FINAL REPORT FOR THE AUSTRALIAN GOVERNMENT DEPARTMENT OF THE ENVIRONMENT AND HERITAGE

# A project that designs and trials a pilot survey to map the distribution of chyridomycosis (caused by the amphibian chytrid fungus) in Australian frogs.


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Cover images: photo of *Litoria wilcoxii* by D. Hall; distribution map by James Cook University.

 This report should be cited as: Speare R, Skerratt L, Berger L, Hines H, Hyatt AD, Mendez D, McDonald KR, Hero J-M, Marantelli G, Muller R, Alford R, Woods R. (2005). ***A project that designs and trials a pilot survey to map the distribution of chyridomycosis (caused by the amphibian chytrid fungus) in Australian frogs..*** Final report for project ID 44381 (tender 63/2003) to the Australian Government Department of the Environment and Heritage.

The views and opinions expressed in this publication are those of the authors and do not necessarily reflect those of the Commonwealth Government or the Commonwealth Minister for the Environment and Heritage. This project was funded by the Australian Government Department of the Environment and Heritage through the national threat abatement component of the Natural Heritage Trust.

Australian Government
Department of the Environment and Heritage

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## Executive Summary

The aim of this tender was to design and trial a pilot survey to map the distribution of chytridiomycosis in Australian frogs. The project has 5 components: Scope Items 1 and 3 being desk top synthesis and evaluation activities, Scope Item 2 being a data gathering and proof of concept activity, Scope Item 4 being a field and laboratory based project testing the protocol developed in Scope Item 3, and Scope Item 5 assessing and evaluating the previous outputs and providing recommendations that need to be addressed to allow a national survey for chytridiomycosis to proceed in an effective manner.

***Diagnostic Tests:***
An analysis of published and unpublished literature identified that chytridiomycosis could be diagnosed by at least 11 tests, that can be collapsed into 5 categories; culture, microscopical examination of unstained epidermis, histology, capture ELISA and PCR. Although each diagnostic test has detection of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), as its final outcome and could on superficial assessment appear to be competing with each other, we discovered that this was true only in part. The tests have different functions and complement, rather than exclude, one another. For example, examination of fresh tissue is an essential test when culture of *B. dendrobatidis* from infected skin is being attempted in the laboratory. Histology is the diagnostic test of choice for retrospective surveys of archived specimens, and the sensitivity of diagnosis is increased by immunohistochemistry using polyclonal antibodies. For the proposed mapping survey, real-time PCR is the recommended diagnostic technique owing to its increased sensitivity and non-invasiveness.

An estimate of costs revealed that any diagnostic test will have direct costs of at least $12.50 per sample, with real time PCR at $30.75, being almost two and a half times more expensive than the lowest test. The real-time PCR direct cost estimate also does not include royalty, payable on commercial use of the test. The test with the lowest cost is histology, but if labour costs are ignored, the microscopic examination of skin sloughs or digital web, has the lowest costs for consumables.

***Review of Current Mapping Protocols:***
Review of the literature and personal enquiries to colleagues overseas revealed that no country had developed a protocol for systematically mapping the distribution of chytridiomycosis at a regional or national level. This highlights the global significance of this study.

***Feasibility of Snapshot Distribution Map for Chytridiomycosis:***
Creating a snapshot distribution map was easily achieved. Collation of data into a national database was more difficult due to problems with the current database structure being inadequate, inability of some researchers to locate data and concerns from some researchers about making unpublished data available to the general public.

***Development of a Mapping Protocol:***
We developed an adaptable protocol to map chytridiomycosis in any region of Australia inhabited by amphibians. The recommended diagnostic test is the real-time PCR with samples obtained by swabbing amphibians using a standardised technique. The chosen end point of the survey is presence / absence of *B. dendrobatidis* to detect a prevalence of 2% and greater in the selected population or area. For broad scale sampling, Australia should be surveyed using the 65 interim biogeographic regions. Within these regions the survey strategy will classify amphibians into four categories or ecological guilds and select amphibians for sampling from at least two of the guilds. The four ecological guilds are; Stream Breeders, Permanent Water Body Breeders, Ephemeral Breeders, and Terrestrial Breeders. To be confident of detecting the desired minimum prevalence, 149 animals need

to be sampled from each guild. Other criteria which need to be considered in surveying for chytridiomycosis are season, life cycle stage and availability of amphibians. Sick and dead frogs should always be sampled as their probability of being infected is roughly 50%. A survey of all 65 interim biogeographic regions will require testing of 19,370 specimens (65 regions x 2 guilds x 149 samples) at a total estimated cost of $595,627, using the estimate of $30.75 direct cost per sample. However, if the prevalence of chytridiomycosis detected by real-time PCR is higher than that detected by histology, the number of samples required to be tested may be reduced. In addition if the current predictive model of the distribution of chytridiomycosis is validated then areas unlikely to be infected do not need to be tested when they are surrounded by uninfected areas, which are more likely to harbour *B. dendrobatidis* based on their climate and habitat.

***Testing the mapping protocol*:**

Batching for PCR. The first stage of testing the mapping protocol was to evaluate whether PCR testing of samples in batches was a cost effective strategy. In one trial using specimens collected from a known positive area, batch testing in units of five gave a negative result although prevalence on individual testing was at least 5% and possibly 10% if suspicious positives are counted. All intensities of infection were <1 zoospore equivalent. It appears that batch testing of amphibian populations with low intensity of chytridiomycosis may lead to false negatives. Hence, at this stage it is not recommended for mapping surveys.

Comparing PCR and histology. Trials by Alex Hyatt et al. at AAHL had shown that in experimentally infected frogs, PCR was more sensitive in lightly infected frogs than histology. In surveys in north Queensland and southeast Queensland of wild frogs in which a toe tip was used for histology and a swab for PCR, the PCR test was approximately 6 times more sensitive than histology. Hence, the recommendation to use the real-time PCR test as a survey tool was supported by this result.

To further support the increased sensitivity of the PCR test for use on wild populations, comparing current and historical results are informative. In the Wet Tropics past surveys of clinically normal frogs using histology of top tips had found overall prevalences of 7%. Using the PCR test of swabs collected from frogs from the Wet Tropics, including many of the sites sampled in the past, the overall prevalence was 61%, a result consistent with PCR being a much more sensitive test than histology in these populations with low intensity of infection.

Field testing of the proposed mapping protocol. The mapping protocol is feasible. The most significant problem in field trials was obtaining sufficient samples for testing. However, since the prevalence of chytridiomycosis in positive populations tested by PCR is higher than that shown by histology, it seems appropriate to move the minimum threshold upwards from 2% to 5% or possibly even 10%. The protocol required that 149 frogs be tested to detect one positive frog if prevalence was 2% or higher. To detect a 5% and 10% or higher prevalence, 59 and 30 frogs respectively should be tested. Since these numbers of samples are more feasible, we recommend that 60 frogs be tested using the real-time PCR to detect one positive frog when the prevalence is 5% or greater.

***Gaps in knowledge that should be addressed to improve mapping survey:***

The cost/efficiency of batch testing versus loss of sensitivity needs to be evaluated. The best method of swabbing different species of frogs for PCR testing needs to be determined. There is also a need to confirm that swabs collected in the field can be stored at ambient temperatures in the field and back in the laboratory without loss of sensitivity. There is a need to obtain more evidence that the prevalence of *B. dendrobatidis* when present is at least or greater than 5% in Australian frog populations using the real-time PCR test. The feasibility of surveying tadpoles instead of adult frogs to map *B. dendrobatidis* in amphibian populations needs to be tested for more species. The current

predictive model of the distribution of *B. dendrobatidis* in Australia is largely based on potentially biased data and needs to be validated.

***Publications from this Tender***

To date (December 2005) three publications have been generated by this project and others are in preparation. The three publications are in press. Kriger et al compare real-time PCR with histology in a natural population and demonstrates the greater sensitivity of the former test. Berger et al describe the distribution of Bd on the skin of severely infected green tree frogs and highlights the importance of sampling skin from the feet and ventral surface for the best sensitivity. Webb et al demonstrate that the standard amphibian anaesthetic, MS-222, does not kill Bd and can be safely used on frogs in experimental studies on chytridiomycosis.

***Recommendations:***

Recommendations that need to be addressed to allow the survey protocol developed in Stage 3 to be adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus fall into four domains.

**Dissemination and Concensus:** To be adopted nationally the survey protocol should be disseminated to individuals and groups within Australia most likely to undertake the work and those most likely to fund such surveys. Dissemination has already commenced through conference presentations in 2004 and 2005. Publication in scientific journals is the next key step, particularly the proof of concept study reviewed in section 5.1. Three papers relevant to diagnosis of chytridiomycosis are currently in press from this tender and others are in preparation. The next step is to achieve concensus among key stakeholders. A national workshop to discuss the implementation of the protocol would assist this. The workshop could be run in conjunction with a conference such as the Australian Society for Herpetology in April 2006. Groups and individuals likely to be interested in participating could be invited such as the Victorian Frog Group, Frog Decline Reversal Project, other community based frog groups and wildife managers at state, territory and national levels. The workshop should also include training for aspects of the protocol that require skill and consistency such as swabbing frogs and tadpoles. This workshop could also be used to accredit the competency of individuals and groups to carry out survey work.

**Funding for a National Survey:** Funds should be provided to undertake the national survey. A national survey using the protocol proposed will involve testing of 19,370 specimens and an approximate cost of $600,000 for real-time PCR tests. This estimate does not include the costs associated with sample collection. Field based costs vary with the remoteness of the sites sampled and the availability of amphibians.

**Quality Assurance of Diagnostic Results:** Since multiple laboratories are now performing diagnostic tests for chytridiomycosis, ensuring a high standard of sensitivity and specificity is essential for confidence in reported results. The most critical element is implementation and ongoing support for a quality assurance (QA) system. The two key tests are real-time PCR and histology. The QA system could have both proactive and passive components. The proactive component could involve twice yearly dissemination of samples from a central body to diagnostic laboratories that test the samples without being aware of their status. The actions to follow incorrect results would have to be determined by concensus among the participating laboratories or could be proscribed by the body funding the scheme. For PCR tests, swabs and DNA extracts could be sent out to participating laboratories. For histology, stained and unstained slides of amphibian skin could be distributed. The passive component of the QA scheme could consist of laboratories submitting a proportion of positive diagnoses, particularly highly critical ones such as new species and new locations, and some negative ones for confirmation. Although participating laboratories should pay to participate in such

a QA scheme, DEH should consider providing funding for its initial establishment for 5 years. Funding will be required initially as start-up costs will involve establishing protocols, credibility of a QA scheme and incentives for laboratories to participate. Laboratories in New Zealand should be asked to participate and the scheme could possibly be extended to other countries. Extension of such a QA scheme beyond Australia will increase its reputation and validity, even within Australia.

**Data Management:** The current database should be updated regularly and a web based interface for data entry should be implemented. The knowledge and skills to do this are located within the Australian Wildlife Health Network (AWHN) and the Amphibian Disease Ecology Group (ADEG) at JCU. The AWHN already have a web data entry and database system (Wildlife Health Information System (WHIS)) in place to capture wildlife health information and this could be adapted to capture information on chytridiomycosis. We recommend that funding be provided to employ a database programmer located within the AWHN who can adapt WHIS for chytridiomycosis reports. We also recommend that a database manager be employed for three years to ensure that all available data on chytridiomycosis testing of wild amphibians is captured and analysed to provide relevant information on the distribution of chytridiomycosis both spatially and temporally for each species of amphibian and that a predictive model for the disease is produced to enable mangers to take preventative action to stop the spread of *B. dendrobatidis*.

## Scope Items

**Objective 1.1: Undertake a comprehensive audit and analysis (including contacting relevant government agencies, researchers and industry organisations) of the current tools, techniques and practices for surveying and mapping chytrid fungus both in Australia and in other countries.**

Activity 1.1.1: Assemble published and unpublished literature on diagnostic tests and techniques for surveying and mapping chytridiomycosis.

Activity 1.1.2: Conduct an audit of practices in other countries on mapping chytridiomycosis.

**Objective 1.2: This audit and analysis will include providing a clear description of each of these current tools, techniques and practices. It will also discuss the advantages and disadvantages of each tool, technique and practice along with relative costs.**

Activity 1.2.1: Clear description of current diagnostic techniques, survey techniques and practices will be provided.

Activity 1.2.2: The analysis of advantages and disadvantages and relative costs will be made based on available evidence.

**Objective 1.3: Provide a bibliography of the reference material audited.**

Activity 1.3.1: Provide one copy of all literature used in the audit as best quality photocopies organised by first author.

Activity 1.3.2: Provide an electronic copy as PDF files on CD ROM of all literature used in audit.

Activity 1.3.3: Provide an electronic copy on CD ROM of an Endnote database containing all reference material.

**Objective 2.1: Determine if the data that is currently available to map the distribution of chytrid fungus in Australia could be (a) brought together and stored on a single national database and (b) used to create a snapshot map of the current distribution of chytrid fungus in Australia.**

Activity 2.1.1: Review current national database held by Amphibian Diseases Group at School of Public Health and Tropical Medicine to identify likely locations of additional records and feasibility of obtaining these records.

Activity 2.1.2: Review best technique for creating a snapshot map from this database.

**Objective 2.2: If it is determined that (a) and (b) of the point above can be achieved, undertake this task so that a national database on the distribution of chytrid fungus in Australia is created. Ensure that permission is obtained from the original data custodians for their data to be used to produce the national snapshot distribution map, noting that the map will be made available to the public.**

Activity 2.2.1: Identify and approach owners of additional records currently not included in national database held by Amphibian Diseases Group at School of Public Health and Tropical Medicine for permission to include their data.

**Objective 2.3: Provide the national database to an appropriate institution/agency. The role of this institution/agency will be to ensure the safe keeping and possible future updating of the database. It will also produce and publish (via the internet) the national snapshot distribution map which is to be made available to the public free of charge.**

Activity 2.3.1: Amphibian Diseases Group at SPHTM will update the national database and publish on the internet the current national snapshot map available free of charge to the general public.

Activity 2.3.2: Investigate in collaboration with the Australian Wildlife Health Network (AWHN) the feasibility of establishing a national surveillance system which involves collection, collation and analysis of data and timely distribution of information for action.

**Objective 2.4: Identify the limitations of producing the distribution map using the process outlined in the points above.**

Activity 2.4.1: Identify the next steps in developing a more sophisticated and interactive distribution map on the Internet.

**Objective 3.1: Design feasible survey protocols with sufficient power for surveying all amphibian populations in Australia to determine whether they are infected or not infected with chytrid.**

Activity 3.1.1: Design survey protocols to detect presence/absence at a cut off prevalence of 2%.

Activity 3.1.2: Design survey protocols that address 1) most appropriate diagnostic tests, 2) minimum number of amphibians to sample, 3) season of sampling to optimise chance of finding both frogs and *B. dendrobatidis*, 4) species to sample, 5) life stage to sample, 6) combining results from different species at sites, 7) choice of site, 8) spacing of sites in a region, 9) randomization of sites and individuals, 10) feasibility of protocols for a wide-range of Australian amphibian populations.

**Objective 3.2: In designing the survey protocols, include a process that will compare the efficiency, effectiveness and costs of the main diagnostic tools used to detect chytrid fungus.**

Activity 3.2.1: Include in survey protocols a comparison of the cost-efficiency, ease of use and feasibility of the diagnostic tests.

**Objective 4.1: Test the survey protocols designed in Stage 3 in the field by undertaking a pilot survey.**

Activity 4.1.1: Investigate the role of batching samples as a cost effective strategy in determining presence / absence of chytridiomycosis in amphibian populations.

Activity 4.1.2: Test the survey protocols in a range of amphibian populations in a range of environments to determine suitability of protocols as recommended national standards.

Activity 4.1.3: Provide a report addressing Objectives 3 and 4.

**Objective 5.1: Review the pilot survey and identify and prioritise the gaps in existing knowledge concerning the effectiveness of the survey protocol developed in Stage 3 and provide recommendations as to areas of future research activity that will address those gaps identified.**

Activity 5.1.1: Review results of pilot survey and make recommendations about areas of future research needed to address gaps in knowledge.

Activity 5.1.2: Discuss the possibility of the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.

Activity 5.1.3: Provide comment on the suitability of the survey protocols for a standard way of mapping amphibian populations in Australia for chytridiomycosis.

**Objective 5.2: Based on the discussion above, provide recommendations that would need to be addressed to allow the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.**

Activity 5.2.1: Provide recommendations that would need to be addressed to allow the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.

Activity 5.2.2: Final report will contain an executive summary of the work undertaken and key findings of project.

# 1. Scope Item 1

**Objective 1.1: Undertake a comprehensive audit and analysis (including contacting relevant government agencies, researchers and industry organisations) of the current tools, techniques and practices for surveying and mapping chytrid fungus both in Australia and in other countries.**

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## 1.1 Comprehensive Audit and Analysis of Diagnostic Tests

Chytridiomycosis can only be diagnosed by laboratory tests. Healthy infected frogs show no signs of disease, and the clinical signs of severe chytridiomycosis are non-specific with similarities to those caused by other diseases, such as iridoviral infection and bacterial septicaemia ("red leg") (Cunningham et al. 1996). Hence laboratory tests are needed to make a diagnosis in sick frogs and frogs showing no clinical signs.

Diagnosis of chytridiomycosis involves the identification of *B. dendrobatidis*, either by light microscopy to visualize sporangia or by polymerase chair reaction (PCR) to detect DNA. Culture is difficult and is not used for diagnosis. ELISA protocols have been developed but are comparatively insensitive and non-specific compared to PCR (A. Hyatt, unpub). Electron microscopy is used mainly for research applications and is not used routinely for diagnosis.

Microscopy includes examination of wet skin preparations (scrapings, smears or whole skin), histology on skin sections, and immunohistochemistry on skin sections. These routine tests have a high positive predictive value and when used on diseased frogs with heavy infections also have a high sensitivity. However, healthy frogs typically have light infections and only small samples can be obtained without sacrificing the animal. As the PCR tests are more sensitive than microscopy (Boyle et al. 2004; Annis et al. 2004), they are rapidly replacing the use of microscopy particularly for surveys of large numbers of healthy, live frogs. Real-time PCR is highly sensitive and can detect *B. dendrobatidis* within one week of experimental infection. PCR on toe samples was 8 times as sensitive as histology in early infection (Boyle et al. 2004). It is a quantitative test, giving an indication of the levels of infection (Boyle et al. 2004). However each technique requires different skills and facilities and may be appropriate under different situations.

Here we describe methods to diagnose the presence or absence of *B. dendrobatidis* in skin of healthy, sick or dead frogs.

### 1.1.1 Sampling

Sampling for diagnosis of chytridiomycosis requires knowledge of the anatomical sites most heavily infected and an understanding of the test being performed. The type of test determines how samples should be collected, transported and processed.

### 1.1.2 Sites of Infection

***Adults:***
In adults *B. dendrobatidis* is restricted to superficial keratinized epidermis. Infection in frogs with severe chytridiomycosis is heaviest on ventral surfaces of the feet, abdomen and limbs (Berger et al. 2005; see Appendix 1). Sampling can be done by collecting skin scrapings or smears, excising pieces of skin, or by swabbing. Toe-webbing, and/or toes can be excised from live or dead frogs, and strips of skin from the inguinal area (pelvic patch) can be collected from dead animals. Details on swabbing are contained within the section on PCR.

***Tadpoles:***
The mouthparts of apparently healthy tadpoles can be infected with *B. dendrobatidis* (Berger et al. 1998). Infected tadpoles display abnormalities of the jaw sheath and tooth rows (Fellers et al. 2001), which may be visible grossly. These abnormalities are indicative of infection, and diagnostic tests should be done to confirm chytridiomycosis, as there are other causes of these abnormalities (Rachowicz 2002). For microscopy whole tadpoles can be collected and preserved. Tail stumps in metamorphs are a sensitive site to sample (Marantelli et al. 2004). Swabbing, which is non-

destructive and therefore useful in monitoring the status of threatened or captive populations held for breeding, can be used to sample tadpole mouthparts for PCR (Hyatt, Boyle and Olsen, unpub). The prevalence of chytridiomycosis in tadpoles may be high in some populations with long lived tadpoles, and sampling tadpoles may be a sensitive way of assessing whether *B. dendrobatidis* is in a water body (Berger et al. 1999).

### 1.1.3 Collection of Samples for Histology and Immunohistochemistry

Amphibians degenerate rapidly after death. To preserve morphology the animal must be kept cool if not immediately transferred into an appropriate fixative (10% buffered formalin or 70% ethanol for light microscopy).

Although the quality of the morphological preservation is reduced in frozen, decomposed or mummified animals, it may still be possible to detect the presence of the fungus by histopathology and immunohistochemistry. These techniques work well on formalin-fixed paraffin embedded samples and museum samples, however, if samples have been stored in formalin for excessive periods of time immunohistochemical studies may be unsuccessful (Hyatt, Boyle and Olsen unpub). However, the oldest global record of 1938 was confirmed by an immunoperoxidase technique (Weldon et al. 2004).

### 1.1.4 Collection of Samples for PCR

A range of samples can be collected for diagnosis by PCR, including those collected for microscopy such as toe–clips, skin scrapings or excised strips of skin from the webbing or inguinal regions. Fresh, frozen or ethanol-fixed samples are preferable. Fixation in formalin for more than a short time (< three months) will prevent the detection of *B. dendrobatidis* by real-time PCR. This holds for both formalin-fixed-paraffin-embedded sections and ‘bulk’ skin samples (Hyatt and Boyle, unpub).

In addition swabs and filtrates collected non-destructively can be tested by PCR. The use of swabs (e.g. Medical Wire & Equipment Co (UK) MW 100-100 sourced from Biomirieux Australia) is as sensitive as any other protocol for the detection of *B. dendrobatidis* (Hyatt, Boyle and Olson unpub). The underside of the feet (especially webbing), limbs and abdomen should be swabbed twice. Swabs appear to be the best method of sampling in the field because fixatives or additional solutions are not required, and permits for toe-clipping are becoming increasingly difficult to obtain. Samples can be stored at 23°C for one month, although < 4°C is preferable (Hyatt unpub).

Great care is needed to prevent cross-contamination between samples because the PCR assay detects tiny amounts of DNA, which may remain even after sterilization. New gloves for handling an animal and new instruments for collecting each sample must be used.

### 1.1.5 Sampling Populations and Individuals

***Populations:***
Sampling of animals is a complex procedure. For example, if populations are to be surveyed for the presence/absence of *B. dendrobatidis* then any of the techniques will yield pertinent information provided the correct number of animals is sampled. For example, to determine whether *B. dendrobatidis* is present or absent in a population, a sampling protocol must be used that will detect the lowest level of infection expected with a high degree of likelihood. According to DiGiacomo and Koepsell (1986), to achieve a 95% probability of detection, 149 animals must be tested to detect one

infected animal in a population that has a 2% infection rate, assuming random sampling. To process this number of samples the applied assay must have the capacity of accommodating large numbers in a short period of time (high throughput), such as real-time TaqMan PCR

More than one positive test per frog population may be needed, this will depend on both the estimated prevalence of chytridiomycosis and the false positive error rate of the test.

To optimize the chance of detecting infection, sampling should be planned for a time when frogs will be readily available and temperatures will be between 17°C and 25°C, or as close to being within this range as possible.

The sampling of populations is discussed more extensively in Section 3.1.

***Individuals:***
Sampling of individual animals invokes a different set of rules. Although the different assays vary in sensitivity and specificity, false negatives may occur with any test if the sampling procedure misses the site of infection or if the epidermis has sloughed just before sampling (Boyle et al. 2004). Therefore, to increase the accuracy of diagnosis of a single animal when there is a high suspicion of chytridiomycosis, samples should be collected at least a second time if results from the first sample are negative.

## 1.2 Identification of *B. dendrobatidis*

### 1.2.1 Skin Scrapings and Smears

Examination of skin scrapings or smears by light microscopy is a quick and simple method of diagnosis, and can be done on fresh, frozen or fixed samples. With some practice, accuracy of diagnosis in frogs with severe chytridiomycosis is similar to that when using histology (Berger 2001), and suitable samples are easily obtained from such frogs. Shedding skin is lifted or scraped off the frog, using a scalpel or sterile plastic spoon (Berger 2001; Briggs and Burgin 2003), spread out flat on a slide with a drop of water, a cover-slip is placed on top and the preparation is examined under a compound light microscope. Ideally, an even monolayer of keratinized epidermal cells is obtained. Magnification of 100x is used initially to scan a section, then 400x is used to confirm the presence of sporangia. The refractile walls of the sporangia are more distinctive if the condenser is racked down.

The round to oval intracellular sporangia (5 - 13 μm) occur in clumps. Old empty sporangia are the most prevalent stage in shedding skin, although sporangia containing zoospores are commonly found (Fig. 1). Discharge tubes usually point perpendicularly to the skin surface and thus appear as small circles, which can be difficult to discern. The observation of internal septa within sporangia increases confidence in the diagnosis. Epidermal cell nuclei are a similar size to sporangia but can be differentiated by their irregular, indistinct membranes and flat, granular, grey appearance.

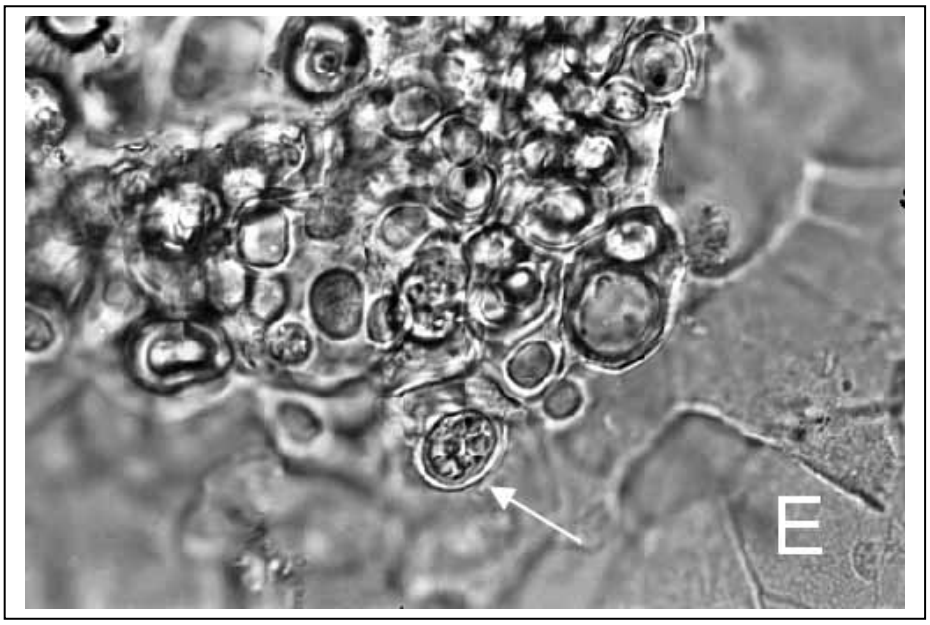



***Figure 1:*** Unstained wet mount of shedding skin from an infected adult of *Litoria caerulea.* Note refractile round and oval sporangia. Most are empty but one contains developing zoospores (arrow). E = epidermal cell.

The advantages of wet preparations are that preparation of the slide is much quicker and cheaper than preparing a histological section, and this method allows a non-invasive ante mortem diagnosis. A disadvantage is that interpretation is much more difficult for an inexperienced worker. Although a larger surface area of skin can be checked by examination of skin scrapings or whole skin, compared to a 5 µm histology section, sporangia may be more difficult to identify.

***Tadpoles – Wet Preparation:***

Likely infected hosts can be identified in the field by the presence of focal dekeratinization of the jaw sheaths, which can be seen with the aid of the 10X magnification of a hand lens (Fellers *et al.* 2001, Rachowicz 2002, Rachowicz and Vredenburg 2004) (Fig. 2). Tadpole mouth-parts can then be examined by cutting off pieces of the pigmented horny jaw sheaths or teeth rows and squashing under a cover-slip (Fig. 3).

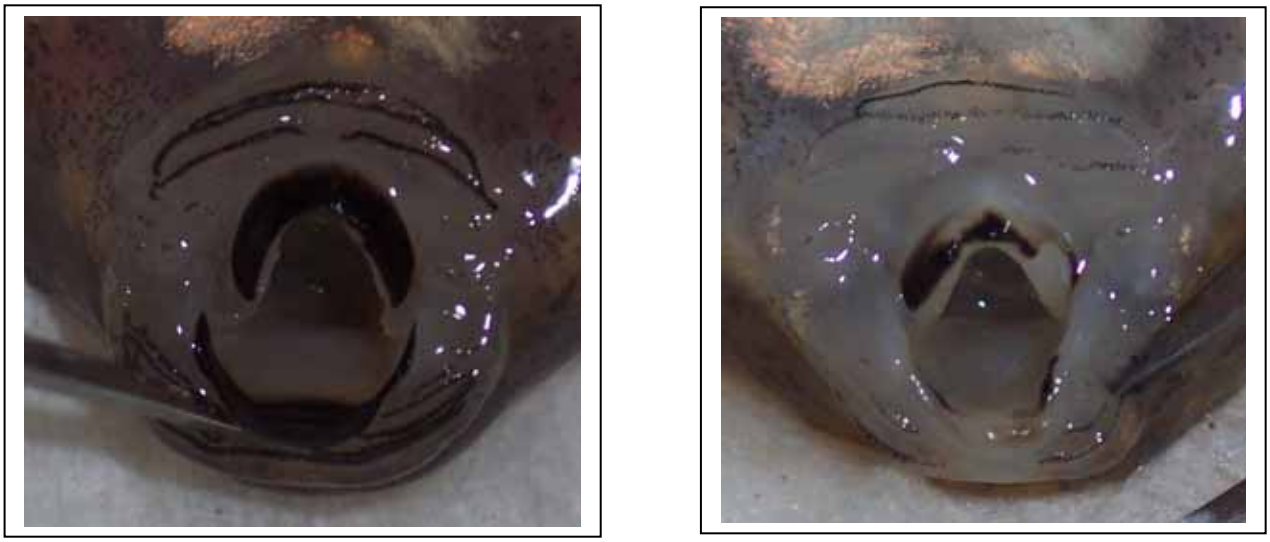


***Figure 2:*** Mouths of *Afrana fuscigula* tadpoles from Namaqualand, South Africa. The mouth on the left is normal while that on the right shows severe depigmentation of rostrodonts (beaks) and denticles. Loss of pigmentation in an irregular pattern is the hallmark of chytridiomycosis in the mouths of tadpoles (Rachowicz and Vredenburg 2004). Similar effects have been seen in tadpoles from Southeast Queensland (Pearl Symmonds, pers comm.), North Queensland (Diana Mendez, pers comm.) and Tasmania (Obendorf 2005).

***Figure 3*:** Unstained squash preparation of pigmented keratinised denticles from an infected tadpole of *Mixophyes fasciolatus*. The arrows indicate clusters of sporangia of *B. dendrobatidis.*

***Stains:***

Diagnosis of chytridiomycosis by staining skin scrapings or smears has also been described. A 1:1 mixture of cotton blue (Parker ink) and 10% aqueous KOH is an effective stain in wet preparations (Mazzoni et al. 2003). Congo red dye stains chitinous components of *B. dendrobatidis* (Briggs and

Burgin 2003). Following 20 to 30 minutes staining with 0.01% Congo red the walls of empty sporangia and exposed discharge tubes stain brick-red. After 45-60 minutes staining the walls of most immature, mature and empty sporangia were stained, but zoospores are not stained by this procedure (Briggs and Burgin 2003). Epidermal cell nuclei stain pale orange with Congo red if cells are damaged. DipQuick (Jorgensen, USA) was used to stain dried smears resulting in staining of cytoplasm, zoospores and walls, as well as host nuclei (Nichols et al. 2001). These stains may improve accuracy and ease of diagnosis, but comparisons have not been done.

### 1.2.2 Wet Preparation of Whole Skin

Samples of full thickness skin, from webbing or elsewhere, can be examined unstained (Joyce Longcore, pers comm.). This technique maintains the skin anatomy and a large surface area can be examined. The advantage of this technique is that the sample can be orientated and the location of the suspected agent can aid in identification (e.g., are the suspected fungal profiles within superficial epithelial cells, indicative of *B. dendrobatidis*, or in deeper layers indicating that the profiles may be normal amphibian morphology). This technique is quick, inexpensive and, when used by skilled observers, is equivalent in sensitivity to basic histology with haematoxylin and eosin staining. It is useful in healthy frogs when sheets of shedding skin cannot be obtained. *B. dendrobatidis* is routinely identified in fresh mounts before attempting to isolate the fungus into pure culture.

### 1.2.3 Histology

Histological sections are prepared from tissue preserved in 10% formalin or 70% ethanol, then dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin. A vertical section through the skin is achieved. Digits are examined by sectioning a whole foot ventral side down or by sectioning a single toe. For toes the maximum length of *stratum corneum* is obtained from a longitudinal section rather than a cross section. Larger digits, for example from amphibians with a snout-vent length > 60 mm, require removing skin from the underlying phalanx and sectioning the skin without bone. Digits from smaller amphibians are decalcified in EDTA for 48 hours at 37ºC or in 10% formic acid for 3-5 days before processing (Berger *et al.* 2000).

In the *stratum corneum* the chytrid is spherical or oval with discharge papillae projecting from the surface (Fig. 4). Discharge papillae can be seen in histological sections, but they are not common. Zoospores that develop in the zoosporangium escape through the open discharge tube. The wall of the zoosporangium is smooth, uniform in thickness and usually stains eosinophilic. The contents of the zoosporangia vary with the developmental stage of the chytrid. Four stages can be identified. The earliest stage contains a central basophilic, fairly homogenous mass. Zoosporangia become multinucleate and then the cytoplasm divides to form zoospores. Zoospores are basophilic and appear in cross-section as round or oval bodies (Fig. 4), usually numbering about 4 to 10 depending on the plane of section. Once the zoospores are released via the discharge papilla, the empty zoosporangia remain. In some empty colonial stages, thin septa are visible dividing the sporangium into internal compartments. The empty sporangium may collapse into an irregular shape (Fig. 5). During this terminal stage the empty shell sometimes becomes colonised by bacteria, and these are seen in section as basophilic rods or cocci (Fig. 5). Empty sporangia are the most common stage present, particularly in the sloughing surface layer (Berger et al. 2000).

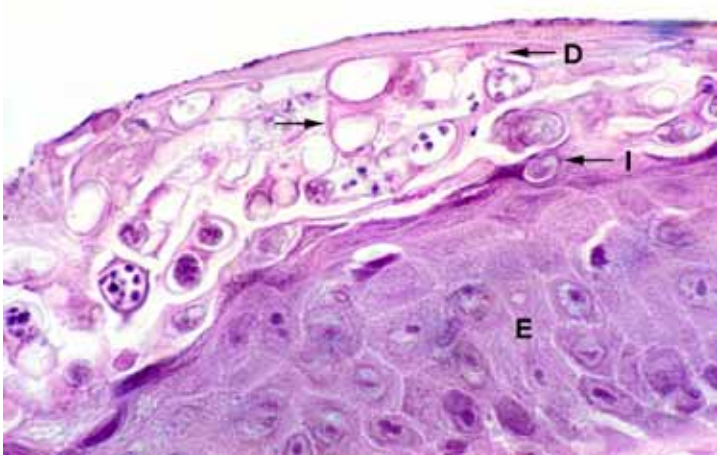



***Figure 4:*** Section of skin from a heavily infected adult of *Litoria caerulea*. Note homogenous immature stage (I), larger multinucleate stages (M), zoosporangium with discharge tube (D) containing zoospores, and empty zoosporangium after zoospores have discharged (arrow). E = epidermis. (H&E)

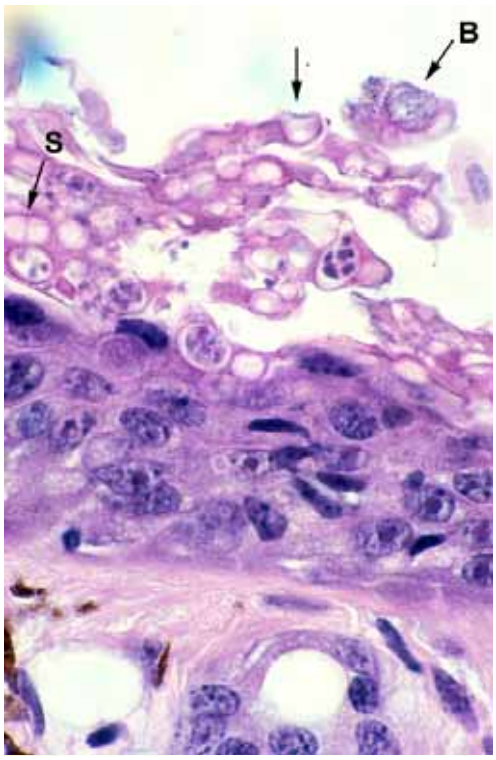



***Figure 5:*** Section of skin from a juvenile of *Mixophyes fasciolatus* with mostly empty sporangia. Note empty collapsing sporangium (arrow) and one containing bacteria (B). A sporangium is divided by an internal septum (S). (H&E)

In histological sections the diameter of zoosporangia varies from 5 to 13 µm. They are a similar size to epidermal cell nuclei. Discharge tubes have a diameter of 2 µm and a variable length, usually between 2 to 4 µm, but up to 10 µm. Zoospores are about 2 µm in diameter (Berger et al. 2000).

Infection is usually associated with skin pathology, and these changes can be used to detect, at low magnification, areas likely to be infected. Focal hyperkeratosis and erosions are common in the area

adjacent to the organisms. Irregular thickening of the epidermis (hyperplasia) may be present. In some fatal cases extensive sloughing of the hyperkeratotic layer leaves the epidermis with few organisms. However, in these cases chytrids can be detected in low numbers in the slightly keratinised surface layer, or may be seen in large numbers if the sloughed skin is examined. Sporangia are not present in areas of extensive ulceration.

Occasionally other fungi invade the epidermis of amphibians, for example cutaneous mucormycosis (Taylor et al. 1999). Most have thread-like hyphae, but in cross-section the hyphae can often have a circular appearance, and if the number of planes of section are few, the inexperienced examiner may confuse hyphae with chytrid sporangia. If special fungal stains (PAS, silver stain) are used in these cases, the filamentous nature of the hyphae may be more apparent.

Ducts from dermal glands often appear as spherical spaces between epidermal cells and may be confused with empty zoosporangia although they lack a distinct and complete wall, and are extracellular (Fig. 6) (Berger et al. 2000). The basophilic immature stages of the chytrid in the subsurface layer can appear similar to epidermal cell nuclei, but are often surrounded by a clear halo. Special stains demonstrate the fungal wall around immature chytrids.

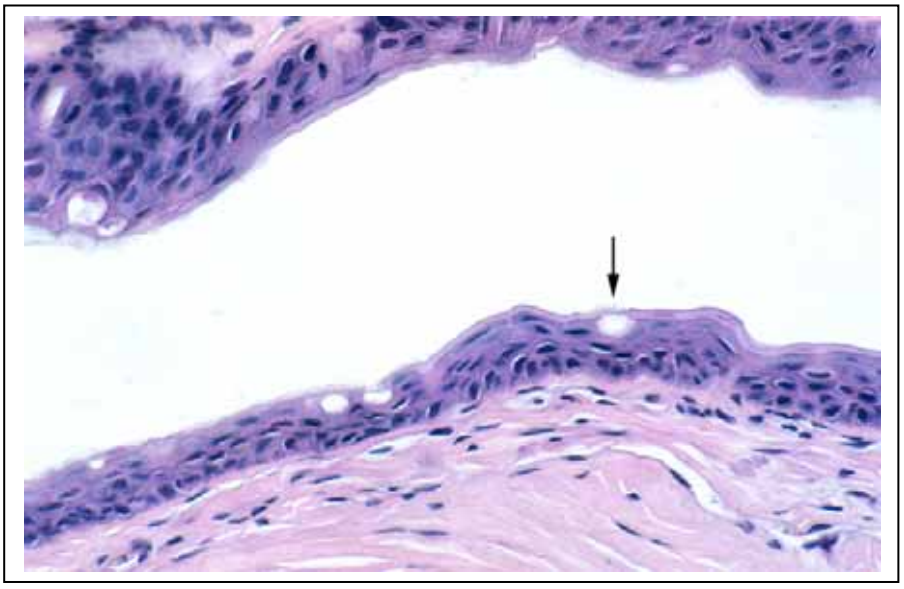


***Figure 6:*** Normal toe skin from a *Litoria chloris* with vesicular structures in the epidermis (arrow). These are probably ducts from dermal glands but appear similar to empty sporangia. Note that the clear spherical space lacks a cell wall and the adjacent epidermal cells are not hyperkeratotic. (H&E)

In tadpoles, a section through the mouthparts including the dark brown keratinized jaw sheaths or tooth rows is required for diagnosis. Large tadpoles are cut longitudinally through the midline with a scalpel and embedded with the cut surface downwards. Small tadpoles are best embedded whole on their side in the paraffin block, and then serially sectioned to reach the mouthparts. The size of the mouth varies between species, which affects the ease of obtaining a suitable section. The usual range of fungal stages may be present in tadpole mouthparts. They can occur on all surfaces of the jaw sheaths, and on caudal surfaces of the tooth rows (Marantelli et al. 2004).

***Special Histological Stains:***
Special fungal stains, such as periodic acid-Schiff (PAS) or silver stains (Swisher 2002) may be used to highlight sporangia. With optimal silver staining, sporangia are clearly differentiated from frog skin. This technique is comparable to immunohistochemistry, although other fungal species are also stained. These stains are useful for confirming infection in cases where a few indistinct stages are present because low numbers of sporangia can be easily spotted at low magnification.

The carbohydrate component of the fungal walls is stained with the Grocott-Gomori methenamine silver method and the Gridley's modified aldehyde fuchsin-PAS protocols (Swisher 2002). With the former method the reaction involves a reduction of the silver by the aldehyde groups produced after oxidation of the fungal wall components with chromic acid. The fungi are stained black against a light green background. The second method reveals more detail of the internal structure of the fungus but is considered less value when looking for low numbers of fungi.

### 1.2.4 Immunohistochemistry

Antibodies can be used in an immunoperoxidase test that specifically highlights sporangia of *B. dendrobatidis*. The sensitivity and ease of diagnosis is increased, and an untrained investigator can detect the presence of the fungus (Fig. 7). Polyclonal (PAbs) and monoclonal (MAbs) antibodies were generated against *B. dendrobatidis* by inoculating homogenized whole culture into rabbits, sheep and mice (Berger *et al.* 2002; Hyatt and Olsen unpub). When used in an immunoperoxidase test fungal sporangia are stained brick-red (Berger et al. 2002). Antisera reacted strongly with all stages of *B. dendrobatidis* and stained the walls, cytoplasm, rhizoids and zoospores. Immmunostaining kits with other chromagens have also been used successfully.

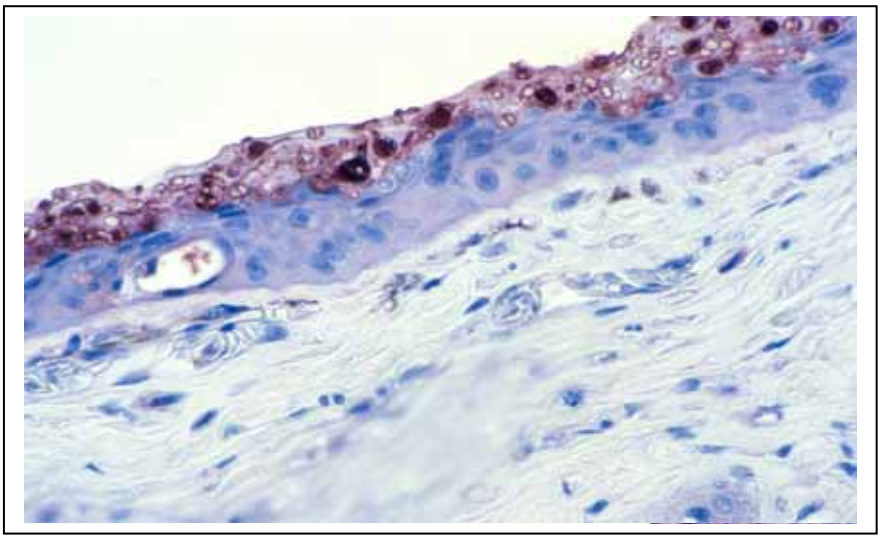


***Figure 7:*** Immunoperoxidase stain on skin of a *Litoria caerulea* with a heavy infection of *B. dendrobatidis*. Fungal walls, cytoplasm, zoospores and septa stain strongly. Antiserum from rabbit 667 was used at 1:1000. Scale bar = 80 μm.

Both the PAbs and MAbs produced some cross-reactivity, but it was only with other Chytridiomycetes, which are not animal pathogens. The polyclonal antibodies cross-reacted with two tested chytrids in the order Chytridiales (Berger et al. 2002), and Mab 19G6 also cross-reacted with

three tested chytridialeans (Boyle and Hyatt, unpub). Fungi from other phyla were not stained. The only other spherical fungus known to infect amphibian skin is *Mucor amphibiorum* (Speare et al. 1997) and this fungus is not labeled with the antibodies.

The immunoperoxidase stain with polyclonal antibodies against *B. dendrobatidis* has greater sensitivity than the H & E staining. At 19 days post experimental exposure, 61.8% (n=55) of toeclip samples were evaluated as lightly infected with the immunoperoxidase staining protocol, whereas 52.7% of the same toeclip samples were rated as positive using H&E staining (Berger et al. 2002).

The immunoperoxidase stain is useful when combined with recognition of the morphology and infection site of *B. dendrobatid*s. Immunohistochemistry can be used instead of, or as an adjunct to, conventional histological staining when increased sensitivity of testing is required. It is useful for screening toe-clip samples from healthy frogs where only a few sporangia may be present, and these can be detected at low magnification. The stain is also useful for testing necrotic or autolysed samples from sick frogs that may contain few distinctive sporangia.

The benefits of increased sensitivity and quicker examination of slides stained by the immunoperoxidase test must be weighed against the greater complexity of the staining method, which takes about four hours and requires more expensive and specialized reagents. However many diagnostic laboratories conduct immunohistochemistry and various methods can easily be adapted for use with the *B. dendrobatidis* antibodies.

### 1.2.5 Detection of *B. dendrobatidis* with Immunohistology and a Keratin Specific Stain

Histological identification of *B. dendrobatidis* can be complicated by the sloughing of the superficial keratinized layer (*stratum corneum*) leading to misdiagnosis since the zoosporangia have been lost with the skin slough. Combining immunostaining for *B. dendrobatidis* with Hollande's Trichrome keratin stain helps determine whether a negative result could be due to loss of the keratin layer (Olsen et al. 2004). In this technique the polyclonal antibody attaching to *B. dendrobatidis* is detected with acid phosphatase and appears blue-purple.

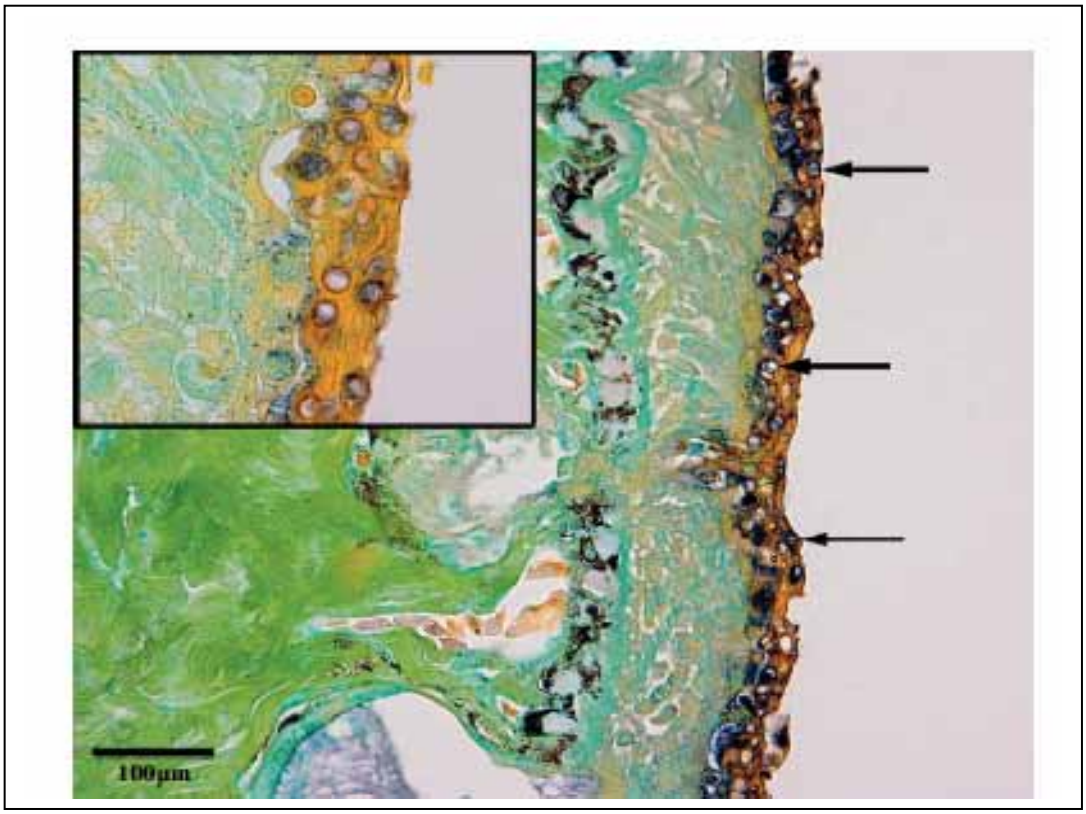



***Figure 8.*** Combined immunoperoxidase and keratin stain. Keratin stains orange and *B. dendrobatidis* stains blue (Olsen et al 2004 Fig 4).

### 1.2.6 Polymerase Chain Reaction (PCR)

***Real-time PCR:***
Real-time PCR is a method based on conventional PCR, which is a technique for amplifying DNA sequences by separating DNA into two strands and incubating them with oligonucleotide primers and DNA polymerase. The basic technique amplifies a specific sequence of DNA by as many as one billion times to produce a PCR product. Conventional detection of these PCR products uses electrophoresis and ethidium bromide, southern blots or direct sequencing. Advantages of real time TaqMan PCR include increased sensitivity, processing of large numbers of samples (96 well plates) in short time periods and generation of quantitative data.

Real-time PCR differs from conventional PCR in that the PCR product is monitored in real time via the detection of fluorescence emission. The Taqman probe has two fluorescent tags attached to it. One is a reporter dye that has its emission spectra quenched due to the spatial proximity of a second fluorescent dye (quencher). Degradation of the Taqman probe, by the Taq DNA polymerase, frees the reporter dye from the quenching activity of the quencher dye and thus the fluorescent activity increases with an increase in cleavage of the probe, which is proportional to the amount of PCR product formed. The Taqman probe is located between the two PCR primers. The specificity of this technique is attributed to the combined use of specific primers and a specific Taqman probe.

Boyle et al. (2004) developed a real-time Taqman PCR for the detection of *B. dendrobatidis* with a sensitivity of 0.1 zoospore equivalents. PCR amplification and sequencing of chytrid fungi rDNA

which included the 18S, ITS-1 and 5.8S regions, led to the generation of a forward primer (ITS-1 Chytr), a Taqman probe (Chytr MGB2) located within the ITS -1 region, and a reverse primer (5.8S) located partially within the 5.8S region. The authors tested four isolates of *B. dendrobatidis* and five other Chytridiomycetes: from the order Chytridiales- *Rhizophydium* sp. (JEL136), *Rhizophlyctis–Rhizophydium*-like (JEL142), *R. haynaldii* (JEL151); the order Monoblepharidales- *Gonapodya* sp. (JEL183) and the order Blastocladiales- *Allomyces macrogynus* (JEL204) and found the assay to be specific for *B. dendrobatidis*. Standards were generated that permitted the quantitation of *B. dendrobatidis* within a sample. As there is a high copy number of the ITS-1 region it is possible to detect low numbers of organisms; for example the authors demonstrate a sensitivity of 0.1 genome equivalents. This technique can therefore detect infection at much lower levels than any other technique. However, preliminary data suggests that frog secretions may interfere with the test leading to false negatives if they are not initially diluted (Hyatt et al, unpub).

The assay has been used to successfully detect *B. dendrobatidis* in the mouth parts of tadpoles using swabs in live animals and excising mouth parts in fixed specimens, mummified frogs and in formalin-fixed paraffin embedded samples. With an effective sensitivity of one zoospore, laboratory experiments have demonstrated that *B. dendrobatidis* can be detected as early as seven days post-infection (Hyatt, Boyle and Olsen, unpub).

***Standard PCR:***
A conventional DNA-based assay has also been developed for the detection of *Batrachochytrium dendrobatidis* (Annis et al. 2004). This assay uses primers designed from the ITS1 and ITS2 regions and produces a 300bp product compared with 146bp for the real-time assay (Boyle et al. 2004). The standard assay was used with small amounts of skin and had a sensitivity of 10 zoospores per sample and detected all 52 isolates of *B. dendrobatidis* examined. The assay did, however, produce a faint band with *Podochytrium dentatum* (a chytrid that grows on chitin and is not known to grow on amphibians). This assay is based on older technology and lacks the capacity for accurate quantitation and is less sensitive than the real-time PCR. However, the equipment required is cheaper and more readily available. Swabs may be used and good results were achieved when these were stored in ethanol and the ethanol included in the DNA extraction process (John Wood, pers comm). Standard PCR has been used to diagnose infection in samples that have been stored in formalin for years (E. Sadic, unpub), so is very useful in testing archived samples.

A disadvantage of all PCR tests is that expensive laboratory equipment is needed and a high level of care is needed in collection of samples and in running the tests to ensure contamination with DNA does not occur.

## 1.3 Relative Costs of Diagnostic Tests

Costing for tests has been calculated assuming that amphibians or appropriate samples from amphibians are available for examination and discounting any costs in obtaining these samples. Costs of collecting samples can be very high per sample depending on remoteness of sample site and availability of amphibians at a site; e.g., 20 samples collected from frogs at McIllraith Range, Queensland, may cost up to $20 each to collect.
Costs for each test involve a cost for materials and a cost for labour with labour including in some cases interpretation by a scientist other than the scientist or technician performing the test. In estimating costs, we have charged preparation time at $30 per hour (includes oncosts of 19%) and interpretative time at $55 per hour (also including oncosts). The differential refers to the higher level of skill required for interpretation. For wet mounts since interpretation requires a high level of skill and requires on average 15 minutes per sample, costs are largely due to labour. Some tests are done as individual tests and cannot be automated; e.g., wet mounts, and consequently have a high labour

cost per individual test. Others can be run in batches with automatic processing during some phases of the test; e.g., histology and PCR. For tests that are automated and run in batches in part, labour cost has been shared between tests to calculate an individual test cost. Time given for tests has been calculated from actual measurements of tests done on survey material which included positive and negative cases. Since standard PCR test is not performed in Australia, times have been estimated, not measured. Time in performing a test assumed no unexpected delays. Time will also vary between laboratories and the skill and speed of the operator. The estimate of costs given in Table 1 does not make allowance for replacement of equipment (PCRs) or for royalty costs (TaqMan PCR) if charged to outside users. These estimates are indicative only and in reality costs will vary markedly between institutions. However, they do allow comparison and are best viewed as allowing comparison for costs.

Overall, these estimates show that any test for chytridiomycosis will cost at least $12.50 per sample. TaqMan real-time PCR is the most expensive test at over $30 per sample, but has a major advantage in surveys of live animals in terms of higher sensitivity and non-invasive sampling regime. Standard PCR also has these characteristics, probably with a lower sensitivity than real-time-PCR, but also at a lower cost. Using histology as the baseline, Taqman real-time PCR is at least two and a half times as expensive, and the other tests are from 8% to 24% more expensive. Wet mounts have the lowest cost for consumables.

**Table 1:** Estimate of direct costs for diagnostic tests for chytridiomycosis. Labour charged at $30 and $55 per hour for preparation and interpretation respectively. This cost does not include indirect costs for replacement of equipment, royalties, etc.

| Item | Wet mount | Histology | Immunohistology | Standard PCR | TaqMan-rt-PCR |
|---|---|---|---|---|---|
| Preparation (hrs) | 0.05 | 0.125 | 0.14 | 0.25 | 0.5 |
| Interpretation (hrs) | 0.25 | 0.083 | 0.075 | 0.02 | 0.05 |
| Consumables | $0.25 | $4.25 | $6.25 | $5.00 | $13.00 |
| Preparatory labour cost | $1.50 | $3.75 | $4.25 | $7.50 | $15.00 |
| Interpretative labour cost | $13.75 | $4.50 | $4.00 | $1.00 | $2.75 |
| Total cost | $15.50 | $12.50 | $14.50 | $13.50 | $30.75 |
| Relative cost to histology | 1.24 | 1.0 | 1.16 | 1.08 | 2.46 |

## 1.4 Summary of Diagnostic Tests

Each test has a role in the diagnosis of chytridiomycosis. Choosing the most suitable diagnostic test for the specific purpose it very important.

*Wet mounts* have a critical role in isolation attempts. Suitable sporangia containing zoospores can be selected, processed and cultured (Longcore 2000). No other test can fill this role. A wet mount also has a major advantage of rapidity of diagnosis. It is by far the quickest technique to detect positives with a diagnosis being made within 15 minutes or less. Surveys done using examination of wet mounts of intact web edge used by an expert can achieve the same or better sensitivity than histology

(Joyce Longcore, pers comm., 2004). However, this requires a high level of operator expertise to be useful.

*Histology* is the best test for surveys of archived amphibians such as those held in museums. Histology of toes or digital webbing has proven very useful in retrospective surveys (Bonaccorso et al. 2003; Weldon et al. 2004; Retallick et al. 2004; McDonald et al. 2005). Since it is usual practice for amphibians to be stored in museums as multiple specimens in common containers, PCR is not recommended owing to the high risk of cross-contamination.

*Immunohistochemistry* adds value to histology by increasing sensitivity if low numbers of sporangia are present (Berger et al. 2002) or in confirming critical specimens detected on H&E histological slides as chytridiomycosis; for example, the oldest global record (Weldon et al. 2004). The addition of a keratin stain (Olsen et al. 2004) aids an assessment of how intact the superficial epidermis is and improves assessment of negative results.

Most frogs sick with chytridiomycosis have heavy infections with *B. dendrobatidis* that are easily recognised by standard histopathological techniques (Berger et al. 2000; Pessier et al. 1999). However, histological diagnosis is less sensitive when dealing with light infections in healthy animals, autolysed samples, or if the examiner has limited experience in chytridiomycosis diagnosis. False negatives may occur when prevalence of infection is low and clusters of sporangia are scattered because sections may miss the sporangia of *B. dendrobatidis.* The immunohistochemical methods increase sensitivity and confidence in the diagnosis, but are also limited by the fact that selected sections may not contain sporangia (Berger et al. 2002; Boyle et al. 2004). As histology is a routine and widely used method, it will continue to be useful for diagnosis.

PCR is much more sensitive than microscopy, and as larger numbers of samples can be tested rapidly, it is clearly the best choice for surveying healthy, wild frogs that typically have light infections. It also works well for sick frogs. Sampling by swabbing is quick, effective and non-invasive. The non-invasive nature of the swabbing technique versus the need to remove one or more toe tips for histology has, owing to ethical changes, resulted in histology becoming a much less defensible technique in population surveys. Of the two types of PCR tests available, the real-time PCR is in theory more sensitive and quantitative. However, since standard PCR and real-time PCR have not been compared either in the laboratory or on naturally infected populations, there is no evidence to favour one test or the other.

## 1.5 Amphibian Anaesthetic and Euthasania Solution (MS-222)

MS-222 (tricaine methane sulfonate) is an agent commonly used to anaesthetise or euthanize amphibians used in experiments. It is administered by immersing the animal to allow absorption through the skin. Since Bd is located in the animal's skin, it should come into contact with MS-222 when used for euthanasia or for anaesthia in experiments. Bd is a sensitive organism which could possibly be killed by MS-222. Hence, results of chytridiomycosis studies in which MS-222 is used could be unreliable. A concentration of 2 g l–1 and an exposure duration of of 1 h is at the high end of the range at which MS-222 would be most commonly used. Exposure to 2 g l–1 MS-222 for 1 h does not kill Bd cultures, suggesting that MS-222 is safe to use in chytridiomycosis studies. A paper reporting this work is in press in Diseases of Aquatic Organisms

**Table 2:** Comparison of the characteristics of each diagnostic test. All four methods of diagnosis are useful, although they have various advantages and disadvantages.

| | Wet preparations | Histology | Immunostaining | Standard PCR | Taqman-real-time PCR |
|---|---|---|---|---|---|
| Complexity of preparation | + | ++ | +++ | ++++ | ++++ |
| Cost per test | $15.50 | $12.50 | $14.50 | $13.50 | $22.50 |
| Timeliness of diagnosis | Most rapid (15 min) | 3 days | 3 days | 1 day | 8 hours |
| Facilities required | + | ++ | +++ | +++++ | +++++ |
| Ease of interpretation | + | ++ | +++ | +++++ | +++++ |
| Sensitivity | ++ | ++ | +++ | +++++ | +++++ |
| Use on live frogs | Yes | Yes | Yes | Yes | Yes |
| Use for healthy frogs | Less useful | Less useful | Less useful | Very useful | Very useful |
| Specimens required | Smear, scrape, web, other skin | Toe, web, other skin | Toe, web, other skin | Scrape, toe, web, other skin, swab, filtrate | Scrape, toe, web, other skin, swab, filtrate |
| Use on formalin fixed | Yes | Yes | Yes | Yes | < 3 months for real-time PCR |
| Use on ethanol fixed | Yes | Yes | Yes | Yes | Yes |
| Use on frozen | Yes | Yes | Yes | Yes | Yes |
| Use on decomposed | Possible | Possible | Possible | Yes | Yes |
| Use in archival surveys | Yes | Yes | Yes | No, unless each specimen is stored separately throughout | No |
| Quantifiable | Yes | Yes | Yes | Yes | Yes |

## 1.6 Diagnostic Test Recommended for Mapping Chytridiomycosis in Australia

For Australia the real-time PCR is the recommended technique for mapping chytridiomycosis. The main reasons for this is 1) it has the highest sensitivity and specificity; 2) it is non-invasive and will be acceptable to animal ethics committees; 3) it is available and the owner, AAHL, will transfer technology to other institutions and support the technology by providing standards, advice and training.

The increased sensitivity of the real-time PCR over histology has been demonstrated in experimentally infected frogs (Boyle et al. 2004). However, this sensitivity needs to be proven in

field conditions. Section 3.2 provides evidence of the increased sensitivity of this test in naturally infected amphibian populations.

## 1.7 Mapping Techniques

No country has published a national mapping protocol for chytridiomycosis to provide a snapshot of country-wide distribution. Individual researchers or research groups have done studies on the prevalence of chytridiomycosis in populations at particular sites, but there have been no systematic studies at a state/province or national scale. Weldon et al. (2004) in their study of museum specimens extending back to the nineteenth century in South Africa demonstrated that chytridiomycosis is widespread. However, this was an opportunistic study that provided a national snapshot geographically and chronologically, but it was not designed as a systematic national survey.

A mapping project is being undertaken in Europe (Walker 2005), but is not yet complete. This is using real-time PCR as the major diagnostic tool combined with predictive modelling. However, the sampling strategy used to select populations for testing is unknown.

The lack of published protocols highlights the need for the systematic approach proposed under Scope Item 3.

## 1.8 Bibliography of Reference Material

As a requirement of Scope Item 1 a bibliography of literature consulted in included in Section 6, Literature Cited in This Report. Hard copies and electronic copies of all papers have been provided with this report.

# 2. Scope Item 2

**Objective 2.1: Determine if the data that is currently available to map the distribution of chytrid fungus in Australia could be (a) brought together and stored on a single national database and (b) used to create a snapshot map of the current distribution of chytrid fungus in Australia.**

Activity 2.1.1: Review current national database held by Amphibian Diseases Group at School of Public Health and Tropical Medicine to identify likely locations of additional records and feasibility of obtaining these records.

Activity 2.1.2: Review best technique for creating a snapshot map from this database.

**Objective 2.2: If it is determined that (a) and (b) of the point above can be achieved, undertake this task so that a national database on the distribution of chytrid fungus in Australia is created. Ensure that permission is obtained from the original data custodians for their data to be used to produce the national snapshot distribution map, noting that the map will be made available to the public.**

Activity 2.2.1: Identify and approach owners of additional records currently not included in national database held by Amphibian Diseases Group at School of Public Health and Tropical Medicine for permission to include their data.

**Objective 2.3: Provide the national database to an appropriate institution/agency. The role of this institution/agency will be to ensure the safe keeping and possible future updating of the database. It will also produce and publish (via the internet) the national snapshot distribution map which is to be made available to the public free of charge.**

Activity 2.3.1: Amphibian Diseases Group at SPHTM will update the national database and publish on the internet the current national snapshot map available free of charge to the general public.

Activity 2.3.2: Investigate in collaboration with the Australian Wildlife Health Network (AWHN) the feasibility of establishing a national surveillance system which involves collection, collation and analysis of data and timely distribution of information for action.

**Objective 2.4: Identify the limitations of producing the distribution map using the process outlined in the points above.**

Activity 2.4.1: Identify the next steps in developing a more sophisticated and interactive distribution map on the Internet.

## 2.1 Review the existing national database on the distribution of amphibian chytrid fungus in Australia

### 2.1.1 Existing structure and content of the database

The existing database is an Excel spreadsheet comprising the fields listed in table 3 and some additional fields derived from these (e.g. month, year, season).

**Table 3:** Fields in the existing national database.

| Field name | Field description |
|---|---|
| Record ID | A unique number for each record in the database |
| Species | The scientific name of the frog species sampled |
| Family | The taxonomic family the species belongs in |
| Genus | The genus the species belongs in |
| Site of collection | A written description of the collection site |
| State | The state in the which the collection site falls |
| Bioregion | The biogeographic region in which the collection site falls |
| Latitude | Decimal degrees latitude for the location of the site |
| Longitude | Decimal degrees longitude for the location of the site |
| Latlong source | The source of the latitude and longitude (e.g. from collector, looked up web based gazetteer) |
| Elevation | Elevation of the site (metres above sea level) |
| Map sheet | Map sheet name and or number on which the site falls |
| Date | The date of collection |
| Collector/source | The collector and or source of the record |
| Sex | Sex of the animal(s) sampled |
| Number of individuals | The number of individuals included in the sample |
| Database | The database the record was sourced from |
| Chytrid? | Presence of chytrid in sample: positive, negative, suspicious positive, suspicious negative, unknown, no result |
| Source reference | The sample reference identifier used by the sample collector |
| Notes | Any additional notes about the samples or animals sampled |

The database is mostly made up of the results of histological sections of skin or toe clip samples. There are 4326 records in the database, with samples from 83 species with representatives from the four families of frogs that occur naturally in Australia and the one introduced family, Bufonidae. Table 4 summarises the number of samples for each species that could confidently be assigned as negative or positive for chytrid fungus.

**Table 4:** The number of frogs found to be negative or positive for chytrid fungus.

| Scientific name | Common name | Family | negative | positive | % positive |
|---|---|---|---|---|---|
| *Bufo marinus* | Cane Toad | Bufonidae | 29 | 3 | 9% |
| *Cyclorana alboguttata* | Striped Burrowing Frog | Hylidae | 12 | | 0% |
| *Cyclorana brevipes* | Short-footed Frog | Hylidae | 3 | | 0% |
| *Cyclorana novaehollandiae* | New Holland Frog | Hylidae | 3 | | 0% |
| *Litoria adelaidensis* | Slender Tree Frog | Hylidae | | 7 | 100% |
| *Litoria andiirrmalin* | Cape Melville Tree Frog | Hylidae | 9 | | 0% |
| *Litoria aurea* | Green and Golden Bell Frog | Hylidae | 3 | 6 | 67% |
| *Litoria brevipalmata* | Green-thighed Frog | Hylidae | 1 | | 0% |
| *Litoria caerulea* | Green Tree Frog | Hylidae | 32 | 47 | 59% |
| *Litoria chloris* | Red-eyed Tree Frog | Hylidae | 69 | 9 | 12% |
| *Litoria dentata* | Bleating Tree Frog | Hylidae | 1 | | 0% |
| *Litoria eucnemis* | Fringed Tree Frog | Hylidae | 75 | | 0% |
| *Litoria ewingii* | Brown Tree Frog | Hylidae | 1 | 2 | 67% |
| *Litoria fallax* | Eastern Dwarf Tree Frog | Hylidae | 94 | | 0% |
| *Litoria genimaculata* | New Guinea Tree Frog | Hylidae | 566 | 73 | 11% |
| *Litoria gracilenta* | Dainty Green Tree Frog | Hylidae | 240 | 2 | 1% |
| *Litoria inermis* | Peters' Frog | Hylidae | 15 | | 0% |
| *Litoria infrafrenata* | White-lipped Tree Frog | Hylidae | 287 | 5 | 2% |
| *Litoria latopalmata* | Broad-palmed Frog | Hylidae | 7 | | 0% |
| *Litoria lesueuri* | Lesueur's Frog | Hylidae | 85 | 32 | 27% |
| *Litoria longirostris* | Long-snouted Frog | Hylidae | 31 | | 0% |
| *Litoria moorei* | Moore's Frog | Hylidae | 1 | 13 | 93% |
| *Litoria nannotis* | Torrent Tree Frog | Hylidae | 246 | 16 | 6% |
| *Litoria nasuta* | Rocket Frog | Hylidae | 11 | 1 | 8% |
| *Litoria pallida* | Pale Frog | Hylidae | 3 | | 0% |
| *Litoria pearsoniana* | Pearson's Frog | Hylidae | 31 | 13 | 30% |
| *Litoria peronii* | Peron's Tree Frog | Hylidae | 3 | 9 | 75% |
| *Litoria raniformis* | Southern Bell Frog | Hylidae | | 2 | 100% |
| *Litoria revelata* | Revealed Frog | Hylidae | 53 | | 0% |
| *Litoria rheocola* | Creek Frog | Hylidae | 409 | 105 | 20% |
| *Litoria rothii* | Roth's Tree Frog | Hylidae | 28 | | 0% |
| *Litoria rubella* | Desert Tree Frog | Hylidae | 36 | | 0% |
| *Litoria spenceri* | Spotted Tree Frog | Hylidae | 8 | 8 | 50% |
| *Litoria tornieri* | Tornier's Frog | Hylidae | 1 | | 0% |
| *Litoria verreauxii* | Verreaux's Frog | Hylidae | 1 | | 0% |
| *Litoria xanthomera* | Orange-thighed Frog | Hylidae | 1 | | 0% |
| *Nyctimystes dayi* | Australian Lacelid | Hylidae | 245 | 17 | 6% |
| *Austrochaperina robusta* | Robust Frog | Microhylidae | 8 | | 0% |
| *Adelotus brevis* | Tusked Frog | Myobatrachidae | 9 | 13 | 59% |
| *Crinia deserticola* | Desert Froglet | Myobatrachidae | 3 | | 0% |
| *Crinia georgiana* | Tschudi's Froglet | Myobatrachidae | | 3 | 100% |
| *Crinia glauerti* | Glauert's Froglet | Myobatrachidae | | 1 | 100% |
| *Crinia insignifera* | Sign-bearing Froglet | Myobatrachidae | | 2 | 100% |
| *Crinia parinsignifera* | Eastern Sign-bearing Froglet | Myobatrachidae | 2 | | 0% |
| *Crinia pseudinsignifera* | False Western Froglet | Myobatrachidae | | 1 | 100% |
| *Crinia riparia* | Streambank Froglet | Myobatrachidae | 9 | | 0% |
| *Crinia signifera* | Common Froglet | Myobatrachidae | 3 | | 0% |
| *Geocrinia rosea* | Karri Frog, Roseate Frog | Myobatrachidae | | 1 | 100% |

| Scientific name | Common name | Family | negative | positive | % positive |
|---|---|---|---|---|---|
| *Heleioporus australiacus* | Giant Burrowing Frog | Myobatrachidae | | 1 | 100% |
| *Heleioporus eyrei* | Moaning Frog | Myobatrachidae | | 2 | 100% |
| *Lechriodus fletcheri* | Fletcher's Frog | Myobatrachidae | | 1 | 100% |
| *Limnodynastes dorsalis* | Western Banjo Frog | Myobatrachidae | | 4 | 100% |
| *Limnodynastes dumerilii* | Eastern Banjo Frog | Myobatrachidae | 2 | 5 | 71% |
| *Limnodynastes ornatus* | Ornate Burrowing Frog | Myobatrachidae | 7 | | 0% |
| *Limnodynastes peronii* | Brown-striped Frog | Myobatrachidae | 6 | 1 | 14% |
| *Limnodynastes tasmaniensis* | Spotted Grass Frog | Myobatrachidae | 13 | 8 | 38% |
| *Limnodynastes terraereginae* | Northern Banjo Frog | Myobatrachidae | 7 | 1 | 13% |
| *Mixophyes fasciolatus* | Great Barred Frog | Myobatrachidae | 17 | 3 | 15% |
| *Mixophyes fleayi* | Fleay's Barred Frog | Myobatrachidae | 14 | 6 | 30% |
| *Mixophyes iteratus* | Giant Barred Frog | Myobatrachidae | 16 | | 0% |
| *Mixophyes schevilli* | Northern Barred Frog | Myobatrachidae | 12 | | 0% |
| *Mixophyes sp.* | | Myobatrachidae | 2 | 10 | 83% |
| *Neobatrachus pelobatoides* | Humming Frog | Myobatrachidae | | 1 | 100% |
| *Neobatrachus sudelli* | Sudell's Frog | Myobatrachidae | 1 | | 0% |
| *Philoria kundagungan* | Mountain Frog | Myobatrachidae | 2 | | 0% |
| *Pseudophryne bibronii* | Brown Toadlet | Myobatrachidae | 2 | | 0% |
| *Pseudophryne corroboree* | Southern Corroboree Frog | Myobatrachidae | 85 | 17 | 17% |
| *Pseudophryne pengilleyi* | Northern Corroboree Frog | Myobatrachidae | 170 | 16 | 9% |
| *Taudactylus acutirostris* | Sharp-snouted Torrent Frog | Myobatrachidae | 3 | 4 | 57% |
| *Taudactylus eungellensis* | Eungella Torrent Frog | Myobatrachidae | 268 | 60 | 18% |
| *Taudactylus liemi* | Liem's Frog | Myobatrachidae | 14 | | 0% |
| *Taudactylus rheophilus* | Tinkling Frog | Myobatrachidae | 2 | | 0% |
| *Uperoleia altissima* | Montane Toadlet | Myobatrachidae | 1 | | 0% |
| *Uperoleia laevigata* | Smooth Toadlet | Myobatrachidae | | 1 | 100% |
| *Uperoleia mimula* | Mimic Toadlet | Myobatrachidae | 5 | | 0% |
| *Rana daemeli* | Water Frog, Wood Frog | Ranidae | 3 | | 0% |
| *Indeterminate or unidentified* | | | 1 | 1 | 50% |
| **Total** | | | **3327** | **533** | **14%** |

**Table 5:** The distribution across Australian states of samples found to be negative or positive for chytrid fungus.

| State | negative | positive | % positive |
|---|---|---|---|
| ACT | 31 | 3 | 9% |
| NSW | 278 | 60 | 18% |
| NT | 13 | | 0% |
| QLD | 2969 | 409 | 12% |
| SA | 22 | 15 | 41% |
| VIC | 13 | 11 | 46% |
| WA | 1 | 35 | 97% |
| **Total** | **3327** | **533** | **14%** |

### 2.1.1. Identification of significant datasets not contained within the existing database and feasibility of obtaining them for inclusion.

The most important datasets not yet contained within the database are those currently being generated through the use of the real time PCR test. We are aware of the following projects using this technology in Australia:

- David Obendorf's and Alexander Dalton's study on the distribution of chytrid in Tasmania;
- The results from the pilot survey of chytrid distribution in Australia (see section 4).
- Kerry Kriger's PhD study on the altitudinal and latitudinal distribution of chytrid fungus in eastern Australia.
- Studies by the Amphibian Disease Research Group at JCU and collaborative groups.

Some of these records could be obtained relatively easily. Other records would not be made available due to the concern that some individual researchers had about making unpublished data available to the public. Likely constraints on distribution of data arising from intellectual property issues and unpublished datasets could be resolved through data licensing.

In addition the large dataset used by Aplin and Kirkpatrick (2000) to map chytrid in southwest Australia could not be located for incorporation despite the willingness of the owners of the data to incorporate it. This highlights the need for a central database and caretaker to prevent the loss of datasets.

There are other smaller datasets that need to be captured and incorporated too such as PhD studies like those of Pearl Symonds at University of Queensland. The number of these studies is expected to increase exponentially over the next few years. Whilst these studies may have different aims to this mapping protocol, they may provide useful information on distribution of *B. dendrobatidis*. The importance of a national database needs to be promoted to these researchers so that they incorporate their data.

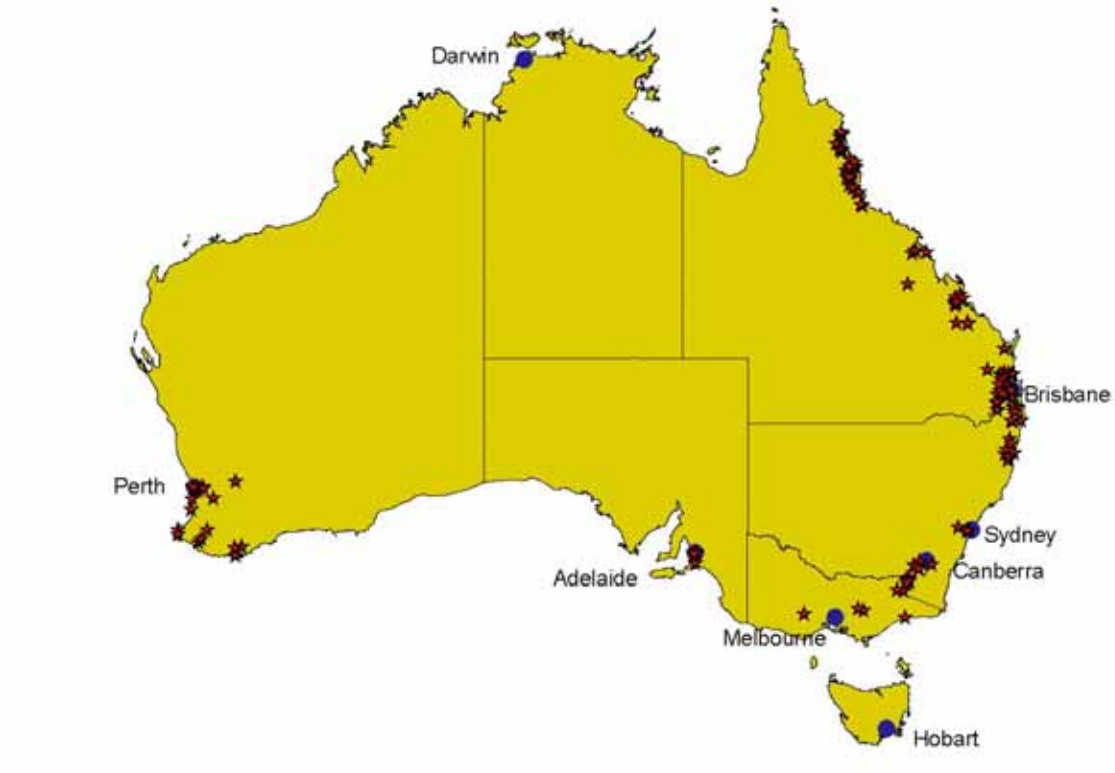
Some of the datasets that have already been incorporated may need to be updated such as records from the Hunter Valley, which were collected by Dr Michael Mahony.

### 2.1.2 Review best technique for creating a snapshot map from the existing database

The records from the existing database were imported into an Access database and linked to an ARCVIEW version 3.2 GIS database using Microsoft's ODBC to produce a snapshot of the distribution of chytrid in Australia. Site of collection details were compared with the location plotted on maps within the GIS database to check for coarse georeference errors. The map was exported in JPEG format.

## 2.2: Produce the National Snapshot Distribution Map from the National Database.

***Figure 9:*** Map of the distribution of the amphibian chytrid fungus in Australia from records currently held in the national database.




## 2.3: Make the National Snapshot Distribution Map Available to the Public

The snapshot map has been incorporated into a WWW page at
http://www.jcu.edu.au/school/phtm/PHTM/frogs/chyspec.htm

## 2.4 Limitations of the Existing Database

The principal limitation of the existing database is that it contains mostly records from histological examination of toe clips (see section 2.1.2). However expanding the database in its current form to include the additional datasets being generated through other methods, principally real time PCR, requires additional fields and coding of the records and requires a substantial effort.

In addition more than one analytical test can be performed on a sample, or samples, collected from an individual frog (or on batches of samples from the same species from a site). It is therefore critical that the database can deal with results from multiple techniques for determining the presence of chytrid and that these tests may be undertaken on the same individual on more than one occasion (i.e. during mark-recapture studies).

Another limitation is missing data, or inconsistent or erroneous data in fields. These problems can be easily rectified through the use of well designed data entry forms that do not allow records to be saved if critical information is missing and only allows set codes, values or formats to be entered.

There have also been some recent taxonomic changes within Australian frogs and some of these have not been dealt with in the current dataset (e.g. the splitting of *Litoria lesueuri* into three species with narrowly overlapping ranges).

## 2.5 Developing a New National Database

Key considerations in developing a new database are:

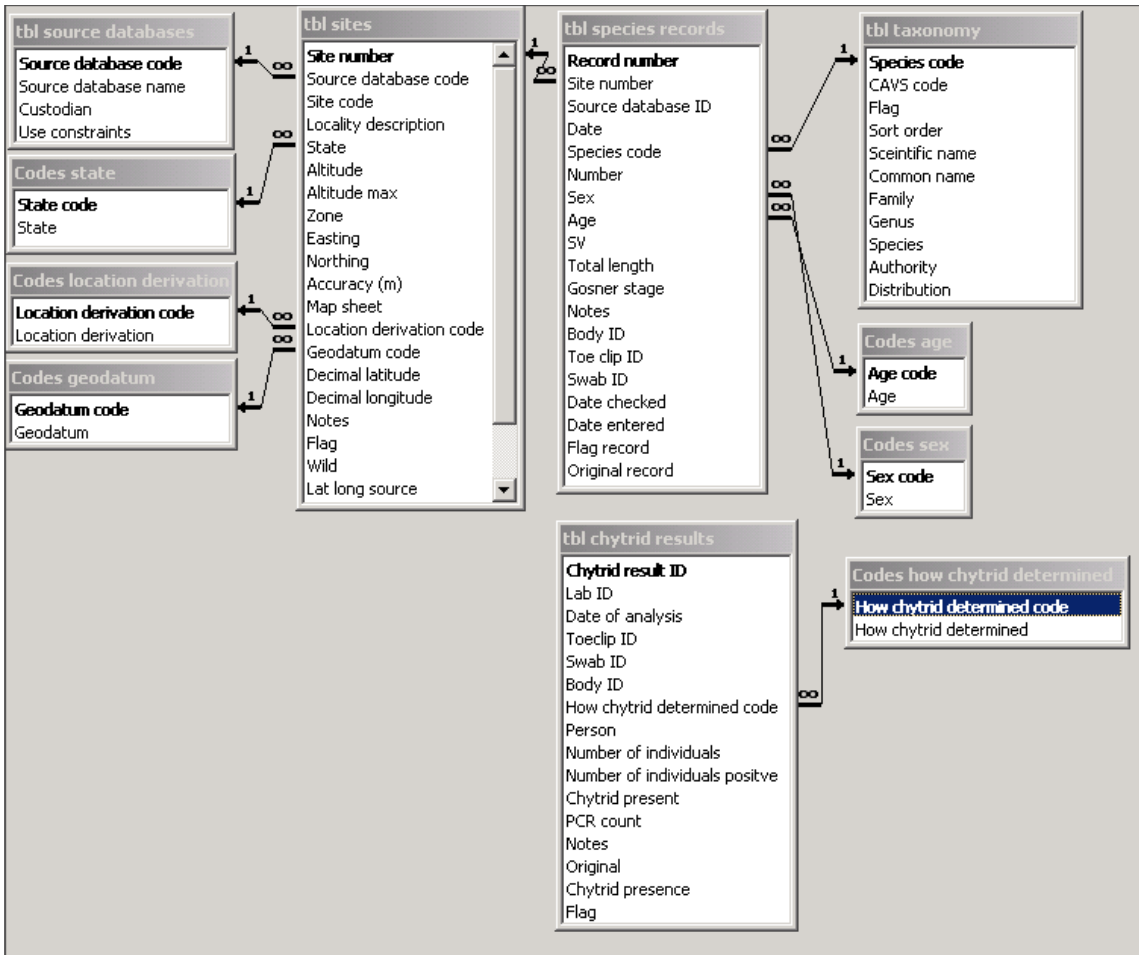
- Errors, inconsistency and omissions in the dataset are minimised.
- That data from various sources can be readily identified in the collated dataset and that any limitations on their use are clearly stated within the database and queries/reports designed to ensure that these data are not used for any purpose not permitted.
- Data entry and editing is accurate and efficient.
- Maps showing the distribution of chytrid within Australia area easily updateable
- Reporting, both within the database and against mapped information available within geographical information systems, is simple.

### 2.5.1 Database structure

Many of the limitations of the existing database are due to its simple, flat structure (i.e. a two dimensional table with each row representing a single sample tested for chytrid and each column providing a piece of information about that sample such as date of sample, species, presence of chytrid in sample). Flat databases become very large and cumbersome and prone to errors as each record (i.e. the information pertaining to each sample) has to contain all the data required for interpretation, summation and analysis. For example site information such as locality description, georeference and state has to be entered for every sample collected at that site whether it is one sample or thousands. By establishing a separate table containing site details, the potential for data entry and editing errors in the latter case, is greatly reduced, consistency is maximised and the size of the database is minimised. The information in the site table and the sample table are related through the use of a unique site code or identifier for each site. Simple queries are used to extract the required information from each table, also allowing tailoring of output for specific purposes.

Relational databases allow for much more complex relationships between pieces of information than illustrated above with the ability to link numerous tables. Figure 10 below shows the structure of the new database and the relationships between the tables. There are a variety of relational database packages available. We have chosen Microsoft Access due to its wide availability and ease of use. At this stage the structure has been developed and data from the existing database imported. Further testing and refinement of the structure is required, through the incorporation of datasets not yet captured, before data entry forms and reports are developed. An alternative software package will be required if web-based data entry and reporting is required and external expertise will be required for this development. However the database structure, data and codes, as outlined below, can be easily transferred to other applications in the future.

***Figure 10:*** The principal data tables, lookup tables and their relationships within the new database. Refer to tables 6, 7 and 8 for details of the structure of the principal data tables.




The three principal tables in the database are ‘tbl sites’, ‘tbl species records’ and ‘tbl chytrid results’. These tables contain the information on when, where, by whom and what species were sampled, the type of tests carried out and the result of the test or tests. The other tables contain codes and descriptions of codes used in the three principal tables. For each of the three principal tables the fields and their descriptions are listed in tables 6, 7 and 8.

**Table 6:** The structure of the database table ‘tbl sites’.

| Field name | Field description |
|---|---|
| Site number | An automatically assigned unique numeric identifier for each site samples were collected from. |
| Source database code | Abbreviation of name of database record was sourced (as per table 'tbl source databases') |
| Site code | Site code used by sample collector. |
| Locality description | A written description of the location of the site. |
| State | Abbreviated Australian state name where sample was collected from. |
| Decimal latitude | Decimal degrees latitude, using the Geodetic Datum of Australia 1994 (GDA94). |
| Decimal longitude | Decimal degrees longitude, using the Geodetic Datum of Australia 1994 (GDA94). |
| Accuracy | Accuracy of the georeference (lat/long) in metres. This value represents the radius of a circle that encloses the areas in which the sightings were made as well as the error inherent in the calculation of the georeference. |

| | |
|---|---|
| Map sheet | Mapsheet name and or number the site falls on. |
| Location derivation code | Location derivation code for how site georeference (lat/long) was determined, as per table 'Codes location derivation'. |
| Geodatum code | Datum of georeference, as per table 'Codes geodatum'. Note that all georeferences should be converted to or calculated as GDA94 prior to entry. |
| Altitude | Altitude in metres or minimum altitude if a range provided |
| Altitude max | Maximum altitude in metres if a range provided. |
| Notes | Additional information about the site. |
| Flag | Field for temporarily flagging sites for custom queries, etc. |
| Wild | A field to denote whether the site is in the wild or at a captive husbandry or research facility or is the location of a pet frog. |

**Table 7:** The structure of the database table 'tbl species records'.

| Field name | Field description |
|---|---|
| Record number | An automatically assigned unique numeric identifier for each sample. |
| Site number | The unique numeric identifier for the site where the sample was collected. |
| Source database ID | The record identifier used in the source database. |
| Date | Date of record. |
| Species code | Unique species abbreviation for the taxa, as per the table 'tbl taxonomy', comprised of first the four letters of the genus and the first four letters of the species. |
| Number | The number of individuals sampled. |
| Sex | Code for the sex of the animals sampled as per table 'Codes sex'. |
| Age | Code for the age of the animals sampled as per table 'Codes age'. |
| SV | Snout-vent length (mm). |
| Total length | Total length of tadpole (mm). |
| Gosner stage | Tadpole development stage after Gosner (1960). |
| Animal ID | A number or code (e.g. toe clip number or PIT tag code) that uniquely identifies the individual at that site. Used in mark-recapture studies. |
| Notes | Notes about the animal sampled or the samples collected. |
| Body ID | The unique identifier the collector associated with a whole animal sample at the time of collection (e.g. field number N73058). |
| Toe clip ID | The unique identifier the collector associated with the toe clip sample at the time of collection (e.g. toe clip vial number HH03-056). |
| Swab ID | The unique identifier the collector associated with a skin swab sample at the time of collection (e.g. swab number HH03-S0121). |
| Date entered | Date sample record originally entered (automatically assigned in data entry form). |
| Date checked | Date sample record checked and final editing completed. |
| Flag record | Field for temporarily flagging sample records. |
| Original record | The record as provided from the source dataset. Fields are comma delimited. |

**Table 8:** The structure of the database table 'tbl chytrid results'

.

| Field name | Field description |
|---|---|
| Chytrid result ID | An automatically assigned unique numeric identifier for each result from analyses of samples for chytrid fungus. |
| Lab ID | The sample identifier the analysing laboratory used. |
| Date of analysis | Date sample analysed or reported on. |
| Toeclip ID | The sample identifier for toeclip samples (e.g. toe clip vial number HH03-056). |
| Swab ID | The sample identifier for skin swab samples (e.g. swab number HH03-S0121). |
| Body ID | The sample identifier for whole animals (e.g. field number N73058). |
| How chytrid determined code | What technique was used to determine whether chytrid fungus was present, as per table "How chytrid determined". |
| Person | The name of the person how carried out the analysis. |
| Number of individuals | The number of individuals in the sample analysed (usually equal to 1, but in some cases toe clips from multiple individuals of the same species are included in the one vial, or PCR analyses performed as a batch). |
| Number of individuals positve | The number of individuals in the sample analysed that were positive for chytrid. |
| Chytrid present | Indicate whether chytrid fungus was found in the sample, whether one or more individuals were included in the sample, using one of the following: "Present" Or "Absent" Or "Probable" Or "Possible" Or "Uncertain". |
| PCR count | Taqman real-time PCR assay result, which is the number of zoospore equivalents in the sample. |
| Notes | Any notes on the results from the sample or the technique used. |
| Original | A code for uniquely identifying the hard copy of the results (e.g. HHCR01, for Harry Hines chytrid results page 1), or a file reference. |
| Chytrid presence | A standard description for reporting results from tests (see 'Chytrid presence' table). |
| Flag | A field for temporarily flagging records of interest. |

### 2.5.2 Database management

Management of the database will involve the following main tasks:

1. Updating frog taxonomic changes. While the taxonomy of Australian frogs has been relatively stable, in recent years new species have been described and this trend is likely to continue. This is most likely to involve the description of 'cryptic' species within currently recognised widespread taxa. Consequently there is a need to review the contents of the 'tbl taxonomy' periodically and when there have been taxonomic changes modify the table and any related records in 'tbl species records' accordingly. It is recommended that changes to the taxonomy in 'tbl species records' be carried out only following consultation with the custodian of the original datasets.

2. Ongoing collation or updating of datasets. This is the largest task and will require regular attention if the database is to represent a real-time picture of the distribution of chytrid. It requires tapping into the network of researchers and veterinarians across Australia that are currently sampling for chytrid to ensure timely provision of results to the national database.

As the majority of data in the foreseeable future will be coming from researchers it will be an important task to liase with these researchers to ensure data are provided in a digital format that will allow easy and reliable uploading into the database.

**3.** Database development. There will be a need to continually develop the database in order to respond to changes in the type of data collected and queries asked.

**4.** Database management agency. The current database is maintained by the ADEG at the School of Public Health, Tropical Medicine and Rehabilitation Sciences at James Cook University. AWHN is the body responsible for recording wildlife diseases in Australia, particularly those like chytridiomycosis that are on the World Organization for Animal Health Wildlife Diseases List. Initial discussions with AWHN occurred before they had finalised their national database protocols for monitoring wildlife diseases and consequently discussions were premature. This role of AWHN is now further advanced and discussions have recommenced.

# 3. Scope Item 3

**Objective 3.1: Design feasible survey protocols with sufficient power for surveying all amphibian populations in Australia to determine whether they are infected or not infected with chytrid.**

Activity 3.1.1: Design survey protocols to detect presence/absence at a cut off prevalence of 2%.

Activity 3.1.2: Design survey protocols that address 1) most appropriate diagnostic tests, 2) minimum number of amphibians to sample, 3) season of sampling to optimise chance of finding both frogs and B. dendrobatidis, 4) species to sample, 5) life stage to sample, 6) combining results from different species at sites, 7) choice of site, 8) spacing of sites in a region, 9) randomization of sites and individuals, 10) feasibility of protocols for a wide-range of Australian amphibian populations.

**Objective 3.2: In designing the survey protocols, include a process that will compare the efficiency, effectiveness and costs of the main diagnostic tools used to detect chytrid fungus.**

Activity 3.2.1: Include in survey protocols a comparison of the cost-efficiency, ease of use and feasibility of the diagnostic tests.

## 3.1 Design Feasible Survey Protocol to Map Chytridiomycosis in Australia

### 3.1.1 Background

The aim of this survey protocol is to provide a simple and standard method for sampling all frog populations in Australia for infection with *Batrachochytrium dendrobatidis*. A simple protocol will be more readily adopted and a standard protocol will enable comparison among frog populations. The survey protocol aims to maximise the chances of detecting chytridiomycosis in frog populations if it is present. This will be achieved by sampling frog populations at times of the year when climatic conditions are most favourable for chytridiomycosis. However, this is constrained by the biology of frogs and the fact that some frog species can only be readily detected at certain times of the year such as during the monsoon season for frogs that inhabit arid parts of northern Australia. Sick frogs should be tested opportunistically to increase the probability of testing frogs that are infected with *B. dendrobatidis*, as a previous study found that >50% had chytridiomycosis (Berger et al. 2004). The survey protocol aims to provide an acceptable level of confidence (95%) to detect chytridiomycosis if it is in a specific frog population. To date data suggests that chytridiomycosis occurs at prevalences of >2% in frog populations using histological examination of toe tips and consequently 2% was used as the base prevalence for sample size calculations (Retallick et al. 2004; McDonald et al. 2005).

The task of surveying all Australian frog populations is large. For many frog populations information on the extent of their distributions is unknown. Therefore, this survey protocol aims to take into account the feasibility of such an undertaking. An efficient approach would be to sample for *B. dendrobatidis* in those frog populations near to or sympatric with known infected populations working outwards incrementally from known infected populations This is because *B. dendrobatidis* has been found in most areas in Australia where it is predicted to occur based on modelling the climatic variables that describe the distribution of *B. dendrobatidis* (Retallick 2003). Although this

model may reflect some of the collection biases, recent surveys have detected chytrid in areas where the model predicted a high likelihood of occurrence such as Tasmania and failed to detect it in areas where it is unlikely to occur such as the Northern Territory. However systematic sampling across a range of environments and species is necessary to validate or refine the model.

There are a number of aspects of surveying for *B. dendrobatidis* that we considered. These included which diagnostic test should be used, the number of frogs that need to be sampled, the time of year to sample, the life-stage to sample, the method of sampling and the logistics of sampling. Finally we designed the survey protocol so that it could be used by professional and amateur herpetologists and volunteers working with conservation agencies and other institutions.

### 3.1.2 Key Aspects to Consider When Surveying

**Area**

To survey systematically throughout all frog populations throughout Australia is logistically a major task. In order to make sampling feasible we propose to use the interim biogeographic regionalisation of Australia as defined by geology, climate and vegetation for our sampling scale and to prioritise sampling (Environment Australia 2000).

**Species**

There are 217 frog species in Australia. Therefore, there is a need to group frog species with similar risk of exposure to *B. dendrobatidis*. Because *B. dendrobatidis* requires moisture for survival, frogs have been grouped according to the type of water body they occupy, which is usually dependent on their breeding strategy. There are four groups or "ecological guilds": Stream Breeders (eg. *Litoria wilcoxi, L. rheocola*), Permanent Water Body Breeders (eg. *Litoria peronii, L. fallax, Limnodynastes peroni*), Ephemeral Breeders (eg. *L. caerulea, L. chloris*, most *Uperoleia* spp, *Cyclorana* spp.) and Terrestrial Breeders (eg. microhylids such as *Cophixalus ornatus*). These guilds broadly represent the different breeding strategies of frogs and how they relate to types of water bodies. Some frog species have a strategy that uses more than one type of water body such as permanent and ephemeral water bodies (eg. *L. caerulea* and *L. inermis*). If sampling these species, then the type of water body that they are associated with should be recorded. Direct developers would be included in the guild best reflecting the breeding sites that they occupy eg the extinct *Rheobatrachus silus* in Stream Breeders and *Assa darlingtoni* in Terrestrial Breeders. Once at least two species have been selected to represent each ecological guild then one needs to sample a significant number of each species throughout the bioregion (n=149, see below). If there are not enough individuals from each representative species then individuals from both species from the same guild may be pooled (Although, not ideal, combined probability methods can be used when equally low numbers of various species are collected, however, it is better to exclude negative samples from 1 or 2 individuals from a species when there are many individuals from other species).

**Life stage**

Testing of tadpoles may be more sensitive as high prevalence occurs in *Mixophyes* spp. from SE Qld and species in NE Qld (Pearl Symonds personal communication, Berger et al. 1999, Woodhams 2003). However, data is required from a range of species such as species with different breeding strategies and duration of tadpole stage. Duration of tadpole stage is probably significant as higher prevalences have been detected in older tadpoles as determined by size. Tadpoles that overwinter may be more likely to be infected than a cohort of the same species completing the tadpole stage in summer (Pearl Symonds personal communication).

Therefore, we suggest collecting tadpoles as well as swabbing frogs when possible. Collection and testing of tadpoles with abnormal mouthparts is a higher priority as this will increase the chance of obtaining positive results (Fellers et al. 2001, Rachowicz 2002, Rachowicz and Vredenburg 2004).

**Minimum number**

The expected prevalence of chytridiomycosis when it occurs in Australian and overseas frog populations is ≥2% based on the minimum prevalence detected in frog populations to date (Department of Environment and Heritage 2005). One needs to test 149 individuals to be 95% certain of detecting one positive frog from an infinite population given random sampling from a binomial distribution when the prevalence is ≥2% (Cannon and Roe 1982; DiGiacomo and Koepsell 1986).

However, as sampling is not random and the nature of the distribution of chytridiomycosis is unknown, one cannot be 95% certain of detecting at least one positive frog. If we assume that *B. dendrobatidis* spreads throughout a population rather than forming discreet clusters of infection and we sample systematically throughout the distribution of a population, then the certainty of detecting at least one positive frog will closely approximate 95%. If swabs analysed with the TaqMan PCR test developed by Boyle et al. (2004) consistently prove to be more sensitive than histological examination of toes then the expected prevalence may rise and the number of individuals that need to be tested will decrease. For example if the expected prevalence is 4% then 74 frogs need to be tested to be 95% certain of detecting at least one positive frog. A close approximation to the number of individuals that need to be tested to be 95% certain of detecting at least one positive frog is $n = 3/p$ where p is the prevalence.

**Season**

Which season to sample frogs will vary throughout Australia and with frog species. Sampling success will depend on when frogs are detectable. Ideally temperature should be considered given that optimal temperatures for *B. dendrobatidis* are between 17 and 25°C (Berger 2001, Woodhams et al 2003, Berger et al 2004, Piotrowski et al. 2004). For example this would mean sampling in Spring in southern Queensland when most frogs are detectable and temperatures are similar to those preferred by *B. dendrobatidis*. In contrast, in monsoonal Australia sampling must occur in summer when ephemeral breeders are detectable. This type of sampling depends on rainfall events and accessibility to these inundated areas. Temperatures at these times may be suboptimal for detecting chytridiomycosis.

### 3.1.3 Diagnostic tests

The ideal diagnostic method for this survey is likely to be swabbing frogs for the TaqMan PCR test (Boyle et al. 2004; Section 1.5). The PCR test is more sensitive than histology when conducted on toe tips (Boyle et al 2004). It is important to confirm the effectiveness as well as compare the cost, efficiency, ease of use and feasibility of the different diagnostic tests as part of this survey protocol (Objective 3.2). Therefore, we recommend collection of toe tips for histology until it is clear which diagnostic test is superior. Any concurrent collection of toe tips will also be useful in helping to confirm a suspect positive PCR test by histology. In addition, histology may be used infrequently to check the sensitivity of the PCR, which relies on homogeneity and conservation of the *B. dendrobatidis* DNA sequence where the primers and probe bind. To date, there is extremely low variation of DNA sequence among strains of *B. dendrobatidis* from different countries. We do not recommend systematic concurrent collection of toe tips with swabs since this would significantly increase the survey effort and raise ethical concerns.

Preliminary results indicate that the PCR test is highly specific (Boyle et al. 2004, Alex Hyatt, unpublished observations). Contamination of samples with *B. dendrobatidis* is the most likely cause of false positives, and could occur during field collection or during laboratory testing. Adherence to hygiene protocols in the field, such as wearing gloves/bags, holding frogs individually (Speare et al. 2004) will reduce contamination during collection. In the laboratory, negative controls will detect any systematic contamination although they may not indicate whether infrequent contamination is occurring. The PCR test is run in triplicate for each sample and all three tests must be positive before the swab is regarded as positive. This will reduce the likelihood of infrequent contamination leading to false positives (Boyle et al 2004). It is important to intermittently test the specificity of the PCR test to make sure that *B. dendrobatidis* DNA is being detected in positive tests. This will require sequencing of a PCR product from the sample to make sure it is *B. dendrobatidis* DNA.

Batch testing would reduce costs and preliminary trials indicate that up to 5 swabs from separate individuals may be combined with little loss of sensitivity (Alex Hyatt unpublished observations). When testing in batches, and there is a positive batch, then individual samples could be tested to ensure at least one tests positive. This will reduce the likelihood of contamination biasing results.

There is an urgent need to determine if different methods of storage of samples collected from the field affect PCR results. Preliminary results indicate that storing swabs at 23˚C in the laboratory for at least six months does not reduce sensitivity (Alex Hyatt unpublished observations). Until the best method of storage is determined we recommend storage of swabs at 4˚C in the field and freezing at <-20˚C in the laboratory. There is no loss of sensitivity of frozen swabs stored at <-20˚C for one year (Kriger and Hero unpublished observations). There is also an urgent need to agree on a standard swabbing technique under field conditions for the mapping protocol. Until a superior method of swabbing has been demonstrated, we recommend the method agreed upon at the Husbandry and Hygiene Conference, 10-14 Dec, Amphibian Research Centre, Werribee be used.

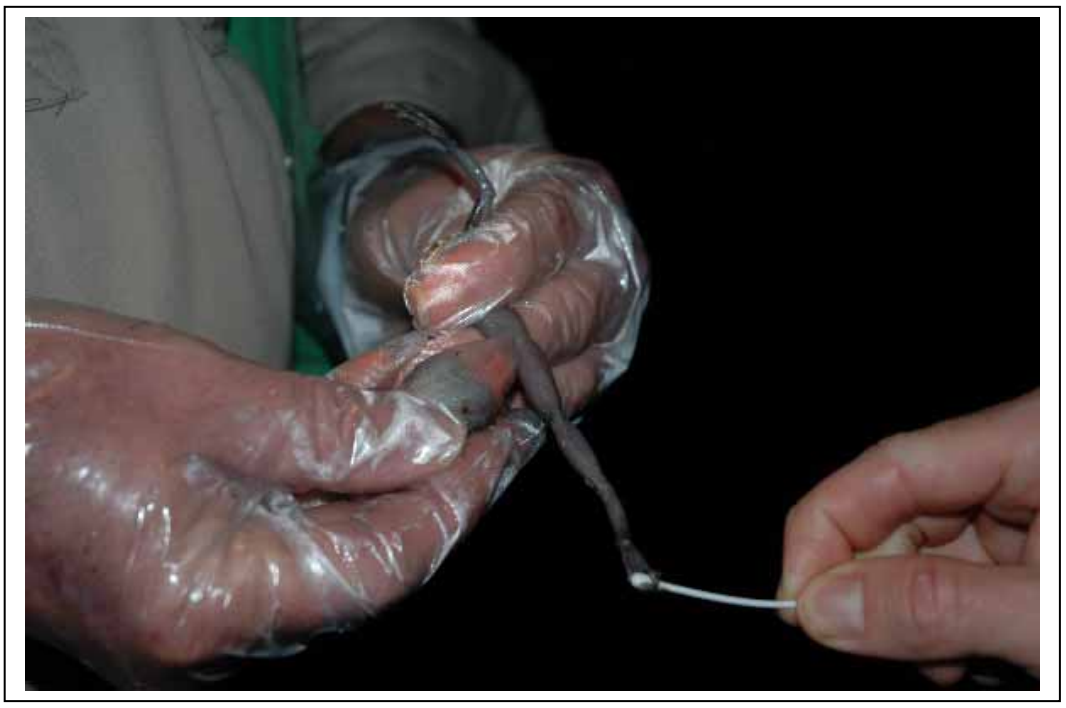


***Figure 11:*** Swabbing the hindfoot of a *Litoria nannotis*. Note how the frog grasps the swab with its toes enabling substantial pressure to be applied to the skin as the swab is drawn over its surface. The handler is wearing plastic food handling gloves, the swabber is holding the swab halfway along its length to avoid contaminating the tip but allowing better dexterity.

### 3.1.4 Surveying strategy

Surveys for chytridiomycosis in threatened species/populations in infected bioregions are the initial priority (use interim biogeographic regionalisation of Australia for bioregions, Environment Australia 2000). This has mostly been done in northern Australia and is currently underway in southern Australia. Unsurveyed bioregions, which are next to bioregions that have chytridiomycosis, would be surveyed next. Subsequently we would survey outwards incrementally from the known infected bioregions. This is because chytridiomycosis has been found within coastal bioregions of Australia, which appear to be the preferred habitat and climate for *B. dendrobatidis* i.e. lower maximum temperatures and higher rainfall. Moving inland the habitat and climate for chytridiomycosis becomes less preferable and therefore it is less likely to be present. Within each bioregion we aim to sample two species representative from each ecological guild throughout the distribution of that guild in the bioregion (some guilds will be absent from some bioregions). It is better to use the same species where possible throughout the distribution of the guild for consistency. For many species of amphibians, population, metapopulation or ecologically significant unit data are not available and populations will be defined based on the species distribution patterns (see Cogger 2000 for a summary of distributions of species). For this survey, the population will be defined by the distribution of the species within a bioregion. Stream breeding frogs should be targeted as these have been shown to be infected regularly with Bd (Berger et al. 2004).

The spacing of sampling of the population of interest will vary depending on distribution and accessibility of the population within the bioregion and the size of the bioregion. It is important to sample as widely as possible for each population of interest within the bioregion. This should result in sampling from heterogeneous areas and provide a non-biased sample of the population. For example, if the population is spread over 100 km within the bioregion, then the aim is to collect periodically over the 100 km, ie. every 10 km collect 15 individuals, and not to collect all tadpoles or frogs from the same location. Active males will be more readily observed and captured. However, there was no evidence of a difference in prevalence between males and females in *Taudactylus eungellensis* and *Litoria jungguy*/*wilcoxii* (n=71) (Retallick et al. 2004). Bioregions found to be infected can be surveyed subsequently on a smaller scale if necessary.

It is important to test all sick frogs seen. It is also recommended that any ill or recently dead frogs be retained as whole specimens for necropsy, with a toe clip stored in 70% ethanol and the body fixed in 10% buffered neutral formalin or frozen at -20˚C. Populations from different ecological guilds may overlap and therefore it will be possible to sample more than one ecological guild concurrently. A population should only be regarded as positive when at least two separate samples test positive. This will reduce the likelihood of false positives impacting on the distribution of Bd. If a population is positive for chytridiomycosis, one may stop testing from that population (there is no need to test the remaining samples) and sympatric populations belonging to that same ecological guild in the bioregion. For large bioregions, such as the Southern Brigalow Belt, which are 1000 km in length, it may be necessary to divide the bioregion into smaller sub-regions or areas, such as 200 km in length, to be sampled and prioritise areas to be sampled first.

Using this strategy the approximate number of samples for Australia could be (at most): 65 bioregions x 4 ecological guilds x at least two species per guild x 149 individuals per species = 38,740. However, this is likely to change dramatically as more is known about the distribution and biology of Bd and the real time PCR test. The number of samples required will be greatly reduced if the prevalence of chytridiomycosis is high using the PCR test, if some guilds are absent from bioregions and if some bioregions do not need to be sampled due to the current predictive model of the distribution of Bd being validated or improved. For example, if areas with low probability of

having Bd continually test negative then the current predictive model of the distribution of Bd is correct and only a proportion of these areas will need to be tested. The number of samples will increase if several species within an ecological guild within a bioregion need to be sampled, i.e. if the distribution of an ecological guild within the bioregion is determined by several species due to the heterogeneity of the bioregion.

### 3.1.5 Priorities

Surveying threatened species that have not been sampled previously within these infected and surrounding bioregions is a major priority to assist management. The next highest priority is surveying bioregions surrounding known infected bioregions for Bd. The next priority is sampling ecological guilds that have not been sampled previously within infected bioregions. The last priority is to sample species in infected regions that have not been sampled.

### 3.1.6 Other outcomes of the survey

The aim of this mapping protocol is to obtain the geographic distribution of chytridiomycosis in a range of species representing broadly different life strategies in Australia. While fulfilling this aim the survey may result in additional information on habitat preferences, prevalence and seasonal occurrence of chytridiomycosis. However, these issues are more complex than mapping, requiring greater sample sizes, so precise data will not be obtained.

**Survey Protocol Methodology**

**A.** Choose bioregions based on priorities of threatened species, regions next to infected regions, ecological guilds not sampled and species not sampled. (Sample susceptible guilds and species as first priority such as stream frogs)**.**

**B.** Determine which ecological guilds and species are present within the bioregion.

**C.** Determine the distribution of species within the ecological guilds present. Choose species (at least two), which represent the ecological guild to be sampled throughout the distribution of the ecological guild within the bioregion (choose species that are easy to find, widely distributed and are likely to be infected with Bd).

**D.** Select time of year to sample those species. Will have to wait for rain for opportunistic breeders.

**E.** Determine accessibility to the distribution of those species within the bioregion.

**F.** Sample systematically throughout accessible parts of the distribution for each species to be sampled within the bioregion. May need to divide distribution of species within bioregion up into smaller areas if the bioregion is large and prioritise sampling of areas based on likelihood of being infected with Bd. Recommended minimum area within a bioregion to be surveyed is 20% of accessible distribution of targeted species. Accessible areas may vary throughout the year, for example access is lower in the peak of the monsoonal wet compared to the early and late monsoon when species are active.

**G.** Initially sample species likely to be infected such as stream breeding species followed by ephemeral and permanent pond species unless the stream guild does not occur in the bioregion.

**H.** Collect swabs from at least 149 individual frogs from each species to be sampled. (May need to sample more frogs from some parts of the distribution compared with others depending on number of frogs present).

**I.** Collect tadpoles in 70% alcohol concurrently when swabbing frogs where possible. Collect 149 individual tadpoles for each species to be sampled.

**J.** Take a GPS reading at each collection point. A collection point is defined by a 1 km radius and a maximum of 15 individuals to be collected from each collection point. Space collection points evenly throughout the area to be surveyed for target species (this may not be possible if the species and preferred habitat is not distributed evenly across the landscape and environmental conditions vary within the collection period eg rainfall event).

**K.** Combine sampling of species from different ecological guilds where possible.

**L.** Test the 149 samples until at least two positives are detected. The remaining samples may be tested if additional information on the distribution of Bd within a bioregion is needed.

# 4. Scope Item 4

**Objective 4.1: Test the survey protocols designed in Stage 3 in the field by undertaking a pilot survey.**

Activity 4.1.1: Investigate the role of batching samples as a cost effective strategy in determining presence / absence of chytridiomycosis in amphibian populations.

Activity 4.1.2: Test the survey protocols in a range of amphibian populations in a range of environments to determine suitability of protocols as recommended national standards.

## 4.1 Batching of Samples for PCR Tests

Batching is a cost effective strategy for surveying frog populations which have moderate to high intensities of infection with Bd (at least 1 zoospore equivalent detected) (Hyatt et al unpublished observations). This is because any negative samples included in the batch have a minimal dilution effect on the sensitivity of the PCR. There was no loss of sensitivity when one positive swab (spiked with one zoospore) was batched with four negative swabs. This was replicated 13 times. When higher numbers of swabs were batched, the sensitivity of the assay decreased.

Batching has yet to be shown as cost effective when intensities of infection are lower. In this survey a known infected population, *M. fleayi* in South East Queensland, was negative when samples were batched as groups of 5. After retesting of individuals (n=39) the prevalence was 5% (n=2) although intensities of infection were very low, <1 zoospore detected (Table 1). There were also two suspicious positive samples, 2 samples that had 2 of 3 wells positive, so the prevalence could have been 10%. All 4 samples had a zoospore concentration of less than 1. Initially the samples were run as 7 batches of 5 plus 4 individuals. When the 7 batches were retested, 1 swab from each of 4 batches returned a positive or suspicious positive. Therefore areas that test negative with batch testing may have low intensities of infection. These results are not surprising, even though this was a known positive population, as prevalence and intensity of infection of Bd decline in summer in Queensland, which was when these samples were collected. The Australian Animal Health Laboratory has also had a similar experience with loss of sensitivity with batching. The batching of five swabs failed to detect a suspicious positive, i.e. when one swab, which was positive in one of three wells, was included with four *B. dendrobatidis*-negative swabs, the batched sample returned a negative result.

Field samples were collected by Keith McDonald, Harry Hines, Lee Skerratt, Andrea Phillott[1], Kerry Kriger and Jean-Marc Hero. Samples were analysed by Ruth Campbell[2], Diana Mendez, Andrea Phillott, Lee Skerratt, Donna Boyle[3] and Kerry Kriger[4].

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## 4.2 Feasibility of Undertaking the Survey Protocol

**A. Choose bioregions based on priorities of threatened species, regions next to infected regions, ecological guilds not sampled and species not sampled. (Sample susceptible guilds and species as first priority such as stream frogs).**

**Priorities:**

1) **Threatened species/populations**
   Most of these have been or are about to be surveyed. Survey protocols employed to date have been very effective in detecting Bd in these species and populations. This is because Bd is the threatening process for most of these species and populations. To test this survey protocol on threatened species and populations we chose the bioregions South East Queensland and Wet Tropics where most threatened species have been recorded. These are bioregions known to have frog populations infected with Bd and serve as positive controls for this survey protocol.

2) **Uninfected bioregions next to infected bioregions**
   We chose the Einasleigh Uplands and Cape York next to the infected Wet Tropics. We also chose the Southern Brigalow Belt next to the infected bioregion South East Queensland.

3) **Ecological guilds not sampled in infected bioregions**
   We chose the Wet Tropics and South East Queensland and NSW North Coast bioregions.

4) **Regions not next to infected regions**
   We chose the Gulf Plains and Tasmania, which was subsequently surveyed by Alexander Dalton and David Obendorf.

**B. Determine which ecological guilds and species are present within the bioregion.**

All four ecological guilds were present in each bioregion chosen apart from the stream breeders and terrestrial breeders in the drier bioregions such as the Gulf Plains and Einasleigh Uplands.

**C. Determine the distribution of species within the ecological guilds present.**

Queensland Museum and literature records and personal knowledge of frog field biologists were used for species distributions by bioregion.

**D. Choose species (at least two), which represent the ecological guild to be sampled throughout the distribution of the ecological guild within the bioregion (choose species that are easy to find, widely distributed and are likely to be infected with Bd).**

This step in the protocol applies to guilds not previously surveyed in both infected and uninfected bioregions. For the Einasleigh Uplands we chose *L. caerulea* and *L. rubella* to represent permanent and ephemeral breeding guilds. For the Gulf Plains we chose *Cyclorana novaehollandiae* and *L. inermis* to represent ephemeral breeders. We also chose *L. caerulea* and *L. latopalmata* for the Southern Brigalow Belt and South East Queensland to represent permanent and ephemeral breeding guilds. We chose *L. chloris* and *L. gracilenta* to represent permanent and ephemeral breeding guilds for the NSW North Coast. These choices were made before any field work was attempted. Subsequently, we found that it was hard to find

some of these targeted species when the field work was conducted. The detectibility of permanent water and ephemeral breeders was particularly hard to predict. Often non-targeted species from the same ecological guilds were more easily detected, for example *C. alboguttata* was found when looking for *L. caerulea*. Environmental conditions at the time of collection, particularly rainfall in the monsoonal areas, greatly affected detection of species and species numbers. Rainfall events maybe very localised and influence the presence and detection of species within the area. Therefore, we recommend that surveyors be prepared to collect species that are readily detectable and in sufficient numbers while in the field rather than to predetermine targeted species, especially for non-stream breeders in more xeric habitats.

**E. Select time of year to sample those species. Will have to wait for rain for opportunistic breeders.**

We surveyed stream breeders throughout the year in the mesic Wet Tropics to investigate the effect of season on detectibility of Bd. For the remaining frog populations we surveyed when frogs were most readily found, in spring in the upland rainforests of Cape York and ephemeral breeders in the monsoonal summer in the Gulf Plains, Einasleigh Uplands and the Southern Brigalow Belt. We surveyed permanent water breeders in spring and summer in South East Queensland and the NSW North Coast.

**F. Determine accessibility to the distribution of those species within the bioregion.**

Accessibility was determined by availability of vehicle access such as roads and walking access within frog distributions. Prevailing climatic conditions also affected access with flooded areas in the Gulf Plains being inaccessible during the monsoon.

**G. Sample systematically throughout accessible parts of the distribution for each species to be sampled within the bioregion. May need to divide distribution of species within bioregion up into smaller areas if the bioregion is large and prioritise sampling of areas based on likelihood of being infected with Bd. Recommended minimum area within a bioregion to be surveyed is 20% of accessible distribution of targeted species. Accessible areas may vary throughout the year, for example access is lower in the peak of the monsoonal wet compared to the early and late monsoon when species are active.**

Threatened populations and species were not sampled systematically throughout their distribution as these species have already been identified as being infected with Bd. Rather a few known infected populations were sampled, *Mixophyes fleayi* at Cunningham's Gap and Goomburra in the bioregion South East Queensland and populations of *L. rheocola*, *L. nannotis*, *L. genimaculata* and *Nyctymystes dayi* at Tully Valley, Nandroya, Dinner Creek, Breeden Creek, Big Tableland and Mt Misery in the bioregion the Wet Tropics. These are known infected populations for Bd and serve as positive controls for this survey protocol. Transects were walked to keep searching effort constant among species and over time. For ecological guilds not sampled in infected bioregions we chose small areas likely to be infected within the bioregions such as the Numinbah and Brisbane Valleys in South East Queensland and NSW North Coast bioregions. We also sampled stream dwelling frogs likely to be infected in these areas to act as positive controls. Similarly with uninfected bioregions next to infected bioregions we chose small areas most likely to be infected such as Millstream Falls National Park in the Einasleigh Uplands, the McIllwraith Range and Endeavour Valley in Cape York. For regions not next to infected bioregions such as the Gulf Plains we sampled as systematically and widely as possible throughout the distributions of targeted guilds.

**H. Initially sample species likely to be infected such as stream breeding species followed by ephemeral and permanent pond species unless the stream guild does not occur in the bioregion.**

Published data was examined to determine species likely to be infected with Bd (Berger et al 2004, Speare and Berger 2005).

**I. Collect swabs from at least 149 individual frogs from each species to be sampled. (May need to sample more frogs from some parts of the distribution compared with others depending on number of frogs present).**

It was not always possible to sample this number of frogs because it was too dry at the time of sampling and species were not easily detected in sufficient numbers.

**J. Collect tadpoles in 70% alcohol concurrently when swabbing frogs where possible. Collect 149 individual tadpoles for each species to be sampled.**

It was not always possible to sample this number of tadpoles. For instance at the time of sampling on the Gulf Plains very few tadpoles were detected as breeding had not peaked or the early breeding had resulted in rapid metamorphosis of tadpoles.

**J. Take a GPS reading at each collection point. A collection point is defined by a 1 km radius and a maximum of 15 individuals to be collected from each collection point. Space collection points evenly throughout the area to be surveyed for target species (this may not be possible if the species and preferred habitat is not distributed evenly across the landscape and environmental conditions vary within the collection period eg rainfall event).**

This was relatively easily achieved.

**K. Combine sampling of species from different ecological guilds where possible.**

This was possible.

**L. Test the 149 samples until at least two positives are detected. The remaining samples may be tested if additional information on the distribution of Bd within a bioregion is needed.**

For this pilot study we tested all samples collected in order to gain additional information on chytridiomycosis in frog populations.

**M. Compare the efficiency, effectiveness, costs, ease of use and feasibility of the main diagnostic tools used to detect chytrid fungus.**

This was conducted on four species within the Wet Tropics and *Mixophyes iteratus* in South East Queensland. Taqman real-time PCR from swabs was compared with histology of toe tips for adult frogs. Collected tadpoles have not been tested yet.

## 4.3 Results

### 4.3.1 Testing Priority Categories

**Threatened species**
The survey of stream breeding species previously known to be infected with Bd in the Wet Tropics showed that all five species surveyed were positive for Bd by both histology for toe tip clips and PCR. Abundance of species differed by site and season (Table 1). Prevalence of Bd also differed by site and season. Prevalences were similar among species. These are similar results to those of McDonald et al (2005) and Woodhams (2003), who examined these species in the Wet Tropics by histology of toe tip clips alone. It also concurs with the work of Berger et al (2004). Generally prevalence of Bd increases with cooler temperatures in the Wet Tropics such as at higher altitudes and during the cooler months. However, abundance of frogs can have an inverse relationship to these factors and therefore there is a trade off between ease of finding frogs and likelihood of being infected with Bd. Similar trade offs are likely to occur elsewhere in Australia. Regardless Bd is easily detected in these populations with minimal survey effort. Populations elsewhere in Australia with similar high prevalence of Bd should also be relatively easy to confirm as positive for Bd.

**Bioregions not surveyed next to infected bioregions**
The guilds most likely to be infected such as stream and pond breeders were targeted in these surveys. In addition, areas on the border with infected bioregions or areas more likely to be infected in non surveyed bioregions were surveyed, eg high altitude wet sclerophyll forest in north east Queensland. These bioregions remained negative.

**Guilds not surveyed in infected bioregions**
Guilds such as pond and ephemeral breeders remained negative despite stream breeders within 1km being positive for Bd in Queensland (Table 1). Pond breeders in mesic habitats in Queensland have been reported with Bd previously, however, these results suggest that stream breeders are more likely to be infected in Queensland and should be targeted first when surveying non surveyed areas. It also suggests that Bd may not be as important a pathogen in these guilds compared with stream breeders in northern Australia. This concurs with the severe effects that Bd appears to have had on the abundance of stream breeders compared with the other guilds although much of this information is anecdotal. In southern Australia, these relationships between Bd and frog guilds may differ due to lower temperatures and different rainfall patterns.

**Bioregions not surveyed and not next to infected bioregions**
Stream and pond breeders were not present in this bioregion, the Gulf Plains. Therefore, ephemeral breeders were targeted and all were negative. Whilst a positive record from one of these drier bioregions would drastically change the predicted distribution of Bd (Retallick 2003), it appears that these regions are likely to be negative for Bd. Extensive sampling in these regions is likely to be unrewarding and it is recommended that bioregions next to infected bioregions that have not been surveyed have the highest priority.

**Table 9:** Presence of *Batrachochytrium dendrobatidis* within frog species within bioregions

| Location | Species | Date | # Swabs | # Positives | % Percentage |
|---|---|---|---|---|---|
| Camp Creek, McIllwraith Ranges, **Cape York** | *Litoria eucnemis* | Sep-04 | 27 | 0 | 0 |
| | *Litoria longirostris* | Sep-04 | 41 | 0 | 0 |
| First Creek, McIllwraith Ranges, **Cape York** | *Litoria eucnemis* | Sep-04 | 45 | 0 | 0 |
| | *Litoria longirostris* | Sep-04 | 79 | 0 | 0 |
| Transect Creek, McIllwraith Ranges, **Cape York** | *Litoria eucnemis* | Sep-04 | 22 | 0 | 0 |
| | *Litoria longirostris* | Sep-04 | 94 | 0 | 0 |
| | *Rana daemeli* | Sep-04 | 1 | 0 | 0 |
| Solander Road, Endeavour Valley, **Cape York** | *Litoria inermis* | Jan-05 | 1 | 0 | 0 |
| Burke and Wills Roadhouse, **Gulf Plains** | *Cyclorana brevipes* | Jan-05 | 4 | 0 | 0 |
| | *Cyclorana novaehollandiae* | Jan-05 | 11 | 0 | 0 |
| Burke Development Road near Normanton, **Gulf Plains** | *Cyclorana alboguttata* | Jan-05 | 1 | 0 | 0 |
| | *Cyclorana novaehollandiae* | Jan-05 | 2 | 0 | 0 |
| | *Litoria caerulea* | Jan-05 | 1 | 0 | 0 |
| Gulf Development Road near Normanton, **Gulf Plains** | *Cyclorana alboguttata* | Jan-05 | 1 | 0 | 0 |
| | *Cyclorana brevipes* | Jan-05 | 1 | 0 | 0 |
| Burketown Road near Normanton, **Gulf Plains** | *Cyclorana novaehollandiae* | Jan-05 | 41 | 0 | 0 |
| | *Litoria caerulea* | Jan-05 | 12 | 0 | 0 |
| | *Litoria dahli* | Jan-05 | 1 | 0 | 0 |
| | *Litoria electrica* | Jan-05 | 1 | 0 | 0 |
| Barrett's Lagoon, Endeavour Valley, **Cape York** | *Limnodynastes convexiusculus* | Jan-05 | 1 | 0 | 0 |
| | *Litoria caerulea* | Jan-05 | 1 | 0 | 0 |
| | *Litoria inermis* | Jan-05 | 2 | 0 | 0 |
| | *Litoria infrafrenata* | Jan-05 | 1 | 0 | 0 |
| | *Litoria nigrofrenata* | Jan-05 | 1 | 0 | 0 |
| | *Litoria rothi* | Jan-05 | 1 | 0 | 0 |
| Jensen's Crossing, Endeavour Valley **Cape York** | *Litoria inermis* | Jan-05 | 9 | 0 | 0 |
| | *Litoria nasuta* | Jan-05 | 3 | 0 | 0 |
| Tumoulin, **Wet Tropics** | *Litoria fallax* | Jan-05 | 3 | 1[1] | 33 |
| | *Litoria genimaculata* | Jan-05 | 3 | 0 | 0 |
| | *Litoria gracilenta* | Jan-05 | 3 | 0 | 0 |
| | *Litoria rubella* | Jan-05 | 1 | 0 | 0 |
| Millstream National Park, **Einasleigh Uplands** | *Litoria gracilenta* | Jan-05 | 40 | 0 | 0 |
| | *Litoria gracilenta* | Jan-05 | 1 | 0 | 0 |
| | *Litoria inermis* | Jan-05 | 3 | 0 | 0 |
| | *Litoria latopalmata* | Jan-05 | 1 | 0 | 0 |
| | *Litoria rubella* | Jan-05 | 18 | 0 | 0 |
| Breeden, **Wet Tropics**[*] | *Litoria genimaculata* | Oct-04 | 8 | 7 | 88 |
| | | Dec-04 | 7 | 1 | 14 |
| | *Litoria lesueuri* | Oct-04 | 5 | 5 | 100 |
| | *Litoria nannotis* | Jun-04 | 1 | 0 | 0 |
| | | Aug-04 | 1 | 0 | 0 |
| | | Oct-04 | 3 | 3 | 100 |
| | | Dec-04 | 2 | 1 | 50 |
| Dinner Falls, **Wet Tropics**[*] | *Litoria genimaculata* | Oct-04 | 8 | 4 | 50 |
| | | Dec-04 | 0 | 0 | #DIV/0! |
| Mt Misery, **Wet Tropics**[*] | *Litoria genimaculata* | Jun-04 | 1 | 1 | 100 |
| | | Aug-04 | 13 | 10 | 77 |
| | | Oct-04 | 1 | 0 | 0 |
| | *Litoria lesueuri* | Oct-04 | 3 | 2 | 67 |
| | *Litoria rheocola* | Jun-04 | 24 | 23 | 104 |

| | | | | | |
|---|---|---|---|---|---|
| | | Aug-04 | 15 | 15 | 100 |
| | | Oct-04 | 25 | 6 | 24 |
| | | Dec-04 | 25 | 13 | 52 |
| Nandroya, **Wet Tropics*** | *Litoria genimaculata* | Dec-04 | 2 | 1 | 50 |
| | *Litoria nannotis* | Dec-04 | 2 | 2 | 100 |
| | *Litoria rheocola* | Aug-04 | 3 | 3 | 100 |
| | | Dec-04 | 2 | 2 | 100 |
| O'Keefe, **Wet Tropics*** | *Litoria genimaculata* | Jun-04 | 21 | 15 | 71 |
| | | Aug-04 | 38 | 26 | 68 |
| | | Oct-04 | 38 | 17 | 45 |
| | | Dec-04 | 3 | 0 | 0 |
| | *Litoria leseuri* | Oct-04 | 1 | 0 | 0 |
| | *Litoria rheocola* | Jun-04 | 19 | 18 | 95 |
| | | Aug-04 | 10 | 3 | 30 |
| | | Oct-04 | 8 | 3 | 38 |
| | | Dec-04 | 1 | 0 | 0 |
| Tully, **Wet Tropics*** | *Litoria genimaculata* | Aug-04 | 11 | 9 | 82 |
| | | Oct-04 | 5 | 2 | 40 |
| | | Dec-04 | 4 | 0 | 0 |
| | *Litoria lesueuri* | Oct-04 | 3 | 0 | 0 |
| | *Litoria nannotis* | Jun-04 | 12 | 10 | 83 |
| | | Aug-04 | 1 | 1 | 100 |
| | | Oct-04 | 4 | 1 | 25 |
| | | Dec-04 | 2 | 1 | 50 |
| | *Litoria rheocola* | Jun-04 | 14 | 9 | 64 |
| | | Dec-04 | 4 | 4 | 100 |
| | *Nyctimystes dayi* | Jun-04 | 3 | 2 | 67 |
| | | Aug-04 | 4 | 4 | 100 |
| | | Oct-04 | 1 | 0 | 0 |
| | | Dec-04 | 16 | 4 | 25 |
| Gap Creek West, Main Range National Park, **SE Qld*** | *Mixophyes fleayi* | Jan-05 | 39 | 4 | 10 |
| Haigslea, **SE Qld** | *Litoria caerulea* | Jan-05 | 8 | 0 | 0 |
| Pine Mountain, **SE Qld** | *Litoria caerulea* | Jan-05 | 12 | 0 | 0 |
| Kurumbul - Wondalli Road, near Kurumbul, **S Brig Belt** | *Cyclorana alboguttata* | Jan-05 | 16 | 0 | 0 |
| | | Feb-05 | 28 | 0 | 0 |
| | | Mar-05 | 6 | 0 | 0 |
| | *Litoria latopalmata* | Jan-05 | 15 | 0 | 0 |
| Numinbah Valley, **SE Qld and NE NSW** | *Litoria gracilenta* | | 40 | 0 | 0 |
| | *Litoria latopalmata* | | 14 | 0 | 0 |
| | *Mixophyes fasciolatus* | | 12 | 0 | 0 |
| | *Litoria pearsoniana* | | 374 | 178 | 48 |
| | *Litoria wilcoxii* | | 414 | 112 | 27 |
| | *Litoria chloris* | | 170 | 15 | 9 |

*All samples, except for those from Gap Creek West and Wet Tropics, were analysed in batches of 5. Where samples could not be equally divided into batches of 5, the remainder were analysed individually. All samples from the Wet Tropics were analysed individually. Samples from Gap Creek West were initially batched, but were re-analysed individually when all batches presented negative for the presence of chytrid. As this location is a know chytrid infection area it was decided to re-test the swabs to determine the sensitivity of batching. When retested, samples presented a low positive for the presence of chytrid. As all samples were tested in triplicate, those with only 1-2 positive wells will be retested. These suspicious positives were counted as positives in the table.

[1]Only 1 well from 3 presented positive for chytrid. This sample is viewed as a suspicious positive and will be retested.

Not all of the frogs sampled from the Wet Tropics were tested.

### 4.3.2 Comparison of PCR and Histology

The PCR test conducted on swabs was shown to be overall six times (range 2-6 times for individual species of frogs) more sensitive compared with histology of toe clips for detection of Bd in stream breeding frogs in the Wet Tropics including *L. rheocola*, *L. nannotis*, *L. genimaculata* and *Nyctimystes dayi* and in South East Queensland, *M. iteratus* (Tables 2 and 3, see Appendix 2). Many frogs tested positive by PCR, but were negative by histology (n = 119). Only nine animals tested positive by both PCR and histology although an additional nine that were positive for PCR were suspect positive for histology. There were a number of suspicious positive PCR results (n = 23). Not all three wells in the PCR tested positive for these animals and zoospore counts were less than 1. Only one of these was also a suspicious positive by histology. These samples will be retested by PCR and immunoperoxidase. Vary rarely frogs were positive on histology but negative by PCR (n = 3). There were an additional 6 suspicious positives by histology but negative by PCR. Overall the PCR detected 161 frogs as being positive or suspect positive whereas histology detected 28. There is little increase in sensitivity if both tests are used.

**Table 10:** Comparison of the sensitivity of diagnostic tests, Taqman PCR of swabs and histology of toe clips, in detecting *Batrachochytrium dendrobatidis* in stream breeding species including *L. rheocola*, *L. nannotis*, *L. genimaculata* and *Nyctimystes dayi* in the Wet Tropics.

| | **PCR** | | | |
|---|---|---|---|---|
| **Histology** | Negative | Positive | Susp.Positive | **TOTAL** |
| Negative | 80 | 105 | 14 | 199 |
| Positive | 2 | 9 | 0 | 11 |
| Susp. Positive | 1 | 8 | 1 | 10 |
| **TOTAL** | 83 | 122 | 15 | **220** |

**Table 11:** Comparison of the sensitivity of diagnostic tests, Taqman PCR of swabs and histology of toe clips, in detecting *Batrachochytrium dendrobatidis* in *Mixophyes iteratus* in South east Queensland (n = 101).

| | **PCR** | | | |
|---|---|---|---|---|
| **Histology** | Negative | Positive | Susp.Positive | **TOTAL** |
| Negative | 72 | 14 | 8 | 94 |
| Positive | 1 | 0 | 0 | 1 |
| Susp. Positive | 5 | 1 | 0 | 6 |
| **TOTAL** | 78 | 16 | 8 | **101** |

# 5. Scope Item 5

**Objective 5.1: Review the pilot survey and identify and prioritise the gaps in existing knowledge concerning the effectiveness of the survey protocol developed in Stage 3 and provide recommendations as to areas of future research activity that will address those gaps identified.**

Activity 5.1.1: Review results of pilot survey and make recommendations about areas of future research needed to address gaps in knowledge.

Activity 5.1.2: Discuss the possibility of the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.

Activity 5.1.3: Provide comment on the suitability of the survey protocols for a standard way of mapping amphibian populations in Australia for chytridiomycosis.

**Objective 5.2: Based on the discussion above, provide recommendations that would need to be addressed to allow the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.**

Activity 5.2.1: Provide recommendations that would need to be addressed to allow the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.

Activity 5.2.2: Final report will contain an executive summary of the work undertaken and key findings of project.

## 5.1 Review of the Pilot Survey

The following review of the pilot survey was provided by the authors of this report and Andrea Phillott, Ruth Campbell, Kerry Kriger and Rick Speare.

Whilst the aim of this mapping protocol is to map the distribution of Bd in all Australian frog populations, it is clear from this pilot study that priority setting is important. This pilot study suggests that Bd has higher prevalences in species that have experienced known declines. Preliminary evidence suggests that drier and/or warmer bioregions next to currently known infected bioregions may not have Bd or it may occur at very low prevalences. Non stream breeding guilds may have lower prevalences of Bd in northern Australia. Therefore when surveying frog populations in northern Australia it is important to start with stream breeding species, especially those that have declined, in mesic environments experiencing moderate temperatures for at least part of the year. It appears that the probability of detecting Bd declines as the environment becomes more xeric, temperatures increase (for example Bd declined in prevalence in the wet tropics during summer) and frog species spend less of their life history associated with permanent water. Within these general trends there may be minor exceptions such as when drier and warmer conditions result in frogs being closer to permanent water and increasing their risk of exposure to Bd. If Bd is absent in a bioregion in frog populations likely to be infected, then it would not be worthwhile surveying sympatric

species that have a lower risk of being infected. Similarly if Bd is absent from a bioregion, then it would not be worth surveying surrounding bioregions that are less likely to be infected. However, more work is needed to confirm that the ecological and environmental behaviour of Bd is consistent throughout Australia.

When present, chytridiomycosis occurred at prevalences of >5% in stream, pond and ephemeral breeding species in this pilot survey using swabbing of skin and Taqman PCR as a diagnostic test (Boyle et al. 2004). The PCR test was shown to be at least twice and up to six times as sensitive compared with histology of toe clips for detection of Bd in stream breeding frogs in the Wet Tropics and South East Queensland. Vary rarely were frogs positive by histology, but negative by PCR. Therefore, using both tests does not increase sensitivity significantly. Assuming that the PCR test is consistently more sensitive than histology by a factor of two for all frog species then the minimum prevalence at which Bd will occur as detected by PCR is expected to be 5% based on the minimum 2.7% prevalence detected in *Xenopus* in southern Africa by histology (Weldon *et al*. 2003). Subsequently, using a 5% prevalence as a minimum cut off for detection of Bd using PCR will reduce the number of individuals required to sample to 59 to be 95% confident of detecting at least one positive. Thus, using the PCR is far more achievable and practical for mapping Bd as compared with histology. However, it is possible that Bd occurs at prevalences lower than 5% in some frog species. For this reason we recommend that further work be done to establish that Bd as detected by Taqman PCR does not occur at prevalences of 2% and lower in some species and environments.

Batch testing would reduce costs if intensities of infection were ≥1 zoospore. However, batch testing will increase costs if batches need to be retested to detect lower intensities of infection. At the moment we recommend the maximum number of swabs to be batched as five based on this loss of sensitivity. More work needs to be done to improve the sensitivity of batch testing samples which have low intensities of infection. Running samples in single wells will reduce costs, but will also reduce the sensitivity of the PCR in detecting suspicious positives. A cost/benefit analysis needs to be done on these different strategies in relation to the prevalence and intensity of infection of Bd in Australian frog populations. The swabbing of tadpole mouthparts shows promise as an alternative strategy to swabbing frogs to map Bd (Alex Dalton and David Obendorf unpublished observations). More work should be done to confirm this on a range of species. There is also preliminary evidence that abnormal mouthparts may be used as an indicator of prevalence of Bd in tadpoles (Fellers et al. 2001; Alex Dalton and David Obendorf unpublished observations).

There is an urgent need to standardise swabbing techniques through experiments comparing the sensitivity of different swabbing techniques. Currently, all swabbing methods aim to sample a constant proportion of a frog. Kerry Kriger and Jean-Marc Hero swab some dorsal and ventral surfaces five times - swabbing the back, ventral sides, back of each thigh, “drink patch" 5 times - (up and down), then swabbing the webbing between each toe on the hind feet (outwards only). Keith McDonald and Lee Skerratt swab ventral surfaces of the abdomen, hindlimbs, hands and feet twice. Alex Hyatt recommends swabbing the underside of feet, legs and drink patch 2-3 times. In the method of Harry Hines, the frog is held in the left hand with the stifle joints held firmly between the index and middle fingers and the front limbs rest on the thumb. The following areas are then swabbed once (the swab is lightly drawn across the skin once): along the dorsal midline from the urostyle to the dorsal midpoint between the eyes, along the left side of the body from the angle of the stifle to the groin continuing on to the armpit, along the ventral midline from the vent to the sternum and finally from the webbing between the digits on the left foot.

There is also a need to determine the best method of storage of swabs in the field. Work by AAHL in the laboratory situation has shown that Bd DNA is remarkably stable even at room temperature (Alex Hyatt, pers comm.). This should be confirmed in field studies particularly in higher than laboratory temperatures.

In sick frogs the highest intensity of infection occurs on ventral surfaces (Berger 2001). In healthy frogs infection occurs mainly on the feet and hands (Rick Speare unpublished observations; Weldon 2005). There is a need to look at the distribution of Bd on several species of amphibians from different ecological guilds over the course of infection especially during subclinical infection, which areas are the best to swab for detecting Bd and how many times those areas should be swabbed.

For the mapping protocol, a technique that combines simplicity, speed, reproducibility between species and individuals and maximises detection probability would be best. For results to be comparable between frog species of different body sizes then the proportion of the body sampled should be consistent.

For some research projects, there is a need to maximise detection probabilities and be able to quantitate infection at the expense of other attributes of the technique. Swabbing the entire frog comprehensively may achieve this. However, evidence to date suggests that swabbing of ventral surfaces will provide the same sensitivity and relative quantification of infection levels compared with swabbing the entire frog. This is because infection does not readily occur on dorsal surfaces and has only been reported in frogs when ventral surfaces are infected (Berger 2001, Rick Speare unpublished observations). In addition, there is preliminary evidence that dorsal skin secretions may interfere with the real-time PCR and therefore the sensitivity of the detection test (Alex Hyatt unpublished observations). Although the real-time PCR is reported in zoospore units, it is essential to bear in mind that there has been no work published to show that the number of zoospore units is directly correlated to the number of sporangia per unit of skin or the number of total sporangia on the amphibian's body. Severe chytridiomycosis on histology does give a higher zoospore unit value on real-time PCR. At present the real-time PCR test should be regarded as semi-quantitative until the degree of correlation with intensity of infection and degree of activity of infection has been demonstrated by experimentation.

These issues were discussed at the Husbandry and Hygiene Conference at the Amphibian Research Centre, December 11-14, 2004. It is recommended that we use the technique agreed upon at that conference where only the ventral surfaces of the lower abdomen, thighs, and hands and feet are swabbed twice for surveying frog populations (see photos, video and description of technique available on the Amphibian Diseases Homepage). This technique may be modified once further data is obtained on the best swabbing method.

An experiment comparing ideal versus possible worse case storage methods for swabs in the field would be worthwhile to see if there is any loss of sensitivity. This will involve storing samples on ice in the field and freezing at –80 C upon return to the laboratory versus keeping samples at ambient temperature in the field for four weeks and in an office for two weeks.

Activity 5.1.2: Discuss the possibility of the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.

Activity 5.1.3: Provide comment on the suitability of the survey protocols for a standard way of mapping amphibian populations in Australia for chytridiomycosis.

The survey protocols appear suitable for use as a standard way of mapping amphibian populations in Australia for chytridiomycosis. Acceptance of these protocols was tested by presenting them at two conferences, the Husbandry and Hygiene Conference at the Amphibian Research Centre, December 11-14, 2004 and the Australian Society for Herpetology Conference in Springbrook, February, 2005 (Skerratt et al 2004, 2005). On both occasions the protocols were well received. The protocol should be continually adjusted to account for new findings as knowledge about chytridiomycosis is rapidly increasing. As crucial information on the distribution of chytridiomycosis is lacking in some species and guilds, we recommend more detailed surveying in the first year of implementation of this protocol, then reassessing the protocol to increase its efficiency.

Using these protocols the approximate number of samples for Australia could be (at most): 65 bioregions x at least two species per guild x 149 individuals per species = 38,740. The number of samples required will be greatly reduced if the prevalence of Bd is higher using the PCR test =7,800. In addition, not all samples will need to be analysed given that once two positives are detected, the remaining samples from the positive population are no longer tested. This will further reduce costs. Some guilds in some bioregions will not need to be surveyed if sympatric populations of species at higher risk of infection with Bd are negative. The cost of sample collection includes salary, vehicle, field equipment and consumable costs. This approximates $20 / sample in bioregions close to population centres such as the wet tropics where frog numbers are higher and found over a smaller distance. Field collection costs will rise in remote areas such as Cape York and the Gulf Plains. Laboratory costs for testing samples are a minimum of $30.75 per sample using the Taqman RT-PCR.

**Objective 5.3: Based on the discussion above, provide recommendations that would need to be addressed to allow the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.**

Activity 5.3.1: Provide recommendations that would need to be addressed to allow the survey protocol developed in Stage 3 being adopted nationally as the standard way of surveying Australian amphibian populations for chytrid fungus.

**Dissemination and Concensus:** To be adopted nationally the survey protocol should be disseminated to individuals and groups within Australia most likely to undertake the work and those most likely to fund such surveys. Dissemination has already commenced through conference presentations in 2004 and 2005. Publication in scientific journals is the next key step, particularly the proof of concept study reviewed in section 5.1. Three papers relevant to diagnosis of chytridiomycosis are currently in press from this tender and others are in preparation. The next step is to achieve concensus among key stakeholders. A national workshop to discuss the implementation of the protocol would assist this. Participants would get a sense of contribution and ownership of the protocol and would therefore be likely to implement it. The workshop could be run in conjunction with a conference such as the Australian Society for Herpetology in April 2006. Running a workshop in conjunction with a relevant conference would increase attendance and reduce costs. Groups and individuals likely to be interested in participating could be invited such as the Victorian Frog Group, Frog Decline Reversal Project, other community based frog groups and wildife managers at state, territory and national levels. The workshop should also include training for aspects of the protocol

that require skill and consistency such as swabbing frogs and tadpoles. This workshop could also be used to accredit the competency of individuals and groups to carry out survey work.

**Funding for a National Survey:** Funds should be provided to undertake a national survey. A national survey using the protocol proposed will involve testing of 19,370 specimens and an approximate cost of $600,000 for real-time PCR tests. This estimate does not include the costs associated with sample collection. Field based costs vary with the remoteness of the sites sampled and the availability of amphibians.

**Quality Assurance of Diagnostic Results:** Since multiple laboratories are now performing diagnostic tests for chytridiomycosis, ensuring a high standard of sensitivity and specificity is essential for confidence in reported results. The most critical element is implementation and ongoing support for a quality assurance (QA) system. The two key tests are real-time PCR and histology. The QA system could have both proactive and passive components. The proactive component could involve twice yearly dissemination of samples from a central body to diagnostic laboratories that test the samples without being aware of their status. The actions to follow incorrect results would have to be determined by concensus among the participating laboratories or could be proscribed by the body funding the scheme. For PCR tests, swabs and DNA extracts could be sent out to participating laboratories. For histology, stained and unstained slides of amphibian skin could be distributed. The passive component of the QA scheme could consist of laboratories submitting a proportion of positive diagnoses, particularly highly critical ones such as new species and new locations, and some negative ones for confirmation. Although participating laboratories should pay to participate in such a QA scheme, DEH should consider providing funding for its initial establishment for 5 years. Funding will be required initially as start-up costs will involve establishing protocols, credibility of a QA scheme and incentives for laboratories to participate. Laboratories in New Zealand should be asked to participate and the scheme could possibly be extended to other countries. Extension of such a QA scheme beyond Australia will increase its reputation and validity, even within Australia.

**Data Management:** The current database should be updated regularly and a web based interface for data entry should be implemented. The knowledge and skills to do this are located within the Australian Wildlife Health Network (AWHN) and the Amphibian Disease Ecology Group (ADEG) at JCU. The AWHN already have a web data entry and database system (Wildlife Health Information System (WHIS)) in place to capture wildlife health information and this could be adapted to capture information on chytridiomycosis. We recommend that funding be provided to employ a database programmer located within the AWHN who can adapt the WHIS for chytridiomycosis reports. We also recommend that a database manager be employed for three years to ensure that all available data on chytridiomycosis testing of wild amphibians is captured and analysed to provide relevant information on the distribution of chytridiomycosis both spatially and temporally for each species of amphibian and that a predictive model for the disease is produced to enable mangers to take preventative action to stop the spread of *B. dendrobatidis*.

Activity 5.3.2: Final report will contain an executive summary of the work undertaken and key findings of project.

Provided at front of this document.

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# 7. Appendices

## APPENDIX 1: Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis

This paper has been published as:

Berger L[1], Speare R[1], Skerratt L[2] (2005). Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis. Diseases of Aquatic Organisms, Vol. 68: 65-70.

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ABSTRACT: Although histopathology is used routinely for diagnosis of chytridiomycosis in live and dead amphibians, there are no quantitative data on the distribution of the causative fungus, Batrachochytrium dendrobatidis, in the skin. We performed quantitative histological examinations on 6 sites on the body and 4 toes of 10 free-ranging adult green tree frogs Litoria caerulea found recently dead or dying from chytridiomycosis. Large numbers of sporangia occurred in all areas of ventral skin and toes; on average there were 94.3 sporangia mm–1 of superficial epidermis. The number of sporangia was highly variable and this appeared to be related to the stage in the cycle of sloughing. The stratum corneum tends to build up with high intensities of infection and then sheds entirely rather than being shed continuously. Very few or no sporangia occurred on dorsal skin. This distribution could be explained by the dryness of the dorsal skin or possibly by the greater number of serous glands, which produce antifungal peptides, on the dorsum. In some frogs, ulceration and erosions occurred on skin on the back in the absence of sporangia. Other pathological changes such as hyperkeratosis and congestion occurred much more frequently on ventral surfaces.

## APPENDIX 2: Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR

The manuscript of this paper is under review at the time of writing this final report. Consequently, this paper could not be presented as part of this final report. It is anticipated that this paper will be published in a journal in the near future.

## APPENDIX 3: MS-222 does not kill *Batrachochytrium dendrobatidis*.

This paper has been published as:

Webb R, Berger L, Mendez D, Speare R (2005). MS-222 (tricaine methane sulfonate) does not kill the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms, Vol. 68: 89-90.

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ABSTRACT: MS-222 (tricaine methane sulfonate) is an agent commonly used to anaesthetise or euthanize amphibians used in experiments. It is administered by immersing the animal to allow absorption through the skin. Chytridiomycosis is an important disease of amphibians and research involves experiments with live animals. *Batrachochytrium dendrobatidis,* the fungus which causes chytridiomycosis, is located in the skin and therefore the organism should come into contact with MS-222 when it is used. *B. dendrobatidis* is a sensitive organism which could possibly be killed by MS-222. Hence, results of chytridiomycosis studies in which MS-222 is used could be unreliable. A concentration of 2 g l–1 and an exposure duration of 1 h is at the high end of the range at which MS-222 would be most commonly used. Exposure to 2 g l–1 MS-222 for 1 h does not kill *B. dendrobatidis* cultures, suggesting that MS-222 is safe to use in chytridiomycosis studies.