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Research Article

Isolation and identification of fungal species from the skin of Bull frog (*Hoplobatrachus tigerinus*): prevalence and ecological implications

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Abstract

Amphibians, especially frogs, play a critical role in ecosystems, acting as prey and predator and influencing nutrient cycling between aquatic and terrestrial habitats. This study aimed to isolate and identify fungal species present on the skin of the bullfrog (*Hoplobatrachus tigerinus*) in District Kasur, Pakistan. A total of 20 bullfrogs were sampled, and mucus swabs were taken from their skin to analyze fungal communities. The swabs were cultured on Sabouraud Dextrose Agar, Potato Dextrose Agar, and Brain Heart Infusion Agar. Morphological and biochemical techniques were used for the identification of fungal species. A total of 35 fungal isolates representing four species including *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus terreus*, and *Rhizopus stolonifer* were identified. *Aspergillus niger* (60%) was the most prevalent species, followed by *Aspergillus fumigatus* (50%), *Rhizopus stolonifer* (30%) and *Aspergillus terreus* (15%). It can be concluded that the presence of Aspergillus spp., may have significant effects on health of *Hoplobatrachus tigerinus*. However, more research is needed to determine whether these fungi are benign, symbiotic, or pathogenic in amphibians. Future studies should aim to investigate the specific interactions between these fungi and their amphibian hosts, especially under various environmental stress conditions, such as pollution or climate change.

Keywords: *Aspergillus niger*, *Aspergillus fumigatus*, *Rhizopus stolonifer*, Ecological role, Environmental monitoring

Introduction

Amphibians are considered environmental indicators and play a major role in many ecosystems. A co-evolutionary relationship exists between amphibians and the diversity of animals and plants, and their existence is intertwined (Hashim et al., 2016). Amphibians are important components of food pyramids because they provide food for a variety of predatory animals and control the populations of many insects and pests (Ali et al., 2018). Amphibians bridge the evolutionary gap between aquatic and land animals. Amphibians make an important contribution to the transfer of nutrients from aquatic to terrestrial ecosystems, and their exclusion from any ecosystem can have an impact on populations of invertebrates, algae communities, nutrient cycling, leaf litter decomposition, and dynamics of the predator (Ali et al., 2018).

Amphibians and wetlands are intimately connected. Several factors such as the type of aquatic habitat for species that are only found in water, the type of substrate, vegetation, and the dependence of mature individuals on water affect the distribution of amphibian species in a given area. Numerous abiotic factors, including temperature, hydro-period, water quality, and biotic factors such as vegetation structure in and around the pond, are likely to have an impact on the existence and richness of amphibians (Akram et al., 2015). The *Hoplobatrachus tigerinus* commonly known as bullfrog belongs to the genus Hoplobatrachus and family Dicroglossidae. It is the largest frog found in Pakistan. It hibernates by burrowing in the soil during winter as well as during the drought period. Breeding activity is primarily confined to monsoons (July-September). Bullfrogs have dull, olive-green or brown-green coloration, which helps them camouflage with their environment. However, the color of breeding males is lemon yellow with deep blue vocal sacs, while females remain dull-colored. The tadpole of the Bull Frog has a cylindrical body with a muscular tail (Tabassum et al., 2011). Frogs serve as biological pest control agents and biological indicators, commonly used as dissection animals and food for other organisms, and their larvae filter water (Hashim et al., 2016).

Frogs have a thin and persistently moist layer of skin. The frog skin is a complex organ that performs several functions such as protective barrier, osmoregulation, cutaneous respiration, and water intake because it is permeable to water and capable of gas exchange (Xu & Lai, 2015). Skin can quickly absorb toxic substances that make them very sensitive (Samojeden et al., 2022). Furthermore, their skin has a sugar-rich mucosal layer that can act as a growth substrate for pathogenic bacteria and fungi (Ross et al., 2019).

Fungi are heterogeneous, eukaryotic and unicellular to filamentous organisms that produce spores and are chemoorganotrophic. Fungi are classified into three types based on their morphology: filamentous mold, unicellular yeast, filamentous mold, unicellular yeast, and yeast-like form (pseudohyphae form). Depending on the temperature, dimorphic fungi can produce both forms (yeast and mold). The yeast form is produced inside the host's body, whereas the mold form can only be found in natural environments or on artificial culture media. Pseudo hyphae are networks of elongated ellipsoidal cells with gaps between them (Samanta, 2015).

Fungi are a diverse group that plays important roles in ecosystems as pathogens, parasites, and decomposers (Kirk et al., 2008; Fierer, 2017). Fungi are common in nature and can affect individuals who are stressed, injured or immuno-suppressed. Some fungi are opportunistic or secondary invaders but others are severe primary pathogens. *Batrachochytrium dendrobatidis*, a chytrid fungus, is currently amphibians' most significant and well-reported pathogen. The fungal pathogens *Phialophora spp.*, *Fonseca spp.*, *Rhinocladiella spp*., and *Cladosporium spp.* are responsible for chromomycoses (Krzysciak et al., 2014).

Furthermore, fungi form complex symbiotic relationships that assist them in nutrient acquisition and host defense (Philippot et al., 2013). Non-chytrid fungi have been found on frogs and other non-chytrid fungi have been shown to cause disease in frogs. Non-pathogenic fungi have also been found in frogs, and these fungi have shown potential defensive roles in frogs. Despite a wealth of knowledge about chytrid-host interactions, little is known about the diversity and metabolic capabilities of non-pathogenic fungi associated with amphibians. The aim of the study is to isolate and identify fungal communities living on frog skin using microscopic observation and culture-based methods and to analyze their significance as components of the frog skin microbiome.

Material and methods

Sampling

The present one-year study was conducted in the Postgraduate Lab, Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki. A total of 20 bullfrog (*Hoplobatrachus tigerinus*) specimens were collected from selected sites in District Kasur using hand nets. The GPS coordinates of each captured specimen were noted in the log book for

GIS-based mapping (Fig. 1). The map was created using ArcGIS software at the GIS and Remote Sensing Lab of the Department of Wildlife and Ecology, UVAS, Ravi Campus. The captured specimens were brought to the lab in sterilized boxes for further processing. Mucus samples were obtained by swabbing the frog's skin. The entire specimen body was swabbed with a sterile cotton swab. Each frog was swabbed 20 times with a sterilized cotton swab (five streaks each on the dorsal side, ventral side, and hind limb). After sampling, all frogs were released into their natural habitat.

Isolation of Fungal Species

Swabbed samples were cultured using three mediums such as Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA), and Brain Heart Infusion (BHI) Agar supplemented with antibiotics at concentrations of chloramphenicol 25.0 mg/L and 100 µg/mg streptomycin in each Petri dish. Parafilm was used to seal the Petri dishes, and they were incubated for 4 to 7 days at a temperature of 27°C ± 2°C. The incubation period was recorded for each sample. It was nearly identical for similar species. The day of the first visible growth observed after plating was considered an incubation period for growth. To obtain pure cultures for identification, mycelium tips were isolated from the master plates and transferred to fresh agar plates without the addition of antibiotics.

Morphological identification

The fungal isolates were identified using morphological and biochemical methods. Both microscopic and macroscopic characteristics were used to identify isolated fungi. An identification key described by (Klich, 2002;Samson and Varga, 2007) aided in the identification.

Macroscopic identification

Macroscopic identification was done after 4 to 5 days and morphological characteristics such as colony growth pattern, texture, edges and color of the colony were used for identification.

Microscopic identification

For microscopic identification, the slides were prepared by the coverslip method and tape lift method. The lactophenol cotton blue stain technique and India ink were used to identify the isolated fungi. Morphological characteristics such as the morphology of conidial shape, conidial head, conidiophore and hyphae were used to identify the species levels of fungi (Klich, 2002).

Tape lift method

The adhesive tape method (tape lift method) was used for microscopic examination after the colony grew (Forbes et al., 2002). A transparent adhesive portion of Scotch tape was applied on the surface of the colony and then the tape was gently pressed into the fungal colony. A few drops of India ink or lactophenol blue stain were applied on the glass slide. The tape was adhered to the surface of a glass slide with a stain. Slides were examined by using a light microscope with 10X and 40X lenses.

Cover slip method

The slide was prepared by applying a drop of the stain on a clean glass slide with the help of a micropipette. From the fungal culture, a small portion of the mycelia was taken out and put in a drop of stain. The mycelium was evenly distributed on the slide with the mounting needle. A cover slip was placed gently with little pressure to remove air bubbles. The slide was examined under the light microscope with 10X and 40X objective lenses.

Biochemical identification

Biochemical methods, such as carbon fermentation, carbon and nitrogen utilization, and enzymatic activities, such as cellulase, urease, caseinase, and lipase, were used to identify fungi (Sangeetha & Thangadurai, 2013).

Results

In this study, twenty bullfrogs (*Hoplobatrachus trigeminus*) were collected from selected sites in District Kasur to investigate skin microflora. A GIS-based map was created to depict the study area and the spatial distribution of the sampling sites. Mucus samples were collected by swabbing the skin of bullfrogs. Swabbed samples were processed, and 35 fungal isolates were obtained overall. The isolated fungal isolates underwent morphological analyses to identify their species. The obtained results revealed the presence of four distinct species belonging to two genera including Aspergillus and Rhizopus. Within the genus Aspergillus, three species were identified. These included *Aspergillus niger, Aspergillus fumigatus* and *Aspergillus terreus*. Additionally, one species belonging to the genus Rhizopus was identified as *Rhizopus stolonifer* (Figure 3).

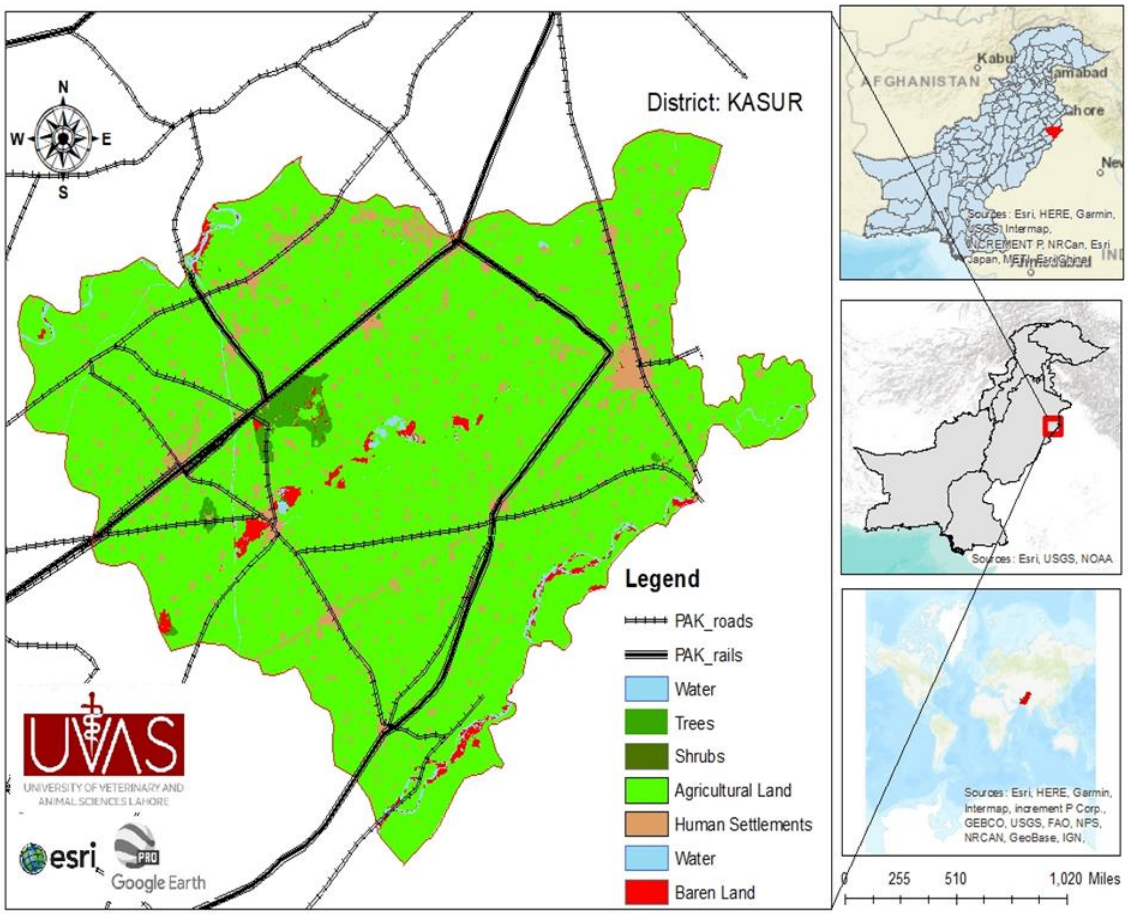

Figure 1. GIS-based map of District Kasur

Prevalence of Fungi

During the Present study, *Aspergillus niger* was the most prevalent species, occurring in 12 out of the twenty samples, representing a prevalence rate of 60%. Following closely behind, *Aspergillus fumigatus* was found in 10 out of the twenty samples, with a prevalence rate of 50%. *Rhizopus stolonifer*, another fungal species, occurred in 6 out of the twenty samples, with a prevalence rate of 30%. *Aspergillus terreus*, was less prevalent than the other species, was identified in 3 out of the twenty samples, representing a prevalence rate of 15% (Figure 2)

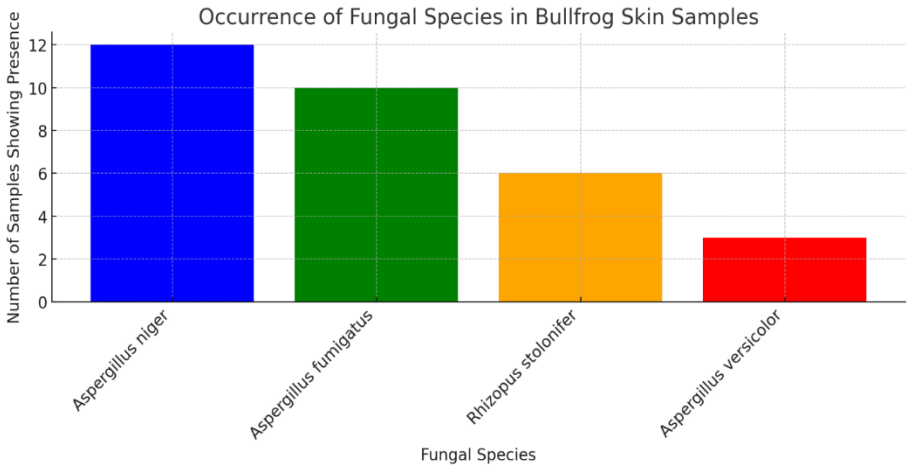

Figure 2. Bar graph showing the number of samples where each fungal species was identified.

Morphological characterization of isolated Fungi

Rhizopus stolonifer

When grown on Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA), the colonies of *Rhizopus stolonifer* were observed to be very fast growing, displaying a tendency to coalesce. The aerial mycelium growth on the media initially appeared as white and cottony, which gradually turned brownish-grey to blackish-grey. Throughout the colony, black globule sporangia were observed. The reverse side of the plate exhibited a combination of white and light brown colors. Remarkably, the fungus was capable of covering the entire petri dish within a span of just three days. Under microscopic examination, *Rhizopus stolonifer* displayed distinct structures and features. Sporangiophores, which were smooth-walled and non-septate were observed. These sporangiophores were simple in structure and occurred either singly or in clusters. They were connected to one another by septate hyphae known as Stolons. Large saclike sporangia that contain sporangiospores arises from long sporangiophore. Sporangia were located opposite the Rhizoids, which are root-like structures. Sporangiospores are hyaline, grey or brownish, globose to ellipsoidal, and smooth-walled.

Aspergillus fumigatus

The surface color of *A. fumigatus* colonies was white initially then turned to dark green, gradually turning black with age. The texture of the colonies was powdery. On the reverse side of the plate, a pale yellow coloration was observed. Additionally, *A. fumigatus* colonies exhibited a fast-growing nature. Under microscopic examination, *A. fumigatus* displays distinct structures and features. The conidiophores were short, smooth, and unbranched. At the top of the conidiophores,

a hemispherical capsule or vesicle was observed. The vesicles were either completely or partially covered with flask-shaped phialides. Around the phialides, numerous conidia were spread. These conidia were single-celled and nearly spherical in shape. They possessed a rough outer surface and appear green in color. The conidia were produced in long chains that may diverge or coalesce, forming compact columns.

Aspergillus niger

In both Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) media, the growth of *Aspergillus niger* colonies was observed to be slow. Initially, the colonies appeared white, but later they turned into roundish black colonies with whitish margins. The reverse side of the Petri plate exhibited a yellow coloration and the colony's surface seems to be powdery. Microscopic examination revealed distinctive structures of *Aspergillus niger*. The mycelium of *Aspergillus niger* is septate, branched and characterized by long smooth-walled conidiophores. The vesicle is covered by metulae and phialides. The conidia of *A. niger* are globose in shape and possess a dark brown coloration.

Aspergillus terreus

The fungal colonies displayed a remarkable growth rate, expanding rapidly over the substrate. Their surface had a distinct cinnamon-brown color and a velvety texture, providing a unique visual appearance. Conversely, when the colonies were observed from the reverse side, they exhibited a whitish-brown coloration. The conidial heads of the fungus are densely packed in a columnar shape. The arrangement of the conidia is biseriate, forming two parallel rows. The metulae, which are structures on the conidiophore, are as long as the phialides. The conidiophore stipes are transparent (hyaline) and have a smooth surface. The conidia themselves are round to ellipsoidal. They appear hyaline to slightly yellow and have a smooth outer surface.

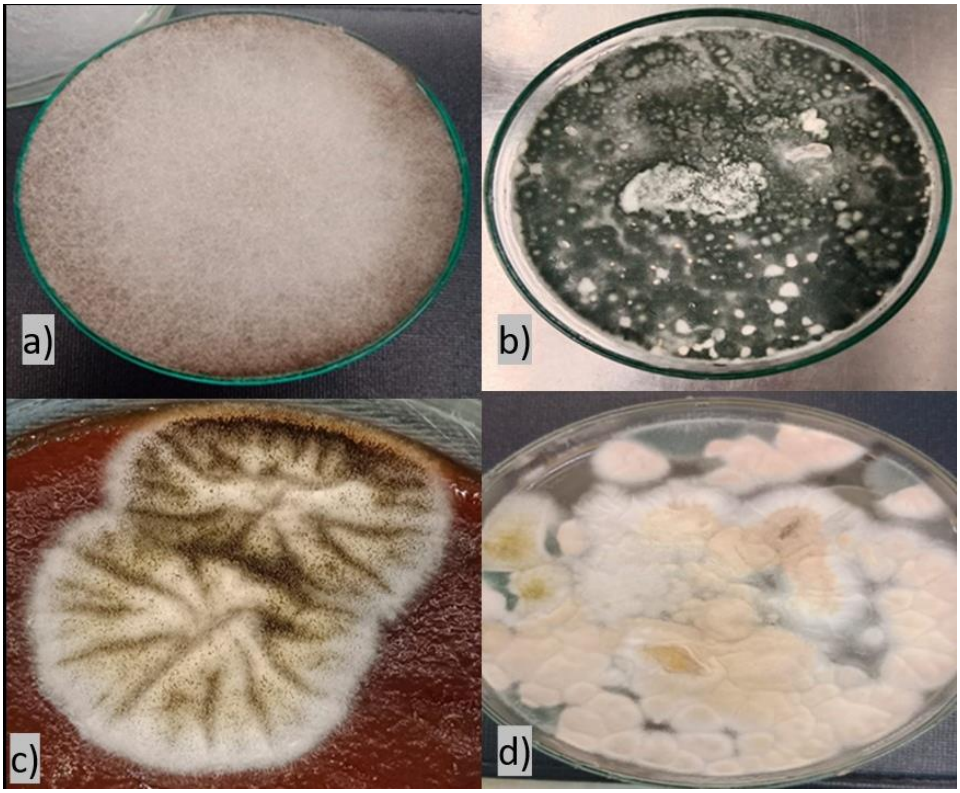

Figure 3. Fungal colonies showing distinct growth patterns: (a) *Rhizopus stolonifer*, (b) *Aspergillus niger*, (c) *Aspergillus terreus*, and (d) *Aspergillus fumigatus*.

Discussion

Fungi are opportunistic organisms that can rapidly develop in favorable temperature and moisture conditions (Ab Majid et al., 2015). They produce abundant spores, allowing them to survive in the environment even under unfavorable circumstances. The amphibian skin provides an ideal habitat for microorganisms, including fungi and bacteria, due to its moisture and nutrient-rich environment derived from mucous secretions and metabolic processes (Culp et al., 2007; Bletz et al., 2017). So the present study was conducted to isolate and characterized the fungus from bullfrog skin.

Fungal species, particularly those from the Aspergillus genus, are known to inhabit a variety of environments, ranging from soil to decaying organic matter. The presence of Aspergillus species on amphibians may reflect environmental exposure, given their ubiquity in terrestrial ecosystems. However, amphibians' moist skin provides an ideal substrate for fungal colonization, and some

species may form part of the natural microbiota of amphibians, potentially contributing to the ecosystem's nutrient cycling through their roles in organic decomposition.

The results obtained in this study showed that the total four species of fungi *Rhizopus stolonifer*, as well as Aspergillus sp. namely *A. niger, A. fumigatus* and *A. terreus,* were isolated from the skin of bullfrogs (*Hoplobatrachus tigerinus*) in District Kasur. Our findings regarding the isolation of fungal species from the skin of bullfrogs are consistent with several published studies on fungal diversity in amphibians. (Douglas et al., 2017) documented the presence of Aspergillus species and *Rhizopus stolonifer* in a study on fungal diversity in the skin microbiota of various amphibians.

The highest isolation frequency was documented for *A. niger* and *A. fumigatus.* Similar composition of cultivable mycobiota was reported by (Peixoto et al., 2020) for the integument of the toad species *Rhinella major* from northern Brazil. This suggests that fungal spores, particularly from the Aspergillus and Trichoderma genera, may be commonly present in close contact with amphibian skin worldwide, making them easily detectable through culture-based methods. However, the isolation frequency of *A. terreus* was found to be very low. While the occurrence of these fungi in amphibians is common, there is currently no evidence linking them to infections in these animals.

Previous research has indicated that the microbial community structure of amphibian skin is influenced by the surrounding environment (Jani & Briggs, 2018). The environment likely serves as a reservoir from which potential skin microbes are recruited. A long-term study conducted by (Falvey & Streifel, 2012) also suggested that it is nearly impossible for any environment to be completely free of Aspergillus spores, making exposure to Aspergillus difficult to avoid. Conidia of Aspergillus species can be deposited on frog skin from the air, as they are lightweight and easily dispersed, and their presence may be more prominent in habitats with greater plant diversity (Samson et al., 2019). It is worth noting that Aspergillus species are not only considered environmental fungi but are also known to produce toxins that can pose health risks to humans and animals (Zain, 2011). However, the present study did not investigate the production of toxins by these organisms.

Our findings on the isolation of *Rhizopus stolonifer* from the skin of bullfrogs align with previous studies that have reported similar fungal species associated with amphibians. For instance, (Stupar et al., 2022) documented the presence of *Rhizopus stolonifer* in a study on fungal diversity in the

skin microbiota of various amphibians. No cases have been reported that evidence of their relationship to these animal infections. It is commonly found throughout the environment, causing different rots in fruits and vegetables and infection in people with weakened immunity (Aslam et al., 2018). However, the present study did not carry out studies related to the production of toxins by these organisms.

Conclusion

It can be concluded that the presence of Aspergillus spp., may have significant effects on health of *Hoplobatrachus tigerinus*. However, more research is needed to determine whether these fungi are benign, symbiotic, or pathogenic in amphibians. Future studies should aim to investigate the specific interactions between these fungi and their amphibian hosts, especially under various environmental stress conditions, such as pollution or climate change. Additionally, expanding the scope of research to include other amphibian species in different regions could provide a more comprehensive understanding of the fungal communities associated with amphibians and their environmental roles. Molecular techniques, such as DNA sequencing, would also enhance the ability to detect and identify fungal species with greater accuracy and assess their potential impacts on amphibian populations. In conclusion, this study highlights the need for continued research on amphibian-associated fungi and their ecological significance. Given the global decline in amphibian populations and the potential role of fungal pathogens in amphibian diseases, this area of research is crucial for both amphibian conservation and broader environmental monitoring efforts.

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