

Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes

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ABSTRACT: *Batrachochytrium dendrobatidis* is a major pathogen of frogs worldwide. It has been associated with catastrophic declines of frog populations including those in pristine habitats in Queensland, Australia. To facilitate genetic and disease studies of this fungus and related species, it is essential to have a reliable long-term storage method to maintain genetic integrity of isolates. We have adapted well-established techniques used for the long-term storage of tissue-culture cell lines to the preservation of *B. dendrobatidis* and other chytridiomycetes. This simple method has allowed us to recover these fungi from storage at -80°C and in liquid nitrogen over an extended period. With this technique it is now possible to preserve saprobic and parasitic isolates from a variety of environmental and disease situations for comparative genetic and biological studies.

KEY WORDS: Chytrids · *Batrachochytrium dendrobatidis* · Storage · Cryopreservation · Amphibian declines

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INTRODUCTION

Chytridiomycosis is a fatal disease of amphibians caused by the fungus *Batrachochytrium dendrobatidis*, the only member of the Chytridiomycota that causes disease in vertebrates (Berger et al. 1998, 1999). This fungus has a broad amphibian host range and occurs worldwide (Daszak et al. 1999). As of the year 2000, a total of 2 amphibian orders (Anura and Caudata), 15 families and 94 species from Australia, South America, Central America, North America, Europe, New Zealand and Africa have been diagnosed as being infected with *B. dendrobatidis* (see www.jcu.edu.au/school/phtm/PHTM/frogs/ampdis.htm, Speare 2001). Within Australia, 46 of the 214 described species are known to be infected with *B. dendrobatidis*.

Chytridiomycosis has been associated with regional and global declines of amphibian populations (Berger et al. 1999, Daszak et al. 1999). Whilst many species are known to be infected with *Batrachochytrium dendrobatidis*, species which have restricted geographical

ranges, live at high altitudes (or cooler climates), have a riparian terrain and are of low fecundity are the most susceptible to population declines and, in the worst-case scenario, extinction. To date, our understanding of *B. dendrobatidis* and chytridiomycosis is limited. Infected animals vary in clinical signs; some are described as lethargic, subject to fitting, lack the 'righting reflex' and exhibit unusual behavior such as sitting in open areas. The disease is typified by a hyperkeratotic and hyperplastic response of the stratum corneum and stratum granulosum. This pathology is useful in diagnosing the disease but does not provide a definitive insight into understanding the changes in physiology that lead to the death of infected animals.

To date, 80 isolates of *Batrachochytrium dendrobatidis* have been made (J. E. Longcore, unpubl. data) from North America and Australia. The life cycle of the fungus has been described by Longcore et al. (1999) in terms of the presence of zoospores and sporangia; however no resting stage has been identified. If the pandemic is to be understood then questions relating

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to the origin and spread/transmission of disease and specific cause of death must be addressed. To address these questions it is essential that the many isolates be effectively frozen and archived until required. The development of such a procedure would be invaluable for future research as questions relating to attenuation (via passage), contamination and alterations in the genome (e.g. site mutations) can be minimized. *B. dendrobatidis* can be isolated from infected frogs using TGhL agar and then maintained on agar or in TGhL broth at 23°C (Longcore et al. 1999). Cultures also grow well at 15°C and survive many months at 4°C. These procedures are, however, labour intensive since cultures require regular examination for contamination, growth failure and passage every 14 to 21 d on nutrient agar plates at room temperature or every 4 to 5 mo when grown in liquid medium and stored at 4 to 6°C.

Previous methods for long-term storage of *Chytridiomycetes* at low temperatures have produced survival rates of only 16% (Smith 1982) while Hohl & Iselin (1986) had limited success with storage in liquid nitrogen. We have developed a simple method of freezing, based on established methods for freezing tissue-culture cell lines that have had 100% success in retrieval of *Batrachochytrium dendrobatidis* from storage over 12 mo. The method was also successfully applied to other fungi from the phylum Chytridiomycota.

MATERIALS AND METHODS

Culture and harvest of *Batrachochytrium dendrobatidis*. TGhL (13 ml) broth (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 1000 ml distilled water) in a 25 cm² disposable tissue-culture flask was inoculated with 2 ml of an actively growing 7 d old broth culture of *B. dendrobatidis* (Isolate A98 1810/3) and incubated at 17 to 23°C for 3 to 21 d (Longcore et al. 1999). All cultures contained active released zoospores and sporangia (empty or containing zoospores), both in the media and attached to the plastic. Sporangia were scraped off the flask and centrifuged at 1700 \times g for 10 min (thereby removing most zoospores). The supernatant (which contained large numbers of active zoospores) was decanted and the pellet resuspended in 1 ml cryoprotectant (see below).

A zoospore suspension was prepared by seeding TGhL agar plates (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose, 10 g agar, 1000 ml distilled water) with 2 ml of actively growing broth culture. The plates were then left until most of the solution had diffused into the agar at which time the sealed plates were inverted and incubated at 23°C for 4 d. Zoospores

were harvested by flooding the plate (¥2) with 2 ml of DS solution (a weak salt solution resembling pond water, comprising 10⁻³ M KH₂PO₄, 10⁻⁴ M MgCl₂ and 2 \times 10⁻⁵ M CaCl₂), following which the solution was removed and placed in a 1 or 2 ml cryotube. Zoospore numbers were counted in a haemocytometer.

Cryoprotectants. The following cryoprotectants were evaluated: (a) TGhL broth only (control), (b) 10% (v/v) glycerol in 1.3% (w/v) skim milk powder, (c) 10% DMSO (dimethyl sulfoxide)/TGhL broth or (d) 10% DMSO/10% foetal calf serum (FCS) in TGhL broth, (see Table 1). The optimum cryo-protectant was found to be 10% DMSO/10% FCS in TGhL broth and this was subsequently used to freeze all samples.

Freezing of samples. Pellets of zoosporangia were suspended in cryopreservation media and frozen in 1 ml cryotubes in a cryocontainer (Nalgene Cryo 1°C Freezing Container, Cat. No. 5100-0001, Nalgene Nunc Int.) containing 100% isopropanol, which gives a 1°C min⁻¹ rate of cooling when placed at -80°C. Note: the absolute number of zoosporangia is not critical for this process and the above protocol was used for all species of fungi assessed within this study. These frozen samples were either stored at -80°C or transferred to liquid nitrogen cabinets within 5 d. A culture of zoospores only (collected as described above, pelleted in a microfuge [13 \times 10³ \times g] and resuspended in the optimal cryoprotectant) was harvested from 4 d old agar plates and frozen as described above to assess whether released zoospores could be cryopreserved and revived. To assess the efficiency of the optimal method, samples containing mixtures of zoospores and sporangia (within cryo-tubes) were also frozen by placing the cryotubes directly in liquid nitrogen and reviving by the method described below.

Revival of *Batrachochytrium dendrobatidis*. Samples were removed monthly from -80°C and immersed immediately in water at 43°C for 30 s to 1 minute, or until nearly thawed. Placing the samples at 43°C hastens thawing without the risk posed by a rapid uncontrolled rise in temperature, whereas temperatures >43°C kill the organism. The sample was divided between 2 TGhL plates and either 1 ml DS solution or 1 ml TGhL broth was added to dilute any possible detrimental effects of the DMSO. The plate was subsequently left until most of the liquid had diffused into the agar, sealed with parafilm, inverted and incubated at 23°C.

The plates were assessed daily for growth of sporangia and presence of zoospores. It was not possible to count the number of sporangia. Sporangia growth was characterised by an increase in colony size and zoospore activity around the edges of the colonies. The efficiency of revival was judged by the number of actively growing colonies per plate.

Table 1. *Batrachochytrium dendrobatidis*. Assessment of cryopreservation protocols using 4 d old cultures. Quantification of revival (presence of colonies of sporangia with active zoospores; i.e. motile zoospores indicative of viable sporangia) is expressed as either '-', '+', '++' or '+++', where '-' indicates no growth and '+++' represents growth equivalent to that in non-frozen cultures. ND: not done, as skimmed milk/glycerol was the least optimal cryopreservative. DMSO: dimethyl sulfoxide; FCS: foetal calf serum

| Method of freezing | | Freezing media | | | |
|------------------------------------|-------------------------------------|----------------|------------------------------|------------------------|----------------------------------|
| | | TGhL broth | 10% glycerol in skimmed milk | 10% DMSO in TGhL broth | 10% DMSO + 10% FCS in TGhL broth |
| Cryotube in cryo-container (-80°C) | Time post-planting (d) | 14 | 4–5 | 4–5 | 3–4 |
| | Number of actively growing colonies | – | + | ++ | +++ |
| Cryotube in liquid nitrogen | Time (d) | 14 | ND | 14 | 7 |
| | Number of actively growing colonies | – | ND | – | + |

Initially, cultures of *Batrachochytrium dendrobatidis* were used in establishing the optimum method. During the course of this study other Chytridiomycetes, namely *Rhizophydium* sp. (JEL136), *Rhizophlyctis-Rhizophydium*-like (JEL142) and *Rhizophydium ?haynaldii* (JEL151) (order Chytridiales), *Gonapodya* sp. (JEL183) (order Monoblepharidales) and *Allomyces macrogynus* (JEL204) (order Blastocladales) were imported. Following the establishment of optimal cryoarchiving protocols, these additional fungi (sporangia, fruiting bodies and zoospores) were also frozen from 4 d old cultures in 10% DMSO/10% FCS in TGhL broth using a cryocontainer to assess the broader application of the protocol.

Cryo-electron and light microscopy. Viability of cultures was assessed by light microscopy and electron microscopy. For electron microscopy a segment of agar from a TGhL plate was placed onto the specimen stage (adhered with 'TISSUE-Tek™ O.C.T. Compound', ProSciTech) of a JEOL 6340F field emission scanning electron microscope fitted with an OXFORD 1500 cryo (high resolution) system. The samples were prepared by plunging the sample into nitrogen slush, transferred to the OXFORD 1500 chamber and etched for 1 min at -95°C. Samples were coated with 2 nm Au-Pd and examined at 2 kV and 12 µA. For light microscopy cultures were viewed with an Olympus Tokyo CK microscope.

RESULTS

Initial attempts at freezing the chytrids involved scraping the sporangia off cultures grown on agar plates and resuspending in skimmed milk/glycerol as the cryoprotectant. This method was unwieldy and gave very poor recovery (data not shown). Growth of the sporangia was very slow and few colonies grew. Sub-

sequently, *Batrachochytrium dendrobatidis* was grown in broth culture. Manipulation of broth culture was considerably easier than scraping cultures from agar plates. Various cryoprotectants for freezing were tested (Table 1) in addition to rapid freezing in liquid nitrogen.

The DMSO/FCS mixture in TGhL broth, using a cryocontainer to control the freezing rate, produced the best revival (Table 1). Typically, many colonies were obvious and zoospore activity was apparent at 4 to 7 d after thawing, although sometimes at 1 to 2 d (Fig. 1). Of the cultures frozen directly in liquid nitrogen, active cultures were only revived from those with DMSO/FCS as cryoprotectant. (Table 1). Fewer colonies revived from such freezing than in those cultures that were frozen slowly using the cryocontainer and

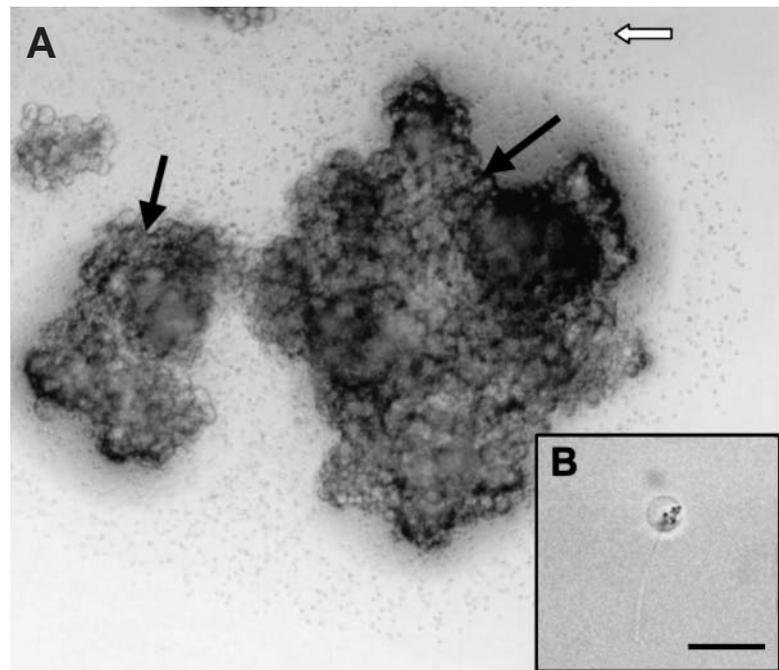


Fig. 1. *Batrachochytrium dendrobatidis*. Light micrographs of a zoospore 7 d post defrosting. (A) Micrograph of active zoospores (open arrow) around colonies of zoosporangia (closed arrow). (B) Higher magnification of a single cultured zoospore. Dark droplets are probably lipid globules. Scale bar = 6 µm

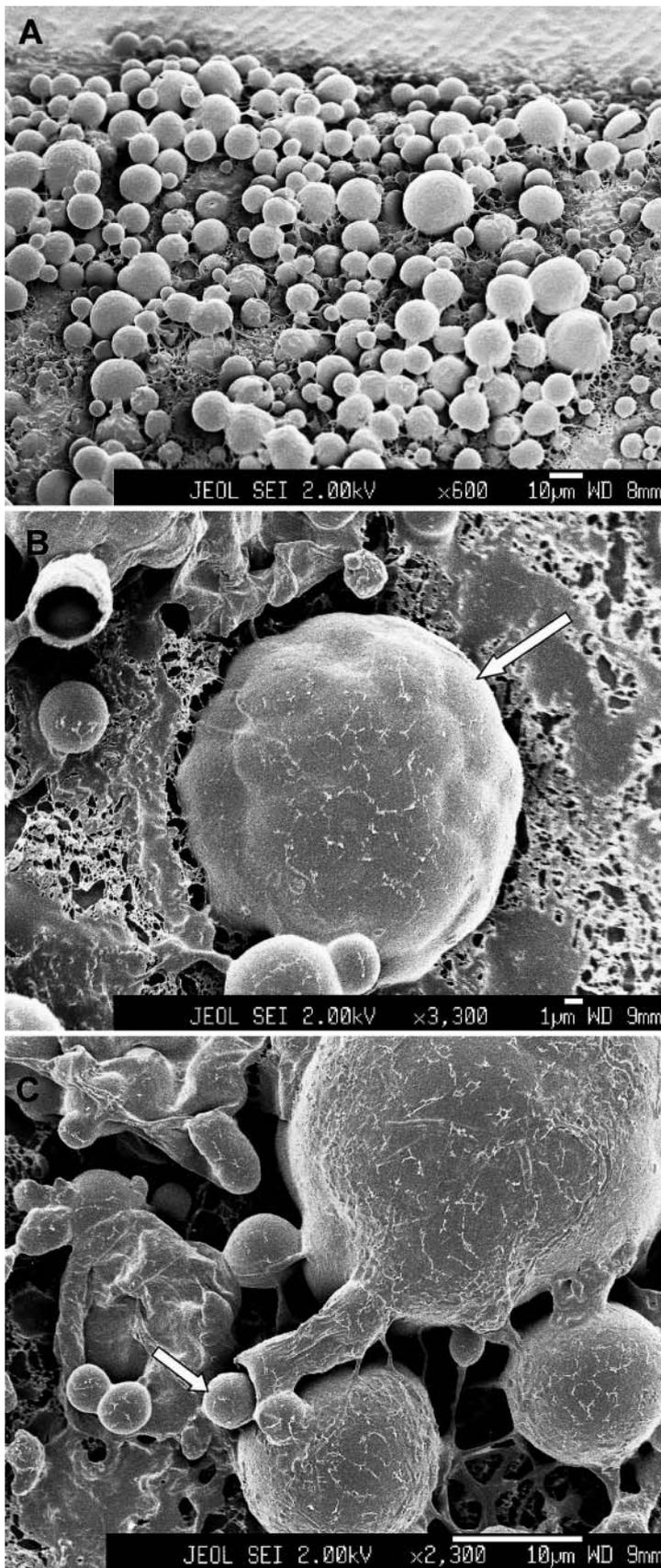


Fig. 2. *Batrachochytrium dendrobatidis*. Scanning electron micrograph of a revived culture. (A) Low magnification image of a culture illustrating a large number of developing sporangia. (B) Sporangia containing zoospores (open arrow); the internal zoospores are obvious from the surface topography of the zoosporangia. (C) Zoospore (open arrow) being released from sporangia

the same cryoprotectant (Table 1). The age of the agar plates used to revive the thawed cultures was also important. *Batrachochytrium dendrobatidis* could be most successfully revived using plates up to 2 mo of age (Figs. 2 & 3). Recovery of *B. dendrobatidis* decreased when plates beyond this age were used. Examination of the older plates by cryo-field emission scanning electron microscopy revealed the presence of surface-associated crystals (data not shown).

Recovery of zoospores (identified by the presence of zoosporangia with free-swimming zoospores) was optimal when cultures less than 8 d old were frozen. The freezing of cultures greater than 8 d yielded poor recovery rates (Fig. 4). Fig. 4 also indicates that zoospore activity could be observed as early as 1 d post-plating. Examination of young (<3 to 7 d old) cultures by light and scanning electron microscopy revealed that they contained developing and mature sporangia (Fig. 3A); i.e. zoospores were still contained within the

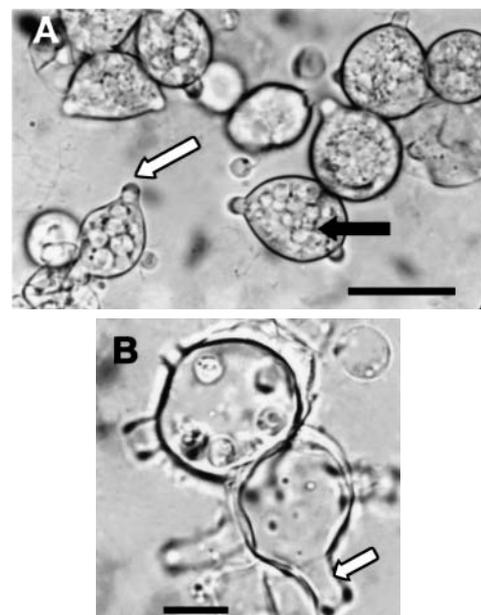


Fig. 3. *Batrachochytrium dendrobatidis*. (A) Light micrograph of young (<3 to 4 d old) sporangium. Sporangium is filled with developing zoospores (solid arrow); discharge papillae are apparent (open arrow). Scale bar = 20 µm. (B) Light micrograph of an older sporangium (14 d), which is representative of older cultures where the zoospores have been released; discharge papillae are also apparent (open arrow). Scale bar = 10 µm

sporangia. Examination of older colonies (7 to 8 d old) revealed the presence of large numbers of empty sporangia (Fig. 3B), i.e. zoospores had been released. It is therefore probable that early activity may be attributable to the release of zoospores from young plated sporangia.

Using the optimal protocol, *Batrachochytrium dendrobatidis* was successfully recovered over a period of 12 mo from storage at -80°C (Table 2). This chytrid, frozen as per the optimum protocol, was also successfully revived from storage in liquid nitrogen after 9 mo; there was no difference in revival between these suspensions and those stored at -80°C . Suspensions of frozen zoospores (without sporangia) were also successfully revived (7 d post plating) from storage at -80°C using the optimum protocol (Table 3). Table 3 also shows that a suspension of sporangia can be revived within 1 d of plating. The data, however, in association with that presented in Fig. 4, is suggestive that zoospores upon revival, or during the process of cryo-preservation, encyst and do not persist as motile structures.

Of the other fungi, 4 were successfully recovered after storage at -80°C (Table 2). Preliminary attempts to recover 204 *Allomyces macrogynus* using this method were unsuccessful.

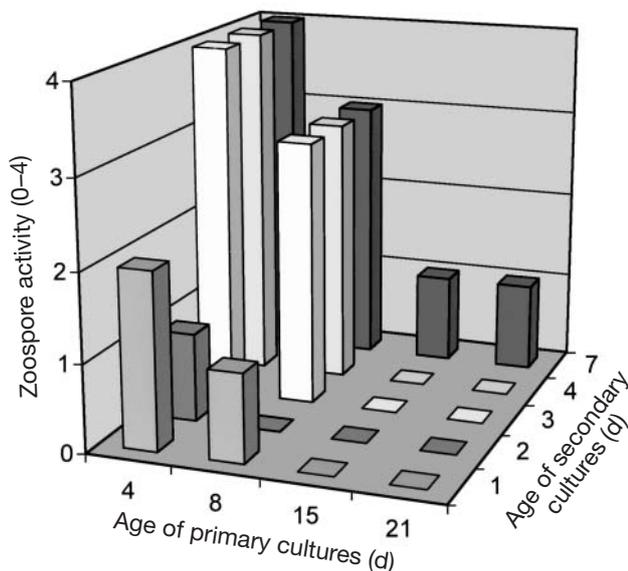


Fig. 4. *Batrachochytrium dendrobatidis*. Bar graph illustrating the comparative revival of 4 to 21 d old cultures following freezing of suspensions using the optimum protocol. Zoospore activity (% motile zoospores) was scored from '-' to '++++', where '-' indicates no growth and '++++' indicates maximum zoospore activity. A score of '-' is represented as '0' and '++++' as '4'

Table 2. Recovery of different fungi from storage at -80°C over 12 mo. '+' indicates revival of zoospores from frozen stock whilst '-' indicates non-revival of zoospores from frozen stock. This experiment was not repeated for cultures frozen directly in liquid nitrogen as the data in Table 1 indicate that this form of freezing is sub-optimal

| Order | Species | Time (mo) | Recovery |
|-----------------|---|-----------|----------|
| Chytridiales | <i>Batrachochytrium dendrobatidis</i> | 1-12 | + |
| | <i>Rhizophydium</i> (136) | 5 | + |
| | <i>Rhizophlyctis-Rhizophydium</i> -like (142) | 5 | + |
| | <i>Rhizophydium ?haynaldii</i> (151) | 5 | + |
| Monoblepharides | <i>Gonapodya</i> sp. (183) | 5 | + |
| Blastocladales | <i>Allomyces macrogynus</i> (204) | 5 | - |

Recommended protocol. For simplicity, the optimal protocol for successful preparing, freezing, and thawing of *Batrachochytrium dendrobatidis* is presented below in point form.

Preparation of *Batrachochytrium dendrobatidis* for freezing from broth culture: (1) Take 2 ml of actively growing broth culture (1 wk old) and add to 13 ml of fresh broth in a 25 cm² flask. (2) Incubate for 3 to 4 d ensuring the flasks remain in a horizontal position (17 to 23°C). (3) Aseptically scrape the sides of the flask, transfer contents to a 15 ml plastic centrifuge tube and spin at 1700 \times g (10 min).

Freezing the cultures: (1) In a Biosafety (Class 2) cabinet, or equivalent (to maintain sterility), resuspend the pellet in 1 ml 10% DMSO, 10% FCS in broth and transfer to a 1 or 2 ml cryotube. (2) Place preparation in an isopropanol-containing plastic cryocontainer in a -80°C freezer overnight, then transfer the cryotubes to a liquid nitrogen cabinet for permanent storage.

Thawing: (1) Fill a container with water at 43°C. (2) Place cryo-tubes directly from liquid nitrogen into the warm water. Thaw until samples begin to liquefy (30 s to 1 min). Stop warming at this stage. (3) Divide thawed chytrid between 2 TGH plates, add 1 ml of

Table 3. *Batrachochytrium dendrobatidis*. Revival of zoospores vs sporangia following cryopreservation using the optimum protocol, indicated by % zoosporangia associated with external motile zoospores

| Time (age of secondary culture in d) | Frozen sample | |
|--------------------------------------|------------------|---------------|
| | Zoospores only | Sporangia (%) |
| 1 | 0 ^a | 50 |
| 2 | 0 ^a | 6 |
| 3 | 0 ^a | 84 |
| 4 | 0 ^a | 86 |
| 7 | 43% ^b | 100 |

^aNon-motile zoospores observed on the agar
^bNon-active zoospores had encysted and developed into sporangia, which were associated with external motile zoospores

DS salt solution or TGhL broth, air dry until most of the liquid has diffused into the agar, seal plate with parafilm. (4) Invert plate and incubate at 23°C.

DISCUSSION

We have successfully established methods for the long-term storage of *Batrachochytrium dendrobatidis*. The assay was also successfully applied to the freezing and revival of some related fungi. The technique is simple and readily applied in most microbiological laboratories. The methodology provides the opportunity for maintaining the integrity of the genome and biological activity of field isolates by obviating the need for continuous passage.

The described technique is based on well-established tissue culture freezing and recovery methods that have been used to store cell cultures for many years. These methods have provided easy and convenient methods for long-term storage of continuous cell lines or short-term cell cultures from a wide variety of species including insects, mammals, amphibians and fish. Unlike Hohl & Iselin (1986), we found that *Chytridium* and *Rhizophyidium* species were successfully preserved using a simple freezing device with a near constant rate of cooling. The success is most likely attributable to the cryoprotectant used and the use of cultures less than 8 d old. Data from this study indicate that motile zoospores are not themselves revived following cryopreservation, and that they encyst either during the cryopreservation protocol or immediately upon revival. The rapid revival of cultures of *Batrachochytrium dendrobatidis* is therefore, in all likelihood, dependent upon the presence of young sporangia-containing viable zoospores. Observations of older cultures showed that many of them contained large numbers of sporangia where the zoospores had been released; this was indicated by the absence of mucus plugs and collapsed appearance of sporangia.

Results from the study also showed that aged TGhL plates were less successful in supporting cultures of *Batrachochytrium dendrobatidis*. The crystalline surface structure of the agar plates most likely represents dehydration of the agar and the subsequent crystallisation of the associated salts. Such a salt-rich (hypertonic) local environment would be detrimental to supporting the growth of cultures, as prolonged exposure of zoospores to increased saline conditions is detrimental to the active reproduction of *B. dendrobatidis* (Boyle & Hyatt unpubl. obs.).

The method described in this paper will allow the preservation of isolates of *Batrachochytrium dendro-*

batidis and related fungi from the environment or from animals. The archiving of isolates of *B. dendrobatidis* is essential for both genotypic and phenotypic comparisons. The ability to cryo-archive isolates will facilitate investigations into the origin of *B. dendrobatidis* and thereby the pandemic. The procedure will also enable investigations to be undertaken with isolates that have undergone minimal (or no) passages on TGhL agar. It should, therefore, now be possible to undertake infectivity trials involving a range of isolates and species of amphibia without major concern of isolate attenuation.

The method described in this paper for long-term storage of isolates represents a major advance in studies involving *Batrachochytrium dendrobatidis* and the associated decline of amphibians throughout the world.

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