis of ecological competition is that species that are more closely related to each other phylogenetically will be more likely to have a fundamental niche overlap (phylogenetic niche conservatism (Fountain-Jones *et al*., 2018)) and thus competitive exclusion may be more probable (Venail *et al*., 2014; Godoy, Kraft & Levine, 2014). Further, it has been suggested that higher species diversity in ecosystems may hinder the ability of emerging microbial pathogens to invade a system (Belden & Harris, 2007; Antwis *et al*., 2015). If a chytrid on Madagascar already exploits the amphibian skin niche sufficiently, interspecific competition dynamics could impact the ability of *Bd* to colonise the island, even if under experimental conditions Malagasy species are susceptible to *Bd* infection.

In this chapter, I identify where the Malagasy chytrids fall in relation to *Bd* in the phylogeny of the Chytridiomycota, develop a diagnostic to aid further research on these novel chytrids and begin to explore what, if any, interactions exist between potentially invasive *Bd* and the putatively new chytrid species.

5.3 Methods

5.3.1 Collection of chytrid isolates

Between August and September 2015, five sites in Madagascar – Andasibe, Ankarafantsika, Ankaratra, Antoetra and Ranomafana (Figure 5.1) – were sampled opportunistically in night and day searches to attempt isolation of *Bd* via toe clipping of adult amphibians (Section 2.3). Sites were selected on the basis of where was logistically feasible to reach, while also falling under the remit of the Cellule d'Urgence Chytride Madagascar who facilitated the fieldwork. Samples from Ankaratra, Antoetra and Ranomafana (53% of samples) were processed as described in Section 2.3. Due to a lack of resources, samples from Andasibe and Ankarafantsika were placed directly into nutrient broth with antibiotics. Samples were processed in the field up until the stage of being transferred to an Eppendorf tube containing TGhL broth, at which point they were kept as cool as possible until they could be transported to ICL for further processing under sterile conditions as described in Section 2.3.

One sample (R160) appeared to contain a chytrid-like organism, but also serious bacterial contamination. This sample was transferred directly into an Eppendorf tube and centrifuged at 7,000rpm for 10 minutes. The supernatant was removed, taking care not to disturb the pellet which was then resuspended by vortexing in the same amount of TE buffer as removed supernatant, and then frozen until further analysis could take place.

Figure 5.1. Map of chytrid isolation field sites on Madagascar. Map generated in Tableau using the Satellite base map sourced from Mapbox OpenStreetMap.

5.3.2 Phylogenetic analyses

DNA was extracted from two Malagasy samples (one live culture, Md210, and one frozen isolate, R160, preserved with TE buffer) observed to contain chytrid-like organisms, 10 isolates of *Bd*, one isolate of *Bsal* and one isolate of an unidentified chytrid from Taiwan (TW16459) using DNeasy™ Blood and Tissue Kit (Qiagen, Venlo, Netherlands) (Section 2.6) (isolate metadata provided in Appendix 11). Immediately following DNA extraction, both Malagasy chytrids were subjected to pan-lineage *Bd* qPCR assay (Boyle *et al*., 2004). They were then TA-cloned and Sanger sequenced to obtain consensus ITS sequences along with the other chytrids listed above. The Malagasy ITS sequences were searched against the Basic Local Alignment Search Tool (BLAST) database, and a phylogeny was constructed to identify where the putatively new chytrids fell within the Chytridiomycota. As analyses indicated that these were likely to be the same and a new species, a novel TaqMan MGB qPCR diagnostic assay based on the sequenced ITS region was designed to detect the new chytrid from field samples.

5.3.3 TA cloning and Sanger sequencing

Following DNA extraction, all isolates underwent PCR amplification targeting ITS1, the 5.8S gene and ITS2 using primers ITS1 and ITS4 in a 50µl reaction (primer starting concentration 10µM). The reaction profile was as follows: 35 seconds at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 45°C, then 45 seconds at 68°C and finally 15 minutes at 72°C. Gel electrophoresis was carried out on 10µl of PCR products obtained to confirm amplification had been successful before beginning TA cloning. The TA cloning reaction was carried out using Invitrogen Life Sciences TOPO TA Cloning Kit (Invitrogen, ThermoFisher Scientific, Massachusetts, USA) followed by transformation into One Shot competent cells (Invitrogen, ThermoFisher Scientific, Massachusetts, USA) following the regular chemical transformation protocol. To analyse transformants, eight colonies per isolate of *Bd* and *Bsal* and 10 colonies of TW16492 and the Malagasy chytrids were cultured overnight in LB medium containing 50µg/mL kanamycin before isolating the plasmid DNA using PureLink Quick Plasmid Miniprep Kit (ThermoFisher Scientific, Massachusetts, USA). The plasmids were screened for inserts by PCR in a 50µl reaction using primers ITS1 and ITS4 at 10µM starting concentration in a reaction mix consisting of 0.5µl Taq polymerase, 5µl buffer solution, 1µl dNTPs starting at 10µM concentration, 1µl of each primer, and 36.5µl of filtered water. The reaction profile was: 95°C for 30 seconds, followed by 30 cycles of 95°C for 30 seconds, 45°C for 30 seconds and 68°C for 45 seconds, then five minutes at 68°C. 5µl of PCR product was used for gel electrophoresis to confirm successful amplification.

DNA extracts were quality checked on a Qubit™ fluorimeter (ThermoFisher Scientific, Massachusetts, USA) and on a TapeStation™ 2200 (Agilent Technologies, California, USA) and then Sanger sequenced following the protocol described in Section 2.7. Sequencing files were quality checked and trimmed in CodonCode v4.2.5 (CodonCode Corporation, Massachusetts, USA), and contigs were assembled from which consensus sequences were generated, using Phred base calling to assign bases.

ITS consensus sequences generated through TA cloning for the unidentified Malagasy chytrids were BLASTn searched against the NCBI nr-database. The Malagasy chytrid consensus ITS sequences, excluding any contaminator non-chytrid ITS sequences, were imported into MEGA v7.0 for alignment and analysis. Consensus sequences from all other chytrid isolates that were TA cloned were also imported, as well as the ITS sequence for *Kappamyces laurelensis*, the closest match to the Malagasy chytrids according to the nr- database, which was downloaded from NCBI. A 1,346bp alignment was generated from consensus sequences using MUSCLE (Appendix 12) after which MEGA v7.0 was used to identify the best supported maximum likelihood model for the data. A maximum likelihood unrooted tree bootstrapped 500 times was computed using a Tamura Nei model, with rates among sites set to gamma distributed and missing data treatment set to partial deletion (Hall, 2013). The phylogeny was imported into FigTree v1.4.4 (Rambaut, 2009) for presentation.

The ITS sequence alignment used to generate the Chytridiomycete phylogeny was manually searched for SNPs specific to the Malagasy chytrids. 100bp sequences surrounding identified SNPs were then imported into Primer Express v3.0 and the software was directed to identify candidate probes and primers. TaqMan MGB probes and primers with the lowest penalty score were chosen and ordered from Applied Biosystems.

To identify contaminating fungi present in the cultures and confirm the position of the Malagasy chytrids among the Chytridiomycota, DNA was sequenced from the isolate Md210 using Illumina HiSeq, and assembled using DiSpadesv3.8.0 (Bankevich *et al*., 2012). The assembly consisted of long contigs with low depth and short contigs with high depth (chytrid genome N50 = 53514, N90 = 9826; basidiomycete genome N50 = 797040, N90 = 473191). Gene sets were obtained for the following chytrid genome assemblies: *Bd* (JEL423), *Bsal*, *Homolaphlyctis polyrhiza* (*Hp*), *Spizellomyces punctatus* (*Sp*), *K. laurelensis* and *Gonapodya prolifera* (Farrer *et al*., 2017), and orthologs were identified using OrthoMCL v1.4 (Li, Stoeckert & Roos, 2003). Additionally, we

constructed a Synteny plot (Farrer, 2017) using the same method for four basidiomycete genome assemblies, including the contaminating *Cryptococcus* species identified through searching the BLAST database.

To confirm that the novel TaqMan assay was specific to the Malagasy chytrids, a DNA testing panel comprising all four known lineages of *Bd* at the time of testing (*Bd*GPL, *Bd*CAPE, *Bd*ASIA-1, *Bd*ASIA-2/BRAZIL, *Bd*CH), two isolates of *Bsal*, *K. laurelensis*, both Malagasy chytrid isolates and another unknown chytrid isolated from Taiwan, TW16459, was prepared (isolate metadata provided in Appendix 13). DNA was extracted from live cultures for all isolates, except for R160 from Madagascar and TW16459 from Taiwan, following the *RACE* protocol described in Section 2.6. DNA extract previously used for TA cloning and extracted as described above was used for isolates R160 and TW16459.

All isolates in the DNA testing panel were subjected to two qPCR diagnostic assays; once using the *Bd* pan-lineage qPCR protocol (Boyle *et al*., 2004) and once with the novel TaqMan MGB probe described here. DNA extracts were diluted 1 in 10 with filtered water prior to testing. The novel qPCR assay followed the pan-lineage qPCR protocol, using the same reaction mix and the same reaction profile (Section 2.6). Primers were used at a starting concentration of 10µM and the TaqMan probe at a starting concentration of 100µM. DNA quantification standards for standard curve analysis were prepared using isolates Md210 for the novel Malagasy chytrid diagnostic assay and IA042 for the *Bd* diagnostic assay (Section 2.5).

5.3.4 Validation of optical density (OD) as a tool to record chytrid growth

The ability of a microplate reader to record chytrid growth accurately was tested in a series of pilot experiments run on a FLUOstar® Omega filter-based multi-mode microplate reader (BMG Labtech, Ortenberg, Germany). Quantitation standards of IA042, a vigorous and well established *Bd*GPL isolate were prepared by collecting and harvesting live zoospores as described in Section 2.5, and then creating a 1 in 2 five-fold dilution series starting at 2 million zoospores per ml. The

dilution series was initially read using an absorbance spectrum setting, to assess what wavelength was appropriate for reading the optical density at, followed by being read at 492nm, in line with previous work (Stevenson *et al*., 2013; Muletz-Wolz *et al*., 2019). Each zoospore quantitation standard was replicated four times and remaining wells were filled with 1ml of sterile TGhL broth. The OD was measured using a spiral setting, taking 76 readings per well, in a spiral pattern from the centre of the well with a scan diameter of 5mm (thus excluding the edges of the well from the readings) (Figure 5.2) in order to account for the patchy distribution of chytrid zoospores throughout the well. The mean OD recorded for each quantitation standard was used to generate a standard curve, from which the efficacy of optical density as a tool for quantifying chytrid zoospores was assessed. Plates were run with four blank wells and final OD readings for a zoospore concentration represent the mean, blank corrected value for all OD readings (each well reading represents the mean of the 76 readings per well, each quantitation standard reading represents the mean of all four replicates values, blank corrected). An R^2 value close to 1 indicated that the plate reader was able to quantify reliably the number of zoospores present in a well.

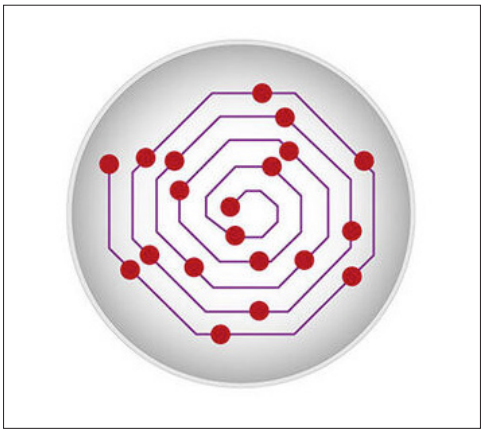


Figure 5.2. Diagram of a spiral well scan on a BMG Labtech microplate. Red dots indicate an individual reading. The final OD reading for the well is the mean of all well readings taken. Image taken from www.bmglabetch.com.

5.3.5 *In vitro* co-culture of a Malagasy chytrid with *Bd*GPL

SA-NC8, a *Bd*GPL isolate that displayed vigorous growth *in vitro* (personal observation) and Md210, the only Malagasy chytrid surviving *in vitro*, were selected for an *in vitro* co-culture experiment. Both isolates were collected in the same month (September 2015), were cryopreserved soon after collection and so had undergone comparable and minimal numbers of passages and were collected from Southern Africa (Md210 collected from Ankaratra in Madagascar and SA-NC8 collected from the Northern Cape Province of South Africa). The isolates were thawed from cryopreservation (Section 2.4) and then incubated at 18^{o}C until healthy growth was established. At this point, 500µl of growing culture was transferred to a TGhL 1% agar plate to harvest zoospores, as described in Section 2.5. Multiple agar plates for zoospore harvesting were prepared over several days to maximise the chances of both isolates producing zoospores for harvesting simultaneously, for which plates were examined daily under a light microscope. Due to the low level of zoospore production by isolate Md210, a lower concentration of zoospores than planned for both isolates was used. Standardised solutions of 10,000 zoospores per ml were prepared (Section 2.5) of each isolate from the collected supernatant.

Three 48-well sterile culture plates each containing four replicates of three experimental treatment groups and one blank group were set up in a randomised block design (excluding outer wells due to the higher risk of evaporation from these wells) as shown in Figure 5.3.

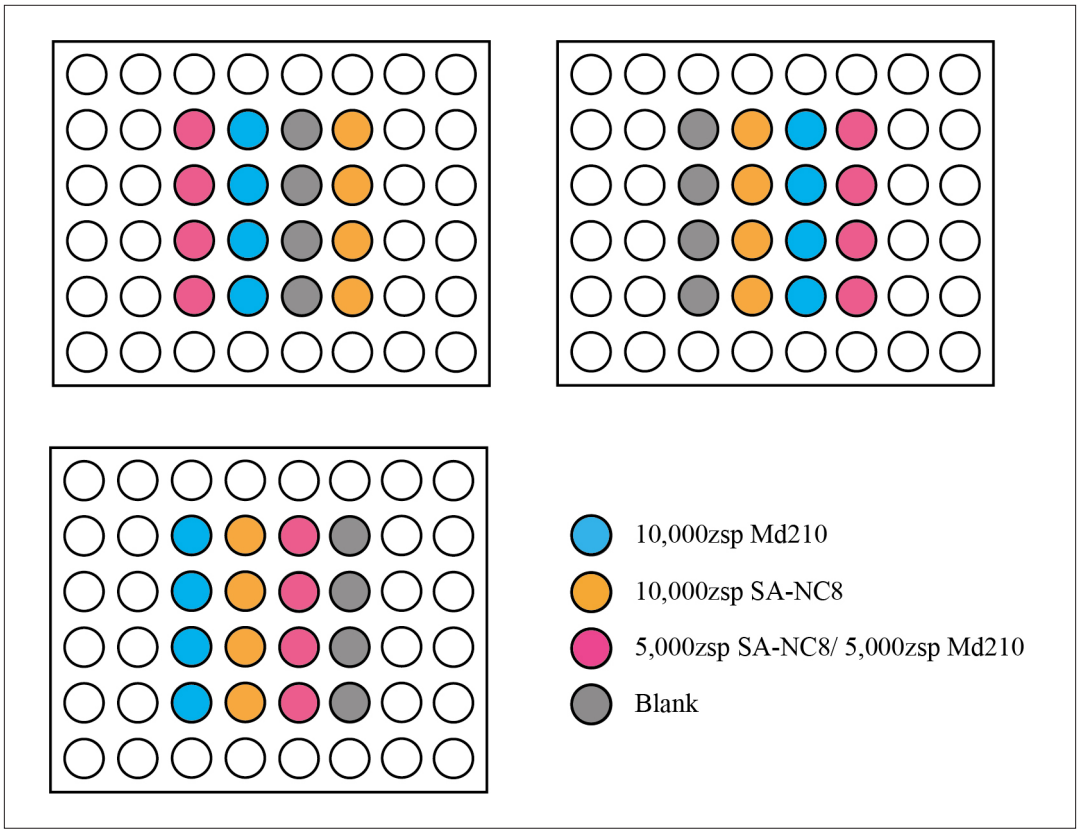


Figure 5.3. Experimental set up for Md210 and SA-NC8 *in vitro* co-culture experiment

Treatments consisted of 1) 10,0000 zoospores of Md210 2) 10,000 zoospores of SA-NC8 and 3) 5,000 zoospores of Md210 and 5,000 zoospores of SA-NC8. All experimental wells contained a total of 2ml of TGhL broth containing the relevant number of zoospores and blank wells were filled with 2ml of TGhL broth. All remaining wells were topped up with approximately 2ml of TGhL broth to reduce the effect of evaporation.

The plates were placed in a Tupperware box, to reduce the effect of evaporation on the experiment, centrally in an 18^{o}C incubator and left undisturbed for 24 hours. Plates were rotated within the Tupperware box every day. After the initial 24 hours of incubation, plates were read using a FLUOstar® Omega microplate reader at 24-hour time points for a total of 10 days' growth at which point the OD plots indicated that growth in some experimental wells was plateauing.

At this point, the experiment was ended and the base of the treatment wells was thoroughly scraped with a pipette to dislodge any sporangia before the entire contents of each well were transferred to individual 2ml Safe-Lock Eppendorf tubes prepared with 0.03 to 0.04g of 0.5mm silica homogenisation beads. The DNA was extracted according to the *RACE* protocol for extracting DNA from live cultures (Section 2.6). DNA extracts were tested with both the pan-lineage *Bd* diagnostic (Section 2.6) and the novel Malagasy chytrid-specific diagnostic described here using DNA quantitation standards prepared from the *Bd* isolate IA042 and the Malagasy chytrid isolate Md210. Tableau v2019.3 was used to produce graphs of the mean change in chytrid concentration for each treatment.

5.3.6 Back-screening of NMP samples for Malagasy chytrids

Swab samples collected in 2014 and 2016 from five sites in Madagascar (Figure 5.4) and stored as DNA extracts at Fisher Lab, ICL were thawed and rescreened for the presence of the new Malagasy chytrid. DNA extracts had been stored at -80^{o}C since they were initially processed and had not undergone any freeze-thaw cycles since initial testing for *Bd*, up to four years prior to this work. All samples were thawed on ice and a 1 in 10 dilution with filtered water was prepared. The samples were pooled and tested in duplicate (two samples per reaction well; two reaction wells per sample) using the novel TaqMan MGB qPCR assay described in this chapter. Quantification was carried out via a standard curve analysis, with DNA quantitation standards prepared from live Md210 (Section 2.5). The same threshold for positivity was used as for the NMP project, namely amplification in duplicate with a mean GE of at least 0.1, after accounting for dilution. Sample selection was largely opportunistic depending on what samples, mostly from past NMP sampling, were accessible. Samples from 2014 and 2016 were targeted as these were the closest years available to 2015, when the Malagasy chytrid isolates were collected.

Figure 5.4. Source locations on Madagascar of swabs back-screened to detect *BxMada.* the Satellite base map sourced from Mapbox OpenStreetMap.

5.4 Results

5.4.1 A novel Malagasy chytrid

In total, 1,454 adult amphibians from 37 species or species complexes were sampled from the five sites. The Mascarene grass frog (*Ptychadena mascariensis*) dominated sampling (n=426, 29.29% of all samples collected) and was found at all sites except for Ranomafana. *Mantidactylus ulcerosus* (n=223), *Heterixalus luteostriatus* (n=181), *Mantidactylus pauliani* (n=144) and *Mantidactylus betsileanus* (n=107) were also all well represented. Ranomafana led in terms of species diversity, with 25 species sampled, more than double that in the next two diverse sites, Antoetra and Ankaratra, from which nine species were collected each. Only three species of amphibian were collected at Ankarafantsika and two at Andasibe. The balance of species diversity throughout the sites is consistent with previous work on amphibian diversity in Madagascar; Ranomafana National Park harbours the highest known diversity of amphibians in Madagascar, with approximately 129 recorded species (Rosa *et al.*, 2015). Two samples, one collected from a *M. pauliani* in Ankaratra and one collected from a *M. betsileanus* in Ranomafana, showed evidence of the growth of a chytrid-like organism (Figure 5.5).

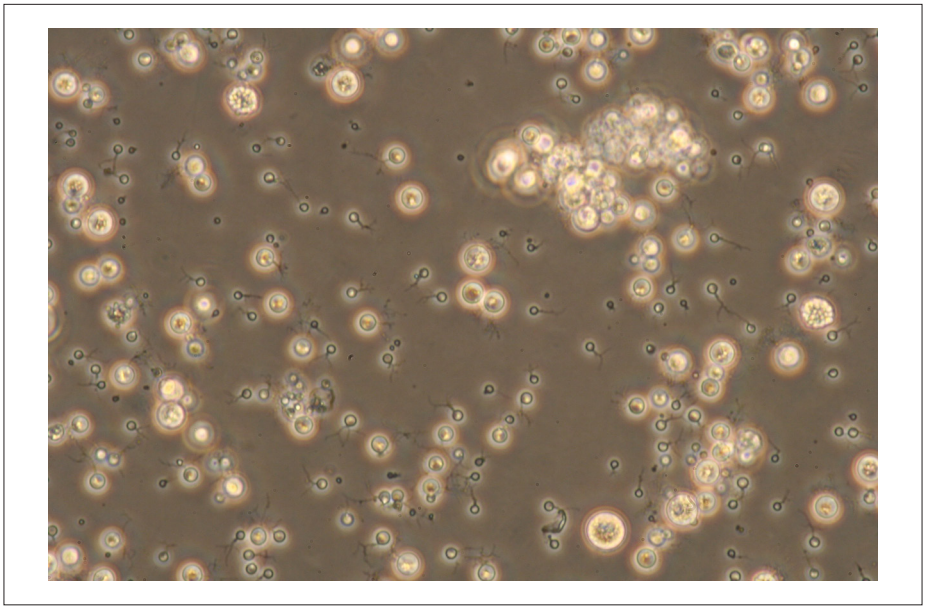


Figure 5.5. *BxMada* in culture, sample Md210 immediately following transfer into a culture well plate during the isolation process.

Sample Md210 was successfully transferred into *in vitro* culture, while the sample from Ranomafana (R160) was cryopreserved due to bacterial contamination prior to downstream analyses. Initial qPCR results from the pan-lineage *Bd* diagnostic indicated that neither isolate was *Bd.*

Following TA cloning, consensus sequences of the ITS region for both Malagasy chytrids were searched against the NCBI nr-database and *K. laurelensis*, a Rhizophydiales chytrid, was returned as the closest match. *K. laurelensis* was isolated from soil in the USA and was first described in 2005, and assigned its own genus (Letcher & Powell, 2005). However, query coverage was extremely low (mean of 31% across the two isolates), indicating that this result was unlikely to be a correct identification of species. Additionally, Md210 sequences also returned a high proportion of reads attributed to an uncultured *Cryptococcus* isolate (clone PPO 12-21), although no second fungus was visible under a light microscope and its presence did not seem to hinder the ability of the chytrid to persist *in vitro*. Therefore, due to the difficulties inherent in separating the two fungi, the culture was maintained with no further interference, presumably with both fungi persisting, which should be noted when interpreting these results.

Phylogenetic analysis of the ITS sequences (following exclusion of *Cryptococcus* reads) revealed R160 and Md210 to be the same species, most closely related to *K. laurelensis* and somewhat distantly related to *Bd* and *Bsal* (Figure 5.6). The low bootstrap values within the *Bd* complex reflects the inability of the ITS region to resolve sub-specific lineages within the Chytridiomycota. Diagnostic primer and TaqMan MGB probe sequences are detailed in Figure 5.7.

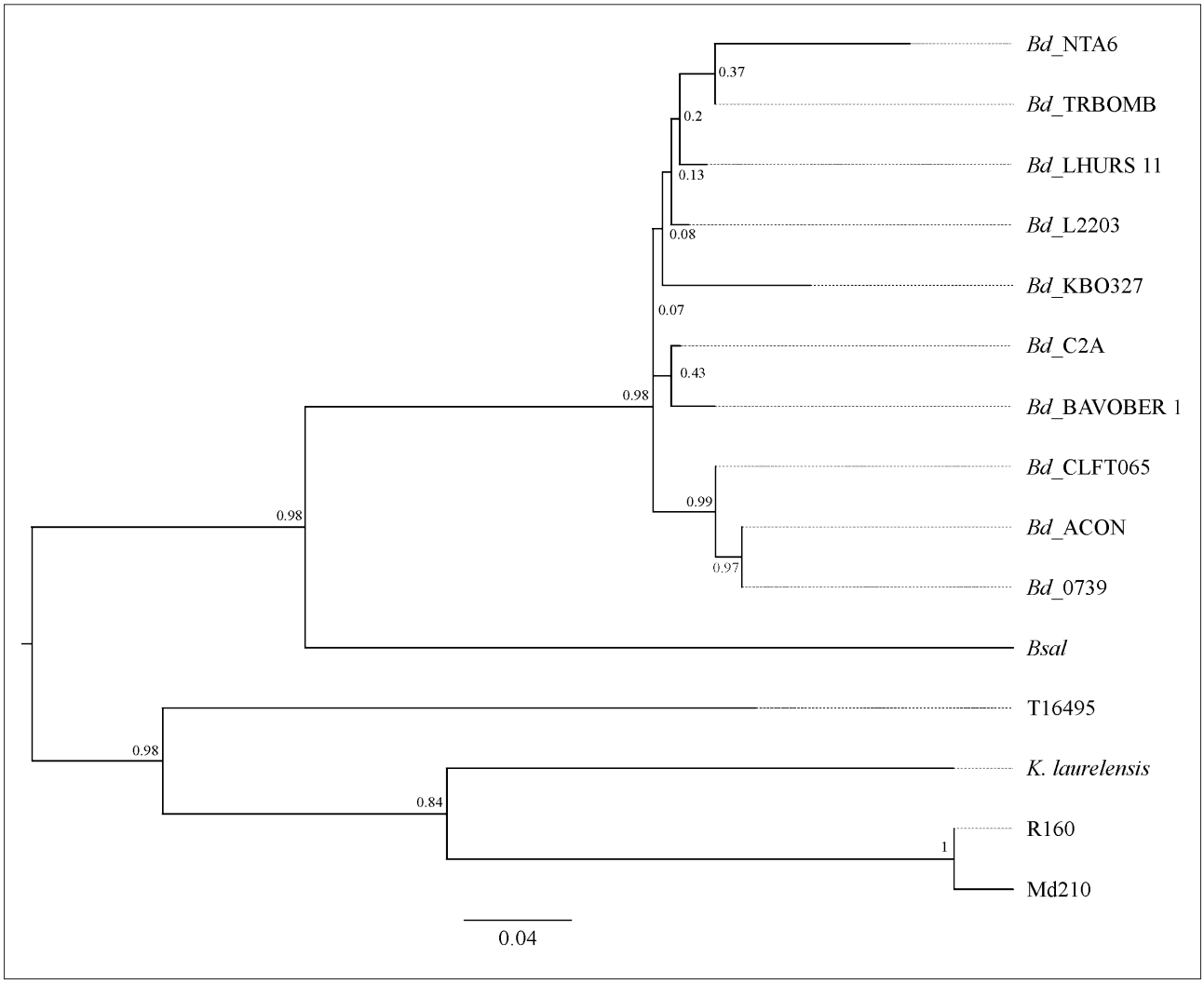


Figure 5.6. Unrooted phylogeny of the Chyridiomycota, showing placement of novel Malgasy chytrids Md210 and R160.

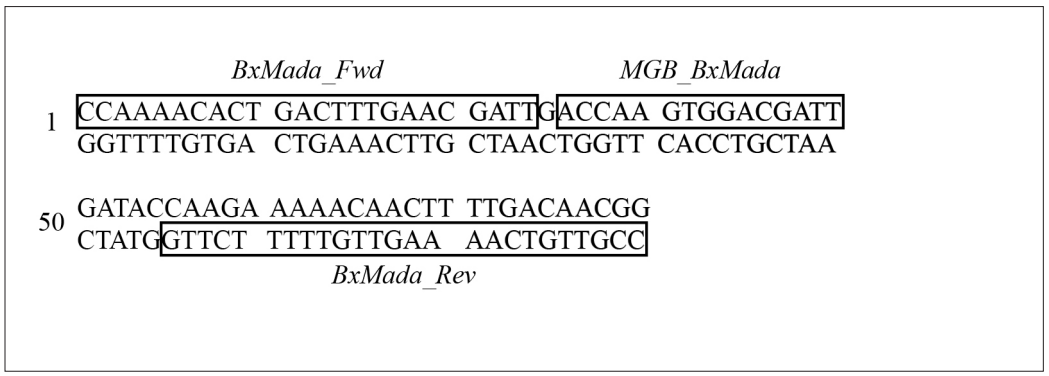


Figure 5.7. Nucleotide sequences for novel *BxMada*-specific primer and TaqMan MGB probes.

Assembling the Illumina HiSeq sequences suggested two separate species were present (approximately half of the contigs were long and had a low depth of coverage, and the other half had short contigs that had a higher depth of coverage). Furthermore, by annotating the protein-coding genes encoded by the contgis using the Broad Institute's Eukaryotic Genome Annotation Pipeline, and identifying orthologs using the Synima (Farrer, 2017) pipeline, we found that the short contigs had orthologs with the chytrids, while the long contigs had orthologs with Tremellaceae species including *Cryptococcus neoformans*. We subsequently divided the assembly into these two putative species, re-annotated and re-ran the Synima pipeline, thereby confirming the presence of two separate species: the unidentified chytrid (referred to as putatively *BxMada* from here onwards) (Figure 5.8a) and a basidiomycete from the Tremellaceae family (referred to as putatively *Tremella madagascari* from here onwards) (Figure 5.8b). Md210 henceforth refers to the *in vitro* maintained culture still presumed to be a mixture of *BxMada* and *T. madagascari*.

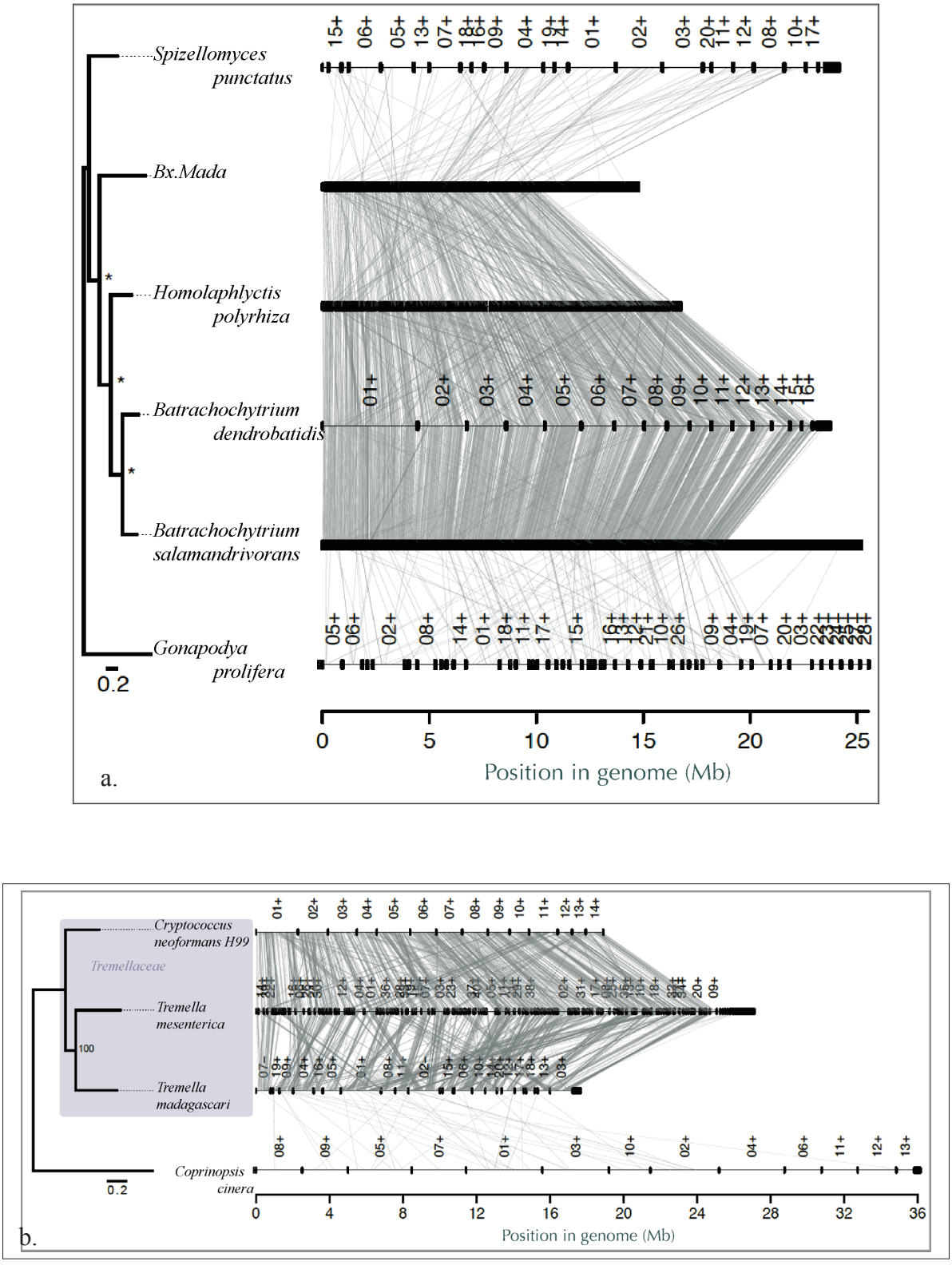
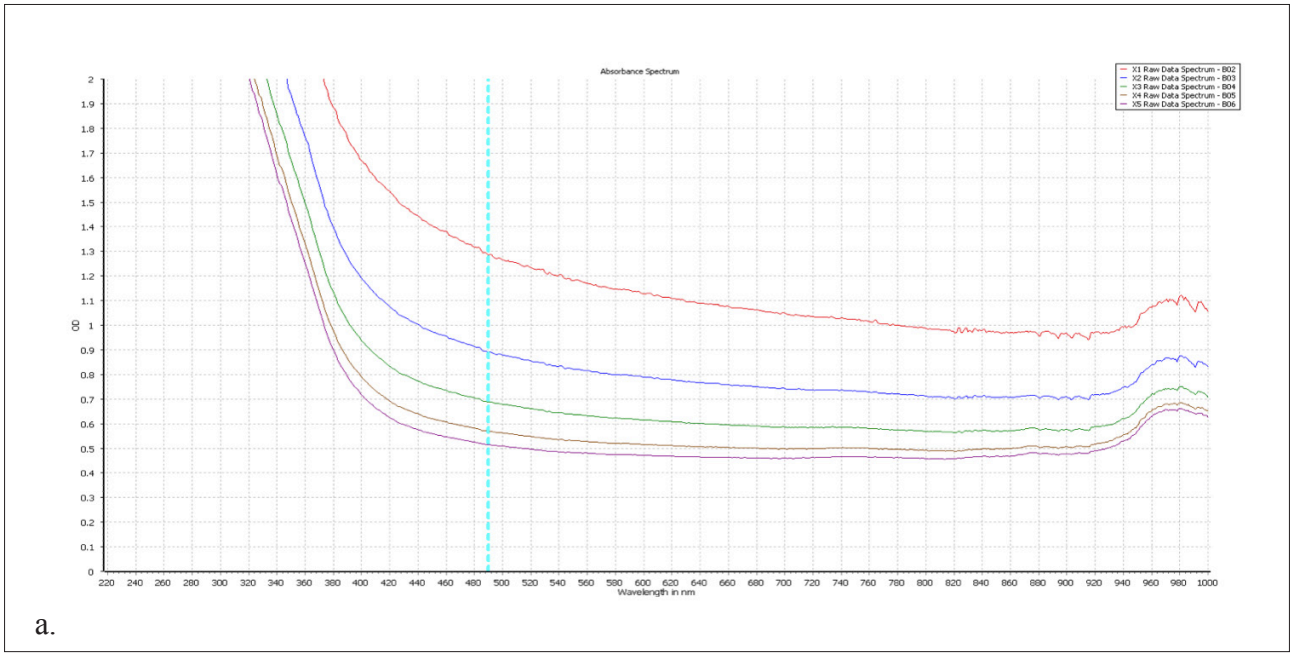


Figure 5.8. Synteny plots for (a) *BxMada* and (b) *T. madagascari.*

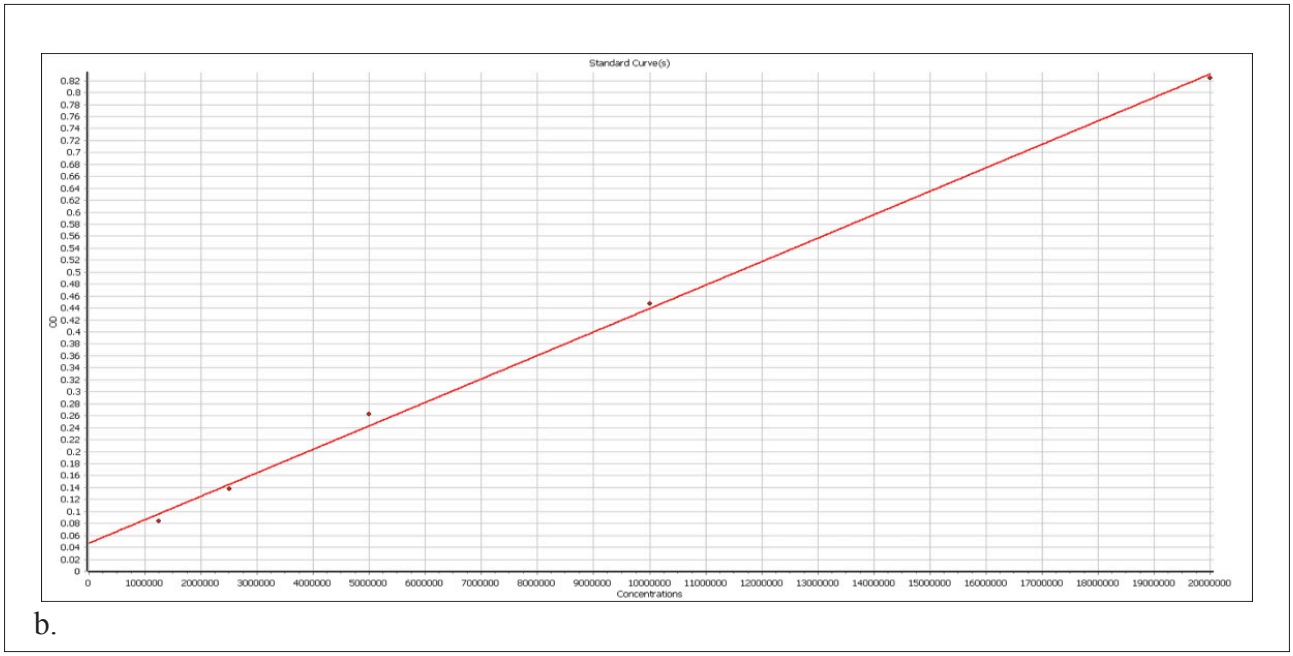
The novel TaqMan assay detected both isolates of *BxMada* (R160 and Md210) but not any other isolates on the specificity panel. The *Bd* pan-lineage specific qPCR did not detect Md210, *Bsal*, or TW16495, but did detect R160 as well as the *Bd* isolates on the panel. Following this result, the ITS sequence data for R160 was unsuccessfully manually searched for the pan-lineage *Bd* TaqMan MGB probe sequence in MEGA v7.0, leading to the conclusion that the R160 DNA extract had become contaminated with *Bd* at some point during the three years between collection and the Malagasy chytrid-specific TaqMan MGB probe being tested.

5.4.2 OD as a tool to record chytrid growth

An absorbance spectrum plot of the 1 in 2 five-fold dilution series starting at 2,000,000 zoospores (Figure 5.9a) showed that absorbance for all zoospores concentrations was low but relatively even throughout the entire spectrum. As a result, a wavelength of 492nm was selected for all further readings to stay in line with current literature which normally records *Bd* growth at between 490nm to 495nm (Piotrowski, Annis & Longcore, 2004; Stevenson *et al*., 2013; Voyles *et al*., 2017). The standard curve calculation (Figure 5.9b) for the same dilution series had an R^2 value of 0.99, showing that although the OD readings were very low, the plate reader was able to quantify accurately the number of zoospores present in a well.



a.



b.

Figure 5.9. Microplate reader plots showing (a) absorbance spectrum for *Bd* and (b) *Bd* standard curve.

5.4.3 Inhibition of *Bd*GPL by Md210

In vitro co-culture of isolate Md210 with *Bd*GPL isolate SA-NC8 showed that SA-NC8, while growing very well in isolation, was unable to grow in the presence of Md210 and exhibited a significant and large drop in GE. In contrast, in isolation Md210 showed a decrease in concentration and appeared to grow better in the presence of SA-NC8. Figure 5.10 illustrates the substantial reduction in growth undergone by SA-NC8 in the presence of Md210. In co-culture with Md210, SA-NC8 decreased from 5,000 zoospores at the start of the experiment to less than 100GE at the end. In contrast, in pure culture the number of SA-NC8 GEs increased over five times, to a mean of over 50,000GE. Md210 showed a mean reduction in GE of just over 2,200 in pure culture, although when examined under a microscope, the culture appeared to be growing, with dense sporangial growth visible. In co-culture Md210 showed marginal growth, with a mean increase of approximately 500GE compared with the start of the experiment, again despite dense sporangial growth being visible under a light microscope.

5.4.4 *BxMada* is not detectable on NMP field swabs

703 DNA samples were extracted from swabs taken through NMP monitoring from Ankaratra, Ankarafantsika, Antoetra, Ivoloina and Andasibe and tested for presence of *BxMada* using qPCR. The majority of samples (n=521, 74.1%) were collected in 2016 and 182 (25.9%) were collected in 2014. In the case of some samples the species was unidentified, but 14 named species were represented. No swabs returned a positive result for *BxMada*.

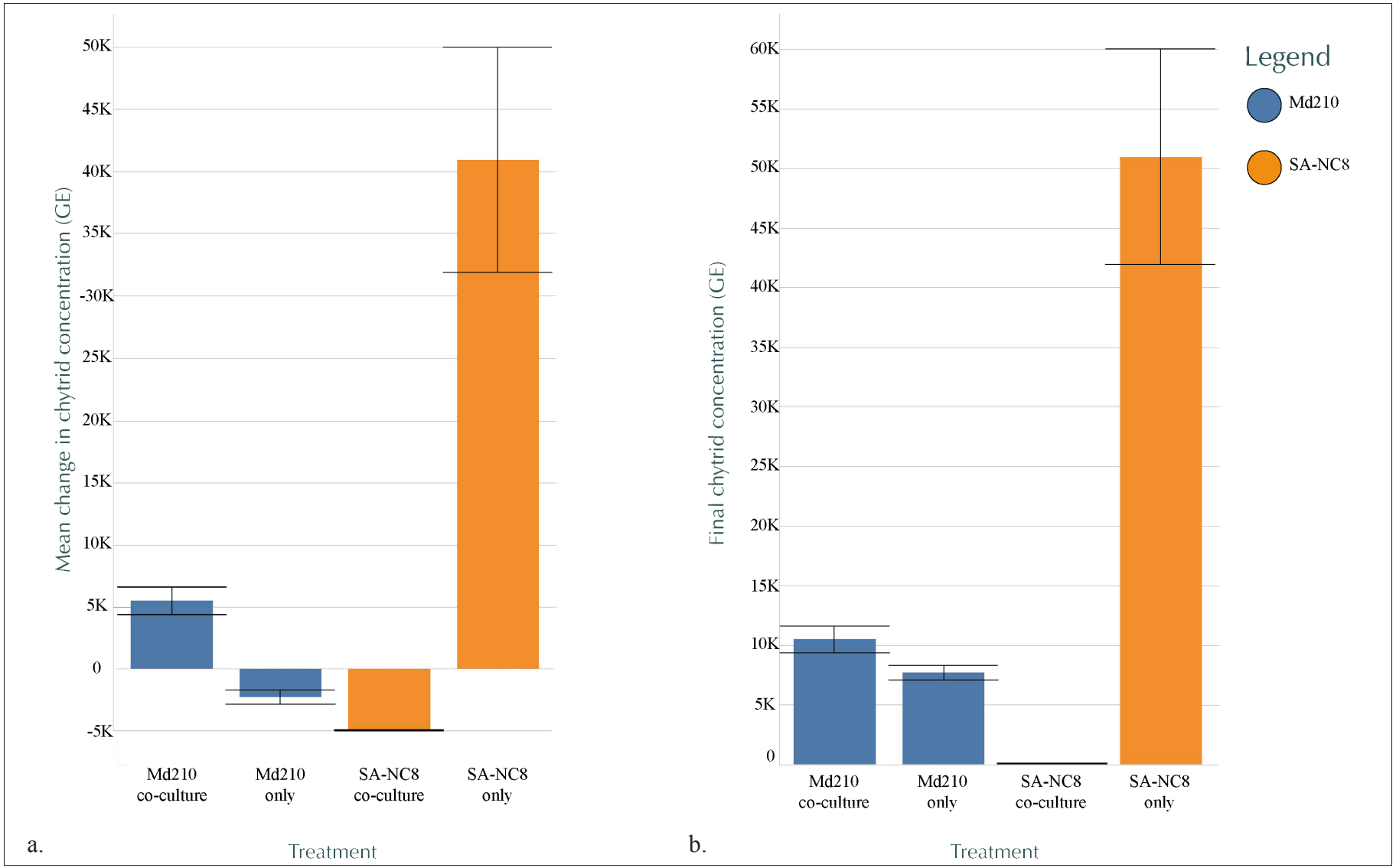


Figure 5.10. Bar plots of results of *in vitro* co-culture of Md210 and SA-NC8 (*Bd*GPL) showing (a) mean change in GE over the course of the experiment and (b) the mean chytrid concentration at the end of the experiment. Error bars represent upper and lower 95% confidence intervals.

5.5 Discussion

5.5.1 Discovery of a novel Malagasy chytrid

Phylogenetic analyses conducted here indicate that Md210 and R160 are likely the same, undescribed chytrid species in the same order (Rhizophydiales) as *Bd* and *Bsal*. Their apparent association with amphibian skin (a speculation strengthened by the fact that they were isolated from two amphibian species in very different environments) raises the intriguing possibility that niche competition among chytrids could hinder the ability of *Bd* to invade the mycobiome of Malagasy amphibians. While the possibility that the amphibian skin microbiome could provide a probiotic answer to the *Bd* epidemic has been the subject of much research (Bletz *et al*., 2013; Woodhams e*t al.*, 2016; Rebollar *et al*., 2016; Antwis & Harrison, 2018), comparatively little attention has been paid to the amphibian skin mycobiome. However, recent research suggests that exploiting inter-fungal competition dynamics may be more effective in the fight against *Bd* than exploiting the microbiome (Maherali & Klironomos, 2009; Kearns *et al*., 2017).

The basidiomycete discovered to be present in the Md210 isolate via sequencing must also be considered for any hypotheses generated. This new fungus was grouped within the Tremellaceae, a fungal family that has been demonstrated to exhibit microparasitism (Zugmaier, Bauer & Oberwinkler, 1994), and thus there is also a strong possibility that the basiodiomycete may impact growth of *Bd* as well as the novel chytrid species. A priority in taking this work forward is to attempt separation of these two species (bearing in mind that it is possible that the basidiomycete is an obligate parasite of the chytrid) and investigate the impact of the two fungi on *Bd*, both together and as pure isolates.

5.5.2 OD as a tool for recording *Bd* growth

Recording optical density of a well in a microplate reader is commonly used to measure microbial growth (Biesta-Peters *et al*., 2010; Hall *et al*., 2013; Peñuelas-Urquides *et al*., 2013), and has

been used to measure *Bd* growth rates *in vitro* (Piotrowski, Annis & Longcore, 2004; Stevenson *et al*., 2013; Muletz-Wolz *et al*., 2019). However, I was unable to find studies that had validated this for *Bd*. Moreover, the patchy nature of *Bd* zoospore distribution in liquid culture, and the two-phase life cycle of the fungus, means that the use of OD as a measure of growth is unlikely to be as straightforward as it is for many other microbes, particularly bacteria. Traditionally, microplate readers record a single OD measure per well, usually at the centre of the well, making the method unsuitable for organisms with a patchy or highly dispersed distribution, such as *Bd*. New technology in the form of well scanning (BMG Labtech, 2019), where multiple reads are systematically taken from a single well and averaged to obtain an optical density reading for each well, offer the possibility of much more accurate assessments of *Bd* growth than have been possible until now. We found that measurements of zoospore concentration were highly reliable, with an R^2 of 0.99. However, the OD readings overall were extremely low, with 2 million zoospores producing an average OD reading of just 0.82, and 125,000 zoospores (still a substantial infection intensity) producing an OD reading of approximately just 0.08. As a result, we would not recommend this method for assessing *Bd* growth unless the starting inoculate of zoospores is at least 100,000 zoospores per well for a 48 well plate. Another key point to note is that here we have only assessed the accuracy of the plate reader in measuring zoospore concentration. Although growth will be recorded as sporangia germinate, it will need to be assessed whether this growth can be recorded quantitatively in a mixed culture of zoospores and sporangia. If chytrid isolates vary in rate of transition between the two life stages and also in the size and density of those life stages, quantification will be compromised. The extent to which this could be an issue is illustrated in Figure 5.11. The growth curves indicate that SA-NC8 is not growing while Md210 is growing rapidly. However, microscopic photography (taken at a later date due to availability of a microscope with a camera attachment) at different stages of growth shows that the two chytrids are both growing well in pure culture, but Md210 is forming densely clustered sporangia at a much faster rate and few zoospores. SA-NC8, with growth trending towards zoospores and fewer, larger

sporangia, simply fails to reach sufficient densities for the microplate reader to detect the growth within the time period, despite healthy growth taking place.

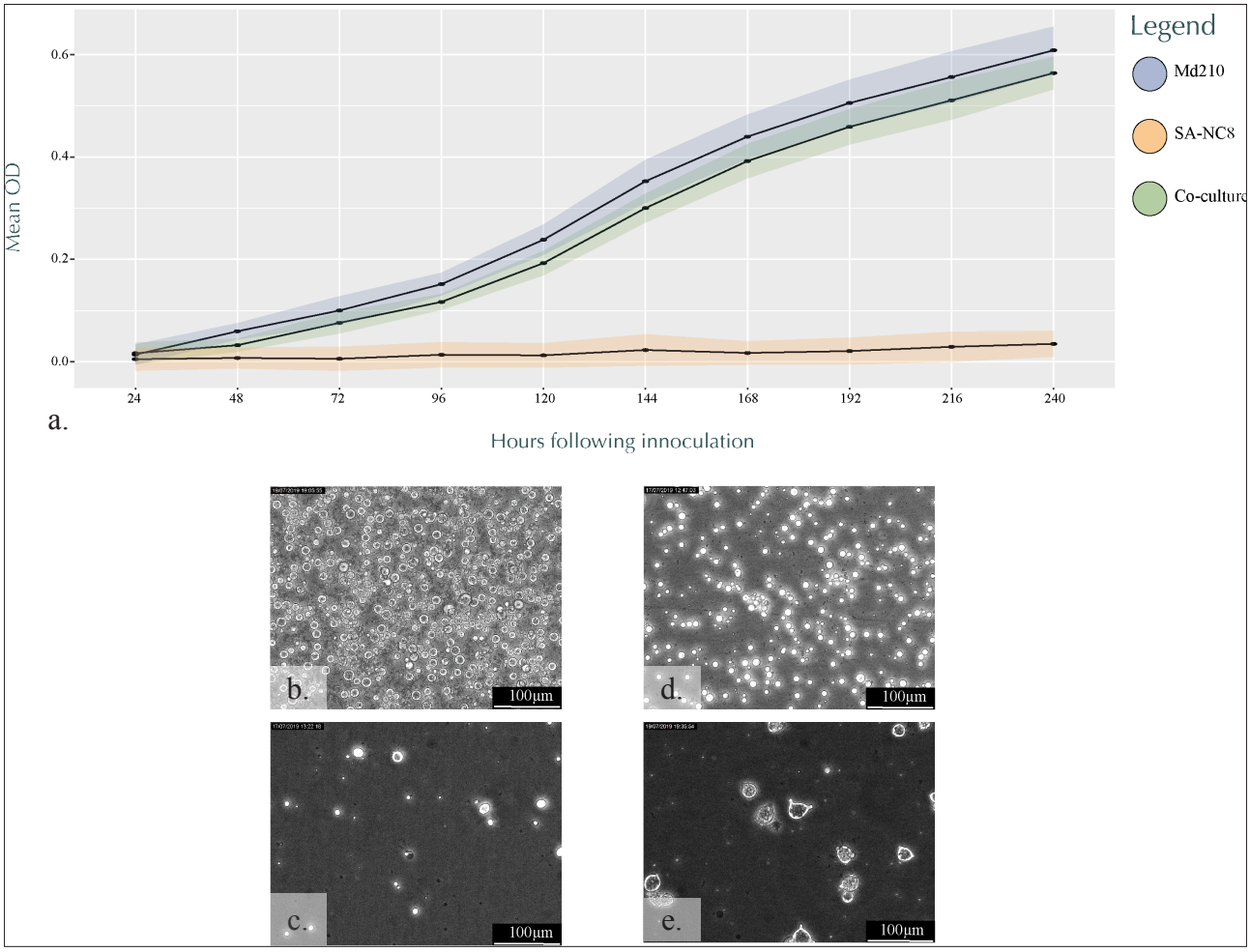


Figure 5.11. Images demonstrating different growth of Md210 and SA-NC8: (a) an OD plot of chytrid growth over 240 hours; microscopy images showing growth at 48 hours following inocluation with 10,000 zoospores for (b) Md210 and (c) SA-NC8; microscopy images showing growth at 120 hours following innoculation with 10,000 zoospores for (d) Md210 and (e) SA-NC8.

5.5.3 Inhibition of *Bd*GPL by Md210, and potential problems with surveying for *BxMada*

Experimental co-culture of the *Bd*GPL isolate SA-NC8 and the Malagasy chytrid isolate Md210 showed dramatic inhibition of *Bd*GPL. A curious result was that Md210 appeared to display some, but limited, growth in co-culture and decrease in pure culture, despite appearing to grow well when examined under a light microscope. What was clear, however, was that growth of SA-NC8 was severely limited, if not prevented altogether in the presence of Md210. There are four next steps that would be valuable to take as a priority with this research. Firstly, due to the nature of Md210's growth, with small dense sporangia produced and very limited numbers of zoospores, it would be valuable to compare the quantification accuracy of the TaqMan MGB probe described here when analysing a sporangial-heavy solution of Md210 and a zoosporangial heavy solution of Md210 in case this is causing artificially low quantification of Md210; secondly, the ability of *BxMada*, both with and without the associated *T. madagascari* to colonise amphibian skin should be investigated; thirdly, through genomic interrogation and experimental manipulation establish whether *BxMada* is a pathogenic fungus; and finally undertake experimental work to assess whether the *in vitro* result seen here can be reproduced *in vivo*. Previous work has shown that *Bd*'s *in vitro* response to experimental conditions is not always reflected when tested *in vivo*; for example, recent work has demonstrated that the temperature at which *Bd* grows fastest differs *in vitro* and *in vivo* (Sonn, Berman & Richards-Zawacki, 2017), so this is a crucial step to take.

This work highlights the importance of considering the amphibian mycobiome and the environmental microbial diversity beyond the bacteria when investigating the ability of a microbial community to protect amphibians from *Bd*. The attempt to survey how widespread *BxMada* is on Malagasy amphibians here did not result in detection of the chytrid in any of the over 700 samples tested. This is somewhat surprising, as chytrid isolation is time consuming and challenging (Fisher *et al*., 2018); only reasonably high infection loads are likely to result in the chytrid being isolated. To isolate *BxMada* twice in a single field trip from two different species in two different regions

would suggest that the chytrid might be expected to be detectable easily. Investigation into the ability of the TaqMan qPCR used here to quantify and detect *BxMada* sporangia, as described above would also be valuable in this case. If the qPCR is not able to detect sporangia efficiently, and this is the life stage that Md210 is most likely to be existing in, detectability may be extremely low with high numbers of false negatives. An alternative possibility is that the DNA present in the sample has degraded in storage, resulting in false negatives. Although generally freezing at -20^{o}C is accepted to be a stable storage solution for DNA extracts (Oxford Gene Technology, 2011), there is evidence that degradation results over longer time periods, with estimates of DNA stability at -20^{o}C ranging from two months to decades (Oxford Gene Technology, 2011; Romanazzi *et al*., 2015)

The risk that an invasion by *Bd* poses to the hyper diverse Malagasy amphibian fauna has been of huge concern to conservationists globally ever since the extreme virulence potential of the pathogen was realised (Andreone *et al*., 2008; Rabemananjara, Andreone & Rabibisoa, 2011; Rakotomanana, Jenkins & Ratsimbazafy, 2013; Weldon *et al*., 2013; Rosa *et al*., 2015). Given the relatively high level of wildlife trade in Madagascar, and the fact that *Bd*GPL has dispersed widely across the globe in a relatively short space of time (Skerratt *et al*., 2007; Farrer *et al*., 2011; O'Hanlon *et al*., 2018), it is somewhat surprising that *Bd* does not appear to have colonised the island stably, particularly despite controversial evidence of occasional occurrence (by qPCR) (Bletz *et al*., 2015b; Kolby & Skerratt, 2015; Byrne *et al*., 2019) and the fact that Malagasy frogs are susceptible to the fungus under experimental conditions (Bletz *et al.*, 2015b). Several avenues of recent research in the country have focussed on the potential protective properties of amphibian skin-associated bacteria may have in enabling Malagasy amphibians to resist *Bd* invasion, but no research to date has investigated how diversity within fungi and particularly among the Chytridiomycota themselves may protect amphibian populations. The closer two species are phylogenetically, theory predicts that the more likely it is that they will overlap in their fundamental niche requirements and thus the more likely that they will be forced to compete over resources, with the potential to restrict each other's ability to expand. Here, we have isolated a novel chytrid from amphibian skin in

Madagascar, which if shown to be obligatorily amphibian-associated, would be only the third chytrid discovered to be so. As such, it may already sufficiently occupy the fundamental niche of *Bd* to prevent a *Bd* invasion on the island. The discovery of the impact that *BxMada* has on *Bd*GPL growth illustrates the importance of examining the mycobiome, as well as the microbiome, when considering the microbial interactions of pathogens.

5.6 Acknowledgements and contributions

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Chapter 6

General Discussion

It is now clear that as rates of globalisation increase and we transport ourselves, our goods and hitchhiking microbial communities around the world at an ever-increasing pace, we are driving a re-emergence of infectious disease as a global health problem (Daszak *et al*., 2000; Gibbs, 2005; Semenza *et al*., 2016; Richardson *et al*., 2016; Bloom, Black & Rappuoli, 2017). Fungi in particular seem to be capitalising on our highly connected and warming world (Slippers, Stenlid & Wingfield, 2005; Gurr, Samalova & Fisher, 2011; Fisher *et al*., 2012; Fisher, Gow & Gurr, 2016; Fones, Fisher & Gurr, 2017; Ghosh, Fisher & Bates, 2018). Furthermore, there is growing recognition that human health is intrinsically linked to ecosystem and biodiversity health and that understanding pathogens in the wider ecosystem can enable us to protect ourselves against future emerging infectious diseases (Degeling *et al*., 2015; Lebov *et al*., 2017; Cunningham, Daszak & Wood, 2017; Patil, Kumar & Bagvandas, 2017). Although historically disease has not been considered a primary threat to wildlife populations, it is now clear that under certain conditions, pathogens can be critical proximate causes of population decline, with the corresponding ecological disruption further impacting ecosystem health (McCallum, 2012; Heard *et al*., 2013).

To manage the increasing threat of emerging infectious diseases, we need to be able to map disease risk across a landscape as a first step towards identifying which of the myriad potential epidemiological factors are driving disease dynamics (Ostfeld, Glass & Keesing, 2005; Pigott *et al*., 2015; Stephens *et al*., 2016). Many attempts have been made to quantify *Bd* distribution and chytridiomycosis risk across multiple scales from the global to the local, but these have often produced conflicting results and exceptions to the general trends continue to emerge (; Kriger & Hero, 2007, 2008; Puschendorf *et al*., 2009; Murray *et al*., 2011; Olson *et al*., 2013; Olson & Ronnenberg, 2014; Xie, Olson & Blaustein, 2016; Sonn, Berman & Richards-Zawacki, 2017). Two

aspects of *Bd* biology that have not to date been fully explored and may help to explain some of these discrepancies are the influence of *Bd* lineages and lineage interactions, and the interactions of *Bd* within the wider fungal community, on *Bd* distribution. The lack of appropriate tools for identifying *Bd* lineage has greatly hampered research into this interesting facet of *Bd* ecology to date by making it near impossible to generate enough baseline data to develop and test hypotheses. Broadly, this research highlights three main points with which to move forward within the *Bd* research community. Firstly, that diagnostic tool selection is critical to epidemiological research and that without appropriate tools in place, it will not be possible to develop meaningful hypotheses or gather informative baseline data. Secondly, that the lineages of *Bd* represent the next frontier for the *Bd* research community. Although lineage is widely acknowledged to be epidemiologically important, there has been limited progress in quantifying exactly how important they are, or how lineage interactions are likely to impact disease dynamics. Finally, marrying the insightful work that has been carried out on chytrid comparative genomics with an understanding of ecological niche theory, and considering the impact not only *Bd* strains but the wider fungal community may have on *Bd* distributions and virulence, may open up a new avenue of research into how hosts can resist disease where the mechanism of resistance is currently cryptic.

6.1 Tool selection is critical to informative spatial epidemiology

The ability to identify a disease accurately and economically at an epidemiologically relevant taxonomic level is crucial for mapping disease risk and for investigating drivers of disease dynamics (Peeling, Smith & Bossuyt, 2006; Ghosh, Fisher & Bates, 2018). Therefore, the development of appropriate diagnostic tools is the foundation of effective spatial epidemiology. It is now clear that the response of *Bd* to a host population, or the response of the host population to *Bd*, is so variable that simply knowing that *Bd* is present is not indicative of whether a population is threatened, or whether the *Bd* is an endemic long-term associate of the host population or an invasive pathogen (Berger *et al*., 2005; Rodriguez *et al*., 2014; Becker *et al*., 2017; O'Hanlon *et al*., 2018).

Now that it is clear that *Bd* lineage is very likely to be an important variable in these responses, it is necessary to distinguish routinely between them. WGS, the gold standard for *Bd* lineage identification, is prohibitively time-consuming, expensive and skill-intensive for application in most instances where lineage identification is required. Although there have been previous attempts to develop *Bd* lineage-specific diagnostics, the qPCR assay developed here holds significant advantages over other approaches in that it a) uses technology already present in any laboratory that works with *Bd* and thus is easy to adopt, b) is highly sensitive even in the case of asymptomatic individuals, with a limit of detection of 1 GE and c) targets a region that has been shown to be reliable at resolving lineages in agreement with WGS analysis, rather than utilising the ITS region (Boyle *et al*., 2004; Rodriguez *et al*., 2014; Byrne *et al*., 2017). Without the development of the lineage-specific qPCR described here it would not have been possible to generate such a large volume of data as that in Chapter 4 so quickly and within a reasonable budget.

Critically, the ability to screen field samples using a tool that diagnosed at a finer taxonomic resolution revealed previously hidden distribution patterns that may impact broader estimates of the *Bd* niche, highlighting the importance of selecting a tool that operates at an epidemiologically relevant scale. Similarly, the ability to pre-screen archived specimens for a positive result with the lineage-specific qPCR meant that time and financially costly shotgun sequencing could be targeted towards specimens most likely to yield useful data (Chapter 3). The concurrence of the shotgun sequencing with the qPCR lineage identification also served to strengthen confidence further in this novel method.

The utility of this tool also extends beyond spatial epidemiology and into experimental work, enabling mixed-lineage infections to be detected *in vivo* (Chapter 2) which should facilitate future work into lineage interactions in hosts. Furthermore, it is increasingly recognised that species level-based biosecurity is not always appropriate for microbes (McTaggart *et al*., 2016). As it becomes increasingly likely that this is the case for *Bd*, it makes sense to adjust the taxonomic level at which

we monitor the pathogen accordingly.

6.2 *Bd* lineage is the next research frontier

Substantial progress has been made in the field of *Bd* research by taking a comparative genomics approach (Farrer *et al.*, 2011; Rosenblum *et al*., 2013; Farrer *et al*., 2017; Farrer & Fisher, 2017; O'Hanlon *et al*., 2018). Comparative genomics has revealed the existence of *Bd* lineages; demonstrated that lineage hybridisation has occurred in nature; revealed the varying levels of diversity among the lineages; indicated that only one lineage, *Bd*ASIA-1, shows evidence of endemism with native hosts; and shown that the global panzootic lineage is greatly lacking in genetic diversity compared with other lineages (Farrer *et al*., 2011; O'Hanlon *et al*., 2018).

The time is now right to push forward in addressing the question of exactly how important *Bd* lineage is epidemiologically and how much the lineages differ ecologically, which will require substantial research effort. The greatest progress in this direction has been made in the Brazilian Atlantic Forest, where *Bd*GPL and *Bd*ASIA-2/BRAZIL are both found (Rodriguez *et al*., 2014; Jenkinson *et al*., 2016). However, the frequency of *Bd*ASIA-2/BRAZIL in the wild is reasonably low, particularly in comparison with the much more widely found *Bd*GPL (Rodriguez *et al*., 2014; Jenkinson *et al*., 2016), so natural environmental associations are difficult to define, and the lack of another well-defined lineage co-occurrence region with which to compare the Brazilian Atlantic Forest makes it impossible to say whether any associations that are seen may reflect *Bd* dynamics globally.

Even after over 20 years of intensive research, the environmental envelope of *Bd* has not been satisfactorily resolved, and incorporating *Bd* lineage is a clear next step to take to address this (James *et al*., 2015; Ghosh & Fisher, 2016). Understanding how and when lineages interact, the epidemiological importance of lineages and whether they tend to occupy different environmental niches will be necessary for understanding the evolution of *Bd* as a species, and for developing accurate species distribution models and risk assessments for hosts. In Chapter 4, South Africa is

presented as a complementary study system to that in place in the Brazilian Atlantic Forest. This research showed that *Bd*GPL and *Bd*CAPE are associated with different environments and that they have very different population structures which hint at the differences in their evolutionary pasts. The survival of multiple *Bd* isolates at temperatures 8°C above the generally accepted thermal maximum illustrates the highly plastic nature of the fungus and the next step is to establish whether that plasticity exists at the isolate level or the lineage level — or both — and how that is likely to impact disease dynamics.

A key finding of this research was the identification of a zone of low prevalence lineage co-occurrence, at the very local scale of a single pond in which both lineages infect the same population of frogs. This presents an exciting opportunity to track lineage interactions in nature at a scale that has not previously been possible. The baseline data and patterns described in this research lay strong foundations on which to build investigations into the significance of lineage in the *Bd* story, using Southern Africa as a model system.

6.3 Linking niche and phylogenetic relatedness to understand disease dynamics and explore novel pathogen control mechanisms

The interactions between amphibian skin-associated bacteria, the microbiome, and *Bd* and their impact on host survival have been the subject of substantial research effort, and it has been shown multiple times that *Bd* infection is associated with a perturbed amphibian microbiome, and that some amphibian-associated bacteria are able to inhibit *Bd in vitro* and show a degree of host protection *in vivo* (Harris *et al*., 2009; Vredenburg, Briggs & Harris, 2011; Bletz *et al*., 2013; Jani & Briggs, 2014). Progress has stalled, however, when it has come to demonstrating that amphibian skin-associated bacteria can protect amphibian communities in a meaningful way on a large scale, and the effects are not consistent between host populations or *Bd* isolates (Antwis *et al.*, 2015; Antwis & Harrison, 2018).

In comparison, very little research has explored how amphibian skin-associated fungi might

impact *Bd* disease dynamics (Kearns *et al*., 2017). This is despite the fact that phylogenetic niche conservatism theory predicts that organisms that are most closely related to each other are most likely to overlap in their fundamental niche resource requirements, thus leading to elevated competition and a greater probability of competitive exclusion (Maherali & Klironomos, 2009; Godoy, Kraft & Levine, 2014; Venail *et al*., 2014; Fountain-Jones *et al*., 2018). Although the applicability of this theory to nature has been widely debated (Venail *et al.*, 2014; Godoy, Kraft & Levine, 2014), there is some evidence from among fungi that this concept holds true (Maherali & Klironomos, 2009).

As such, it would seem that the mycobiome as a modulating force on *Bd* dynamics merits further investigation. The chance isolation of an amphibian skin-associated non-*Bd* chytrid from Madagascar in 2015 presented an exciting opportunity to investigate this possibility. In Chapter 5, *in vitro* experimental work showed that an isolate of *Bd*GPL, selected for its vigorous growth, was unable to persist in co-culture with *BxMada*, and if this can be replicated *in vivo*, it is a candidate mechanism for how the amphibians of Madagascar have so far resisted *Bd* infection despite many being susceptible under laboratory conditions. If the amphibian skin-associated microbiome is providing a protective effect, this will not necessarily be seen in exposure trials as even animals taken directly from the wild experience rapid and significant reduction in skin-associated fungal diversity following captivity.

Phylogenetic niche conservatism also points to why we might expect *Bd* lineages to have a particularly strong impact on each other's dynamics when they encounter each other. If true, then lineages of the same species, due to their extremely close phylogenetic relatedness, will be most likely to undergo strong competition on meeting. This has been shown to be a strong force in disease dynamics in other pathogens (Gower & Webster, 2005; Koskella, Giraud & Hood, 2006; Pepin, Lambeth & Hanley, 2008; Pepin & Hanley, 2008; Cobey & Lipsitch, 2013; Hecht *et al*., 2016), and it stands to reason that the same may be the case for *Bd* as well. It was notable

in Chapter 4 that cold spots for prevalence of both lineages coincided with where their ranges overlapped (this is in contrast to what is seen in Brazil, where although *Bd*ASIA-2/BRAZIL is of low prevalence, *Bd*GPL is ubiquitous (Jenkinson *et al.*, 2016)). One explanation for this pattern, could be that the lineages are experiencing mutual competitive suppression. The parallels between the results of Chapters 4 and 5, where in both cases it appears that *Bd* experiences population suppression in the presence of competition, raises the exploration of competitive chytrid interactions as an exciting research avenue. Combining an understanding of *Bd* phylogeny with *Bd* ecology explores a new facet of the disease triangle at the pathogen biology/environmental interface and adopting this eco-phylogenetic approach could lead to new insights into how to manage *Bd* and explore its evolutionary pathway.

6.4 Future directions

Delineating *Bd* lineage distributions globally is a critical next step in the world of *Bd* research. To do this, it will be necessary to design diagnostic tools similar to those presented here for the remaining four known *Bd* lineages, *Bd*ASIA-1, *Bd*ASIA-2/BRAZIL, *Bd*ASIA-3 and *Bd*CH, and to view the screening of *Bd* surveillance samples for lineage as a standard protocol. The development of these tools will also enable the vast trove of information currently hidden inside museum archive specimens to be unlocked, shedding light on historical *Bd* distributions and generating valuable data on the realised niche of pathogens prior to modern-day high rates of pathogen pollution. A key priority is to define the environmental envelopes for the *Bd* lineages and assess whether a failure to account for lineage has affected the accuracy of *Bd* SDMs and risk assessments to date.

Similarly, experimental work both *in vitro* and *in vivo* will be necessary to understand how lineages interact with each other when they meet in a host or in a landscape, and whether this must also be accounted for when modelling *Bd* distributions. Understanding *Bd* lineage interactions will also be critical for identifying whether there is a risk of hypervirulent recombinant offspring arising from *Bd* lineage contact, and if so, where in the world this is most likely to happen. At a broader scale,

this research has highlighted that investigating the amphibian mycobiome and how it may impact *Bd* population dynamics represents an intriguing prospective research avenue.

6.5 Final comments

In this thesis I sought to design a tool that would enable the diagnosis of *Bd* lineage from amphibian skin swabs, museum specimens and tissue samples in order to facilitate the generation of baseline data of lineage distributions and enable mixed-lineage infections to be detected under experimental conditions. Using this novel diagnostic, I then explored Southern Africa as a candidate model study system for *Bd* lineage ecology and asked whether lineages that are known to be epidemiologically relevant differed in their distributions across a landscape. Finally, I expanded the line of inquiry to look at whether the wider chytrid community could also impact *Bd* distribution and thus whether the amphibian mycobiome may have a role to play in protecting amphibians from *Bd* invasion in naïve areas.

This study has opened up many exciting new research possibilities, from exploiting novel data sources, such as museums, to indicating how focussing on an eco-phylogenetic approach to unpicking the *Bd* disease triangle may shed fresh light on old problems of understanding *Bd*'s apparently hyperplastic nature. The *Bd* system is clearly highly complex, but untangling the drivers behind the dynamics of the most destructive pathogen the world has encountered to date is a critical issue not only in addressing the 21st century's ongoing biodiversity crisis, but to creating a framework through which to view other pathogens which are likely just around the corner. A multi-disciplinary research approach, combined with an appreciation of the highly complex nature of interactions between hosts, their pathogens and the wider microbial community in nature will be necessary going forward to protect biodiversity and human health in our hyper-globalised world.

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Appendices

Appendix 1. Recipe for Tryptone-based nutrient broth and 1% agar plates (TGhL) (makes 1L of broth or approx. 40 plates)

Ingredients

8g Tryptone

2g Gelatin hydrosolate

4g Lactose

10g Agar (for plates only)

0.2g Streptomycin sulfate

0.2g Penicillin-G

Protocol

Dissolve all ingredients in 1L of filtered water (only add agar if making plates). Autoclave to sterilise and cool to 18^{o}C before using. Store the broth or plates at 4^{o}C. For antibiotic-infused broth and plates, add 950ml of filtered water, instead of 1L, to the dry ingredients, and autoclave to sterilise. When the broth has cooled to 50^{o}C, dissolve 0.2g (200mg/L) streptomycin sulfate and 0.2g (200mg/L) penicillin-G in 50ml of distilled water in a BioSafety ACDP Class 2 cabinet or equivalent. Pass the dissolved antibiotics through a 0.2 micrometer millipore filter and add to the autoclaved broth. Mix for two minutes using a magnetic stirrer, then prepare plates and broth once the solution has cooled to 18^{o}C.

Appendix 2. Metadata for isolates used to make DNA quantitation standards

Isolate name	Source location	Lineage	Year isolated	Host species
TF5a1	Mallorca	*Bd*CAPE	2007	*Alytes muletensis*
IA042	Spain	*Bd*GPL	2004	*Alytes obstetricans*

Appendix 3. Metadata for isolates used for broad-range DNA specificity testing panel

Isolate name	Source Location	Lineage	Year Isolated	Host Species
ACON	Switzerland	*Bd*ASIA-2/Brazil	2007	*Alytes obstetricans*
UM142	Brazil	*Bd*ASIA-2/Brazil	2009	*Lithobates catesbeianus*
CLFT065	Brazil	*Bd*ASIA-2/Brazil	2013	*Hylodes japi*
TRBOOR'11	Trade	*Bd*ASIA-1	2012	*Bombina variegata*
KBO317	South Korea	*Bd*ASIA-1	2014	*Bombina orientalis*
KBO327	South Korea	*Bd*ASIA-1	2014	*Bombina orientalis*
KBO319	South Korea	*Bd*ASIA-1	2014	*Bombina orientalis*
TF5a1	Mallorca	*Bd*CAPE	2007	*Alytes muletensis*
NTA6	Cameroon	*Bd*CAPE	2012	*Ophisthothylax immaculatus*
TRGSCM12	Trade	*Bd*CAPE	2007	*Geotrypetes seraphini*
SA1D	South Africa	*Bd*CAPE	2010	*Hadramophryne natalensis*
SA4C	South Africa	*Bd*CAPE	2010	*Amietia angolensis*
MG01	South Africa	*Bd*CAPE	2008	*Amietia vertebralis*
ETH4	Ethiopia	*Bd*GPL	2011	*Leptopelis spp.*
ETH2	Ethiopia	*Bd*GPL	2011	*Afrixalus enseticola*
MG08	South Africa	*Bd*GPL	2008	*Amietia angolensis*
UG3	Uganda	*Bd*GPL	2012	*Amietophrynus spp.*
BEW2	Switzerland	*Bd*GPL	2010	*Alytes obstetricans*
BLI1	Switzerland	*Bd*GPL	2010	*Alytes obstetricans*
BR1	Switzerland	*Bd*GPL	2011	*Alytes obstetricans*
RC5	France	*Bd*GPL	2010	*Rana catesbeiana*
LHURS 11	France	*Bd*GPL	2011	*Alytes obstetricans*
LHURS 11'7	France	*Bd*GPL	2011	*Alytes obstetricans*
LHURS 11'11	France	*Bd*GPL	2011	*Alytes obstetricans*
PUITS'11	France	*Bd*GPL	2011	*Alytes obstetricans*
PUITS' 12' 3	France	*Bd*GPL	2012	*Alytes obstetricans*
PUITS' 12' 4	France	*Bd*GPL	2012	*Alytes obstetricans*
PUITS' 12' 5	France	*Bd*GPL	2012	*Alytes obstetricans*
PUITS' 12' 7	France	*Bd*GPL	2012	*Alytes obstetricans*
C2A	Spain	*Bd*GPL	2002	*Alytes obstetricans*
PICOS A01	Spain	*Bd*GPL	2010	*Alytes obstetricans*
AD MALA	Spain	*Bd*GPL	2012	*Alytes dickilleni*
IA042	Spain	*Bd*GPL	2004	*Alytes obstetricans*
IA043	Spain	*Bd*GPL	2004	*Alytes obstetricans*
IA' 12 1	Spain	*Bd*GPL	2012	*Alytes obstetricans*
IA' 12 3	Spain	*Bd*GPL	2012	*Alytes obstetricans*
TORO' 11	Spain	*Bd*GPL	2011	*Alytes obstetricans*
IA' 11	Spain	*Bd*GPL	2011	*Alytes obstetricans*

Appendix 3 (cont.) Metadata for isolates used to make broad-range DNA specificity testing panel

Isolate name	Source location	Lineage	Year isolated	Host species
BAVOBER 1	Germany	*Bd*GPL	2012	*Alytes obstetricans*
BAVOBER 2	Germany	*Bd*GPL	2013	*Alytes obstetricans*
BAVOBER 3	Germany	*Bd*GPL	2013	*Alytes obstetricans*
MODS 26.1	Sardinia	*Bd*GPL	2010	*Discoglossus sardus*
MODS 27	Sardinia	*Bd*GPL	2010	*Discoglossus sardus*
AP15.2	Sardinia	*Bd*GPL	2010	*Discoglossus sardus*
SP10.2	Sardinia	*Bd*GPL	2010	*Discoglossus sardus*
UKBELV'12	UK	*Bd*GPL	2012	*Lissotriton vulgaris*
UKMABC'12	UK	*Bd*GPL	2012	*Bufo calamita*
UKSHBC12	UK	*Bd*GPL	2012	*Bufo calamita*
L2203	Montserrat	*Bd*GPL	2009	*Leptodactylus fallax*
JEL261	Canada	*Bd*GPL	1999	*Lithobates catesbeianus*
JEL270	USA	*Bd*GPL	1999	*Lithobates catesbeianus*
JEL274	USA	*Bd*GPL	1999	*Bufo boreas*
JEL423	Panama	*Bd*GPL	2004	*Hylomantis lemur*
0739	Switzerland	*Bd*CH	2007	*Alytes obstetricans*

Appendix 4. Accession numbers for archive specimens from the Natural History Museum, London and NWU

Accession number	Collection	Host species	Year collected	Source location
1985.1373	Natuural History Museum, London	*Xenopus gilli*	1982	South Africa
1935.10.10.290	Natural History Museum, London	*Xenopus laevis bunyoniensis*	1934	Uganda
1935.10.10.295	Natural History Museum, London	*Xenupus laevis bunyoniensis*	1934	Uganda
1985.1374	Natural History Museum, London	*Xenopus gilli*	1982	South Africa
1948.1.8.74	Natural History Museum, London	*Xenopus fraseri*	1933	Cameroon
AC040803_A	Prof. C. Weldon, NWU	*Nectophrynoides asperginis*	2003	Tanzania
AC290703_A1	Prof. C. Weldon, NWU	*Nectophrynoides asperginis*	2003	Tanzania
AC290703_A2	Prof. C. Weldon, NWU	*Nectophrynoides asperginis*	2003	Tanzania
AC0290703_A3	Prof. C. Weldon, NWU	*Nectophrynoides asperginis*	2003	Tanzania

Appendix 5.Primer sequences for Kihansi spray toad DNA Index PCR (shotgun sequencing library preparation)

Sample	Oligo ID	Oligo Sequence (5′ - 3′) (index in lower case)
AC040803_A	P7 primer BEST_701	CAAGCAGAAGACGGCATACGAGATcctgcgaGTGACTGGAGTTCAGACGTGT
	P5 primer BEST_501	AATGATACGGCGACCACCGAGATCTACACcctgcgaACACTCTTTCCCTACACGACGCTCTT
AC290703_A2	P7 primer BEST_702	CAAGCAGAAGACGGCATACGAGATtgcagagGTGACTGGAGTTCAGACGTGT
	P5 primer BEST_502	AATGATACGGCGACCACCGAGATCTACACtgcagagACACTCTTTCCCTACACGACGCTCTT

Appendix 6. Metadata for South African and Lesotho isolates lineage typed by WGS

Isolate name	Year collected	Source location	Latitude	Longitude	Host species	Lineage
SAKN-1	2016	Mont Aux Sources	-28.75231	28.894361	*Amietia hymenopus*	*Bd*CAPE
SAKN-4	2016	Royal Natal Natioanl Park	-28.71981	28.923611	*Hadramoprhyne natalensis*	*Bd*CAPE
SAKN-5	2016	Royal Natal National Park	-28.71981	-28.71981	*Hadramophryne natalensis*	*Bd*CAPE
SAKN-6	2016	Royal Natal Natioanl Park	-28.71981	28.923611	*Hadramophryne natalensis*	*Bd*CAPE
SAFS-1	2016	Mont Aux Sources	-28.75064	28.871361	*Amietia hymenopus*	*Bd*CAPE
DB8-4	2016	Mont Aux Sources	-28.760278	28.896389	*Amietia hymenopus*	*Bd*CAPE
SA152	2017	Ukhahlamba	-29.39124	29.73853	*Amietia delalandii*	*Bd*CAPE
SA186	2017	Lotheni	-29.45001	29.51483	*Amietia delalandii*	*Bd*CAPE
SA190	2017	Lotheni	-29.45001	29.51483	*Amietia delalandii*	*Bd*CAPE
SA211	2017	Sani Pass	-29.60708	29.33532	*Amietia delalandii*	*Bd*CAPE
SA243	2017	Cobham	-29.69575	29.4129	*Amietia delalandii*	*Bd*CAPE
SA266	2017	Garden Castle	-29.74601	29.20829	*Amietia delalandii*	*Bd*CAPE
SA280	2017	Garden Castle	-29.74601	29.20829	*Amietia delalandii*	*Bd*CAPE
SA286	2017	Garden Castle	-29.74601	29.20829	*Amietia delalandii*	*Bd*CAPE
SA546	2017	Douglas	-29.08682	23.79704	*Amietia delalandii*	*Bd*CAPE
SA1D	2010	Royal Natal National Park	-28.71986	28.92375	*Hadramophryne natalensis*	*Bd*CAPE
SA3E	2010	Pinetown	-29.02242	30.58106	*Amietia angolensis*	*Bd*CAPE
SA4C	2010	Pinetown	-29.02242	30.58106	*Amietia angolensis*	*Bd*CAPE
SA5C	2010	Pinetown	-29.02242	30.58106	*Amietia angolensis*	*Bd*CAPE
SA6E	2010	Pinetown	-29.02242	30.58106	*Amietia angolensis*	*Bd*CAPE
MG1	2008	Mont Aux Sources	-28.844605	29.054547	*Amietia vertebralis*	*Bd*CAPE
SA499	2017	Douglas	-29.08682	23.79704	*Amietia delalandii*	*Bd*GPL
SA523	2017	Douglas	-29.08682	23.79704	*Amietia delalandii*	*Bd*GPL

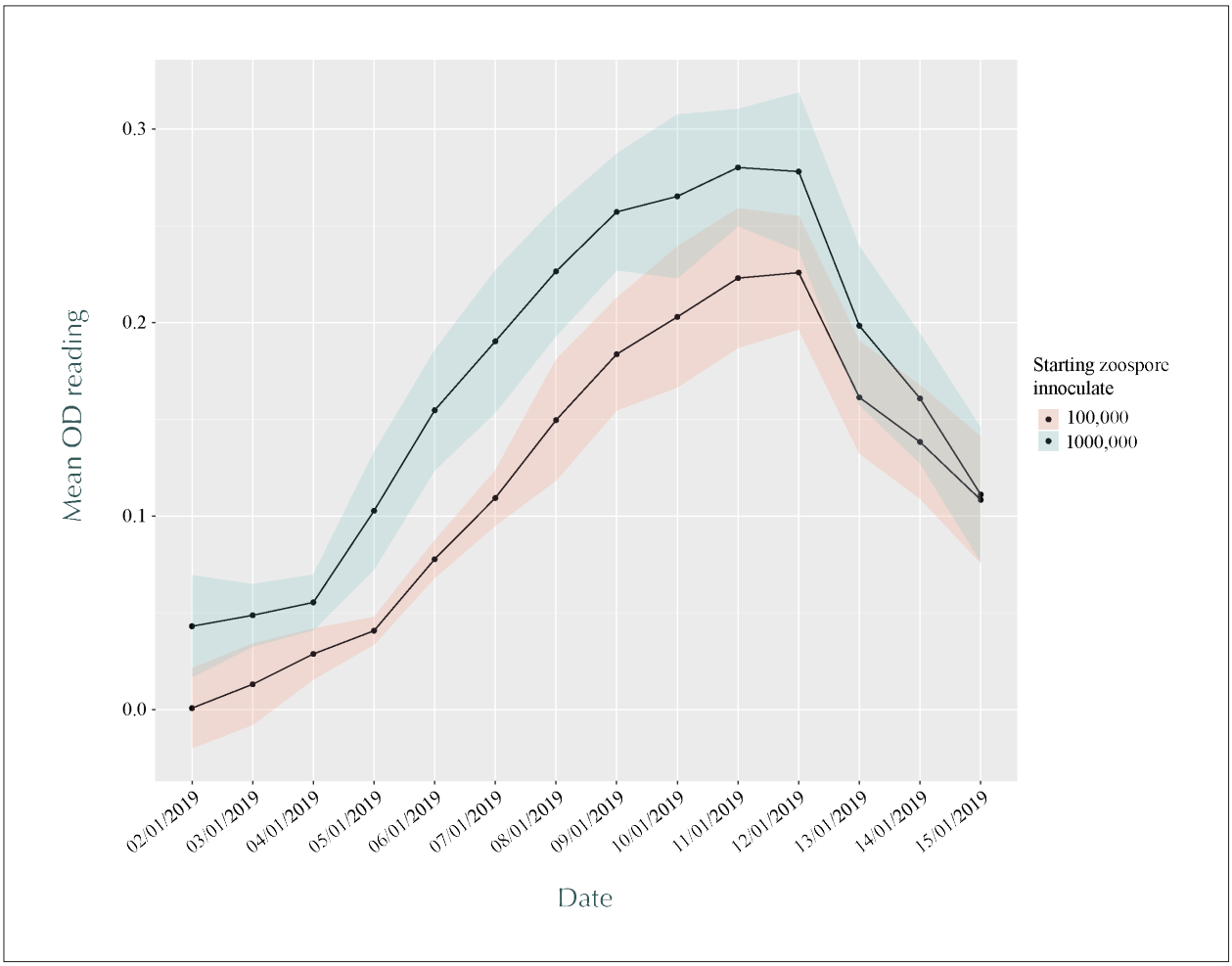
Appendix 6 (cont.). Metadata for South African and Lesotho isolates lineage typed by WGS

Isolate name	Year collected	Source location	Latitude	Longitude	Host species	Lineage
SA-EC1	2015	Aliwal North	-30.598095	29.894511	Unidentified	*Bd*GPL
SA-EC6	2016	Aliwal North	-30.72189	26.9064555	*Amietia quecketti*	*Bd*GPL
SA-EC7	2016	Aliwal North	-30.72189	26.9064555	*Amietia quecketti*	*Bd*GPL
SA-NC1	2015	Pella	-28.91356	19.0014167	*Amietia quecketti*	*Bd*GPL
SA-NC2	2015	Pella	-28.91356	19.0014167	*Amietia quecketti*	*Bd*GPL
SA-NC3	2015	Pella	-28.91356	19.0014167	*Amietia quecketti*	*Bd*GPL
SA-NC4	2015	Pella	-28.91356	19.0014167	*Amietia quecketti*	*Bd*GPL
SA-NC5	2015	Pella	-28.91356	19.0014167	*Amietia quecketti*	*Bd*GPL
SA-NC6	2015	Onseepkans	-28.73725	19.299222	*Amietia quecketti*	*Bd*GPL
SA-NC7	2015	Onseepkans	-28.73725	19.299222	*Amietia quecketti*	*Bd*GPL
SA-NC8	2015	Vioolsdrift	-28.69598	17.597361	*Amietia quecketti*	*Bd*GPL
SA-NC9	2015	Vioolsdrift	-28.69598	17.597361	*Amietia quecketti*	*Bd*GPL
SA-NC10	2015	Vioolsdrift	-28.69598	17.597361	*Amietia quecketti*	*Bd*GPL
08MG02	2008	Mont Aux Sources	-28.844605	29.054547	*Amietia vertebralis*	*Bd*GPL
MG3	2008	Memel	-27.682836	29.57863	*Amietia angolensis*	*Bd*GPL
MG4	2008	Silvermine	-34.093316	18.42167	*Amietia fuscigula*	*Bd*GPL
08MG05	2008	Silvermine	-34.093316	18.42167	*Amietia fuscigula*	*Bd*GPL
MG8	2008	Magoebaskloof	-23.85	30.033333	*Amietia angolensis*	*Bd*GPL
MCT8	2008	Silvermine	-34.093316	18.42167	*Amietia fuscigula*	*Bd*GPL
MC94	2008	Luvuvhu	-22.901675	-22.901675	*Xenopus laevis*	*Bd*GPL
JEL433	2005	Namaqualand	-29.983039	19.374335	*Xenopus laevis*	*Bd*GPL
SA-EC3	2015	Hogsback	-32.51572	26.934611	*Amietia quecketti*	Recombinant
SAEC-5	2016	Aliwal North	-30.72189	26.9064555	*Amietia quecketti*	Recombinant

Appendix 7. Metadata for experimental isolates used for *in vitro* assessment of lineage heat shock response

Isolate name	Year collected	Source location	Latitude	Longitude	Host species	Lineage
SAKN-1	2016	Mont Aux Sources	-28.75231	28.894361	*Amietia hymenopus*	*Bd*CAPE
SAKN-2	2016	Mont Aux Sources	-28.75231	28.894361	*Amietia hymenopus*	*Bd*CAPE
SA152	2017	Ukhahlamba	-29.39124	29.73853	*Amietia delalandii*	*Bd*CAPE
SA280	2017	Garden Castle	-29.74601	29.20829	*Amietia delalandii*	*Bd*CAPE
SA546	2017	Douglas	-29.08682	23.79704	*Amietia delalandii*	*Bd*CAPE
SA-NC2	2015	Pella	-28.91356	19.0014167	*Amietia quecketti*	*Bd*GPL
SA-NC4	2015	Pella	-28.91356	19.0014167	*Amietia quecketti*	*Bd*GPL
SA-NC8	2105	Vioolsdrift	-28.69598	17.597361	*Amietia quecketti*	*Bd*GPL
SA-EC6	2016	Aliwal North	-30.72189	26.9064555	*Amietia quecketti*	*Bd*GPL
SA523	2017	Douglas	-29.08682	23.79704	*Amietia delalandii*	*Bd*GPL
SA-EC5	2016	Aliwal North	-30.72189	26.9064555	*Amietia quecketti*	Recombinant

Appendix 8. Example *Bd* growth curve generated using a BMG Labtech microplate reader with spiral well scanning, isolate IA042



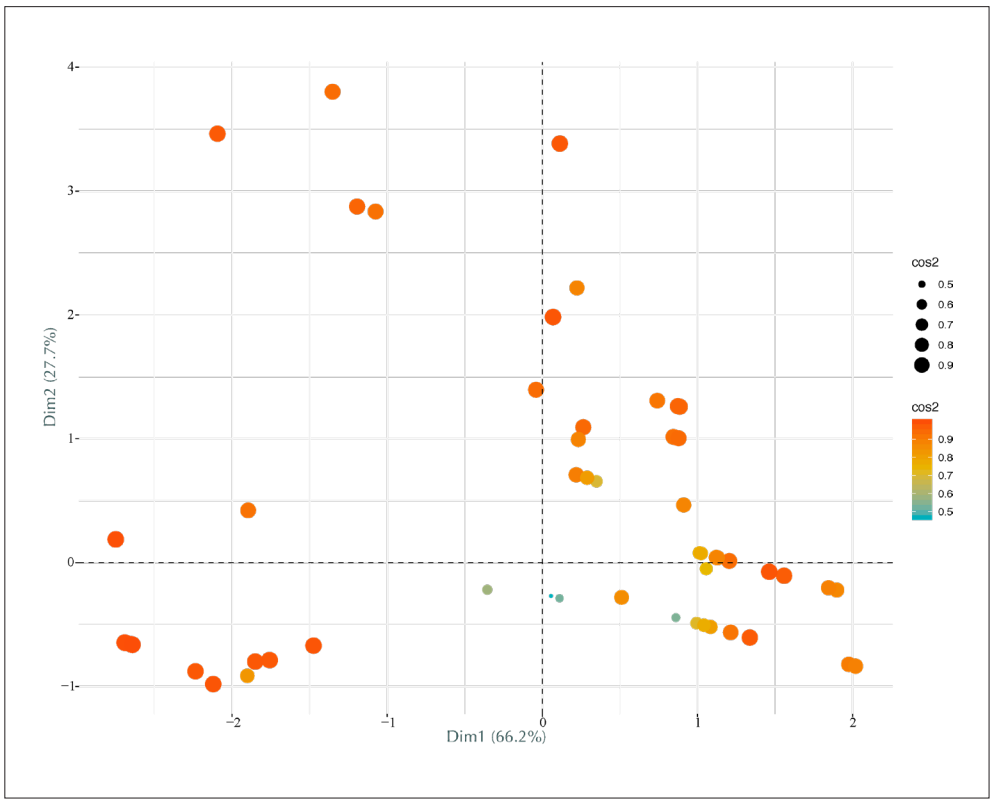
Appendix 9a. Eigenvalues for PCA of lineage identification and environmental variables

Dimension	Eigenvalue	Percentage of variance explained
Dimension 1	2.65	66.24
Dimension 2	1.11	27.67
Dimension 3	0.19	4.70
Dimension 4	0.06	1.40

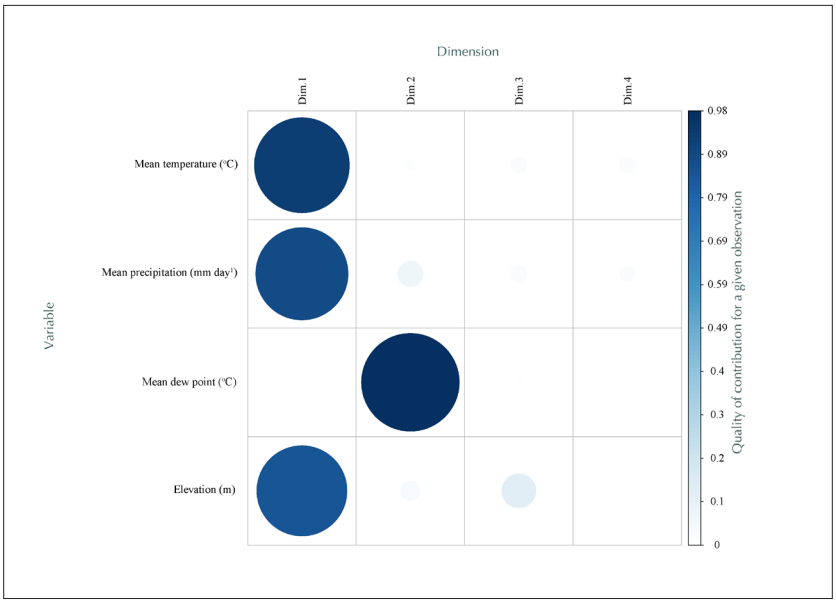
Appendix 9b. Dimension descriptions for PCA of lineage identification and environmental variables

Dimension	Variable	Correlation	P-value
Dimension 1	Mean annual precipitation (mm $year^{-1}$)	0.94	<0.05
	Elevation (m)	0.92	<0.05
	Mean annual temperature (^{o}C)	-0.96	<0.05
Dimension 2	Mean annual dew point (^{o}C)	0.99	<0.05
	Mean annual precipitation (mm $year^{-1}$)	0.26	<0.05
	Elevation (m)	-0.20	<0.05

Appendix 9c. PCA showing quality of dimensions' contribution to indvidual data points



Appendix 9d. Visualisation of variables' contributions to PCA dimensions



Appendix 10. Post-hoc multiple comparisons test results (Tukey HSD)

Comparison	Estimate	Std. Error	z value	Pr(>|z|)	Significance code
28°C - 18°C	-0.030	0.614	-0.049	0.961	
32°C - 18°C	-1.423	0.614	-2.317	0.026	*
36°C - 18°C	-1.935	0.614	-3.152	0.002	**
40°C - 18°C	-4.479	0.614	-7.295	2.23×10^{-12}	***
70°C - 18°C	-4.422	0.633	-6.989	1.04×10^{-11}	***
32°C - 28°C	-1.393	0.595	-2.34	0.026	*
36°C - 28°C	-1.906	0.595	-3.202	0.002	**
40°C - 28°C	-4.450	0.595	-7.475	1.15×10^{-12}	***
70°C - 28°C	-4.391	0.614	-7.153	4.25×10^{-12}	***
36°C - 32°C	-0.513	0.595	-0.862	0.449	
40°C - 32°C	-3.057	0.595	-5.135	8.44×10^{-7}	***
70°C - 32°C	-2.999	0.614	-4.884	2.59×10^{-6}	***
40°C - 36°C	-2.544	0.595	-4.274	4.12×10^{-5}	***
70°C - 36°C	-2.486	0.614	-4.049	9.64×10^{-5}	***
70°C - 40°C	0.0677	0.614	0.094	0.961	

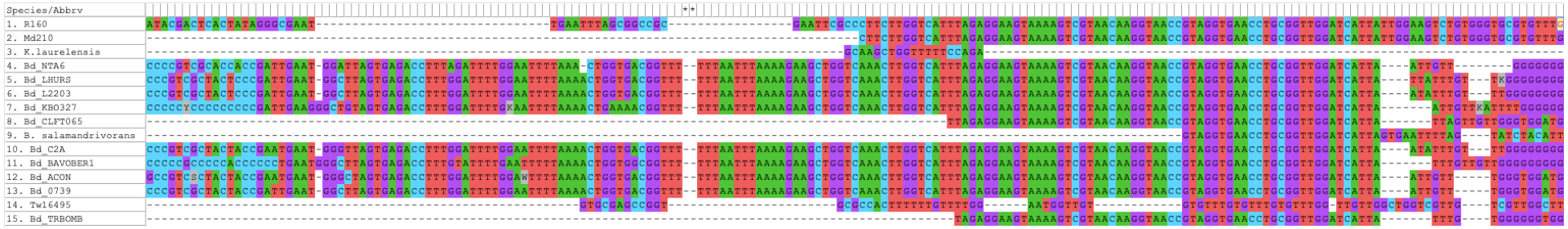
Significance codes: 0 = '*', 0.001 = '**', 0.01 = '*"**

Adjusted p values reported, fdr methods

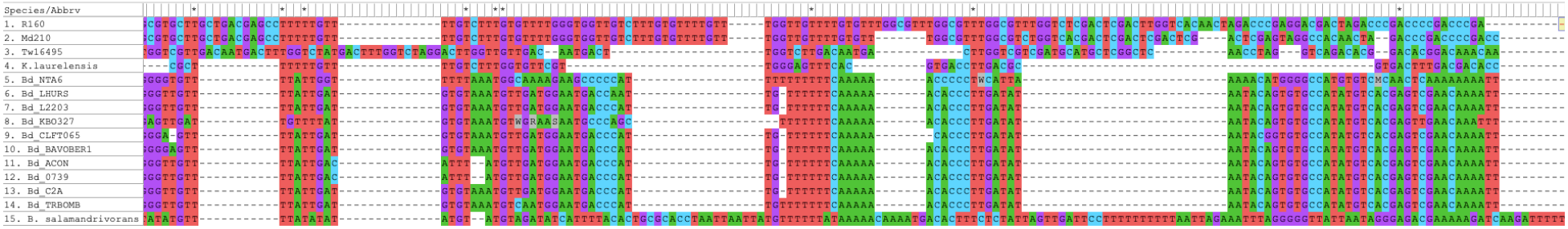
Appendix 11. Metadata for chytrid ITS phylogeny including novel Malagasy chytrids

Isolate name	Source location	Species/lineage	Year collected	Host species
NTA6	Cameroon	*Bd*CAPE	2012	*Afrixalus spp.*
TRBOMB	Belgium, trade	*Bd*CAPE	2011	Unknown
L2203	Montserrat	*Bd*GPL	2009	*Leptodactylus fallax*
KBO327	South Korea	*Bd*ASIA-1	2014	*Bombina orientalis*
C2A	Spain	*Bd*GPL	2002	*Alytes obstetricans*
BAVOBER1	Germany	*Bd*GPL	2012	*Alytes obstetricans*
CLFT065	Brazil	*Bd*ASIA-2/Brazil	2013	*Hylodes japi*
ACON	Switzerland	*Bd*ASIA-2/Brazil	2007	*Alytes obstetricans*
0739	Switzerland	*Bd*CH	2007	*Alytes obstetricans*
LFRC1	UK breeder	*B. salamandrivorans*	2015	*Salandra atra*
TW16495	Taiwan	Unknown	2016	*Bombina adenopleura*
K. laurelensis	USA	*K. laurelensis*	2005	N/A
R160	Madagascar	*BxMada*	2015	*Mantidactylus betsileanus*
Md210	Madagascar	*BxMada*	2015	*Mantidactylus pauliani*

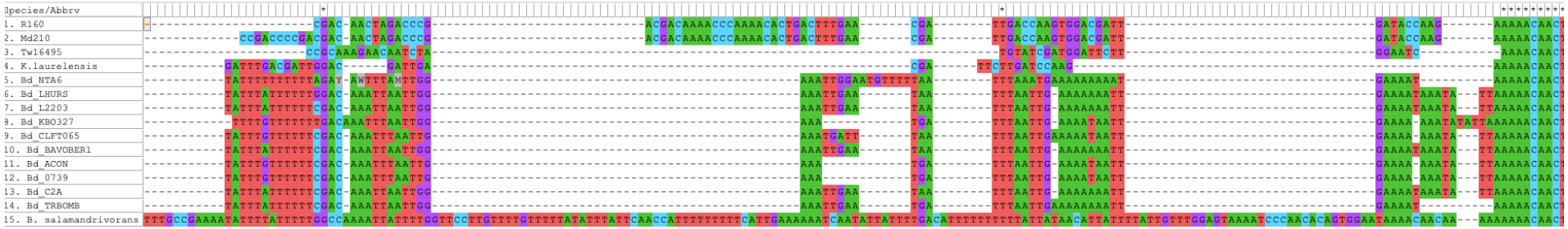
Appendix 12. ITS alignment for chytrid ITS phylogeny including novel Malagasy chytrids



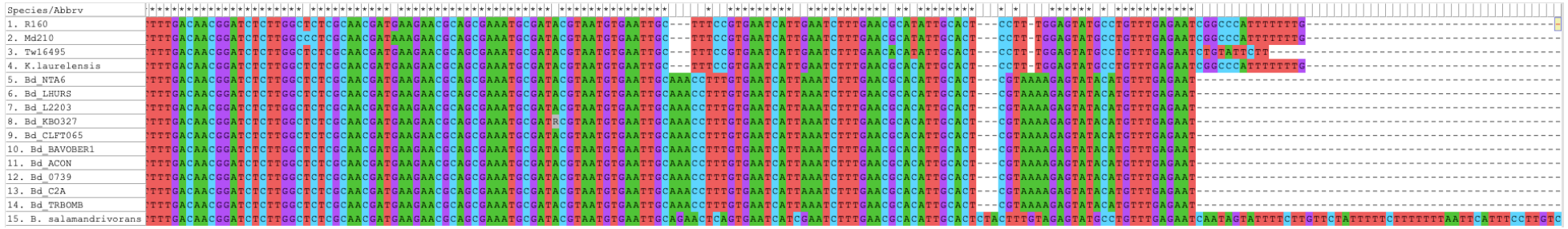
Appendix 12a. Alignment section 1 of 7, base pairs 1 - 193



Appendix 12b. Alignment section 2 of 7, base pairs 194 - 386

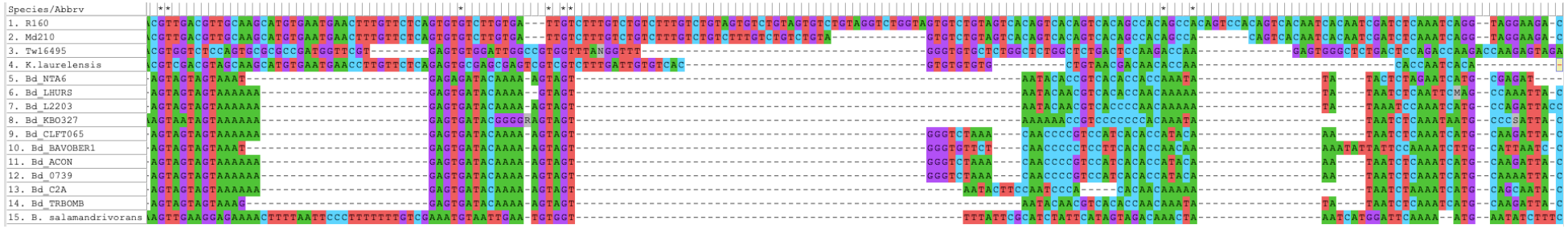


Appendix 12c. Alignment section 3 of 7, base pairs 387 - 579

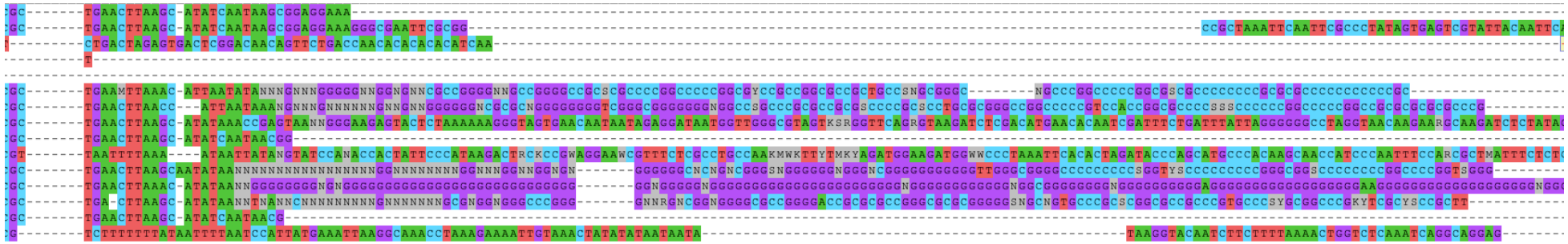


Appendix 12d. Alignment section 4 of 7, base pairs 580 - 772

Appendix 12e. Alignment section 5 of 7, base pairs 773 - 964



Appendix 12f. Alignment section 6 of 7, base pairs 965 - 1157



Appendix 12g. Alignment section 7 of 7, base pairs 1158 - 1346

Appendix 13. Isolate metadata for *BxMada*-specific primers and TaqMan MGB probe specificity testing panel

Isolate name	Source location	Species/lineage	Year collected	Host species
IA042	Spain	*Bd*GPL	2004	*Alytes obstetricans*
KB72	South Korea	*Bd*ASIA-2/BRAZIL	2014	*Lithobates catesbeianus*
ETH4	Ethiopia	*Bd*GPL	2011	*Leptopellis spp.*
08MG08	South Africa	*Bd*GPL	2008	*Amietia angolensis*
KB23	South Korea	*Bd*ASIA-2/Brazil	2014	*Lithobates catesbeianus*
KBO317	South Korea	*Bd*ASIA-1	2014	*Bombina orientalis*
NTA6	Cameroon	*Bd*CAPE	2012	*Afrixalus spp.*
0739	Switzerland	*Bd*CH	2007	*Alytes obstetricans*
KB45	South Korea	*Bd*Asia-2/BRAZIL	2014	*Lithobates catesbeianus*
LFRC1	UK breeder	*B. salamandrivorans*	2015	*Salamandra atra*
Bsal	The Netherlands	*B. salamandrivorans*	2013	*Salamandra salamandra*
TF5a1	Mallorca	*Bd*CAPE	2007	*Alytes muletensis*
SA4c	South Africa	*Bd*CAPE	2010	*Amietia angolensis*
TRBOOR	Germany, Trade	*Bd*ASIA-1	2012	*Notophthalmus viridescens*
K. laurelensis	USA	*K. laurelensis*	2015	N/A
KBO327	South Korea	*Bd*ASIA-1	2014	*Bombina orientalis*
TW16495	Taiwan	Unknown	2016	*Babina adenopleura*
R160	Madagascar	*BxMada*	2015	*Mantidactylus betsileanus*
Md210	Madagascar	*BxMada*	2015	*Mantidcatylus pauliani*

Appendix 14. Co-authored papers published during Ph.D.

Doherty-Bone, T.M., Cunningham, A.A., Fisher, M.C., Garner, T.W.J., **Ghosh, P.N**., Gower, D.J., Verster, R., Weldon, C. (2019) Amphibian chytrid fungus in Africa - realigning hypotheses and the research paradigm. *Animal conservation*. Published ahead of print. DOI:10.1111/acv.12538

Ghosh, P.N., Fisher, M.C., Bates, K.A. (2018) Diagnosing Emerging Fungal Threats: A One Health Perspective. *Frontiers in Genetics* **9**: 376

Fisher, M.C., **Ghosh, P.N**., Shelton, J.M.G. *et al.* (2018) Development and worldwide use of non-lethal, and minimum population-level impact, protocols for the isolation of amphibian chytrid fungi. *Nature Scientific Reports* **8** (1): 4 - 11

O'Hanlon, S.J., Rieux, A., Farrer, R.A., ...**Ghosh, P.N.**..*et al*., (2018) Recent Asian origin of chytrid fungi causing global amphibian declines. *Science* **360** (May): 621 - 627

Ghosh, P.N. & Fisher, M.C. (2016) Dr Jekyll and Mrs Hyde: Risky hybrid sex by amphibian-parasitizing chytrids in the Brazilian Atlantic Forests. *Molecular Ecology* **25**: 2961 - 2963