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MUCOR AMPHIBIORUM IN THE TOAD, *BUFO MARINUS*, IN AUSTRALIA

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ABSTRACT: *Mucor amphibiorum*, a fungus previously found in captive amphibians in Europe and the platypus in Australia, was observed in free-ranging toads, *Bufo marinus*, in Australia. In tissues the fungus occurred as sphaerules 4.9 to 36.4 μm in diameter; hyphae were not formed. Some sphaerules developed two to 11 daughter sphaerules internally and these were released into tissues by dissolution of the outer wall. Infected toads were found at 11 sites from nine locations in northern and eastern Australia. The overall prevalence of infection in 3,518 toads was 0.71%. *Mucor amphibiorum* was isolated from soil at one location.

Key words: *Mucor amphibiorum*, giant toad, *Bufo marinus*, Australia, epidemiology, identification, fungus, amphibia.

INTRODUCTION

A species of *Mucor* spp. isolated from captive amphibians in Europe produced a fatal fungal septicemia in natural and experimental infections in anurans (Frank et al., 1974; Frank, 1975). The *Mucor* sp. existed in tissues in a unique spherical form, which Frank et al. (1974) called a "sphaerule." The species could not be identified in the original publication, but the fungus subsequently was described by Schipper (1978) as a new taxon, *Mucor amphibiorum*. There were no further reports in refereed journals until Obendorf et al. (1993) reported *M. amphibiorum* as the causative agent of the ulcerative dermatitis and septicemia in wild platypus, *Ornithorhynchus anatinus*, in Australia. This condition originally had been reported by Munday and Peel (1983); but while *M. amphibiorum* occurred in the tissues, it was not identified at that time.

Bufo marinus, the marine, giant or cane toad, is a native inhabitant of southern North America, central America and northern South America (Zug and Zug, 1979). In Australia, 101 toads were released in coastal north Queensland in 1935 from an introduced population in Hawaii (USA) (Sabath et al., 1981). With human assistance, the distribution of the cane toad in Australia expanded to include coastal Queensland, coastal northern New South Wales and part of the north eastern seaboard of the Northern Territory (van Beurden and Grigg, 1980; Easteal et al., 1985; Freeland and Martin, 1985). *Bufo marinus* in Australia is regarded as a pest and a serious threat to the ecological integrity of wilderness areas.

Large numbers of parasites and pathogens have been reported from *B. marinus* (Speare, 1990). Some of the protozoan parasites introduced with *B. marinus* have infected native Australian amphibians, while some protozoan parasites of Australian frogs have crossed to the toad (Delvinquier, 1986, 1987). One of the possible detrimental effects of the introduced cane toad could be to carry potential pathogens into newly colonized areas and thereby introduce disease into populations of native Australian animals in these areas.

During a survey of diseases of free-ranging *B. marinus* in Australia, toads from several locations were found to be infected naturally with *M. amphibiorum*. Our objective was to determine the prevalence of *M. amphibiorum* in toads, and to describe the diagnostic features of *M. amphibiorum* in culture and in tissues.

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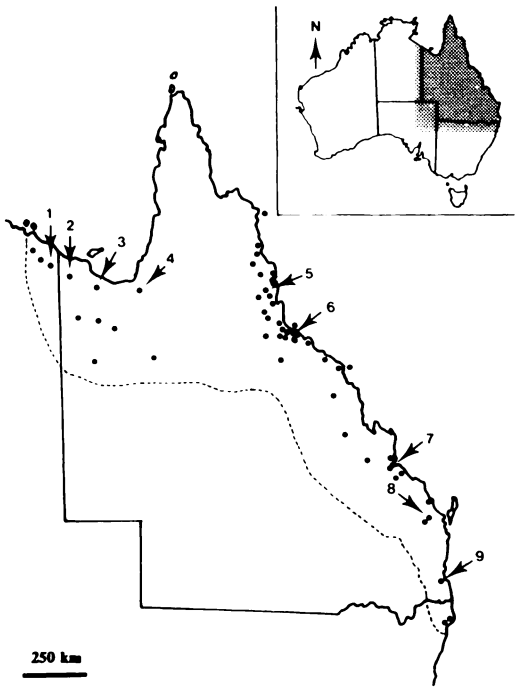

FIGURE 1. Collection sites of cane toads, *Bufo marinus*, in Australia. Key to locations of toads infected with *Mucor amphibiorum*: 1 = Calvert Hills Station, 2 = Westmoreland Station, 3 = Escott Station near Burketown, 4 = Normanton, 5 = Innisfail, 6 = Townsville, 7 = Rockhampton, 8 = Oowongalaema Station near Munduberra, 9 = Brisbane. Dotted line indicates range of *B. marinus* in Australia.

MATERIALS AND METHODS

We collected 3,518 toads from 100 sites at 59 locations in Australia (Fig. 1); ten toads also were collected in Hawaii and 31 toads were collected in Costa Rica. Toads were killed either by pithing or injection of pentobarbitone sodium (324 mg/ml) (Arnolds of Reading Pty., Ltd., Boronia, Victoria, Australia) into the subcutaneous lymph sinuses. Carcasses were opened along the midventral line, viscera removed and examined grossly for lesions (Frank et al., 1974). The occurrence of *M. amphibiorum* in tissues was confirmed by histology or by culture. For most specimens mycological or histological examinations were not done if gross lesions were not seen.

Blocks of tissue were collected aseptically and placed in sterile plastic vials prior to evaluation. Tissue and gut contents were inoculated into Sabouraud's dextrose agar (Oxoid (Aust.) Propriety Ltd., West Heidelberg, Victoria, Australia) with added benzyl penicillin (20 IU/ml) (CSL, Parkville, Victoria), and streptomycin sulphate (40 IU/ml) (Sigma Chemical Company, St. Louis, Missouri) and Mycological agar (Difco Laboratories, Detroit, Michigan, USA) with added thiamine hydrochloride (1 μg/ml) (Sigma Chemical Company) either by placement into the agar or spreading by means of an inoculating loop. Plates were incubated at 28 C and checked daily for growth. A culture also was made by inoculating infected liver homogenized in normal saline onto the surface of autoclaved soil.

Five to ten gram samples of soil and substrate were collected in sterile plastic vials from two locations. Twenty-eight samples from Townsville (19°16'S, 146°47'E) were obtained from a greenhouse where infected toads had been collected on several occasions over a 12 mo period. Of these, 23 were of soil collected from ground level, while the remainder consisted of two samples of soil collected from pot plants above the ground and three samples of material scraped from wooden structures above ground level. Fifteen samples of soil and two of water were obtained from a waterhole and its environs on Westmoreland Station (17°18'S, 138°18'E) where infected toads were found. Particles of substrate were implanted in Sabauraud's agar and water samples were collected on a loop, smeared over the surface of Sabouraud's dextrose agar and both cultured as for tissue samples. Fungi were identified to genus using characteristics found in McGinnis (1980) and Carmichael et al. (1980). For identification to species, the criteria of Roper and Fennell (1973) were used for *Aspergillus* spp. and the criteria of Fassatiova (1986) for *Paecilomyces* spp. *Mucor* spp. were identified using the criteria of Schipper (1978). The identification of *Mucor amphibiorum* was confirmed by the CAB International Mycological Institute, Bakerham Lane, Egham, Surrey, United Kingdom.

Detailed studies of fungal morphology and mating reactions were carried out on potato dextrose agar (Oxoid (Aust.) Pty Ltd., West Heidelberg, Victoria). Measurements were taken from 10 new isolates from toads, one from a platypus from the South Esk River, Tasmania (specimen received from R. Mason, reported in Obendorf et al., 1993) and from two mating strains. The mating strains, CBS 763.74 (type strain, positive) and CBS 185.77 (negative strain) were obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. Twenty-five measurements were made for each isolate and are presented as mean ± standard error (range).

Samples of tissue were preserved for histological examination in 10% buffered neutral formalin, embedded in paraffin; 5 μm sections were prepared and stained with hematoxylin and eosin (H&E). In addition some sections were stained with periodic acid Schiff (PAS), Alcian blue PAS

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TABLE 1. Mating types of 11 strains of *Mucor amphibiorum* isolated from Australia contrasted with type specimens, and negative (isolate 11) and positive (isolate 12) controls. The production of zygospores occurs in a band when opposite types mate. The strength of mating is expressed in terms of the width (mm) of the band of zygospores formed on potato dextrose agar at 25 C after 10 days.

Isolate number	Isolate number[a]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
13	0[b]	0	0	0	16.5	0	16.5	0	17.5	11.0	14.0	0	0
12	0	0	0	0	18.5	0	27.0	0	21.5	11.0	22.5	0	
11	12.0	12.0	11.5	10.0	0	13.5	0	12.0	0	0	0		
10	8.5	8.0	9.0	9.5	0	10.5	0	8.5	0	0			
9	18.0	17.0	13.0	17.0	0	16.0	0	13.0	0				
8	0	0	0	0	19.5	0	14.5	0					
7	17.0	19.0	14.5	18.5	0	14.5	0						
6	0	0	0	0	19.0	0							
5	16.0	16.0	13.5	14.0	0								
4	0	0	0	0									
3	0	0	0										
2	0	0	0										
1	0												
Mating type	pos[c]	pos	pos	pos	neg	pos	neg	pos	neg	neg	neg	pos	pos

[a] Origin of isolates: 1, Garbutt, Townsville (soil); 2, Oonoonba, Townsville (toad); 3, Calvert Hills Station; 4, Brisbane (toad); 5, Escott Station (toad); 6 and 7, Oonoonba, Townsville (toads); 8, South Esk River (platypus); 9, Escott Station (toad); 10, Westmoreland Station (toad); 11, CBS 185.77 negative mating type (South America); 12, CS 763.74 positive mating type (Australia); 13, Oowongalaema Station (toad).

[b] Width of zygospore band (mm); each value is a mean of two replicates.

[c] pos, positive; neg, negative.

(ABPAS), Gram, Giemsa, Gomori's silver methanamine (GSM) (Culling et al., 1985) and Martius scarlet blue (MSB) (Drury and Wallington, 1967). Squash preparations were made by placing a small sample of tissue on a microscope slide in 0.85% saline and after teasing, applying a coverslip and examining unstained.

RESULTS

After 6 days at 28 C, pure cultures of flat, cream to off-white, suede-like colonies, 50 to 56 mm in diameter, were observed. These had an outer ring of substrate mycelium, but little to no available aerial mycelium. At 21 days the colonies had filled the petri dish and the centers were slightly raised with a white aerial mycelium. Color on the reverse ranged from bright yellow in the center to pale yellow at the periphery. In the culture using sterile soil incubated at 22 to 25 C, an aerial mycelium developed within 2 days and sporangia were present within 7 days.

On potato dextrose agar at 25 C, colonies were pale smoke gray to pale yellowish, the reverse side was pale yellowish. Colony height was up to 16 mm, rhizoids were present. Mean ± SD (range) diameters for sporangiophores were 10.1 ± 0.19 μm (4.6 to 26.0 μm), unbranched. Sporangia at first were pale yellow becoming dark brown, globose to sub-globose, 61.6 ± 0.53 μm (26.2 to 113.4 μm) in broadest dimension. Columella were elipsoidal-obovate, pyriform, or subglobose to ovate, and were 35.3 ± 0.51 μm (10.4 to 67.6 μm) wide by 41.5 ± 0.66 μm (14.3 to 87.1 μm) high, with a small collarette. Sporangiospores were mostly globose, measuring 5.1 ± 0.06 μm (3.2 to 11.7 μm) in broadest dimension.

Zygospores were light to dark brown, globose to subglobose 41.9 ± 0.40 μm (26.0 to 62.4 μm) in broadest dimension with projections ≤6.5 μm; suspensors were inflated or not, generally unequal. Larger suspensors were 19.4 ± 0.36 μm (7.8 to 41.6 μm) in diameter; smaller suspensors were 8.7 ± 0.15 μm (4.6 to 20.8 μm) in diameter. The suspensors sometimes were short, and sometimes rich yellow in color. Strong mating reactions were observed

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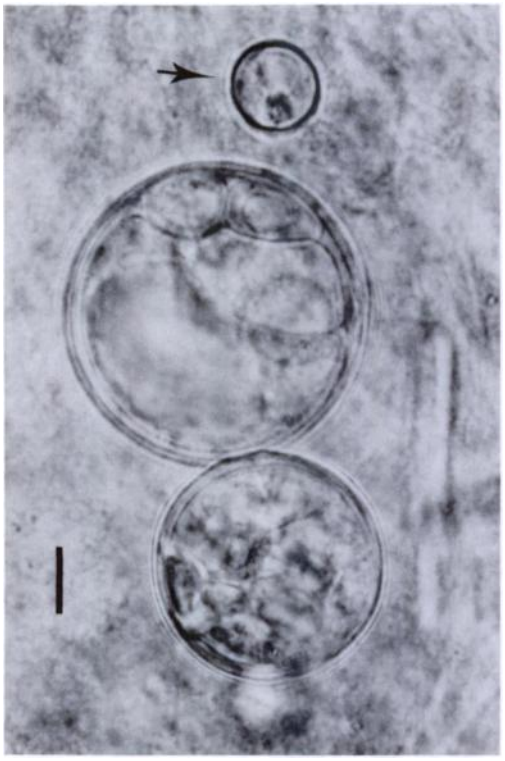

FIGURE 2. Sphaerules of *Mucor amphibiorum* from squash preparation of granuloma in bladder of *Bufo marinus*. Simple sphaerule indicated by arrow; other sphaerules have internal structures which develop into daughter sphaerules. Unstained. Bar = 10 μm.

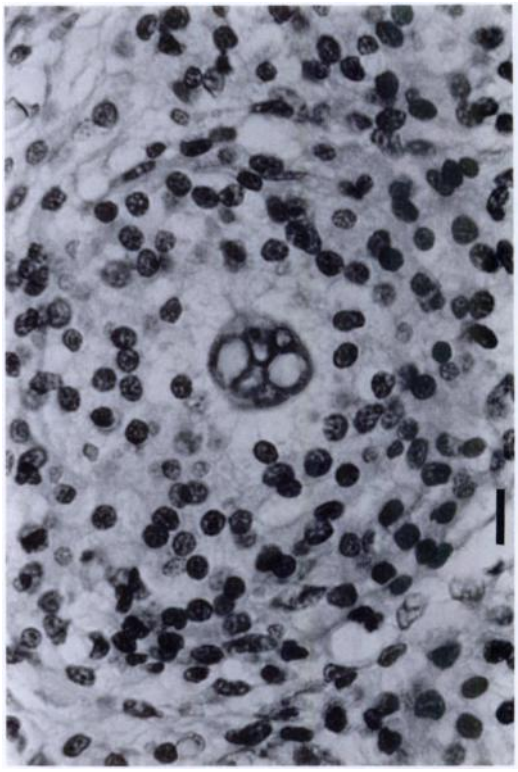

FIGURE 4. Mother with daughter sphaerules of *Mucor amphibiorum* in section of granuloma in liver of *Bufo marinus*. Sphaerule is surrounded by lymphocytes. H&E. Bar = 10 μm.

with all isolates in contrast to *M. amphibiorum* CBS 763.74 (type strain, positive) or CBS 185.77 (type strain, negative) (Table 1). Dense bands of zygospores were produced with no band being <8 mm wide after 10 days. Seven isolates had a positive mating type and the remaining four were negative. Around half the isolates showed some growth at 36 C and several strains grew well at this temperature.

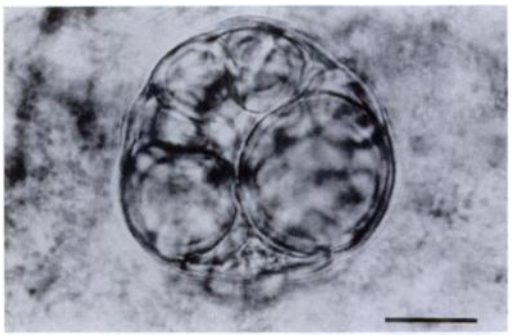

FIGURE 3. Sphaerule of *Mucor amphibiorum* containing daughter sphaerules from squash preparation of granuloma in bladder of *Bufo marinus*. Unstained. Bar = 10 μm.

Mucor amphibiorum in toads occurred in granulomas in many tissues. Walls of the sphaerules were refractile in squash preparations and could be seen easily. The same morphological features could be seen in both unstained squashes and histological sections. Sphaerules had several different morphologies. They could be roughly spherical with no internal structure (Fig. 2) or be roughly spherical with internal structures, either daughter sphaerules (Fig. 3) or other structures (Fig. 2); some had other shapes.

Roughly spherical sphaerules with no

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TABLE 2. Staining characteristics of *Mucor amphibiorum* sphaerules in tissue sections.

Stain	Wall	Contents
Alcian blue periodic acid Schiff	Violet	Purple or maroon
Giemsa	Dark blue or purple	Blue or purple
Gomori's silver methanamine	Dark brown or black	Brown
Gram's stain	Pink or red	Pink
Hematoxylin and eosin	Red	Blue-red
Martius scarlet blue	Dark blue	Dark blue
Periodic acid Schiff	Violet	Purple

internal structure measured 10.6 ± 2.8 μm (14.6 to 4.9 μm) by 9.9 ± 2.6 μm (14.6 to 4.9 μm) (n = 15). The smaller diameter was 7.0 ± 7.0% (0 to 20%) less than the larger diameter. Walls were thin (<1 μm) and in H&E were eosinophilic with a basophilic tinge, particularly in the smaller sphaerules (Fig. 4). The material just medial to the wall was slightly basophilic and in some sphaerules formed a narrow band with a distinct inner margin and a central clear area. In some the poorly staining area was eccentric. The contents of other sphaerules were diffusely basophilic, particularly in sphaerules with diameters less than 10 μm. Staining characteristics with other stains are given in Table 2. A greater proportion of the contents stained with GSM, than with other stains, but the walls of sphaerules showed a greater tendency to shatter and sphaerules to be displaced in GSM stained sections.

Sphaerules which were roughly spherical with internal structure contained either daughter sphaerules or other structures. Mother sphaerules, sphaerules containing daughter sphaerules (Fig. 3), measured 21.1 ± 5.3 μm (37.2 to 14.6 μm) by 19.3 ± 5.8 μm (36.4 to 14.6 μm) (n = 19). The outer wall was thick and stained eosinophilic with a basophilic tinge (H&E). Daughter sphaerules developed inside the outer wall and when they were fully developed appeared similar to the small simple sphaerules. The mean number of daughter sphaerules per mother sphaerule was 7.4 ± 2.8 (2 to 11) (n = 19). The mean maximum diameter of daughter sphaerules was 5.3 ± 1.7 μm (10.5 to 2.4 μm) (n = 69) and within a single mother sphaerule the diameter of daughter sphaerules varied (Fig. 3). In tissue squashes and histological sections examples of daughter sphaerules in close proximity to ruptured or collapsed larger sphaerules were seen. The staining characteristics of mother and daughter sphaerules were similar. Sphaerules occasionally contained structures that appeared to be developing daughter sphaerules, but the features were unclear (Fig. 2).

Oval and crescent shaped forms occasionally were seen and appeared to be collapsed sphaerules, often with an obvious disruption of the wall. On occasion a mother sphaerule was seen with a ruptured wall and with smaller sphaerules still contained within the broken larger sphaerule as well as outside it.

Infected toads were found only in Australia. Twenty-five toads were infected with *M. amphibiorum* (Table 3), giving a prevalence of 0.71% in the Australian survey. Infected toads were found at nine (13%) of the 59 locations (Fig. 1); mean prevalence of infection at positive locations was 1.2% ranging from 0.4% to 16.7% (Table 3). At Townsville, infected toads were found at three sites each separated by a minimum of 10 km. Toads with *M. amphibiorum* were found in both tropical and temperate zones. The isolation from Calvert Hills Station was from the large intestinal contents of a toad with no pathological changes attributable to *M. amphibiorum*. All other occurrences were in toads with pathological changes attributable to the fungus.

Mucor amphibiorum was isolated from

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TABLE 3. Locations and prevalences of *Mucor amphibiorum* in *Bufo marinus*.

Locations[a]	Number of sites[a]			Number of toads from location		Prevalence in toads (%)
	Total	Neg[b]	Pos[b]	Total	Infected	
Brisbane (27°25'S, 153°6'E)	1	0	1	6	1	17
Calvert Hills Station (17°12'S, 137°19'E)	6	5	1	223	1	0.4
Oowongalaema Station (25°28'S, 151°15'E)	1	0	1	46	1	2.2
Escott Station (17°45'S, 139°35'E)	1	0	1	116	5	4.3
Innisfail (17°32'S, 146°2'E	2	1	1	9	1	11
Normanton (17°43'S, 141°5'E)	1	0	1	13	1	7.7
Rockhampton (23°20'S, 150°28'E)	1	0	1	10	1	10
Townsville (19°16'S, 146°47'E)	18	15	3	1,597	12	0.75
Westmoreland Station (17°18'S, 138°18'E)	1	0	1	129	2	1.6
	Combined prevalences					
Australian positive locations[c] (n = 9)	32	21	11	2,149	25	1.2
Australian negative locations[d] (n = 50)	68	68	0	1,369	0	0
Australian total (n = 59)	100	89	11	3,518	25	0.71
Overseas[e] negative locations (n = 2)	2	2	0	41	0	0

[a] Locations: towns and regions where toads were collected; sites: discrete points within locations where toads were collected; e.g., at the location Townsville there were 18 collection sites.
[b] Neg: no toads with *M. amphibiorum* collected from site; Pos: at least one toad with *M. amphibiorum* collected from site.
[c] Positive locations: locations in Australia at which one toad infected with *M. amphibiorum* was found.
[d] Negative locations: locations in Australia at which no infected toads were found.
[e] Observed locations: Hawaii and Costa Rica.

two of the 20 soil samples collected from ground level at Townsville (Table 4) with no isolations from samples collected above ground level; *M. amphibiorum* was not isolated from soil or water samples collected at Westmoreland Station.

DISCUSSION

The isolates from infected toads were identified initially as *M. amphibiorum* on the basis of their asexual structures and cultural characteristics. These features agreed well with the data of Schipper (1978). The sporangiospore size range was greatly extended in this study, but some of the larger spores (sparse in number) were noted in the type strain as well as in the strain from the platypus. Some of the new isolates from toads had colonies with little of the characteristic yellowish coloration, but these gave strong mating reactions. Both mating types occurred in Australia and there appeared to be no geographic pattern; one site in Townsville had both types. We also noted that some isolates grew strongly at an elevated temperature in contrast to Schipper's (1978) experience with a limited range of isolates. The strong mating reactions of all strains left no doubt that the organism isolated from toads, the platypus and infected soil was *M. amphibiorum*.

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In tissues of amphibians and the platypus the morphological features characteristic of *M. amphibiorum* are the absence of hyphae, the occurrence of sphaerules and the presence of daughter sphaerules (Frank et al., 1974; Munday and Peel, 1983; Frank, 1975; Obendorf et al., 1993). Although other fungi occur in tissues solely as spherical forms (Anthony, 1973), few have smaller spherical forms formed within larger ones. The morphology of *Coccidioides immitis* in tissues is similar to *M. amphibiorum*; *C. immitis* forms thick-walled sphaerules 30 to 60 μm in diameter containing many endospores 2 to 5 μm in diameter (Rippon, 1974). The sphaerules stain well with H&E, PAS and silver stains (Rippon, 1974). The smaller size of the endospores of *C. immitis* and the large number per sphaerule distinguish it from *M. amphibiorum*. Two cases of infection with *C. immitis* have been reported from Australia (Symers, 1971; Steele et al., 1977), but the organism is not considered endemic to Australia. *Pneumocystis carinii* in tissues forms intracystic bodies (Campbell, 1972; Farrow et al., 1972) and may be confused with the spores of zygomycetes (Reinhardt et al., 1977); *P. carinii* differs from the sphaerules of *M. amphibiorum* in its smaller diameter (1.5 to 10 μm), thinner walls and poor staining with H&E. The alga, *Prototheca* sp., occurs in tissues as spherical or oval forms with endosporulation, and may be confused with *M. amphibiorum*. The cells of various species of *Prototheca* are spherical to oval, hyaline, thick walled, 1.3 to 16.1 μm in diameter, have two to eight spherical endospores and stain with H&E, GSM and Gridley stains (Sudman, 1974; Tyler et al., 1980). The mother sphaerules of *M. amphibiorum* are larger than the cells of *Prototheca* although the smaller sphaerules may be the same size. When cultured in vitro on Sabouraud's dextrose agar and blood agar, *Prototheca* retain their spherical form (Sudman, 1974) unlike *M. amphibiorum* which forms a mycelium (Frank et al., 1974). In the first reported instance of infection with *M. amphibiorum* in Australia in ulcerative dermatitis in wild Platypus, Munday and Peel (1983) were unaware of the characteristic morphology of *M. amphibiorum* in tissues and considered *Prototheca* sp. the most likely etiological agent. This error is understandable since *M. amphibiorum* had been reported only from amphibians in captivity in Europe. Subsequently Obendorf et al. (1993) demonstrated that *M. amphibiorum* was the infectious agent in the ulcerative skin disease in platypi.

TABLE 4. Fungi isolated from 23 specimens of substrate in Townsville greenhouse and environs (ground = at ground level; elevated = above ground level).

Species	Number of isolates		
	Ground	Elevated	Total
Alternaria sp.	3	0	3
Aspergillus candidus	3	0	3
Aspergillus flavus	1	0	1
Aspergillus glaucus	0	2	2
Aspergillus niger	9	4	13
Aspergillus sp.	1	0	1
Cladosporium sp.	2	1	3
Conidiobolus sp.	1	0	1
Curvularia sp.	1	1	2
Fusarium sp.	11	0	11
Mucor amphibiorum	2	0	2
Mucor sp.	2	1	3
Nigrospora sp.	0	1	1
Paecilomyces lilacinus	17	4	21
Penicillium sp.	5	3	8
Rhizopus sp.	5	0	5
Trichoderma sp.	17	3	20
Trichopsporon sp.	2	0	2
Unidentified fungi	1	1	2
	83	21	104

The multiplication of *M. amphibiorum* within the amphibian host occurs solely by formation of daughter forms and release by dissolution and rupture of the wall of the mother sphaerule. Frank et al. (1974) noted that in vitro simple sphaerules from amphibian tissue could give rise to hyphae while mother sphaerules did not do so. This suggests that at some point the sphaerule becomes committed to reproduction either by hyphae or by formation of daughter sphaerules. Our findings support the ob-

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servations of Frank et al. (1974) that sphaerules do not occur in vitro and hyphae do not occur in vivo.

Mucor amphibiorum grows well in soil and sporulates rapidly. Based on the isolation of *M. amphibiorum* from soil at the Townsville location where infected toads were collected over 12 mo we speculate that *M. amphibiorum* can persist in soil; *B. marinus* commonly ingests soil when it feeds (Zug and Zug, 1979) and the fungus could be ingested with soil contaminating prey items. *Mucor amphibiorum* has been isolated from the feces of toads with internal lesions and a toad with no pathological changes. Therefore, *B. marinus* has the potential to disseminate *M. amphibiorum*.

Prior to the reports of Munday and Peel (1983) and Obendorf et al. (1993), *M. amphibiorum* was unknown from a natural environment. One of the original isolations was from a green tree frog, *Litoria caerulea* (reported as *Hyla caerulea*), held captive in Europe (Frank et al., 1974). Other infected anurans in the same collection were *Aparasphenodon* sp. and *Trachycephalus* sp. from South America (Frank et al., 1974). *Litoria caerulea* is native to a vast area of northern and eastern Australia (Cogger, 1979), and Frank et al. (1974) speculated that *M. amphibiorum* may have been imported either from Australia or from South America. Although infection with *M. amphibiorum* is endemic in *B. marinus* in Australia, the possibility exists that it was introduced into Australia with *B. marinus*. However, the discovery of *M. amphibiorum* in the platypus in Tasmania in the absence of *B. marinus* (Obendorf et al., 1993) supports the argument that *M. amphibiorum* is native to Australia.

The importance of *M. amphibiorum* to free-ranging native Australian amphibians is unknown. Many amphibians have been collected and killed for zoological purposes in Australia. Apart from reports of minor pathological changes associated with parasitic infections, no diseases have been described in Australia from free-ranging amphibians other than *B. marinus*. The absence of *M. amphibiorum* from native amphibians could be more apparent than real and due to a lack of detailed study of diseases of these amphibians in Australia. Conversely, this may not be the case and *M. amphibiorum* may not be a natural pathogen in Australian native anurans. However, with the decline of amphibian populations in Australia, it is vital that further study is carried out to determine the importance of *M. amphibiorum* as a natural pathogen and the role of the introduced cane toad in its dissemination.

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