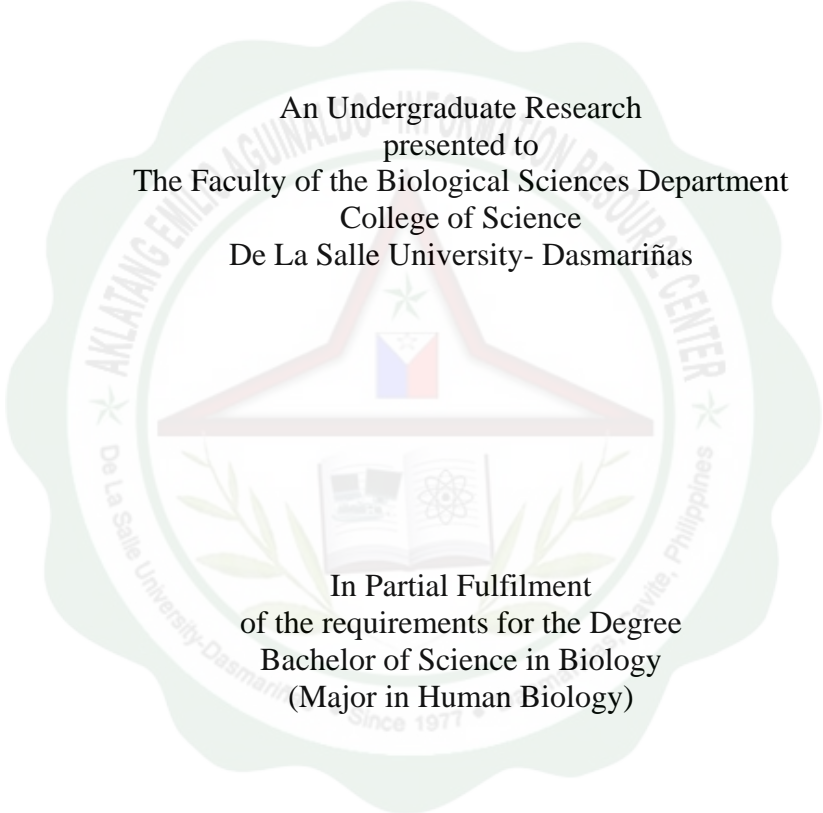


**DETECTION OF CHYTRID FUNGI IN THE ANURAN SPECIES OF MTS.  
PALAY-PALAY, MATAAS NA GULOD PROTECTED LANDSCAPE**



An Undergraduate Research  
presented to  
The Faculty of the Biological Sciences Department  
College of Science  
De La Salle University- Dasmariñas

In Partial Fulfilment  
of the requirements for the Degree  
Bachelor of Science in Biology  
(Major in Human Biology)

Ancheta, Joanna Marie A.

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# DETECTION OF CHYTRID FUNGI IN THE ANURAN SPECIES OF MTS. PALAY-PALAY, MATAAS NA GULOD PROTECTED LANDSCAPE

Author :

Ancheta, Joanna Marie A.

Coauthor: Ronaldo D. Lagat, MS

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## Abstract

This study confirmed the presence of Chytridiomycosis in two species of frogs found in Mts. Palay-Palay Mataas-na-Gulod Protected Landscape. Specimens were collected from the area around the DENR station. A total of 14 individuals were collected on October 2009, belonging to two species namely; *Rana similis* (12 individuals) and *Limnonectes woodworthi* (2). Toe clippings were collected and tissues were processed histopathologically and interpreted by a pathologist. *R. Similis* was observed to have 58% of the samples positive for Chytridiomycosis and *L. woodworthii* was observed to have 50% positive. The presence of chytrid infection was confirmed by the formation of zoospores within the zoosporangium in the *stratum corneum* of the infected frog skin.

## Introduction

Chytridiomycosis is a disease caused by the amphibian chytrid fungus. *Batrachochytrium dendrobatidis* is responsible for widespread global declines in amphibian populations.

*Batrachochytrium dendrobatidis*, the causative agent of chytridiomycosis, has been implicated in widespread amphibian declines. It is currently the largest infectious disease threat to biodiversity. It affects the frogs by interfering with skin functions including maintenance of fluid balance, electrolyte homeostasis, respiration and role as a barrier to infections.

The study was primarily concerned with the detection of fungus in frog species in Mts. Palay-Palay Mataas-na-Gulod Protected Landscape. Specifically, the study aims to achieve the following:

(1) To detect the presence of chytrid fungi in the anurans of Mts. Palay-Palay Mataas-na-Gulod Protected Landscape. (2) To determine what species of frogs are infected with *Batrachochytrium dendrobatidis*. (3) To

determine how many of the individuals are affected by the infection.

The researcher detected the fungi causing disease in the integument system of the frog species in Mts. Palay-Palay Mataas-na-Gulod Protected Landscape.

The main site for collection is on Site 4. It is 300-400 meters above sea level. The DENR station is located here. It also served as a camp and this site is adjacent to the main road which is covered with tall grasses. Inward the forest is the understory consists of a dense growth of shrubs and generally secondary growth forest.

Chytrid fungi invade the *stratum corneum* and *stratum granulosum* only. Therefore, the required organ for the study is the skin particularly in the toe area.

There will be no isolation and identification of the fungus from the internal organs of the species.

The gross and microscopic pathological examinations, treatment of the infection and bacteriological examination

characterization of the skin disease is not included in the study. The origin, type of skin sample and species will be recorded. Random frog species from a specific part of the mountain will be the organism under study.

Decalcified toe clippings were brought to De La Salle University Medical Center Pathology Department to check for the presence of fungi.

Hematoxylin and Eosin staining is used in the histological process.

Amphibian such as frogs plays an important role in the ecosystem. They act as predator and prey. The tadpoles act as filter feeders that play an important role in aquatic ecosystems. Adult frogs regulate insect populations that can be a vector of a disease or pests on crops. Their decline in number and extinction signals that the biosphere is changing.

Chytridiomycosis is an emerging disease is a major threat to survival of the amphibians.

Through this study, increased awareness on Chytridiomycosis will be achieved. It will help expand understanding the causes of declines and extinction in frog species.

Anuran conservation and protection is the key to its survival. It will help in getting the protected area effective conservation measures and control the harvesting of the species to help prevent decline in population.

The study involved is a Descriptive Research Design concerned with Diagnosis of Chytridiomycosis. The method of data collection that will be used is Monitory method including library collection, observation, histological process and slide preparation.

It is a descriptive study wherein the control of variables will not be manipulated so the sample sizes and number of

representative species per test organisms is random. These subjected random organisms that are candidate to toe clipping are taken from Anurans that are situated in Mts. Palay Palay Mataas na Gulod Protected Landscape.

Non Random sampling was used in this study. The sample focuses on easily available units or those that just happen to be present when the research is done. Convenience sample is also called accidental sample. The researcher selects units that are convenient, close at hand and easy to reach.

This method is useful for quick and cheap studies, case studies, qualitative research, pilot studies and for developing hypothesis for future research.

Mts. Palay Palay, Mataas na Gulod Protected Landscape has the coordinates of 121°51' East longitude and 14°16' North latitude (Lagat 2009), it is situated within the municipalities of ternate and Maragondon in Cavite and Nasugbu in Batangas.

The main site for collection is on Site 4. It is 300-400 meters above sea level. The DENR station is located here. It also served as a camp and this site is adjacent to the main road which is covered with tall grasses. Inward the forest is the understory consists of a dense growth of shrubs and generally secondary growth forest (Causaren, 2009).

Collected specimens underwent histotechnique. It includes 1.) Smear Preparation, 2.) Tissue preparation, fixation and preservation, 3.) Dehydration, clearing and paraffin impregnation, 4.) Embedding, Sectioning and affixing sections on slides, 5.) Staining and Coverslipping.

## Results and Discussion

Table 4.1 Frogs collected from Mts. Palay – Palay, Mataas na Gulod Protected Landscape and analyzed for *Batrachochytrium dendrobatidis*

Species	Positive(+)	Negative (-)
<i>Rana similis</i>		
Total	$\frac{7}{12}$	$\frac{5}{12}$
	$\times 100 = 58.33\%$	$\times 100 = 41.67\%$
<i>Limnonectes woodworthi</i>		
Total		
<i>Limnonectes woodworthi</i>	$\frac{1}{2}$	$\frac{1}{2}$
	$\times 100 = 50\%$	$\times 100 = 50\%$

A total of 14 organisms from two different species were captured on one night sampling-12 organisms belonging to *Rana similis* and 2 organisms belonging to *Limnonectes woodworthi*.

Seven out of the twelve slides belonging to *Rana similis* were positive for the chytridiomycosis while there are five slides are negative for the infection. Out of the two *Limnonectes woodworthi* slides, only one is positive for the chytridiomycosis infection.

The collected species *Rana similis* and *Limnonectes woodworthi* are endemic to Luzon (Causaren 2009).

Chytridiomycosis is caused by the chytrid fungi *Batrachochytrium dendrobatidis* (Kilpatrick A. et al. 2009). Chytrids are ubiquitous fungi that develops without hyphae. It is found in moist soil and aquatic habitats where they degrade cellulose, chitin and keratin ( Dazsak et al, 1999).

The presence of zoosporangia and zoospores are signs of infection (Berger et al. 1999) Zoosporangia or thalli and zoospores were detected on the toe clippings. Absence of these structures means it does not qualify to be diagnosed as positive for the infection.

Zoospores are basophilic bodies; round or oval in structure. Zoosporangia are eosinophilic to slightly basophilic. These structures are spherical in shape and wall off the zoospores. Through the discharge openings, the zoospores escape is not readily visible on Hematoxylin and Eosin stained slides. (Longcore et al. 1999)

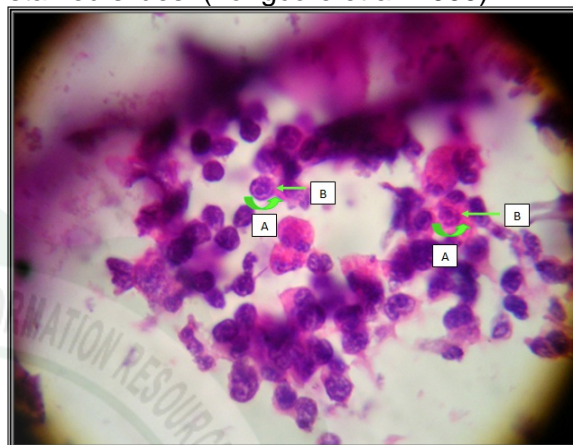


Figure 4.2 above shows the cells of frog toe clipping in Oil Immersion Objective. It is highly infected with chytridiomycosis. Spherical Zoosporangium or thalli is labelled as A whereas the zoospore is labelled B. The inside part of the zoospore is seen.

Diagnosis of *Batrachochytrium dendrobatidis* done by observing the shape of the zoosporangium. It can be spherical, balloon shaped or collapsed sphere. The nature of the wall is also considered as a basis. It can be thin, has a uniform smooth outer and inner surfaces. Contents of the zoosporangium are also noted. It varies from bacteria, single central body and zoospores. The size is useful for distinguishing zoosporangia. It is the guide for differentiating other bodies in the stratum corneum. The size of the zoosporangia is less than 15  $\mu$  m in diameter.

The observed toe clipping can be qualified as positive when the observed structure is highly spherical in the stratum corneum. It has a discharged papilla projecting from the surface. This structure is the zoosporangium. The walls must have a smooth inner and outer layer. The thickness must be uniform. Its characteristic when

stained can be eosinophilic and slightly basophilic. Keratin may be present in variable amounts surrounding the organism. The contents of the zoosporangia vary depending on the developmental stage of the chytrid. Four stages can be identified through the histological sections. Early stage has a central mass which is basophilic and roughly spherical or oval. This mass is homogenous in staining characteristics and divides to form zoospores. Zoospores are basophilic and appears as round or oval bodies in cross section. The margin are poorly defined. When the zoospores are released via the discharged papilla, the empty zoosporangium retains its spherical shape. Thin septae may divide the zoosporangium into internal compartments in some empty stages. The next stage is the collapse of the empty zoosporangium. It turns into an irregular shape. In this terminal stage, bacteria sometimes colonise the empty shell. These are seen as basophilic rods or cocci in the section of the empty zoosporangium. Bacteria can be mistaken as zoospores. Bacteria are less numerous and smaller than zoospores. Frogs in terminal stages of chytridiomycosis, large number of bacteria may be observed between layers of sloughing keratin. The most common stage present are the empty zoosporangia particularly in the layer of the sloughing surface. Empty zoosporangia must be looked for carefully for it may be missed. A useful guide for detection when examining is the presence of clear spaces in the epithilium surface. Examination should be made at high power and oil immersion when these are seen (Berger *et al.* 1999).

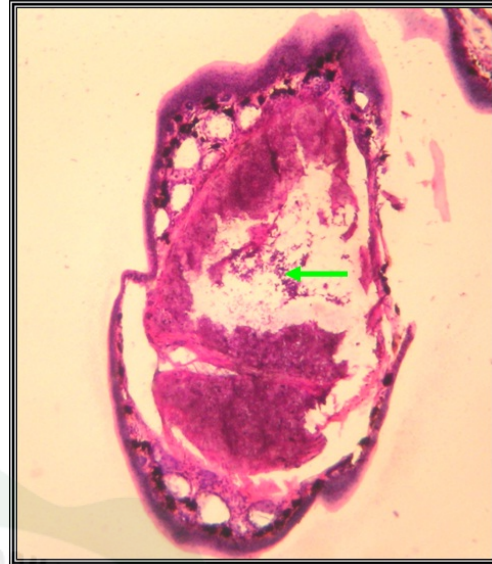


Figure 4.3 shows the low power magnification of the frog's toe clipping, particularly on the nail part.

The arrow is pointed at the suspicious part where there is a possible presence of zoosporangia.

Suspicious areas or organisms are scanned at x200 power and easily detected by experienced observers. To confirm the diagnosis, these suspicious areas should be examined at x400 power. When in doubt of the identity of the suspicious structure, examination should be done under oil immersion or x1000 power. This will improve the confidence of the decision. Oil immersion is not required in heavy infections. In light infections with few chytrids, oil immersion may be required to achieve definite diagnosis (Berger *et al.* 2000).

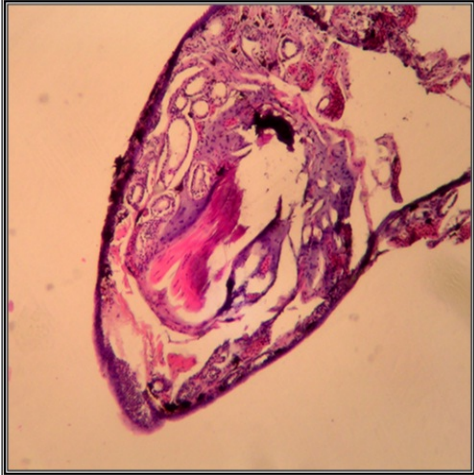


Figure 4.4 above shows low power magnification of a frog's normal toe clipping.

In a normal toe clipping, absence of zoosporangia and zoospores were observed. Therefore it is negative for chytridiomycosis infection. Six organisms of the collected specimens were negative for chytridiomycosis. Of all the fourteen slides which have frog toe clippings, a total of eight slides were observed and showed evidence for chytridiomycosis.

Based on the values of the result, the computed percentage for the *Rana similis* chytridiomycosis infection in Mts. Palay - Palay, Mataas na Gulod Protected Landscape is 58.33%. Its prevalence among the collected samples of anurans affects more than half the number of *Ranasimilis*. The percentage of infection for *Limnolectes woodworthi* on the site is 50% on the site.

The species of *Rana similis* is highly infected with chytridiomycosis, with the percentage of 58.33% as well as *Limnolectes woodworthi* with 50%. These values indicate that the samples collected are threatened due to the high prevalence of chytridiomycosis among the species gathered from Site 4. These results apply to the populations of frogs in Site 4 only where the prevalence of chytridiomycosis was identified.

Chytridiomycosis exists on the anurans of Mts. Palay – Palay, Mataas na Gulod Protected Landscape during the wet

season of 2009. *Rana similis* and *Limnolectes woodworthi* toe clippings were collected from Site 4. These species are endemic to Luzon.

Seven out of eight *Rana similis* samples were positive for the infection while one out of two *Limnolectes woodworthi* were negative for chytridiomycosis.

It is recommended to use at least thirty frog samples. Extend the area or site of the study to locate more of the different frog species found in Mts. Palay-Palay Mataas na Gulod Protected Landscape.

Polymerase Chain Reaction is recommended for definitive diagnosis. Research on the ability of *Batrachochytrium dendrobatidis* to survive in a laboratory culture. The skin on ventral part of frogs is recommended as the target organ. Higher probability of infection from this target organ is expected for it is in contact with the ground.

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