DIAGNOSIS, IDENTIFICATION AND ANTIFUNGAL SUSCEPTIBILITY OF CUTANEOUS FUNGI ISOLATED FROM KIHANSI SPRAY TOADS AND GIRAFFES

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ABSTRACT

Fungi is a large group of eukaryotic organisms that can be either pathogenic or non-pathogenic. Pathogenic fungi cause a range of diseases in plants and animals that can either be systematic or localized to specific organs or tissues. Among the diseases caused by pathogenic fungi that affect the skin, chytridiomycosis in amphibians and giraffe skin disease (GSD) in giraffes (*Giraffa camelopardalis*) are the two most important diseases that affect wild animals. Chytridiomycosis is caused by chytrid fungus *Batrachochytrium dendrobatidis* and has been associated with global amphibian population decline. The extinction of Kihansi spray toads (KST) in the wild is also attributed to chytridiomycosis. Furthermore, 351 toads died at the Kihansi captive breeding facility with clinical signs typical for chytridiomycosis. GSD which affects giraffes is a cutaneous fungal diseases characterized by hair loss followed by rising of the affected skin and later wrinkling, cracking and encrustation mainly on the caudal aspects of the carpal joint. GSD was first observed in 2000 in giraffes of Ruaha National Park and has now spread to Tarangire and Lake Manyara National Parks. Although GSD does not cause any mortality, affected giraffes are reluctant to use their legs making them more vulnerable to predation or poaching. Up till now, the aetiology of GSD remains unknown. The aim of this study was to identify *B. dendrobatidis* and fungi associated with GSD by sequencing of internal transcribed spacer (ITS1 and ITS2) and 5.8s rRNA gene. In addition, the antifungal susceptibility testing of the fungi associated with GSD was investigated. Analysis of the 5.8S rRNA and ITS regions obtained in this study showed that of *B. dendrobatidis* were not 100% identical to any other *B. dendrobatidis* nucleotide sequences at GenBank but were closely identical to isolates from Equador, Japan, USA, Brazil, Korea and South Africa. Possible introduction of *B. dendrobatidis* into the Udzungwa mountains arising from stocking of rainbow trout (*Oncorhynchus mykiss*) has

been speculated, although further fieldwork and re-sampling is necessary before conclusions can be reached about the nature and origin of *B. dendrobatidis* in the Udzungwa mountains. Further studies on the epidemiology of *B. dendrobatidis* in the Udzungwa mountains are recommended in order to understand the origin, prevalence and molecular characteristics in wild amphibian populations. This will be important for conservation of several endemic amphibian species in the Udzungwa Mountains in Tanzania, which are part of the Eastern Arc Mountains, a global biodiversity hotspot. Based on nucleotide sequences of 5.8S rRNA and ITS regions, fungi associated with GSD were identified to be *Aspergillus fruticulosus, Aspergillus multicolor, Aspergillus niger, Aspergillus sydowii, Aureobasidium pullulans, Botryosphaeria sp.,* Cochliobolus *lunatus, Cylindrocladium canadense, Dothideomycete sp., Epicoccum sorghinum, Fennellia nivea, Fusarium equiseti, Fusarium sp., Leptosphaerulina chartarum, Montagnulaceae sp., Penicillium citrinum, Penicillium commune, Penicillium simplicissimum, Pestalotiopsis clavispora, Phoma sp.* and *Setosphaeria* rostrata. The susceptibility of fungi against antifungal, including amphotericin B, clotrimazole, fluconazole, itraconazole, ketoconazole and nystatin was investigated. No fungus was resistant to all tested antifungal and no antifungal was able to inhibit the growth of all fungi. This finding indicates that commonly available antifungal can be used in the treatment of GSD. Further studies on the role of fungi in the pathogenesis of GSD are recommended in order to ensure the conservation of giraffes.

DECLARATION

I, **Mariam Richard Makange**, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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The above declaration is confirmed

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25 Nov 2014

Prof. Gerald Misinzo — Date

(Supervisor)

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DEDICATION

I dedicate this work to my aunt Monika Makange, my father Richard Makange and my brothers Nelson and Lugano for their love, encouragement and support.

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Makange M., Kulaya N., Biseko E., Kalenga P., Mutagwaba S. and Misinzo G. (2014). *Batrachochytrium dendrobatidis* detected in Kihansi spray toads at captive breeding facility (Kihansi, Tanzania). *Diseases of Aquatic Organism.* 111: 159–164.

Makange M., Muse E. A., Josephat A., Mwamengele G. L., Mngumi E B., Mpanduji D. G. and Misinzo G. (2014). Isolation, Identification and Antifungal Susceptibility of Fungi Associated with Giraffe Skin Disease in Giraffes of Ruaha National Park, Tanzania. Submitted to PLOS ONE Journal

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1.0 INTRODUCTION

1.1 Background Information

Fungi are important causes of diseases in wildlife animal species (Milton, 1999). Among the diseases caused by fungi, chytridiomycosis in amphibians and giraffe skin disease (GSD) in giraffes are the two most important cutaneous fungal diseases affecting wildlife population in Tanzania.

Chytridiomycosis caused by *Batrachochytrium dendrobatidis* is the single most important infectious amphibian disease that has commonly been associated with significant amphibian mortalities (Daszak *et al.*, 1999; Longcore *et al.*, 1999; Lips *et al.*, 2006). Clinical signs of amphibian chytridiomycosis include abnormal posture, lethargy, and loss of righting reflex while gross morphological lesions consist of abnormal epidermal sloughing, cutaneous hemorrhages and hyperemia of digital and ventral skin (Daszak *et al.*, 1999). *B. dendrobatidis* disrupts cutaneous osmoregulatory function, leading to electrolyte imbalance, asystolic cardiac arrest and death (Voyles *et al.*, 2007, 2009). In addition *B. dendrobatidis* produce toxic factors that inhibit protective host immune response (Fites *et al.*, 2013) and cause slower rehydration across the skin (Carver *et al.*, 2010).

Mass mortalities of Kihansi spray toad (KST) were observed in 2003, crashing the wild KST population by early 2004 with the last sighting of this toads recorded in 2005 (Weldon and du Preez 2004; Krajick 2006). Some of the KST specimens recovered from the 2003 mass mortalities in Kihansi spray wetlands were diagnosed with chytridiomycosis (Weldon and Du Preez 2004) and chytridiomycosis in KST has been recorded in captivity at the Bronx Zoo (McAloose *et al.*, 2008). Amphibian chytridiomycosis, stress due to drought caused by the failure of the sprinkler system,

predation by safari ants (*Dorylus* sp.) and/or pesticides in flushed dam sediments caused the KST population extinction in the wild (Weldon and du Preez, 2004; Channing *et al.*, 2006; Krajick, 2006).

At the moment, KST is extinct in the wild and the species survives in captivity at the Wildlife Conservation Society's Bronx Zoo and the Toledo Zoological Society and at Kihansi and University of Dar es Salaam captive breeding facilities in Tanzania (Channing *et al.*, 1999; Krajick, 2006). In November 2012, 351 Kihansi spray toad died at the Kihansi captive breeding facility. Dead Kihansi spray toad showed typical chytridiomycosis signs including epidermal sloughing, and hyperemia of digital and ventral skin. The cause of KST mass mortalities at the Kihansi captive breeding facility has not yet been established.

GSD which affects giraffes (*Giraffa camelopardalis*) is a cutaneous fungal diseases characterized by hair loss followed by rising of the affected skin and later wrinkling, cracking and encrustation. GSD was first observed in 2000 in giraffes of Ruaha National Park in the northern eastern section (Lunda) and has now spread to Tarangire and Lake Manyara National Parks (Mlengeya and Lyaruu, 2005). Affected skin appears itchy, making the giraffe frequently scratch by rubbing against branches of smaller bushes. Some affected giraffe are reluctant to use their legs; they stand at one place for long periods and show signs of lameness, possibly making them more vulnerable to predation or poaching.

Ruaha National Park and its surrounding wildlife protected areas support a large population of giraffes and other wildlife species, including the highest antelope diversity of any park in Tanzania. In Ruaha National Park, within a population of approximately

2000 giraffes, the prevalence of GSD has reached 80% (Muse, 2009). Surveys conducted between 2002 and 2003 found that 85% of the giraffes sighted were affected (Mlengeya and Lyaruu, 2007). Another survey conducted in 2005 reported the variation in the prevalence of the disease in different months and seasons of the year (Mlengeya and Lyaruu, 2007). It was reported that in the wet season (February), 82% of the giraffes sighted were affected, while in the dry season (October), 63% were affected; suggesting the prevalence of the disease to be high during the rainy season and subsides during dry months. The aetiology of GSD is unknown at the moment.

The objective of this study is to perform confirmatory diagnosis of chytridiomycosis in KST that died during the November 2012 mass mortalities at the Kihansi captive breeding facility by detecting *B. dendrobatidis* using PCR and subsequent sequencing of the 5.8S rRNA gene and internal transcribed spacer regions (ITS1 and ITS2). Information obtained from this study will be useful in establishing appropriate disease preventive measures to be observed at the KST biosecure captive breeding facility. In addition, the isolation of the fungi from skin scrapping obtain from giraffes with GSD in Ruaha National Park will be performed. Isolated fungi will be identified by sequencing of the 5.8S rRNA gene and franking ITS1 and ITS2 regions, and antifungal sensitivity of the isolates will be determined. The results obtained from the present study will assist in understanding the aetiology and control of GSD.

1.2 Chytridiomycosis

1.2.1 Chytridiomycosis and amphibian population decline

Chytridiomycosis is an amphibian disease caused by the pathogenic fungus *B. dendrobatidis* that cause the population decline of many amphibians in the world (Longcore *et al.*, 1999). Amphibians are currently facing an extinction crisis; of the over

six thousand species of amphibian and around 30% are listed as vulnerable, endangered or critically endangered (IUCN Red List, 2008) with population declines occurring in 43% of species (Gascon *et al.*, 2007). Disease has been implicated as a factor in the decline of amphibian populations worldwide (Berger *et al.*, 1998; Daszak, 2000). Other factors including habitat loss and fragmentation, chemical pollution, climate change, introduction of exotic species, increased ultraviolet radiation, and natural population fluctuations (Pechmann *et al.*, 1991). Infectious diseases reported in amphibians with an emphasis on diseases associated with population decline. While many diseases have been observed within amphibian populations, the pathogenic chytrid fungus *B. dendrobatidis* that cause chytridiomycosis has been identified as having severe impacts upon amphibian populations at a number of locations around the world. *B. dendrobatidis* has several mechanisms that allow it to have these catastrophic effects on its hosts, its ability to infect hosts at several life stages (Blaustein *et al.*, 2005) and its potential to exist in the environment for up to several weeks without a host (Johnson and Speare, 2003). This fungus affects the epithelium and epidermal cells and cause extinction of many amphibians in the world (Berger *et al.*, 1998; Longcore *et al.*, 1999). Mortalities in affected amphibian results from the disruption of normal epidermal functioning that lead to osmotic imbalance through loss of electrolytes and destruction of cutenous osmoregulation (Voyles *et al.*, 2007).

In several cases, chytrid fungus was found on dead and dying toads suggesting that chytridiomycosis was likely to be the primary cause of death (Berger *et al.*, 1998; Bosch *et al.*, 2001). This has been supported by a number of experiments where inoculation with chytrid fungus spores caused death in amphibians (Nichols *et al.*, 1998). The impacts of infection with *B. dendrobatidis* on the host vary greatly (Kriger and Hero, 2006). It has been observed that it is able to drive populations down to extremely low levels (Daszak *et*

al., 1999; Bosch *et al.*, 2001) and may even be able to drive species to extinction (Daszak *et al.*, 1999). Amphibian susceptibility to *B. dendrobatidis* infection varies with species and some amphibian species have been shown to be carriers of chytridiomycosis (Daszak *et al.*, 2004; Blaustein *et al.*, 2005).

1.2.2 Classification of *Batrachochytrium dendrobatidis*

This amphibian chytrid is in the genus *Batrachochytrium*, Phylum Chytridiomycota, Class Chytridiomycetes and Order Chytridiales having characteristics separating it from all other chytrid fungi (Longcore *et al.*, 1999). Order and genera in Phylum Chytridiomycota are classified by ultrastructural morphology of zoospore especially the flagella and molecular characteristic. *B. dendrobatidis* was originally isolated from and named for a blue poison dart frog (Mueller *et al.*, 2001) and is unique in the Chytridiomycota in that it invades the skin of amphibians and its zoospore occurs on amphibian host. Zoosporangia grow in the superficial keratinised layers of the epithelium only. *B. dendrobatidis* causes a potentially fatal epidermal infection of amphibians and has caused mass mortality, population declines and extinctions due to its ability of replicate within the skin (Berger *et al.*, 1999; Speare *et al.*, 2001; McDonald *et al.*, 2005).

1.2.3 Life cycle of *Batrachochytrium dendrobatidis*

B. dendrobatidis appears in two main forms; a spherical sessile zoosporangium 10 - 40 μm in diameter, and a motile, flagellated zoospore, about 2 μm in diameter. The zoospores are known be active only for a short period of time, and can travel short distances of one to two centimeters (Garner *et al.*, 2006). However, the zoospores are capable of chemotaxis, and can move towards a variety of molecules that are present on the amphibian surface, such as sugars, proteins and amino acids (Moss *et al.*, 2008). The motile zoospore attaches to the substrate, develops rhizoids, and becomes a

zoosporangium. Zoospores form within the zoosporangium and are released into the external environment via discharge tubes.

In culture, *B. dendrobatidis* grows slowly at 6 °C, develops most rapidly at 23 °C but dies if kept at temperatures above 29 °C (Johnson *et al.*, 2003; Berger 2005). In culture, the lifecycle takes about 4 - 5 days at room temperature (Longcore *et al.*, 1999). After a period of motility, zoospores encyst, reabsorb their flagella and form germlings. Sporangia grow larger and mature over 4 - 5 days. The sporangia become multinucleate by mitotic divisions and the entire contents cleave into zoospores while the discharge tubes form. The discharge tube is closed by a plug that absorbs water and dissolves when zoospores are ready to release. Some thalli develop colonially with thin septa dividing the contents into multiple sporangia each with their own discharge tube. In summary, once the zoospore reaches its host, it forms a cyst underneath the surface of the skin and initiates the reproductive portion of its life cycle. The encysted zoospores develop into zoosporangia, which may produce more zoospores that can reinfect the host or be released into the surrounding aquatic environment (Berger *et al.*, 2005). The amphibians infected with these zoospores are shown to die from cardiac arrest.

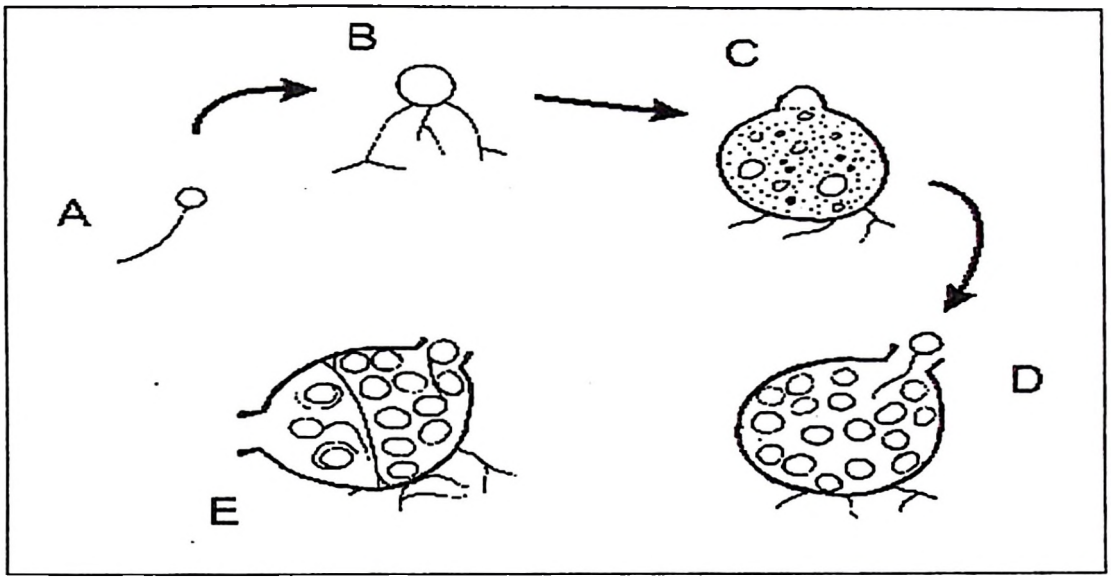

Figure 1: Batrachochytrium dendrobatidis life cycle in culture starting from zoospore (A), germling (B), immature sporangium (C), monocentric zoosporangium(D), and finally to colonial thallus.

1.2.4 Epidemiology of chytridiomycosis

1.2.4.1 Distribution

B. dendrobatidis has been found in archived specimens as far as 1934 (Weldon *et al.*, 2004). Chytriomycosis has been recorded in Australia since 1989 and has been observed in various regions including rainforest of Southern, Central and Northern Queensland and New Zealand, Europe, Africa and South, Central and North America from a broad range of habitats (Berger *et al.*, 1998). Epidemiological evidence suggests it was introduced into naïve populations in many countries. There is evidence that Africa is the origin (Weldon *et al.*, 2004).

1.2.4.2 Transmission

It has been shown that *B. dendrobatidis* may be translocated by movement of moist river sand and that birds may carry the amphibian chytrid between frog habitats (Johson and

Speare, 2005). *B. dendrobatidis* infection can spread by animal to animal contact or via contact with waterborne motile zoospores. Long distance transmission is understood to occur by means other than water; including translocation of animals during international trade (Rowley *et al.*, 2007) and potentially by movement of contaminated water or moist soil.

1.2.4.3 Hosts

B. dendrobatidis are waterborne can live for over 24 hrs (Berger unpubl) and are infective to frogs and tadpoles.

1.2.5 Clinical signs

Infected animals vary in clinical signs where by some are scibed as lethergic, subjected to fitting, lack the righting reflex, redening of vetral skin, accumulation of sloughed skin over the body and exhibit unusual behavior such as sitting in open areas.

1.2.6 Isolation and cryopreservation

The chytrid fungus is isolated from the hind toe webbing of the adult amphibians either by swabbing or by taking a piece of the skin. Toe webbings of the dead amphibians are excised using a scalpel blade and dipped in tryptone agar or liquid medium containing gelatin and lactose (ThGL). *B. dendrobatidis* grows in 1% tryptone agar (1 g peptonized milk, 1 g tryptone, 5 g glucose, 10 g agar, 1 L distilled water, with 400 mg streptomycin sulfate and 200 mg Penicillin-G) at 23 °C for 5 days. Rate of growth influenced by temperature where by optimal temperature range appears to be 17 - 23 °C (Piotrowsk *et al.*, 2004). The collected sample with the addition of phosphate buffer saline was preserved in crayol vial tube in -80 freezers.

1.2.7 Diagnosis of *B. dendrobatidis*

The most reliable methods for diagnosis are quantitative polymerase chain reaction (qPCR, or "real-time" PCR), convectional PCR and histology.

1.2.7.1 Convectional polymerase chain reaction

Polymerase Chain Reaction (PCR) refers to a method of heat cycling to amplify DNA. It was made possible by the discovery of a thermophilic (heat loving) bacterium: *Thermus aquaticus*. The DNA polymerase from this bacterium made DNA replication at high temperatures (60-70 degrees Celsius) possible. PCR works by the cycling of several temperatures which are melting, annealing and elongation. The sample can either be a cotton swab that was run across the amphibian or a piece of amphibian skin (Kniger *et al.*, 2006). PCR is generally the preferred method of testing live amphibians for chytridiomycosis as it is rapid, cost-effective, and harmless to amphibians (Boyle *et al.*, 2004; Kriger *et al.*, 2006). It is amplify highly conserved region of the genome using specific primers for these genomic regions. Primers for diagnosis used are ITS-1/5.8S and Bd1a/Bd2a primers which targets the conserved regions (Annis *et al.*, 2004).

1.2.7.2 Real-time Taqman PCR

Real-time Taqman PCR (qPCR) is a newly described technique for the detection of *B. dendrobatidis* (Boyle *et al.*, 2004; Hyatt *et al.*, 2007). It use a primer/probe which set designed to target a highly conserved region 5.8, 18 and 28S DNA separated by internal transcribed spacers (ITS-1 and ITS-2) and an intergenic spacer to detect *B. dendrobatidis* from swabs, toe clips, filters and tadpole oral discs (fresh or desiccated). Sequences of 5.8, 18 and 28S rRNA are highly conserved, whereas the ITS region and intergenic spacer units evolve quickly. The assay has a sensitivity of 0.1 zoospore equivalents. It has been shown to be the most rapid, sensitive and specific method available. It is capable of

quantitatively detecting one chytrid zoospore where by only *B. dendrobatidis* is identified. Furthermore, real-time Taqman PCR can detect *B. dendrobatidis* infection in newly-infected frogs 7 to 14 days earlier than can histological methods (Boyle *et al.*, 2004).

1.2.7.3 Histopathology

Histopathology is a method that relies on the microscopic examination of amphibian skin to detect the chytrid fungus. Diagnostic to date has largely related upon histological examination via haematoxylin and eosin staining of toe clips or skin scrapings (Daszak *et al.*, 1999). It determines the cause of death as the degree of damage to the skin can be assessed, and other potential causes of death in amphibian. This method detecting *B. dendrobatidis* on dead or dying amphibian but not for live, apparently healthy individuals or biologically important individuals such as those in endangered species in captive breeding programs. This is due to the necessity of skin samples which can harm the amphibian and raise ethical concerns. Furthermore, histology has a high chance of yielding a false negative result due to the difficulty in testing a large portion of the amphibian's skin.

1.2.8 Control of chytridiomycosis

1.2.8.1 Biological control

B. dendrobatidis infection is not lethal to all amphibians because several cutaneous bacteria of some amphibians inhibit the growth of *B.dendrobatidis* (Woodhams *et al.*, 2003; Banning *et al.*, 2008; Lauer *et al.*, 2008). Such cutaneous bacteria produce metabolites that inhibit fungal growth including 2, 4-diacetylphloroglucinol (DAPG) and Phenazine-1-carboxylic acid (PCA) (Brucker, 2007; Brucker *et al.*, 2008). Examples of cutaneous antifungal bacteria include *Lysobacter gummosus* isolated from the red-backed

salamanders, *Plethodon cinereus* from four-toed salamanders and *Janthinobacterium lividum*, a commensal bacterium isolated from redback salamanders (*Plethodon cinereus*) produces an antifungal metabolite called violacein (Becker *et al.*, 2009).The transfer of cutaneous antifungal bacteria onto amphibians sensitive to *B. dendrobatidis* confers chytridiomycosis resistance to these amphibians to these amphibians forming the basis of the biological control against *B. dendrobatidis.*

1.2.8.2 Chemical control

Disnfectants such as sodium chloride, household bleach, potassium per manganate, formaldehyde solution, Path-XTM agricultural disinfectant, quarternancy ammonium compound 128(DDAC), Dithane, Virkon ethanol and Benzalkoniumchloride have fungicidal against *B. dendrobatidis* which has been used at 2mg/L to successfully treat a similar superficial mycotic dermatitis in dwarf African clawed frogs. The use of disinfectants for the control of *B. dendrobatidis* can only be employed in captive husbandry and laboratory settings. Also cutaneous itraconazole, amphotericin and fluconazole have also been used to treat *B. dendrobatidis* infections (Taylor *et al.*, 1999).

1.2.8.3 Antifungal treatment

Following the diagnosis, chytridiomycosis in amphibians can be treated using antifungal drugs. The cutaneous itraconazole, chloramphenicol, amphotericin and fluconazole are antifungal drugs used to treat *B. dendrobatidis* infections (Taylor *et al.*, 1999). Itraconazole is widely used in a broad number of species, including some critically endangered, in zoos and amphibian conservation programs and is the most commonly used agent in experimental . Some of these drugs are antibiotic that acts by inhibiting protein synthesis but found to also be active against *B.dendrobatidis*, although only reported in a few animals (Young *et al.*, 2012, Baitchman and Pessier, 2013).

1.2.9 Kihansi spray toads *(Nectophrynoides asperginis)*

The Kihansi spray toad (KST) *Nectophrynoides asperginis* is an endemic ovoviviparous amphibian species found in the Kihansi gorge along the Udzungwa Mountains in Tanzania. *N. asperginis* was first seen in Kihansi spray wetlands in late 1996 and was formally described as a new species in 1998 (Poynton *et al.*, 1998).This species occur in the spray wetlands created by the falls of the Kihansi river. In 1999, constructions of the Lower Kihansi Hydroelectric Plant (LKHP) reduce the water flow over Kihansi gorge. The reduced water flow led to a change and loss of Kihansi spray wetlands that resulted into *N. asperginis* population decline (Poynton *et al.*, 1998). In 2000, an artificial spray irrigation system was installed in the gorge in order to maintain the Kihansi spray wetland habitat and *N. asperginis* population recovery. Meanwhile, approximately 500 *N. asperginis* were translocated into captivity in the USA (Bronx and Toledo zoos) with the purpose of establishing a captive-bred line and as an "insurance policy" in case species become extinct in the Kihansi gorge. Mass mortality of *N .asperginis* was observed in the Kihansi gorge in 2003 and by 2004 no *N. asperginis* sighted within the Kihansi spray wetlands and the species was classified as extinct in the wild by IUCN (Weldon *et al.*, 2004). At the moment, the species survives in captivity at the Wildlife Conservation Society's Bronx Zoo and the Toledo Zoological Society in the US, at Kihansi and University of Dar es Salaam captive breeding facilities in Tanzania (Channing *et al.*, 1999; Krajick 2006). In 2012 2000 *N. Asperginis* in the Kihansi captive breeding facilities were reintroduced to their natural habitat (IUCN, 2012).

1.2.10 Chytridiomycosis in amphibian of the Kihansi gorge

Surveillance of B. dendrobatidis in the Kihansi gorge has detected in B. dendrobatidis in amphibian species including Arthroleptides yakusini, Ptychadena anchietae and Kihansi spray toads Nectophrynoides asperginis (Weldon, 2004; Weldon and Mtui, 2006).

A. yakusini is thought to serve as a carrier host of B. dendrobatidis because the species has persisted in the Kihansi gorge despite the presence of the chytrid and infected individuals do not show any obvious adverse effects (Weldon, 2004; Weldon and Mtui, 2006).

1.2.11 Chytridiomycosis in N. Asperginis

N. Asperginis population in the Kihansi spray wetlands collapsed in the year 2003. During the mass mortality outbreak in 2003, dead *N. asperginis* were collected in upper spray wetlands and Mhalala in the Kihansi gorge and were confirmed with chytridiomycosis. Although pesticides and habitat change may contribute to *N. Asperginis* extinction in wild, chytridiomycosis seems to be the most probable cause of *N. Asperginis* extinction. *N. asperginis* seems to be highly susceptible to Chytridiomycosis which has been identified as the causal agent of amphibian population decline and extinctions (Berger *et al.*, 2007).An outbreak of chytridiomycosis in *N. Aspergins* in captivity at Bronx zoo killed the entire 49 individual in the affected cages, indicating the high susceptibility of this spicies to chytridiomycosis.

1.3 Giraffe Skin Disease

1.3.1 Giraffe

The giraffe (*Giraffa camelopardalis*) is an African even-toed ungulate mammal, the tallest living terrestrial animal and the largest ruminant. Its species name refers to its camel-like appearance and the patches of color on its far. Its chief distinguishing characteristics are its extremely long neck and legs, its horn like ossicones and its distinctive coat patterns. It stands 5-6 m (16-20 ft) tall and has average weight of 1600 kg (3500 lb) for males and 830 kg (1830 lb) for females. It approximately 4.3 meters to 5.2 meters tall, although the tallest male recorded stood almost 6 meters (Wilson and Reeder

2005). It is classified under the family *Giraffidae,* along with its closest extant relative, the okapi. The nine sub species are distinguished by their coat patterns. Giraffes usually inhabit savannas, grasslands, and open woodlands. They are not present in deserts, dense forest and mountains (Fennessy *et al.*, 2001). Their primary food source is acacia leaves which they browse at heights most other herbivores cannot reach.

Giraffes are preyed by lions, and adult giraffes do not have strong social bonds, though they do gather in loose aggregations if they happen to be moving in the same general direction. Males establish social hierarchies through 'necking' which are combat bouts where the neck is used as a weapon. Dominant males gain mating access to females, which bear the sole responsibility for raising the young. The giraffe is a protected species in most of its range. Although it has been evaluated by IUCN at the least concern species, it does not qualify as threatened, near threatened or conservation dependant (Fennessy and Brown 2008).

1.3.2 Diseases of giraffes

The giraffe is susceptible to infections and non infectious diseases that are seen in domestic and common in relative the okapi (Raphael *et al.*, 1986). It also susceptible to viral diseases among them is rinderpest, malignant catarrhal fever, cutaneous viral papilomas and lump skin disease (Jolly *et al.*, 2003). Bacterial such as such as *Pseudomonas species*, *Corynebacterium haemalyticus* and *Staphylococcus aureus* has been incriminated in causing disease to giraffe (Jensen *et al.*, 1999). Cutaneous skin disease that is grossly characterized by proliferative and crusty lesions observed in giraffes in the National Park. This disease suggested to be called Giraffes Skin Disease (GSD) based on the description of lesions observed in affected giraffe (Muse *et al.*, 2012).

1.3.3 Giraffe skin disease

1.3.3.1 Epidemiology and prevalence of giraffe skin disease

In 2009, a cross sectional road transect survey was used to determine the prevalence and spatial distribution of diseased giraffe in Ruaha National Park. Disease status, age, sex, herd size, vegetation type and presence of ox-peckers were recorded for each systematically selected animal. Overall prevalence of GSD was 80%, with 109 giraffes from 36 herds assessed. Adult animal were more commonly affected (84.6%) compared to sub-adult and young. Most lesions observed involved the caudal aspects of the carpal joint. Nearly all (99%) of the skin lesions on affected giraffe appeared chronic in nature and more than half (51.7%) of animals were classified as severely affected. Approximately 10% of affected giraffe had gait abnormalities (lameness, stiff gait, slow gait). Although giraffes were observed in close association with other species (impala, zebra, elephant, warthogs and dikdik), no other species exhibited skin lesions. Ox-pecker birds which have been associated with spread of other infections, were observed on 20.3% of the giraffes examined (Mlengeya, 2002).

1.3.3.2 Proposed aetiology of giraffe skin disease

Preliminary investigation of affected giraffe suggested the condition to be caused by bacteria known as *Dermatophillus congolensis* (Mlengeya and Lyaruu, 2007). Infection in wild animals was first described in America in a woodchuck (*Marmota monax*) and in a striped skunk (*Mephitis mephitis*). This bacterium was also recovered from a raccoon (*Procyon lotor*) carcass that had been frozen for a year.

The most important epidemiological factor in dermatophilosis infection is presence of predisposing factors causing skin damage e.g. prolonged wetting during rainy season thorny bushes and other objects likely to cause trauma which make transmission of *D.*

congolensis from carriers to susceptible animals a likely consequence. Other factors that can cause skin damage include biting flies and ticks, especially *Amblyomma variegatum* (Msami *et al.*, 2001).

1.3.3.3 Transmission of giraffe skin disease

The mechanism of transmission of skin disease in giraffe population is unknown. It is speculated that the disease might be transmitted by contact with other affected animals, spores in grass, oxpeckers, biting fliesor other insects. During dry season animals have been to congregate together at the remaining waterholes (Epaphras *et al.*, 2008), the behaviour which is likely to increase chances of contact among animals and may thereby increase disease transmission if a direct transmission route is involved. The presence of giraffes browsing vegetation in the habitat influences giraffe distribution in the park. High numbers of animals in one area may increase the rate of disease transmission in animals.

1.3.3.4 Clinical signs of giraffe skin disease

In giraffes, GSD is characterized by skin lesions localized at the flexor side of carpal and/or hock joints of one or both forelimbs and hind limbs. In same occasion, GSD has been seen on the lateral and medial aspects of the carpal joints as well as the sternum. Three giraffe in Tarangire National Park has lesions extending to as far as the lateral aspects of the abdomen, thorax, hind quarters, perineal area and all sides, (lateral, medial, cranial and caudal) of the hock joints, in the hind limbs. The lesions start as small skin nodules of about 2-3 cm in diameter with raised hair, that later coalesce to form a large round or oval patch up to 10-16 cm in diameter. Hardening of the skin, drying, and scaling then follow (especially during the dry season), and the skin finally cracks resulting in raw fissures (Mlengeya and Lyaruu, 2005; Muse, 2009). The affected area initially appears raised, collapses and looks like a flap of skin protruding on either side of

the hock/carpal joint. The dorsal surface of the hock and medial aspects of the elbow joints have been evolved in some cases and more advanced stages, skin lesions have been observed on the medial aspects of upper forelimbs, the brisket and neck (Mlengeya and Lyaruu, 2005).

Also affected skin appears itchy making giraffe frequently scratch by rubbing against branches of smaller bushes and trees. Some affected giraffe are reluctant to use their legs; they stand at one place for a long period and when disturbed they use their legs with great care (lameness) possibly making them more vulnerable to predation or poaching.

1.3.4 Fungi isolation and identification of fungal species

1.3.4.1 Isolation and cryopreservation of fungi

Fungi are isolated using Sabouraud dextrose agar (SDA) which contains agar, glucose and mycological peptone. SDA is an acidic pH medium (pH 5.6 ± 0.2 at 25°C) for the isolation of dermartophytes, other fungi and yeast (Jarett *et al.*, 1980). Fungal colonies are sub cultured in order to obtain pure cultures. The low pH favours fungal growth and inhibits contaminating bacteria from clinical specimens (Murray *et al.*, 2003). The acid reaction of the final medium is inhibitory to a large number of bacteria making it particularly useful for cultivating fungi and aciduric microorganisms.

1.3.4.2 Identification of fungi

Fungal can be identified by three different methods such as macro morphological, micro morphology and molecular methods.

(a) Macromorphology of fungal species

Fungi macro morphology exist in two fundamental forms; the filamentous (hypha) and single celled budding forms (yeast). A mass of hypha is known as mycelium. Fungal

mycelia can be observed on various surfaces and substrates, where they are commonly called molds. It is the hypha that is responsible for the filamentous nature of mould. Yeasts are unicellular spherical to ellipsoid cells. They reproduce by budding, which result in blastospore (blastoconidia) formation. In some cases, as the cells buds fail to detach and elongate thus forming a chain of elongated hyphae like filament called pseudohyphae. Yeast colonies generally look similar to bacterial colonies. Mycelia grown on solid agar media in laboratory petri dishes are usually referred to as colonies. These colonies can exhibit growth shapes and colors (due to spores or pigmentation) that can be used as diagnostic features in the identification of species or groups.

(b) Micromorphology of fungal species

Most fungi grow as hyphae at their tips by a process called branching. The combination of apical growth and branching leads to the development of a mycelium, an interconnected network of hyphae. Hyphae can be either lack cross walls (coenocytic) or have cross walls (septate) depending on the species. In some cases septate hyphae develop clamp connections at the septa which connect the hyphal elements (Harris, 2008). Aerial hyphae often produce asexual reproduction propagules termed conidia (synonymous with spores). This morphology can be identified by standard morphology using the light microscopy where by fungi colonies are initially stained by using lactophenol cotton blue (Guarro *et al.*, 1999).

(c) Molecular characterization of fungal species.

Characterization of the fungi is done by intergenic spacers within the ribosomal DNA. The nuclear-encoded ribosomal RNA genes (rRNA) of fungi exist as a multiple-copy gene family comprised of highly similar DNA sequences (typically from 8-12 kb each) arranged in a head-to-toe manner. Each repeat unit has coding regions for one major

transcript (containing the primary rRNAs for a single ribosome), punctuated by one or more intergenic spacer (IGS) regions.

The internal transcribed spacer regions within the ribosomal DNA were used as molecular target to detect and identify the different fungal species. Although the ITS region is not translated into proteins, the regions has a critical role in the development of functional rRNA. Variation in this region makes it useful as a signature region for molecular assays for the characterization and identification of fungi.

Polymerase chain reaction (PCR) are reliable methods of diagnosis by amplify 5.8 ribosomal DNA and its flanking intergenic spacers, ITS-1 and ITS-2. The amplification done by a PCR of the 5.8 ribosomal DNA by using specific primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Balajee et al., 2007; Iwen et al., 2002). In addition to the standard ITS1+ITS4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences primers.

1.3.5 Antifungal sensitivity testing

One of the selections treatments for fungal infections is by using antifungal susceptibility testing. Susceptibility testing of fungi used different methods including disc diffusion, agar dilution and broth dilution procedures. Numerous *in vitro* factors such as media, buffer, inoculums, incubation and end point criteria can affect results significantly.

Antifungal agents commonly used are like Polyene group such as Amphotericin B acts by binding to ergosterol in the cell membrane causing loss of membrane integrity and results in osmotic instability. Amphotericin has fungicidal activity and minimum inhibitory

concentration (MIC) end points are read at complete inhibition of growth (Odds *et al.*, 2004; Espinel-Ingroff A, 2003). Another group of antifungal is Azoles such as ketoconazole, fluconazole, itraconazole and Clotrimazole which have a broad spectrum of activity against yeasts and dimorphic fungi. Azoles inhibit fungal cytochrome P450 dependant enzymes, impairing ergosterol synthesis thus depleting ergosterol in the cell membrane. Activity is fungistatic, partial inhibition of fungal growth occurs over a wide concentration range.

2.0 Conclusion and Recommendations

2.1 Conclusion

Clinical signs, morphological lesions and PCR results confirm that that cutenious skin disease is the one which caused to mortalities of animals in the wildlife especially in amphibians. For amphibian of Kihansi gorge, molecular diagnosis investigates the presence of *B. dendrobatidis* as a possible cause of mortalities in the KST, *N. asperginis,* at the Kihansi captive breeding facility in November 2012. It is possible that *B. dendrobatidis* may have been introduced into the captive toad population from wild amphibians in the vicinity of the facility that harbour the pathogen. This study also determine the aetiology of GSD on Ruaha's giraffes population through morphological lesion observed in giraffes

2.2 Recommendations

It is recommended that strict biosecurity measures be adhered to by personnel at the facility in order to avoid further introductions of amphibian diseases to the captive KST population. Similarly, personnel monitoring the reintroduced KST in the Kihansi spray wetlands have to observe strict biosecurity measures in order to avoid introducing *B. dendrobatidis* to this only remaining wild KST population. Further studies on the

epidemiology of *B. dendrobatidis* in the Udzungwa mountains are recommended in order to understand the origin, prevalence and molecular characteristics in wild amphibian populations. This will be important for conservation of several endemic amphibian species in the Udzungwa Mountains in Tanzania, which are part of the Eastern Arc Mountains, a global biodiversity hotspot. Given that GSD is now present and highly prevalent throughout Ruaha National Park, surveys in other protected areas is required. This is essential to determine the geographic distribution of GSD and to identify GSD free giraffes populations that could serve as refugia and comparison populations for population studies.

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NOTE

Batrachochytrium dendrobatidis detected in Kihansi spray toads at a captive breeding facility (Kihansi, Tanzania)

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ABSTRACT: The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is the aetiological agent of amphibian chytridiomycosis, a disease associated with global amphibian population declines. In November 2012, mass mortalities of Kihansi spray toads *Nectophrynoides asperginis* were observed at the Kihansi captive breeding facility, located in the Udzungwa Mountains, Tanzania. Mortalities increased rapidly, and dead toads showed typical clinical signs of chytridiomycosis, including reddening of the skin that was especially evident on the toe pads. Treatment of toads with itraconazole rapidly reduced mortalities. Dead toads (n = 49) were collected and used to perform *Bd*-specific polymerase chain reaction and subsequent nucleotide sequencing. All toads collected at the facility were positive for *Bd*. The obtained *Bd* 5.8S rRNA gene and flanking internal transcribed spacer regions (ITS1 and ITS2) were not 100% identical to any other *Bd* sequences in GenBank, but closely resembled isolates from Ecuador, Japan, USA, Brazil, Korea, and South Africa. To our knowledge, this is the first study reporting molecular characteristics of *Bd* isolated from the Udzungwa Mountains. Strict biosecurity measures at the breeding facility and in Kihansi spray wetlands where toads have been reintroduced have been implemented. Further studies on *Bd* epidemiology in the Udzungwa Mountains are recommended in order to understand its origin, prevalence, and molecular characteristics in wild amphibian populations. This will be important for conservation of several endemic amphibian species in the Udzungwa Mountains, which are part of the Eastern Arc Mountains, a global biodiversity hotspot.

KEY WORDS: Chytridiomycosis · *Bd* · Itraconazole · *Nectophrynoides asperginis* · 5.8S rRNA · Internal transcribed spacer region

INTRODUCTION

The Kihansi spray toad (KST) *Nectophrynoides asperginis* is a small-sized (snout–vent length: 10–18 mm; body mass: 0.45–0.66 g) ovoviviparous bufonid that was first recorded in the Kihansi spray wetlands in 1996 (Poynton et al. 1998, Lee et al. 2006). The spray wetlands in the Kihansi gorge are generated by falls of the Kihansi River along the steep edge of the eastern escarpment of the southern Udzungwa Mountains in Tanzania (Channing et al. 2006). At the time of species description and immediately afterwards, the KST was classified as Critically Endangered due to its restricted geographical range and the

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decreased water flow caused by the commissioning of the Lower Kihansi Hydropower Project (Poynton et al. 1998, Channing et al. 2006). The loss and modification of habitat and associated toad population fluctuations led to the translocation of 499 toads from the Kihansi spray wetlands to zoos in the USA in 2000 (Krajick 2006, Lee et al. 2006). Mass mortalities of toads were observed in 2003, leading to a crash in the wild toad population by early 2004, with the last sighting of toads recorded in 2005 (Weldon & du Preez 2004, Krajick 2006). Currently, the KST is considered Extinct in the Wild, but the species survives in captivity at the Wildlife Conservation Society's Bronx Zoo and the Toledo Zoological Society, and at Kihansi and the University of Dar es Salaam captive breeding facilities in Tanzania (Krajick 2006, Channing et al. 2009). Attempts to re-introduce the KST to its natural habitat in the Kihansi spray wetlands have been underway since 2012 (www.iucn.org).

The amphibian disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) together with stress due to drought caused by the failure of the artificial sprinkler system that irrigates the wetlands, predation by safari ants (*Dorylus* sp.), and/or pesticides in flushed dam sediments have contributed to the KST population extinction in the wild (Weldon & du Preez 2004, Channing et al. 2006, Krajick 2006). Some of the KST specimens recovered from the 2003 mass mortalities in the Kihansi spray wetlands were diagnosed with chytridiomycosis (Weldon & du Preez 2004), and chytridiomycosis in KST has been recorded in captivity at the Bronx Zoo (McAloose et al. 2008).

Chytridiomycosis caused by *Bd* is the single most important infectious amphibian disease that has commonly been associated with significant amphibian mortalities (Daszak et al. 1999, Longcore et al. 1999, Lips et al. 2006). Clinical signs of amphibian chytridiomycosis include abnormal posture, lethargy, and loss of the righting reflex, while gross morphological lesions consist of abnormal epidermal sloughing, cutaneous hemorrhages, and hyperemia of digital and ventral skin (Daszak et al. 1999). *Bd* disrupts cutaneous osmoregulatory function, leading to electrolyte imbalance, asystolic cardiac arrest, and death (Voyles et al. 2007, 2009). In addition, *Bd* produces toxic factors that inhibit protective host immune responses (Fites et al. 2013) and cause slower rehydration across the skin (Carver et al. 2010). In captivity, chytridiomycosis in amphibians can be treated with either chloramphenicol or itraconazole, the latter being the drug of choice (Forzán et al. 2008, Bishop et al. 2009, Young et al. 2012, Baitchman & Pessier 2013). A diagnostic polymerase chain reaction (PCR) assay for sensitive and specific detection of *Bd* has been developed using primers that amplify a region of the 5.8S ribosomal RNA gene and the flanking internal transcribed spacer regions (ITS1 and ITS2; Annis et al. 2004). Sequencing of this fragment of the 5.8S rRNA gene has been previously used to infer phylogeny of *Bd* (Gaertner et al. 2009, Goka et al. 2009, Schloegel et al. 2012, Bataille et al. 2013).

In November 2012, 351 toads died at the Kihansi captive breeding facility. Dead toads showed typical signs of chytridiomycosis, including epidermal sloughing and hyperemia of digital and ventral skin. The aim of this study was to perform confirmatory diagnosis of chytridiomycosis as the cause of death using PCR and subsequent 5.8S rRNA gene sequencing.

MATERIALS AND METHODS

Study site, sample collection, and storage

This study was conducted at the KST captive breeding facility located in Kihansi, Mlimba, Tanzania, following mass mortality of toads in November 2012. Seven dead toads were collected from each of the 7 affected cages, resulting in a total of 49 samples. Toads from each of the cages were pooled into sterile 50 ml Falcon tubes and placed on ice for transport to the laboratory within 24 h. Afterwards, 2 ml of sterile phosphate-buffered saline (PBS) filtered through a 0.22 µm Minisart syringe filter (Sartorius Stedim Biotech) were added into each tube with the toads. Each tube with the toads was inverted several times before PBS was pipetted out and collected into an Eppendorf tube. Washings from each of the tubes were pelleted by centrifugation at 13 000 × *g*, and the pellet was resuspended in 200 µl PBS and stored at −80°C. A total of 38 *Phrynobatrachus mababiensis* frogs collected in the vicinity of the facility were swabbed in order to screen for *Bd*. Before DNA extraction, 500 µl PBS were added into the swabs followed by vortexing. Afterwards, swab suspensions were pelleted by centrifugation at 13 000 × *g*, and the pellet was resuspended in 200 µl PBS and stored at −80°C.

DNA extraction, PCR amplification, and sequencing

Toad washings and frog swab suspensions were thawed and 150 µl of thawed washings and suspensions were incubated with 20 µl of 20 mg ml^{-1} Pro-

teinase K (Macherey-Nagel) at 55°C for 1 h. DNA was then extracted from Proteinase K-digested washings and suspensions using QiaAmp nucleic extraction kits (Qiagen), following the manufacturer's instructions. During nucleic acid extraction and PCR, a culture (MCT08) of *Bd* from South Africa and PBS were used as positive and negative controls, respectively. PCR detection of *Bd* was performed using primers Bd1a and Bd2a that amplify the 5.8S rRNA gene and flanking ITS1 and ITS2, as previously described by Annis et al. (2004), using AccuPower PCR premix (Bioneer). The expected size of the PCR product using primers Bd1a and Bd2a is approximately 300 bp (Annis et al. 2004). PCR amplification was performed by an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min using GeneAmp PCR systems 9700 (Applied Biosystems). Afterwards, PCR products were electrophoresed in a 2% agarose gel mixed with GelRed nucleic acid stain (Phenix Research Products) before visualization and imaging using a BioDoc-It imaging system (UVP). In order to verify the retrieval of fragments representing *Bd*, PCR fragments were purified from agarose gels using a NucleoSpin gel and PCR clean-up kit (Macherey-Nagel) and subjected to dideoxynucleotide cycle sequencing by using Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems). Products from the dideoxynucleotide cycle sequencing reaction were purified by ethanol precipitation and separated on a 3500 Genetic Analyzer (Applied Biosystems).

Nucleotide sequence similarity search

The nucleotide sequence of the 5.8S rRNA *Bd* gene and partial flanking ITS1 and ITS2 regions obtained from the toads was submitted to GenBank, and compared with other sequences using BLASTn (Altschul et al. 1990). BLASTn compares nucleotide sequences to sequence databases and calculates the statistical significance of matches and can be used to infer functional and evolutionary relationships between sequences.

Treatment of chytridiomycosis

In an attempt to reduce mortalities, and due to the unavailability of itraconazole at the start of the outbreak, all toads at the facility were initially treated with a 0.002% chloramphenicol bath beginning on Day 18 after the start of the outbreak. Chloramphenicol was only used as a temporary measure while waiting for an itraconazole supply. On Day 23, chloramphenicol was replaced by a 10 min daily bath in amphibian Ringer's solution containing 0.1 mg ml^{-1} (0.01%) itraconazole (Sporanox, Janssen Pharmaceutica) for 11 d.

RESULTS

KST mortality

The Kihansi breeding facility housed 562 toads in 17 cages prior to the mortalities observed in late 2012. The highest mortalities were observed between 18 and 26 d after initial toad deaths in early November, and declined noticeably within 3 d of the itraconazole treatment (Fig. 1). Toad mortalities continued to increase throughout the treatment with chloramphenicol that lasted for 5 d (Fig. 1). Mortalities were observed in all 17 cages at the facility. By late December, when mortalities completely ceased, the captive breeding population had been reduced to 211 toads, with most survivors being juveniles and sub-adults.

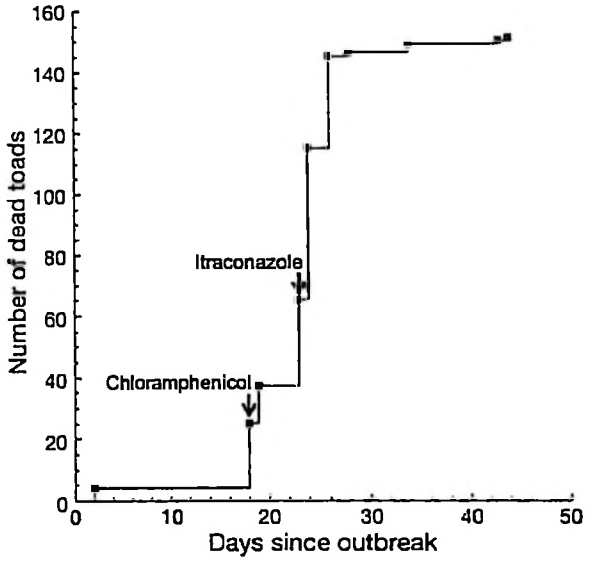

Fig. 1. Kihansi spray toad *Nectophrynoides asperginis* mortality at the Kihansi captive breeding facility. The cumulative number of dead toads is plotted against the days since the start of the chytridiomycosis outbreak. Mortalities of toads at the facility increased sharply with peak mortalities around Day 24 of the outbreak, followed by a sharp decrease 3 d after administration of itraconazole

Gross morphological lesions in dead toads

Gross lesions in dead toads consisted of variable hyperemia (reddening) especially evident on the toe pads and the inguinal area, sloughing of the superficial epidermis of the feet, and abdominal skin discolouration (Fig. 2). The severity of the gross morphological lesions varied between individual toads.

Diagnosis of *Bd* using PCR

Agarose gel electrophoresis of PCR products revealed that all washings from 7 dead toads sampled were positive for *Bd*, producing a 300 bp PCR product as observed in the positive control. No PCR product was observed in the negative controls. In addition, PCR screening of wild *Phrynobatrachus mababiensis* from areas surrounding the facility revealed 9 out of 38 (24%) frogs with *Bd*.

Similarity of KST *Bd* 5.8S rRNA sequence to GenBank sequences

All 7 sequences of 5.8S rRNA and partial flanking ITS1 and ITS2 nucleotides of *Bd* from the toads were identical. The obtained nucleotide sequence was submitted to GenBank under accession number KF702314. BLASTn of KF702314 showed high identity with a number of published *Bd* sequences, although it was not 100% identical to any of the *Bd* sequences available in GenBank (Table 1). KF702314 showed the highest similarity to *Bd* from Ecuador, Japan, and the USA (Table 1).

DISCUSSION

We investigated the presence of *Bd* as a possible cause of mortalities in KST at the Kihansi captive breeding facility in November 2012. Morphological lesions and PCR results confirmed that chytridiomycosis caused the observed mortalities. Preliminary analysis showed that the *Bd* 5.8S rRNA gene and partial flanking ITS1 and ITS2 nucleotide sequences obtained in this study were not 100% identical to any other *Bd* nucleotide sequences available in GenBank, although they were very similar to isolates from Ecuador, Japan, USA, Brazil, Korea, and South Africa (James et al. 2006, Goka et al. 2009, McCracken et al. 2009, Schloegel et al. 2012, Bataille et al. 2013). Possible introduction of *Bd* into the Udzungwa Mountains arising from stocking of rainbow trout *Oncorhynchus mykiss* has been speculated, although further fieldwork and re-sampling is necessary before conclusions can be reached about the nature and origin of *Bd* in this area (Moyer & Weldon 2006).

Prior to treatment, mortalities of KST increased rapidly, indicating susceptibility and vulnerability to chytridiomycosis. Mortality of all KST following a chytridiomycosis outbreak has previously been observed at the Bronx Zoo (McAloose et al. 2008). This susceptibility, and the rapid deaths observed at the Kihansi facility, support the sharp population declines of wild toads observed in 2003 and their proposed chytridiomycosis-mediated extinction (Weldon & du Preez 2004, Channing et al. 2006). Chloramphenicol and itraconazole have been successfully used in treating amphibian chytridiomycosis (Forzán et al. 2008, Tamukai et al. 2011, Young et al. 2012, Baitch-

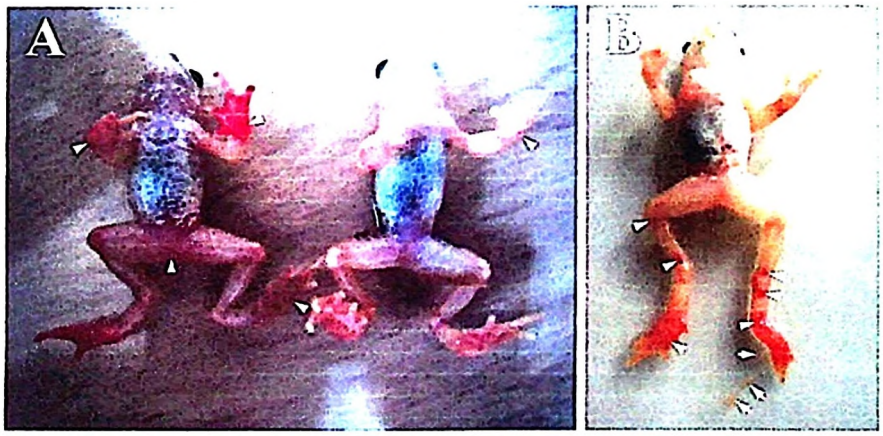

Fig. 2. Gross morphological lesions of chytridiomycosis in dead Kihansi spray toads *Nectophrynoides asperginis*. (A) Dead toads with variable hyperemia (reddening, indicated by arrowheads) that is especially evident on the toe pads and the inguinal area. The toad on the left shows greater hyperemia than the toad on the right. (B) A toad with hyperemia of the skin on the hind feet (arrowheads) and sloughing of the superficial epidermis of its left hind foot (arrows)

Table1. Closest matches in GenBank (as determined by a BLASTn search) to the nucleotide sequence of the *Batrachochytrium dendrobatidis* (*Bd*) 5.8S rRNA gene and partial flanking ITS1 and ITS2 regions (filed under accession number KF702314) from Kihansi spray toads *Nectophrynoides asperginis*

Bd isolate/strain	Geographic origin	Year of isolation	Nucleotide identity (%) ITS1-5.8S rRNA-ITS2	Nucleotide identity (%) 5.8S rRNA	Accession no.	Reference
MF20242	Ecuador	2004–2006	250/251 (99)	150/150 (100)	FJ232006	McCracken et al. (2009)
Bd-21	Japan	2008	250/251 (99)	150/150 (100)	AB435231	Goka et al. (2009)
Bd-30	Japan	2010	249/251 (99)	150/150 (100)	AB723966	Goka et al. unpubl.
Bd-17	Japan	2007	249/251 (99)	150/150 (100)	AB435227	Goka et al. (2009)
AD27_Bd_JG77_78	USA	2008	248/251 (99)	150/150 (100)	FJ373881	Gaertner et al. unpubl.
JEL648 clone L	Brazil	2010	281/288 (98)	150/150 (100)	JQ582893	Schloegel et al. (2012)
JEL648 clone B	Brazil	2010	281/288 (98)	150/150 (100)	JQ582889	Schloegel et al. (2012)
CW34 clone D	South Africa	2005	279/286 (98)	150/150 (100)	JQ582906	Schloegel et al. (2012)
Bd-20	Japan	2008	248/252 (98)	150/150 (100)	AB435230	Goka et al. (2009)
KR11	Korea	2010–2011	246/252 (98)	149/150 (99)	JX983051	Bataille et al. (2013)
CW34 clone F	South Africa	2005	285/293 (97)	150/150 (100)	JQ582927	Schloegel et al. (2012)
CW34 clone L	South Africa	2005	285/293 (97)	150/150 (100)	JQ582916	Schloegel et al. (2012)
CW34 clone T	South Africa	2005	284/293 (97)	150/150 (100)	JQ582937	Schloegel et al. (2012)
CW34 clone P	South Africa	2005	284/293 (97)	150/150 (100)	JQ582915	Schloegel et al. (2012)
CW34 clone G	South Africa	2005	284/293 (97)	150/150 (100)	JQ582904	Schloegel et al. (2012)
CW34 clone J	South Africa	2005	284/293 (97)	150/150 (100)	JQ582903	Schloegel et al. (2012)
JEL648 clone F	Brazil	2010	280/288 (97)	149/150 (99)	JQ582892	Schloegel et al. (2012)
JEL648 clone E	Brazil	2010	280/288 (97)	150/150 (100)	JQ582891	Schloegel et al. (2012)
JEL648 clone N	Brazil	2010	280/288 (97)	149/150 (99)	JQ582890	Schloegel et al. (2012)
JEL197 (AFTOL-ID 21)	USA	1997	284/293 (97)	150/150 (100)	AY997031	James et al. (2006)

man & Pessier 2013). In our study, toads were initially treated using chloramphenicol before itraconazole was made available. Chloramphenicol was administered for 5 d, slowing the rate of deaths. It is possible that chloramphenicol would have been effective if the drug had been administered for a longer duration. Chloramphenicol has been shown to be effective against chytridiomycosis when administered for at least 2 wk (Young et al. 2012, Baitchman & Pessier 2013). However, treatment of KST with itraconazole rapidly reduced mortalities, supporting previous observations of the high efficacy of this drug against chytridiomycosis in amphibians (Forzán et al. 2008, Tamukai et al. 2011, Baitchman & Pessier 2013). However, peak mortalities of KST were observed between 1 and 72 h following treatment. The concentration of itraconazole used in this study is commonly administered (Baitchman & Pessier 2013) but was 4 times higher than the concentrations recommended by Brannelly et al. (2012), and this may have contributed to drug-induced mortalities.

Previous surveillance of *Bd* in the Udzungwa Mountains found widespread distribution of *Bd* in amphibian populations (Moyer & Weldon 2006). In the present study, *Bd* was found in 24% of amphibians in the vicinity of the facility using testing with *Bd*-specific PCR. Therefore, *Bd* is present in the vicinity of the facility, and local infected amphibians may be a possible source of the chytridiomycosis outbreak at the facility. Sequencing of *Bd* from amphibians in the vicinity of the facility is required in order to affirm this possibility. Biosecurity measures at the facility have been implemented to prevent accidental introduction of disease, including restricted entry, changing of footwear, soaking of footwear in a hypochlorite foot bath, and wearing protective clothing and gloves while handling toads. It is recommended that strict biosecurity measures be adhered to by personnel at the facility in order to avoid further introductions of amphibian diseases to the captive KST population. Similarly, personnel monitoring the reintroduced KST in the Kihansi spray wetlands have to observe strict biosecurity measures in order to avoid introducing *Bd* to this only remaining wild KST population.

To our knowledge, this is the first study reporting the molecular characteristics of *Bd* isolated from captive KST at Kihansi within the Udzungwa Mountains of Tanzania. Further studies on the epidemiology of *Bd* in the Udzungwa Mountains are recommended in order to understand its origin, prevalence, and molecular characteristics in wild amphibian populations. This will be important for conservation of several endemic amphibian species in the Udzungwa Mountains, which are part of the Eastern Arc Mountains, a global biodiversity hotspot.

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Isolation, Identification and Antifungal Susceptibility of Fungi Associated with Giraffe Skin Disease in Giraffes of Ruaha National Park, Tanzania

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Abstract

Giraffe skin disease (GSD) affects giraffes causing hair loss, raising, wrinkling, cracking and encrustation of skin mainly on flexor side of carpal joints and medial side of scapula. GSD was first seen in Ruaha National Park in 2000 and has now spread to Tarangire and Lake Manyara national parks in Tanzania. In present study, fungi associated with GSD were isolated from giraffes with or without GSD, identified and tested for their antifungal susceptibilities. The following fungi species including *Aspergillus fruticulosus, Aspergillus multicolor, Aspergillus niger, Aspergillus sydowii, Aureobasidium pullulans, Botryosphaeria sp., Cochliobolus lunatus, Cylindrocladium canadense, Dothideomycete sp., Epicoccum sorghinum, Fennellia nivea, Fusarium equiseti, Fusarium sp., Leptosphaerulina chartarum, Montagnulaceae sp., Penicillium citrinum, Penicillium commune, Penicillium simplicissimum, Pestalotiopsis clavispora, Phoma sp.* and *Setosphaeria rostrata* were identified to be associated with GSD lesions in giraffes. However, *Aureobasidium pullulans* and *Epicoccum sorghinum* were also isolated from healthy giraffes. Most of the fungi associated with GSD have been described in opportunistic infections, naturally occur in the environment or are etiological agents of plant diseases. When susceptibility of fungi against antifungals, including amphotericin B, clotrimazole, fluconazole, itraconazole, ketoconazole and nystatin was investigated, no fungi was resistant to all tested antifungals nor a single antifungal was able to inhibit the growth of all fungi. This finding suggests that a combined antifungal treatment can be used in treating of GSD. The isolated fungi are postulated to be secondary etiological agents that contribute towards the development of clinical GSD, and not necessarily the primary cause of GSD.

Introduction

Giraffe skin disease (GSD) is a cutaneous infection of giraffes (*Giraffa camelopardalis*). GSD cutaneous lesions start as small skin nodules of about 2 to 3 cm in diameter with raised hair, which later coalesce to make a large round or an oval patch of 10 to 16 cm in diameter. Afterwards, the affected skin becomes raised, discolored, thickened, hardened, wrinkled and alopecic. Finally, the skin sloughs off and cracks resulting in raw fissures that may ooze blood [1], [2]. The lesions are typically located on flexor side of carpal joints and occasionally on medial side of the scapula area. In more advanced stages, skin lesions may be observed on the medial aspect of upper forelimbs, the brisket and even the neck. GSD was first observed in 2000 in giraffes of Ruaha National Park in the northern eastern section (Lunda) and has now spread to Tarangire and Lake Manyara national parks [1]. In Ruaha National Park with a population of approximately 2000 giraffes, the prevalence of GSD is estimated to be 80% [2]. It has been reported that the prevalence of the GSD is higher during the rainy season and subsides during dry months. Although giraffes often occur in close association with other animals such as zebra and impala, no GSD have been observed in other wildlife species suggesting that the disease affects only giraffes [3]. Affected giraffes stand at the same place for long time due to reluctancy to use their legs, and when disturbed, they use their legs with great care. GSD may impact giraffe populations indirectly through increased losses from predation on lame giraffe or increased vulnerability to other environmental stressors like drought and fire [2].

The range of giraffe once covered most of Africa, but is presently patchy as a result of the rinderpest pandemic and anthropogenic activities such as poaching, increased human settlement and expansion of agriculture. The remaining isolated populations

64 are more vulnerable to stochastic factors such as diseases, including GSD, which
65 negatively impact giraffe populations and adversely affect economic revenues
66 derived from tourism in Tanzania. Understanding the etiology of GSD is important in
67 the development of effective control measures. The purpose of the present study was
68 to isolate and identify fungi present in skin scrapings collected from giraffes with or
69 without GSD in Ruaha National Park. Fungi were isolated by cultivation in
70 microbiological media and identified using morphological and molecular techniques.
71 The identification of fungi is done by observation of differentiating morphological
72 structures and/or by DNA amplification and nucleotide sequence analysis of 5.8S
73 rRNA and flanking internal transcribed spacers (ITS1 and ITS2) [4]–[6]. Afterwards,
74 the antifungal susceptibility of isolated fungi was determined. Information obtained
75 from the present study will assist in understanding the etiology of GSD and provide
76 recommendations on the appropriate control of GSD in affected giraffes.

77 **Materials and Methods**

78 **Study area, sample collection and storage**

79 This study was conducted at Ruaha National Park located in central Tanzania
80 between Latitudes 7° 30' and 8° 00' S and Longitudes 33° 50' and 35° 25' E about
81 130 kilometers west of Iringa. Ruaha National Park is the largest national park in
82 Tanzania. Skin scrapings were collected from affected skin of giraffes with GSD and
83 from unaffected skin of healthy giraffes after immobilization of giraffes. Giraffes
84 were immobilized using etorphine hydrochloride (M99®, Novartis, Johannesburg,
85 South Africa) at a dose ranging between 0.0225 to 0.025 mg/kg. Immobilization was
86 conducted in the morning and in evenings when temperatures were lower to avoid
87 hyperthermia, exhaustion and/or shock. Giraffes were darted and once signs of
88 immobilization were exhibited, giraffes were roped to lower them to the ground. The

89 handling time of giraffes was limited to 15 minutes to avoid complications from
90 prolonged recumbence. Skin scrapings from individual giraffes were collected into
91 separate containers without any preservative. Fourteen samples from giraffes with
92 GSD and two samples from giraffes without GSD were collected. After sampling,
93 anesthesia was reversed with intravenous injection of diprenorphine hydrochloride
94 (M5050®, Novartis, Johannesburg, South Africa) at a dose ranging between 0.0675
95 to 0.075 mg/kg. The containers containing the skin scrapings were transported to the
96 laboratory and stored at room temperature before performing *in vitro* cultivation of
97 fungi.
98
99 **Fungi isolation and subcultivation**
100 Fungi were isolated by inoculation of skin scrapings into Sabouraud dextrose agar
101 (SDA). SDA was prepared by adding 65 g of SDA powder (Oxoid, Berkshire, UK)
102 to 1 litre of distilled water followed by boiling to dissolve the powder completely.
103 Afterwards, boiled SDA was sterilized by autoclaving at 121 °C for 15 minutes.
104 Molted SDA was cooled and molten agar were mixed with skin scrapings in 50 ml
105 tubes (BD Falcon, San Jose, CA) and poured into 150 mm diameter sterile cell
106 culture dishes (Corning Incorporated, Corning, NY). Skin scrapings were incubated
107 at room temperature for one week before primary fungi colonies were subcultured in
108 SDA using 9 mm diameter Petri dishes (MLS, Menen, Belgium). Fungi colonies
109 were passaged four times in order to obtain pure cultures of the different fungi
110 isolated from GSD skin scrapings.

Fungi identification

Fungi identification based on morphology

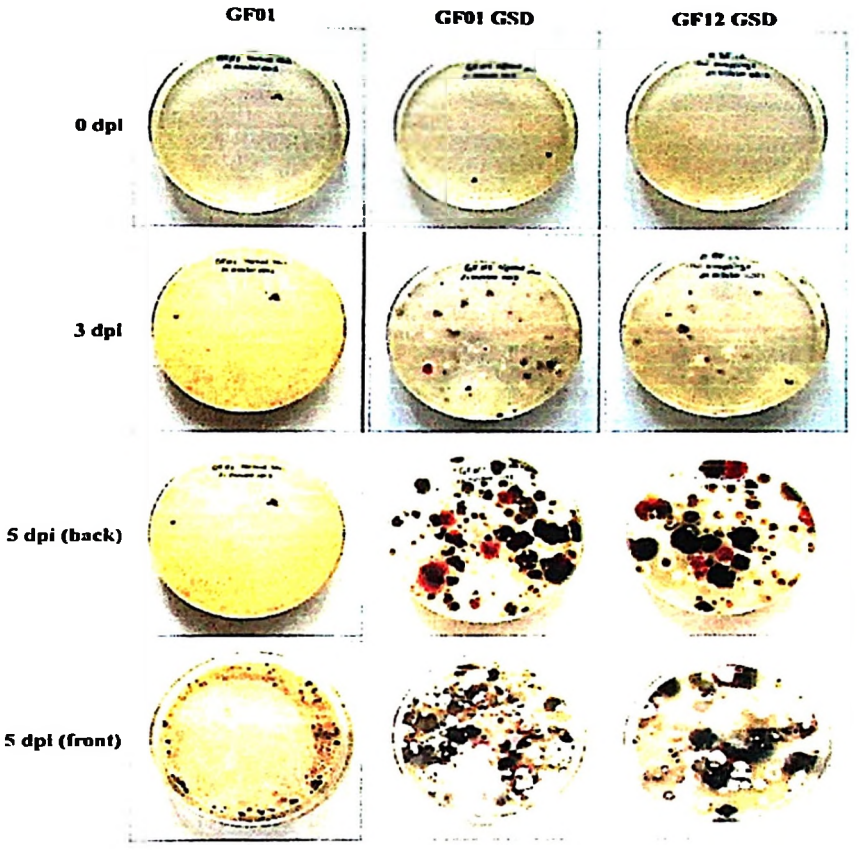
Fungi identification was performed using macromorphological observation of colony colour, texture and topology using naked eye and micromophological observation of fungi under the light microscope using a cell tape technique or needle mount technique. Before micromophological observation fungi were stained using lactophenol cotton blue containing phenol that kills fungi, lactic acid that preserves fungal structures and cotton blue which stains the chitin in the fungal cell walls. Afterwards, stained fungi were observed under the light microscope (Olympus, Tokyo, Japan) and images acquired using a digital camera (Nikon, Japan) mounted to the microscope.

Fungi identification using rRNA sequencing

Pure colonies of isolated fungi were picked using a sterile pipette tip and mixed with 140 µl of phosphate-buffered saline (PBS; Sigma, St. Louis, MO). Afterwards, fungi were incubated with 20 µl of 20 mg/ml proteinase K (Macherey-Nagel, Düren, Germany) at 50 °C for 2 hours. DNA was extracted from pure colonies of fungi digested with proteinase K using QiaAmp DNA extraction kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. Amplification of the 5.8S rRNA gene and flanking ITS1 and ITS2 (ITS1-5.8S-ITS2 region) was performed using ITS1F (5'- CTT GGT CAT TTA GAG GAA GTA A -3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') primers [7]. PCR was performed using a high fidelity DNA polymerase (Thermo Scientific, Pittsburgh, PA). PCR amplification conditions included an initial denaturation at 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C 45 seconds, annealing at 55 °C 45 seconds and

136 extension at 72 °C for 60 seconds and a final extension at 72 °C for 10 minutes using
137 GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Afterwards,
138 PCR products were electrophoresed in a 2% agarose gel mixed with GelRed nucleic
139 acid stain (Phenix Research Products, Candler, NC) before visualization and imaging
140 using a GelDoc-EZ Imager (Bio-Rad Laboratories, Hercules, CA).
141 In order to verify the retrieval of fragments of fungi, PCR fragments were purified
142 from agarose gels using a NucleoSpin gel and PCR clean-up kit (Macherey-Nagel,
143 Düren, Germany) and subjected to dideoxynucleotide cycle sequencing by using Big
144 Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City,
145 CA). Products from dideoxynucleotide cycle sequencing reaction were purified by
146 ethanol precipitation and separated on a 3500 Genetic Analyzer (Applied
147 Biosystems, Foster City, CA). The nucleotide sequences of ITS1-5.8S-ITS2 region
148 obtained from the isolated fungi were submitted to GenBank. Fungi species
149 identification was performed by comparing the obtained nucleotide sequences with
150 other nucleotide sequences of fungi at GenBank using BLASTn [8].
151
152 **Antifungal sensitivity testing**
153 Fungi sensitivity to ketoconazole (15 µg), nystatin (50 µg), fluconazole (25 µg),
154 clotrimazole (10 µg) and amphotericin B (10 µg) (Rosco, Taastrup, Denmark) was
155 performed using a simple agar diffusion method. Briefly, SDA agar was prepared by
156 weighing 32.5 g of SDA powder into 500 ml of distilled water. The mixture was
157 heated to dissolve completely and autoclaved at 121 °C for 15 minutes. Afterwards,
158 fungi were suspended in warm agar in sterile tubes containing 10 ml for each isolated
159 fungi. The fungi-SDA mixture was poured into petri dish and allowed to solidify
160 followed by placement of tablets into the surface of SDA. Fungi were incubated at

161 room temperature for 7 days. The diameter of zone of inhibition was recorded in mm
162 so as to determine sensitivity of each isolated fungi to the different antifungal agents.
163 **Results**
164 **Fungal isolates macromorphology and micromorphology**
165 Fungi were isolated by inoculating skin scrapings from healthy and sick giraffes into
166 SDA growth media. Fungi germination started after 24 hours of incubation with
167 complete germination of fungi by day 5 (Figure 1). Fungi with different colony
168 topology, color and texture were observed. Light yellow pigments diffused into the
169 growth medium after inoculation with skin scrapings from healthy giraffes while
170 different pigments including light yellow, cream, sandybrown, peru, different shades
171 of brown, black, maroon, darkolivegreen and white pigments diffused into the
172 growth medium in plates inoculated with skin scrapings from giraffes with GSD
173 (Figure 1; Table 1). Sub-cultivation of fungi from healthy giraffes led to the isolation
174 of two different fungi isolates that were also isolated from giraffes with GSD (Table
175 1). One of these fungi produced light yellow pigment into the growth media and had
176 darkslategray colonies while the other produced yellow pigment and had black
177 colonies (Figure 2). The color of fungi colonies and pigments produced into the
178 growth media of all other fungi isolated only from giraffes with GSD are shown in
179 Table 1 and Figure 2. The micromorphology of fungi was examined under the light
180 microscope for fungi identification. The different micromorphological features of
181 fungi observed under the light microscope are shown in Table 1.
182 **Figure 1. Isolation of fungi from skin scrappings of girrafes with or withour**
183 **giraffe skin disease (GSD).**

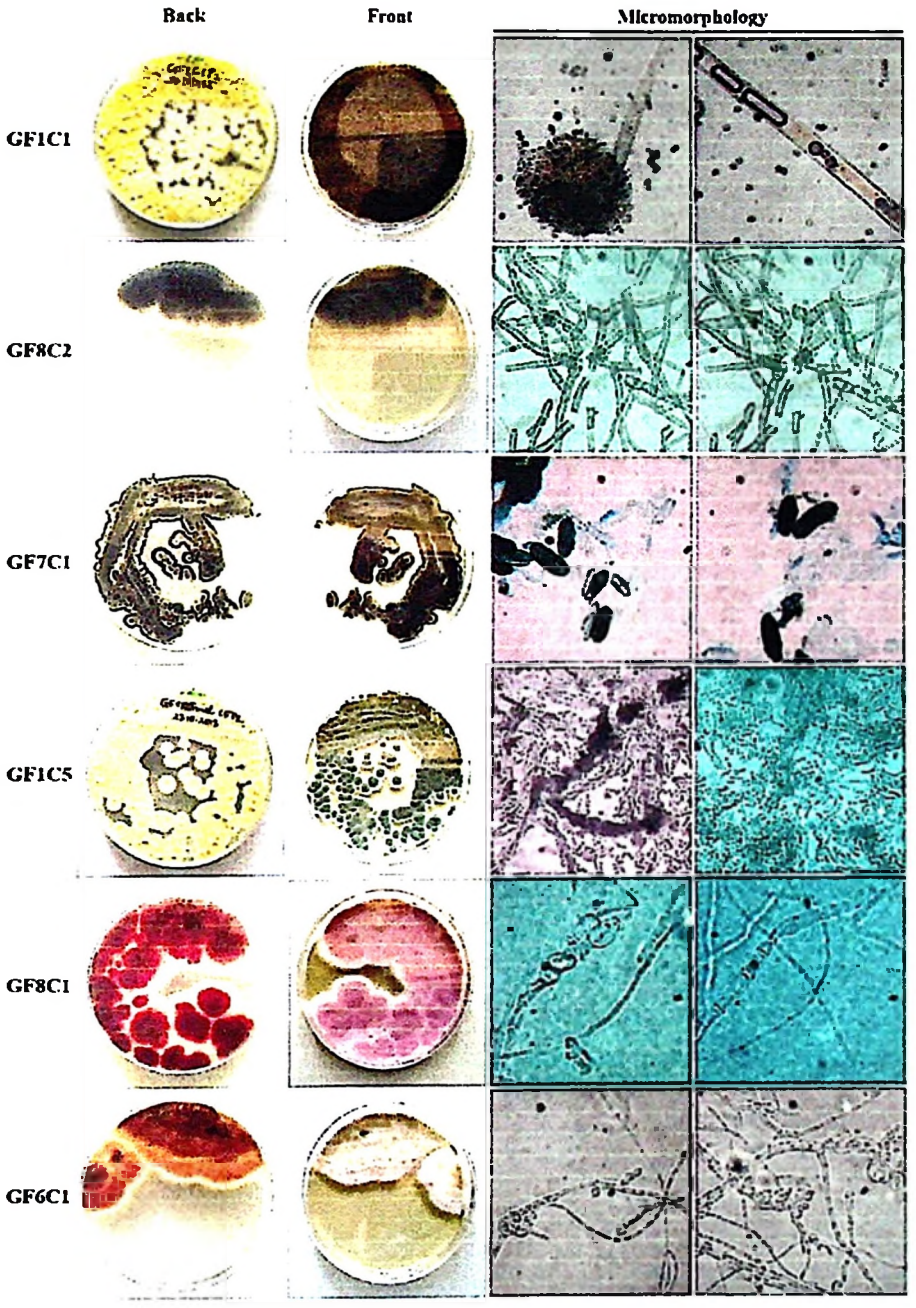


184

185 Skin scrappings from a giraffe without GSD (GF01) and giraffes with GSD (GF01
186 GSD and GF12 GSD) were inoculated into Saborauds dextrose agar (SDA). The
187 growth of fungi in SDA is shown after 0 and 5 days post inoculation (dpi). The front
188 and the reverse side of the plates are shown. The colony color and topology differed
189 between plates inoculated with skin scrapings from giraffes with and those without
190 GSD.

191 **Figure 2. The macromorphology and micromorphology of fungi isolated from**

192 **giraffes.**



193

194 Different fungi were isolated from skin scrapings of giraffes. The front and reverse

195 view of macromorphology of fungi is shown for each isolate together with the

micromorphological features observed under the light microscope at 400x magnification.

Genetic identification of fungi

DNA was extracted from pure colonies of fungi isolates followed by DNA amplification and nucleotide sequencing of ITS1-5.8S-ITS2 region using primers ITS1F/ITS4. DNA fragments with 275 to 650 base pairs in size were obtained after PCR and sequencing. The obtained nucleotide sequences were used to determine the fungal species using BLASTn. The different fungi identified based on nucleotide identity and their accession numbers after submission of nucleotide sequences at GenBank are shown in Table 1. Fungi identity based on ITS1-5.8S-ITS2 region corresponded well with the micromorphological features of fungi. Identified fungi included *Aspergillus fruticulosus, Aspergillus multicolor, Aspergillus niger, Aspergillus sydowii, Aureobasidium pullulans, Botryosphaeria sp., Cochliobolus lunatus, Cylindrocladium canadense, Dothideomycete sp., Epicoccum sorghinum, Fennellia nivea, Fusarium equiseti, Fusarium sp., Leptosphaerulina chartarum, Montagnulaceae sp., Penicillium citrinum, Penicillium commune, Penicillium simplicissimum, Pestalotiopsis clavispora, Phoma sp.* and *Setosphaeria rostrata.*

215 **Table 1. The macromorphology, micromorphology, molecular identity and accession numbers of fungi isolated from skin scrappings**
216 **of giraffes with or without giraffe skin disease.**

Fungi isolated from giraffes						Molecular identity (BLASTn)			
Fungi isolate	**GSD**	**Media color**	**Colony color**	**Micromorphology**	**Accession number**	**Species (isolate/strain)**	**Accession number**	**Nucleotide identity (%)**	**Gaps (%)**
GF1C5	+/-	Light yellow	Darkslat egray	Multicellular filamentous hyphal structures of varying sizes; budding ellipsoidal cells	KM979488	*Aureobasidium pullulans* (LKF08-138)	JX171163	534/536 (99%)	0/536 (0%)
GF1C1	+/-	Yellow	Black	Solitary, subspherical, verrucose and muriform conidia	KM979489	*Epicoccum sorghinum* (EpSo 3)	KM212176	377/377 (100%)	0/377 (0%)
GF1C1 GSD	+	Cream	White	Biverticillate conidiophores; flask-shaped phialides; spherical to subspherical conidia	KM979490	*Penicillium citrinum* (JF 11)	KM226953	550/552 (99%)	0/55 (0%)

GF2C1	+	Sandybrown	Mistyrose	Flask shaped and terminally flattened vesicles; globose or slightly subglobose and irregularly echinulate conidia	KM979491	*Aspergillus fruticulosus* (LCF10)	FJ867933	532/532 (100%)	0/532 (0%)
GF2C2	+	Peru	Darkslategray	Effuse conidial heads with translucent and smooth conidiophores stalks, roughly spherical and spiky conidia	KM979492	*Aspergillus sydowii* (DTO:26616)	KJ775571	275/275 (100%)	0/275 (0%)
GF6C1	+	Medium brown	White with light pink edges	Biseriate, large, globose conidial heads; smooth-walled, transluscent conidiophores	KM979493	*Aspergillus niger* (NJA-1)	KJ365316	602/602 (100%)	0/602 (0%)
GF7C1	+	Black with	Black	Biverticillate conidiophores;	KM979494	*Penicillium*	KM226953	565/565	0/56

		white edges	with white edges	flask-shaped phialides; spherical to subspherical conidia		*citrinum* (JF 11)		(100%)	(0%)
GF8C2	+	Black	Black	Macro- and microconidia produced from slender phialides	KM979495	*Fusarium sp.* (P47E2)	JN207347	522/522 (100%)	0/522 (0%)
GF8C1	+	Maroon	Pink	Fusiform and straight conidia	KM979496	*Pestalotiopsis clavispora* (TA-6)	AY924263	524/524 (100%)	0/524 (0%)
GF8C4	+	Black	Black	Septate hyphae, unicellular, hyaline, and oval-shaped conidia	KM979497	*Phoma* (TVD)	KF493958	502/505 (99%)	0/505 (0%)
GF9C1	+	Dark brown	Dark brown	Ellipsoidal and distoseptate conidia with protruding and truncate hilum	KM979498	*Setosphaeria rostrata* (ITC3)	KJ887577	530/530 (100%)	0/530 (0%)

GF9C2	+	Black with white edge	Black with white edge	Bitunicate ascus	KM979499	*Dothideomycete sp.* (7685)	EU680546	567/567 (100%)	0/567 (0%)
GF9C3	+	Black with yellow edges	Pale brown	Cylindrical, straight and rounded at both ends conidia	KM979500	*Cylindrocladium canadense* (SDAU08-72)	FJ557245	456/534 (85%)	40/534 (7%)
GF9C4	+	Cream	White	Macro- and microconidia produced from slender phialides	KM979501	*Fusarium sp* NRRL 43631	GQ505459	576/578 (99%)	2/578(0%)
GF9C5	+	Medium brown	Mistyros e	Globose conidial heads; long and nonseptate conidiophores; hemispherical vesicles; subglobose conidia	KM979502	*Aspergillus multicolor* (NRRL 4775)	EF652477	533/537 (99%)	4/537 (0%)

3C1	+	Darkolive green	Darkoliv egreen	Phialidic conidiogenous cells, globose and elliptical when young conidia	KM979503	*Fennellia nivea strain CH-Y-1043*	FJ155814	611/612(99%)	0/612(0%)
3C4	+	Cream	Cream	Macro- and microconidia produced from slender phialides	KM979504	*Fusarium sp. GrS5*	FJ904916	582/583(99%)	0/583(0%)
3C6	+	Cream	Cream	Septate, falcate, with a distinctive curvature macroconidia; foot-shaped basal cell; very elongated apical cell; branched or unbranched monophialides conidiophores	KM979505	*Fusarium equiseti isolate ATT040*	HQ607811	557/557(100%)	0/557(0%)
3C8	+	White	White		KM979506	*Montagnulaceae*	KJ780753	414/421(98%)	2/421(0%)

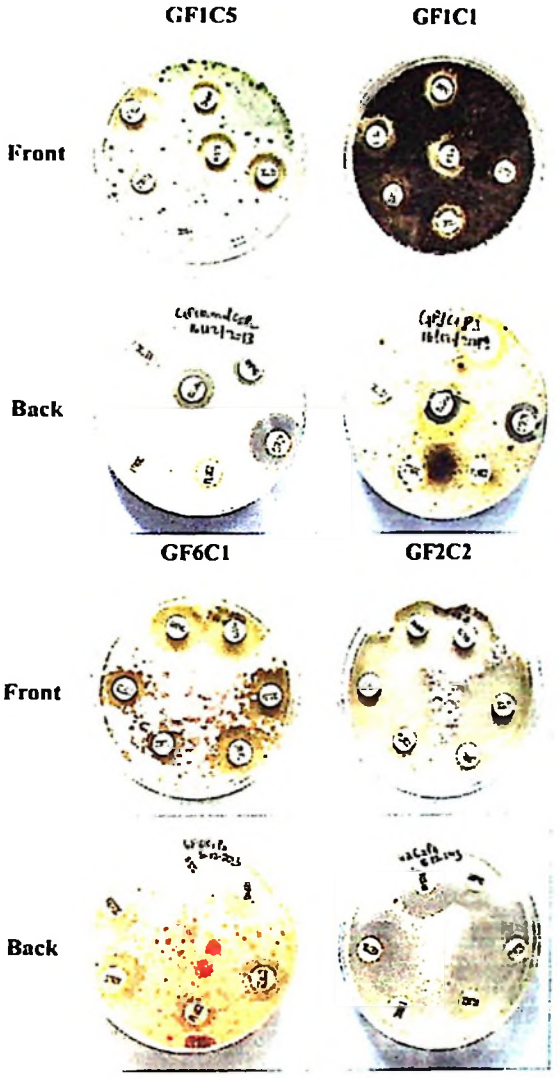
						sp. A1S3-D34			
3D4	+	White	White	Hyaline and aseptate conidia	KM979507	*Botryosphaeria sp. ATT043*	HQ607814	525/530(99%)	2/530(0%)
3D7	+	Light yellow	Darkslat egray	Mostly biverticillate, sometimes monoverticillate, occasionally terverticillate conidiophores; conidia mostly ellipsoidal, often subspherical, pyriform	KM979508	*Penicillium simplicissimum strain LAHC-FFPK-M12*	KF815055	556/559(99%)	0/559(0%)
3D8	+	Darkolive green	Darkoliv egreen	Terverticillate conidiophores; globose to subglobose conidia; cylindrical tapering to a distinct collulum conidia phialides	KM979509	*Penicillium commune strain ATCC 10428*	AY373905	546/553(99%)	1/553(0%)

3E4	+	Brown with white edge	Cream	Bitunicate asci, ellipsoid, cylindrical or oblong ascospores	KM979510	*Leptosphaerulina chartarum strain SJTU59*	KC879283	651/652(99%)	0/652(0%)
3E6	+	Pale brown	Pale brown	septate conidiophores, curved conidia with 3 septa and 4 cells	KM979511	*Cochliobolus lunatus strain IP 613.60*	DQ836798	591/591 (100%)	0/591 (0%)

Antifungal susceptibility of fungi isolated from GSD

The results from the *in vitro* antifungal susceptibility test varied among the different fungal isolates (Table 2). GF9C3 was sensitive to all antifungals tested in the present study. Isolates GF1C1 GSD and GF1C1 were resistant to fluconazole, itraconazole and ketoconazole. In addition, GF1C1 was also resistant to clotrimazole while GF2C1 was resistant to amphotericin B, itraconazole and nystatin. Other fungal isolates were susceptible, with varying degrees, to all antifungal tested (Table 2).

242 **Figure 3: Antifungal susceptibility of fungi isolated from skin of giraffes.**



243

244 Fungi were allowed to grow in the presence of antifungal agents including
245 ketoconazole (15 µg), nystatin (50 µg), fluconazole (25 µg), clotrimazole (10 µg)
246 and amphotericin B (10 µg) and allowed to grow for 5 days. The different isolates
247 (GF1C5 and GF1C1 from a giraffe without GSD and GF6C1 and GF2C2 from
248 giraffes with GSD) had different susceptibilities to the different antifungal agents
249 tested. The front and the reverse side of the plates are shown.

250 **Table 2. Antifungal susceptibility of fungi isolated from giraffes.**

Fungi isolate	Antifungal sensitivity (Ø in mm)					
	AMPHO	CTRIM	FLUCZ	ITRAC	KETOC	NYSTA
GF1C5	15	26	14	12	17	17
GF1C1	14	17	10	10	10	17
GF1C1 GSD	12	10	10	10	10	16
GF2C1	10	14	15	10	17	10
GF2C2	28	22	21	22	26	30
GF6C1	23	16	13	21	20	28
GF7C1	24	18	26	20	26	26
GF8C2	44	33	34	34	30	35
GF8C1	17	20	24	14	24	27
GF9C1	25	21	22	24	30	26
GF9C2	25	23	20	24	25	26
GF9C3	90	90	90	90	90	90
GF9C4	55	44	19	29	44	58
GF9C5	21	21	10	19	19	26

251

252 Antifungal susceptibility of fungi from giraffes with or without giraffe skin disease
253 (GSD) after incubation with antifungal agents including amphotericin B
254 (AMPHO), clotrimazole (CTRIM), fluconazole (FLUCZ), itraconazole (ITRAC),
255 ketoconazole (KETOC) and nystatin (NYSTA). The zone of growth inhibition was
256 measured in mm and 10 mm represents the diameter of the tablets and resistance to such
257 an antifungal.

258

Discussion

In this study, the fungi associated with GSD in Ruaha National Park were isolated from giraffes with or without GSD, identified and tested for their antifungal susceptibilities. Fungi were isolated from 16 animals using standard mycological culture techniques while identification was performed using rDNA (ITS1-5.8S-ITS2 region) sequencing. The following fungi including *Aspergillus fruticulosus, Aspergillus multicolor, Aspergillus niger, Aspergillus sydowii, Aureobasidium pullulans, Botryosphaeria sp.,* Cochliobolus *lunatus, Cylindrocladium canadense, Dothideomycete sp., Epicoccum sorghinum, Fennellia nivea, Fusarium equiseti, Fusarium sp., Leptosphaerulina chartarum, Montagnulaceae sp., Penicillium citrinum, Penicillium commune, Penicillium simplicissimum, Pestalotiopsis clavispora, Phoma sp.* and *Setosphaeria rostrata* were identified to be associated with GSD lesions in giraffes. *Aureobasidium pullulans,* and *Epicoccum sorghinum* were also isolated from both healthy and sick giraffes indicating that they were contaminants from the environment or part of the cutaneous normal flora. *Aureobasidium pullulans* is a ubiquitous fungi commonly considered to be a contaminant that can be found in different environments, plants and animals without causing any symptoms of disease [9], [10]. *Epicoccum sorghinum* has been isolated from phaeosphaeria leaf spot disease lesions of maize [11]. Most of the fungi associated with GSD have been associated with opportunistic infections especially in immunocompromised individuals [12]–[19], naturally occurring in the environment as contaminants [20]–[23] or etiological agents of plant diseases [24], [25]. The pathogenic nature of these fungi species may indicate their possible role in GSD etiology. However, it can be assumed that these fungi colonize GSD lesions after primary wounding of the skin.

284

285 The different fungi showed differing antifungal susceptibilities. No single fungi
286 was resistant to all tested antifungals (Table 2). In addition, no single antifungal was
287 able to inhibit the growth of all fungi (Table 2). This finding indicates that commonly
288 available antifungals can be used in the treatment of GSD. However, a combined
289 treatment is desirable in order to eliminate all fungi associated with GSD.

290

291 Giraffes suffer from another cutaneous infection called giraffe pinna dermatitis
292 (GPD) that has only been described in Mikumi-Selous ecosystem [26]–[28]. By 2001,
293 90% of the giraffe population in Mikumi-Selous ecosystem was affected with GPD,
294 since the first sighting of the disease in 1999 in Mikumi National Park. Giraffes with
295 GPD had enlarged, distorted, thickened, pendulous and necrotic ears that attracted
296 swarms of flies. Initial investigations of GPD and GSD in giraffes have suggested skin
297 damage followed by nematodes infection predisposing the skin to secondary infections
298 although the exact etiology and pathogenesis of these diseases remain unknown [2],
299 [27]. The nematodes in GPD lesions have been partially identified to be spirurid
300 nematodes closely related to *Dirofilaria immitis* and *Thelazia gulosa* [27]. It is
301 hypothesized that the nematodes are the primary causes of GPD and skin injury caused
302 by these nematodes predisposes the skin to secondary bacterial and fungal infections.
303 Flies have been suggested to be responsible for the spread of GPD in between giraffe
304 populations. Similarly, GSD lesions are itchy making the giraffes to frequently scratch
305 by rubbing against branches of smaller bushes and trees. As a result, the affected area
306 may develop cracks, ooze blood and becomes prone to secondary bacterial and/or
307 fungal infection that leads to formation of pus. Preliminary investigation of affected
308 giraffes suggested that GSD is a bacterial infection caused by *Dermatophillus*

309 *congolensis* [1] and nematodes have been observed in GSD lesions. The results
310 presented in this study indicate that GSD lesions are highly contaminated with fungi
311 that naturally occur in the environment. The role of nematodes in the primary
312 development of GSD lesions and the identification of flies responsible for the spread of
313 GSD between giraffes is worthy further investigating.

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318

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