Project Update: July 2018

Introduction

Amphibians have been declining faster than other groups of vertebrates, in fact, it is thought that more than 50% of all species are critically endangered [1]. In general, the decrease of amphibian populations is largely attributed to the pathogen *Batrachochytrium dendrobatidis* (Bd) [2]. This fungus causes chytridiomycosis, an emerging disease that is capable of infecting tadpoles, metamorphs and adults [3]. This fungus can infect a large number of amphibian species, causing an osmotic imbalance that leads to death [4] and in addition it can impair the immune response[5]. However, not all populations and species have the same susceptibility to Bd, nor all life stages [6]. Bd apparently does not affect the survival rate in tadpoles, therefore this stage could be an intraspecific reservoir [7]. However, Bd seem to reduce the survival of post-metamorphic individuals [8].

Bd is responsible for mass mortality, global and local extinctions, and loss of phylogenetic diversity [9,10], however, a method that helps to mitigate the effects of this disease on wild populations has not been established yet [11]. Additionally, the effectiveness to reduce the prevalence of infection will depend on the host life history and the stages of the disease [12]. To treat this pathogen, several alternatives have been proposed, such as identifying the host's stage that endanger the viability of the population and temporarily keep them in captivity until they overcome that stage [13]. Also, it has been proposed the use of fungicides such as itraconazole [11,14,15]. Nevertheless, these strategies are complicated and difficult to carry out in the field.

To propose new strategies to control Bd it is necessary to understand the host defense mechanisms. The microbiota and mucous membranes found in the amphibians are important for host health and there are an essential part of the innate immune system of these organisms[11,16], which influences the development, behaviour, metabolism and inflammatory response of the animal [17]. The mucosome has different independent factors such as mucosal antibodies, antimicrobial peptides, lysosomes, alkaloids, among others [11]. The microbiota is composed by fungi, bacteria, and secondary metabolites produced by these organisms [11] that can change during the life history of the host [18,19]. The interactions between microbiota and mucosome provides protection to the host against pathogens [20,21] and can result in resistant phenotypes [22]. In the case of the infection caused by Bd, the bacteria associated with the skin can provide protection through the production of antifungal metabolites, limiting pathogen adhesion to host cells, resource competition or interacting with the immune system [23,24]. Furthermore, antimicrobial peptides secreted by the skin of some frogs are able to inhibit the growth of Bd *in vitro* [25]. In addition, fungi play a key role in the defense against pathogens, for example, it is known that these microorganisms are capable of producing antimicrobial compounds such as *Penicillium* [26]. However, the effects and interactions that fungi have on the amphibians health are unknown, and few studies on the interaction between the cutaneous fungi of amphibians and Bd have been carried out [27].

Fungi are a very diverse group of microorganisms, with distinct roles in ecosystems such as decomposers, pathogens and/or parasites[28,29]. Moreover, in nature there are several examples of fungi that perform symbiosis with other organisms to be part of the host's defense against pathogens, for example the mycorrhizal symbiosis [30]. Some fungi have the ability to inhibit infectious agents [27]; in the case of Bd there was found numerous taxa capable of inhibiting the growth of Bd [27]. Kearns et al. (2017) concluded that fungi abundance contributes more in the defense against Bd than the bacterial community. Also, it has been suggested that the use of a therapy with fungal probiotics does not induce an endocrine-immune response, in contrast to bacterial probiotics that can suppress the response of antimicrobial peptides in frogs [27]. There is an increase in the literature that suggest the use of bacteria as probiotics against Bd [31–33], but little is known about the interaction between fungal microbiota and Bd.

Due to the importance that fungi can have in the defense against Bd, this study intends to test the hypothesis that skin fungi microbiota play a role in the defense against Bd. To test these hypothesis, the cutaneous fungi of two different species of frogs, *Dendropsophus molitor* and *Rheobates palmatus,* were isolated and challenge against to Bd. The populations of these frogs have been exposed to Bd for a long period of time, and they have not been apparently affected. Additionally, studies on these frogs populations show that these two species harbour a high proportion of bacteria with antifungal abilities, moreover, peptides secreted by their granular glands are inhibiting Bd growth [34,35]. This study provides fundamental information on the role of fungal community, ecology and interactions among skin-associated fungi and Bd, these will serve as a basis of future applied research and potential interventions aimed at amphibian conservation.

Methods

Sample collection and fungi isolation

We studied two sympatric species from the Andes of Colombia: *Rheobates palmatus* and *Dendropsophus molitor* (previously known as *D. labialis*) which inhabit a permanent pond located near Cáqueza, department of Cundinamarca (04º26'12" N, 73º55'10" W), 56 km east of Bogotá, at approximately 1970 m. We visited the site once in December 2017. Individuals were collected using a net or by hand and kept in individual plastic bags until processed.

We aimed to determine the role of the fungal community as a barrier against Bd. To accomplish this, we took skin samples to isolate fungi and then, through challenge assays we determine the anti-Bd activity of each isolate. To isolate fungi microbiota, each individual was rinsed twice with sterile water (50 mL) to remove transient microbiota [36]. In order to recover the highest number of fungi morphotypes we used two methods, (1) Individual skin impressions were taken in petri dishes with Potato Dextrose Agar (PDA) and incubated at 25°C for 8 days. Then each morphotype were transferred to a new petri dish. (2) Skin swabs were performed using a synthetic rayon swab (MWE 100). The left, right and ventral surfaces were swabbed, samples were preserved in a 2 mL tube in 0.85% saline solution at -20 °C until processed. To isolate fungal colonies, dilutions of 1:10, 1:100 and 1:1000 of the saline solution in which the swabs were stored, were plate on PDA with antibiotic (Rifampicin), Malt Extract Agar

and Sabouraud Agar incubated at 25 °C for 8 days. Afterwards, each morphotype obtained in each method were plated in PDA with antibiotic (Rifampicin) and incubated at 25°C.

Fungi identification

For strain identification, we used morphological and molecular approaches. 1) Cultural characteristics such as texture, pigmentations and obverse and reverse colour of the colony were observed after 7 days of incubation on PDA (with rifampicin) plates. For microscopic characteristics such as colour, septation, branching patterns, conidia and chlamydospores were registered 2) To identify each isolate, the ITS region was amplified and will be sequenced in the next month. Isolates were grown in Sabouraud´s dextrose with yeast (SDY) medium with glucose (2%), yeast extract (1%) and peptone (1%) in agitation for 7 days at 25°C. Then fungi were filtered using a 25 µm pore filter paper and lyophilized. DNA was extracted using phenol-chloroform method described by Goodwin, Drenth, & Fry (1992). DNA extractions were verified using a 1% agarose gel electrophoresis. The ITS region was amplified using ITS1F (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers [38]. The PCR parameters were: denaturation step at 94°C for 5 min; 30 cycles of 94°C for 30s, 59°C for 30s and 72°C for 1 min; and a final extension step of 7 min at 72°C. The results were checked by electrophoresis in 1% agarose gels. Products will be sent to Macrogen (Korea) for sequencing. DNA sequences will be cleaned and assembled using Geneious (Kearse et al., 2012). Sequences will be identified using Blast in NCBI and sequences will be uploaded to Mycobank.

Bd diagnosis and quantification

To determine infection status, Bd presence/absence, as well as the intensity of infection (as zoospore loads), each animal was swabbed by running a synthetic rayon swab (MWE 100) ten times over the ventral surface, the inner thigh area and the plantar surface of the hind feet webbing for a total of 50 times. Swabs were stored dry at -20 °C until processed. Presence of Bd will be diagnosed and quantified using qPCR (Real-Time PCR). For the qPCR Bd assay, DNA will be extracted using PrepMan Ultra. Extractions and qPCR's will be performed following the methods described by Hyatt et al. (2007) and Boyle et al. (2004). Each reaction consists of 0.45 ml of each primer (50 mM), 5.975 ml of water, 12.5 ml of TaqMan, 0.625 ml of MGB Probe and 5 ml of the DNA template. To quantify infection intensity (as number of zoospores) standards of known concentrations and negative controls will be used.

Growth inhibition assays

To test if the skin fungi can inhibit or facilitate Bd growth, challenge assays will be performed using each isolated morphotype. To perform the growth inhibition assays, Bd strain EV001 (Flechas et al 2013) will be plated in TGhL agar (Tryptone 10 g, Agar 10 g, Gelatine hydrolisate 4 g, 1 l distilled water), and allow to grow at 23 ºC for at least three days or until maximum zoospores production. Bd will be harvested from Petri dishes, re-suspended in TGhL broth and the solution will be filtered in order to allow the passage of the zoospores and retain the zoosporangia. The filtered zoospores are count on a haemocytometer and re-suspend in sterile TGhL broth to a concentration of $1x10^6$ zoospores/mL. We are going to run two kinds of challenge assays: (1) Each isolated morphotype will be tested against Bd in plates with TGhL agar in order to determine if

there is a mechanical response of fungi, for example under the microscope we can observe hyphae surrounding Bd zoosporangia. (2) Each fungal morphotype will be growth in petri plates in PDA agar, then each sample will be rinsed with 3 ml water for 20 min, and filter-sterilizing the secondary metabolites. Then in 96-well plates we will add 50 µl of a zoospore solution and 50 µl of the secondary metabolites. The positive control plates containing Bd and 50 µl of sterile water, and the negative control plates containing 50 µl of heat-killed Bd and 50 µl of sterile water. The plates will be incubated for 7 days at 23°C and measure at 490 nm absorbance the first day, third day and seventh day to see the change in optical density.

Preliminary results

Morphological descriptions

We captured 10 tadpoles, 8 juveniles and 2 adults of *Rheobates palmatus* (Aromobatidae), and one juvenile of *Dendropsophus molitor* (Hylidae). A total of 39 cultivable fungi morphotypes were isolated. From isolate fungi, 14 were yeast and 25 were moulds. For each morphotype we registered the following characteristics: texture, colony colour, pigmentations and obverse and reverse colour of the colony, and mycelium characteristics as colour, septation, branching patterns, conidia and chlamydospores (Table 1).

Table 1. Macroscopic and microscopic morphological characteristics.

Fungal isolation ID	Type of fungi	Color	Reverse color	Pigmentation to the medium	Texture	Mycelium
1	Mold	Olive green	Beige	None	Powdery	Hyaline, non-septate and thick hyphae. Conidiogenous arrangement with phialides and basipetal chains. Green round spores.
2	Yeast	White	White	None	Creamy	No pseudomycelium formation. Division by bipolar budding.
3	Yeast	Orange	Orange	None	Creamy	No pseudomycelium formation.
5	Yeast	Creamy yellow	Creamy yellow	None	Creamy	No pseudomycelium formation.
9	Mold	Olive green	White	None	Powdery	Hyaline, non-septate and thin hyphae. Conidiogenous arrangement with phialides and basipetal chains.

						Hyaline round spores.
10	Mold	Olive green	Beige	None	Powdery	Hyaline, non-septate and thin hyphae. Conidiogenous arrangement with phialides and basipetal chains. Hyaline round spores.
11	Yeast	Pastel pink	Pastel pink	None	Creamy	No pseudomycelium formation.
12	Yeast	Creamy orange	Creamy orange	None	Creamy	No pseudomycelium formation.
13	Mold	Olive green	Beige	None	Powdery	Hyaline, non-septate and thick hyphae. Conidiogenous arrangement with phialides and basipetal chains. Hyaline round spores.
15	Mold	Dark green in the middle and white at border	Brown in the middle and beige at border	Orange	Cotton	Hyaline, non-septate and thin hyphae.
16	Mold	Reddish brown	Brown	None	Leathery	Hyaline, septate and thin hyphae. Round ameroconidia.
17	Yeast	Pastel pink	Pastel pink	None	Creamy	No pseudomycelium formation.
18	Yeast	Creamy white	Creamy white	None	Creamy	No pseudomycelium formation.
20	Yeast	Creamy white	Creamy white	None	Creamy	No pseudomycelium formation.
22	Mold	Brown with white dots	Brown	None	Floccose	Hyaline, non-septate and thin hyphae.
23	Mold	Olive green	Olive green	None	Floccose	Hyaline, non-septate and thin hyphae. Ovoid ameronidia.
24	Mold	Light green in the middle	Brown in the middle and	None	Cotton, Floccose	Phaeoid, non-septate and think hyphae. Intercalary and end round chlamydospores.

		and brown at border	black at border			
25	Mold	Olive green with dots	Creamy	None	Powdery	Hyaline, non-septate and think hyphae. Conidiogenous arrangement with phialides and basipetal chains. Green round spores.
26	Mold	Green, white, yellow and pink from the center to the edge.	Green	None	Floccose	Hyaline, septate and thin hyphae. Intercalary and end round chlamydospores.
27	Mold	Black, olive green and grey from the center to the edge.	Black	Brown	Floccose	Phaeoid, septate and think hyphae.
28	Mold	Brown	Brown	None	Leathery	Hyaline, non-septate, and thin hyphae.
29	Mold	Peach	Peach	None	Leathery	Hyaline, septate and thin hyphae. Conidiogenous aggregates whiteout arrangement.
31	Yeast	White	White	None	Creamy	No pseudomycelium formation.
32	Yeast	Creamy yellow	Creamy yellow	None	Creamy	No pseudomycelium formation.
33	Mold	Olive green in the middle and creamy orange	Olive green	Orange	Floccose	Hyaline, septate and thin hyphae. Intercalary and end round chlamydospores.

		at border				
34	Mold	Creamy orange	Orange	Reddish brown	Floccose	Hyaline, septate and thin hyphae. Intercalary and end round chlamydospores.
35	Mold	White	Yellow	Yellow	Cotton	Hyaline, non-septate, and thin hyphae. Fusiform, slightly curved with septa macroconidia, monophialides are present.
36	Mold	White	Yellow	Yellow	Cotton	Hyaline, non-septate, and thin hyphae. Fusiform, slightly curved with septa macroconidia, monophialides are present.
37	Mold	Grey in the middle and white at border	Yellow	None	Cotton	Hyaline, septate, and thin hypahe.
38	Mold	Creamy orange	Vermilion	Orange	Floccose	Hyaline, non-septate, and thin hyphae.
39	Mold	Black	Black	Black	Cotton	Phaeoid, septate and think hyphae, thickened at the tip with a black color.
40	Mold	Olive green with white dots	Olive green	Yellow	Powdery, floccose	Hyaline, septate, and thin hypahe. Ameroconidia in chains of approximately 5 conidia each with a union of 8 chains forming a "flower". They come straight out of the hypha, bate shape phialides.
41	Mold	White	Creamy yellow	None	Cotton	Hyaline, septate, and thin hypahe.
42	Mold	Royal yellow	Royal yellow	Red wine	Cottony	Phaeoid, septate and thick hyphae. Intercalary and end round chlamydospores. The hyphae and chlamydospores are orange.

43	Mold	Olive green with black dots	Olive green with black dots	None	Leathery	Hyaline, septate and thick hyphae whit thin and smooth walls. Intercalary and end round chlamydospores. Hyaline round ameroconidia
44	Yeast	Creamy white	Creamy white	None	Creamy	No pseudomycelium formation.
45	Yeast	Creamy white	Creamy white	Yellow	Creamy	No pseudomycelium formation.
46	Yeast	Creamy white	Creamy white	None	Creamy	No pseudomycelium formation.
47	Yeast	Creamy white	Creamy white	None	Creamy	No pseudomycelium formation.

Morphotypes examples

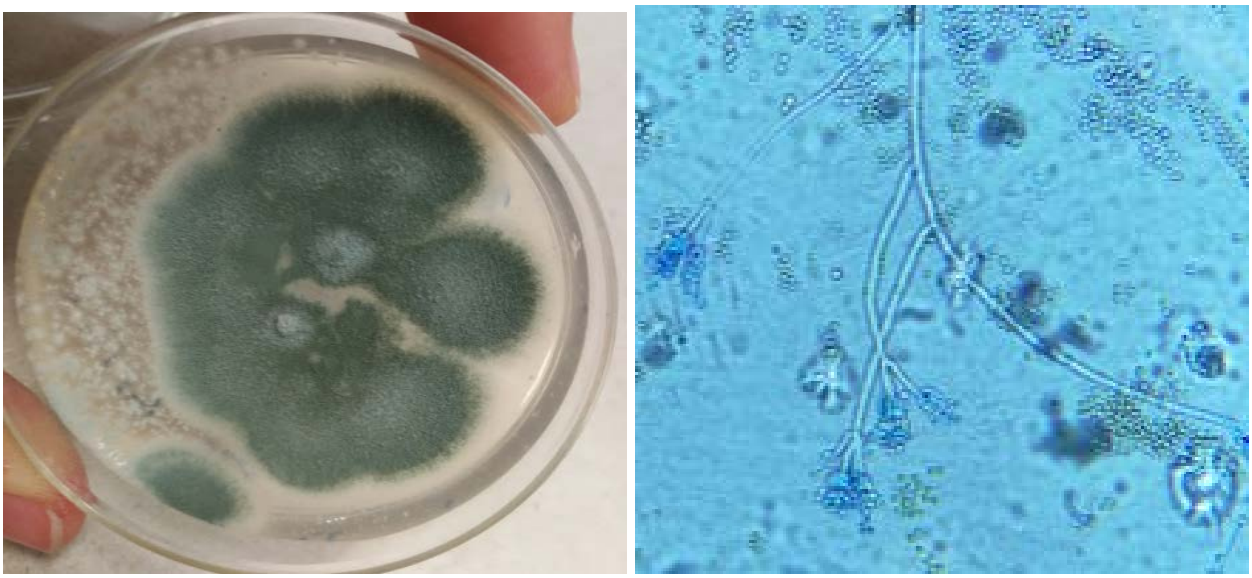

Figure 1. Morphotype 1, in the left macroscopic and in the rigth microscopic charactheristics.

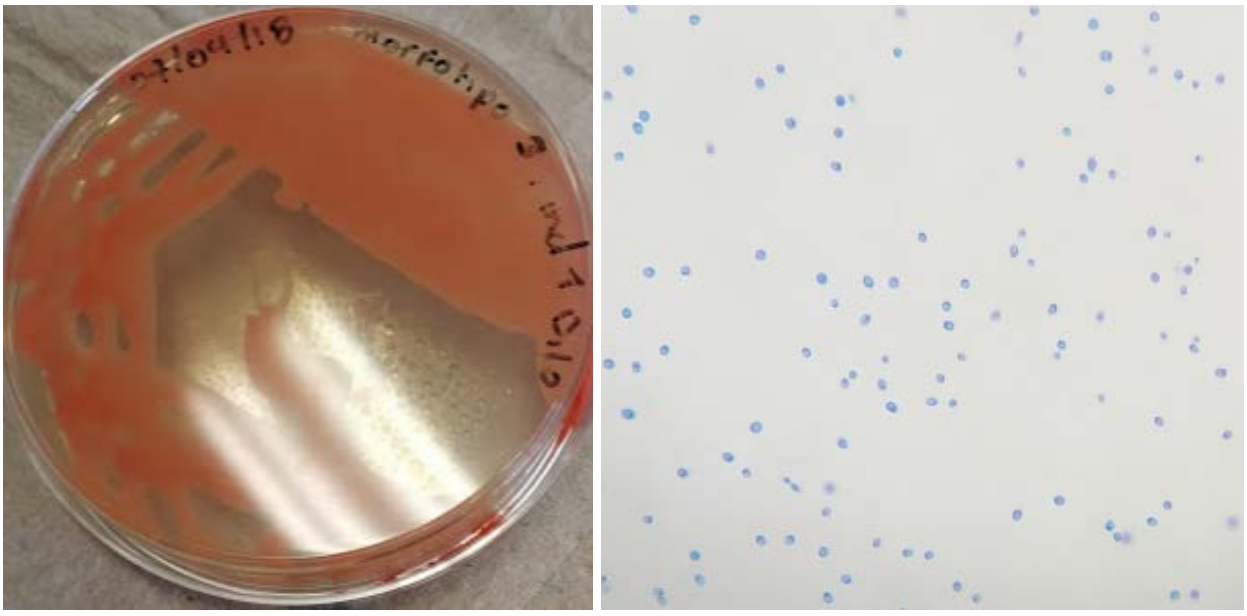

Figure 2. Morphotype 3, in the left macroscopic and in the rigth microscopic charactheristics.

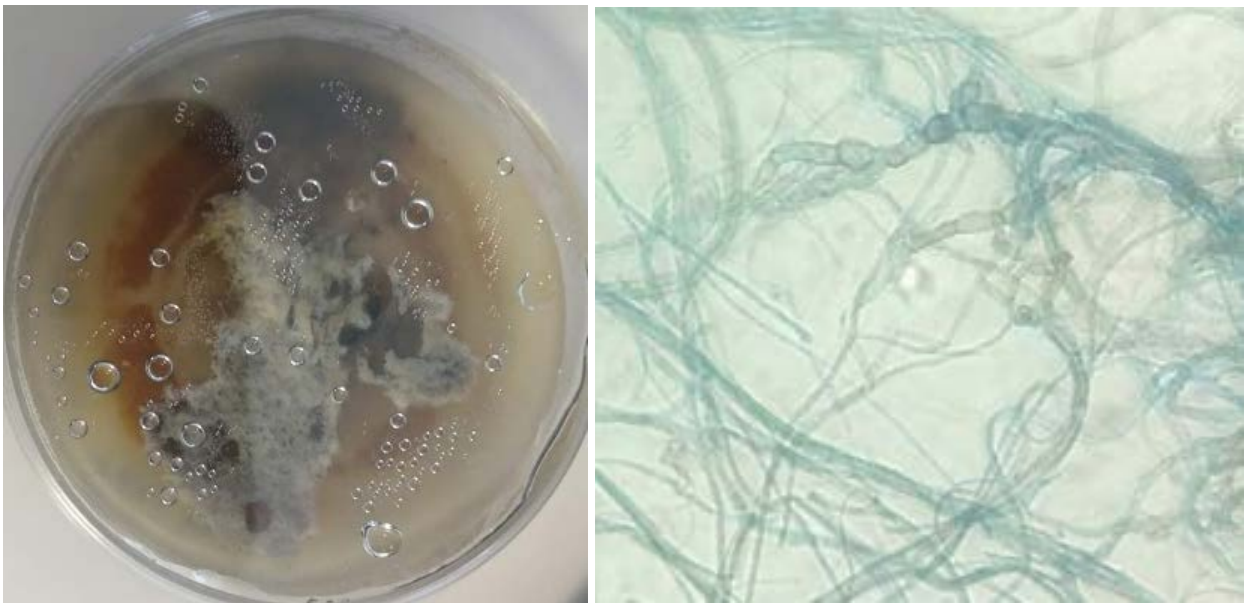

Figure 3. Morphotype 24, in the left macroscopic and in the rigth microscopic charactheristics.

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