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Phylogeny and PCR Identification of the Human Pathogenic Fungus *Penicillium marneffei*

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The phylogenetic position of the human pathogenic fungus *Penicillium marneffei* was assessed from the nucleotide sequences of the nuclear and mitochondrial ribosomal DNA regions. Phylogenetic analysis determined that *P. marneffei* is closely related to species of *Penicillium* subgenus *Biverticillium* and sexual *Talaromyces* species with asexual biverticillate *Penicillium* states. Knowledge of the phylogenetic position of *P. marneffei* facilitated the design of unique oligonucleotide primers, from the nuclear ribosomal DNA internal transcribed spacer region, for the specific amplification of *P. marneffei* DNA. These primers were successful at selectively amplifying DNA from six isolates of *P. marneffei* and excluding the other species tested, which included *Penicillium* subgenus *Biverticillium* and *Talaromyces* species and several well-known fungal pathogens, namely, *Aspergillus fumigatus*, *Coccidioides immitis*, *Histoplasma capsulatum*, and *Pneumocystis carinii*. The primers that we have developed for the specific amplification of *P. marneffei* have the potential to be incorporated in a PCR identification system which could be used for the identification of this pathogenic agent from clinical material.

The fungus *Penicillium marneffei* Segretain, Capponi & Sureau has become a well-recognized human pathogen since the first reported case of a natural human infection in 1973 (8). Typically, it infects humans afflicted with diseases involving cell-mediated immunity such as Hodgkin's disease, tuberculosis, corticosteroid therapy, and AIDS, although infections in apparently immunocompetent individuals have been reported (7, 14, 32). In fact, because of the increasing frequency with which it affects AIDS patients, it is considered an "AIDS defining pathogen" (29). *P. marneffei* causes severe infections that can be focal or disseminated and can affect the bone marrow, intestine, kidneys, lungs, lymph nodes, liver, spleen, skin, and soft tissue (14, 18, 31, 32). The clinical manifestations most commonly described for *P. marneffei* mycosis are fever, weight loss, anemia, skin lesions, cough, hepatomegaly, adenopathies, and pulmonary infiltrates (14). Common among *P. marneffei*-infected patients is travel in Indonesia or Southeast Asia, where this fungus is endemic (32) (but see Hilmarsdottir et al. [14]). Apparently, several routes of entry are possible for *P. marneffei*, as infections via skin trauma, inhalation, and the digestive tract have been reported (14).

This fungal pathogen is unique among *Penicillium* species because it is the only species known to exhibit a temperature-dependent dimorphic growth habit. A yeast-like growth phase occurs in human tissue and in cultures at 37°C, while at 24°C, the mycelial phase, bearing conidiophores typical of other *Penicillium* species, predominates (15). The diagnosis of *P. marneffei* infection has proven difficult in many cases because symptoms of this mycosis may be suggestive of tuberculosis or infections by other fungal pathogens such as *Histoplasma capsulatum* and *Pneumocystis carinii* (5–7, 14, 15, 32). Identification of *P. marneffei* is further complicated by the coexistence of opportunistic infections frequently found among patients with AIDS infections (32). The ability to identify *P. marneffei* accurately in tissue samples is very important from a treatment point of view. Tuberculosis and *P. carinii* mycosis, for example, require completely different treatments than do *P. marneffei* infections (5, 32). Delay in diagnosis and treatment of this disease has contributed to a high mortality rate among patients (6), while early diagnosis followed by antifungal treatment (e.g., amphotericin B, itraconazole, and fluconazole) is reportedly quite successful in reversing the clinical manifestations of this mycosis (14, 18, 29, 32).

In the first mycological description of *P. marneffei* by Segretain (28), *P. marneffei* was classified among *Penicillium* species in the section Asymmetrica subsection Divaricata of Raper and Thom's (26) taxonomic treatment of *Penicillium* species; Raper and Thom's (26) section Asymmetrica subsection Divaricata is equivalent to Pitt's (24) *Penicillium* subgenus *Furcatum*. However, the most recent taxonomic scheme for *Penicillium* species by Pitt (24) assigned *P. marneffei* to subgenus *Biverticillium* rather than subgenus *Furcatum* because the conidiophores of *P. marneffei* are most frequently biverticillate and growth on G25N medium is weak, as is typical of other species in the subgenus *Biverticillium* (24). In agreement with Pitt (24), Frisvad and Filtenborg (12) suggested that *P. marneffei* be reclassified from subgenus *Furcatum* to subgenus *Biverticillium* on the basis of similar physiology and secondary metabolite profiles between *P. marneffei* and species of *Penicillium* subgenus *Biverticillium*. We were interested in determining whether *P. marneffei* had phylogenetic affinities with *Penicillium* subgenus *Biverticillium* as these investigators had suggested (12, 24).

Nucleotide sequences from the mitochondrial small subunit ribosomal DNA (mtSrDNA) and the nuclear rDNA region containing the internal transcribed spacers and 5.8S rRNA gene (ITS1-5.8S-ITS2) were obtained to examine the phylogenetic position of *P. marneffei*. Nucleotide sequence data from the ITS1-5.8S-ITS2 rDNA region were further analyzed to identify sequences unique to *P. marneffei*. We demonstrate in this paper the ability to design oligonucleotide primers from sequence data which can be used in the PCR to selectively amplify DNA of *P. marneffei* and differentiate it from other fungi. Similar PCR identification systems have been developed for other fungal pathogens such as *H. capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Trichophyton rubrum*, *Cryptococcus neoformans*, and *P. carinii* (3, 4, 19, 21).

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TABLE 1. Design of *P. marneffei*-specific oligonucleotide primers

Primer	Primer location[a]	Species	Nucleotide sequence (5′ to 3′)[b]
PM1 (5′)	ITS1 (bp 125–142)	*P. marneffei*	TCGCCGGGGGACGTTTGT
		T. thermophilus	C.....GGTCG.CC[c]
		T. trachyspermus	.....AG..C.ATG
		T. stipitatus	.....A...C...
		T. flavus	.....A.A.CC..
		P. purpurogenum	.....CA.TGCGCC
		P. funiculosum	.....A....C..
		H. capsulatum	CT.....AGC..CTC
		C. immitis	CT.....AT..CCC..
PM2 (5′)	ITS1 (bp 187–205)	*P. marneffei*	GATGGACTGTCTGAGTACC
		T. thermophilus	CGA...T.....GAT
		T. trachyspermus	..GA.G.....C..
		T. stipitatus	.....G.....GAT
		T. flavus	.....G.....TT
		P. purpurogenum	.....G.....GTG
		P. funiculosum	.....G.....T
		H. capsulatum	TGATTGGC.....C.TG
		C. immitis	AGATTGGC.....C.TG
PM4 (3′)	ITS2 (bp 498–519)	*P. marneffei*	ATGGTGGTGACCAACCCCCGCA
		T. thermophilus	TGACCTT...GGCTATGG.C.C
		T. trachyspermus	TAA.G.....A.CG...AC..
		T. stipitatus	G.....T.....
		T. flavus	G.....
		P. purpurogenum	TC.TG....G.....
		P. funiculosum	G.....C
		H. capsulatum	AGATG.TG.G..GGAG.CGG.C
		C. immitis	AGATC..CCC.GAT.GGGTCTT

[a] Primer location is based on *P. marneffei* ITS sequence, GenBank accession no. L37406.
[b] Primer sequences are aligned with sequences of two *Penicillium* species, four *Talaromyces* species, *H. capsulatum*, and *C. immitis.*
[c] A period (.) indicates an identical nucleotide to the *P. marneffei* sequence.

MATERIALS AND METHODS

The six isolates of *P. marneffei* included in this study and their origins are as follows. Strains from the American Type Culture Collection (Rockville, Md.) included ATCC 18224 (type culture; isolated from a bamboo rat in Indonesia), ATCC 24100 (isolated from a human spleen infection in the United States [infection with *P. marneffei* occurred during patient's travel to Vietnam]), ATCC 64101 (isolated from human tissue in China), and ATCC 64102 (isolated from a bamboo rat in China). Strains from the Commonwealth Scientific and Industrial Research Organization (Food Research Laboratory, North Ryde, New South Wales, Australia) included FRR 3841 (CBS 122.89; isolated from a leg infection in an AIDS patient in France) and FRR 4059 (isolated from a skin biopsy from an AIDS patient in Australia). DNA isolation of *P. marneffei* mycelium grown on malt extract agar at 25°C followed the procedure described by LoBuglio et al. (16). DNA from *Penicillium citrinum* FRR 806, *Penicillium crustosum* FRR 1809 (Commonwealth Scientific and Industrial Research Organization, Food Research Laboratory), *Penicillium chrysogenum* UCB 81-4 (University of California, Berkeley Microgarden, Berkeley) and *Aspergillus fumigatus* LCP 2642 (Laboratorie Cryptogamie Paris, Paris, France) was obtained as described above. DNAs of *H. capsulatum* and *P. carinii* were gifts from D. Carter and J. Niemiec, respectively, of Roche Molecular Systems Inc., Alameda, Calif.

Primers utilized in the PCR amplifications were MS1 and MS2 for the mtSrDNA and ITS2, ITS3, ITS4, and ITS5 for the ITS1-5.8S-ITS2 rDNA region (33). The PCR assay conditions were as described by White et al. (33) and as modified by LoBuglio et al. (16).

Nucleotide similarity among six isolates of *P. marneffei* was analyzed initially by restriction fragment length polymorphisms of the ITS1-5.8S-ITS2 rDNA PCR products. The ITS1-5.8S-ITS2 rDNA PCR products were digested singly with 1 to 3 U of the four-base-cutter restriction enzymes *Ava*I, *Ava*II, *Hha*I, *Mbo*I, and *Taq*I (Bethesda Research Laboratories, Gaithersburg, Md.) with the buffers and temperatures recommended by the manufacturer. These enzymes were selected because they were shown to generate ITS1-5.8S-ITS2 rDNA restriction fragment length polymorphism patterns that delimited each *Penicillium* and *Talaromyces* species examined previously, as determined by analysis of restriction sites in ITS1-5.8S-ITS2 rDNA sequences using the SITE command in the Seq option of the Intelligenetics software package (Intelligenetics, Inc., Mountain View, Calif.) (16, 17). Results of PCR amplifications and digested amplified products were assayed by gel electrophoresis in 1 to 2% agarose gels.

The mtSrDNA and ITS1-5.8S-ITS2 rDNA regions of *P. marneffei* (isolates ATCC 18224 and FRR 3841) were sequenced from single-stranded template generated from asymmetric PCR amplifications and by use of the parameters described previously by LoBuglio et al. (16). The sequences have been submitted to GenBank and are identified by the accession numbers L37407 and L37406. Nucleotide sequences from other species used in phylogenetic analyses (depicted in Fig. 1; see references 16 and 17 for GenBank accession numbers) were determined from previous studies (16, 17). DNA sequences were initially aligned by use of the Genalign option in the Intelligenetics software package and then by visual optimization. Only regions without ambiguity were included in the analysis (alignment is available upon request).

The heuristic method using the stepwise addition approach in the Phylogenetic Analysis Using Parsimony Program (PAUP 3.1) (30) was used to generate parsimony trees from sequence data. One hundred different replications under the random addition sequences option were used in the heuristic search to evaluate the effectiveness of the heuristic searches in finding the most parsimonious tree(s) (30). Under this option, each replication step randomly selects taxa to initiate tree building so that different starting trees are obtained for each replication. Bootstrap analysis (10) was used to evaluate the strength of the internal branches in the resulting parsimony trees by performing 500 bootstrap replications with the heuristics option in PAUP 3.1 (30). Phylograms resulting from parsimony analysis were tested against hypothetical phylogenies by use of the maximum likelihood program from Phylip 3.3 (11).

On the basis of a ITS1-5.8S-ITS2 rDNA sequence comparison of *P. marneffei* with its most closely related taxa, three nucleotide regions approximately 20 bp in length were identified as being unique to *P. marneffei*. Two of the regions are located in the ITS1 rDNA region and were selected as the 5′ direction primers PM1 and PM2. The 3′ primer PM4 was identified in the ITS2 rDNA region. The sequences of PM1, PM2, and PM4 and the fungal species included in the evaluation of PCR-based identification of *P. marneffei* are listed in Table 1.

The specificity of the *P. marneffei* primers was tested in a nested fashion. All fungal isolates included in the evaluation were first amplified with primers designed as general fungal primers, namely, ITS5 and ITS4 (33), with an annealing temperature of 48°C. The ITS1-5.8S-ITS2 rDNA PCR products were subsequently agarose purified to eliminate primers. These PCR products were diluted 1,000-fold and then amplified with two primer combinations, PM1-PM4 and PM2-PM4, at annealing temperatures of 60 or 65°C. PCR amplifications from *P. marneffei* miniprep DNA diluted 1,000-fold were also tested with PM1-PM4 and PM2-PM4 to ensure that the primers amplified genomic DNA.

RESULTS

Restriction enzyme digestions of the ITS1-5.8S-ITS2 rDNA PCR products for six *P. marneffei* isolates (see above) were conducted with the enzymes *Ava*I, *Ava*II, *Hha*I, *Mbo*I, and *Taq*I. The restriction fragment length polymorphism patterns generated were identical among the six *P. marneffei* isolates. On the basis of this initial screening, two isolates of *P. marneffei* were selected for nucleotide sequencing, namely, the type isolate ATCC 18224, which originated from a bamboo rat, and isolate FRR 3841, which originated from an AIDS patient (see above).

The mtSrDNA and ITS1-5.8S-ITS2 rDNA sequences generated from the two *P. marneffei* isolates ATCC 18244 and FRR 3841 were 100% identical. Phylogenetic analyses of the combined mtSrDNA and ITS1-5.8S-ITS2 rDNA data using heuristic random addition searches of 100 replications (PAUP 3.1) (30) produced 45 equally parsimonious trees of 618 steps (the 45 trees obtained varied only among the arrangement of taxa within the *Eupenicillium* and *Talaromyces* clades). There were no new trees produced after the first replication under the heuristic random addition sequences option, indicating that all of the most-parsimonious trees had been identified in this heuristic search. The phylogram in Fig. 1 is the strict consensus tree of the 45 heuristic trees generated by PAUP 3.1 (30). In all 45 heuristic trees, *P. marneffei* clustered with *Penicillium* subgenus *Biverticillium* and their related *Talaromyces* species in one clade. Bootstrap analysis of this data set indicated that the basal branch of this clade is supported by 96% of the bootstrapped data sets.

Maximum likelihood analysis (11) was used to test the hypothesis that *P. marneffei* is more closely related to *Eupenicillium* than *Talaromyces* species as would be predicted from Segretatin's (28) classification of this species. Reanalysis of the combined data set using the constraint option in PAUP 3.1 to enforce the phylogenetic position of *P. marneffei* within the *Eupenicillium* clade rather than within the *Penicillium* subgenus *Biverticillium* and *Talaromyces* clade produced 92 most-parsimonious trees of 660 steps, 42 steps longer than the parsimony tree depicted in Fig. 1. Maximum likelihood analysis indicated that the fit of the sequence data to the most-parsimonious tree (Fig. 1; log likelihood = −4772.27) was 5 standard deviations better than the fit of the data to the hypothesis tree positioning *P. marneffei* within the *Eupenicillium* clade (log likelihood = −4960.14; standard deviation = 37.62). Since a 2-standard deviation difference in log likelihood corresponds to a 95% confidence level, we can reject the tested hypothesis.

Knowledge of the phylogenetic position of *P. marneffei* facilitated the design of unique oligonucleotide primers for use in the PCR to identify *P. marneffei*. Nucleotide sequence comparisons of the ITS1-5.8S-ITS2 rDNA region among *P. marneffei* and closely related species identified three nucleotide regions unique to *P. marneffei* (Table 1). These regions were selected for the development of *P. marneffei*-specific oligonucleotide primers, namely PM1, PM2 (both 5′ primers), and PM4 (a 3′ primer). The mtSrDNA sequence was not variable enough to design species-specific primers. The selectivity of the primer combinations PM1-PM4 and PM2-PM4 was tested by amplifying DNA from closely related *Talaromyces* and *Penicillium* species (Fig. 2; Table 2) as well as from the six *P. marneffei* isolates. These species were selected because they grow well at 37°C and some have been isolated from clinical material (e.g., *Talaromyces thermophilus*, *Penicillium purpurogenum*, *Penicillium minioluteum*, and *Penicillium funiculosum*) (14a, 25). The ability of these primers to select against DNA from the well-known fungal pathogens *A. fumigatus*, *C. immitis*, *H. capsulatum*, and *P. carinii* as well as three *Penicillium* species that are considered common air and clinical contaminants (i.e., *P. citrinum*, *P. crustosum*, and *P. chrysogenum*) (24, 27) was also tested (Fig. 2; Table 2).

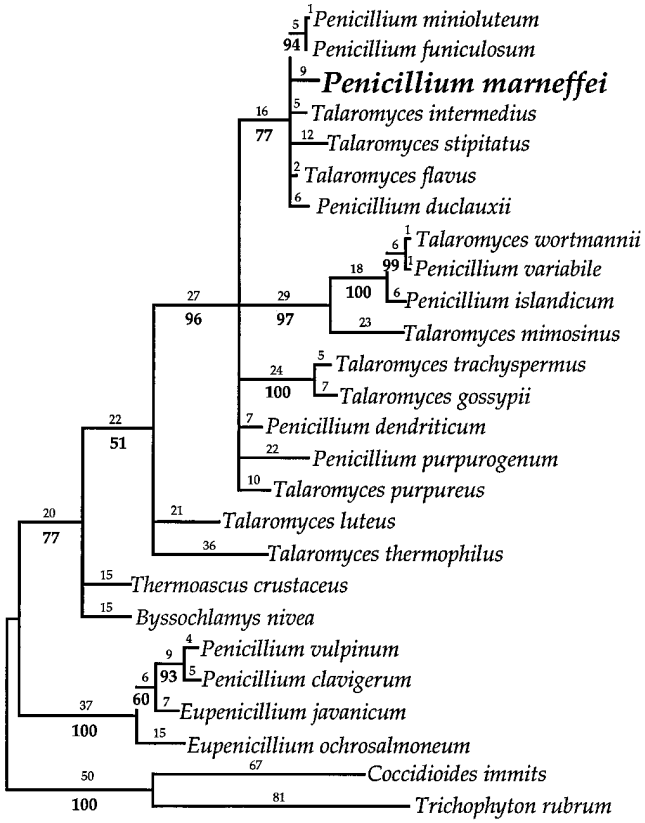

FIG. 1. Phylogenetic relationship of *P. marneffei* to *Penicillium*, *Talaromyces*, and *Eupenicillium* species inferred from combined nucleotide sequence of the internal transcribed spacers and 5.8S rRNA gene (ITS1-5.8S-ITS2) and mitochondrial small subunit rRNA gene (mtSrDNA). The phylogram presented is the strict consensus of 45 most-parsimonious trees (each 618 steps) generated from heuristic random addition searches of 100 replicates in PAUP 3.1 (30). The percentages below the branches are the frequencies with which a given branch appeared in 500 bootstrap replications (10) when the heuristics option in PAUP 3.1 was used (30), and the numbers above the branches are the total nucleotide changes assigned by PAUP 3.1 (30). Bootstrap values below 50% are not displayed. *C. immitis* and *T. rubrum* were chosen to root the tree as a result of previous phylogenetic analysis of 18S rRNA sequence data (2).

The primer combination PM2-PM4 was 100% successful at amplifying *P. marneffei* DNA (whether agarose-purified ITS5-ITS4 PCR products or genomic DNA was used) and selecting against the amplification of all other species tested at an annealing temperature of 60°C (Fig. 2; Table 2). The primer combination PM1-PM4 was less selective at an annealing temperature of 60°C because two of the fungi tested, *P. funiculosum* and *Talaromyces stipitatus*, showed positive amplification. A higher annealing temperature of 65°C was successful at excluding amplification of *P. funiculosum* but not amplification from *T. stipitatus* (Table 2). It should be noted that of these two species, only *P. funiculosum* has been isolated from clinical material (25). As shown in Fig. 2, when *P. marneffei* DNA was amplified with PM1-PM4 or PM2-PM4 at an annealing temperature of 65°C, PCR products of the expected sizes (403 and 347 bp for PM1-PM4 and PM2-PM4, respectively) in addition to PCR products of lower molecular weight were produced (Fig. 2). The PCR products of PM1-PM4 and

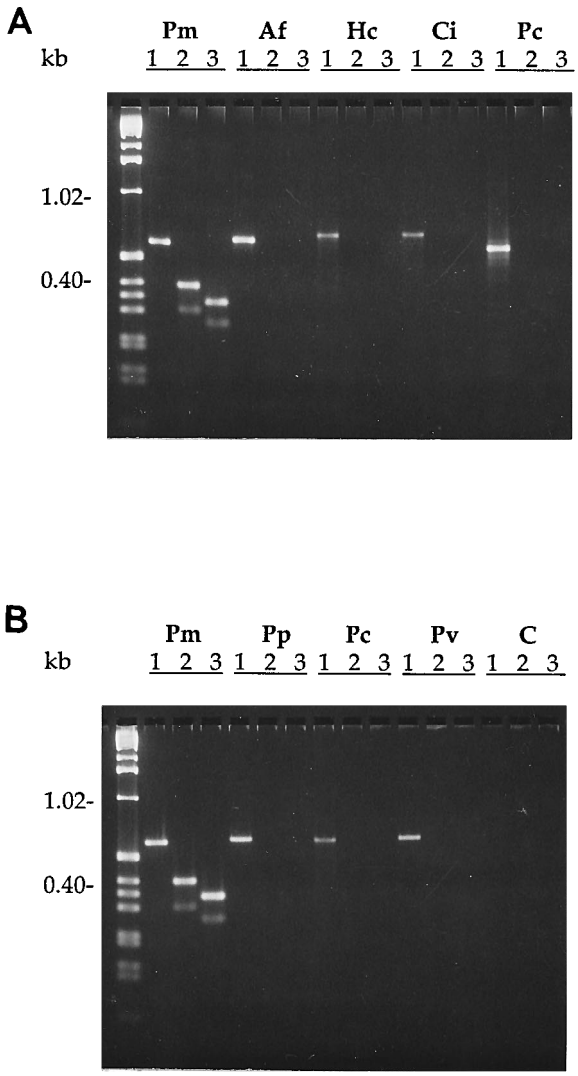

FIG. 2. A 3% agarose gel demonstrating the selective amplification of *P. marneffei* DNA using specific primer pairs, PM1-PM4 and PM2-PM4, at an annealing temperature of 65°C. (A) Lanes: Pm, *P. marneffei* ATCC 18224; Af, *A. fumigatus*; Hc, *H. capsulatum*; Ci, *C. immitis*; Pc, *P. carinii*. (B) Lanes: Pm, *P. marneffei* FRR 3841; Pp, *P. purpurogenum*; Pc, *P. chrysogenum*; Pv, *Paecilomyces variotti*; C, negative controls. (A and B) Lanes: 1, amplification from miniprep DNA with primers ITS5-ITS4 (which serves as a positive control); 2, amplification from agarose-purified ITS5-ITS4 PCR products with primers PM1-PM4; 3, amplification from agarose-purified ITS5-ITS4 PCR products with primers PM2-PM4. The unlabeled first lane on each gel contains the 1-kb marker from Bethesda Research Laboratories.

PM2-PM4 amplifications were each sequenced with their respective 5′ and 3′ primers. Each sequencing reaction yielded one sequence that was identical to the expected *P. marneffei* sequence. These results indicate that the secondary bands represent single strands of the PM1-PM4 and PM2-PM4 PCR products. Apparently, in PCR reactions using these primer combinations, there is unequal primer annealing so that an assymmetric amplification of the target region occurs and results in the production of single strands. At annealing temperatures of 40 or 45°C, the amount of single strands was greatly reduced in PCR PM1-PM4 and PM2-PM4 amplifications.

The efficiency of the primer pair PM2-PM4 in amplifying only *P. marneffei* DNA and excluding all of the other DNAs

TABLE 2. Specificity test of *P. marneffei*-specific primers

Species and isolate no.[a]	Amplification[b] by:			
	ITS5-ITS4[c] at 48°C[d]	PM1-PM4		PM2-PM4 at 60°C
		60°C	65°C	
Penicillium marneffei ATCC 18224, ATCC 24100, ATCC 64101, ATCC 64102, FRR 3841, FRR 4059	+	+	+	+
Penicillium funiculosum FRR 1823	+	±	−	−
Penicillium purpurogenum FRR 1147	+	−	−	−
Penicillium chrysogenum UCB 81-4	+	−	−	−
Penicillium citrinum FRR 806	+	−	−	−
Penicillium crustosum FRR 1809	+	−	−	−
Talaromyces thermophilus FRR 1791	+	−	−	−
Talaromyces trachyspermus FRR 1792	+	−	−	−
Talaromyces stipitatus FRR 2166	+	+	+	−
Talaromyces flavus FRR 2386	+	−	−	−
Paecilomyces variotti FRR 1658	+	−	−	−
Geosmithia argillaceae FRR 3098	+	−	−	−
Aspergillus fumigatus LCB 2642	+	−	−	−
Histoplasma capsulatum[e]	+	−	−	−
Coccidioides immitis FRR 3841	+	−	−	−
Pneumocystis carinii[f]	+	−	−	−

[a] Collection abbreviations: ATCC, American Type Culture Collection; FRR, Commonwealth Scientific and Industrial Research Organization, Food Research Laboratory; UCB, University of California, Berkeley Microgarden; LCP, Laboratorie Cryptogamie Paris.
[b] Symbols: +, positive amplification; ±, weak amplification; −, no amplification.
[c] Primers.
[d] Annealing temperature.
[e] DNA gift from Dee Carter, Roche Molecular Systems Inc.
[f] DNA gift from John Niemiec, Roche Molecular Systems Inc.

tested can be attributed to the greater number of mismatches between the sequence of PM2 and the sequences of the other species examined. As shown in Table 1, the corresponding sequences of the species compared are more dissimilar to the 3′ end of the primer sequence PM2 than to that of primer sequence PM1. A greater number of 3′ mismatches between primer and template will prevent complete primer annealing to template DNA during the PCR and subsequently inhibit in vitro DNA synthesis.

DISCUSSION

The exclusion of *P. marneffei* from the *Eupenicillium* lineage and its inclusion in the clade of *Penicillium* subgenus *Biverticillium* and *Talaromyces* species indicates that *P. marneffei* has phylogenetic affinities with the subgenus *Biverticillium* as determined by Pitt (24) and Frisvad and Filtenborg (12) and not with subgenus *Furcatum* as originally proposed by Segretain (28). Peterson (22) has demonstrated through nucleotide sequence analysis of the 25S rDNA gene and ITS1-5.8S-ITS2 rDNA region that of the sexual genera with *Penicillium* asexual states, *Eupenicillium* and *Talaromyces*, the asexual species in *Penicillium* subgenera *Furcatum*, *Aspergilloides* and *Penicillium* are more closely related to the sexual genus *Eupenicillium*. Thus, morphological (24), physiological (12), and nucleotide sequence data (present study) all support the contention that *P. marneffei* is phylogenetically related to *Talaromyces* species with biverticillate *Penicillium* asexual states and strictly asexual species of *Penicillium* subgenus *Biverticillium*.

It is interesting to note that over the past 3 years, other species of *Penicillium* subgenus *Biverticillium* have been isolated from clinical material at the Commonwealth Scientific and Industrial Research Organization (14a, 25). The ability of

a number of species in *Penicillium* subgenus *Biverticillium* and their sexual *Talaromyces* relatives to grow relatively rapidly at 37°C (necessary for fungi pathogenic to humans) may indicate that this group of fungi represents another group of opportunistic human pathogens warranting further attention (25a).

As many researchers have pointed out, an increase in fungal infections along with an increase in immunocompromised patients has occurred (1, 3, 20, 23). Fungal infections can proceed rapidly, and if not detected early enough, can be fatal in short periods of time as has occurred in *P. marneffei* infections (6). Identification of *P. marneffei* from infected human tissues is often difficult because histological staining does not routinely reveal the fungus and results can be misinterpreted as revealing tuberculosis or other fungal pathogens (9). Immunohistochemical identification of *P. marneffei* has been demonstrated; however, the monoclonal antibody (EB-A1) used also detects *Aspergillus* species and thus is not genus specific (9). A PCR-based identification system offers the potential to be a rapid and accurate diagnostic method for the identification of infectious agents from clinical samples (3, 13). PCR identification systems have been successful on DNA extracted from a variety of tissue sources, including embedded tissue, cytology, blood, or bone marrow smears, fixed chromosome spreads on glass slides, and from specimens many decades old (13). The primers that we have developed for the specific amplification of *P. marneffei* could be used to identify this pathogenic agent from clinical tissue samples as has been achieved for the fungal pathogen *P. carinii* (21).

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