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MP1 Homologue-Based Multilocus Sequence System for Typing the Pathogenic Fungus *Penicillium marneffei*: a Novel Approach Using Lineage-Specific Genes[▽]

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A highly reproducible and discriminative typing system is essential for better understanding of the epidemiology of *Penicillium marneffei*, the most important thermal dimorphic fungus causing respiratory, skin, and systemic mycosis in Southeast Asia. The sequences of 11 housekeeping genes were identical among 10 strains of *P. marneffei*, but those of *MP1* and its 13 homologues, a novel superfamily of mannoproteins in the subdivision Pezizomycotina of Ascomycetes, mostly species of *Penicillium* and *Aspergillus*, showed significant variations. Therefore, a multilocus sequence typing (MLST) system for *P. marneffei* was constructed using *MP1* (549 bp) and the four of its homologues (*MPLP4* [337 bp], *MPLP7* [347 bp], *MPLP10* [546 bp], and *MPLP13* [422 bp]) that showed the greatest variations. Among the 2,201 bp of the five loci, 183 polymorphic sites were observed in 44 strains of *P. marneffei*. The median number of alleles at each locus was five (range, 5 [*MPLP4*, *MPLP7*, and *MPLP13*] to 15 [*MPLP10*]). Four of the five genes had nonsynonymous substitution/synonymous substitution (d_n/d_s) ratios of >1. A total of 35 different sequence types (STs) were assigned to the 44 *P. marneffei* isolates, with 28 of the 35 STs identified only once. The discriminatory power was 0.9884. *MP1* and its homologues were better than housekeeping genes for MLST in *P. marneffei*. Due to their more rapid evolutionary rates, lineage-specific genes may be better candidates than housekeeping genes for sequence-based typing, especially in microbes that evolve slowly or have evolved recently.

Penicillium marneffei is the most important thermal dimorphic fungus causing respiratory, skin, and systemic mycosis in Southeast Asia (17, 31, 39, 45). *P. marneffei* was first discovered in Chinese bamboo rats, *Rhizomys sinensis*. Subsequently, it was also recovered from other species of bamboo rats in the Rhizomyinae subfamily, including hoary bamboo rats (*Rhizomys pruinosus*), large bamboo rats (*Rhizomys sumatrensis*), and lesser bay bamboo rats (*Cannomys badius*) (8, 11). After the discovery of *P. marneffei* in 1956, only 18 cases of human disease were reported until 1985 (10). The appearance of the human immunodeficiency virus (HIV) pandemic, especially in Southeast Asian countries, saw the emergence of the infection as an important opportunistic mycosis in HIV-positive patients. About 8% of AIDS patients in Hong Kong are infected with *P. marneffei* (24). In northern Thailand, penicilliosis is the third-most-common indicator disease of AIDS, following tuberculosis and cryptococcosis (31). Besides in HIV-positive patients, *P. marneffei* infections have been reported in other immunocompromised patients, such as transplant recipients, patients with systemic lupus erythematosus, and patients on corticosteroid therapy (23, 37, 40, 43). Despite its medical importance, the ecology, epidemiology, and mode of transmission of *P. marneffei* remain largely unknown.

A highly reproducible and discriminative typing system is essential for a better understanding of the epidemiology of *P. marneffei*. Trewatcharegon et al. have used pulsed-field gel electrophoresis (PFGE) and Fisher et al. have used multilocus microsatellite typing for typing *P. marneffei* (13, 14, 36). However, due to experimental variations, PFGE patterns are difficult to compare among different laboratories. As multilocus sequence typing (MLST) is well known to be highly reproducible and discriminative for bacteria and has also been used for a number of fungal pathogens, we started to develop such a typing system for *P. marneffei* 3 years ago. Recently, Lasker has published a nucleotide sequence-based system for typing *P. marneffei* (21). In this article, we report the difficulty in using housekeeping genes and the subsequent success in using lineage-specific loci—a set of homologous genes belonging to a novel mannoprotein superfamily—for MLST in *P. marneffei*. The potential application of using genes of higher lineage

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TABLE 1. Characteristics of *P. marneffei* strains used in the present study[a]

Strain	Yr of isolation	Origin		Patient		ST	BURST group	Allelic profile
		Country	Type of clinical specimen	Sex/age (yr)	HIV status			
PM1	NA	Hong Kong	Blood	M/2	−	ST-1	Singleton	1,1,1,1,1
PM2	1994	Hong Kong	LN biopsy	M/34	+	ST-2	1	2,2,1,1,1
PM3	1994	Hong Kong	Blood	M/51	+	ST-3	Singleton	3,1,2,2,4
PM4	1993	Philippines	Blood	F/30	−	ST-2	1	2,2,1,1,1
PM5	NA	Hong Kong	BAL fluid	M/23	−	ST-4	2	3,2,2,12,1
PM6	NA	Hong Kong	Blood	M/NA	+	ST-5	3	4,1,2,4,1
PM7	NA	Hong Kong	Blood	M/NA	+	ST-6	2	3,2,1,9,1
PM8	NA	Hong Kong	Blood	M/NA	+	ST-6	2	3,2,1,9,1
PM9	NA	Hong Kong	Bone marrow	F/21	−	ST-7	1	4,1,1,13,1
PM10	NA	Hong Kong	Bone marrow	M/3	−	ST-8	Singleton	10,1,1,14,1
PM11	1998	Hong Kong	Pleural fluid	F/65	−	ST-9	1	3,1,1,2,1
PM12	NA	Hong Kong	Blood	NA	NA	ST-1	Singleton	1,1,1,1,1
PM13	1996	Hong Kong	Blood	F/45	−	ST-10	Singleton	7,2,2,7,1
PM14	1996	Hong Kong	Blood	M/52	+	ST-11	Singleton	7,2,1,4,4
PM15	NA	Hong Kong	Blood	NA	NA	ST-7	1	4,1,1,13,1
PM16	NA	Hong Kong	Blood	NA	NA	ST-7	1	4,1,1,13,1
PM17	1998	Hong Kong	Sputum	M/60	−	ST-1	Singleton	1,1,1,1,1
PM18	1999	Thailand	Blood	F/22	NA	ST-12	Singleton	5,3,3,3,1
PM19	1999	Hong Kong	Blood	M/35	+	ST-13	Singleton	2,2,1,2,2
PM20	1999	Thailand	Blood	F/26	+	ST-14	3	6,2,2,4,1
PM21	1999	Hong Kong	Blood	M/39	+	ST-13	Singleton	2,2,1,2,2
PM22	1999	Hong Kong	Blood	M/73	−	ST-15	1[b]	4,1,1,2,1
PM23	2000	Hong Kong	Blood	M/63	+	ST-16	3	6,2,2,1,1
PM24	2001	Hong Kong	Blood	M/39	+	ST-17	Singleton	2,1,2,8,1
PM25	2002	Hong Kong	Blood	M/47	+	ST-18	Singleton	3,2,1,2,3
PM26	2003	Philippines	Blood	F/35	+	ST-19	1	4,2,1,2,1
PM27	2004	Hong Kong	Blood	M/25	+	ST-20	1	4,2,1,1,1
PM28	2004	Hong Kong	Blood	M/57	−	ST-21	Singleton	3,1,4,9,4
PM29	2005	Hong Kong	Blood	F/45	+	ST-22	Singleton	7,4,1,10,3
PM30	2005	Hong Kong	Blood	M/33	+	ST-14	3	6,2,2,4,1
PM31	NA	Hong Kong	Skin biopsy	F/38	−	ST-23	4	3,2,4,7,1
PM32	2005	Hong Kong	Blood	F/40	+	ST-24	Singleton	4,1,1,4,4
PM33	2005	Vietnam	Blood	M/22	+	ST-25	Singleton	8,2,2,5,1
PM34	2004	Hong Kong	Blood	M/48	+	ST-26	Singleton	9,5,2,11,5
PM35	2005	Hong Kong	Blood	M/41	NA	ST-27	2	3,2,1,6,1
PM36	2005	Hong Kong	Blood	M/48	+	ST-28	3	4,2,2,4,1
PM37	2005	Hong Kong	Blood	F/48	−	ST-29	2[b]	3,2,2,6,1
PM38	2006	Hong Kong	Blood	M/46	+	ST-30	2	3,2,2,10,1
PM39	2006	Hong Kong	Blood	F/52	−	ST-31	Singleton	11,2,1,4,1
PM40	2006	Hong Kong	Blood	M/37	+	ST-15	1[b]	4,1,1,2,1
PM41	2006	Vietnam	Blood	M/30	+	ST-32	Singleton	10,2,2,15,1
PM42	2006	Vietnam	Blood	M/34	+	ST-33	4	3,2,4,1,1
PM43	2006	China	Blood	M/35	+	ST-34	3	4,1,2,1,1
PM44	2006	Philippines	Blood	F/44	+	ST-35	3	6,2,5,4,1

[a] NA, not available; LN, lymph node; BAL, bronchoalveolar lavage; M, male; F, female.
[b] Ancestral type in BURST analysis.

specificity with accelerated evolutionary rates for typing fungal pathogens is also discussed.

MATERIALS AND METHODS

***P. marneffei* strains.** Forty-four nonduplicated strains of *P. marneffei* collected from five hospitals in Hong Kong were used in this study (Table 1). All strains were identified by a combination of cultural and microscopic characteristics. When cultured on Sabouraud dextrose agar at 25°C, the colonies were mycelial in nature, were greenish-yellow in color, and produced a diffusible red pigment. Microscopically, the mycelia bore numerous penicilli, characteristic of other *Penicillium* species. When cultured at 37°C, yeast-like colonies were formed without pigment production. A single colony of each strain grown on Sabouraud dextrose agar at 37°C was inoculated into yeast-peptone broth and incubated in a shaker at 37°C for three days. Ten microliters of each *P. marneffei* culture was used for DNA extraction.

DNA extraction. DNA extraction was performed by using a DNeasy plant mini kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The extracted DNA was eluted in 50 μl of AE buffer (QIAGEN, Hilden, Germany), the resultant mixture was diluted 10 times, and 1 μl of the diluted extract was used for PCR.

PCR amplification and sequencing. In the first part of the study, the extracted DNA of 10 of the 44 strains of *P. marneffei* was used as the templates for the amplification of 11 housekeeping genes and 14 lineage-specific genes. The 11 housekeeping genes were mannose phosphate isomerase (MPI), plasma membrane H+ ATPase (PM-ATPase), pyruvate kinase (PK), glutamate dehydrogenase (GDH), phosphoglucomutase (PGM), ribonucleoside-diphosphate reductase (rNDP reductase), glutamate synthase precursor (GltS precursor), ribonucleotide reductase (RNR), transcription factor PacC, carbon catabolic repressor protein (CCR), and DNA topoisomerase II (TOP2). MPI and PM-ATPase were used in the MLST scheme of *Candida albicans*; PK, GDH, and PGM were used in the multilocus enzyme electrophoresis scheme of *C. albicans*; CCR and TOP2 were known housekeeping genes of *Penicillium verruculosum* and *Talaromyces flavus*, respectively; and rNDP reductase, GltS, RNR, and PacC were genes found in our random exploration of the *P. marneffei* genome project (29, 30, 33, 47). None of these seven genes was the

TABLE 2. Primers for amplification and sequencing of 11 housekeeping genes and *MP1* homologues in *P. marneffei*

Gene product or locus	Primers		Amplicon size (bp)
	Forward	Reverse	
Housekeeping genes			
MPI	LPW1920, 5′-AAYAGCTAYGAYTGGGGTA-3′	LPW1921, 5′-GGYTTGTGGTTGTCATCTG-3′	539
PM-ATPase	LPW1924, 5′-CAGTCCGCCATCACTGGTG-3′	LPW1925, 5′-CTCACGRATRGAGAGCTGGT-3′	516
PK	LPW1949, 5′-ACTCCGTYGAGAARATCAATG-3′	LPW1950, 5′-ATGTTGCCRTTGTTVAGGCA-3′	679
GDH	LPW1951, 5′-CAACTCYGCYCTHGGTCC-3′	LPW1952, 5′-TTGSWGTCGGARAGYGAGAC-3′	660
PGM	LPW1953, 5′-GTCACCAAYAAGATCTACGA-3′	LPW1954, 5′-TAGTCGTARCGMGTGAAGAA-3′	863
rNDP reductase	LPW2018, 5′-ACMTACTCYCTBCTSATTGA-3′	LPW2019, 5′-TTCTCVAASAAGTTSGTCT-3′	620
GltS precursor	LPW2020, 5′-CACGTKATCAACTTCTTCTAC-3′	LPW2021, 5′-ACCRCGGAAGWAGCAGGTA-3′	630
RNR	LPW2076, 5′-GAGACMTACTCYCTBCTSAT-3′	LPW2077, 5′-CACCRGCCTTCTGGTAGT-3′	652
PacC	LPW2083, 5′-GCHGATGAYTCGGTSCT-3′	LPW2084, 5′-TCATTGGDGGDARATGRTA-3′	572
CCR	LPW2154, 5′-TGCWCRAARCGBTTCAGTCG-3′	LPW2155, 5′-GAGTCGTGRGAGAADGTAGG-3′	575
TOP2	LPW2156, 5′-AYGGTTWCGGTGCBAAGC-3′	LPW2157, 5′-ATYTGRTCNGCRATGTAGTT-3′	523
MP1 homologues			
MP1	LPW2562, 5′-CGTTAATCAACATGAAGTTC-3′	LPW4800, 5′-GCTCAATATCAACATTAAACT-3′	657
MPLP4	LPW4801, 5′-GACAAAATTGACCGCAGT-3′	LPW4802, 5′-TCCTTGATATACCGATCAAA-3′	398
MPLP7	LPW4803, 5′-GATTCAGAAATTCCGACG-3′	LPW2736, 5′-GCTCTAAGCTGCACCAGTG-3′	415
MPLP10	LPW4804, 5′-CGTAGGGTTTTTGCGAAT-3′	LPW4805, 5′-TGTCGTCCAACTATCACAAA-3′	613
MPLP13	LPW3510, 5′-AAGCAACCTACAGCACCTTA-3′	LPW3512, 5′-TCGAAATCTATAGAAGCTATC-3′	491

same as the four housekeeping genes chosen for the recently published nucleotide sequence-based system for typing *P. marneffei* (21). The 14 lineage-specific genes include *MP1* and its homologues that belong to a novel mannoprotein superfamily. In the second part of the study, the extracted DNA of all the 44 strains of *P. marneffei* was used as the templates for the amplification of 5 of the 14 gene loci of the *MP1* homologues (*MP1*, *MPLP4*, *MPLP7*, *MPLP10*, and *MPLP13*). The primers used for PCR are listed in Table 2. The primers were designed by multiple alignments of the 14 homologues so that they were specific to each *MP1* homologue.

The PCR mixture (100 μl) contained denatured *P. marneffei* DNA, PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 0.01% gelatin), 200 μM of each deoxynucleoside triphosphate, and 2.5 U *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). The sample was amplified in 40 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min in an automated thermal cycler (Perkin-Elmer Cetus, Gouda, The Netherlands). Ten microliters of each amplified product was electrophoresed in 2% (wt/vol) agarose gel with a molecular size marker (ϕX174 HaeIII digest; Fermentas, Canada) in parallel. Electrophoresis in Tris-borate-EDTA buffer was performed at 100 V for 1 h. The gel was stained with ethidium bromide (0.5 μg/ml) for 15 min, rinsed, and photographed under UV light illumination.

The PCR product was gel purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Both strands of the PCR product were sequenced with an ABI prism 3700 DNA analyzer according to the manufacturer's instructions (Applied Biosystems, Foster City, CA), using the PCR primers.

Allele and ST assignment. The nucleotide sequences of the five gene loci used for MLST in all the *P. marneffei* strains were aligned and compared with those of strain PM1 using ClustalX 1.83 (35). An arbitrary number was assigned to each distinct allele in a locus. Therefore, five numbers, representing the sequence type (ST), were given to each strain. Each ST was numbered in the order of identification (ST-1, ST-2, etc.).

Sequence analysis. The proportions of nucleotide alterations that led to a change in the amino acid sequence (nonsynonymous substitution, d_n) and the proportions of nucleotide alterations that did not lead to a change in the amino acid sequence (synonymous substitution, d_s) were calculated with START2 (http://www.mlst.net). Construction of the dendrogram was performed with the unweighted-pair group method with arithmetic mean using MEGA 3.1, excluding insertions and deletions (20). The grouping of STs into lineages was performed with BURST. The members of a BURST lineage were defined as groups of two or more independent isolates where each had alleles at four or more loci that were identical with at least one other member of the group.

Nucleotide sequence accession numbers. The sequences of the *MP1*, *MPLP4*, *MPLP7*, *MPLP10*, and *MPLP13* genes of 44 *P. marneffei* isolates have been deposited in GenBank (accession no. EU002558 to EU002777).

RESULTS

Sequencing of 11 housekeeping genes in *P. marneffei*. Amplification and sequencing of the 11 housekeeping genes (MPI, PM-ATPase, PK, GDH, PGM, rNDP reductase, GltS precursor, RNR, PacC, CCR, and TOP2) from 10 strains of *P. marneffei* showed that the nucleotide sequences of all 11 genes were identical among the 10 strains of *P. marneffei*.

Sequencing of gene loci of *MP1* homologues in *P. marneffei*. Single bands of the expected sizes were observed for each *MP1* homologue amplified using the specific primers. In the first 10 strains of *P. marneffei* sequenced, the sequences of 4 (*MPLP2*, *MPLP3*, *MPLP6*, and *MPLP12*) of the 14 homologues were identical, but the other 10 (*MP1*, *MPLP1*, *MPLP4*, *MPLP5*, *MPLP7*, *MPLP8*, *MPLP9*, *MPLP10*, *MPLP11*, and *MPLP13*) showed variations. Among these 10 loci, 5 (*MP1*, *MPLP4*, *MPLP7*, *MPLP10*, and *MPLP13*) showed more variations than the others (data not shown). Therefore, only these five loci were sequenced for the other 34 strains of *P. marneffei*, and the sequences of these five loci were used for developing the MLST system.

Variations at the five MLST loci. Among the 2,201 bp of the five loci, a total of 183 polymorphic sites were observed in the 44 strains of *P. marneffei* (Fig. 1). Allelic profiles were assigned to the 44 strains of *P. marneffei* (Table 1). The alleles defined for the MLST system were based on sequence lengths of between 337 bp (*MPLP4*) and 549 bp (*MP1*) (Table 3). The median number of alleles at each locus was 5 (range, 5 [*MPLP4*, *MPLP7*, and *MPLP13*] to 15 [*MPLP10*]).

In the *MP1* gene locus, the insertion of two bases (GT) at positions between 255 and 256 resulted in a premature stop codon (TAA) in the *MP1* gene in genotype 11 (Fig. 1). For the 21-bp tandem repeat (positions 499 to 519 in strain PM1), 1 and 23 of the 44 *P. marneffei* isolates possessed three and two copies of the repeat, respectively, whereas 20 isolates had one copy of the repeat only (Fig. 1). In the *MPLP4* and *MPLP7*

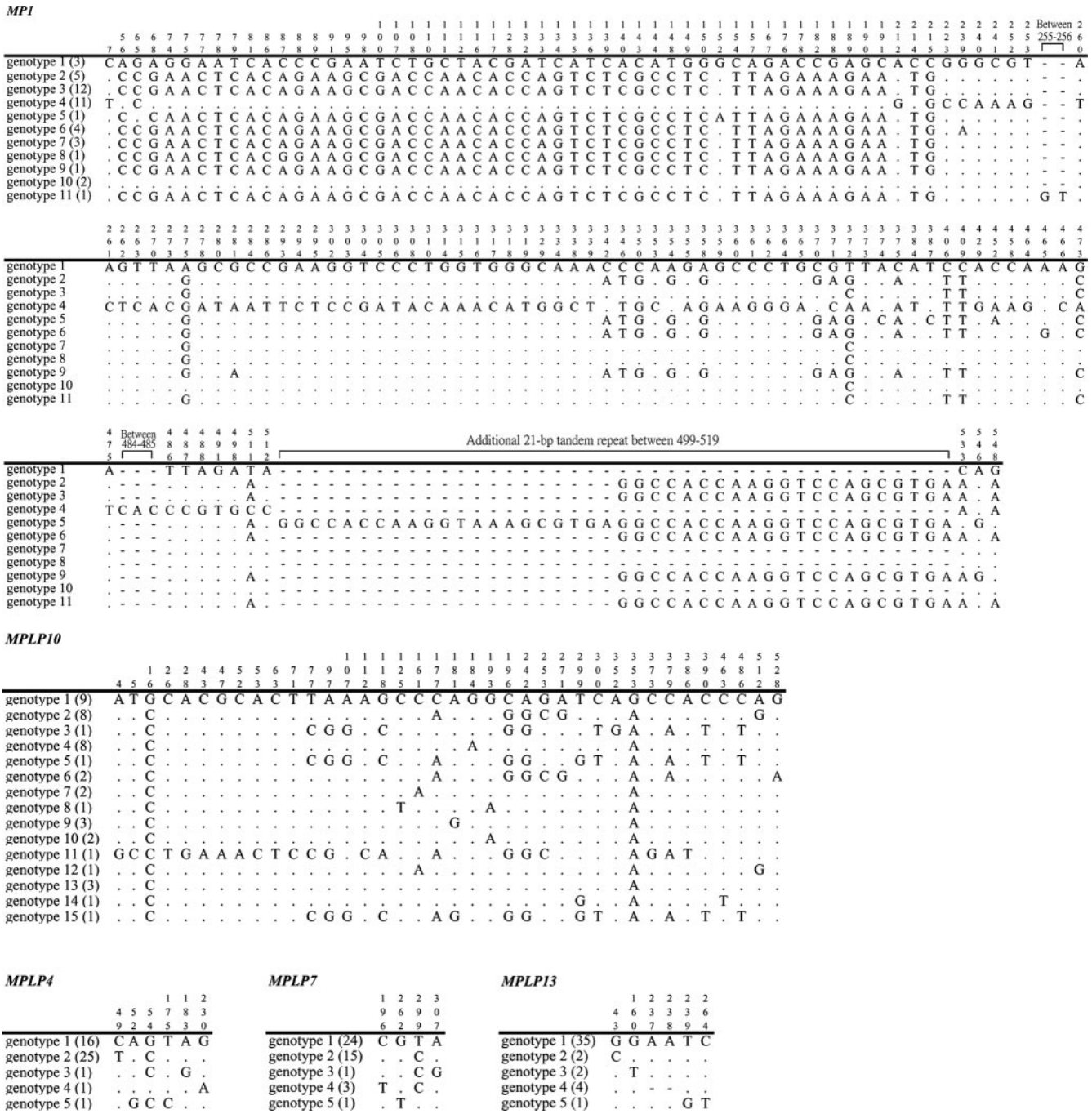

FIG. 1. Polymorphic nucleotide sites in *P. marneffei* MLST genes. Only the variable sites are shown. Sites that are the same as genotype 1 are shown by dots, and gaps are shown by dashes. The numbers of isolates with the same genotype are indicated in parentheses. Numbers shown vertically represent polymorphic nucleotide positions in the GenBank sequences (accession no. for *MP1*, EU002602; for *MPLP4*, EU002646; for *MPLP7*, EU002690; for *MPLP10*, EU002734; and for *MPLP13*, EU002558) of the five genes in strain PM1.

loci, single nucleotide substitutions, at positions 230 (G→A) and 196 (C→T), respectively, resulted in premature stop codons (TAG and TAA, respectively) in genotype 4 (Fig. 1). In the *MPLP13* locus, a single nucleotide substitution at position 160 (G→T) in genotype 3 resulted in a premature stop codon (TAA), whereas deletions at positions 237 and 238 in genotype 4 resulted in frameshift mutation and early truncation of the protein 46 nucleotides downstream from the deletions (Fig. 1). In the *MPLP10* locus, none of the mutations resulted in premature stop codons.

The d_n/d_s ratios for the five gene loci are shown in Table 3. The relatively high d_n/d_s ratios for the five genes (four of the five genes have d_n/d_s ratios of >1) indicate that a strong positive selective pressure is present at these loci.

Relatedness of *P. marneffei* isolates. A total of 35 different STs were assigned to the 44 *P. marneffei* isolates, with 28 of the

TABLE 3. Characteristics of the five loci included in the *P. marneffei* MLST system

Locus	Size of sequenced fragment (bp)	No. of alleles identified	% of polymorphic nucleotide sites	% G+C	d_n/d_s
MP1	549	11	23.9	51.3	0.6482
MPLP4	337	5	1.8	43.3	$d_n = 0.0100$, $d_s = 0$
MPLP7	347	5	1.2	42.7	$d_n = 0.0056$, $d_s = 0$
MPLP10	546	15	7.0	42.4	2.4773
MPLP13	422	5	1.0	44.6	1.3243

35 STs identified only once (Table 1). The most common STs (ST-1 and ST-7) were identified three times in the present data set, followed by ST-2, ST-6, ST-13, ST-14, and ST-15 (two isolates each). The overall discriminatory power for the 44 isolates was 0.9884 and that for the 36 isolates from Chinese patients was 0.9857. The unweighted-pair group method with arithmetic mean was used to construct a dendrogram using the concatenated nucleotide sequences of the five gene loci of the 44 isolates (Fig. 2). BURST grouped the isolates into four lineages, with 6 STs in group 1, 5 STs in group 2, 6 STs in group 3, and 2 STs in group 4, whereas 16 STs did not belong to any of the four groups (Table 1). No relationships were observed among the 44 *P. marneffei* isolates, their years of isolation, or the countries of origin or HIV status of the patients (Table 1 and Fig. 2).

DISCUSSION

Traditionally, typing of pathogenic bacteria and fungi was carried out using various phenotypic and genotypic methods. Before the invention of MLST, PFGE of restriction enzyme-digested genomic DNA was the most widely used method because of its high discriminatory power. However, due to interlaboratory variations, the results obtained using PFGE from different laboratories cannot be easily compared. Therefore, comparison of bacterial and fungal strains from different localities cannot be performed easily, unless all strains are sent to the same laboratory for PFGE. Since the invention of MLST in 1998, this technique has been confirmed to be highly reproducible and objective, and it can be performed easily by different laboratories to type strains collected in different localities (26). In the past 10 years, MLST has been used widely for the typing of pathogenic bacteria by sequencing multiple housekeeping genes (25). For fungi, MLST has also been used for the typing of various yeasts, molds, and dimorphic fungi (1, 2, 3, 12, 16, 18, 19, 21, 22, 27, 30, 32, 33). However, in general, it has been found that MLST is less discriminative for fungi than for bacteria.

The sequences of *MP1* homologues are more variable than those of housekeeping genes in *P. marneffei*. In 1998, we reported the cloning and characterization of *MP1*, which encodes an abundant, secreted, and cell wall immunogenic mannoprotein, Mp1p, from *P. marneffei* (5). Subsequently, we have documented that Mp1p-based enzyme-linked immunosorbent assays for antibody and antigen detection are very useful for serodiagnosis of active *P. marneffei* infections, and an *MP1*-based DNA vaccine produced a protective immune response against *P. marneffei* challenge in a mouse model (6, 7, 38). Recently, we discovered that genes with higher lineage specificity in Ascomycetes evolved at a much faster rate than those with lower lineage specificity (4). Therefore, genes with higher lineage specificity are potentially more useful targets than housekeeping genes for typing pathogenic fungi. *MP1* homologues have so far only been found in the subdivision Pezizomycotina of Ascomycetes, mostly in species of *Penicillium* and *Aspergillus* (5, 9, 41, 46). Furthermore, Mp1p is a cell wall immunogenic protein located on the surface of *P. marneffei* and, hence, is subject to strong selective pressure by the immune system (5). Therefore, *MP1* homologues are potentially more rapidly evolving than housekeeping genes. Recently, from the data acquired in our complete genome sequence project (42, 44), we discovered that there are more than 10 *MP1* homologues in the *P. marneffei* genome (unpublished data). The characterization of these loci will be reported elsewhere. Therefore, we hypothesized that it would be more discriminatory if this set of gene targets were used for MLST in *P. marneffei*. To test this hypothesis, we sequenced 11 housekeeping genes, some of which have been used for targets of MLST in other fungal pathogens, and the *MP1* homologues of *P. marneffei*. The results showed that the sequences of the 11 housekeeping genes were identical among the 10 strains of *P. marneffei* sequenced but that remarkable variations exist in the sequences of the *MP1* homologues. Therefore, the *MP1* homologues were used as targets for building the MLST system in *P. marneffei*.

Using two times the number of human isolates, we found that the present MLST system appears to be more discriminative than another MLST system recently published for molecular typing of *P. marneffei* (21). In the recently published article on nucleotide sequence-based analysis of *P. marneffei* isolates, nine gene loci were sequenced (21). Among these nine loci, five showed sequence variations. When these five loci (2,089 bp) were used for building the MLST system, four showed minimal variations. These four loci were housekeeping genes. The only locus that showed significant variation was *MP1*, which is in line with the results observed in the present study, that only the *MP1* homologues, but not the 11 housekeeping genes, showed significant variation. In the present study, the region of the *MP1* gene used was from nucleotide 57 to 626, whereas in the other study (21), the region used was from nucleotide 508 to 957, using strain PM4 as the reference. This is probably the explanation of the higher variation in the *MP1* gene in the present study. The high sequence variation in 5 of the 14 *MP1* homologues is probably because they are evolving rapidly, as shown by their relatively high *dn*/*ds* ratios (Table 3). These resulted in the relatively high discriminatory power in the present MLST system (0.988) compared to that in the other one (0.939) (21). In fact, with *MP1* eliminated from the two MLST systems, the discriminatory power of the present

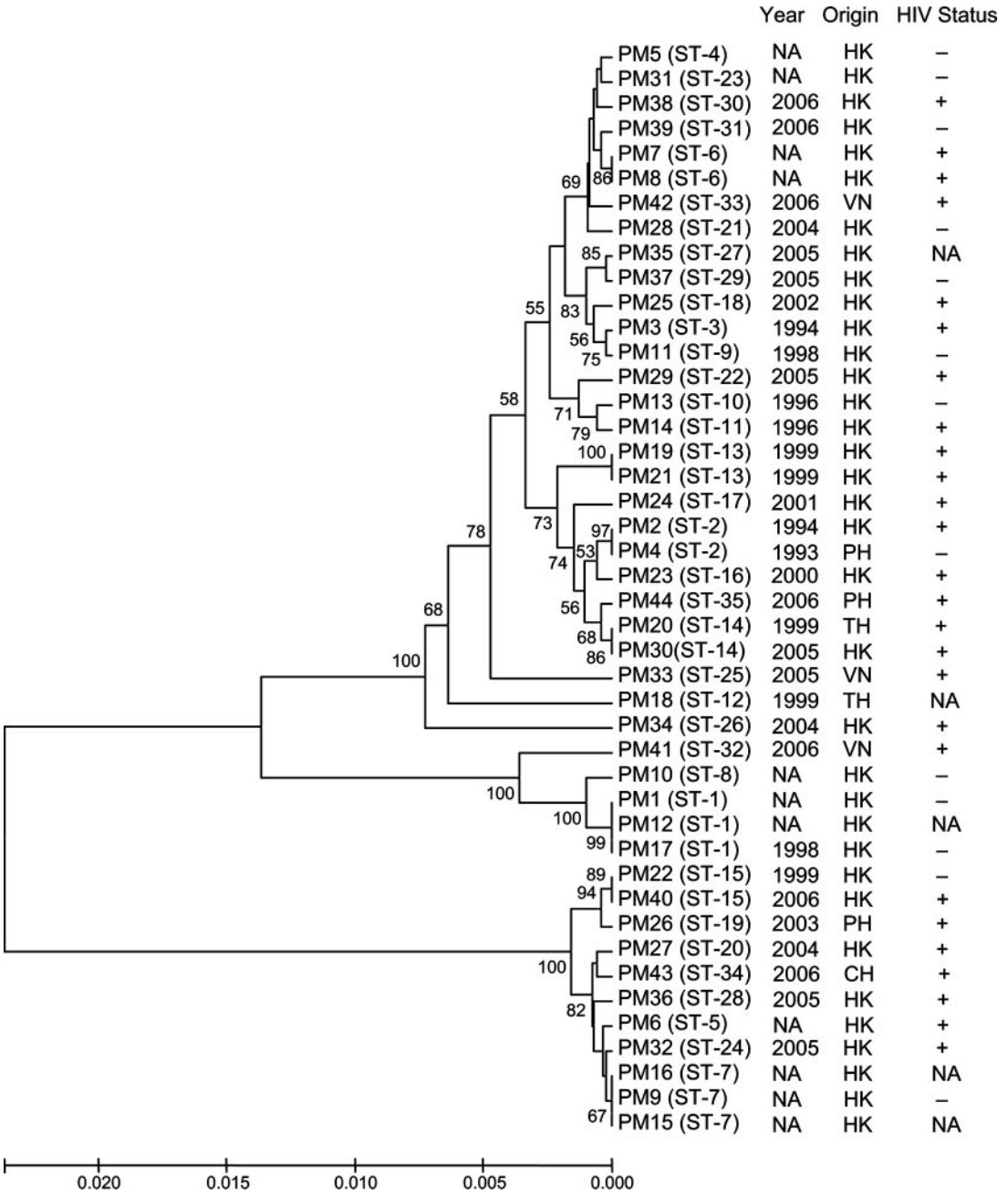

FIG. 2. Phylogenetic tree showing the relationships of the 35 STs of *P. marneffei* in this study. The tree was inferred from concatenated nucleotide sequence data of the five MLST gene loci by the Kimura two-parameter method, and bootstrap values calculated from 1,000 trees. The analysis included 2,201 nucleotide positions in each concatenated nucleotide sequence. Only bootstrap values of >40 are shown. The scale bar shows the genetic distance estimated using the Kimura two-parameter substitution model. NA, not available; HK, Hong Kong; VN, Vietnam; PH, Philippines; TH, Thailand; CH, mainland China.

MLST system is still 0.982, whereas that of the other MLST system dropped to 0.747, suggesting that the incorporation of *MP1* or its homologues is important in setting up an MLST system with good discriminatory power for *P. marneffei*. When the same *P. marneffei* strain was subcultured 10 times, no difference was observed between the sequences of the five gene loci in the original strain and in the strain after 10 subcultures (data not shown). Therefore, these five loci are discriminative enough for typing but not evolving so rapidly as to mask genetic relatedness. Interestingly, a discriminatory power of only 0.8 was achieved by the MLST system of the other study when only isolates from Thailand were included (21). When only strains of Chinese patients were analyzed, the present study is only slightly more discriminatory, by 0.0221 (0.9857 versus 0.9636), than the other study. The apparent difference in the overall discriminatory powers of the MLST systems in the present study and the previous one could be due to a genuine difference in their discriminatory powers or to the difference in

the collections of strains. Further studies using more isolates from other countries are required to determine whether the discriminatory power of the present MLST system is universally high for *P. marneffei* isolates of different origins.

Lineage-specific genes may be better candidates than housekeeping genes for sequence-based typing, especially in microbes that evolve slowly or have evolved recently. When MLST was first designed as a sequence-based typing method for bacteria, housekeeping genes were used as the targets for amplification and sequencing (26). Subsequently, most researchers who set up MLST systems for other bacteria followed this tradition. However, when MLST systems were used in fungal pathogens, it was observed that the discriminatory power was often not satisfactory when only housekeeping genes were used (1, 27, 34). On the other hand, when one or two nonhousekeeping genes were also included as targets, the discriminatory power was improved (19, 21). In fact, the same phenomenon was also observed in bacterial pathogens, and it has been shown that the same discriminatory power can be achieved by using fewer targets, hence decreasing the labor and consumable costs considerably, if nonhousekeeping genes are used (15, 28). This is in line with our recent observation that lineage-specific genes were associated with more rapid evolutionary rates (4). Hence, more mutations would be expected in these genes than in housekeeping genes, which are not lineage specific, between different strains. In *P. marneffei*, the small amount of variation in its housekeeping genes could be because of its slow evolutionary rate, because it has evolved as a new species recently, or both. Therefore, lineage-specific genes may be better targets for sequence-based typing systems, such as MLST, for slowly evolving or recently evolved pathogens.

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