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First record of *Saprolegnia* sp. in an amphibian population in Colombia

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Abstract

Objective: Most research related to the decline of amphibians has been focused on the detection of the pathogenic fungus *Batrachochytrium dendrobatidis*. This fungus is the main pathogen detected around the world. However, research has shown the presence of another fungus, *Saprolegnia ferax*, as a cause of mortality in amphibians in North America. Our study suggests a possible interspecific transmission caused by the presence of rainbow trout; thus, amphibian declines may not be attributable only to the presence of a single pathogen, but to other organisms and factors. **Materials and methods.** Our study revealed the presence of *Saprolegnia* sp. in the Andean frog *Atelopus mittermeieri* using the imprinting technique with lactophenol blue staining, which allowed the typical structures of this fungus to be observed. **Results.** The importance of this discovery is the presence of two pathogenic fungi, *B. dendrobatidis* and *Saprolegnia*, which affecting simultaneously a population of amphibians. This finding brings attention to the eventual presence of other microorganisms that might be involved individually or collectively in the decline of amphibian species. **Conclusions.** This record suggests a possible transmission between rainbow trout (*Oncorhynchus mykiss*), an introduced species in the highlands of Colombia, which shares the same habitats with different species of amphibians in the Sanctuary of Flora and Fauna Guanentá in the upper river Fonce in the mid Cordillera Oriental of Colombia.

Key words: decline, amphibians, Saprolegnia, fishes, Atelopus, Colombia.

Resumen

Primer registro de *Saprolegnia* sp. en una población de anfibios en Colombia. Objetivo. La mayoría de investigaciones en relación al declive de anfibios se han enfocado en la detección del hongo patógeno *Batrachochytrium dendrobatidis*. Este es el principal patógeno detectado en el mundo. Sin embargo, otras investigaciones han demostrado la presencia de *Saprolegnia ferax* como un vector mortal en anfibios de Norteamérica. Este trabajo establece una posible transmisión entre especies causada por la presencia de la trucha arcoíris, sugiriendo que el declive de anfibios no solo se atribuye a la presencia de un solo patógeno, sino que puede ser causado por otros patógenos y factores. **Materiales y métodos.** El estudio revela la presencia de *Saprolegnia* sp. en la rana de los Andes *Atelopus mittermeieri* mediante la técnica de impronta con tinción de azul de lactofenol, que permitió la observación de las estructuras típicas de este hongo. **Resultados.** La importancia de este descubrimiento es la presencia de dos hongos patógenos, *B. dendrobatidis* y *Saprolegnia*, afectando simultáneamente una población de anfibios. Este hallazgo enfoca la atención en la eventual presencia de otros microorganismos que podrían estar involucrados individualmente o colectivamente en el declive de especies de anfibios. **Conclusiones.** Este registro sugiere una posible transmisión entre la trucha arcoiris (*Oncorhynchus mykiss*), una especie introducida en las montañas de Colombia, que comparte los mismos habitats con diferentes especies de anfibios en el Santuario de Flora y Fauna de Guanentá en el alto río Fonce en la parte media de la Cordillera Oriental de Colombia.

Palabras clave: declive, anfibios, Saprolegnia, peces, Atelopus, Colombia.

Resumo

Primeiro registro de *Saprolegnia* sp. numa população de anfíbios na Colômbia. Objetivo. A maioria das pesquisas sobre o declínio dos anfíbios tem-se centrado na detecção do fungo patogênico *Batrachochytrium dendrobatidis*. Este é o principal patógeno encontrado no mundo. No entanto, outros estudos têm demonstrado a presença de *Saprolegnia ferax*, como vetor mortal de anfíbios de América do Norte. Esta pesquisa estabelece uma possível transmissão entre espécies causada pela presença de truta arco-íris, afirmando que o declínio de anfíbios não só é atribuído à presença de um único patógeno, pudendo ser causado por outros patógenos e fatores. **Materiais e métodos.** A pesquisa indica a presença de *Saprolegnia* sp. em rãs dos Andes, *Atelopus mittermeieri*, pela técnica de impressão com lactofenol-azul-de-algodão, onde observou-se as estruturas típicas deste fungo. **Resultados.** A importância desta descoberta é a presença de dois fungos (*B. dendrobatidis e Saprolegnia*) que simultaneamente afetam uma população de anfíbios, concentrando a atenção na presença de outros microorganismos que podem estar envolvidos individualmente ou coletivamente no declínio das espécies de anfíbios. **Conclusões.** A truta arco-íris (*Oncorhynchus mykiss*), uma espécie introduzida nas montanhas da Colômbia, que compartilha o mesmo nicho ecológico com *Atelopus mittermeieri*, no Santuário de Flora e Fauna de Guanentá no Alto Rio Fonce no centro da Cordilheira Oriental da Colômbia, tem sido sugerida por esta pesquisa como o agente transmissor dos fungos as diferentes espécies de anfíbios.

Palavras-chave: declínio, anfíbios, Saprolegnia, peixes, Atelopus, Colômbia.

Introduction

The study of the decline of amphibian populations in Colombia has focused on the morphological evaluation of the fungal pathogen *Batrachochytrium dendrobatidis* (1, 2). However, several authors have found that this pathogen is not solely responsible for the decline of amphibians (3, 4). *Saprolegnia* is a pathogen common in fish populations and has been found in some amphibian populations around the world (5). *Saprolegnia* has been recognized for years as a major eukaryotic pathogen of freshwater fish, distributed worldwide (6). It causes mortality in fish farms, due to its ability to colonize the eggs and fish in early developmental stages, infect wounds and penetrate the epidermis using enzymes or mycelium. When host defenses are low, infection may produce an osmotic imbalance, other infections, and respiratory failure as a result of primary or opportunistic infection (7). *Saprolegnia* has many species with different morphological, physiological and genetic characteristics which give varying levels of virulence and apparently uses multiple hosts (8). The ability of *Saprolegnia* to produce hydrolytic enzymes that allow the mycelium to penetrate the epidermis of the host causes tissue damage, production of toxins and imbalance of body fluids. As a result, *Saprolegnia* spp. are multihost infectious agents in different species of fish, frogs and salamanders (9, 10).

Blaustein *et al*. (4) studied the presence of the fungus *Saprolegnia ferax.* They identified it as responsible for the mortality of amphibian larvae in the mountains of Oregon, USA. Kiesecker and Blaustein (3) identified the interaction between UV and *Saprolegnia ferax***,** showing a reduction in the number of eggs in two frog species (*Anaxyrus boreas* and *Rana cascadae*). They also studied the incidence of this fungus on egg mortality in some amphibian species in north western United States. Kiesecker *et al*. (5) obtained experimental evidence for the role of rainbow trout (*Oncorhynchus mykiss*) in the transmission of *S. ferax* in populations of the toad *Anaxyrus boreas***.** They demonstrated how fish farms act as vectors and increase the mortality of amphibians in different environmental conditions. In 2002, Blaustein and Kiesecker (10) confirmed that a major cause of larval mortality in local populations of some amphibian species in North America is the oomycete fungus *S. ferax.* They also found that the widespread occurrence of this fungus among these populations and geographic regions may be the result in differences in their degree of virulence. Blaustein *et al*. (11) suggested that some pollutants, such as fertilizers, heavy metals, PHAs and other toxic chemicals may have a synergistic influence on the fungal-amphibian relationship. The same author indicated that embryos exposed to high levels of UV-B resulted with an increased mortality by *S. ferax*. Carey and Alexander (12) classified *S. ferax* as an infectious disease causing the decline of amphibian populations as well as increase in natural areas due to climate change; for instance, low levels of rainfall caused by the "El Niño" phenomenon increased the effect of UV-B. Daszak *et al*. (13) noted the need to develop timing analyses of the incidence of saprolegnosis. Collins and Storfer (14) indicated that transmission of *S. ferax* may increase due to the introduction of foreign species which act as reservoirs for the fungus affecting native species.

Scientific reports of this pathogen in South American amphibians do not exist. There are reports restricted to commercial fish, e.g. there are some records of *S. parasitica* in fish fauna in Argentina and Chile. Consequently, there is no information available regarding its potential role as a causal agent and its mode of transmission in amphibians in South America (15).

In Colombia, the introduction of non-native species into ecosystems has been increasing in recent years. Changes in amphibian populations dependent upon lotic waters by direct competition from non-native species are likely, perhaps including populations of the montane frogs *Atelopus muisca* and *A. lozanoi* in Chingaza National Park, or populations of *A. mittermeieri* in the Alto Río Fonce Flora and Fauna Sanctuary; all of them are locations where rainbow trout has been reported (1).

Such practices may have potentially enabled the introduction in Colombia of new pathogens such as *Saprolegnia*, which moves from different hosts that share the same habitats as the introduced fish *Oncorhynchus mykiss, Oreochromis* spp., *Piaractus brachypomus, Pimelodus clarias* and native species such as *Eremophilus mutisii*, the latter endemic to the Cundinamarca-Boyacá high plain in the Eastern Cordillera of Colombia (16, 17, 18).

The present study reports the discovery of the pathogen (*Saprolegnia* sp.) affecting an amphibian population in Colombia. The fungus was detected in a population of *Atelopus mittermeieri* which had been previously reported to be infected with *Batrachochytrium dendrobatidis* (2). Infection by *Saprolegnia* sp. represents a possible interspecific transmission event caused by the presence of rainbow trout. If this is confirmed, then it is possible that the decline of amphibians in this site is not attributed to the presence of a single pathogen, but to additional organisms and factors.

Materials and methods

The fungus was detected by microscopic observation using the imprinting technique on lactophenol-blue stain on integumentary scrapings from the hind limbs of 37 specimens of *Atelopus mittermeieri*. Specimens previously had been fixed in 10% formalin and preserved in 70% ethanol after being collected from the wild. Specimens are deposited in the reference collection of amphibians of the museum of the Pontificia Universidad Javeriana (MUJ). Glass microscope slides were prepared and microscopic observations were done with objective lens magnifications at 4x, 10x, 40x and 100x. The identification of the fungus was aided with the taxonomic keys of Seymour (19). Morphological characteristics were described at the microscopic level. For each slide on which hyphae were observed, we made a description of pigments in the hyphal body, hyphae form, presence of septa and reproductive structures (zoosporangia). These observations allowed for the morphological identification of the genus. Since our samples were preserved in formalin, it was not possible to conduct a molecular identification or use microbiological techniques based on cultures of the fungus. Information on temperature and humidity of the habitat, where positive samples were obtained, was collected from notes accompanying specimens obtained during field monitoring of *Atelopus mittermeieri* populations between 2005 and 2007 in Aguas Claras creek, a tributary of the Río Negro, in the Río Negro basin in the Guanentá Alto Fonce Flora and Fauna Sanctuary, Department of Santander (**Figure 1**). Data points of the infected specimens were recorded with a data logger TrendReader Express® (**Figure 2**). Specimen MUJ 4367 was obtained between days 25 and 27 of September 2006, at an elevation of 2,900 m; specimen MUJ 4358 was obtained in June 2005 at an elevation of 2,525 m.

Results

During field monitoring of *Atelopus mittermeieri* between 2005–2006 in Aguas Claras creek, a tributary of the Río Negro, in the Río Negro basin in the Guanentá Alto Fonce Flora and Fauna Sanctuary in the Department of Santander, some specimens of this species were found dead with some external signs of fungal infection on ventral surfaces of their thighs (**Figures 3 and 4**). These specimens were preserved in formalin.

Additionally, during surveys for amphibian chytridiomycosis in Colombia, it was decided to evaluate these samples by histological observation of *Batrachochytrium dendrobatidis* (previously reported by Ruiz and Rueda in 2008 (2) in the same population of amphibians). Because of these two reasons, those particular specimens were examined in more detail.

Studying the same specimens collected and reported in 1998 using a scraping technique, previous determinations of infection by *Batrachochytrium dendrobatidis* were confirmed, and additional infection by *Saprolegnia* was identified.

Microscopic evaluation of the growth of the fungal bodies revealed the presence of thin somatic hyaline hyphae, without septa and with few branches. It additionally revealed an extensive and dense mycelium near the substrate (**Figure 5**). At a higher magnification (40x), the presence of long and cylindrical reproductive structures was observed (oosporangia), which were separated from the basal somatic hyphae by a small septum.

Oosporangia are 160–220 µm in size, clavate form, thick, and with a smooth wall. Spores are spherical and subglobose,

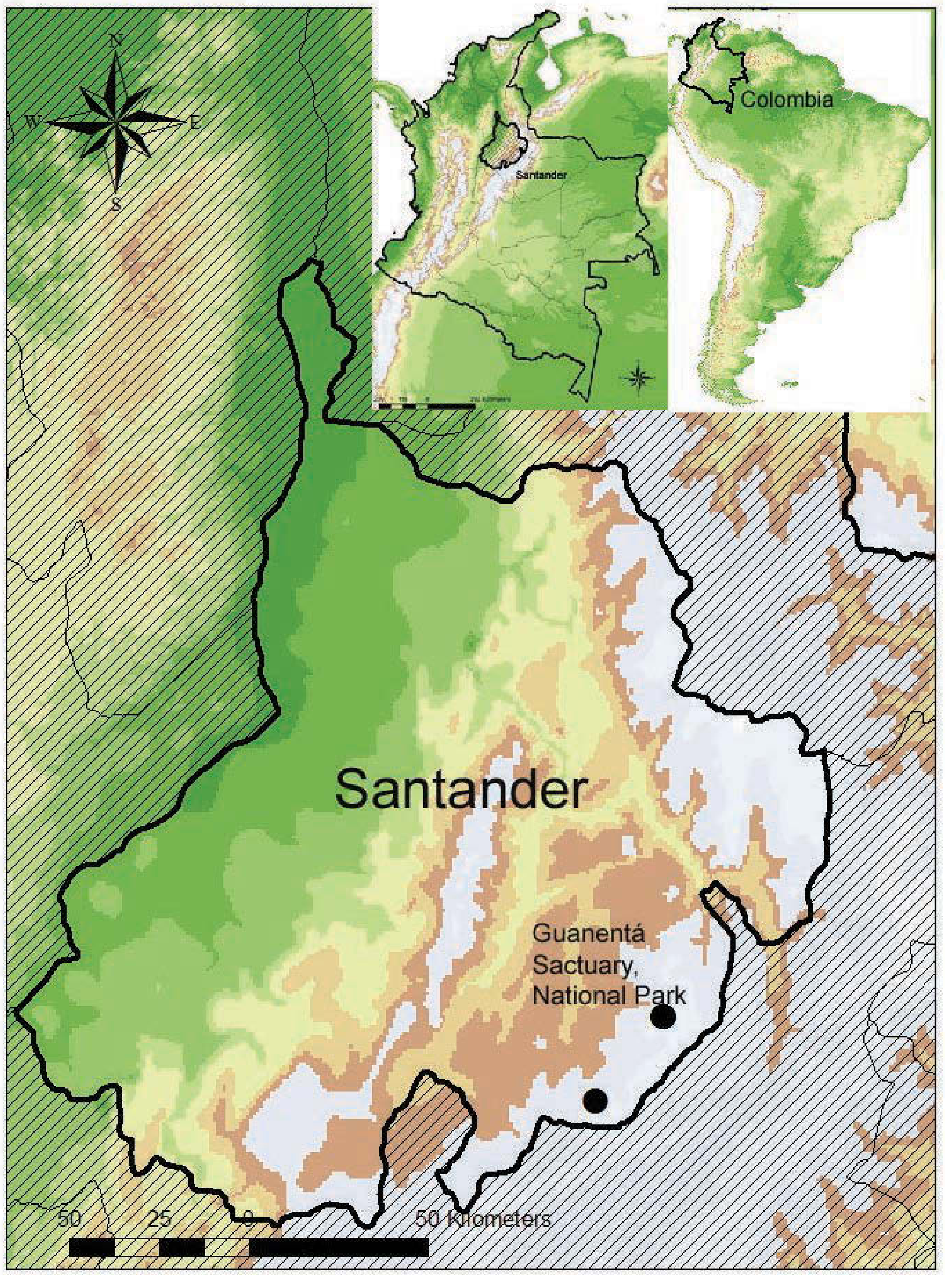

Figure 1. Map showing the locality of *Atelopus mittermeieri* in western Cordillera Oriental in Colombia.

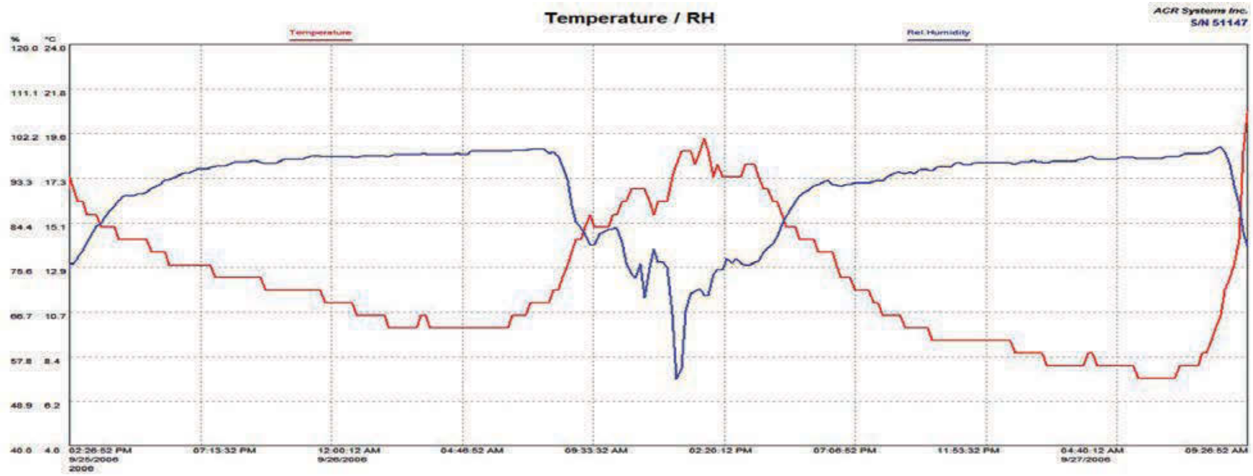

Figure 2. Climograph of Aguas Claras creek. Sampling days which relate relative humidity (blue) and temperature (red). Data were recorded at intervals of 10 minutes.

and were observed in a compact catenula arrangement within the sac or sporangia (fungal propagules) (**Figure 5**).

This study reports for the first time the presence of the oomycete fungus *Saprolegnia* sp. in a population of the highland frog *Atelopus mittermeieri*. Since it was found in association with the chytrid fungus *B. dendrobatidis,* it is noteworthy that two quite unrelated species of pathogenic fungi are affecting amphibian species in Colombia.

Of a total of 37 adult specimens examined from the reference collections, only two of the specimens showed hyphal growth (**Figure 4**). One of the infected amphibians (MUJ 4367) was found dead on the side of the Aguas Claras creek (**Figure 3**) with advanced hyphal growth. Air temperature was between 11.77 °C and 20.55 °C, relative environmental humidity ranged from 53% to 99% (85.73% on average), and water temperature was between 9 °C and 14 °C (**Figure 2**). The second specimen (MUJ 4358) was an adult male and was found dead during the second sampling of this species, with no apparent evidence of hyphal growth.

A more detailed analysis of the incidence of this fungus on adult specimens of *Atelopus mittermeieri* was recorded in a transect at the same site in June 2005. Presence of the pathogen in the specimen MUJ 4358 (**Figure 4**) was found. In the same transect another four specimens were observed, two amplectant individuals were apparently healthy. In another assessment at the same linear distance, three specimens were reported with chytridiomycosis in September 2006; presence of the pathogen *Saprolegnia* sp. was observed only in the specimen MUJ 4367 found dead in the creek (**Figure 3**).

The infected frogs were found in Aguas Claras creek co-occurring with rainbow trout (*Oncorhynchus mykis)*, a finding that suggests a possible vertical transmission of this pathogen among species. During the discovery of dead specimens of *A. mittermeieri* we observed that mycelia had a grayish white colour around the body, especially in the legs. Apparently it was a localized infection because the mycelial growth was observed attached to the limbs skin or hanging from the same skin. In addition, infection was observed in one dead specimen that was missing the right leg (MUJ 4367), and the second specimen infected (MUJ 4358) was in poor physical condition and low weight.

A new survey of three days of search in March 2010 resulted in the discovery of a single specimen of *A. mittermeieri* without symptoms of the disease. We were able to obtain and evaluate several specimens of rainbow trout with negative results. We could also find with the help of locals that introduction of trout was initiated over 30 years ago, upstream in Chontales creek within the same basin of Aguas Claras creek. The few data available allow us to make a preliminary assessment on the prevalence of *Saprolegnia* on local populations of *Atelopus*, but future studies with this population can be further improved, as well as a specific investigation into the possibility of vertical transmission of *Saprolegnia* between trout and *Atelopus* (or other amphibians).

Figure 3. Specimen of *Atelopus mittermeieri* MUJ 4367 found dead in the Guanentá Alto Fonce Flora and Fauna Sanctuary in June of 2005. Specimen was subsequently preserved,

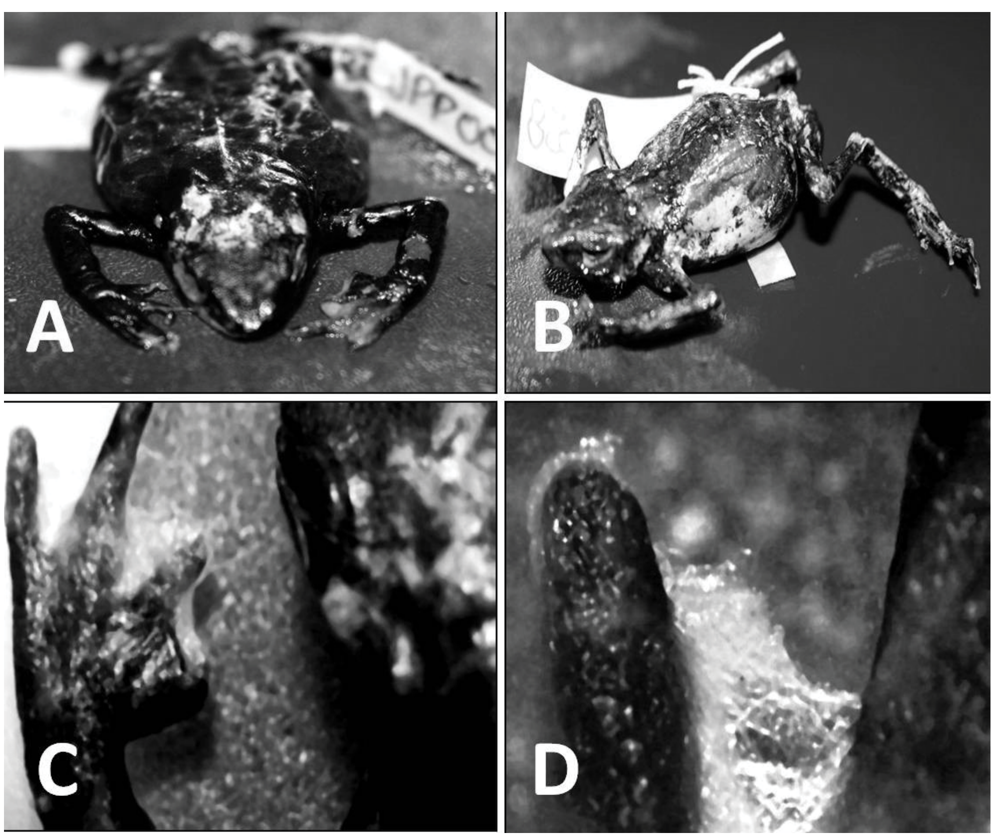

Figure 4. Detail of specimens of *Atelopus mittermeieri* (A) MUJ 4358. (B) MUJ 4367. (C) Sign of *Saprolegnia* infection in the specimen MUJ 4367. (D) Detail of mycelium on *Atelopus mittermeieri*.

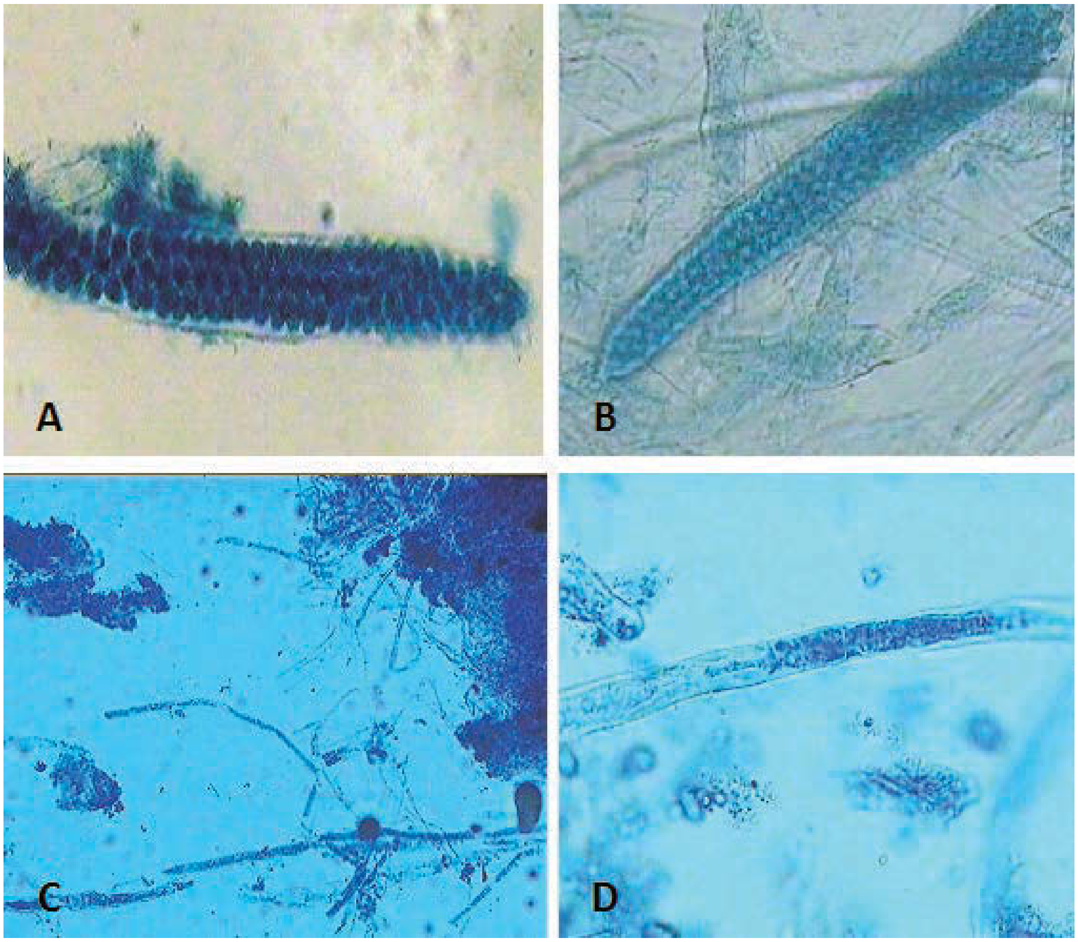

Figure 5 Microscopic slide glass preparations using lactophenol blue stain with integumentary scraping of *Atelopus mittermeieri* MUJ 4367 specimen. (A) spherical spores inside the bag (100x objective). (B) Oosporangia of *Saprolegnia* sp. (objective lens 100x). (C) Extensive and dense mycelium (objective lens10x). (D) somatic hyaline hyphae (objective lens 40x).

Discussion

The decline of amphibians is the result of a complexity of environmental factors that generate variation in the habitat of amphibians (10). Furthermore, other agents may accelerate the decline of amphibians. Examples of this are emerging infectious diseases (13). The most studied disease is chytridiomycosis; however, it is not the only one and does not necessarily act alone in amphibian populations. Amphibians can also be affected by other diseases, such as those caused by different species of the fungal genus *Saprolegnia* which may be considered a secondary disease (5, 18). Therefore, research should be carried out in other infectious agents that directly or indirectly affect amphibian communities.

It has been discussed that interspecies vertical transmission may occur with this fungus: amphibians have a pathogenic fungus prevalent in fish and widely studied in different species of rainbow trout (20). Fungi of the genus *Saprolegnia,* which usually occur when the host immune system is suppressed or compromised, cause most superficial fungal infections affecting fish (21). This fact could had occurred at our study site because specimens were missing limbs or appeared thin and weak, which affect the immunological defense of the specimens and thus could facilitate the infection in both amphibians and not in the other specimens sharing the same habitat.

The taxonomic identification of *Saprolegnia* is traditionally done with a detailed description of morphological characteristics, especially sexual structures as oogonia, antheridia or ornamentation present on the walls of the oogonia. We induced those characteristics by growing the fungus under specific conditions of temperature, prolonged incubation periods and specific nutrients. This classification system based on morphological features alone to identify the genus of the fungus is not fully effective in determining the species in isolates that do not produce any sexual structures typical of this fungus. Consequently, most authors suggest using molecular methods for the confirmatory identification of the fungus (22); however, it was not possible for us to do identification at the species level for the fungus *Saprolegnia* because specimens studied were preserved in formalin, therefore the fungus could not grow in synthetic media. Additionally, due to the preservation of the fungus in formalin, it was not possible to carry out an identification test.

The macroscopic description of the *in vivo* fungus on the specimen of *Atelopus mittermeieri* found dead is similar to that done by Kinkelin et al. (23) in fish. Authors report white aerial mycelium, which is a characteristic manifestation of this disease and the pathogenic fungus *Saprolegnia*. Similar signs were found in the specimens assessed.

Most recent attention to pathogen-driven amphibian decline has been focused on the study of chytridiomycosis as one of the fundamental causes of the decline of amphibians in Colombia and elsewhere (2, 24). In contrast, there are few reports of the presence of other microorganisms that may be involved individually or collectively in the decline of amphibians. Thus, our study points out the importance of research not only on a single agent, but also on the presence of bacteria, fungi and viruses not reported before as pathogenic microorganisms and that might be involved in the drastic decline of some amphibian populations.

Conclusions

Infectious diseases related to the introduction of alien species into natural habitats may be a fundamental cause contributing to the increase of emerging diseases.

Fungi of the genus *Saprolegnia* cause superficial fungal infections in amphibians, and represent a probable cause of decline of amphibians in Colombia in combination to chytrid fungus.

The use of molecular tools for identification of species is of relevance to Colombia, because there are no studies determining which species of *Saprolegnia* parasitize fish in Colombian freshwater ecosystems setting up the possibility of vertical interspecies transmission. Therefore, it is necessary to study in more detail the isolation, culture and molecular identification of the fungus.

The identification of *Saprolegnia* sp. on the body of adult specimens of dead frogs is an indication of pathology, because this fungus has a saprophytic life cycle, but also provides evidence of the ability of this fungus to be an opportunistic parasite of amphibians.

Acknowledgments

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Conflicts of interest

Authors declare that there are no conflicts of interest related to the results obtained in this investigation.

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