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Genomic and experimental evidence for a potential sexual cycle in the pathogenic thermal dimorphic fungus *Penicillium marneffei*

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Abstract **All meiotic genes (except *HOP1*) and genes encoding putative pheromone processing enzymes, pheromone receptors and pheromone response pathways proteins in *Aspergillus fumigatus* and *Aspergillus nidulans* and a putative *MAT-1* α box mating-type gene were present in the *Penicillium marneffei* genome. A putative *MAT-2* high-mobility group mating-type gene was amplified from a *MAT-1* α box mating-type gene-negative *P. marneffei* strain. Among 37 *P. marneffei* patient strains, *MAT-1* α box and *MAT-2* high-mobility group mating-type genes were present in 23 and 14 isolates, respectively. We speculate that *P. marneffei* can potentially be a heterothallic fungus that does not switch mating type.**

Keywords: Genome; Dimorphic fungus; Sexual cycle; Mating type genes; *Penicillium marneffei*

1. Introduction

Penicillium marneffei is the most important thermal dimorphic fungus causing respiratory, skin and systemic mycosis in Southeast Asia [1–5]. After its discovery in 1956, only 18 cases of human diseases were reported until 1985 [6]. The appearance of the 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* [7]. In northern Thailand, penicilliosis is the third most common indicator disease of AIDS following tuberculosis and cryptococcosis [2]. Clinically, penicilliosis manifests as a systemic febrile illness, which results from intracellular infection of the reticuloendothelial cells by the yeast phase of the fungus and the associated inflammatory response of the host. Besides 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 [8–10].

The epidemiology of *P. marneffei* infection is not fully understood. *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*) [11,12]. Although no reports have described the existence of a free living stage or a sexual cycle in *P. marneffei*, the possibility of the presence of a sexual cycle in *P. marneffei* is supported by several lines of evidence. First, since the use of molecular techniques in fungal taxonomy, very similar, or even identical, ribosomal RNA gene sequences were present in asexual and sexual forms of fungi that have never been thought to be related. For *Penicillium* species, their ribosomal RNA gene sequences were found to be clustered with the *Talaromyces* species, and the *Penicillium* species are now considered to be the asexual forms of the *Talaromyces* species [13]. Second, after the availability of complete genome sequence from *Aspergillus fumigatus*, a mold closely related to *Penicillium* [14], ample genomic and other experimental evidence were observed that supported the presence of a sexual form in this opportunistic fungus [15,16]. Third, it has been observed that genes related to the sexual cycle may be present in *P. marneffei*. In our previous study on a set of random sample sequences of *P. marneffei*, it was observed that sequences homologous to pheromone receptors were present [17]. In another study, it was also observed that a gene of the *STE12* family, which can complement the sexual defect of an *Aspergillus nidulans steA* mutant, is present in *P. marneffei* [18].

In 2002, the complete genome sequencing project of *P. marneffei* was started. Recently, we have published the complete mitochondrial genome sequence of *P. marneffei* [14]. At the moment, a 6× coverage of the genome has been completed. Due to the possibility of a sexual cycle in *P. marneffei* and its major clinical and biological significance, we explored genomic and other evidence of the potential and occurrence of a sexual cycle in *P. marneffei*. The implications on reservoir and transmission are also discussed.

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2. Materials and methods

2.1. Strain and DNA extraction for genome sequencing

Penicillium marneffei strain PM1 was isolated from an HIV-negative patient suffering from culture-documented penicilliosis in Hong Kong. The arthroconidia ("yeast form") of PM1 was used throughout the DNA sequencing experiments. Genomic DNA was prepared from the arthroconidia grown at 37 °C. A single colony of the fungus grown on Sabouraud dextrose agar at 37 °C was inoculated into yeast peptone broth and incubated in a shaker at 30 °C for 3 days. Cells were cooled in ice for 10 min, harvested by centrifugation at $2000 \times g$ for 10 min, washed twice and resuspended in ice cold 50 mM EDTA buffer (pH 7.5). 20 mg novazym/ml was added and incubated at 37 °C for 1 h followed by digestion in a mixture of 1 mg proteinase K/ml, 1% *N*-lauroylsarcosine, and 0.5 M EDTA, pH 9.5, at 50 °C for 2 h. Genomic DNA was then extracted by phenol, phenol–chloroform, and finally precipitated and washed in ethanol. After digestion with RNase A, a second ethanol precipitation was followed by washing with 70% ethanol, air-dried and dissolved in 500 μl of TE (pH 8.0).

2.2. Library construction and sequence assembly

A genomic DNA library was made in pUC18 carrying inserts with sizes from 3.0 to 5.0 kb. DNA inserts were prepared by physical shearing using the sonication method. One end of 315 580 clones, representing a 6× coverage of the *P. marneffei* genome, were sequenced. The Phred/Phrap/Consed software package was used for base calling and sequence assembly [19–21].

2.3. Identification of genes in the putative sexual cycle of P. marneffei

Preliminary sequence data from the *P. marneffei* genome were used to identify homologues of genes specific for sexual reproduction. *Saccharomyces cerevisiae*, *A. fumigatus* and *A. nidulans* protein sequences were obtained from NCBI and used as queries for TBLASTN searches against the *P. marneffei* genome sequence. The BLAST results were sorted according to their scores and the E value cutoff for assigning homologues was 1e − 6. This E value cutoff was relaxed in exceptional cases where presence of numerous introns interfered with the translated BLAST search. Reciprocal BLAST against the *S. cerevisiae* protein database was performed for each identified *P. marneffei* homologue using the BLASTP program. The identified homologues were manually re-annotated to minimize the occurrences of false positives, such as cases of orthologues.

2.4. Strains and DNA extraction for PCR amplification

Thirty seven strains of *P. marneffei*, isolated from blood cultures of 37 patients in Hong Kong, were used. The strains were cultured as described above. DNA extraction was performed with 10 ml of *P. marneffei* culture using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The extracted DNA was eluted in 50 μl of AE buffer and the resultant mixture was diluted 10× and 1 μl of the diluted extract was used for PCR.

2.5. PCR amplification and sequencing of MAT1-2-1 high-mobility group mating-type gene in P. marneffei

Extracted DNA of *P. marneffei* strain PM27, a *P. marneffei* strain without the *MAT1-1-1* α box mating-type gene, was used as the template for amplification and sequencing of the *MAT1-2-1* high-mobility group mating-type gene, with primers LPW3358 5′-CATCGTGTAAATTACGCCTA-3′ and LPW3362 5′-GCTTGTCATGGACTTGTCG-3′ (Invitrogen, CA, USA) designed from the sequence information flanking the *MAT1-1-1* α box mating-type gene in the *P. marneffei* genome. 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 triphosphates and 2.5 U *Taq* polymerase (Perkin–Elmer Cetus, Norwalk, USA). The sample was amplified in 40 cycles of 95 °C for 1 min, 60 °C for 1.5 min and 72 °C for 5 min, and with a final extension at 72 °C for 10 min in an automated thermal cycler (Perkin–Elmer Cetus, Gouda, The Netherlands). Both strands of the PCR products were sequenced twice with an ABI 377 automated sequencer according to manufacturers' instructions (Perkin–Elmer, Foster City, CA, USA), using the PCR primers (LPW3358 and LPW3362) and additional sequencing primers (LPW3438 5′-CTCATTCACTTAGACTCACGGA-3′, LPW3439 5′-CATCCTGATCTTGTCCGCTG-3′, LPW3456 5′-CCACACGAGCAACAATCTAA-3′ and LPW3457 5′-GCCTCCTCTCCAACGATCTA-3′) designed from the sequencing results.

2.6. Detection of MAT1-1-1 α box and MAT1-2-1 high-mobility group mating-type genes in P. marneffei strains

Extracted DNA of the 37 strains of *P. marneffei* was used as the templates for amplification of the *MAT1-1-1* α box and *MAT1-2-1* high-mobility group mating-type genes, using primers specific for the *MAT1-1-1* α box mating type gene (LPW3463 5′-CACTGAAGAGCAGGTTCCAA-3′ and LPW3464 5′-CTCAGGTTGAATTAGTCCGAT-3′) and *MAT1-2-1* high-mobility group mating-type gene (LPW3461 5′-CTTATCTGGAACAAAGCCGT-3′ and LPW3462 5′-TGCCCTGAAGTGAGACTTG-3′). 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 triphosphates and 2.5 U *Taq* polymerase (Perkin–Elmer Cetus, Norwalk, USA). The sample was amplified in 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and 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% (w/v) agarose gel, with a molecular size marker (ΦX174 *Hae*III 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 ultraviolet light illumination.

2.7. Phylogenetic analysis

The phylogenetic relationships of the predicted proteins in the putative sexual cycle of *P. marneffei* and the corresponding homologues in other microbes were determined using neighbor-joining method with Clustal X [22].

2.8. Nucleotide sequence accession number

The nucleotide sequences of the *MAT1-1* and *MAT1-2* idiomorphs have been deposited with GenBank under Accession No. DQ340761 and DQ340762, respectively.

3. Results

3.1. Meiotic genes in P. marneffei genome

Based on the meiotic genes of *S. cerevisiae*, the homologues in the *P. marneffei* genome were predicted (Table 1). All genes present in the *A. fumigatus* and *A. nidulans* genomes, except *HOP1*, were also present in the 6× *P. marneffei* genome. Interestingly, two genes, *MSH4* and *MSH5* (gene products forming heterodimer (Msh4p/Msh5p) which interacts with the Mlh1p/Mlh3p heterodimer), present in the *P. marneffei* and *A. fumigatus* genomes, were not found in the completed *A. nidulans* genome.

3.2. MAT1-1-1 α box mating-type gene in P. marneffei genome

TBLASTN searches using the amino acid sequence of *A. fumigatus* isolate AF217 α box mating-type gene (GenBank Accession No. AY898660) [15] as query revealed the presence of a putative *MAT1-1-1* (abbreviated to *MAT-1* for convenience [23]) α box mating-type gene, with one putative conserved intron (62 bp) with typical consensus sites for splicing, in the *P. marneffei* genome (Fig. 1). Protein sequence analysis of the predicted protein, of 348 amino acid residues, revealed the presence of α box, similar to those of the *MAT-1* α box mating-type proteins of other fungi (Fig. 2A). Phylogenetically, the *MAT-1* α box mating-type protein of *P. marneffei* is most closely related to those of *A. fumigatus* and *A. nidulans* (Fig. 2B).

Table 1
Putative meiotic genes in *P. marneffei* genome[a]

Genes	*P. marneffei*			Putative homologue in other fungi				
	Putative homologue	E values	Conserved domain matched	*A. nidulans*	*A. fumigatus*	*N. crassa*[b]	*S. pombe*[b]	*E. cuniculi*[b]
SPO11	**Y**	**6e − 04**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**
MRE11	Y	e − 130	Y	Y	Y	Y	Y	Y
RAD50	Y	0.0	Y	Y	Y	Y	Y	Y
HOP1	**N**	–	**N**	**Y**	**Y**	**N**[c]	**Y**[d]	**N**
HOP2	**N**	–	**N**	**N**	**N**	**N**	**Y**	**Y**
MND1	**Y**	**1e − 12**	**Y**	**Y**	**Y**	**N**	**Y**	**Y**
RAD52	Y	2e − 36	Y	Y	Y	Y	Y	Y
DMC1	**Y**	**8e − 86**	**Y**	**Y**	**Y**	**N**	**Y**	**N**
RAD51	Y	e − 125	Y	Y	Y	Y	Y	Y
MSH4	**Y**	**3e − 62**	**Y**	**N**	**Y**	**Y**	**N**	**N**
MSH5	**Y**	**7e − 33**	**Y**	**N**	**Y**	**Y**	**N**	**N**
MSH2	Y	0.0	Y	Y	Y	Y	Y	Y
MSH6	Y	e − 149	Y	Y	Y	Y	Y	Y
MLH1	Y	e − 154	Y	Y	Y	Y	Y	Y
MLH2	Y	9e − 14	Y	Y	Y	Y	N	N
MLH3	Y	5e − 28	Y	Y	Y	Y	N	N
PMS1	Y	e − 135	Y	Y	Y	Y	Y	Y

Y, present; N, absent.
[a]Meiosis specific genes are in bold.
[b]Data adopted from Ref. [28].
[c]*HOP1* was claimed to be absent in the *N. crassa* genome in Ref. [1] but present in Ref. [28]; our analysis showed that it is absent.
[d]*HOP1* was claimed to be absent in the *S. pombe* genome in Ref. [24] but present in Ref. [28], which was confirmed by in situ immunostaining.

3.3. PCR amplification and sequencing of *MAT1-2-1* high-mobility group mating-type gene in P. marneffei

Using primers designed from the sequence information flanking the *MAT-1* α box mating-type gene in the *P. marneffei* genome, a 3600-bp fragment was amplified from a *P. marneffei* strain without the *MAT-1* α box mating-type gene and was sequenced. Sequence analysis revealed the presence of a putative *MAT1-2-1* (abbreviated to *MAT-2* for convenience [23]) high-

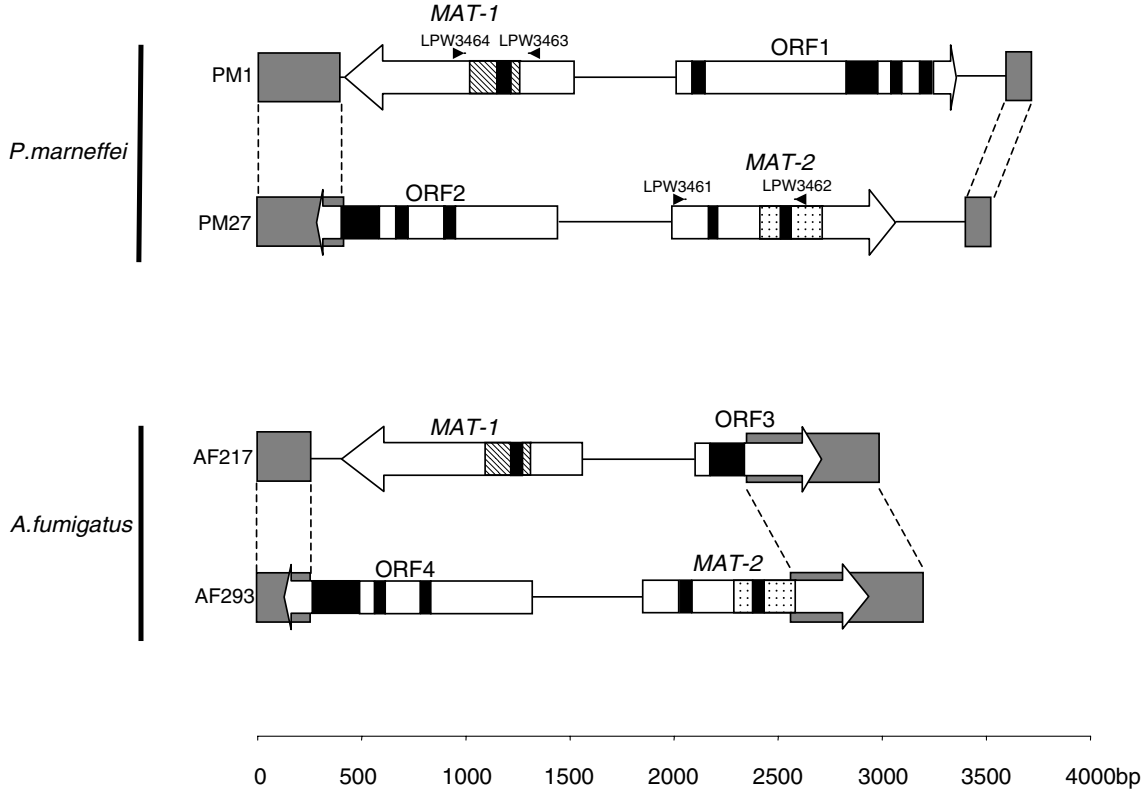

Fig. 1. Schematic representation of the *MAT-1* (strain PM1) and *MAT-2* idiomorphs (strain PM27) in *P. marneffei*. Almost identical flanking regions of the two loci were depicted in gray, predicted introns are in black, α box in strips and HMG domain in dots. The two pairs of PCR primers for amplification of the *MAT-1* (LPW3463 and LPW3464) and *MAT-2* (LPW3461 and LPW3462) genes were also shown. The corresponding *MAT-1* and *MAT-2* idiomorphs in *A. fumigatus* are shown for comparison. ORF1, ORF2, ORF3 and ORF4 are four predicted open reading frames of unknown functions.

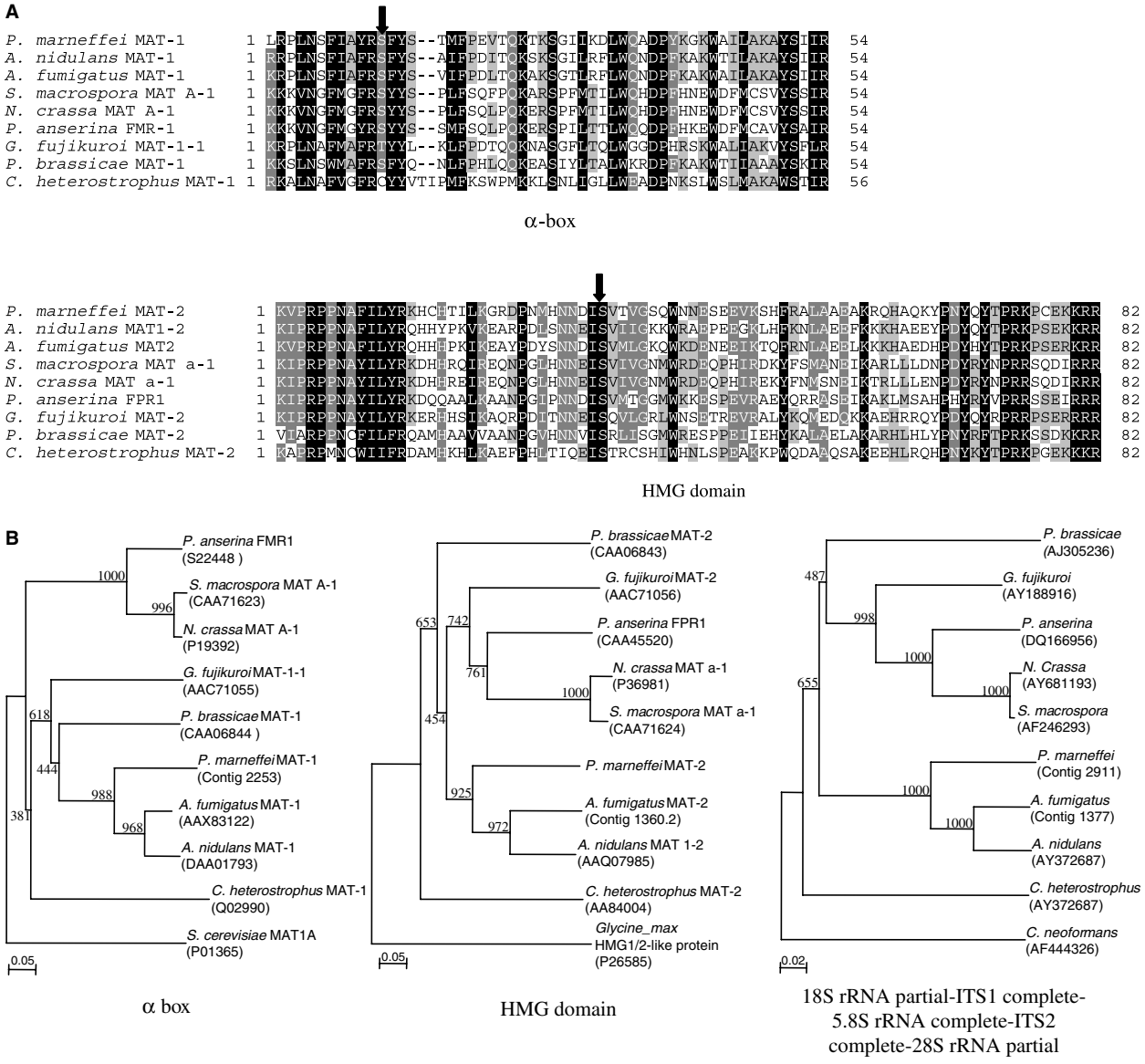

Fig. 2. (A) Multiple alignment of α box of the *MAT-1* gene and HMG domain of the *MAT-2* gene of *P. marneffei* and those of other ascomycetes. The arrows depict the positions of the conserved introns in the α box and HMG domain. (B) Phylogenetic analysis of α box, HMG domain and 18S rRNA-ITS1-5.8S rRNA-ITS2-28S rRNA region of *P. marneffei*. The trees were constructed by neighbor joining method using Jukes–Cantor correction and bootstrap values calculated from 1000 trees. Fifty-six and eighty-two amino acid positions in α box and HMG domain, respectively, and 623 nucleotide positions in 18S rRNA-ITS1-5.8S rRNA-ITS2-28S rRNA region were included in the analysis. The scale bars indicate the estimated number of substitutions per 20 amino acids and 50 nucleotides. Names and accession numbers are given as cited in the GenBank database.

mobility group mating-type gene with two putative conserved introns with typical consensus sites for splicing (Fig. 1). The first putative intron (52 bp) is upstream of the HMG domain and the second (46 bp) is between two codons encoding two conserved amino acids (isoleucine and serine). As in *A. fumigatus*, two flanking regions, almost identical between the *MAT-1* and *MAT-2* idiomorphs, were present upstream and downstream of the *MAT-1* α box and *MAT-2* high-mobility group mating-type genes in *P. marneffei* (Fig. 1). ORF2 and ORF4, but not ORF1 and ORF3, are homologous. The C-terminal 121 amino acids of ORF3 are homologous to the corresponding region in *MAT-2* of *A. fumigatus*, but the same phenomenon is not observed between ORF1 and *MAT-2* of *P. marneffei*. These almost identical flanking regions were absent around the *MAT-1* α box and *MAT-2* high-mobility group mating-type genes in *A. nidulans*.

Protein sequence analysis of the predicted protein, of 328 amino acid residues, revealed the presence of HMG domain, similar to those of the *MAT-2* high-mobility group mating-type proteins of other fungi (Fig. 2A). Phylogenetically, the *MAT-2* high-mobility group mating-type protein of *P. marneffei* is most closely related to those of *A. fumigatus* and *A. nidulans* (Fig. 2B).

3.4. Detection of *MAT-1* α box and *MAT-2* high-mobility group mating-type genes in P. marneffei strains

Among the 37 *P. marneffei* strains isolated from blood cultures of patients, 387-bp *MAT-1* α box mating-type gene spe-

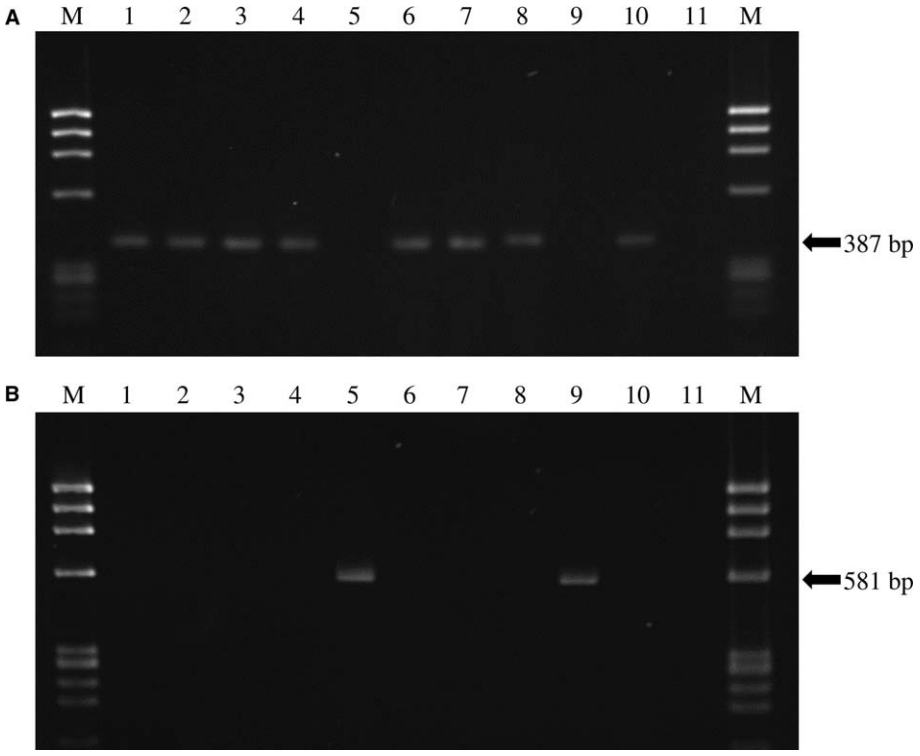

Fig. 3. DNA products from PCR of the *MAT-1* (A) and *MAT-2* (B) gene in 10 *P. marneffei* isolates. Lane M, molecular marker ΦX-174 DNA Hae*III* digest; lanes 1–10, *P. marneffei* strains PM1–10; lane 11, negative control containing distilled water.

cific bands were present in 23 isolates, whereas 581-bp *MAT-2* high-mobility group mating-type gene specific bands were present in the other 14 isolates (Fig. 3).

3.5. Genes encoding pheromone precursors and pheromone processing enzymes in P. marneffei genome

Identification of an α-factor-like pheromone precursor in *P. marneffei* genome was attempted with TBLASTN searches using amino acid sequences of known fungal α-factor-like pheromone precursors as queries. No significant hits were found. Identification of an a-factor-like pheromone precursor was attempted with a different strategy. All possible open reading frames with sizes within the range of 45–135 bp were extracted from the (complete and incomplete) genomes of *P. marneffei*, *A. fumigatus*, *A. nidulans*, *Aspergillus flavus*, *Aspergillus terrus*, *Aspergillus clavatus* and *Neosartorya fischeri*. These were translated to amino acid sequences and then selected for the presence of a CAAX motif at the carboxy-terminus. Sequences that were part of a larger predicted protein were excluded. Finally, BLASTCLUST was used for clustering the remaining sequences. The procedure did not produce any cluster of sequences from 4 or more species, whilst the clusters with sequences from 2 or 3 species did not show any significant homology with sequences of known a-factor-like pheromone precursors.

All genes that encode putative pheromone processing enzymes in *S. cerevisiae*, except *STE13*, were all present in the *P. marneffei*, *A. nidulans* and *A. fumigatus* genomes (Table 2). Although conserved domains showed that *STE13* were "present" in the genomes of *P. marneffei*, *A. nidulans* and *A. fumigatus*, reciprocal BLAST to *S. cerevisiae* genome and manual annotation revealed that the gene with the dipeptidyl pep-

Table 2
Putative pheromone processing genes in *P. marneffei* genome

Genes	*P. marneffei*			Putative homologue in *Aspergillus* species	
	Putative homologue	E values	Conserved domain matched	*A. nidulans*	*A. fumigatus*
STE23	Y	e − 176	Y	Y	Y
STE24	Y	7e − 78	Y	Y	Y
RCE1	Y	2e − 15	Y	Y	Y
RAM1	Y	5e − 18	Y	Y	Y
RAM2	Y	3e − 38	Y	Y	Y
STE14	Y	1e − 42	Y	Y	Y
STE6	Y	e − 120	Y	Y	Y
KEX1	Y	1e − 40	Y	Y	Y
KEX2	Y	e − 156	Y	Y	Y
STE13	N[a]	e − 121	Y[a]	N[a]	N[a]

[a]Although conserved domains showed that *STE13* were "present" in *P. marneffei*, *A. nidulans* and *A. fumigatus*, reciprocal BLAST to *S. cerevisiae* genome and manual annotation revealed that the gene with the dipeptidyl peptidase IV (DPP IV) N-terminal region was in fact dipeptidyl aminopeptidase.

tidase IV (DPP IV) N-terminal region was in fact dipeptidyl aminopeptidase, but not *STE13*.

3.6. Genes encoding pheromone receptors and pheromone response pathways in P. marneffei genome

Except *FAR1*, *STE5*, *KSS1*, *DIG1* and *DIG2*, the other genes that encode proteins of the pheromone response pathway in *S. cerevisiae* were present in the *P. marneffei*, *A. nidulans* and *A. fumigatus* genomes (Table 3). *FAR1* is a scaffold protein used for anchoring *CDC24* and *STE5* is a scaffold protein for anchoring *STE11*, *STE7* and *FUS3*. Since they were apparently absent in the *P. marneffei*, *A. nidulans* and *A. fumigatus* genomes, these fungi may be using alternative proteins for the anchoring functions. In the putative *STE12* of *P. marneffei*, *A. nidulans* and *A. fumigatus*, no *DIG1* and *DIG2* regulatory regions, that were present in *STE12* of *S. cerevisiae*, were present. On the other hand, an additional zinc-finger motif was present in the putative *STE12* of *P. marneffei*, *A. nidulans* and *A. fumigatus*. As *KSS1* (MAP kinase that controls filamentous growth in *S. cerevisiae*) was also absent in the *P. marneffei*, *A. nidulans* and *A. fumigatus* genomes, this implied that *FUS3* of these fungi may be controlling the function of *STE12* through an alternative pathway. *STE18* was not picked up by using the protein of *S. cerevisiae* to BLAST against the *P. marneffei*, *A. nidulans* and *A. fumigatus* genome database, but by using the protein of *S. cerevisiae* to BLAST against the *P. marneffei*, *A. nidulans* and *A. fumigatus* predicted protein databases.

4. Discussion

Traditionally, clinically important fungi are classified morphologically into three major phyla, Ascomycota, Basidiomycota and Zygomycota, with known sexual stages, and a fourth group deuteromycetes, also named "asexual fungi". However, after the use of molecular techniques in fungal taxonomy, many previously "unrelated" fungal species were found to be the sexual and asexual stages of the same fungus, and many fungi that belonged to deuteromycetes were reclassified into the other major phyla because of the discovery of their sexual stages [24]. For example, using the results of ribosomal RNA gene sequencing, *Penicillium emersonii* is now considered as the anamorph of *Talaromyces emersonii*, a thermophilic fungus usually isolated from soil [25]. After genome sequence data of an increasing number of fungal species became available, genomic evidence for sexual cycles were also observed in both yeasts and molds, such as *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *A. fumigatus* [16,26–28]. In this study, we provide genomic and other molecular evidence for a potential but probably rarely occurring sexual cycle in *P. marneffei*.

Based on our results, we speculate that *P. marneffei* can potentially be a heterothallic fungus that does not switch mating type. In this study, we showed that *P. marneffei* possessed all the crucial genes for meiosis, pheromone processing enzymes, pheromone receptors and pheromone response pathways. Although the genes that encode pheromone precursors were not observed, their apparent absence may be because they are still not covered by the 6× genomic data, or because they were missed by the homology search, as a result of the short length of the genes. In fact, one of the pheromone precursor genes is still "missing" in the completely sequenced genome of *A. fumigatus*, which was recently found to be a probably sexual fungus [15]. As for the mating-type genes, about two thirds of the strains possessed *MAT-1* α box mating-type genes and the other one third possessed *MAT-2* high-mobility group mating-type genes. Our results did not support any silent mating-type cassettes. Although *P. marneffei* isolates with *MAT-1* α box mating-type genes were about twice more abundantly found in our patients' blood culture isolates than those with *MAT-2* high-mobility group mating-type genes, further experiments are necessary to elucidate whether *P. marneffei* with *MAT-1* α box mating-type genes are more virulent than those with *MAT-2* high-mobility group mating-type genes, or alternatively, whether this differential abundance of mating-types is an inherent biological property of *P. marneffei*, as in the case of *Cryptococcus neoformans* [29].

Table 3
Putative genes of pheromone response pathway in *P. marneffei* genome

Genes	*P. marneffei*			Putative homologue in *Aspergillus* species	
	Putative homologue	E values	Conserved domain matched	*A. nidulans*	*A. fumigatus*
STE2	Y	8e − 17	Y	Y	Y
STE3	Y	7e − 15	Y	Y	Y
GPA2	Y	2e − 61	Y	Y	Y
STE18	Y[a]	–	Y[a]	Y[a]	Y[a]
STE4	Y	2e − 60	Y	Y	Y
FAR1	N	–	N	N	N
CDC24	Y	5e − 16	Y	Y	Y
CDC42	Y	6e − 69	Y	Y	Y
BEM1	Y	4e − 59	Y	Y	Y
STE20	Y	e − 127	Y	Y	Y
STE5	N	–	N	N	N
STE11	Y	1e − 93	Y	Y	Y
STE50	Y	4e − 17	Y	Y	Y
STE7	Y	7e − 56	Y	Y	Y
FUS3	Y	e − 115	Y	Y	Y
KSS1	N	–	N	N	N
DIG1	N	–	N	N	N
DIG2	N	–	N	N	N
STE12	Y	6e − 53	Y	Y	Y

[a] *STE18* was not picked up by using the protein of *S. cerevisiae* to BLAST against the *P. marneffei*, *A. nidulans* and *A. fumigatus* genome database, but by using the protein of *S. cerevisiae* to BLAST against the *P. marneffei*, *A. nidulans* and *A. fumigatus* predicted protein databases.

The genes in the putative sexual cycle of *P. marneffei* are most closely related to the homologues in *Aspergillus* species. The first molecular evidence that showed a close relationship between *P. marneffei* and the *Aspergillus* species was from comparison of the ribosomal RNA gene sequences [13]. Subsequently, we showed that the mitochondrial genome of *P. marneffei* most closely related to that of *A. nidulans*, with the set of protein coding genes in the *P. marneffei* mitochondrial genome exactly the same as that in the *A. nidulans* mitochondrial genome, and except for the *atp9* gene, the gene order of the protein genes is also the same as that in the *A. nidulans* mitochondrial genome [14]. Furthermore, their close relationships are also exemplified by the presence of unique antigenic proteins of the "antigenic mannoprotein superfamily" in *P. marneffei* and *Aspergillus* species [30–39], which have never been shown to be present in fungal species of other genera. In this study, all the genes in the putative sexual cycle of *P. marneffei* are found to be most closely related to their orthologues in *Aspergillus* species. Recently, the complete genomes of three *Aspergillus* species have been published [40–42], with *A. nidulans* being a homothallic fungus and *A. fumigatus* and *Aspergillus oryzae* heterothallic fungi. This shows that the sexual cycles in *Penicillium* and *Aspergillus* were probably present in their common ancestor, possibly a homothallic fungus, and then evolved independently after the divergence into the two genera and the individual species, with some of the species becoming heterothallic fungi.

Although *P. marneffei* has the potential for a sexual cycle, population genetics studies revealed that sex, if occurred, was a rare phenomenon in this fungus. A recently published study showed, by population genetics studies using 21 microsatellite loci and *P. marneffei* strains collected in different areas in Thailand, that clusters of genotypes of *P. marneffei* were specific to discrete ecological zones [43]. The authors concluded that the evolution of the *P. marneffei* genome is overwhelmingly clonal, and that *P. marneffei* is perhaps the most asexual fungus yet found [43]. This implies that although *P. marneffei* may have the potential for a sexual cycle, the cycle probably occurs relatively infrequently.

The reservoir and transmission of *P. marneffei* in our locality remains enigmatic. *Penicillium* species, other than *P. marneffei*, and *Aspergillus* species are commonly encountered in air samples. In our previous study, 87% of the exogenous contaminants of the fungal cultures in our bone marrow transplant recipients were *Penicillium* species other than *P. marneffei* [44]. For *Aspergillus* species such as *A. fumigatus* and *A. flavus*, inhalation of asexual conidiospores in the air is a major mode of transmission of these molds. Increase in environmental *Aspergillus* contamination and hospital invasive aspergillosis outbreaks have been associated with renovation and construction of buildings [45]. On the other hand, spores of *P. marneffei* have never been reported in air samples. In our locality, although *P. marneffei* causes infection in about 8% of our AIDS patients, almost none of them had histories of contact with bamboo rats. Furthermore, environmental sampling, such as soil sampling, in multiple sites in Hong Kong did not reveal the presence of any *P. marneffei* (unpublished data). If a sexual stage, possibly as a free-living stage in contrast to the parasitic stage in humans and bamboo rats, is present in the life cycle of *P. marneffei*, this sexual stage may serve as the reservoir of this pathogenic dimorphic fungus in the environment. Although the genomic and experimental evidence in the present study supported the potential for a sexual cycle in *P. marneffei*, it is unlikely that this is a frequent event. More intensive efforts have to be spent to search for the asexual form of *P. marneffei* in specific ecological niches as the reservoir of this fungus in Hong Kong.

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