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GENOTYPIC ANALYSIS OF *MUCOR* FROM THE PLATYPUS IN AUSTRALIA

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ABSTRACT: *Mucor amphibiorum* is the only pathogen known to cause significant morbidity and mortality in the free-living platypus (*Ornithorhynchus anatinus*) in Tasmania. Infection has also been reported in free-ranging cane toads (*Bufo marinus*) and green tree frogs (*Litoria caerulea*) from mainland Australia but has not been confirmed in platypuses from the mainland. To date, there has been little genotyping specifically conducted on *M. amphibiorum*. A collection of 21 *Mucor* isolates representing isolates from the platypus, frogs and toads, and environmental samples were obtained for genotypic analysis. Internal transcribed spacer (ITS) region sequencing and GenBank comparison confirmed the identity of most of the isolates. Representative isolates from infected platypuses formed a clade containing the reference isolates of *M. amphibiorum* from the Centraal Bureau voor Schimmelcultures repository. The *M. amphibiorum* isolates showed a close sequence identity with *Mucor indicus* and consisted of two haplotypes, differentiated by single nucleotide polymorphisms within the ITS1 and ITS2 regions. With the exception of isolate 96-4049, all isolates from platypuses were in one haplotype. Multilocus fingerprinting via the use of intersimple sequence repeats polymerase chain reaction identified 19 genotypes. Two major clusters were evident: 1) *M. amphibiorum* and *Mucor racemosus*; and 2) *Mucor circinelloides*, *Mucor ramosissimus*, and *Mucor fragilis*. Seven *M. amphibiorum* isolates from platypuses were present in two subclusters, with isolate 96-4053 appearing genetically distinct from all other isolates. Isolates classified as *M. circinelloides* by sequence analysis formed a separate subcluster, distinct from other *Mucor* spp. The combination of sequencing and multilocus fingerprinting has the potential to provide the tools for rapid identification of *M. amphibiorum*. Data presented on the diversity of the pathogen and further work in linking genetic diversity to functional diversity will provide critical information for its management in Tasmanian river systems.

Key words: Intersimple sequence repeat (ISSR) analysis, ITS sequencing, *Mucor amphibiorum*, *Mucor*, mucormycosis, *Ornithorhynchus anatinus*, PCR.

INTRODUCTION

Mucor is the largest genus of the mucorales, containing 60 species. Most of the species belonging to this genus are saprophytic, many causing food spoilage, and a few species are pathogens of mammals and plants. *Mucor amphibiorum* is a dimorphic, heterothallic pathogen in this order. It exhibits a yeast-like form in infected tissues (spherule-like structures, which may contain two to 11 daughter spherules) and a nonseptate hyphal form on culture medium or in the environment (Frank et al., 1974). Sporangiospores may develop into the hyphal or yeast morphologic form following germination (Orlowski, 1991).

The first report of *M. amphibiorum* was from a German zoo, in 1972, where it resulted in a disseminated mycosis in a green tree frog (*Litoria caerulea*) imported from Australia and subsequently affected frogs, toads, and salamanders in neighboring exhibits (Frank et al., 1974; Frank, 1975; Schipper, 1978). Experimentally infected reptiles remained healthy with only small lesions seen at necropsy; no lesions were seen in experimentally infected laboratory mice, rats, or guinea pigs (Frank et al., 1974). In Australia, *M. amphibiorum* has been reported from free-ranging cane toads (*Bufo marinus*) from Queensland and the Northern Territory, and from a green tree frog (*L. caerulea*) from Queensland (Speare et al., 1994, 1997; Berger et al., 1997). Captive frogs from other mainland Australian states have also been reportedly infected with *M. amphibiorum*. In 1992, a variety of frog species from the Royal Melbourne

Zoo collection in Victoria were infected by a *Mucor* sp. believed to be introduced by giant tree frogs (*Litoria infrafrenata*, also known as the white lipped tree frog) accidentally shipped in fruit crates from the north (Slocombe et al., 1995). In 1997, slender tree frogs (*Litoria adelaidensis*) from an established colony and white lipped tree frogs (*L. infrafrenata*) imported from Queensland developed mucormycosis at Perth Zoological Gardens, Western Australia (Creeper et al., 1998). Taylor et al. (1999) reported four cases of fatal dermatitis without systemic involvement caused by a *Mucor* sp. in a captive breeding colony of Wyoming toads (*Bufo baxteri*) held in Wyoming, USA.

Mucor amphibiorum is the only disease agent known to cause significant morbidity and mortality in the free-living platypus (*Ornithorhynchus anatinus*) in Tasmania. To date, only the positive mating type has been isolated from infected platypuses. This fungus causes a severe granulomatous and often ulcerative dermatitis, which may progress to involve underlying muscle and may disseminate to internal organs, particularly the lungs (Munday and Peel, 1983; Obendorf et al., 1993; Connolly et al., 1998), leading to the death of the animal. In the absence of the systemic spread of the organism, death could result from secondary bacterial infections or impaired thermoregulation and mobility. The route of infection in the platypus is unknown but is thought to be via skin wounds (Munday and Peel 1983; Obendorf et al., 1993; Connolly et al., 1998), although the respiratory route has also been proposed (Munday et al., 1998). Histologically, skin lesions examined from seven cases of platypus mucormycosis were in the form of discrete, poorly encapsulated granulomas or, more commonly, diffuse granulomatous or pyogranulomatous inflammation (Connolly et al., 2000). Platypuses with mucormycosis had hematologic and serum biochemistry changes when compared with clinically healthy animals from the same Tasmanian sites (Connolly et al., 1999). Affected platypuses were mildly anemic (with reduced packed cell volume and hemoglobin concentration), which was thought to be due to blood loss and/or depression of erythropoiesis as a result of inflammation. Lymphopenia was thought to be due to the stress of this serious disease, although capture stress could also lead to reduced lymphocytes through adrenocorticosteroid release. The elevated globulins were thought to be due to the inflammation and antigenic stimulation associated with mucormycosis. An enzyme-linked immunosorbent assay (ELISA) demonstrated the ability of the platypus to mount a humoral immune response to *M. amphibiorum* (Whittington et al., 2002).

The distribution of Tasmanian platypus populations with mucormycosis has expanded from 1982 to 2006, with at least 11 catchments affected since the index cases were seen in the Elizabeth River at Campbell Town, South Esk River catchment (Munday and Peel, 1983; Connolly 2006). The prevalence of mucormycosis among platypuses captured from Brumby's Creek, Cressy, Tasmania during a 12-mo study was 33% (Connolly et al., 1998). A recent survey across many catchments found a much lower prevalence of mucormycosis throughout Tasmania (Gust, pers. comm.). In addition to the presence of *M. amphibiorum*, *M. circinelloides* has also been isolated from an ulcer in one affected platypus (Stewart et al., 1999). Despite the presence of *M. amphibiorum*, mucormycosis has not been confirmed in platypuses on the Australian mainland to date (Connolly et al., 1998).

Differentiating *Mucor* species has been largely based on phenotypic characters (Schipper, 1978; Scholer et al., 1983). Mating experiments of presumptive isolates with test pairs of *M. amphibiorum* and zygospore formation further aides in identification (Schipper, 1978), as does the production of spherule-like structures on blood agar cultured at 35 C (Muir et al., 1996). Growth temperatures have in the

past been used to assist in identification of *Mucor* species. *Mucor amphibiorum* was initially reported to have a maximum temperature for growth in vitro of 36 C (Schipper, 1978; Scholer et al., 1983; Obendorf et al., 1993), whereas, for *M. circinelloides*, this temperature was from 38 C to 40 C. More recently, two of six *M. amphibiorum* isolates were reported to grow at 38 C (Stewart et al., 1999). Speare et al. (1994) reported half the *M. amphibiorum* isolates having some growth at 36 C, and of these, some grew well at that temperature.

Genotypic analysis has been used to identify species and track introductions of many pathogens, including clinically important Zygomycetes (Voigt et al., 1999; Schwarz et al., 2006). Although *Mucor* spp. have been included in these studies, there has been little genotyping specifically conducted on *M. amphibiorum* (Stewart and Munday 2005). The eukaryotic ribosomal RNA (rRNA) gene includes the internal transcribed spacers 1 and 2 (ITS1 and ITS2), which consist of increased sequence diversity compared with the 28S and 5.8S rRNA regions. For this reason, the region has been used widely for evaluating diversity in fungal genera. However, the regions are not amenable to the study of within-species diversity. Here, multilocus fingerprinting is more useful. Intersimple sequence repeat (ISSR) analysis has been shown to be a robust, versatile tool for assessment of genetic diversity in fungi (Hantula et al., 1997; Stummer et al., 2000; Stodart et al., 2007). The technique does not require prior knowledge of DNA sequence and is reproducible among laboratories; ISSR, therefore, offers a valuable tool for determining genetic differences within *Mucor* spp. isolated from the platypus.

The aims of this study were to 1) confirm the identity of *Mucor* species previously isolated from the platypus, amphibians, and environmental samples; 2) generate molecular data for *M. amphibiorum* based on the rRNA region; and 3) assess the genetic variability of *Mucor* spp. isolated from the platypus.

MATERIALS AND METHODS

Fungal isolates

Isolates were confirmed as *M. amphibiorum* at the Australian Medical Mycology Reference Laboratory (AMMRL, Pacific Laboratory Medicine Services, Royal North Shore Hospital, Sydney, Australia) on the basis of their colonial and microscopic morphology on potato dextrose agar at 28 C, according to the descriptions of Schipper (1978). The *M. amphibiorum* positive mating strain isolated in Germany (type stain Centraal Bureau voor Schimmelcultures [CBS] 763.74) and a negative mating strain (CBS 185.77) isolated in Central America were obtained from the Australian Medical Mycology Reference Laboratory. Most of the Tasmanian isolates (10 of 14) were collected as part of a 12-mo mark-recapture study that commenced in April 1994 (Connolly et al., 1998). All Tasmanian platypus isolates of *M. amphibiorum* cultured to date or obtained from the AMMRL have been identified as the positive mating type. The isolates from the AMMRL were stored as Sabouraud agar culture squares in sterile saline/water in McCartney bottles with parafilm around the cap at room temperature, with longevity of up to 5 yr before requiring subculturing (Smith and Onions, 1983). The four cane toad isolates from the Tropical and Aquatic Animal Health Laboratory, Department of Primary Industries (DPI; Townsville, Queensland, Australia) were collected and isolated as described by Speare et al. (1994) and were stored in 10% glycerol at −80 C. The initial *M. amphibiorum* isolate cultured by the DPI was verified by CABI Bioscience (formerly the International Mycological Institute; Egham, UK). Subsequent identification was performed at the DPI using the isolate as the benchmark.

DNA extraction

Genomic DNA was initially isolated from cultures using the DNeasy Plant Mini Kit (Qiagen, Valencia, California), following the manufacturer's directions. However, inconsistent yield proved an issue for downstream applications. Subsequently, the phenol/chloroform method of Raeder and Broder (1985) was applied and resulted in consistent yields of DNA of suitable quality for polymerase chain reaction (PCR) applications. Briefly, each sample was ground to a fine powder in liquid nitrogen and suspended in 5× volume of

extraction buffer (1.5M NaCl, 0.15M NaOAc, 2.5% sodium dodecyl sulfate, and 0.05M ethylenediaminetetraacetic acid; pH 5.7), and an equal volume of phenol/chloroform (1:1) was added before being centrifuged at 15,800 × G for 15 min. The aqueous phase was removed and re-extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged as before. DNA was precipitated from the aqueous phase at −20 C for 2 hr by the addition of 0.1 volume of 3M ammonium acetate and 0.6 volumes of isopropanol. The DNA was pelleted by centrifugation, washed with 70% ethanol, and air-dried. The final pellet was suspended in 30 µl TE buffer and stored at −20 C. DNA samples were quantified on 0.8% agarose gels in TAE buffer using Bioline Hyperladder II (Bioline, International Rosehill, NSW, Australia) as the standard.

ITS amplification and sequencing

Amplification of the complete ITS1, 5.8S, and ITS2 regions of the ribosomal DNA gene was achieved using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White et al., 1990). Amplification reactions (30 µl) consisted of 1× buffer (Bioline), each 0.2mM dNTP, 1.5mM $MgCl_2$, each 0.25µM primer, 1 unit of Taq DNA polymerase (BioTaq; Bioline), and approximately 40 ng of template DNA. Amplifications were performed on a Hybaid PCR Express thermal cycler (Hybaid Ltd, Basingstoke, Hampshire, UK), with initial denaturing at 95 C for 3 min, followed by 35 cycles of 94 C for 45 sec, 56 C for 45 sec, and 72 C for 1 min 30 sec. A final elongation step of 72 C for 7 min was conducted.

Amplicons were purified using AxyPrep PCR-cleanup kit (Axygen BioSciences, Union City, California, USA) following the manufacturer's directions. Purified products were sequenced in both forward and reverse directions at the Australian Genome Research Facility (Brisbane, Australia) using an AB3730xl sequencer (Applied Biosystems, Scoresby, Victoria, Australia). Forward and reverse ITS sequences were used to construct a single sequence for each *Mucor* isolate examined. Available ITS sequences from *Mucor* and related species were obtained from GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA; Benson et al., 2008) following a BLASTN search (Zhang et al., 2000). Alignments were performed using ClustalW (European Bioinformatics Institute, Hinxton, Cambridge, UK; Higgins et al., 1994), and following trimming to produce sequences of similar length, phylogenies were constructed using the Tamura-Nei model (Tamura and Nei, 1993). Consensus neighbor-joining trees were generated, following 1,000 bootstrap replicates. All analyses were performed with Molecular Evolutionary Genetics Analysis, version 4, software (MEGA4; Tempe, Arizona, USA; Tamura et al., 2007).

ISSR motifs and PCR fingerprinting

A literature survey identified 12 ISSR motifs with common occurrence in fungal genre. These were selected for diversity analysis of *Mucor* isolates. The PCR fingerprinting of isolates was conducted using the motifs as arbitrary primers, similar to the procedure for random amplification of polymorphic DNA PCR. Optimization of the ISSR-PCR was conducted initially on two *Mucor* isolates, with differing annealing temperature (44–55 C), $MgCl_2$ concentration (1.5–3mM), primer concentration (0.2–0.6µM), and template amount (0.1–1ng). Seven of the 12 ISSR primers resulted in amplification products, whereas the remaining five primers did not produce amplification products under any conditions. One primer resulted in inconsistent amplification and was removed from further analysis. In further analysis, using six *Mucor* samples, six ISSR primers resulted in consistent, successful amplification (Table 3). Diversity analysis of 21 *Mucor* isolates by ISSR-PCR was performed using an FTS 960 thermal controller (Corbett Research, Sydney, Australia). Each reaction consisted of 1× amplification buffer (Bioline), 0.2mM each dNTP, 0.5µM primer, 0.5 units of *Taq* DNA polymerase (BioTaq, Bioline), and approximately 0.5 ng of template DNA, in a reaction volume of 25 µl. The cycling conditions consisted of an initial denaturation step of 94 C for 3 min followed by 10 cycles each at 94 C for 45 sec, 35 C for 45 sec, and 72 C for 90 sec. This was followed by 30 cycles each at 94 C for 45 sec, 50 C for 45 sec, and 72 C for 2 min. The procedure was completed with a 5-min final extension at 72 C. Amplification products were separated by electrophoresis at 30 V for 16 hr on 2% agarose gels in 0.5× TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

Fragments amplified by each of the ISSR primers were scored visually as alleles at loci (primer) as either present (1) or absent (0), to form a binomial representation for each isolate. Faint bands were ignored, and only those occurring with a frequency of ≥5% were included in the analysis. Pairwise genetic distances were calculated between isolates

across bands by the Nei and Li (1979) coefficient, and the resulting matrix was used to construct a dendrogram with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. A cophenetic correlation was calculated between the original distance matrix and the cophenetic matrix used to construct the dendrogram. All analysis was conducted using the TreeCon software package (Van de Peer and De Wachter, 1994).

Mantel tests (Mantel 1967) were used to determine the association between the distance matrices generated by individual ISSR primers with the use of Mantel version 2.0 software (Liedloff 1999), with significance determined following 1,000 permutations.

RESULTS

Mucor collection

Isolates of *Mucor* spp. were obtained from a number of sources to form the most extensive culture collection available in Australia. Of the 33 isolates obtained, 21 grew readily and were demonstrated as pure cultures (Table 1 and Fig. 1). Australian isolates were represented by 13 from Tasmanian platypuses (nine ulcer and four fecal specimens), five from frogs or toads (three Queensland, one Northern Territory, one Western Australian), and one environmental sample (Tasmanian soil). The viable isolates included a duplicate subculture each of 94/5236A and 95/0649-2 from platypus feces. Two isolates from CBS were included in the collection as type and reference strains of *M. amphibiorum*. Of the nonviable cultures, seven had been classified as *M. amphibiorum* (four from platypus ulcers, three from diseased frogs) and five from feces were classified as *M. circinelloides*.

ITS Sequencing

Sequencing of the ITS region assisted in the identification of the isolates. Basic Local Alignment Search Tool (BLAST) searches of the GenBank database resulted in the identification of possible matches for the sequences submitted (Table 2). No previous records of *M. amphibiorum* ITS sequences were present in GenBank. Of the 14 isolates identified as *M. amphibiorum* by morphologic characters, 12 returned highest scores for *M. indicus* (88% maximum identity). The remaining two isolates returned scores with high similarity to *M. fragilis* and *M. circinelloides*. Of the five isolates identified by morphology as *M. circinelloides*, three returned maximum identity scores of ≥98% to *M. circinelloides* accessions in GenBank. The two remaining isolates returned scores with high similarity to both *M. ramosissimus* and *M. racemosus* (maximum identity 96%). Two isolates were unable to be classified to species level by morphologic characters. Isolate 88-25005 had its highest similarity to *M. indicus* (88%) and was thus thought to be *M. amphibiorum*, whereas isolate 89-23544 returned its highest similarity to *M. racemosus* (99%).

Given the absence of ITS data in GenBank, the sequences generated from isolates identified as *M. amphibiorum* from platypuses were compared with the neotype culture for *M. amphibiorum*, CBS 763.74, also sequenced in this research. The *M. amphibiorum* isolates appeared to consist of two haplotypes, differentiated by single-nucleotide polymorphisms within the ITS1 and ITS2 regions (positions 190 and 429, respectively). With the exception of 96-4049, all isolates from platypuses were represented by one haplotype, which included CBS 763.74 (*M. amphibiorum* 1; Fig. 2). Comparison between the sequences obtained from isolates of *M. amphibiorum* to that of the GenBank accession for the type culture of *M. indicus* (DQ118994.1, CBS226.29) indicated 67 variations within 529 nucleotides examined, excluding gaps. Twenty-two variables occurred in the ITS1 region, three within the 5.8S gene and 42 within ITS2. An alignment between the two *M. amphibiorum* haplotypes and representative sequences from *M. indicus*, *M. circinelloides*, *M. racemosus*, *M. ramosissimus*, and *M. fragilis* are presented in Figure 3.

A neighbor-joining tree (Fig. 2) based on

TABLE 1. Collection of *Mucor* isolates examined in this study. Genetic identity based on internal transcribed spacer (ITS) sequence information is provided.

Accession no.	Morphologic identity	ITS GenBank accession no.	Genetic identity	Source	Origin
CBS 185.77[a]	*M. amphibiorum*	FJ455860	*M. amphibiorum*	*Dendrobates* sp. frog	Central America, unspecified
CBS 763.74	*M. amphibiorum*	FJ455861	*M. amphibiorum*	Frog	Germany, green tree frog introduced disease to collection
88-19473	*M. amphibiorum*	FJ455864	*M. amphibiorum*	Cane toad gut	Queensland, James Cook University Townsville
89-4052	*M. amphibiorum*	FJ455862	*M. amphibiorum*	Cane toad	Queensland, Oowongalaema Station near Mundubbera
94-4936A	*M. amphibiorum*	FJ455854	*M. fragilis*	Ulcerated platypus	Tasmania, Brumbys Creek, Cressy
94-6450-1	*M. amphibiorum*	FJ455857	*M. amphibiorum*	Ulcerated platypus	Tasmania, Brumbys Creek, Cressy
94-3096	*M. amphibiorum*	FJ455856	*M. amphibiorum*	Ulcerated platypus	Tasmania, Brumbys Creek, Cressy
95-0493-2	*M. amphibiorum*	FJ455858	*M. amphibiorum*	Ulcerated platypus	Tasmania, Brumbys Creek, Cressy
94-5236B	*M. amphibiorum*	FJ455855	*M. amphibiorum*	Ulcerated platypus	Tasmania, Liffey River, Carrick
88-3213	*M. amphibiorum*	FJ455853	*M. amphibiorum*	Ulcerated platypus	Tasmania, South Esk River, Perth
96-4053	*M. amphibiorum*	FJ455873	*M. amphibiorum*	Platypus	Tasmania, unspecified
97-4057	*M. amphibiorum*	FJ455863	*M. amphibiorum*	Ulcerated platypus	Tasmania, unspecified
96-4049	*M. amphibiorum*	FJ455859	*M. amphibiorum*	Platypus	Tasmania, South Esk River
97-3700	*M. amphibiorum*	FJ455868	*M. circinelloides*	Slender tree frog	Western Australia, Perth Zoo
94-4936-6	*M. circinelloides*	FJ455872	*M. circinelloides*	Soil from infected site	Tasmania, Brumbys Creek, Cressy
94-5236A	*M. circinelloides*	FJ455870	*M. circinelloides*	Feces from ulcerated platypus	Tasmania, Liffey River, Carrick
94-5236A1	*M. circinelloides*	FJ455869	*M. circinelloides*	Feces from ulcerated platypus	Tasmania, Liffey River, Carrick
95-0649-2A	*M. circinelloides*	FJ455867	*M. ramosissimus*	Feces from healthy platypus	Tasmania, Mersey River, Weegena
95-0649-2	*M. circinelloides*	FJ455871	*M. ramosissimus*	Feces from healthy platypus	Tasmania, Mersey River, Weegena
88-25005	*Mucor* sp	FJ455866	*M. amphibiorum*	Cane toad liver, skin, feces	Northern Territory, unspecified
89-23544	*Mucor* sp.	FJ455865	*M. racemosus*	Cane toad tadpole	Queensland, Herveys range, Townsville

[a] CBS = Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands

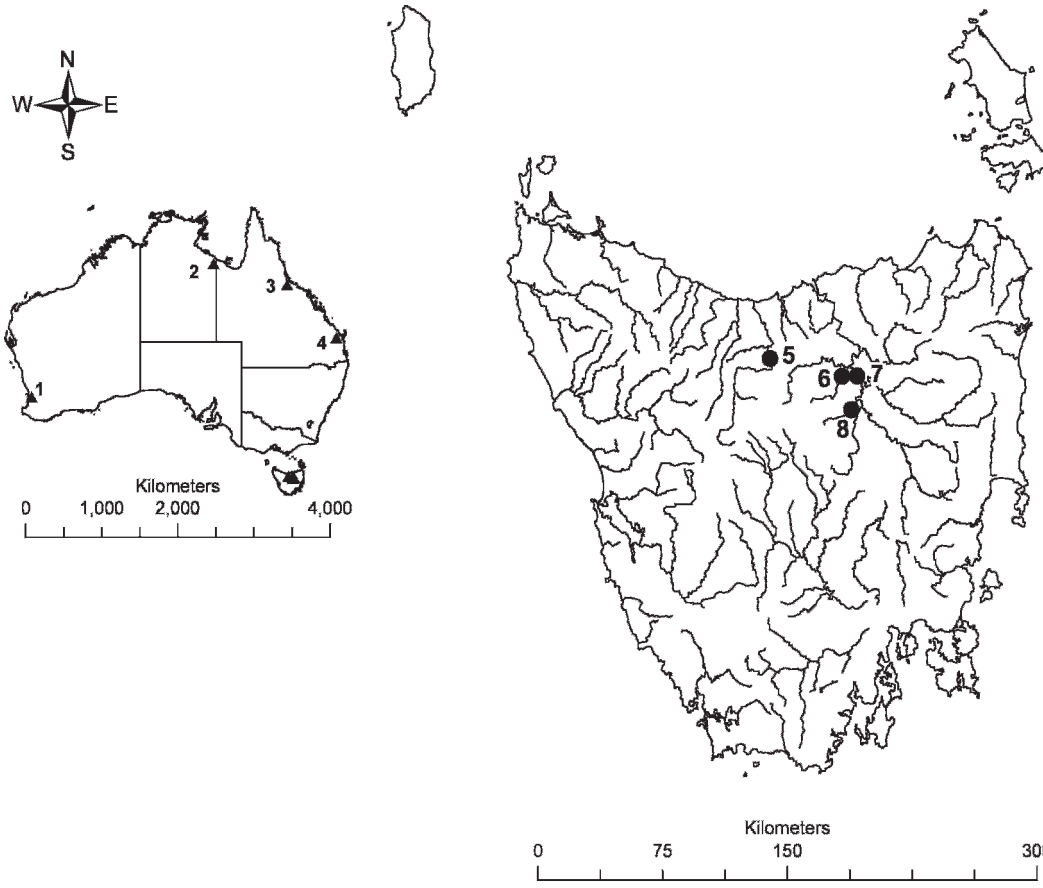

FIGURE 1. Map showing the locations of all Australian *Mucor* spp. isolates used in this study. 1=Perth, Western Australia; 2=Calvert Hills Station, Northern Territory; 3=Townsville, Queensland; 4=Oowongalaema Station, Queensland; 5=Mersey River, Tasmania; 6=Liffey River, Tasmania; 7=South Esk River, Tasmania, 8=Brumby's Creek, Tasmania.

sequence alignments with those available in GenBank supported the BLAST results and placed the 13 isolates of *M. amphibiorum* in a single clade, with bootstrap support of 99%. The sequence of the neotype culture for *M. amphibiorum* (CBS 763.74) was present within this clade, giving support to the identity of isolates from platypuses as *M. amphibiorum*.

Three ITS haplotypes of *M. circinelloides* were evident among the sequences obtained from GenBank. Four of the isolates from this current study, classified as *M. circinelloides* by morphologic characters, were present in two of the three haplotypes. Several isolates classified as *M. circinelloides* (95-0649-2 and 95-0649-2A) clustered with *M. ramosissimus*. These may represent contaminations or misclassification based on morphologic characters.

Suitability of ISSR markers for genetic analysis

The ISSR primers assayed resulted in multiallelic fingerprints for each of the isolates examined. The number of amplified fragments ranged from 19 to 27 resulting in 16 to 18 identifiable genotypes (Table 3). Both Shannon's diversity index (I) and Nei's unbiased genetic diversity (UH) indicated the primers were able to detect moderate levels of diversity within the isolate collection (Table 3). Mantel tests for all

TABLE 2. BASIC Local Alignment Search Tool (BLAST) scores from an internal transcribed spacer (ITS) sequence similarity search of the GenBank database.[a]

Isolate	Description	GenBank No	Total score	Query coverage (%)	E value	Max ident
88-3213 (542)	*M. indicus* CBS 226.29	DQ118994.1	662	99%	0	88
	M. circinelloides CBS 195.68	DQ118991.1	511	99%	2.00E-^{146}	81
94-5236B (542)	*M. indicus* CBS 226.29	DQ118994.1	664	100%	0	88
94-3096 (542)	*M. circinelloides* CBS 195.68	DQ118991.1	513	100%	6.00E-^{147}	81
CBS 763.74 (542)						
97-4057 (542)						
94-6450-1 (542)	*M. indicus* CBS 226.29	DQ118994.1	658	100%	0	88
95-0493-2 (542)	*M. circinelloides* CBS 195.68	DQ118991.1	508	100%	2.00E-^{145}	81
96-4053 (542)						
96-4049 (542)	*M. indicus* CBS 226.29	DQ118994.1	666	100%	0	88
CBS 185.77 (542)	*M. circinelloides* CBS 195.68	DQ118991.1	508	100%	2.00E-^{145}	81
89-4052 (542)						
88-25005 (542)						
88-19473 (542)	*M. indicus* CBS 226.29	DQ118994.1	662	100%	0	88
	M. circinelloides CBS 195.68	DQ118991.1	504	100%	3.00E-^{144}	80
97-3700 (550)	*M. circinelloides* CNRMA 04.805	DQ118990.1	993	100%	0	100
	M. circinelloides CBS108.16	AF412286.1	973	98%	0	100
	M. circinelloides f. circinelloides CBS 478.70	AY243942.1	966	98%	0	99
94-5236A1 (550)	*M. circinelloides* CNRMA 03.371	DQ118988.1	994	100%	0	100
94-5236A (550)	*M. circinelloides* CBS 195.68	DQ118991.1	989	100%	0	99
	M. racemosus f. racemosus RR 218	AJ878933.1	966	100%	0	98
94-4936-6 (551)	*M. circinelloides* CNRMA 03.371	DQ118988.1	994	100%	0	100
	M. circinelloides f. circinelloides CBS 195.68	AY243943.1	993	99%	0	100
	M. circinelloides CBS 195.68	DQ118991.1	989	100%	0	99
	M. racemosus f. racemosus RR 218	AJ878933.1	966	100%	0	98
94-4936A (550)	*M. fragilis* CTSP F1	EU862184.1	975	100%	0	99
	M. circinelloides IP 1873.89	DQ118989.1	915	100%	0	97
89-23544 (542)	*M. racemosus* CTSP F7	EU862189.1	969	100%	0	99
	M. fragilis CTSP F1	EU862184.1	931	100%	0	97
	M. circinelloides CNRMA 04.805	DQ118990.1	904	100%	0	96
95-0649-2 (552)	Species closely related to *M. ramosissimus*	AY213664.1	906	100%	0	96
95-0649-2A (552)	*M. circinelloides* CNRMA 03.154	DQ118987.1	897	100%	0	96
	M. racemosus CTSP F7	EU862189.1	893	100%	0	96

[a] CBS = Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands; CNRMA = National Reference Center for Mycoses and Antifungals, Pasteur Institute, Paris, France; CTSP = College of Agronomy and Biotechnology, China Agricultural University; Beijing, China, IP = Pasteur Institute Collection of Fungi, Institute Pasteur, Paris, France; RR = Plant Pathogen Interactions, Rothamsted Research, Harpenden, United Kingdom.

[b] Numbers in parenthesis indicate the sequence length.

combinations of primers indicated a significant association between all matrices, with the standard normal variate (g) ranging from 2.49 to 8.29 ($P<0.05$; Table 4).

The combined data set consisted of 135 fragments capable of identifying 19 genotypes among the 21 isolates examined. Unweighted Pair Group Method with Arithmetic Mean clustering, based on the

distance matrix, resulted in two major clusters, one containing isolates classified as *M. amphibiorum* and *M. racemosus* by ITS sequencing and the other, isolates classified as *M. circinelloides*, *M. ramosissimus*, and *M. fragilis* (Fig. 4). The structure of the dendrogram was strongly correlated with the original distance matrix, with a cophenetic correlation of $r=0.93$ ($P<0.01$). Within the major clusters a number of subclusters are evident. Seven of the isolates of *M. amphibiorum* from platypuses were present in two subclusters, with the remaining isolate, 96-4053, appearing genetically distinct from all other isolates. The two cultures from the CBS collection fell within the *M. amphibiorum* cluster and were more closely related to platypus isolates than to the three *M. amphibiorum* isolates from cane toads. For the remaining isolates, those classified as *M. circinelloides* by sequence analysis formed a separate subcluster, distinct from isolates classified as *M. ramosissimus* and *M. fragilis* (Fig. 4).

DISCUSSION

Since the index cases of Tasmanian platypuses affected by mucormycosis in 1982 (Munday and Peel, 1983), several studies have been conducted on the prevalence and spread of this disease (Connolly et al., 1998; Stewart, 2001; Connolly, 2006). In most instances, these studies focused on the isolation and morphologic identification of *M. amphibiorum* from lesions in clinically infected platypuses, and genetic diversity of this pathogen within Tasmanian platypus populations was not addressed. Methods allowing rapid identification, along with basic information on the extent of genetic diversity and the distribution of genotypes, provide additional tools to assist in the development of management strategies. This current research has provided information that could form the basis for a rapid, presumptive identification protocol using differences in the ribosomal DNA (rDNA) ITS region. Rapid, presumptive identification would allow for the targeted monitoring of wild populations or potentially quarantine of captured animals, if necessary, particularly in areas in which that the disease has yet to be reported. In addition, this would allow the tracking and monitoring of the spread or decline of the disease in wildlife surveys and assist in confirming diagnosis. Initial information has also been provided on the extent of genetic diversity present within the collection of isolates, based on ISSR amplification. The genetic diversity of fungal pathogens may allow the mode of reproduction to be assessed (sexual or asexual) and the extent to which that occurs. Furthermore, the future linkage of virulence to genotype may allow for the tracking of highly virulent genotypes and their dispersal.

Most studies of the Zygomycetes have concentrated on species involved in human mycoses (Bouza et al., 2006). Several phylogenetic studies concentrating on rDNA regions have been published (Voight et al., 1999; Schwarz et al., 2006; Iwen et al., 2007), and although these studies have provided information that is relevant to diagnostics, they provide limited information on genetic diversity. Before this study, ITS sequences for *M. amphibiorum* were not available, to our knowledge. The ITS region is widely used for molecular-based identifications of fungi, and previous research has indicated its potential in distinguishing species within the *Mucor* genus (Schwarz et al., 2006).

The aim of this present study was to provide molecular data for *M. amphibiorum* based on the rDNA ITS region and to generate initial data to indicate the extent of genetic diversity present amongst isolates from the platypus. The inclusion of the type strain and other reference isolates of *M. amphibiorum* from the CBS collection ensured that the isolates from platypuses were identified correctly. Our results indicate that the isolates of *M. amphibiorum* share high similarity within

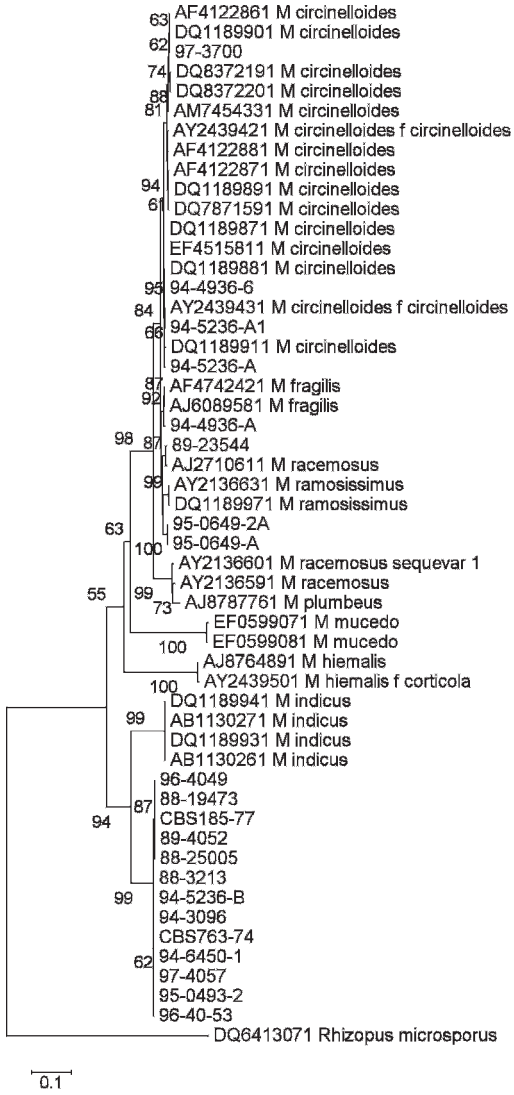

FIGURE 2. Consensus neighbor-joining tree generated from sequence alignments of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions of *Mucor* sp. isolated from platypuses and representative species from the GenBank database. Consensus among 1,000 trees by bootstrap analysis is given for each node, shown as the percentage of supporting trees.

the ITS sequences and are most closely related to *M. indicus*, with 88% similarity. This relationship is consistent with the 18S and 28S rDNA sequence data (Voigt et al., 1999), where *M. amphibiorum* and *M. indicus* were placed in a separate subclade compared with other *Mucor* species examined, sharing 95% and 98% similarity for the 18S and 28S regions, respectively. Unlike *M. circinelloides* ITS sequences, those of *M. amphibiorum* appeared to be homogeneous, with only two haplotypes distinguished by single-nucleotide polymorphisms within ITS1 and ITS2. Seven of eight positive mating type *M. amphibiorum* isolates from platypus ulcers fell within the same haplotype as the positive mating type CBS 763.74 type strain. It is possible that there are differences in pathogenicity for the platypus between the mating types, which require further investigation. Schwarz et al. (2006) found that *M. circinelloides* demonstrates high variability within the ITS regions compared with other Zygomycetes, and that was confirmed in this present study. The apparent differences within the rDNA ITS region among *Mucor* species provide the opportunity to target these regions to provide rapid, species-specific identification protocols.

The ITS sequences provided limited information regarding the genetic diversity present within the collection. To address this issue, ISSRs were chosen. The application of this marker type resulted in the separation of *M. amphibiorum* isolates from those of the other species examined. Furthermore, the isolates from ulcerated platypuses were genetically distinct from those originating from frogs and toads. The isolate collection contained representatives from geographically separated sites. Although there were not enough isolates to determine genetic trends in each region, the results indicated that similar genotypes existed between collection sites and that collection sites could consist of several genotypes. Intersimple sequence repeat markers have been used successfully in a range of fungi and provide the opportunity for further development to target true microsatellites for population analysis (Lian et al., 2001; Rubini et al., 2004; Steimel et al., 2004).

Sequencing of the rDNA ITS region identified several isolates to be unexpected species. *Mucor racemosus*, *M. ramo-*

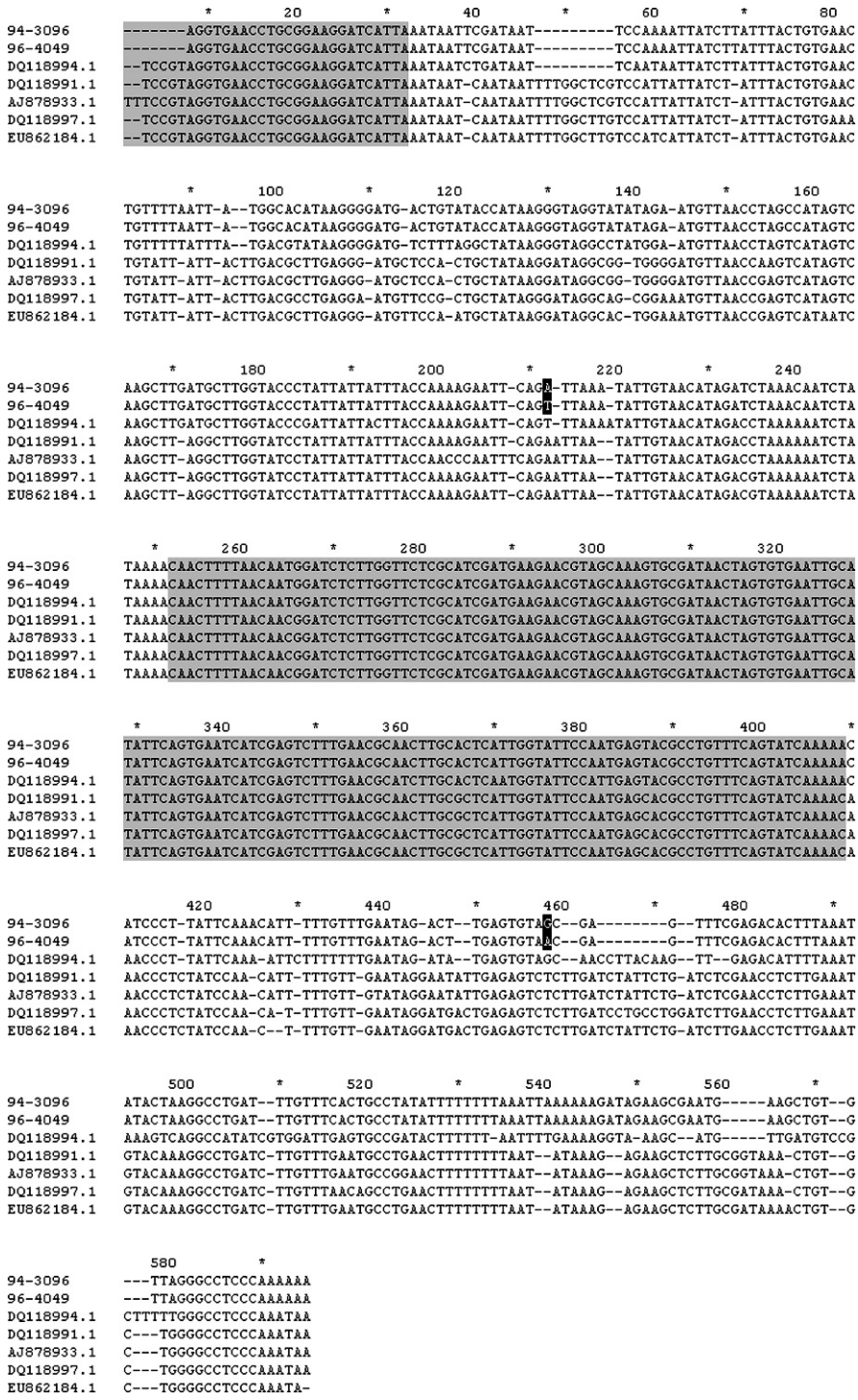

FIGURE 3. Ribosomal DNA (rDNA) internal transcribed spacer (ITS) region sequence alignment among representative isolates of *M. amphibiorum* and sequences of other *Mucor* spp. The 18S and 5.8S gene regions are shaded in grey. The two single nucleotide polymorphisms which separate the identified ITS haplotypes of *M. amphibiorum* are shaded in black. DQ118994.1, *M. indicus*; DQ118991.1, *M. circinelloides*; AJ878933.1, *M. racemosus*; DQ118997.1, *M. ramosissimus*; EU862184.1, *M. fragilis*.

TABLE 3. Statistics for the six intersimple sequence repeat (ISSR) primers used to determine genetic diversity of 21 *Mucor* spp. isolates. For each marker the number of amplified bands and identifiable phenotypes are given. Shannon's diversity index (I) and Nei's unbiased genetic diversity (UH) are presented as means, with standard error in parenthesis.

ISSR motif	Target sequence	Amplified fragments	Identifiable phenotypes	I	UH
AC	5′-(ACACACACACACACAC)-3′	27	17	0.50 (0.03)	0.35 (0.03)
AG	5′-(AGAGAGAGAGAGAGAG)-3′	23	17	0.52 (0.03)	0.37 (0.03)
ACC	5′-(ACCACCACCACCACC)-3′	26	15	0.43 (0.04)	0.29 (0.03)
CAA	5′-(CAACAACAACAACAA)-3′	19	16	0.52 (0.03)	0.36 (0.03)
GACA	5′-(GACAGACAGACAGACA)-3′	19	16	0.51 (0.03)	0.35 (0.03)
GGAT	5′-(GGATGGATGGATGGAT)-3′	21	18	0.42 (0.04)	0.28 (0.03)
Combined		135	19	0.49 (0.01)	0.33 (0.01)

sissimus, and *M. fragilis* were all identified within the collection. When subjected to analysis with the ISSR primers, these isolates were genetically distinct from isolates classified as either *M. amphibiorum* or *M. circinelloides*. These may represent contaminations in the isolate collection. Stewart et al. (1999) proposed the existence of round, spherule structures to be diagnostic for *M. amphibiorum*, despite other *Mucor* spp. producing similar structures in culture. Therefore, this may point to the isolates being misidentified by morphologic characters. A similar situation may be true for isolate 97-3700, identified as *M. amphibiorum* on morphology, but as *M. circinelloides* by ITS sequencing and ISSR analysis. Each of these serve as an example of the care required when maintaining culture collections of this nature and the use of several techniques to provide certainty in diagnosis, particularly within genera consisting of morphologically similar species.

The ecologic niche of *M. amphibiorum* in Tasmania (other than platypus lesions) is unknown, but it has been recovered from soil in regions where frogs are affected in Queensland, Australia. However, *M. amphibiorum* could not be isolated from 14 Tasmanian environmental samples including soil, water, *Ixodes ornithorhynchi* ticks, and frog feces (Connolly et al., 1998). *Mucor circinelloides* and *M. saturninus* were isolated from soil, *M. circinelloides* from frog feces, and *M. circinelloides* and *M. hiemalis* from platypus fecal samples. The potential for other aquatic vectors for *M. amphibiorum* needs to be assessed. Although the agent was not isolated from limited sampling of frogs from an endemic region (Connolly et al., 1998), a more comprehensive frog survey for mucormycosis is required. A study to investigate the potential for salmonid fish species to be another aquatic host and vector of *M. amphibiorum* has been implemented. Future work to further

TABLE 4. Distance matrices obtained from each intersimple sequence repeat polymerase chain reaction correlated using Mantel tests. The standard normal variate (g) is presented below the diagonal, and the correlation coefficient (r) above the diagonal. All data are significant at $P<0.05$ following 1,000 iterations.

	AC	AG	ACC	CAA	GACA	GGAT
AC		0.59	0.42	0.34	0.47	0.35
AG	8.03		0.54	0.42	0.62	0.4
ACC	5.4	6.99		0.27	0.65	0.62
CAA	4.6	5.81	3.23		0.34	0.2
GACA	6.14	8.29	6.87	4.33		0.56
GGAT	4.53	5.19	5.69	2.49	6.18	

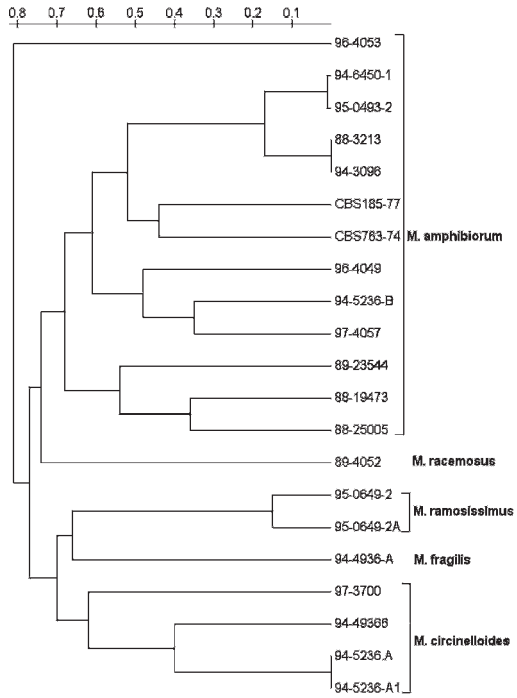

FIGURE 4. Dendrogram based on the genetic differences as determined by analysis of 135 amplified fragments generated from intersimple sequence repeat (ISSR) amplification. Genetic distances were calculated according to Nei and Li (1979), and the resulting distance matrix subjected to Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis. The cophenetic correlation between the original distance matrix and the UPGMA matrix used to construct the dendrogram was 0.93, indicating the dendrogram accurately reflects the original genetic distance between isolates.

develop the data presented here may provide rapid diagnostic tools capable of detecting free-living forms of *M. amphibiorum* in the environment and infected animals. A more extensive survey of *M. amphibiorum* from diseased Tasmanian platypuses and mainland frogs may enable the determination of the role of frogs in the introduction of this disease into Tasmanian platypuses or whether a more virulent endemic exists.

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