SKIN MICROBIOME DIVERSITY OF *BATRACHOCHYTRIUM DENDROBATIDIS*-CHALLENGED PHOFUNG RIVER FROGS (*AMIETIA HYMENOPUS*) IN THE DRAKENSBERG MOUNTAIN RANGE

by

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Declaration of work

I declare that this dissertation, "Skin microbiome diversity of *Batrachochytrium dendrobatidis* challenged Phofung River Frogs (*Amietia hymenopus*) in the Drakensberg Mountain range" is entirely my own work, and that where material could be construed as the work of others, it is fully cited and referenced, and/or with appropriate acknowledgement given.

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Abstract

Infectious diseases are pressuring amphibian populations globally, especially threatened frog species occurring in restricted areas. Chytridiomycosis, which is caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), is arguably the most devastating panzootic disease confronting wildlife today and was determined to be a major driving force of the current amphibian extinction crisis. A healthy amphibian host has a symbiotic relationship with its cutaneous microbial community that benefits the host in many ways including the protection against malicious microbes. The current trend in amphibian skin microbiome studies concentrate predominantly on its bacterial component, while other components of the microbiome such as filamentous fungi and eukaryotes have been neglected. In this study *Amietia hymenopus*, a *Bd*-challenged frog population of southern Africa, was sampled from remote locations of the frog's natural distribution on the Drakensberg mountain range. The cutaneous microbiome diversity was analysed using next generation sequencing and bioinformatics tools, investigating both the bacterial and fungal components of the frogs' skin microbial community. Results indicated high diversity dissimilarities in both *alpha* and *beta* diversity when comparing individual specimens, and sampling areas, respectively. This study touched on an important topic regarding the microbial biodiversity found on frogs, and sheds light on the complex interaction between host and microbial communities in amphibian populations. Knowledge on this intricate relationship between host and their microscopic cutaneous symbionts could help inform amphibian conservation efforts against infectious disease.

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I would like to make it clear that none if this study would have been possible if not for the divine Creator. All praise and acknowledgement belongs to Him, the architect of all. Thanks to Him, we have this incomprehensible creation as our responsibility to care for, learn from, and conserve.

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Table of contents

List of figures

List of tables

List of acronyms

AMSL	above mean sea level
ANOVA	analysis of variance
ºC	degrees Celsius
Bd	*Batrachochytrium dendrobatidis*
*Bd*GPL	global panzootic lineage
DDM	Degrees and Decimal Minutes
Df	degrees of freedom
DNA	Deoxyribonucleic Acid
GPS	Global Positioning System
ITS	Internal Transcribed Spacer
min	Minute/s
MOTU	Molecular Operational Taxonomic Unit
NGS	Next Generation Sequencing
PERMANOVA	Permutational Analysis of Variance
PCR	Polymerase Chain Reaction
rDNA	ribosomal DNA
rtPCR	Real-time Polymerase Chain Reaction
s	Seconds

Chapter 1: Introduction and background

1.1. Amphibian importance

Approximately 360 million years ago, amphibians were the first of the vertebrates to colonize a terrestrial habitat. It can be argued that this invasion of land was the most remarkable evolutionary event in animal history, as it constituted dramatic physiological and morphological adaptations towards an amphibian body (Clack 2009). Since then amphibians have been radiating, and today they can be found on all hospitable continents and land masses including the majority of the larger islands. They exhibit the most appreciable mode of life and display some of the greatest diversities relative to other vertebrates (Meyer 2009). Presently amphibians can be catalogued into three main groups; firstly the order Gymnophiona (Apoda) (Nussbaum and Wilkinson 1989) which are characterized as wormlike, legless amphibians, secondly the Caudata (Urodela) (Baitchman and Herman 2014) which includes all salamanders and newts, and thirdly Anura (Salientia) (DuBois and Frétey, 2020) which features the frogs and toads.

Amphibians play an integral part in many habitats as an intermediate entrant in the food web (Burton and Likens 1975), and have direct relationships with both the biotic and abiotic factors that make up a chaotically balanced ecosystem (Porter 1972). They are a fundamental energy-efficient link in the *Animalia* kingdom and serve as both predators and prey (Seale 1980). Furthermore, tadpoles feature as elemental herbivores in aquatic systems (Dickman 1968; Seale 1980).

Amphibians are excellent indicator species and model organisms to access ecosystem health. They inhabit both the aquatic and terrestrial environments in their different stages of life, which in turn makes them very sensitive to environmental changes (Gendron *et al*. 2003). Amphibians can be regarded as perhaps the most sensitive to both aquatic and atmospheric pollution in the vertebrate group (Blaustein and Wake 1995), further supporting the argument that amphibians can be deemed as valuable early warnings of ecosystems under stress due to environmental changes (Bridges 2000; Blaustein and Johnson 2003; Simon *et al*. 2011). Due to amphibians' semi-permeable skin, chemicals they come in contact with are easily absorbed in both the terrestrial and aquatic environments of their amphibious lifestyle (Blaustein and Johnson 2003; Bridges 2000). Dr. Schmeller and coworkers (2018) provided valuable insight into the direct influence micropollutants have on water quality and their interaction with ecosystem health affecting freshwater biodiversity. The decrease in amphibian population numbers at aquatic sites are clear indications of diminishing ecosystem health.

1.2. Chytridiomycosis

1.2.1. The chytridiomycosis threat

Batrachochytrium dendrobatidis (*Bd*, Chytrid) (Berger *et al*. 1998; Longcore *et al*. 1999) is responsible for arguably the most devastating panzootic wildlife is faced with presently (Scheele *et al*. 2019). Relatively recently (i.e. the turn of the 20th century) the amphibian skin disease chytridiomycosis, caused by a hypervirulent Global Panzootic Lineage (*Bd*GPL) of this pathogenic chytrid fungus, was identified as a proximate driver for the rapid decline in global amphibian populations (Holden *et al*. 2015; O'Hanlon *et al*. 2018). Amphibian chytrid fungus has been implicated in amphibian populations declines of over 500 species and the extinction of at least 90 of them (Olson *et al*. 2013; Scheele *et al*. 2019). As a compounding factor in a global amphibian crisis, *Bd* is at least partly responsible for an estimated 50% of all amphibian species currently being threatened with extinction (Fisher, Garner and Walker, 2009; Venesky *et al.*, 2014; International Union for Conservation of Nature and Natural Resources (IUCN), 2021).

Shortly after the first reported appearance of chytridiomycosis in 1998, *Bd* was isolated during an investigation into enigmatic declines of amphibian populations in North- and Central America and Australia (Berger *et al*. 1998; Longcore *et al*. 1999). Since then more light has been shed on the chytrid's rapid expansion to a global distribution. With the development of high throughput Next Generation Sequencing (NGS) and more advanced bioinformatics tools, knowledge about *Bd* and chytridiomycosis across the world is now at a better output stage (Figure 1.1), but momentum in terms of research effort needs to be maintained (Rebollar *et al*. 2020).

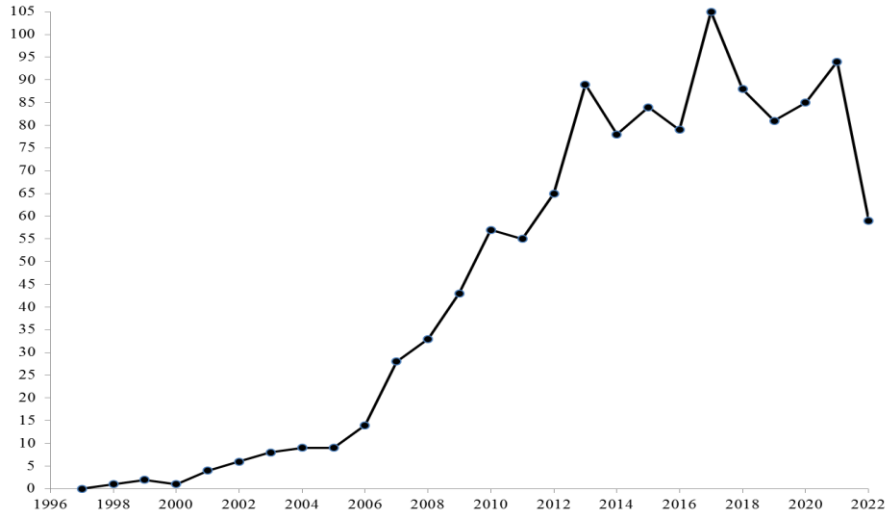

Figure 1.1: The number of publications mentioning chytridiomycosis or *Batrachochytrium dendrobatidis* in the title and/or the abstract per year since first mentioned in 1998 (Berger *et al*. 1998) and classified by Longcore, Pessier and Nichols in 1999 to present (National Center for Biotechnology Information (NCBI), 2022).

1.2.2. Panzootic *Batrachochytrium dendrobatidis* origin

The sudden appearance of chytridiomycosis on several continents suggests that its causative agent, the amphibian chytrid fungus, was introduced into affected regions and can be regarded as invasive (Weldon *et al*. 2004; Doherty-Bone *et al*. 2020). Weldon and collegues (2004) initially proposed in a ground-breaking study that the origin of the amphibian chytrid could have been Africa. In South Africa, the earliest record found was in a *Xenopus laevis* museum voucher collected in 1938 (Weldon *et al*. 2004) which was part of a study surveying 697 southern African specimens belonging to the *Xenopus* genus that were collected and archived between 1879 and 1999. This out-of-Africa hypothesis was supported by key points in the criteria to propose Africa as the source of *Bd*. Their evidence indicated a stable endemic infection and prevalence of chytridiomycosis in the proposed reservoir host (*X. laevis*) since the earliest documented occurrence (1938), and a feasible means of distribution out of Africa through international trade of *X. laevis* beginning in mid-1930 facilitating global dissemination of infections that presently still continues to spread (Weldon *et al*. 2004, Weldon *et al*. 2007).

Since then with the emergence of NGS technology of whole genomes, the uncertainties behind the spatiotemporal origin of the recombinant hyper virulent *Bd*GPL have been better addressed. O'Hanlon *et al*. (2018) found that the hyper virulent *Bd* lineage has an ancestral population of Asian descent, which is supported by previous findings in a study by Bataille and his colleagues (2013), arguing that *Bd* is likely endemic to Asia. This was because the heterogeneity in other lineages could be accounted for by the Asian lineages, and evidence for the dissemination pathways on a temporal scale was also provided. Following studies argued that Africa should thus be considered novel territory for the *Bd* fungus, having potential serious threats associated with invasives to endemic frog species (Doherty-Bone *et al*. 2020). This dire situation is aptly illustrated in the case of the Kihansi spray toad, which has been shown to have gone extinct in the wild due to *Bd* infections (Weldon *et al*. 2020).

1.3. Microbiomes

Multicellular organisms fill the role of host to a diverse suite of microbial life, collectively referred to as a microbiome. This naturally occurring network of symbiotic relationships connecting hosts and microbes are ubiquitous in nature (Antwis and Weldon 2017; Griffiths *et al*. 2018). Studies on the microbial communities inhabiting frog cutaneous tissue indicate that healthy microbiome diversity may counteract the prevalence of *Bd*, which in turn influences the penetrance of chytridiomycosis (O'Hanlon *et al*. 2018; Bletz *et al*. 2017). Definite changes in skin microbiome composition relative to the emergence of a *Bd* infection has been detected in previously naïve frogs subsequently challenged with *Bd* under laboratory conditions w (Jani and Briggs 2014). Similar to the manipulation and supplementation of the

gut microbiome to aid in curing human diseases, it can be hypothesized that manipulation of the skin microbiome of frogs in wild populations can be used to combat chytridiomycosis (Walke and Belden 2016).

1.4. Problem statement and the Drakensberg study area

South Africa is home to 126 species of frogs with 16 of the species categorised as endangered or worse (IUCN 2022). The situation is compounded by the fact that often the habitats these frogs occur in are threatened, for example threatened ecosystem types like the Indian oceanic belt, forests and grasslands (Holness *et al*. 2013). The *Bd* fungus is indeed occurring widely within South Africa (Tarrant *et al*. 2013).

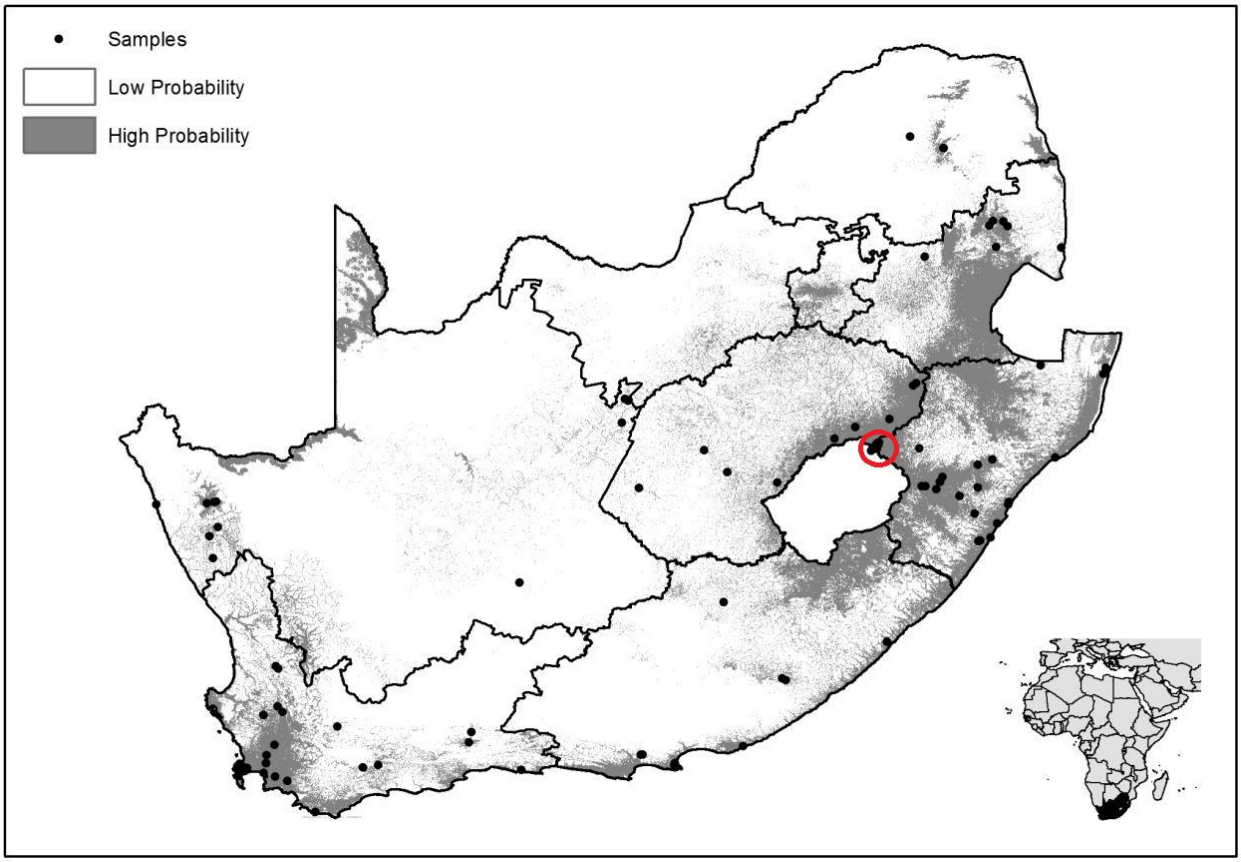

Figure 1.2: Map of South Africa with predicted occurrence of *B. dendrobatidis* indicating high probabilities in grey shading and low probabilities in light areas (Tarrant *et al*. 2013). The marked points refer to the locations where samples were collected. Red highlighted area indicates current study location.

In this study we examined the Phofung river frog (*Amietia hymenopus*). This near threatened (IUCN 2021; Measey 2011) montane frog species occurs in isolated populations with a natural distribution on high grasslands where the North Eastern Lesotho border meets South Africa (Du Preez 2015; IUCN 2021), such as the Drakensberg plateau at Mount-aux-source. This frog species is known to

be affected by *Bd* (Smith *et al*. 2007; Griffiths *et al*. 2018). However, previous studies focused mostly on tadpoles, while the presence on the more mobile adult frogs remains largely unstudied. More emphasis was placed on bacteria during previous studies, while the full fungal component of microbiomes had not yet been characterized.

1.5. Aims and objectives

The aims and objectives for this study are summarised in Table 1.1 below.

Table 1.1: Project aims and objectives

Aims	Objectives
1. Foundational: Establish a skin bacterial and fungal microbiome diversity survey of postmetamorphic *Amietia hymenopus* on Mt. Aux-source (Drakensberg, KZN, RSA).	Determine microbial diversity through targeted sequencing of skin swab samples, and linking Illumina sequencing data with public accessible databases SILVA 132 (Quast *et al*. 2012) and ITS UNITE (alpha version 12_11) (Kõljalg *et al*. 2013; Nilsson *et al*. 2018), respectively.
2. Analyse skin microbiome diversity, species richness and distribution dissimilarity within individual frogs.	Alpha diversity analysis will produce a statistical summary of diversity within the investigated samples: • Rarefaction with Shannon index investigates species richness (number of species). • Distribution diversity represented in a normalised data set, featuring taxa in percentages of relative abundance. • Stacked bar charts will present featured taxa distribution (evenness) on a phylum and genus level. • Alpha diversity boxplot with Kruskal-Wallis pairwise statistical t-tests will indicate significant differences.
3. Compare communal microbiome diversity based on geographical locality. 4. Comparison of *Bd* challenged frogs and frogs with the absence of *Bd* infections.	Beta diversity analysis employs a measurement of similarity/dissimilarity between sample groups or populations: • Principal coordinate analysis plots shows diversity as a Bray-Curtis (1957) distance matrix between samples grouped together according to locality or *Bd* status. • Produce statistical support via Adonis permutation test with the Vegan package (Oksanen *et al*. 2015) in R Studio (RStudio Team 2020). • Plot Venn diagrams to present a schematic of MOTUs shared between- and unique to queried sample groups.

Chapter 2: Literature review

2.1. Introduction

The world today as we know it is driven by humanity's economic goals that neglect conservations' due consideration, which leads management and the conservation of nature and natural resources to be weighed up against their monetary value in light of humans self-centred sentiment. Thus the conservation of amphibians are contingent on human's perception and attitude towards them, for instance, the inclination to support and fund herpetofauna (amphibians and reptiles) conservation movements (Ceríaco 2012). Regrettably humanity considers herpetofauna to be a rather vilified class of vertebrates (Ceríaco 2012; Silva *et al*. 2009). Unfortunately, in the current conservation paradigms highlighting sustainable utilisation of funds, frogs are neglected and considered as low value in regards to ecotourism and their contribution towards uplifting disadvantaged communities. Even though frogs feature eminently in folklore and superstitions, a diverse degree of sociodemographic cultures (Ceríaco 2012; Minter 2004; Silva *et al*. 2009) considers frogs as unimportant and regard them as insignificant to humanity's everyday life.

Fortunately knowledge on amphibians and their paramount role in all ecosystems (López *et al*. 2009; Baffico and Úbeda 2006), as well as their contributions to medicine (Gomes *et al*. 2007; Samgina *et al*. 2012), have encountered a radical increase. Nevertheless, the rates at which amphibian populations are declining, since this was first being recognised as a global phenomenon in early 1990, is disturbing (Scheele *et al*. 2019; O'Hanlon *et al*. 2018; Stuart *et al*. 2004; Blaustein and Wake 1995), and has rapidly increased. This further emphasises the importance for the wellbeing of amphibians, and the serious need for the human society to understand their significance and the value of these vulnerable misunderstood creatures.

This review will briefly summarise the importance and vulnerability of frog species across the world. Frogs are threatened by the chytrid fungus *Batrachochytrium dendrobatidis*, and the review will introduce its effects on frogs, and the threat it poses. Skin microbiomes of frogs have been found to have the potential to aid management of this disease. The essence of these microbiomes, and degree to which they have been studied for frogs will be discussed.

2.2. Importance of amphibians as biological indicators

Frogs play essential roles in ecosystems, but from a conservation perspective their capacity as biological indicators should also be highly emphasised. Due to amphibians' unique physiology, specifically their vulnerable hairless, scale-less and shell-less skin, together with their naked jelly-

encapsulated eggs (Duellman and Trueb 1994), they are exceptionally sensitive to chemical fluctuations in air and water composition (Blaustein *et al*. 1994). As an example, Horne and Dunson (1995) found that increased environmental aluminium and copper significantly decreased Jefferson Salamander (*Ambyostoma jeffersonianum*) and the Wood Frog (*Rana sylvatica*) larval survival.

Frog populations decline and increase due to environmental changes, and they can be biological indicators of water quality, air pollution in the environment, with observable deformities acting as red flags that give early warning to humans on how the health of an environment has deteriorated (Blaustein and Johnson 2003). In addition to being an important ecosystem biological indicator, amphibians are paramount to ecosystems as key trophic links in the food web (Rubbo *et al*. 2006; Davenport and Chalcraft 2012). Therefore, environmental health should have conservation priority to maintain viable amphibian populations. If these warnings are left unaddressed larger and more permanent fallout may ensue, adversely affecting the pan biodiversity in an ecosystem.

2.3. Batrachochytrium dendrobatidis

The chytrid fungus, *Batrachochytrium dendrobatidis*, is the causative agent for the lethal fungal disease chytridiomycosis. *Batrachochytrium dendrobatidis* forms part of the Chytridiomycota, which are ubiquitous organisms and features more than 1,000 species living in water and soil (Barr 1990; Alexopoulos *et al*. 1996). The majority of species categorised within the Chytridiomycota is known to be saprobic decomposers of organic matter, yet some present as obligate or facultative parasites of fungi, invertebrates, and some fauna (Pessier *et al*. 1999). Prior to reports by Berger (1998), Longcore (1999) and Pessier (1999), chytrids were not described as pathogens or parasites to vertebrates (Barr 1990).

2.3.1. Life cycle and transmission

Chytridiomycosis was first discovered in 1997 and mentioned by Berger *et al*. (1998) who hypothesised chytridiomycosis to be a proximate cause for Central American and Australian wild amphibian population declines. Yet *Bd* was only described and identified as its responsible causative agent for chytridiomycosis by Longcore, Pessier and Nichols (1999) the following year. *Batrachochytrium dendrobatidis* is a Chytrid fungus (Chytridiomycota; Chytridiales) that has two life cycle stages and ways of transmission. The first represents as motile, uniflagellated waterborne zoospores released by a zoosporangium, while the second is a stationary monocentric reproductive thallus with a single sporangium stage (Longcore *et al*. 1999; Kilpatrick *et al*. 2010). Motile waterborne zoospores represent the first life stage, where zoospores disperse with the help of a flagellum, staying active for up to 24 hours and covering a much as 2 cm in growth media (Powell 1993; Kilpatrick *et al*. 2010; Berger *et al*. 2005). The stationary monocentric thallus follows, where fruiting bodies called zoosporangia

developed from the zoospores. The contents of the zoosporangia then divide, producing new zoospores (Berger *et al*. 2005; Piotrowski *et al*. 2004).

It is not evident that *Bd* reproduce sexually, yet there is indication that recombination exist (Morgan *et al*. 2007). *Batrachochytrium dendrobatidis* is known to survive and grow between temperatures of 4 and 28 ºC and thrive at temperatures between 17 and 25 ºC (Kilpatrick *et al*. 2010; Longcore *et al*. 1999; Berger *et al*. 2005; Piotrowski *et al*. 2004). It can often be found at high elevations like the Rocky Mountains, Sierra Nevadas and the Andes (Vredenburg *et al*. 2010), which have climates similar to the Drakensberg mountain range from South Africa (Bristow 2003; Souchon 2005). During high temperatures, the chytrid fungus does not thrive (Pounds *et al*. 2006), yet in an environment not conducive for the chytrid growth the fungus may still be capable of surviving for an extended period of time in an arrested state until favourable conditions allows its life cycle to recommence (Johnson and Speare 2005; Johnson and Speare 2003). According to Knapp *et al.* (2011) cold weather environments do not always inhibit *Bd*, although other research argue that it does have an immediate effect on its pathogenesis (Muths *et al*. 2008; Pilliod *et al*. 2010; West *et al*. 2020). The fact remains that there have been substantial amphibian declines at high altitudes where the environment is usually cooler with high rainfall and constant moisture, but the relationship is not always that straight-forward (Fisher *et al*. 2009).

Notably, temperature is not the only factor to consider in regards to *Bds'* responses in degrees of pathogenicity. A study in north eastern Brazil (Valencia-Aguilar *et al*. 2016) showed a clear correlation between *Bd* prevalence and seasonality. *Batrachochytrium dendrobatidis* flourished in the wet summer seasons, and decreased in developmental rate over dry cold seasons (Bosch *et al*. 2007; Valencia-Aguilar *et al*. 2016).

Infections can be transmitted through zoospores in contaminated environments, or through direct contact with tadpoles or other postmetamorphic/adult frogs already challenged with *Bd* fungus infection (Briggs *et al*. 2005). More broadly, environments such as water bodies can become contaminated through alteration. Humans can carry spores and transport them to another water body, or through contaminated water reservoirs that had infected frogs spilling into a natural environment (Kast and Hanna 2012). Transportation of amphibians is another cause of global transmission. An infected amphibian can be traded to other countries to naïve areas, increasing the possibility of infecting other frogs (Scheele *et al*. 2019).

2.3.2. Pathogenicity

Batrachochytrium dendrobatidis is a hyper virulent pathogenic fungus that parasitizes anurans and its implications on this order of amphibians may vary depending on the nature of infection and

ambient aspects. Infection disrupts the cutaneous osmoregulatory process in postmetamorphic individuals, causing an imbalance in electrolytes which in most cases are lethal ending in asystolic cardiac arrest (Voyles *et al*. 2009). Notably, under laboratory conditions some individuals may recover from infection, while infection for others is fatal. The manifestation of chytridiomycosis and survival time was found to be influenced by contact with water, temperature and exposure history (Murphy *et al*. 2011). It does not cause mortality in the larval stage on the account of the relatively small amount of keratinised tissue found in tadpole mouth parts compared to an adult frog's keratinised cutaneous epithelium, but infection may increase larval stage duration and reduce metamorphosis mass, which could affect the frog's survival in the long run (Smith *et al*. 2007).

From a phylogenetic perspective, *Bd* can be classified into multiple lineages (O'Hanlon *et al*. 2018) believed to have emerged out of Asia within the past century. This resulted in complex phylogeographically correlated population genetic structures with each portraying different levels of pathogenicity. Currently there are four distinguishable lineages: *Bd*GPL (Global Panzootic Lineage), *Bd*CAPE (African), *Bd*ASIA1, and *Bd*ASIA-2/*Bd*Brazil. Notably, *Bd*GPL exhibits high virulence and was significantly more lethal than control groups and other lineages. Furthermore, native to the Korean peninsula *Bd*ASIA1 is exhibited as a hyperdiverse lineage with the highest number of segregation sites in a pairwise comparison among lineage isolates. Continued haplotype exchange among lineages after continental invasion is demonstrated by these *Bd* recombinant genomes indicating the constant generation of novel genomic diversity.

2.3.3. Epidemiology in Africa

It is recognised today that *Bd* is a novel pathogen not endemic to the African continent, and that the threat it poses to the African amphibian biodiversity could be greatly underestimated (Doherty-Bone *et al*. 2020). *Batrachochytrium dendrobatidis* has been existent in Africa for almost a century now (Soto-Azat *et al*. 2010), yet little is known about the continent's amphibian population trends. There have been few reports of so-called 'enigmatic declines' where previously abundant amphibians vanish in relatively unchanged habitat. In South Africa, sporadic *Bd*-associated mortality events have been recorded in Poynton's River Frog *Amietia poyntoni* from Namaqualand and the Eastern Cape Province (Lane *et al*. 2003; Hopkins and Channing 2003) and the Phofung River Frog *Amietia hymenopus*, a species inhabiting the high altitudes of the Drakensberg Mountain range (Griffiths *et al*. 2018).

2.4. Amphibian skin microbiomes

Complex organisms serve as hosts to a variety and a diverse suite of microorganisms which include, but are not limited to, bacterial and fungal communities. This is collectively known as a

microbiome. More broadly, microbiomes can be described as the assortment of microorganisms inhabiting a niche, e.g. the skin- or gut microbiome (Griffiths *et al*. 2018), soil or water microbiome (Epp *et al*. 2012), and root or rhizosphere microbiome (Pambuka *et al*. 2021). Symbiotic relationships between microbiome and multicellular host organism are ubiquitous in nature and play a critical role in pathogen resistance, translating to a robust immune system.

In amphibians, the epithelial dermis is an extremely sensitive organ, and by the same token as vital. An amphibian's skin is home to a unique microbiome owing to it being a conducive medium for microbial growth and it constantly being exposed to a manifest of environmental microbiota that includes fungi, bacteria, viruses, eukaryotic organisms, etc. In humans, the gut microbiome enjoys the most attention in research (Weyrich *et al*. 2015), offering compelling evidence to prove their part in everyday life essentials like nutrient absorption, digestion and immune development. Apart from the mutual beneficial relationship between symbiont and host, complex organisms evidently do poses an innate immunological protection against pathogens (Voyles *et al*. 2011). By the same token, amphibian microbiomes' antifungal capabilities have been described both *in vitro* and *in vivo* and promises immense disease prevention properties (Rebollar *et al*. 2020). For example, amphibians produce antifungal metabolites that combats malicious fungal infections and subsequently inhibits pathogen growth (Griffiths *et al*. 2018).

Amphibians' skin microbiome composition can be detrimental in its susceptibility for chytridiomycosis. Chytrids are commonly present in diverse environments, such as rainforests, tundra, and deserts. This hydrophilic phylum prefer to inhabit wet and moist areas (Powell 1993). They facilitate decomposition in keratin, pollen, cellulose, and chitin that is found in dead insects, keratin in skin, pollen and cellulose hair, and vegetative matter (Barr 1990). Factors that affect skin microbiome structure could influence the pathogenicity of *Bd* and in turn the penetrance of chytridiomycosis.

Numerous factors can influence skin microbiomes and their interactions with pathogens such as *Bd*. Griffiths *et al*. (2018) highlighted factors affecting skin microbiome structure such as life stages, genotype, diet, and gender. Post metamorphic amphibians possess a more diverse microbiome than the early tadpole or larval stage in its life cycle. Additionally, the chytrid fungus is more prone to animate on adult amphibians due to post-metamorphic amphibian's keratinized epithelium (Berger *et al*. 1999; Valencia-Aguilar *et al*. 2016; Griffiths *et al*. 2018). Genetic diversity vary among amphibian species', as well as between isolated populations, where gene variations can influence the species susceptibility to diseases and parasites (Belasen *et al*. 2021; Griffiths *et al*. 2018). Genetic diversity is inherited laterally, meaning unique haplotypes are created through the continual transferal from paternal gene variations to offspring (Jani and Briggs 2018). Amphibian diet highly influences the external and internal microbiome

composition, thus playing an important role in the organisms' dynamic defence against pathogens (Hughey *et al*. 2017). Nevertheless, a multifactorial and complex microbial relationship only adds to the network of responses that determines an amphibians' susceptibility to diseases.

Traditionally microbiomes are seen as nongenetic, and its adaption to host remains an evolutionary puzzle. Yet microbial variation have a genetic basis and is capable of evolving (Scheuring and Yu 2012), but does not follow the same inheritance mechanisms as the host's genetics (Henry *et al*. 2021). The microbiomes' hereditary value to the host is correlated to the phenotypic contribution to host. Microbiomes could influence host phenotypic responses to naturally occurring pressures in a combination of two possible ways; host mean phenotype shifting, and variance change in host phenotype. Host mean phenotype shifting is through leverage of host-microbiome to adapt locally, and variance change in phenotype is through buffering against or increasing host variability (Henry *et al*. 2021).

Form an inherent genetic perspective, the individual amphibian's natural immune system coordinates anti-microbial peptide production in its skin through defensive glands (Woodhams *et al*. 2014). This further indicate that variance in immunity on a genetic front has a strong influence on skin microbiomes (Tennessen *et al*. 2009). Inherited genetic diversity may influence microbiome composition, though not all microbial diversity is paternalistic. Diversity in host microbiome composition is largely subject to microbial diversity found in the environment which acts as a microbial reservoir (Loudon, Woodhams, *et al*. 2014). Contact dispersal in amphibians is also common where frogs migrate to and between breeding sites, allowing not only microbial exchange leading to microbiome diversity enriching, but also producing gene flow. This is especially evident where frogs migrate between flooded habitats (Schiesari *et al*. 2003).

In concluding remarks, the *Bd* fungus is a major threat to frog populations across the world. Evidence show that augmenting of a frogs skin microbiome could prove to be a potential method to mitigate the effects of this threatening disease. The fact remains that *Bd* has a pronounced influence on the skin microbiome, thus studies characterizing these microbiomes are paramount. With the advent of next generation sequencing approaches, rapid and extensive screening of frog skin microbiomes is possible. This facilitates studies monitoring possibly deleterious changes, and those pursuing possible beneficial microbes to lessen the effects of *Bd* on frog populations.

Chapter 3: Materials and methods

3.1. Introduction

The methodology for this project was directed at studying the microbial biodiversity of the skin microbiome of *A. hymenopus* in its native environment, the plateau of the Drakensberg Mountain range Amphitheatre (Mont-aux-Source). By employing a cascade of techniques from sample collection to NGS, followed by a pipeline of bioinformatics tools, tests determined whether a significant difference could be detected within and among populations of frog skin microbiome samples. Whether the individuals were exposed to *Bd* infection, was subsequently established using Real-time polymerase chain reaction (rtPCR).

3.2. Sample collection

3.2.1. Sample sites

The 800 m high and 4 km wide Amphitheatre (Souchon 2005) dons characteristic cliff faces, precipices and scattered peaks making up the defining features of the Mont-aux-Source area. Four sites on Mont-aux-Source's plateau were selected that falls in the most Northern part of the natural distribution range of *A. hymenopus*, namely Vemvane GPS (Global Positioning System) coordinates 28º44.907, 28º52.522, Tugela 28º45.156, 28º53.585, Bilanjil 28º45.623, 28º53.766 and Ribbon Falls 28º45.740, 28º55.063 (Figure 3.1). All sites are at an elevation of roughly 2 985 m (±40 m) AMSL (above mean sea level). With an elevation of 3 254 m AMSL, a geological formation named Crow's nest peaks above the sample sites creating a water shed that separates Vemvane and Tugela from Bilanjil and Ribbon Falls.

These sites were ideal for this project for a variety of reasons, including ecosystem stability, habitat consistency, national park protection and human intervention, for example human introduced laundry and washing detergents, and other waste products from urbanised areas close to bodies of water that might influence the freshwater chemistry. The sites were situated in a stable ecosystem close to the origin of the greater Tugela River basin, which obviates the possibility of upstream pollution due to substantial urbanization and anthropogenic activities. With water in the streams always remaining, with exception that Bilanjil sometimes turning to a marsh in the low rainfall months (March to July), *A. hymenopus* tadpoles are present all year round, with high numbers of post-metamorphic individuals. The adult frogs are also reasonably abundant, but require more search effort to collect. Furthermore, although the area is a popular tourist attraction for hikers (Souchon 2005; Bristow 2003), the majority of the general public never encroach upon the area beyond the crest of the Tugela waterfall on account of the

high elevation and the taxing route to summit the plateau. On top of that, human intervention is further regulated thanks to the region falling under the Royal Natal National Park protected area.

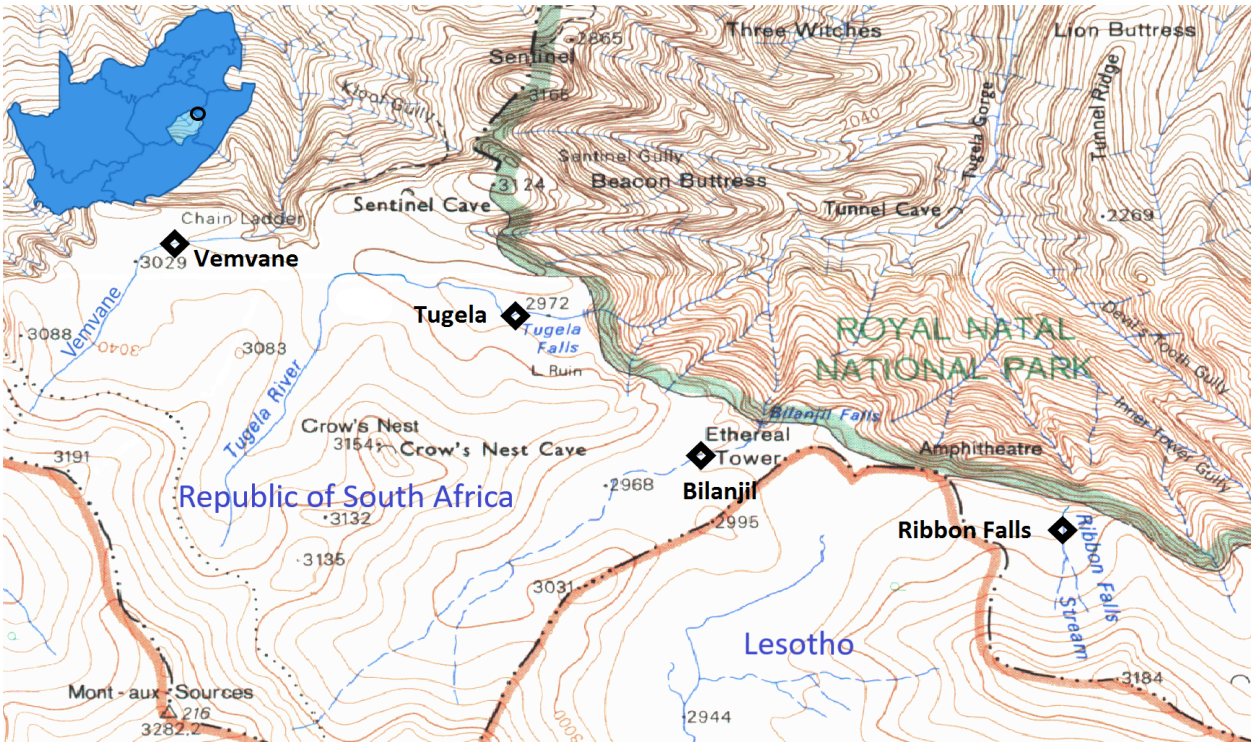

Figure 3.1: Topographical Geographic Information System map showing sample area on the central western border of KwaZulu-Natal, Republic of South Africa, with marked sample sites, namely: Vemvane, Tugela, Bilanjil and Ribbon Falls (Russian Maps - scale 1:500 000 - Southern Africa 1986).

3.2.2. Sample collection protocol

Adult *A. hymenopus* individuals were targeted for sampling to investigate a robust representation of the skin microbiome community. Samples were collected under permit OP 4218/2016 issued in accordance with Nature Conservation Ordinance No 15 of 1974 by Ezemvelo KZN Wildlife. Ethical clearance (UFS-AED2018/0061) was granted by University of the Free State's Animal Research Ethics Committee. In contrast to the exclusive aquatic lifestyle of tadpoles, adult frogs' amphibious lifestyle means that they have been exposed to more of the environment as a consequence of their habits of digging in soil and moving over land and through surrounding vegetation. Arguably, the environment enriches the diversity of their skin microbiome and consequently acts as a microbial reservoir (Jani and Briggs 2018). For each site the aim was to sample ten specimens using the river as a linear transect. Only size as an indication of age was taken into consideration when selecting specimens, avoiding small recently metamorphosed juveniles and thus perusing larger adults only. First ten adult individuals encountered were collected to eliminate sampling bias towards a certain age group or health condition.

This ensured we obtained an accurate representation of *Bd* infection prevalence within the population, as well as a good representation of the mean microbial community associated with the host species.

The following identical procedure was strictly followed at each sampling site to ensure standardized and reproducible results. All Falcon and Eppendorf tubes used in sampling collection process were sterilized through autoclaving in the laboratory prior to the field excursion, and the tubes for each sample site were stored in separate packaging to maximize the compartmentalization of environmental exposure and minimize any potential cross contamination between sites. All sampling instruments were set up and sterilized with a 10% NaClO (Bleach) solution and rinsed with autoclaved double distilled water (ddH_2O). The tubes to store swab samples were numbered, and all consumables were readied as part of the temporary field processing site. GPS coordinates were noted at the base of operation upon arrival at the selected sampling site. Adult *A. hymenopus* frogs were sought no further than 50 meters upstream from the sampling-base and caught by hand wearing sterile examination gloves.

A clean unused pair of sterile examination gloves was used for every specimen of adult *A. hymenopus* that was targeted. Gloves and animals were kept appropriately moist to alleviate any discomfort to subjects during handling. Three replicate swabs were taken from each specimen and stored separately in sterile 1.5 mL centrifuge tubes (Eppendorf, Dubai, United Arab Emirates). The first swab was stored dry, the second swab stored in ddH_2O, and the third swab was stored in 70% C_2H_5OH (Ethanol) solution. The three replicate swabs were stored in this diverse manner to optimise preservation of the microbial integrity of each sample. In an attempt to collect the most complete skin microbiome representation of each specimen for each sample, replicate swabbing started in the folds of the thigh, as it is a prominent area for *B. dendrobatidis* infection (Longcore *et al*. 1999), continued ventrally across the abdominal area and gular region, and ending with the dorsal area. After the sampling procedure was completed, the subjects were released downstream from, but in close proximity to, the sampling base of operation from where the specimen were initially caught. This standardized the release point and prevented the re-sampling of subjects. The swab cotton tips were cut off into afore mentioned appropriately numbered Eppendorf tubes, and stored out of direct sunlight in a cooler with ice packs. They were then moved to a -20 °C freezer as soon as possible until further processing.

3.2.3. Contamination prevention

Each frog was caught by hand while wearing a new sterile pair of examination gloves, as opposed to catching them with nets or other equipment. Doing so minimized the possibility of inadvertent cross contamination of samples due to potential incomplete sterilization of any equipment that came into contact with specimens. All tubes, consumables and sterilizing agents were prepared and packaged in a

clean, sterile laboratory environment to have separate, ready to use field kits for each sample site less than five days before the field excursion. This ensured sampling equipment was only exposed to the sampling site environment that the kit was intended to be used in, minimizing the possibility of cross-site contamination. During wet lab processing of samples, a sterile environment was key to reliable results. Thus, care was taken to wash all surfaces and equipment both before and after experiments in two parts. Firstly, all were wiped off with 10% NaClO (Bleach) solution and rinsed with double distilled water (ddH_2O) to remove bleach, and secondly, all was sterilised with a 70% C_2H_5OH (Ethanol) solution. Single use consumables were all disposed of in a Biohazard bin that gets destroyed by incineration.

3.3. DNA isolation and quantification

A ZymoBIOMICS™ DNA (deoxyribonucleic acid) Miniprep Kit (Lot# ZRC201356) (Zymo Research, Irvine, CA, USA) was used, following the manufacturer's protocol, to extract DNA from cotton swabs (Dryswab™ MW100, Medical Wire & Equipment, Swatar BKR4013, Malta) used for sample collection. In accordance with the "direct swab extraction" method, swabs were placed in supplied 2 mL ZR BashingBead™ Lysis Tubes containing micro bashing beads and 750 µL ZymoBIOMICS™ Lysis Solution was added. Sample tubes were shaken at maximum speed for 5 minutes (min) using the high-speed homogenizer TissueLyser II (Qiagen, Novato, CA, USA). Shaken tubes were centrifuged for 1 minute at 10 000 x g to pellet beads and bashed swabs to prevent "messy practice" and ensure convenient access to the supernatant. Of the supernatant, 400 µL was transferred to the Zymo-Spin™ III-F Filter with 2 mL microcentrifuge tube (Eppendorf) and centrifuged for 1 min at 8 000 x g. Of the provided ZymoBIOMICS™ DNA Binding Buffer, 1 200 µL was added to the ensuing supernatant filtrate and mixed via pulse vortex. The resulting sample solution was then filtered through the same Zymo-Spin™ IICR Column by centrifuging 800 µL portions for 1 minute at 10 000 x g. The flow-through was discarded. Processed Zymo-Spin™ IICR Column was then washed by centrifuging 400 µL ZymoBIOMICS™ DNA Wash Buffer 1 through at 10 000 x g for 1 min. The afore mentioned Zymo-Spin™ IICR Column was washed again with ZymoBIOMICS™ DNA Wash Buffer 2 by centrifuging buffer through the column at 10 000 x g for 1 min in two portions in the order of 700 µL and then 200 µL. The washed Zymo-Spin™ IICR Column was then transferred to a clean sterile 1.5 mL microcentrifuge tube (Eppendorf) where 50 µL ZymoBIOMICS™ DNase/RNase Free Water was added to the column and incubated at room temperature for 5 min before centrifuging for 1 min at 10 000 x g to collect isolated DNA for each sample. As a final wash step, the Zymo-Spin™ III-HRC Filter was prepared by centrifuging 600 µL ZymoBIOMICS™ HRC Prep Solution through the filter at 8 000 x g for 3 min prior to transferring eluted DNA sample to the prepared III-HRC Filter with a clean sterile 1.5 mL

microcentrifuge tube (Eppendorf), which was finally centrifuged at 16 000 x g for 3 min. Isolated DNA was now ready and suitable for further downstream molecular experiments.

Following DNA extraction, the concentration and quality of the isolated DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Republic of Lithuania). No dilution for standardising isolated DNA concentration was necessary as quantities measured within an acceptable range between 1 and 5ng/μL. Isolated DNA was stored at -20 ℃ until further usage in downstream experiments.

3.4. Real-time Polymerase Chain Reactions

In order to categorize all of the collected samples into groups representing frogs with the presence of *Bd*, and those where the chytrid rDNA (ribosomal DNA) was absent, a real-time polymerase chain reaction (rtPCR) experiment was conducted based on specific 5.8S ITS (Internal Transcribed Spacer) 2 region rDNA *Bd* specific primers. These primers included *Bd*_ITS_Fwd 5'CCTTGATATAAT ACAGTGTGCCATATGTC3', and *Bd*_ITS_Rev 5'AGCCAAGAGATCCGTTGTCAAA3' (Boyle *et al*. 2004). Bio-Rad SsoAdvenced™ Universal SYBR® Green Supermix (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's prescribed protocol following recipe depicted in Table 3.1. The final mixture for each reaction consisted of 10 μL Universal SYBR® Green Supermix with 1 μL of both *Bd*_ITS_Fwd and *Bd*_ITS_Rev primers (Boyle *et al*. 2004) with a concentration of 10 μM, 4 μL of isolated DNA template and 4 μL of nuclease-free water to fill reaction mixture up to a total of 20 μL per reaction. A pooled master mixture that excluded any isolated sample DNA template was first prepared in a clean, sterile 1.5 mL microcentrifuge Eppendorf tube covered with foil in a room with the lights dimmed, to protect the photosensitive reagents from external light. After the master mixture was thoroughly blended via vortex to ensure homogeneity, 16 μL aliquots were dispensed into the wells of a Real-Time PCR reaction plate (Thermo Scientific, Republic of Lithuania). Isolated sample DNA template were added to each well containing reaction master mixture and sealed with rtPCR specialised flat cap lids. Plates were again mixed thoroughly via vortex for 30 seconds (s) and spun-down to ensure reaction mixture was collected in the bottom of the well with no bubbles that could interfere with reaction.

Table 3.1: Real-time polymerase chain reaction recipe according to the manufacturer's prescribed protocol.

	Volume (µL)
Universal SYBR® Green Supermix	10
Primer: *Bd*_ITS_Fwd (10 µM)	1
Primer: *Bd*_ITS_Rev (10 µM)	1
DNA template (~1-5 ng/µL)	4
H_2O (molecular grade)	4
Total	**20**

The plate was then loaded onto a QuantStudio5 (Thermo Scientific) thermocycler with the following programmed conditions (Table 3.2): initial denature step at 98 °C for 3 min followed by a 40 replication cycles of 98 °C denaturation for 15 s, a 57 °C annealing and an extension step at 72 °C for 1 min. A melt curve analysis step commenced, stating with an initial denature step at 95°C for 15 s followed by a step-up phase from 60 °C increased with 0.5 °C intervals with 5 s hold until reaching 95 °C. Visualisation and analysis of results were done using QuantStudio™ Design & Analysis Software (Thermo Scientific).

Table 3.2: Real-time polymerase chain reaction thermocycling program.

Amplification step	**Temperature (°C)**	**Time (min:s)**	
Initial denature	98	3:00	
Denature	98	0:15	Repeat x40
Annealing	57	1:00	
Extension	72	1:00	
Melt curve analysis step			
Initial denature	98	0:15	
Step up phase staring temperature	60	Increased with 0.5 °C and held for 0:05	
Step up phase end temperature	95		

3.5. Illumina Next Generation Sequencing Polymerase Chain Reactions

The 5.8S ITS 2 region of the fungal rDNA was amplified by PCRs done on DNA targeting fungi using the ITS3_forward 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGA ACGCAGC3' and ITS4_reverse 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCT CCGCTTATTGATATGC3' universal primer set (White *et al*. 1990; Caporaso *et al*. 2012) specific for fungi in general. Bacterial 16S rDNA was amplified using the 16S342_forward 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG3' and 16S781_reverse 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT

AATCC3' universal primer set (Klindworth *et al*. 2013). All primer sets had over-hanging Illumina adapters (Caporaso *et al*. 2012) which are represented by the underlined regions. PCR amplification was carried out with a final reaction mixture volume of 25 µL (Table 3.3). Final reaction mixture consisted of: 12.5 µL KAPA HiFi Ready-Mix DNA Polymerase (KAPA Biosystems, Lasec, RSA), 1.5 µL 10 mM ITS3_forward/16S342_forward and ITS4_reverse/16S781_reverse primer mixture, 2 µL template DNA which ranged between concentration of 1 to 5 ng/µL and 9 µL nuclease-free water to fill reaction volume to a total of 25 µL. A negative control was included in which the sample template DNA was replaced with PCR-grade water (Merck, Sigma Aldrich, RSA) in the reaction mixture to detect potential contaminants. A positive control with template DNA in the reaction mixture of a previously successful reaction was also included in order to exclude potential false-negative results.

Table 3.3: Illumina Next Generation Sequencing polymerase chain reaction recipe according to the manufacturer's prescribed protocol.

	Volume (µL)
KAPA HiFi Ready-Mix DNA Polymerase	12.5
ITS3_forward/16S342_forward (10 µM)	0,75
ITS4_reverse/16S781_reverse (10 µM)	0,75
DNA template (~1-5 ng/µL)	2
H_2O (molecular grade)	9
Total	**25**

The PCR reactions were performed with the G-Storm GS04822 thermal cycler (Somerton Biotechnology Centre, United Kingdom) using the programmed conditions depicted in Table 3.4. The initial denaturation lasted 3 min at 95 °C, followed by 25 cycles of 95 °C denaturation for 30 s, primer annealing phase lasted 30 s at 58 °C, amplicon extension ensued at 72 °C for 30 s, and lastly a final extension step at 72 °C for 5 min.

Table 3.4: Illumina Next Generation Sequencing polymerase chain reaction amplification program.

Amplification step	**Temperature (°C)**	**Time (min:s)**	
Initial denature	95	3:00	
Denature	95	0:30	Repeat x25
Annealing	58	0:30	
Extension	72	0:30	
Final extension	72	5:00	

The PCR products were run through a gel electrophoresis experiment to ensure the PCR reactions were successful. The gels were prepared at a 2% agarose concentration. A 2 µL portion of each PCR

product was mixed with a loading dye (KAPA Biosystems) and GelRed fluorescent nucleic acid dye solution (Biotium, Inc., Fremont, CA, USA) prior to being loaded into wells of the prepared 2% agarose gel (Cleaver Scientific Ltd, UK) and ran at 120 Volts for 30 min. Visualisation of amplification products were done under a ultraviolet light and photographed in a Gel Doc™ EZ Imaging System (Bio-Rad Laboratories, Inc., Johannesburg, RSA).

At the Next Generation Sequencing Unit (University of the Free State) PCR amplicons were pooled together and purified using the Agencourt AMPure XP bead clean up kit (Beckman Coulter, Atlanta, Georgia, USA) following the manufacturer's protocol. The final library was quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Massachusetts, USA). Verifying that fragment sizes in the final library ranged from 200 bp to 300 bp was validated using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). A Bioanalyzer DNA 1000 chip was loaded with 1 µL of a 1:50 dilution of the final library solution. Purified amplicons were pooled together and normalized into equal concentrations of 8 pmol, finally followed by paired-end sequencing (2 X 300 bp) on an Illumina MiSeq platform (Illumina Inc., San Diego, United States) using a MiSeq v3 reagent cartridge (Illumina Inc.).

3.6. Bioinformatics and statistical pipeline

Sequence data obtained from Illumina sequencing were received in FASTQ format from the Next Generation Sequencing Unit. The raw sequence data was of high quality, allowing strict trimming and quality filtering to bring forth reliable and reproducible results from bioinformatics analysis that followed. Primer and Illumina adapter sequences were trimmed from the 3' end in the range of 50 to 55 bp according to specific primer lengths. Forward sequences were trimmed to 252 bp from the 5' end, while reverse sequences were trimmed to 195 bp for the 16S NGS data. For the 5.8S ITS2 NGS data the forward sequence was trimmed to a length of 245 bp and 180 bp for the reverse. Trimming lengths were selected as such to ensure that the raw sequence data used in the downstream statistical pipeline has a base Phred quality score of >30, meaning the base call accuracy was >99.9% (Shi, Li and Xu, 2016), as well as taking into account that there is still at least a 12 bp overlap of forward and reverse sequences for successful merging.

Microbiome bioinformatics were conducted with Qiime 2 2017.4 (Bolyen *et al*. 2019). Sequence raw data were imported as demultiplexed paired end sequences with quality. Quality filtering and trimming was necessary before any taxonomic classification, or any other analysis could be done. The q2-demux plugin was used to trim off primer ends and truncate raw sequences to filter out small fragments and remove sections with quality scores below desirable (Phred quality score of <30), followed by denoising with DADA2 via q2-dada2 (Callahan *et al*. 2016). Amplicon sequences were aligned using

mafft via q2-alignment (Katoh *et al*. 2002) prior to phylogeny construction via q2-phylogeny using fatsttree2 (Price *et al*. 2010). Sequence variants (Molecular Operational Taxonomic Units, MOTUs) were assigned taxonomy using q2-feature-classifier (Bokulich *et al*. 2018) classify-sklearn naive Bayes taxonomy classifier queried against the appropriate reference database at 99% sequence similarity (Nilsson *et al*. 2011). For fungi (5.8S ITS region 2), the UNITE database (alpha version 12_11) released on 10.10.2017 (Kõljalg *et al*. 2013; Nilsson *et al*. 2018) was used, and for bacterial/archaeal (16S rRNA) the SILVA 132 database (Quast *et al*. 2012) was used. A trial was done with the alternative 97% sequence similarity thresholds (Eevers *et al*. 2016; Solanki *et al*. 2019) for MOTU reference sequence classification (McDonald *et al*. 2012) for both fungal and bacterial/archaeal taxonomy assignment, resulting in minor discrepancies. Only the 99% sequence similarity libraries were used in the downstream analyses. A normalized relative abundance table for all assigned MOTUs was created in Qiime 2 2017.4 (Bolyen *et al*. 2019). To match the species information in the databases to the annotated MOTUs the "collapse taxa" command was used, followed by converting feature table with the assigned MOTUs to a relative abundance table presented in percentages.

Analysis of the abundance table was carried out in R studio (RStudio Team 2020) and the Phyloseq package (McMurdie and Holmes 2013). Rarefaction curve was delineated with a "ggrare" function adapted from the script in the vegan package's (Oksanen *et al*. 2015) "rarecurve" function. Random subsampling was done in a rarefying step (Sanders 1968) using "rarefy_even_depth" function in the Phyloseq package (McMurdie and Holmes 2013) to standardise sample libraries to even depths in preparation for alpha and beta diversity analysis.

For this study, alpha diversity was illustrated by means of Shannon diversity indices of the diversity and richness of fungal and bacterial assigned MOTUs in the sampled microbial community collected at the different localities. Alpha diversity with regards to assigned MOTUs abundance dissimilarities and richness was calculated using Observed MOTUs indices together with the Shannon diversity metrics and plotted as analysis of variance (ANOVA) diversity in RStudio using the "estimate_richness" function. The data input included the group of samples that exhibited an absence of *Bd* specific 5.8S ITS 2 region rDNA (*Bd* -). Shannon diversity illustrates estimations of both the number of different MOTUs and their dissimilarity in abundance presented in the different sample sets. Beta diversity was calculated using Bray-Curtis dissimilarity metrics and visualized in RStudio as a Principle Coordinate Analysis (PCoA) plot (Layeghifard *et al*. 2018) utilizing the "plot_ordination" function from the *Phyloseq* package (McMurdie and Holmes 2013). Beta diversity differences between sample sites was assessed using permutational analysis of variance (PERMANOVA), which were performed using the *adonis* function in the vegan package (Oksanen *et al*. 2015) in R studio. The ggplot2 package (Wickham

2009) in R studio was used to create all visual plots. Venn diagrams were plotted using the function *venn.diagram* in R studio to present a schematic of MOTUs shared between- and unique to sample sites.

Chapter 4: Results

4.1. DNA isolation and quantification

A total of 36 adult *A. hymenopus* frog skin microbiome samples were collected from the four sample sites (Figure 3.1). After DNA was extracted from the cotton swabs containing the microbiome samples, the isolated DNA was quantified, followed by a selection process where 15 samples were selected on merit of the DNA quality yielded from extraction and renamed as AK01 to AK15 (Table 4.1). Of the 15 samples studied, five were collected from Bilanjil, four from Ribbon Falls, three from Vemvane and three from Tugela. According to the mass spectrometry results, potential DNA concentrations showed an average of 3.09 ng/µL, with a range of 1.64 ng/µL at the lowest and 5.00 ng/µL at the highest concentration. The average 260/280 ratio value across samples was 1.69, which is close to the optimal ratio of 1.8 according to the instrument manufacturer. This implies that the ratio on DNA molecules, that absorbs light with 260 nm wavelengths, are almost twice as abundant as the accompanying protein, phenol, or other possible contaminants present in the sample, which absorbs light wavelengths around 280 nm. The average 260/230 ratio value was calculated at 0.26, which is appreciably lower than the optimal range of 1.8 to 2.2. This indicates a relatively high presence of co-purified contaminants smaller than DNA molecules on account of their characteristic absorption of light wavelengths in the spectrum of 230 nm. Relatively low concentrations of DNA molecules are expected as initial amounts of DNA present on the collected swab is low to start with, but based on the results the DNA quality was feasible for downstream experiments.

Table 4.1: Listing of all samples used for next generation sequencing and downstream bioinformatics with their corresponding metadata. The table also presents concentrations of possible [DNA] in nanograms per micro liter, the 260/280 ratio which represents particles absorbing light with waves lengths of 260 nm and 280 nm respectively, and 260/230 ratio which represents particles absorbing light with wavelengths of 260 nm and 230 nm respectively.

Sample	Site	GPS (DMM)	Date sampled	[DNA] ng/µL	260/280	260/230	*Bd*
AK01	Bilanjil	30 45,623 28 53,766	15-May-19	1.64	1.29	0.27	-
AK02	Bilanjil	39 45,623 28 53,766	15-May-19	3.19	2.83	0.26	-
AK03	Ribbon Falls	32 45,740 28 55,063	15-May-19	1.98	2.06	0.35	-
AK04	Vemvane	33 44,907 28 52,522	16-May-19	2.54	1.06	0.17	+
AK05	Vemvane	34 44,907 28 52,522	16-May-19	2.93	1.45	0.18	+
AK06	Vemvane	36 44,907 28 52,522	16-May-19	2.27	1.22	0.11	+
AK07	Tugela	29 45,156 28 53,585	16-May-19	4.11	1.51	0.26	+
AK08	Tugela	30 45,156 28 53,585	16-May-19	2.13	1.70	0.23	+
AK09	Tugela	37 45,156 28 53,585	16-May-19	4.30	1.24	0.39	+
AK10	Bilanjil	31 45,623 28 53,766	15-May-19	2.91	1.46	0.29	+
AK11	Bilanjil	33 45,623 28 53,766	15-May-19	3.32	2.06	0.26	+

Sample	Site	GPS (DMM)	Date sampled	[DNA] ng/µL	260/280	260/230	*Bd*
AK12	Bilanjil	35 45,623 28 53,766	15-May-19	2.53	1.76	0.18	+
AK13	Ribbon Falls	29 45,740 28 55,063	15-May-19	4.66	2.41	0.33	+
AK14	Ribbon Falls	31 45,740 28 55,063	15-May-19	2.85	1.27	0.35	+
AK15	Ribbon Falls	35 45,740 28 55,063	15-May-19	5.00	2.07	0.24	+

***Bd*:** *Batrachochytrium dendrobatidis* 5.8S Internal Transcribed Spacer 2 region rDNA present (+) or absent (-).

4.2. Real-time Polymerase Chain Reactions

All 36 collected samples plus two positive and negative controls were screened for the presence of *Bd* specific 5.8S ITS (Internal Transcribed Spacer) 2 region rDNA. Of the 36 samples, only six failed to show amplification in the rtPCR experiment, excluding the 2 negative controls which also failed to show amplification, indicating the absence of *Bd* in six said samples (Table 4.1). Of the 15 samples selected for downstream bioinformatics analysis, AK01 to AK03 represents samples showing absence of *Bd* specific 5.8S ITS 2 region rDNA amplification, while the rest of the samples, AK04 to AK15, indicated clear amplification of *Bd* specific 5.8S ITS 2 region rDNA. Overall, the average *Bd* infection prevalence across the sampled population was 83.55% (±11.87%). All sample sites indicated high infection prevalence with the highest occurrence of infection presented at Tugela, and the lowest at Vemvane (Table 4.2).

Table 4.2: Summary results of rtPCR analyses of *Batrachochytrium dendrobatidis* infection of *Amietia hymenopus*.

Sample site	Number of individuals sampled	Number of infected individuals	Infection prevalence (%)
Vemvane	9	6	66.67
Tugela	9	9	100
Bilanjil	11	9	81.82
Ribbon Falls	7	6	85.71
Total	36	30	

4.3. Illumina Next Generation Sequencing Polymerase Chain Reactions

Polymerase chain reactions from all 15 selected samples (AK01 to AK15) yielded good quality amplicons with expected fragment lengths of ~400 bp for bacterial 16S rDNA (16S) and ~350 bp for fungal 5.8S ITS 2 region rDNA (5.8S ITS2) according to the molecular size standard (Figure 4.1). There was no unspecific amplification for the 16S reactions with high concentrations of amplicon for all samples, while some minor unspecific amplification were seen in some of the 5.8S ITS2 reactions, specifically in AK01 and AK10 (Figure 4.1, A). Fortunately, it did not influence downstream analysis as targeted region amplicons were still in overpowering abundance and at desirable concentrations. All positive controls (Pos Control) indicated successful reactions while the negative controls (Neg Control)

verified PCR reaction master mixtures were uncontaminated on account of an absolute lack of amplified product.

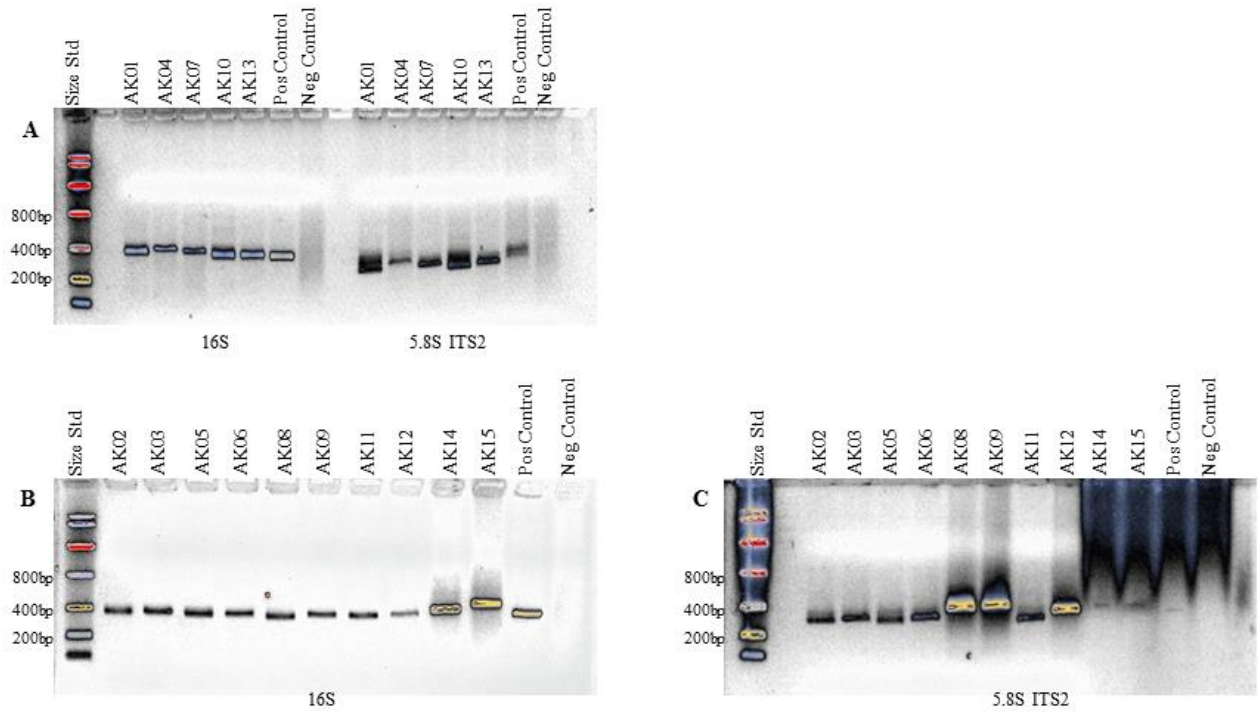

Figure 4.1: Gel electrophoresis images of PCR reaction product visualized on a 2% gel. First lanes present the molecular size standard (Size Std) represented by the KAPA™ Express Ladder (KAPA Biosystems) with size standards for 800 bp, 400 bp and 200 bp molecule lengths indicated. A) 16S region rDNA and 5.8S ITS 2 region rDNA PCR products for AK01, AK04, AK07, AK10, and AK13. B) 16S region rDNA PCR products. C) 5.8S ITS 2 region rDNA PCR products.

4.4. Bioinformatics and statistical pipeline

4.4.1. Sequence quality filtering and rarefying

After quality control a mean number of 57 193 sequence reads per sample were allocated to the fungal (5.8S ITS2) library ranging from a minimum of 20 369 to a maximum of 118 719 sequence reads with an average sequence fragment length of 322 bp. The bacterial library (16S) had a minimum of 29 022 sequence reads, a maximum of 147 660, and a mean number of 72 610 sequence reads with an average fragment length of 415 bp. Initially a total number of 2 228 and 18 656 Molecular Operational Taxonomic Units (MOTUs) were assigned to the fungal and bacterial sequence library, respectively. After filtering out MOTUs assigned to Archaea, chloroplasmic DNA, mitochondrial DNA and MOTUs with unassigned taxonomy in accordance with the relevant database, the fungal component comprised of a total of 743 MOTUs and the bacterial component at a total of 17 217 MOTUs. A number of 8 and 439 doubletons were identified and removed from the fungal and bacterial library, respectively. During the

rarefying step a number of 7 and 886 MOTUs were removed from the fungal and bacterial libraries respectively, because they were no longer present in any sample after random subsampling. Rarefying resamples MOTU tables were done to create consistently sized libraries with even depths as preparation for alpha and beta diversity analysis (Sanders 2014). For the final downstream bioinformatics analysis a total of 728 fungal and 15 938 bacterial unique MOTUs (Table 4.3) assigned at 99% sequence similarity were implored.

Table 4.3: Sequencing results for both the bacterial and fungal component after quality control (QC) and Molecular Operational Taxonomic Units (MOTUs) of all samples' fungal (5.8S ITS2) and bacterial (16S) components.

Sample	Number of reads before QC		Number of reads after QC		Number of assigned MOTUs	
	5.8S ITS2	16S	5.8S ITS2	16S	5.8S ITS2	16S
AK01	128178	85773	45965	69064	125	1844
AK02	66290	78615	32093	62908	113	1317
AK03	55307	180954	38309	147660	60	2668
AK04	37626	138854	25887	127319	31	692
AK05	53578	120904	20369	91843	117	3315
AK06	125589	77986	76776	57954	112	2469
AK07	110816	34531	68933	29022	41	849
AK08	85436	64376	51753	50850	79	1728
AK09	90859	84230	64805	68150	50	1826
AK10	85624	55391	42578	35055	145	1123
AK11	111393	72608	84983	57208	85	1078
AK12	144395	39148	118719	32956	51	961
AK13	79962	112402	48426	92975	47	1713
AK14	81749	125300	39435	94602	87	1863
AK15	125947	87015	98869	71582	82	1340

4.4.2. Rarefaction Curve

Rarefaction curves illustrate alpha diversity indices well. The graph can be interpreted as an estimate of the samples richness in full, with the samples microbial population diversity determined by the number of sequenced taxa (Willis 2019). Rarefaction curve plateaus indicate that sampling reached the saturation point, meaning that the microbial population's diversity is well represented and a good estimate of the taxa diversity presented in the sample (Figure 4.2).

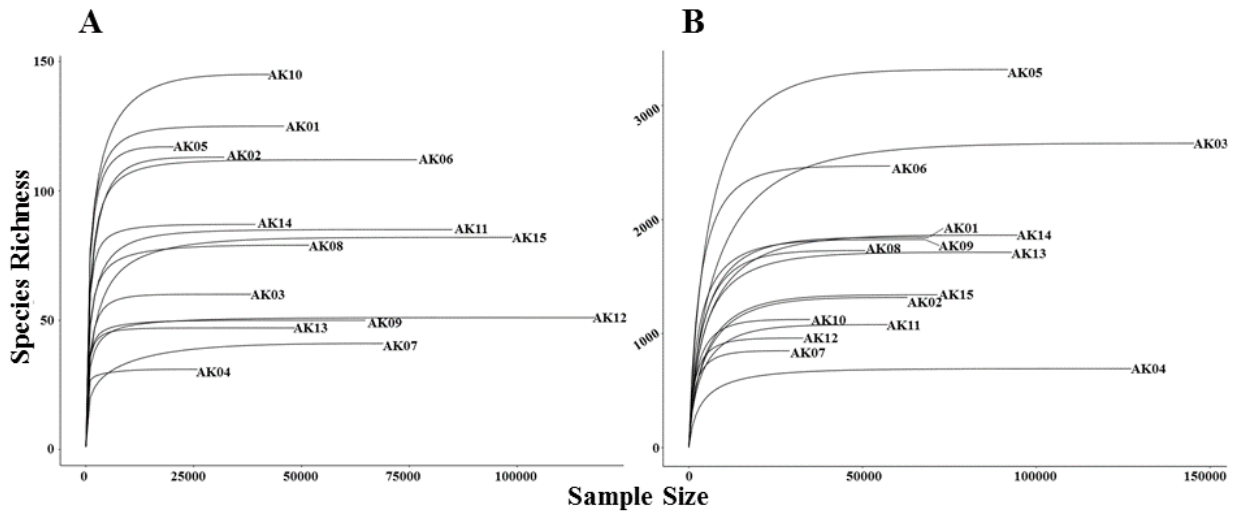

Figure 4.2: Rarefaction analysis of the fungal (A) and the bacterial (B) microbe community richness estimates based on sequences that passed the minimum Phred quality score of 30.

4.4.3. Shannon diversity index

Shannon diversity is a means to illustrate the diversity of species within a given community. For this study, alpha diversity was illustrated by comparison of Shannon diversity indices and richness of fungal (Figure 4.3 A) and bacterial (Figure 4.3 B) assigned MOTUs in the sampled microbial communities. Samples that exhibited an absence of *Bd* specific 5.8S ITS 2 region rDNA (*Bd* -) was also included as a separate group of samples to be compared to samples collected from the different localities.

Shannon diversity is an estimated illustration of both the number of different MOTUs and their dissimilarity in abundance presented in the different sample sets. For the fungal component, the *Bd*- group of samples presented the highest alpha diversity with a mean Shannon diversity index >2.5 (Figure 4.3 A). Ribbon Falls presented the lowest alpha diversity estimate and was the only group of samples exhibiting a mean Shannon diversity index <2.0.

Vemvane indicated the highest mean Shannon diversity index of close to ~6.9 regarding the bacterial component, but also manifested with a strikingly low lower whisker at ≥2.1 while all other groups of samples presented lower whiskers ≥5.0 (Figure 4.3 B). Sample groups Bilanjil and Ribbon Falls both had the lowest mean Shannon diversity indices of <6.0.

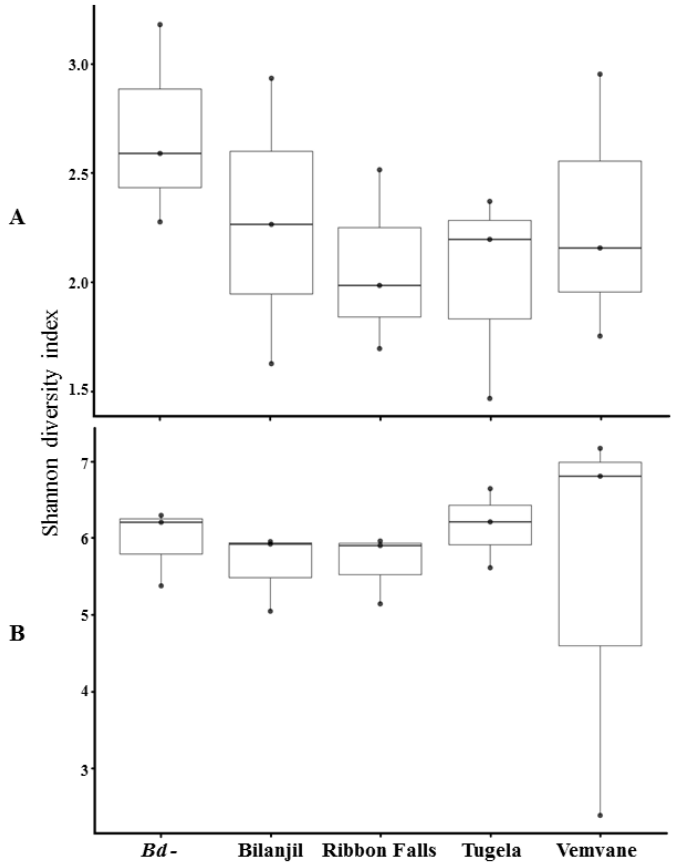

Figure 4.3: Shannon diversity indices of samples that indicated an absence of *Batrachochytrium dendrobatidis* 5.8S ITS 2 region rDNA (*Bd* -) and the four sampling locations for the (A) fungal and (B) bacterial component of the sampled microbiome.

4.4.4. Taxonomic assignment and relative abundance

a. Bacteria

All samples revealed a diverse bacterial community with a variety of phyla in high relative abundance in different samples. The vast majority consisting of thirteen out of the fifteen samples were dominated by Proteobacteria with a relative abundance range of 24.7% to 72.4%, and a mean relative abundance of 37.1% (±14.38%). For the remaining two samples, Bacteroidetes featured as the most abundant in sample AK02 with 41.1% relative abundance, and Verrucomicrobia as predominant phylum in sample AK04 with a relative abundance of 71.6% (Figure 4.4, Table 4.4). Furthermore and in addition to aforementioned preponderance of phyla, Chloroflexi, Cyanobacteria, and Planctomycetes represented samples with relative abundance in the upper 5th percentile (≥16.94%). Firmicutes were the last phylum to feature relative abundance high enough to fall in the upper 10th percentile (≥7.71%) for a single sample, though it is still a cosmopolitan phylum among all samples in low relative abundance. Apart from phyla

in the upper 10th percentile (≥7.71%) relative abundance, Acidobacteria, Actinobacteria, Armatimonadetes, Deinococcus-Thermus, and Patescibacteria were likewise cosmopolitan among samples with a relative abundance range from as low as 0.3% to a maximum of 7.5%, with Acidobacteria rendering the highest mean relative abundance of 4.34% (±3.09%) and Patescibacteria the lowest at 1.7% (±1.65%). There was a remarkably low amount of unassigned MOTUs at phylum level with a mean relative abundance of 0.08% (±0.15%).

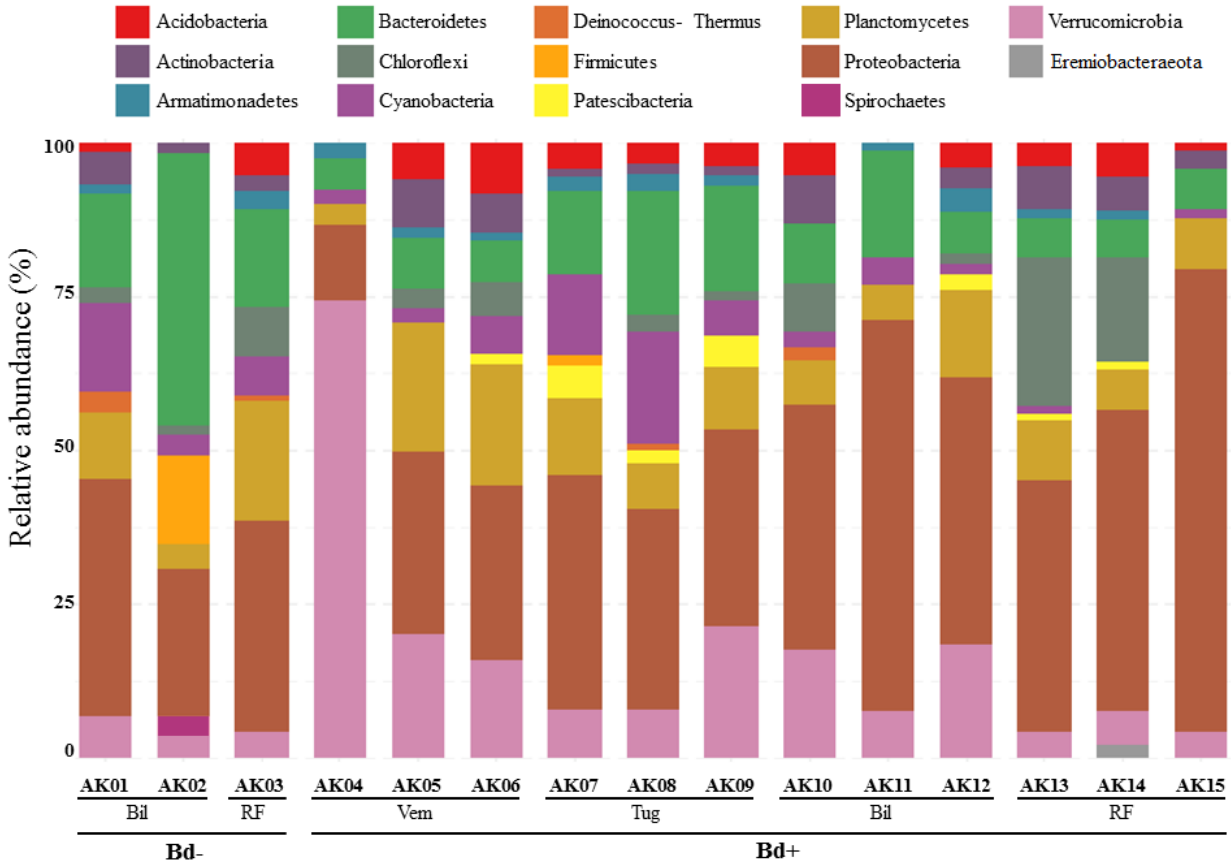

Figure 4.4: Relative abundance (%) of bacterial communities at phylum level present in samples at an abundance of ≥1%. Bil, RF, Vem and Tug are representative for samples collected at Bilanjil, Ribbon Falls, Vemvane and Tugela, respectively. *Bd+* (*Bd–*) indicates that *Batrachochytrium dendrobatidis* was present in (absent from) the sample.

Relative abundance were diversely distributed among genera (Figure 4.5) when considering Table 4.5, which presents genus level assigned MOTUs with relative abundance ≥1%. Proteobacteria featured highest among phyla accounting for close to half the samples' most abundant genus level assigned MOTUs. *Pseudomonas* (Pseudomonadaceae) represented the highest relative abundance of 28.7% and 23.5% for AK11 and AK15, respectively. Additionally, AK12 and AK13 presented relative abundance above the upper 10th percentile threshold of ≥5.9% for *Pseudomonas*. Burkholderiaceae was a well-represented family with *Polaromonas* as most abundant featured genus in AK12 at 9.4% and 14.2% for

AK15 (above upper 5th percentile of ≥8.65%), withal AK11 presented 6.3% (above upper 10th percentile threshold of ≥5.9%) relative abundance for *Polaromonas*. Sp8 uncultured bacterium was the most abundantly featured genus in AK07 and AK08 at 14.9% and 8.1% respectively. Furthermore, regarding Burkholderiaceae; *Duganella*, *Massilia*, and *Noviherbaspirillum* were present in relative abundances above the upper 10th percentile threshold (≥5.9%) for some samples. *Acinetobacter* (Moraxellaceae) featured as most abundant genus level taxa in AK14 with 11.2% relative abundance (above upper 5th percentile of ≥8.65%). *Deefgea* (Chitinibacteraceae) presented abundantly in AK02, AK03, and AK13, with AK02 exhibiting a relative abundance of 7.7% (upper 10th percentile, ≥5.9%), AK03 rendering *Deefgea* as most abundant genus in sample, and AK13 exhibiting a relative abundance of 10.5% (upper 5th percentile, ≥8.65%).

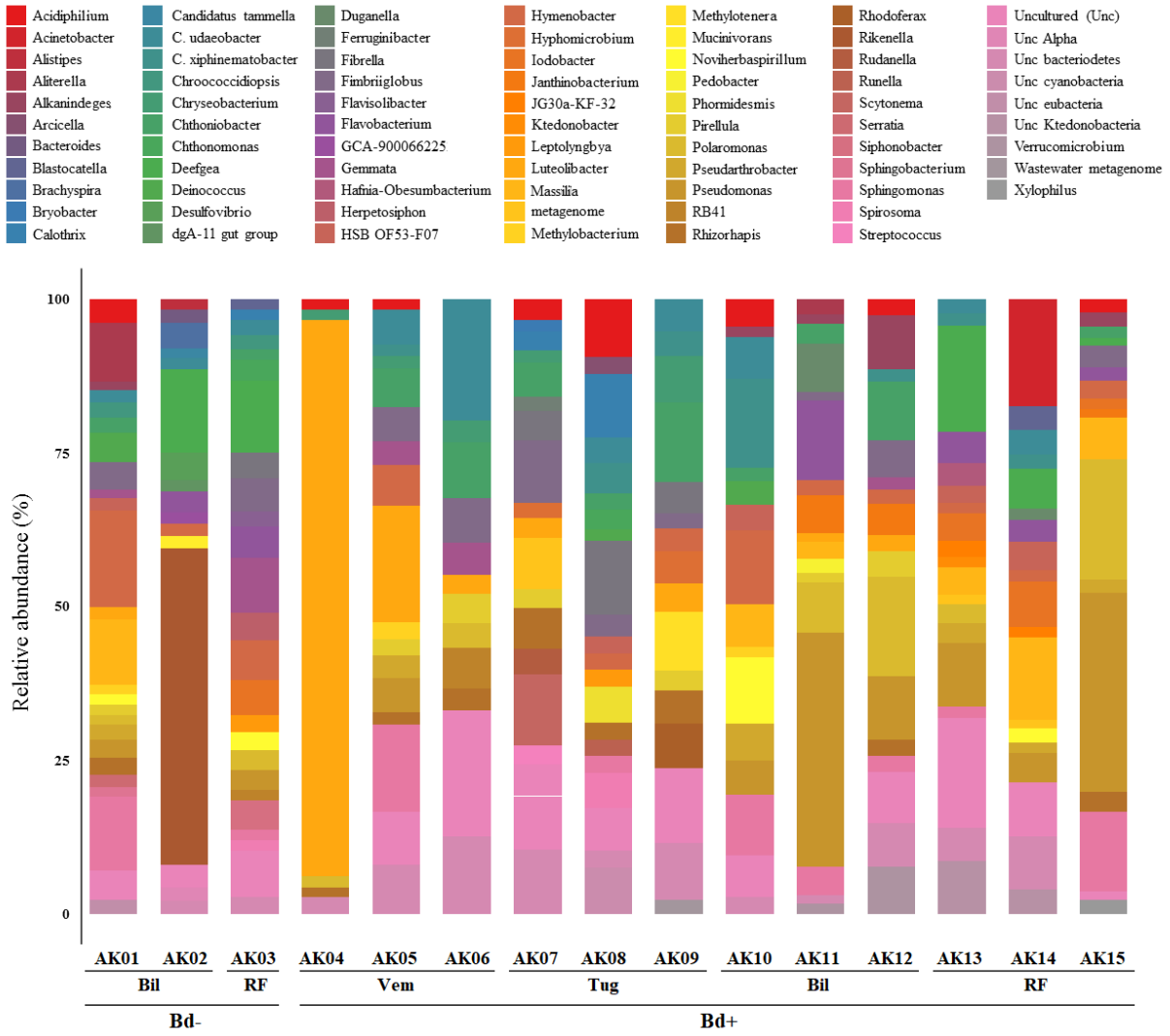

Figure 4.5: Relative abundance (%) of bacterial communities at genus level present in samples at an abundance of ≥1%. Bil, RF, Vem and Tug are representative for samples collected at Bilanjil, Ribbon Falls, Vemvane and Tugela, respectively. *Bd+* (*Bd–*) indicates that *Batrachochytrium dendrobatidis* was present in (absent from) the sample.

Within Bacteroidetes three genera stood out with high relative abundance, namely; *Rikenella* (Rikenellaceae) representing sample AK02's most abundant MOTU, *Hymenobacter* (Hymenobacteraceae) representing AK01's most abundant MOTU as well as 7.3% relative abundance in AK10 which falls within the upper 10^{th} percentile (≥5.9%), and *Flavobacterium* (Flavobacteriaceae) presenting a 9.6% relative abundance in AK11 which is higher than the upper 5^{th} percentile threshold of ≥8.65%. Ktedonobacteraceae (sp4 uncultured bacterium) family within the Chloroflexi phylum was of significance for samples AK13 and AK14 with relative abundance 13.7% (upper 5^{th} percentile threshold of ≥8.65%) and 6.9% (upper 10^{th} percentile threshold of ≥5.9%), respectively. Second to Bacteroidetes, Verrucomicrobia featured most among phyla, represented a third of the sample group's most abundant genus level taxa. Most remarkably AK04 indicated a relative abundance of 68.8% for *Luteolibacter* (Rubritaleaceae), which also presented as AK05's most abundantly featured genus at 9.7% (above upper 5^{th} percentile threshold of ≥8.65%). *Candidatus Xiphinematobacter* (Xiphinematobacteraceae) was most abundant in sample AK10 and was the only other genus within Verrucomicrobia phylum to feature in the upper 5^{th} percentile (≥8.65%). Chthoniobacteraceae's *Candidatus Udaeobacter* and *Chthoniobacter* were most abundantly featured taxa for AK06 (5.3%) and AK09 (4.8%), respectively. Notably, within the Cyanobacteria phylum, *Aliterella* (Chroococcidiopsaceae) rendered at a relative abundance within the upper 10^{th} percentile (≥5.9%) solely for AK01.

Table 4.4: Summary of the relative abundance (%) of bacterial Molecular Operational Taxonomic Units (MOTUs) taxa that were assigned at phylum level. Names used were assigned by 99% similarity threshold to the SILVA 132 database (Quast *et al*. 2012).

99% MOTUs	*Bd*-				Vemvane			Tugela			Bilanjil		Ribbon Falls		
	AK01	AK02	AK03	AK04	AK05	AK06	AK07	AK08	AK09	AK10	AK11	AK12	AK13	AK14	AK15
Bacteria;															
Acidobacteria	2.2	0.7	6.4	0.7	7	13	4.6	3.4	4.1	6.3	1.1	4.1	4.3	5.8	1.4
Actinobacteria	5.2	1.6	2.6	0.4	7.4	5.5	1.3	1.8	1.6	7.5	0.8	3.5	6.6	5.1	2.8
Armatimonadetes	1.6	0.7	2.9	2.6	2	1.1	2.4	2.9	1.5	0.8	1.4	3.6	1.7	1.7	0.8
BRC1 (unclassified Sumerlaeota)		0.1			0.1	0.3	0.4		0.1		0.1	0.1			0.2
Bacteroidetes	**14.8**	***41.1****	**14.9**	4.7	**7.7**	5.8	13	***19.2***	**15.8**	**9.5**	**16.1**	6.4	6	5.9	6.1
Chlamydiae			0.1		0.1	0.5	0.1		0.1	0.1	0.1	0.2			
Chloroflexi	2.4	1.2	8.2	0.1	3.3	5.1	0.6	2.8	1.3	**7.8**	0.5	1.8	***23.7***	**16.8**	0.8
Cyanobacteria	**14.4**	4.9	5.8	2.5	2.6	6.1	13	***19.5***	**8.9**	2.6	5.4	2.3	1.1	1.2	2
Deferribacteres		0.7													
Deinococcus-Thermus	3.2	0.4	1.1	0.4	0.3	0.3	1	1	0.8	2.3	0.5	0.5	0.5	0.3	0.7
Dependentiae						0.1					0.1	0.2			
Elusimicrobia		0.3				0.2			0.3						
Eremiobacteraeota			0.2	0.1	0.1	0.1	0.1	0.1		0.1			0.9	2.3	
Firmicutes	0.3	**13.1**	0.1	0.6	0.3	0.9	1.9	0.7	0.5	0.6	0.4	1	0.2	0.1	0.1
Fusobacteria								0.1	0.2						
Candidate division Galena15						0.2									
Gemmatimonadetes	0.2	0.1	0.1		0.2	0.4						0.1	0.3	0.2	
Hydrogenedentes			0.1			0.1			0.1						
Latescibacteria			0.4		0.1	1.3			0.3				0.2		0.1
Lentisphaerae		0.6													
Nitrospirae			0.1			0.4									
Omnitrophicaeota			0.1			0.1			0.1			0.2			
Patescibacteria	0.3	0.3	0.9	0.8	1	2	5	2.6	5.9	0.4	0.4	2.6	1.3	1.3	0.7
Planctomycetes	**10.5**	3.9	***18.1***	3.2	***19.8***	***16.9***	**11.8**	6.9	9	6.8	5.3	**13.8**	**9.1**	6.3	7.8

99% MOTUs	*Bd-*			Vemvane			Tugela			Bilanjil			Ribbon Falls		
	AK01	**AK02**	**AK03**	**AK04**	**AK05**	**AK06**	**AK07**	**AK08**	**AK09**	**AK10**	**AK11**	**AK12**	**AK13**	**AK14**	**AK15**
Proteobacteria	***38.1****	***22.1***	***33****	**12.1**	***28.8****	***24.7****	***37.1****	***31.2****	***29.9****	***38.4****	***60.5****	***42****	***39.6****	***47.1****	***72.4****
Rokubacteria			0.6		0.1	0.9							0.1		
Spirochaetes		3													
Synergistetes		0.9													
Tenericutes		0.8													
Verrucomicrobia	6.6	3.3	3.9	***71.6****	***18.9***	**13.7**	7.4	7.6	***19.4***	***16.6***	7.2	***17.6***	4	5.2	4.1
Unassigned			0.1		0.1				0.1	0.1			0.2	0.6	

Numerical values in bold represents assigned MOTUs within the upper 10% percentile (≥7.71%) of abundance, whilst numbers in bold and italic represents assigned MOTUs in the upper 5% percentile (≥16.94%). Abundance value followed with superscript asterisk (*) denotes MOTUs featuring as most abundant phyla in relevant sample.

Table 4.5: Summary of the relative abundance (%) of bacterial Molecular Operational Taxonomic Units (MOTUs) taxa that were assigned to genus level with abundance ≥1%. Names used were assigned by 99% similarity threshold to the SILVA 132 database (Quast *et al*. 2012).

99% MOTUs	*Bd-*			Vemvane			Tugela			Bilanjil			Ribbon Falls		
	AK01	**AK02**	**AK03**	**AK04**	**AK05**	**AK06**	**AK07**	**AK08**	**AK09**	**AK10**	**AK11**	**AK12**	**AK13**	**AK14**	**AK15**
Bacteria															
Acidobacteria															
Subgroup 6			1.1			3.9									
Blastocatellia							1								
Holophagae						1									
Blastocatellaceae *Blastocatella*														2.5	
Blastocatellaceae sp.2							1.3					1.1			
Pyrinomonadaceae sp.1						1.8									
Actinobacteria															
Micrococcaceae *Pseudarthrobacter*	1.5				1.8					3.6			2	1.1	1.7

99% MOTUs	*Bd-*			Vemvane			Tugela			Bilanjil			Ribbon Falls		
	AK01	AK02	AK03	AK04	AK05	AK06	AK07	AK08	AK09	AK10	AK11	AK12	AK13	AK14	AK15
Armatimonadetes															
Armatimonadales				2	1		1.4				1.1	2.4			
Chthonomonadaceae *Chthonomonas*			1.8					1.4							
Bacteroidetes															
Bacteroidaceae *Bacteroides*		1.3													
Rikenellaceae *Mucinivorans*		1.1													
Rikenellaceae *Rikenella*		***29.2****													
Rikenellaceae sp.3		1.1													
Chitinophagaceae *Flavisolibacter*			1.3												
Saprospiraceae sp.1							1.9	2.1	2.1						
Hymenobacteraceae *Hymenobacter*	***10.1****	1.1	3.5		3.3			1.1	1.4	**7.3**	1.9	1.3	1	1.2	2.2
Microscillaceae *Siphonobacter*			2.5												
Microscillaceae sp.2									1.3						
Spirosomaceae *Arcicella*								1.2							
Spirosomaceae *Fibrella*			2.2				1.9	5.2	1.9						
Spirosomaceae *Rudanella*							1.7								
Spirosomaceae *Runella*								1.2							
Spirosomaceae *Spirosoma*								2.5							
Spirosomaceae sp.6							1.6								
Flavobacteriaceae *Flavobacterium*		2	2.7								***9.6***		3	2.3	1.5
Weeksellaceae *Chryseobacterium*				1.2	1				2.8						
Weeksellaceae sp.2									1.1						
Sphingobacteriaceae *Pedobacter*											1.7				
Sphingobacteriaceae *Sphingobacterium*	1														

99% MOTUs	*Bd-*		Vemvane				Tugela			Bilanjil			Ribbon Falls		
	AK01	AK02	AK03	AK04	AK05	AK06	AK07	AK08	AK09	AK10	AK11	AK12	AK13	AK14	AK15
Candidatus Dormibacteraeota															
candidate division AD3 bacterium														1.5	
Candidatus Eremiobacteraeota															
candidate division WPS-2 bacterium														2	
Chloroflexi															
Kallotenuales													1.4		
Herpetosiphonaceae *Herpetosiphon*			2.4												
Ktedonobacteraceae *Ktedonobacter*													1		
Ktedonobacteraceae sp.2	1.3							1.2		2.5			1.7	2.9	
Ktedonobacteraceae sp.3													1.6	1.1	
Ktedonobacteraceae sp.4			1.2							2.2			***13.7****	**6.9**	
Cyanobacteria															
Leptolyngbyaceae *Leptolyngbya*			1.5					1.2							
Leptolyngbyaceae *Phormidesmis*								2.6							
Chroococcidiopsaceae *Aliterella*	**6.2**										1.8				
Chroococcidiopsaceae *Chroococcidiopsis*	1.6		1.3												
Chroococcidiopsaceae sp.2	1														
Nostocaceae *Calothrix*							1.2	4.6							
Nostocaceae *Scytonema*							4.7								
Deinococcus-Thermus															
Deinococcaceae *Deinococcus*	3.1									2.3					

99% MOTUs	*Bd*-			Vemvane			Tugela			Bilanjil			Ribbon Falls		
	AK01	AK02	AK03	AK04	AK05	AK06	AK07	AK08	AK09	AK10	AK11	AK12	AK13	AK14	AK15
Firmicutes															
Clostridiales		1.2													
Streptococcaceae *Streptococcus*							1.2								
Ruminococcaceae sp.1		1													
Ruminococcaceae sp.2		1.1													
Ruminococcaceae sp.3		1.2													
Patescibacteria															
Gracilibacteria							3.4						1		
Planctomycetes															
Gemmataceae *Fimbriiglobus*	2.9		2.9		2.9	1.9	4.1	1.6			1.1	3.5			2.6
Gemmataceae *Gemmata*	1		4.8		2	1.4						1.2			
Gemmataceae sp.2	1		2		2.4	2.9				1		2.3	2.2	1.4	1
Pirellulaceae *Pirellula*	1				1.3	1.3	1.3		1.2		1.1	2.3			
Pirellulaceae sp.2					1.1	1.6									
Tepidisphaeraceae sp.1			1.5		3.1	2.6		1.3	3.5	1.7		1.7	1.9	2	
Tepidisphaeraceae sp. 2			4.4		2.4	1.2									
Proteobacteria															
Rhodospirillales bacterium		1.1													
Rickettsiales alpha-proteobacterium		1.3													
Acetobacteraceae *Acidiphilium*	2.5			1.3			1.4	4.1		2.7		1.5			1.6
Acetobacteraceae sp.2	1.3														
Beijerinckiaceae *Methylobacterium*	1				1.4					1					
Hyphomicrobiaceae *Hyphomicrobium*							1		2						
Rhodobacteraceae sp.1			2.1												
Sphingomonadaceae	1.8			1.3	1.1	1	2.7	1.2	2			1.5			2.3

99% MOTUs	*Bd-*				Vemvane			Tugela			Bilanjil			Ribbon Falls	
	AK01	AK02	AK03	AK04	AK05	AK06	AK07	AK08	AK09	AK10	AK11	AK12	AK13	AK14	AK15
Rhizorhapis															
Sphingomonadaceae *Sphingomonas*	**7.8**				**7.1**			1.2		6	3.4	1.5	1.1		***9.4***
Sphingomonadaceae sp.3	1.3														
Desulfovibrionaceae *Desulfovibrio*		2.5													
Burkholderiaceae *Duganella*											**5.9**			1.1	
Burkholderiaceae *Janthinobacterium*											4.6	2.9			1
Burkholderiaceae *Massilia*	**6.8**									4.1	2.1		2.7	**8.6**	4.9
Burkholderiaceae *Noviherbaspirillum*	1.2		1.6							**6.5**				1.4	
Burkholderiaceae *Polaromonas*	1.1		1.7	1.4		1.1					**6.3**	***9.4****	1.8		***14.2***
Burkholderiaceae *Rhodoferax*									2.8						
Burkholderiaceae *Xylophilus*															1.7
Burkholderiaceae sp.8			2.2	1.2	1.8	4.1	***14.9****	**8.1***	2.5				3.4	2.7	1.1
Chitinibacteraceae *Deefgea*		**7.7**	**6.3***										***10.5***	4.1	1
Chitinibacteraceae *Iodobacter*			3.1										2.7	4.7	1.2
Enterobacteriaceae *Hafnia-Obesumbacterium*													2.3		
Enterobacteriaceae *Serratia*	1.3														
Moraxellaceae *Acinetobacter*														***11.2****	
Moraxellaceae *Alkanindiges*										1	1.2	5.1			1.7
Pseudomonadaceae *Pseudomonas*	1.9		1.8		2.8					3.3	***28.7****	**5.9**	**6.2**	3.1	***23.5****
Spirochaetes															
Brachyspiraceae *Brachyspira*		2.3													

99% MOTUs	*Bd-*			Vemvane			Tugela			Bilanjil			Ribbon Falls		
	AK01	AK02	AK03	AK04	AK05	AK06	AK07	AK08	AK09	AK10	AK11	AK12	AK13	AK14	AK15
Verrucomicrobia															
Chthoniobacteraceae *Candidatus Udaeobacter*	1.3	1	1.3		2.8	5.3*		1.8	1.9	4.1			1.4	2.5	
Chthoniobacteraceae *Chthoniobacter*	1.5				3.2	2.5	2.2	1.2	4.8*	1.3	2.4	5.5			1.3
Xiphinematobacteraceae *Candidatus Xiphinematobacter*								2.2	1.5	***8.7****		1.1	1.2	1.5	
Methylacidiphilaceae sp.1												1.4			
Pedosphaeraceae sp.1						1			2.3						
Rubritaleaceae *Luteolibacter*	1.4			***68.8****	***9.7****		1.3		1.7		1	1.6			
Verrucomicrobiaceae *Verrucomicrobium*											1.3	4.4			
Verrucomicrobiaceae sp.2						1	1.2		1.2			1.2			
Verrucomicrobiaceae sp.3									1.9						

Numerical values in bold represents assigned MOTUs within the upper 10% percentile (≥8.65%) of abundance, whilst numbers in bold and italic represents assigned MOTUs in the upper 5% percentile (≥5.9%). Abundance value followed with superscript asterisk (*) denotes MOTU featuring as most abundant genus in according sample.

b. Fungi

For the fungal portion of the sampled microbiome, the mycobiome was overwhelmingly dominated by Ascomycota (Figure 4.6) in all the samples with a mean relative abundance of 65.8% (±10.69%) (Table 4.6). Basidiomycota was also present in high relative abundance in all samples, but not so much so to be included in the upper 10th percentile (≥64.79%). Chytridiomycota and Glomeromycota were present in roughly a quarter of the samples in very low relative abundance (≤0.3%). There was a reasonably low proportion of unassigned and unidentified phyla, exhibiting a mean relative abundance of 5.7% (±4.76%) and 14.1% (±5.98%), respectively.

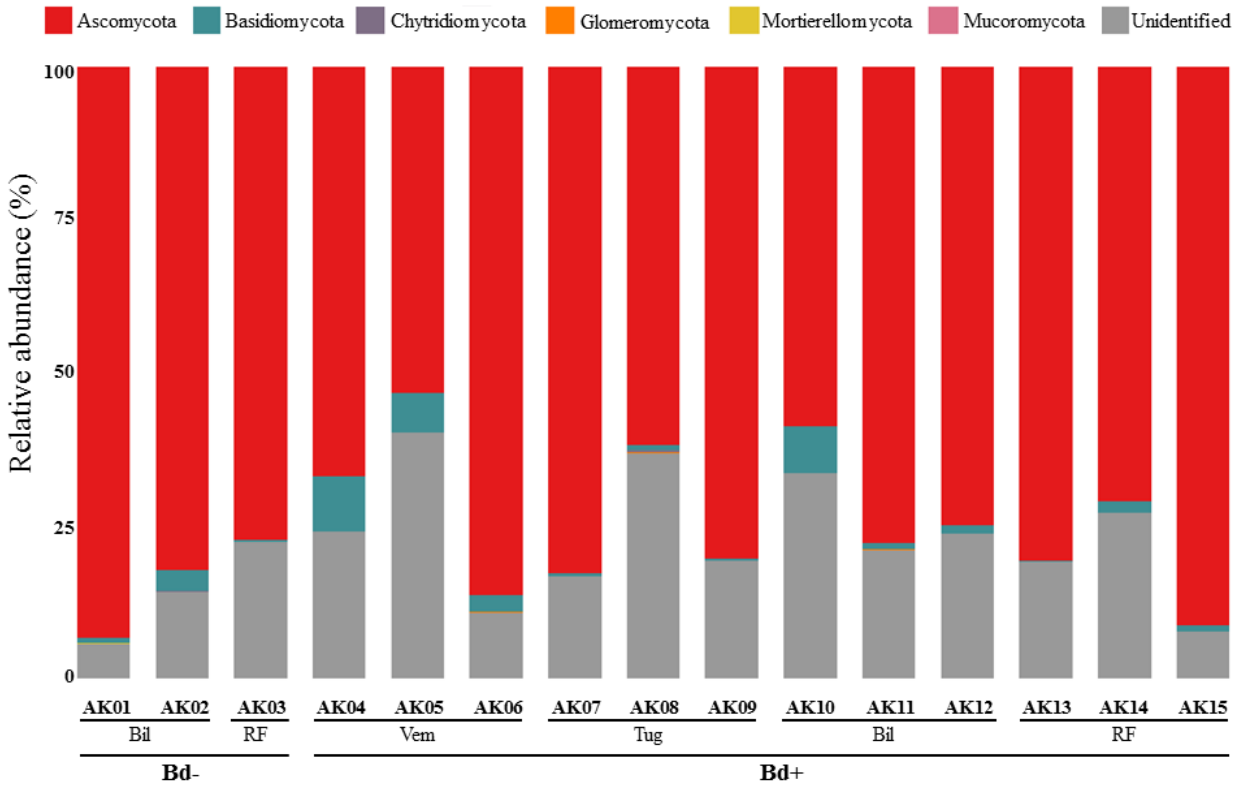

Figure 4.6: Relative abundance (%) of fungal communities at phylum level present in samples. Bil, RF, Vem and Tug are representative for samples collected at Bilanjil, Ribbon Falls, Vemvane and Tugela, respectively. *Bd+* (*Bd–*) indicates that *Batrachochytrium dendrobatidis* was present in (absent from) the sample.

Table 4.6: Summary of the relative abundance (%) of fungal Molecular Operational Taxonomic Units (MOTUs) taxa that were assigned at phylum level. Names used were assigned by 99% similarity threshold to the ITS UNITE database (alpha version 12_11) released on 10.10.2017 (Kõljalg *et al*. 2013; Nilsson *et al*. 2018).

99% MOTUs	***Bd*-**			**Vemvane**			**Tugela**			**Bilanjil**			**Ribbon Falls**		
	AK01	**AK02**	**AK03**	**AK04**	**AK05**	**AK06**	**AK07**	**AK08**	**AK09**	**AK10**	**AK11**	**AK12**	**AK13**	**AK14**	**AK15**
Fungi;															
Ascomycota	**67.4***	**68.4***	**68.1***	62.8*	42.3*	***78.8****	**68.1***	49.5*	***72.9****	50.6*	***76****	***73.3****	**64.5***	58.4*	***85.6****
Basidiomycota	28.2	16.8	12.3	13.3	38.1	8.6	12.4	5.5	4.6	21.3	3.9	3.5	18.7	24.6	4.6
Chytridiomycota		0.1						0.3							
Glomeromycota						0.1		0.1			0.1				
Unassigned	2.1	7	3	5	3.1	5.4	8.4	20.4	7.2	8.4	2.5	2.2	3.9	3	3.2
Unidentified	2.3	7.7	16.6	18.9	16.5	7.1	11.1	24.2	15.4	19.7	17.5	21	12.9	14	6.6

Numerical values in bold represents assigned MOTUs within the upper 10% percentile (≥64.79%) of abundance, whilst numbers in bold and italic represents assigned MOTUs in the upper 5% percentile (≥70.88%). Abundance value followed with superscript asterisk (*) denotes MOTU featuring as most abundant phylum in according sample.

Taking MOTUs with relative abundance of ≥1% into account only (Table 4.7), two taxonomic classes dominated the mycobiome, namely Dothideomycetes of the Ascomycota phylum, and Agaricomycetes of the Basidiomycota phylum. Chytridiomycota was detected in samples AK2 and AK8, however, MOTUs trace back to the genus *Pateramyces* (Pateramycetaceae). Within the Ascomycota phylum, five taxonomic classes were assigned MOTUs, and one group of unassigned taxonomy at class level, with Dothideomycetes being the only class to encompass assigned taxa with relative abundance in upper 5th percentile (≥39.2%). Within Dothideomycetes, *Clohesyomyces* (Lindgomycetaceae) was the only genus featuring in the upper 5th percentile (≥39.2%) (Figure 4.7), in parallel, said genus represents the large majority of the most abundant MOTU assigned to samples. Along with *Clohesyomyces* (Lindgomycetaceae), *Cladosporium* (Cladosporiaceae) were the only genus cosmopolitan among samples, and presented as the highest represented taxa in AK13 with a relative abundance equal to the upper 10th percentile threshold (≥24.94%). Within Basidiomycota, exclusively AK01 and AK05 presented relative abundance higher than the upper 10th percentile threshold (≥24.94%), featuring MOTUs categorised into a group of unassigned taxonomic orders in the Agaricomycetes class. *Cladophialophora* (Herpotrichiellaceae), *Coniochaeta* (Coniochaetaceae), and *Pestalotiopsis* (Sporocadaceae) within the Ascomycota were all nearly cosmopolitan among samples.

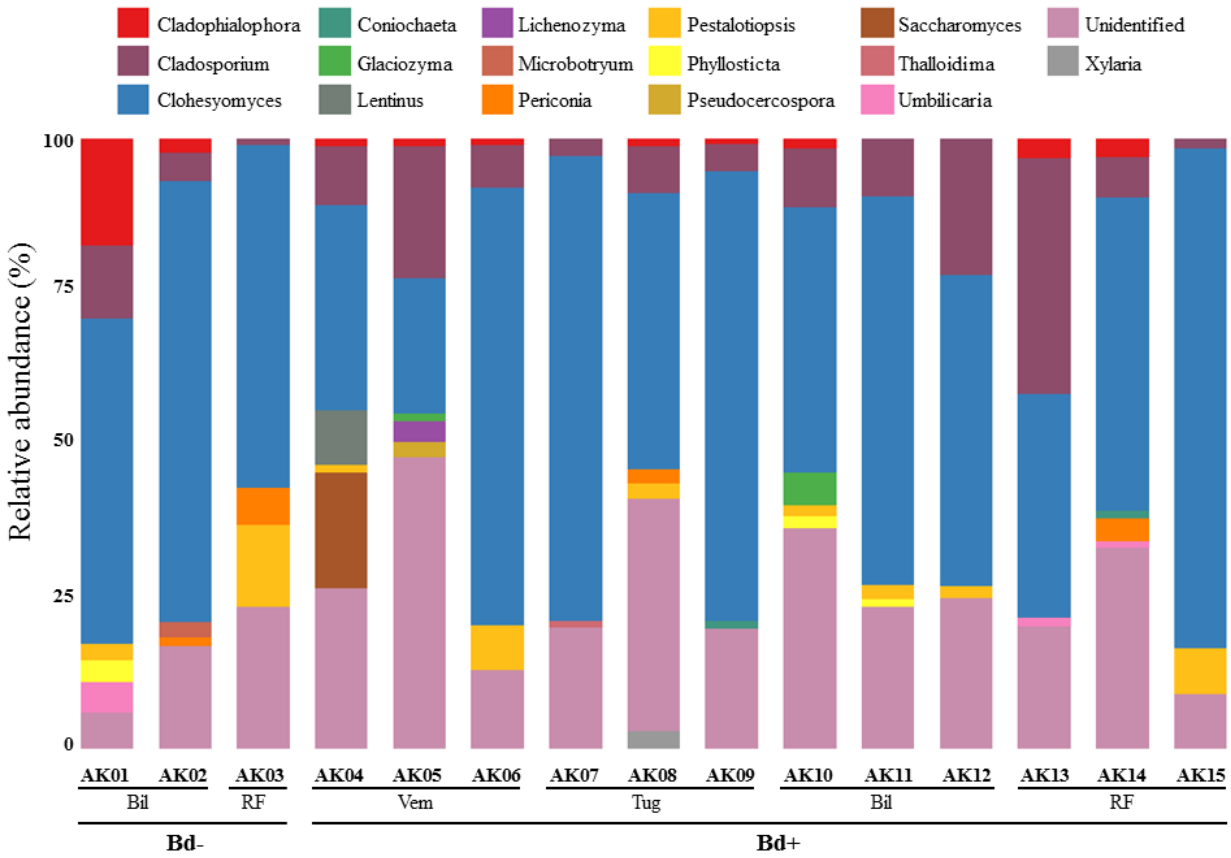

Figure 4.7: Relative abundance (%) of fungal communities at genus level present in samples at an abundance of ≥1%. Bil, RF, Vem and Tug are representative for samples collected at Bilanjil, Ribbon Falls, Vemvane and Tugela, respectively. *Bd+* (*Bd–*) indicates that *Batrachochytrium dendrobatidis* was present in (absent from) the sample.

Table 4.7: Summary of the relative abundance (%) of fungal Molecular Operational Taxonomic Units (MOTUs) taxa that were assigned to genus level with abundance ≥1%. Names used were assigned by 99% similarity threshold to the ITS UNITE database (alpha version 12_11) released on 10.10.2017 (Kõljalg *et al*. 2013; Nilsson *et al*. 2018).

99% MOTUs	*Bd*-			Vemvane			Tugela			Bilanjil			Ribbon Falls		
	AK01	AK02	AK03	AK04	AK05	AK06	AK07	AK08	AK09	AK10	AK11	AK12	AK13	AK14	AK15
Fungi;															
Ascomycota															
Unassigned	**24.9**	22.2	9.4	9.2	19.2	19.4	13.2	8.3	7.9	15.3	10.6	7	7.7	17.5	7
Dothideomycetes															
Unassigned	3.9					1.3									
Pleosporales													2.3		
Phyllostictaceae Phyllosticta	1.3									1					
Cladosporiaceae *Cladosporium*	4.5	2.4		7.5	8.4	4.3	1.9	4.9	3.5	5.3	7.6	19	**24.9***	3.3	1.4
Lindgomycetaceae *Clohesyomyces*	20.2	**37.7***	***40.2****	**26.1***	8.6	***44.5****	***49.9****	**28.7***	***57.6****	23.7*	***50****	***43.2****	23.5	**25.1***	***67.6****
Periconiaceae *Periconia*			4.3					1.5						1.8	
Eurotiomycetes															
Herpotrichiellaceae *Cladophialophora*	6.7	1.3		1									2.2	1.5	
Lecanoromycetes															
Unassigned	1.1														
Umbilicariaceae *Umbilicaria*	1.9												1		
Saccharomycetes															
Saccharomycetales				1.3											
Saccharomycetaceae *Saccharomyces*				14.7											
Sordariomycetes															
Nectriaceae sp.		1			1.1		2								
Lasiosphaeriaceae sp.														2.1	
Coniochaetaceae *Coniochaeta*									1.1						
Sporocadaceae	1		9.7			4.7		1.6		1	1.8	1.5			6.2

99% MOTUs	*Bd-*			Vemvane			Tugela			Bilanjil			Ribbon Falls		
	AK01	**AK02**	**AK03**	**AK04**	**AK05**	**AK06**	**AK07**	**AK08**	**AK09**	**AK10**	**AK11**	**AK12**	**AK13**	**AK14**	**AK15**
Pestalotiopsis															
Xylariaceae *Xylaria*								1.8							
Basidiomycota															
Unassigned														1.8	
Agaricomycetes															
Unassigned	**27.4***	14.8	11.9	6	**29.6***	6	12	3.7	3.7	15.7	3.1	2.4	18.1	21.5	3.6
Agaricales								1.2							
Polyporaceae *Lentinus*				6.9											
Cystobasidiomycetes															
Unassigned					2										
Microsporomycetaceae *Lichenozyma*					1.3										
Microbotryomycetes															
Unassigned					3.6										
Camptobasidiaceae *Glaciozyma*										2.9					
Microbotryaceae *Microbotryum*		1.3													
Unassigned	2.1	7	3	5	3.1	5.4	8.4	20.4	7.2	8.4	2.5	2.2	3.9	3	3.2
Unidentified	2.3	7.7	16.6	18.9	16.5	7.1	11.1	24.2	15.4	19.7	17.5	21	12.9	14	6.6

Numerical values in bold represents assigned MOTUs within the upper 10% percentile (≥24.94%) of abundance, whilst numbers in bold and italic represents assigned MOTUs in the upper 5% percentile (≥39.2%). Abundance value followed with superscript asterisk (*) denotes MOTU featuring as most abundant genus in according sample.

4.4.5. Principle co-ordinate analysis plot and Dendrogram

a. Bacteria

The principle coordinate analysis (PCoA) plot reveals beta diversity as a Brays-Curtis distance matrix (Bray and Curtis 1957) between samples indicating possible clustering and dissimilarities between specimens. Regarding the bacterial portion of the sampled microbiome, the PCoA plot presented principle component 1 on Axis 1 (x-axis), which represented 13.3% of overall diversity among samples, and principle component 2 on Axis 2 (y-axis) that accounted for 11.6% of the diversity between samples (Figure 4.8). The resulting analysis grouped samples that were collected from the same sample sites together into groups 1 to 4, with outliers AK02 and AK15 as the only exceptions. A permutation test indicated that there is a significant difference (p-value <0.05) between groups in regards to locality as represented by groups 1 to 4 (Table 4.8). Furthermore, three clusters can be grouped together as cluster A, B, and C. Cluster A and B corresponds with samples collected from east of Crow's nest, and Cluster C corresponds with samples collected from north-west of Crow's nest (Figure 3.1), with outlier AK02 as the singular exception. A significant difference (p-value <0.05) was also indicated by the permutation test comparing sample groups collected from east of Crow's nest (cluster A and B) and samples collected from north-west of Crow's nest (cluster C) (Table 4.8).

Overall, samples grouped well together in accordance to collection locality, excluding the two outliers (AK02 and AK15). Group 1 (Ribbon Falls) and group 4 (Tugela) clustered together most condensed, and indicate the high similarity between samples, and in the same sense low beta diversity. Group 1 (Ribbon Falls) clustered well with the exception of sample AK15 that plotted closer to group 2 and within cluster B. The same can be said for group 2 (Bilanjil) with a bit more evident separation in the cluster, also with outlier (AK02) plotted alike within cluster C. Group 3 and group 4 clustered very well, with more distinguishable separation in regards to principle component 2, but minimal diversity exhibited according to principle component 1.

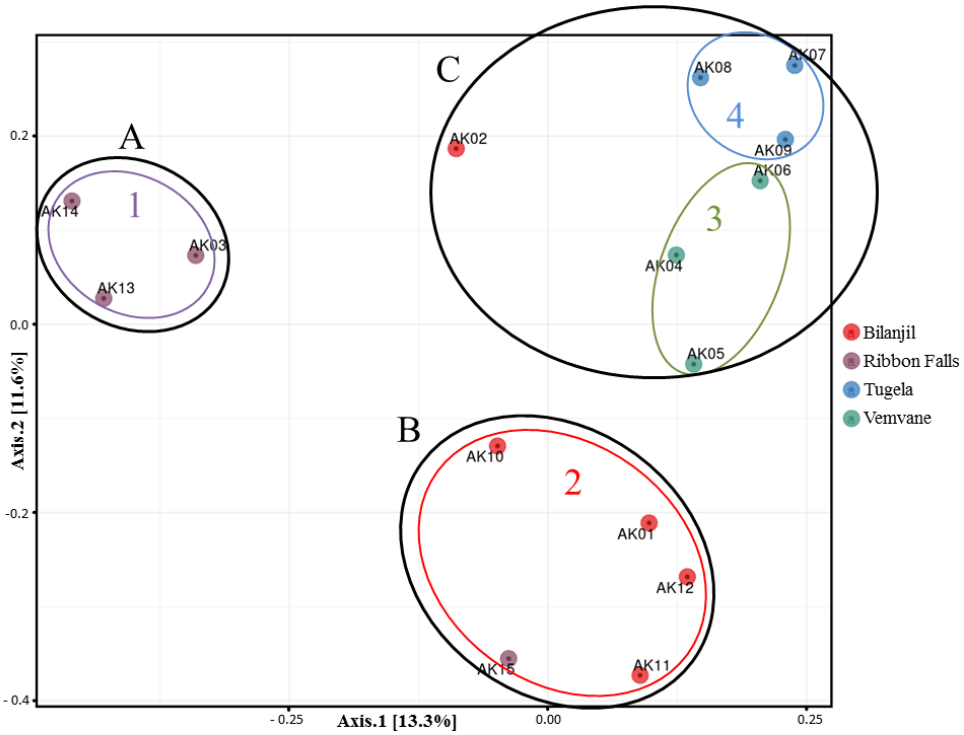

Figure 4.8: Principal co-ordinate analysis of bacterial community, exhibiting two levels of grouping; firstly, colour coded to correspond to sample origen, purple group 1 represents Ribbon Falls, red group 2 represents Bilanjil, green group 3 represents Vemvane, and blue group 4 represents Tugela. And secondly, more broad grouping are presented with black circles A, B, and C corresponding to clades formed in the dendrogram (Figure 4.9).

Table 4.8: Comparison of sampling locality, *Batrachochytrium dendrobatidis* presence/absence, and samples bearing in regards to the Geo-Barrier (Crow's nest geographical structure) for bacterial components according to Adonis permutation test.

Compared Categories	D.F	Sum of Sqs	R^2	*p*-Value
Location	3	1.47505	0.246	0.039
Bd status	1	0.4894	0.08448	0.09
Geo-Barrier	1	0.6065	0.10469	0.002

Bd status refers to the comparison of samples groups with the presence and absence of *Batrachochytrium dendrobatidis* 5.8S ITS 2 region rDNA. Geo-Barrier refers to the samples grouped into those collected from sites north-west of the Crow's nest and samples collected from sites east of the Crow's nest (Figure 3.1), D.F denotes Degrees of Freedom.

The hierarchy clustering dendrogram (Figure 4.9) is in accordance with the clustering presented on the PCoA plot and echoes these results. Three clades (Clades A to C) can be distinguished that represents Cluster A, B, and C. Clade A corresponds with cluster A, likewise for clade B and C in accordance with cluster B and C, respectively. Clade A branches off at the root node separating from the rest of the clades (Figure 4.9). Outlier AK15 groups deep within clade B indicating high similarity and

low beta diversity. Clade C presents discernable grouping of samples collected from sites north-west of the Crow's nest (Figure 3.1) with outlier AK02 branching off as a simplicifolious leaf in the clade.

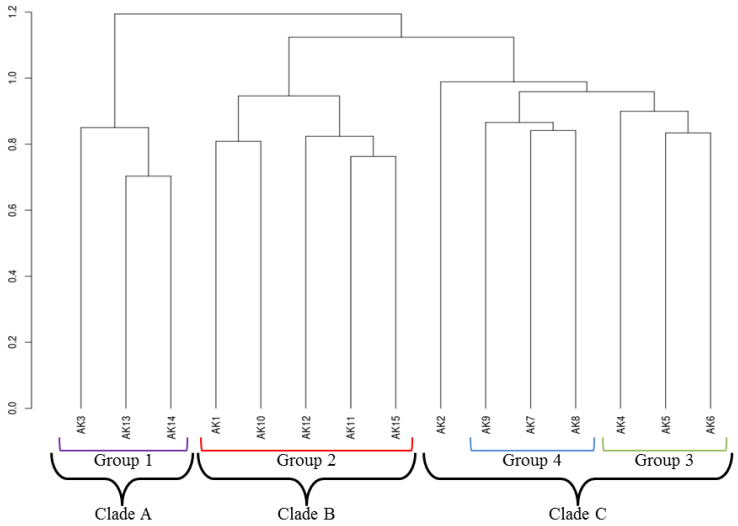

Figure 4.9*:* Hierarchy cluster analysis of beta diversity using Bray-Curtis similarities for bacterial component of sampled microbiome. Groups 1 to 4 are indicated, as well as clades A to C.

The permutation test indicated that there is not a significant difference (*p*-value >0.05) between samples having *Bd* presence, and those with *Bd* absent (Table 4.8).

b. Fungi

In regards to the mycobiome, the PCoA plot indicated that principle component 1 (Axis 1, or x axis) represented 24.2% of overall beta diversity among samples, and principle component 2 (Axis 2, or y axis) accounts for 18.3% of the diversity between samples (Figure 4.10). The fungal PCoA plot showed no clear clustering or grouping in accordance to locality of collected samples. This is also evident in the hierarchical clustering shown by the dendrogram (Figure 4.11) indicating random clustering of samples collected from different locations. The permutation test did however indicate that there is a significant difference (*p*-value <0.05) between the different locality groups where samples were collected (Table 4.9). No significant difference (*p*-value >0.05) was indicated by the permutation test when comparing either sample with *Bd* presence with *Bd* absence, or samples collected from sites north-west of the Crow's nest (Figure 3.1) compared to samples collected east of the Crow's nest (Table 4.9).

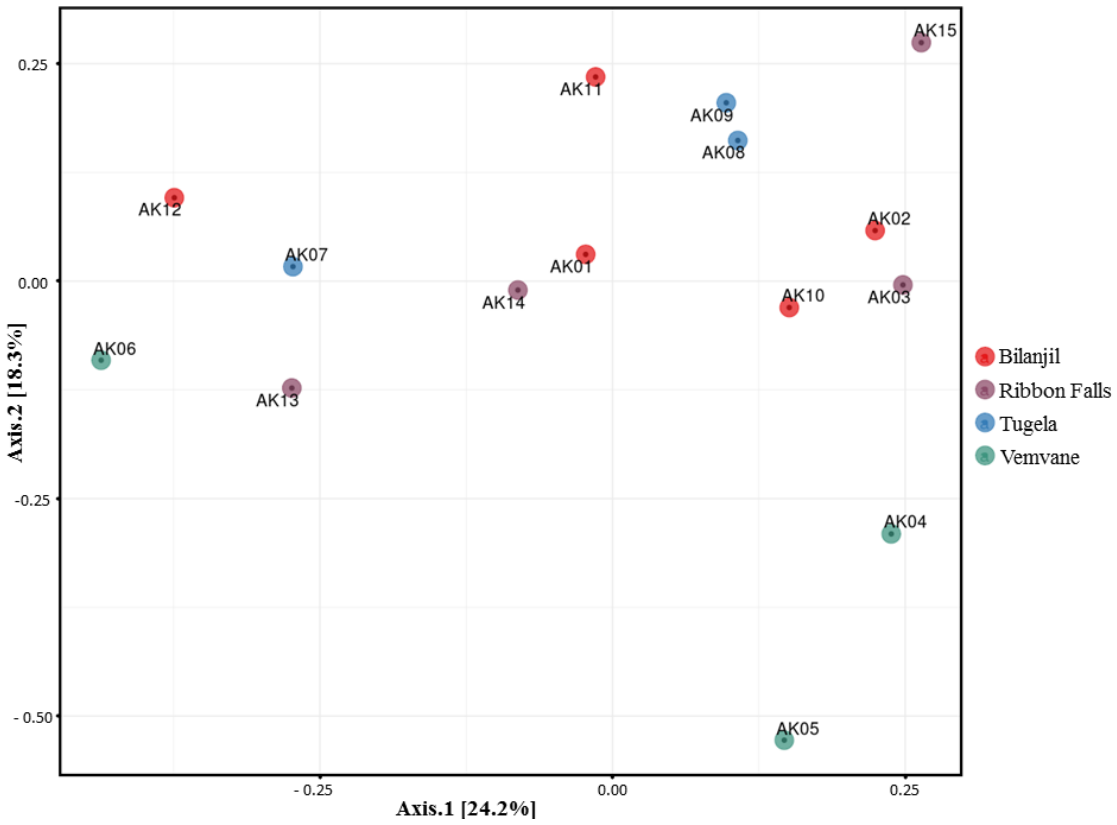

Figure 4.10: Principal co-ordinate analysis of fungal community.

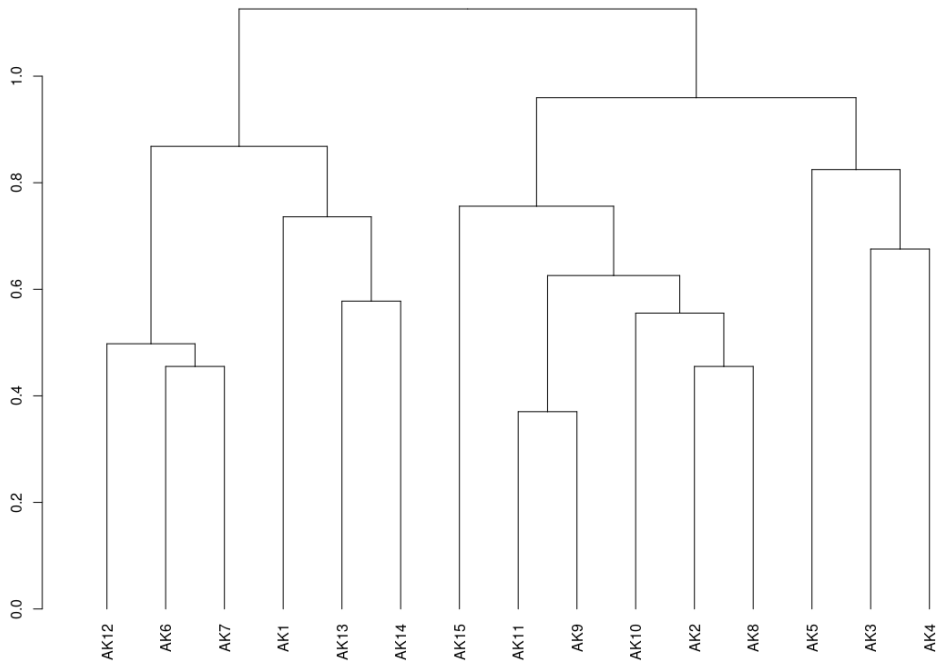

Figure 4.11: Hierarchy cluster analysis of beta diversity using Bray-Curtis similarities for fungal component in sampled microbiome.

Table 4.9: Comparison of sampling locality, *Batrachochytrium dendrobatidis* presence/absence, and samples bearing in regards to the Geo-Barrier (Crow's nest geographical structure) for fungal components according to Adonis permutation test.

Compared Categories	D.F	Sum of Sqs	R^2	*p*-Value
Location	3	1.0047	0.33577	0.007
Bd status	1	0.21456	0.0717	0.45
Geo-Barrier	1	0.1733	0.05792	0.659

Bd status refers to the comparison of samples groups with the presence and absence of *Batrachochytrium dendrobatidis* 5.8S ITS 2 region rDNA. Geo-Barrier refers to the samples grouped into those collected from sites north-west of the Crow's nest and samples collected from sites east of the Crow's nest (Figure 3.1), D.F denotes Degrees of Freedom.

4.4.6. Venn diagram comparison of sample site's bacterial and fungal components

When comparing the number of bacterial MOTUs between sample sites, Bilanjil were assigned the highest number of MOTUs (7 779) from a total of 5 samples, followed by Tugela (4 913) from a number of 3 samples, Ribbon Falls (4 556) from a number of 4 samples, and lastly Vemvane (3 989) from number of 3 samples (Table 4.10). Bilanjil and Ribbon Falls had the most shared MOTUs (724), followed by the amount shared between Bilanjil and Tugela (685) (Table 4.11, Figure 4.12). The least amount of MOTUs shared was between Vemvane and Ribbon Falls (360). The highest number of MOTUs shared only between two sites were between Bilanjil and Ribbon Falls with 309 MOTUs shared (Figure 4.12, A). The lowest number of MOTUs shared strictly between two sites was between Vemvane and Ribbon Falls with only 62 shared MOTUs. Bilanjil was the site representing the highest number of unique MOTUs with a count of 2 758 exclusive MOTUs, while Ribbon Falls exhibited the lowest number of exclusive MOTUs with 1 143 unique MOTUs.

Table 4.10: Summary of total molecular operational taxonomic units (MOTU) assigned to sample sites for both bacterial (16S rRNA) and fungal (5.8S ITS2) libraries.

Sample site	Number of MOTUs	
	16S rRNA	5.8S ITS
Vemvane	3 989	161
Tugela	4 913	215
Bilanjil	7 779	409
Ribbon Falls	4 556	199
Total	15 892	728

Table 4.11: Summary of molecular operational taxonomic units (MOTU) shared between sample collection sites.

	Vemvane	Tugela	Bilanjil	Ribbon Falls
Vemvane		**45**	**58**	**45**
Tugela	*517*		**85**	**54**
Bilanjil	*538*	*685*		**81**
Ribbon Falls	*360*	*526*	*724*	

Values in bold represent MOTUs assigned to fungal library (5.8S ITS2), while values in italic represents MOTUs assigned to bacterial library (16S).

Similar results were presented in the fungal MOTUs, with Bilanjil assigned the highest total number of MOTUs (409) from a total number of 5 samples, followed again by Tugela (215) represented by 3 samples, Ribbon Falls (199) represented by 4 samples, and lastly Vemvane (161) represented by 3 samples (Table 4.10). Deviating from bacterial results, Bilanjil and Tugela shared the highest number of fungal MOTUs (85), followed closely by Bilanjil and Ribbon Falls (81) (Table 4.11). Vemvane had the lowest number of shared MOTUs with both Tugela and Ribbon Falls with 45 shared fungal MOTUs to each. Correlating to above mentioned results, Bilanjil and Tugela shared the highest number of unique MOTUs with 34 shared exclusively (Figure 4.12, B). In accordance to bacterial results, Vemvane and Ribbon Falls shared the least amount of unique MOTUs with only 8 exclusively shared. Similarly to what was exhibited with the bacterial results, Bilanjil presented the largest number of unique MOTUs with 249 exclusive MOTUs, while Vemvane and Ribbon Falls presented the lower margins of unique MOTUs with 70 and 78 exclusive MOTUs respectively.

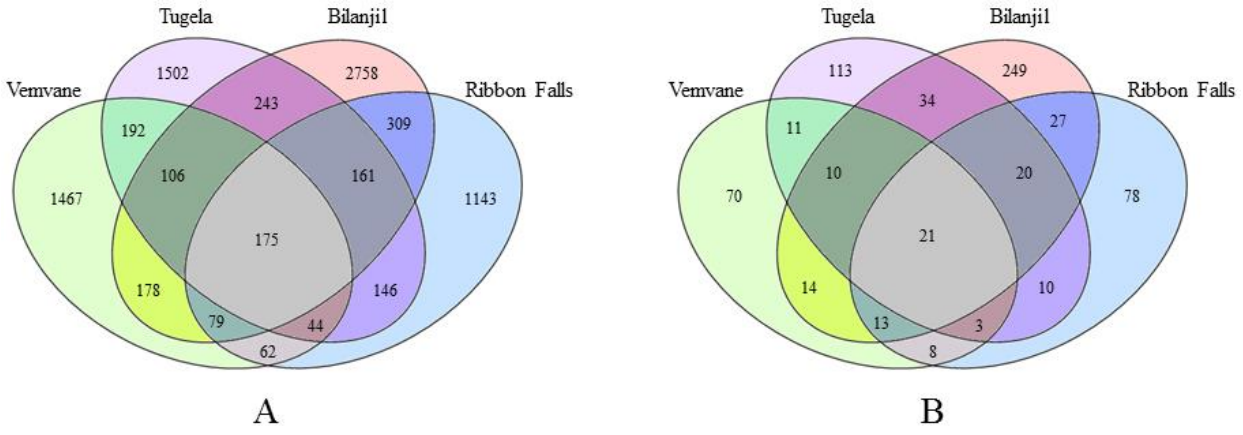

Figure 4.12: Venn diagram depicting (A) bacterial and (B) fungal Molecular Operational Taxonomic Units (MOTUs) unique- and shared between sample sites, namely: Vemvane, Tugela, Bilanjil and Ribbon Falls.

Chapter 5: Discussion

This study focused on adult individuals of the species *A. hymenopus*, which populated an area predicted to have high *Bd* prevalence (Figure 1.2) (Tarrant *et al*. 2013). In the study conducted by Dr. Tarrant and coworkers (2013) some species (*Heleophryne hewitti*) portrayed the prevalence of *Bd* occurring at 79.4%, which is lower than the results exhibited in this study where the sites sampled had a combined average infection prevalence of 83.55% (±11.87%) (Table 4.2). Both *H. hewitti* and *A. hymenopus* are riverine species that naturally inhabit permanent water bodies (Du Preez 2015) with isolated populations, consequently making them more vulnerable to frequent exposure to the hydrophilic *Bd* (Woodhams *et al*. 2016) than terrestrial species.

Research conducted by Griffiths *et al*. (2018) on the same *A. hymenopus* population as this study, showed a strikingly lower infection prevalence of 10.91 (±3.92)%. Albeit, samples were collected from *A. hymenopus* tadpoles' mouthparts, additionally seasonality also has an effect on infection prevalence. Even the two month difference in sample collection dates could account for some of the variation in comparison of results. Furthermore, likelihood of infection is positively correlated with tadpole age (Smith *et al.* 2007), infection prevalence in a tadpole sample is a reflection of the demographic profile of the sample. Thus a sample comprising of tadpoles from an earlier developmental stage is likely to display lower infection prevalence than a sample of later developmental stage tadpoles. In amphibians, *Bd* only infects keratinized cutaneous epithelial tissue, which is only exhibited on a tadpole's mouthpart, explaining the higher numbers of mortalities in postmetamorphic specimens for which the entire skin surface is susceptible to infection (Berger *et al*. 1999). However, infection prevalence on tadpoles is regarded ecologically significant, as infected tadpoles may act as *B. dendrobatidis* reservoirs, allowing chytridiomycosis outbreaks to persist and transmitting infection to postmetamorphic individuals (Rachowicz and Vredenburg 2004). Interestingly, infection prevalence in post-metamorphic frogs is negatively correlated with size (Smith *et al.* 2007), indicating an overall drop in infection in individuals that make up the adult population. Thus even for surveys directed at the post-metamorphic population does demographic composition of the sample influence infection prevalence outcome.

Results showed that the epidermal mycobiomes of all frog samples was dominated by Ascomycota, for the Ascomycocata Dothideomycetes such as Cladosporiaceae (*Cladosporium*), Leptosphaeriaceae (*Plenodomus*), Pleosporaceae (*Curvularia*), and Agaricomycetes in the Basidiomycota had the highest overall relative abundance. Interestingly Chytridiomycota was identified in both *Bd+* and *Bd-*, nevertheless, MOTUs traced back to genus *Pateramyces* (Pateramycetaceae), and thus a false positive for *Bd*. Chytridiomycota was detected in *Bd+* samples at genus level, but in very low abundance

(<1%), and consequently filtered out of Table 4.7. The bacterial component of the microbiome was predominantly dominated by Sphingomonadaceae (*Sphingomonas*) in the Proteobacteria phylum. Furthermore Gemmataceae (*Gemmata*) and Tepidisphaerales in Planctomycetes showed high abundance, as well as Ktedonobacteraceae from the Chloroflexi phylum.

Three fungal MOTUs were the most dominant in the skin microbiome, excluding taxa that could not be assigned. A *Clohesyomyces* MOTU was quite dominant and also present in the microbiomes of all of the sequenced individuals. Species in this genus are aquatic fungi (Zhang *et al*. 2012), and therefore a high probably could be expected for it to form part of the skin microbiome of an aquatic animal. A MOTU of *Cladosporium*, a well-known saprophytic and plant associated genus (Bensch *et al*. 2012) was also present across all of the samples. Lastly, an unassigned MOTU in the Agaricomycetes, a class known to contain mushrooms, was also found across all samples.

Globally, frog skin microbiomes are replete with Proteobacteria, Bacteroidetes, and Actinobacteria. This was also the case in our study. Proteobacteria are abundant in soils, and thereby, associated with tropical frog skins, and the majority of them (80% of the total OTUs present) are in the anti-*Bd* databases that catalogued bacteria taxa presenting antifungal properties against *Bd*. The Proteobacteria are characterized as a major group of purple photosynthetic bacteria along with their relatives within the eubacterial phylum (Woese *et al*. 1985). Bacteroidetes are the most dominant anaerobic bacteria found in the human gut, with its most commonly caused infection resulting in Intra-abdominal sepsis (Wexler 2007). Actinobacteria occur commonly is soils and also water environments, and are well known for their ability to produce various antibiotics (van Bergeijk *et al*. 2020).

The alpha and beta diversity of the adult frogs' skin microbiome were influenced by sample site. Bacterial and fungal components of the microbiome presented a discernable difference in regards to beta diversity. Comparisons between samples showed marked differences in bacterial communities between sample sites, which grouped separately from each other. However, these communities clustered together when taking the larger sampling area and topography into perspective. A geographical barrier present between the populations clearly separated them into two overall groups. It is evident that Ribbon Falls and Bilanjil populations are distinct from Tugela and Vemvane populations, indicating that the mountain range restricts flow between these Eastern and Western populations, which is in agreement with a previous study that show microbiome composition of amphibians is influenced by geographic site, indicating within and among population predictors of diversity seen in microbiome (Griffiths et al. 2018). These results provide additional evidence that amphibian skin microbiomes can be distinguishably related to sample's origin (Kueneman *et al*. 2014; Kruger 2020; Muletz-Wolz *et al*. 2017).

For the fungal beta diversity, all samples were consistently diverse from one another and indicated no trend or clustering in accordance to sample location origin. Therefore the mountain range (Crows nest) did not act as a geographical barrier to the same extent as it did for bacteria. It is already evident through co-occurrence network analyses that fungi shows a much higher network connectivity than bacteria (Xiao et al. 2018). Some fungi, such as *Cladosporium* species that are airborne, are more mobile than bacteria and therefore potentially better at colonizing new habitats. Yet the Adonis significance F-test indicated a significant difference between samples sites. This can be because the Adonis functions by plotting a centroid for each group and then measuring the difference of each sample in the group with the centroid, and comparing that dissimilarity between groups. Thus it is not surprising that the F-test presented a significant difference between groups even though the sample site grouping overlaps conspicuously. The significance shown by Adonis is due to the variance associated with divergent placement of sample group centroids, and the high Bray-Curtis distance between centroids and their according samples. Furthermore, the r^2 value is undesirably low, meaning the observed data does not fit well with the regression model and the majority of the variance observed is not explained.

Chapter 6: Conclusion

This study provided a useful baseline to describe the diversity of the microbiome colonizing adult Phofung River frog epidermis in the species' native range of the Drakensberg mountain range. It is vital to investigate, and continuously monitor the health of the Drakensberg population. A baseline of biodiversity, in this case that of the skins of the frogs, is important to detect future change. In accordance with recent research (Belasen *et al*. 2021; Kueneman *et al*. 2017; Kearns *et al*. 2017) the outcome of this study suggest that investigating the bacterial component of microbiomes alone provides an incomplete representation of host-microbiome interactions. This study's network analyses suggest that there may be important interactions between bacteria and microeukaryotes that have been missed by previous microbiome studies focusing on only one microbial domain or specific microbial interactions.

The use of NGS made it possible to monitor complex microbial communities such as those on the skins of frogs. Next generation sequencing has only recently become economically viable and a robust tool to obtain a significant representation of microbiome diversity, piecing together the dynamic host-symbiont relationships and their responses to pathogens. Knowledge gained in this study is incredibly useful because it is paramount to understand the complexities and functional importance of microbiomes to help mitigate infectious diseases, such as chytridiomycosis. However, a short coming in this study was that NGS provided a culture independent insight, and more conventional technique should also be utilised to generate culture dependant data to strengthen resulting conclusion.

Bacteria, or fungi, with anti *Bd* characteristics safeguard amphibians from the pathogen's infection through producing and releasing antimicrobial metabolites (Piovia-Scott *et al*. 2017). These *Bd* inhibiting bacteria can interact with one another synergistically, producing an enhanced defence (Loudon, Holland, *et al*. 2014). Amphibian immunity is maintained and can possibly evolve through host-mediated selection of *Bd* challenging bacteria (Loudon *et al*. 2016). Given the wide spread use of bacterial probiotic treatments in humans as well as in domesticated and wild animals (Cheng *et al*. 2017; Ghadban 2002; Gram *et al*. 1999) and the interest in expanding these strategies to wild amphibians (Walke and Belden 2016), future studies should prioritize advancing our understanding of interactions between microbiome bacteria and eukaryotes. It is still difficult to extricate the complex cause and effect relationship in microbiome dynamics of amphibians that appear to coexist with *Bd*. However, the baseline established in this study and previous studies from this region, provides useful information to plan future surveys. The information gained about the amphibian microbiome can then be implemented in disease management and conservation strategies of a highly threatened species to maintain the health of amphibians in a world with highly disturbed environments.

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