Identification of MicroRNA-Like RNAs in Mycelial and Yeast Phases of the Thermal Dimorphic Fungus 

*Penicillium marneffei*

Susanna K. P. Lau1,2,3,4*, Wang-Ngai Chow4*, Annette Y. P. Wong4, Julian M. Y. Yeung4, Jessie Bao5, Na Zhang5, Si Lok5, Patrick C. Y. Woo1,2,3,4*, Kwok-Yung Yuen1,2,3,4*

1 State Key Laboratory of Emerging Infectious Diseases, The University of Hong Kong, Hong Kong, China, 2 Research Centre of Infection and Immunology, The University of Hong Kong, Hong Kong, China, 3 Carol Yu Centre for Infection, The University of Hong Kong, Hong Kong, China, 4 Department of Microbiology, The University of Hong Kong, Hong Kong, China, 5 Genome Research Centre, The University of Hong Kong, Hong Kong, China

**Abstract**

**Background:** *Penicillium marneffei* is the most important thermal dimorphic fungus causing systemic mycosis in China and Southeast Asia. While miRNAs are increasingly recognized for their roles in post-transcriptional regulation of gene expression in animals and plants, miRNAs in fungi were less well studied and their potential roles in fungal dimorphism were largely unknown. Based on *P. marneffei* genome sequence, we hypothesize that miRNA-like RNAs (miRNAs) may be expressed in the dimorphic fungus.

**Methodology/Principal Findings:** We attempted to identify miRNAs in *P. marneffei* in both mycelial and yeast phase using high-throughput sequencing technology. Small RNAs were more abundantly expressed in mycelial than yeast phase. Sequence analysis revealed 24 potential miRNA candidates, including 17 candidates in mycelial and seven in yeast phase. Two genes, *dcl-1* and *dcl-2*, encoding putative Dicer-like proteins and the gene, *qde-2*, encoding Argonaute-like protein, were identified in *P. marneffei*. Phylogenetic analysis showed that *dcl-2* of *P. marneffei* was more closely related to the homologues in other thermal dimorphic pathogenic fungi than to *Penicillium chrysogenum* and *Aspergillus* spp., suggesting the co-evolution of *dcl-2* among the thermal dimorphic fungi. Moreover, *dcl-2* demonstrated higher mRNA expression levels in mycelial than yeast phase by 7 folds (P<0.001). Northern blot analysis confirmed the expression of two miRNAs, *PM-miR-M1* and *PM-miR-M2*, only in mycelial phase. Using *dcl-1KO*, *dcl-2KO*, *qde-2KO* and *qde-2KO* deletion mutants, we showed that the biogenesis of both miRNAs were dependent on *dcl-2* but not *dcl-1* or *qde-2*. The mRNA expression levels of three predicted targets of *PM-miR-M1* were upregulated in knockdown strain *PM-miR-M1KO*, supporting regulatory function of miRNAs.

**Conclusions/Significance:** Our findings provided the first evidence for differential expression of miRNAs in different growth phases of thermal dimorphic fungi and shed light on the evolution of fungal proteins involved in miRNA biogenesis and possible role of post-transcriptional control in governing thermal dimorphism.

**Citation:** Lau SKP, Chow W-N, Wong AYP, Yeung JMY, Bao J, et al. (2013) Identification of MicroRNA-Like RNAs in Mycelial and Yeast Phases of the Thermal Dimorphic Fungus *Penicillium marneffei*. PLoS Negl Trop Dis 7(8): e2398. doi:10.1371/journal.pntd.0002398

**Editor:** Joseph M. Vinetz, University of California San Diego School of Medicine, United States of America

**Received** April 5, 2013; **Accepted** July 19, 2013; **Published** August 22, 2013

**Copyright:** © 2013 Lau et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was partly supported by the Strategic Research Theme Fund, Research Grant Council Grant, University Grant Council; Committee for Research and Conference Grant, and University Development Fund, The University of Hong Kong; Shaw Foundation; and Providence Foundation Limited in memory of the late Dr. Lui Hac Minh. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: pcywoo@hkkuc.hku.hk (PCYW); kyyuen@hkkuc.hku.hk (KYY)

* These authors contributed equally to this work.

**Introduction**

*Penicillium marneffei* is the most important thermal dimorphic fungus causing respiratory, skin and systemic mycosis in Southeast Asia [1–4]. Recently, it has been renamed as *Talaromyces* based on phylogenetic analyses [5]. The fungus was first discovered in Chinese bamboo rats, *Rhizomys sinensis*, and subsequently isolated from other species of bamboo rats [6,7]. While only 18 cases of human diseases were reported until 1985 [8], the emergence of the HIV pandemic in the 1980’s has resulted in increasing reports of HIV-associated *P. marneffei* infections in Southeast Asia where the fungus is endemic. Penicilliosis is the third most common indicator disease of AIDS in northern Thailand [2]. In Hong Kong, about 10% of HIV patients are infected with *P. marneffei*, which represents the sixth leading cause of death [9,10]. Cases of imported *P. marneffei* infections have also been reported from countries outside Southeast Asia [11,12]. In addition, *P. marneffei* infections are increasingly reported in other immunocompromised patients, such as transplant recipients and others on immunosuppressant therapy [13–16]. Despite its medical importance, the mode of transmission, and dimorphic and pathogenic mechanisms of *P. marneffei* remain largely unknown. In particular, *P. marneffei* exhibits distinct cellular morphologies in different temperatures, in mycelial phase at 25°C and yeast phase at 37°C. During the
mycelial phase, hyphae can differentiate to produce conidia which are believed to be the infectious form being inhaled to the lungs of infected hosts. When these conidia are phagocytosed by alveolar macrophages, they germinate into yeast cells as the tissue form. Despite the efforts of using various gene knockout experiments in identifying diverse genes and complex mechanisms involved in dimorphic switching in P. marneffei, the signals that trigger the switch in response to temperature and signaling pathways leading to the transition remain elusive [17].

MicroRNAs or miRNAs are small non-coding endogenous RNAs of approximately 22 nt, which play important roles in post-transcriptional regulation of gene expression in animals and plants [18]. They are now known to comprise one of the most abundant classes of gene regulatory molecules in multicellular organisms. The mature miRNAs negatively regulate gene expression by targeting mRNAs mediated through complementary binding to the open-reading frame or untranslated (UTR) regions of specific target genes. Interactions with targets can be through imprecise base pairing leading to translational inhibition in animals, or near-perfect complementarity leading to mRNA cleavage in plants [19,20]. In animals, miRNAs have been shown to play various roles in ranging from cell development, proliferation and differentiation, apoptosis, carcinogenesis to immunity [21–23]. In plants, they are also involved in plant development, stress response and antibacterial resistance [24,25,26].

The first known miRNA lin-4 was discovered in Caenorhabditis elegans in 1993 [27]. However, it was only until 2000 that the second miRNA, let-7, also in C. elegans, was identified [28]. With the advent of molecular and bioinformatics tools, numerous miRNAs have now been identified in animals, plants, viruses and unicellular organisms, with >25,000 miRNAs being currently included in the miRNA database, miRBase release 19.0 [29]. Although small RNA pathways have been found in various fungi, the existence of miRNAs and their roles in fungi has been less well understood. Recently, miRNA-like small RNAs (miRNAs) have been identified in the red bread mold, Neurospora crassa, the plant pathogenic fungus, Sclerotinia sclerotiorum, the entomopathogenic fungus, Metarhizium anisopliae and the human pathogenic yeast, Cryptococcus neoformans [30–33]. However, their existence in thermal dimorphic fungi and potential roles in fungal dimorphism were largely unknown.

In 2002, we started the P. marneffei genome project in an attempt to expedite the study of biology, epidemiology and virulence factors of this dimorphic fungus [34–41]. Based on the available genome sequence data, potential genes encoding proteins important for miRNA biogenesis can be identified in P. marneffei. Since miRNAs are important gene regulatory molecules in multicellular organisms, we hypothesize that miRNAs may be expressed in P. marneffei and involved in the regulation of thermal dimorphism. We attempted to identify miRNAs in P. marneffei in both mycelial and yeast phase using high-throughput Illumina DNA sequencing. Sequence analysis revealed 24 potential miRNA candidates, which were more abundantly expressed in mycelial than yeast phase of P. marneffei. Two genes, dcl-1 and dcl-2, encoding Dicer-like proteins and the gene, qde-2, encoding Argonaute-like protein, were also identified. Phylogenetic analysis showed that dcl-2 of P. marneffei was more closely related to the homologues in other thermal dimorphic pathogenic fungi than to Penicillium chrysogenum and Aspergillus spp. dcl-2 demonstrated higher miRNA levels in mycelial than yeast phase. Northern blot analysis confirmed expression of two miRNAs, PM-miR-M1 and PM-miR-M2, only in mycelial phase, whose expression was dependent on dcl-2 but not dcl-1 or qde-2. The mRNA levels of three predicted targets of PM-miR-M1 were upregulated in knockdown strain PM-miR-M1KO, supporting its regulatory function. This study represents the first discovery of miRNAs in thermal dimorphic fungi, with differential expression in different growth phases.
A strand-specific library construction protocol was used to generate template for Illumina DNA sequencing [42]. An adenylated 3′-adaptor (Integrated DNA Technologies, Coralville, IA) was first ligated to the 3′ ends of a small RNA (≤60 nt) fraction was extracted from 15% denaturing polyacrylamide gel. The 3′-adaptor-ligated small RNAs were then ligated with a 5′-adaptor (Integrated DNA Technologies). Adaptor-ligated small RNAs were reverse transcribed into first-strand cDNA using a primer hybridizing to the 3′-adaptor using SuperScript II reverse transcriptase (Invitrogen). First strand cDNA was amplified by polymerase chain reaction (PCR) from Illumina/Solexa PCR primer binding sites present on the 5′ polymerase chain reaction (PCR) from Illumina/Solexa PCR products for sequencing on the Illumina Genome Analyzer IIx.

**Small RNA analyses**

Sequence reads were processed to remove low quality reads, adaptor and adaptor-dimer sequences, and nuclear and mitochondria RNA sequences to yield 16,479,305 filtered reads for mycelial and 12,754,677 filtered reads for yeast phase respectively. Relative expression levels were estimated by normalizing read counts for each non-redundant small RNA species against RPM (number of reads per million mapped reads) as mapped to the PM1 genome sequence [39]. Small RNA sequences between 17–30 nt were selected to identify perfect matches to the genome using Bowtie (0.12.6) [43].

**Identification of miRNAs and miRNA loci**

To identify miRNA candidates, other non-coding RNAs including tRNAs and tRNA sequences were first excised from the genome with the size of 250 nt flanking to the small RNA sequences with deduced amino acid sequences. Ten potential target sequences were identified using the following criteria: (1) Overlapping sequence regions that were perfectly mapped to no more than 5 different regions in the genome; (2) Potential precursor sequences were excised from the genome with the size of 250 nt flanking to the small RNA sequences, and (3) Hybridization temperatures of 25°C and 37°C were used in the script regarding RNAfold for deep sequencing data from mycelial and yeast form of PM1, with primers shown in Table 1. PCR products of upstream and downstream fragments of dcl-1, dcl-2 and qde-2 were amplified by PCR using DNA extracted from strain PM1 with primers shown in Table 1. PCR products of upstream and downstream fragment sequences were ligated into corresponding restriction sites of plasmid pAN7-1 to generate the knockout plasmids pAN7-dcl-1, pAN7-dcl-2 and pAN7-qde-2 as shown in Fig. 1. The resultant plasmids were linearized with AhdI and transformed to strain PM1 according to previous publications [40,41]. SDA supplemented with 150 μg/ml hygromycin B was used as selection medium. To construct dclDKO mutants, PCR products of dcl-1 flanking fragments were ligated into vector pAN7-1 (a gift from Dr. P. J. Punt) as described previously [46,47]. The flanking sequences upstream and downstream of dcl-1, dcl-2 and qde-2 were amplified by PCR using DNA extracted from strain PM1 with primers shown in Table 1. PCR products of dclDKO mutants were ligated into vector pAN7-dcl-1, pAN7-dcl-2 and pAN7-qde-2 as described previously [46,47].

**Target prediction for miRNAs**

The potential targets of miRNA candidates were predicted using the predicted gene sequences, including their 5′ and 3′ UTRs, of the P. marneffei strain PM1 and ATCC strain 10442 draft genomes by the RNAhybrid program [51] with or without mismatches or insertions at positions 9–11 of the miRNAs and with parameters that encourage complete complementarity at the seed region (positions 2–7 of the miRNA) [52].

**Construction of dcl-1KO, dcl-2KO, dclDKO and qde-2KO mutants of P. marneffei**

Deletion mutants were generated by homologous recombination (Fig. 1). Based on dcl-1, dcl-2 and qde-2 gene sequences from P. marneffei strain PM1, primers were designed to amplify upstream and downstream fragments of dcl-1, dcl-2 and qde-2 for the construction of the corresponding knockout constructs using the vector pAN7-1 (a gift from Dr. P. J. Punt) as described previously [46,47]. The flanking sequences upstream and downstream of dcl-1, dcl-2 and qde-2 were amplified by PCR using DNA extracted from strain PM1 with primers shown in Table 1. PCR products of upstream and downstream fragment sequences were ligated into corresponding restriction sites of plasmid pAN7-1 to generate the knockout plasmids pAN7-dcl-1, pAN7-dcl-2 and pAN7-qde-2 as shown in Fig. 1. The resultant plasmids were linearized with AhdI and transformed to strain PM1 according to previous publications [40,41]. SDA supplemented with 150 μg/ml hygromycin B was used as selection medium. To construct dclDKO mutants, PCR products of dcl-1 flanking fragments were ligated into vector pAN7-1 (a gift from Dr. P. J. Punt) as described previously [46,47] to generate the knockout plasmid pAN7-dcl-1 (Fig. 1). pAN7-dcl-1 was linearized with AhdI and transformed to dclDKO using SDA supplemented with 100 μg/ml phleomycin as selection medium.

**Northern blot analysis**

Northern blot analysis was performed according to published protocols with modifications [30,50]. Briefly, 10–20 μg of small RNAs was separated on 12% denaturing polyacrylamide gel and transferred onto a positively charged nylon membrane (Amersham Biosciences, United Kingdom) with NorthernMax Transfer buffer (Ambion) with NorthernMax Transfer buffer (Ambion) by means of capillary force for 1 h. Crosslinking of RNA probes (Sigma-Aldrich). Detection of the DIG-labeled probe on the blot was performed by using DIG Luminescent Detection kit (Roche).

**Target prediction for miRNAs**

The potential targets of miRNA candidates were predicted using the predicted gene sequences, including their 5′ and 3′ UTRs, of the P. marneffei strain PM1 and ATCC strain 10442 draft genomes by the RNAhybrid program [51] with or without mismatches or insertions at positions 9–11 of the miRNAs and with parameters that encourage complete complementarity at the seed region (positions 2–7 of the miRNA) [52].
Table 1. Primers used in this study.

| Gene Targets | Primers | Purpose |
|--------------|---------|---------|
| Upstream of dcl-1 | LPW10929 5'-GAAGATCTCTCTGTGATCAGTGGATCCTGAG-3' | pAN7-1 cloning |
|              | LPW10930 5'-GAAGATCTCTCTTTTGGCCTGGTTTAAGTCTG-3' | (BglII and HindIII) |
| Downstream of dcl-1 | LPW10931 5'-TGATTGAAGATCCTCCCAAGGTTG-3' | |
|              | LPW10930 5'-GAAGATCTTCTTTTGCGGCCTTTGTAAGTCTG-3' | (BglII and HindIII) |
| Upstream of dcl-2 | LPW13339 5'-GAAGATCTCTGGGAAAACTGGAAAGAGA-3' | pAN7-1 cloning |
|              | LPW13340 5'-TGCTCTCTGGAAGCTCTGCTTACG-3' | (BglII and SfoI) |
| Downstream of dcl-2 | LPW13341 5'-ATAGGCCGCCTTATGCTGATTTATGGTGGATA-3' | |
|              | LPW13342 5'-ATAGGCCGCCTTATGCTGATTTATGGTGGATA-3' | |
| Upstream of qde-2 | LPW12475 5'-ACCCAATAAGGATGAGGAAGTTCGG-3' | qPCR |
|              | LPW12476 5'-GAAGATCTAAGTCAGTCGCAATCTCGTCCGG-3' | (BglII and SbfI) |
| Downstream of qde-2 | LPW12799 5'-ATAGGCCGCCTTATGCTGATTTATGGTGGATA-3' | |
|              | LPW12800 5'-ATAGGCCGCCTTATGCTGATTTATGGTGGATA-3' | |
| dcl-1         | LPW13343 5'-TTTACGGGACGTAAATGGCGGCCTA-3' | qPCR |
|              | LPW13344 5'-AATTCTAGGCGCTGGTAAGTCGGC-3' | |
|              | LPW22067 5'-CTCGGCATTCCATAGTTTGT-3' | cDNA amplification |
|              | LPW22068 5'-CGATGATGAATGGTCGTGAA-3' | cDNA amplification |
|              | LPW22069 5'-GATGCCAAATCTTGGAATGG-3' | & sequencing |
|              | LPW22070 5'-ATATCTTTATTCGTTGGAAGTCCG-3' | |
|              | LPW22071 5'-ACCTTCAGAGACTCCATCGC-3' | |
|              | LPW22072 5'-CTCGGCATTCCATAGTTTGT-3' | & sequencing |
|              | LPW22073 5'-TCGGCCAAAACGTCCCTTTG-3' | |
|              | LPW22074 5'-TCATCTCTGAGCGTGCAC-3' | |
|              | LPW22075 5'-TCTAGCCGAGCCTTGCCCTT-3' | |
|              | LPW22076 5'-CTGTTGGAAATAATCCTCTG-3' | |
|              | LPW22077 5'-ATATCTTTATTCGTTGGAATGG-3' | |
|              | LPW22078 5'-ACCTTCAGAGACTCCATCGC-3' | |
|              | LPW22079 5'-CTGTTGGAAATAATCCTCTG-3' | |
|              | LPW22080 5'-ACCTTCAGAGACTCCATCGC-3' | |
| dcl-2         | LPW13347 5'-GTGTTGAAGTATTGCGCAAAAGG-3' | qPCR |
|              | LPW13346 5'-AATTCTAGGCGCTGGTAAGTCGGC-3' | |
|              | LPW22067 5'-CTCGGCATTCCATAGTTTGT-3' | cDNA amplification |
|              | LPW22069 5'-GATGCCAAATCTTGGAATGG-3' | & sequencing |
|              | LPW22070 5'-ATATCTTTATTCGTTGGAAGTCCG-3' | |
|              | LPW22071 5'-ACCTTCAGAGACTCCATCGC-3' | |
|              | LPW22072 5'-CTCGGCATTCCATAGTTTGT-3' | & sequencing |
|              | LPW22073 5'-TCGGCCAAAACGTCCCTTTG-3' | |
|              | LPW22074 5'-TCATCTCTGAGCGTGCAC-3' | |
|              | LPW22075 5'-TCTAGCCGAGCCTTGCCCTT-3' | |
|              | LPW22076 5'-CTGTTGGAAATAATCCTCTG-3' | |
|              | LPW22077 5'-ATATCTTTATTCGTTGGAATGG-3' | |
|              | LPW22078 5'-ACCTTCAGAGACTCCATCGC-3' | |
|              | LPW22079 5'-CTGTTGGAAATAATCCTCTG-3' | |
|              | LPW22080 5'-ACCTTCAGAGACTCCATCGC-3' | |
| qde-2         | LPW14804 5'-GCCTCATCAAATCCCCGGG-3' | qPCR |
|              | LPW14805 5'-GGAGAAGACCAGCAGGCCACCAT-3' | |
|              | LPW22081 5'-CCTCGTCTCTTCGAGGACT-3' | cDNA amplification |
|              | LPW22082 5'-TTGTTGATAAACATCGTG-3' | & sequencing |
| Actin         | LPW20631 5'-GAACGTGAAATCGTCCGT-3' | qPCR |
| PM-miRNA gene | LPW20160 5'-AGCAAGAATTGAAAGGACAC-3' | |
| locus         | LPW20742 5'-CCGCTCAGGGGACAACAAACAGTGGCAA-3' | pSilent-1 cloning |
|              | LPW20743 5'-GAGGATTCGCCAGGACAAACAAATGCAC-3' | (XhoII-HindIII) |
|              | LPW20740 5'-GGGGTACCAGGCCAAGAAATGCAAC-3' | pSilent-1 cloning |
|              | LPW20741 5'-GAAGATCTGTATTGCGGTATCCATCG-3' | (BglII-KpnI) |
Table 1. Cont.

| Gene Targets | Primers | Purpose |
|--------------|---------|---------|
|              | LPW23656 5'-TTGCCAATAAACAAGACTCTTC-3' | qPCR |
|              | LPW23657 5'-TCTTAGCTGACGACTGCGG-3' | qPCR |
| RanBP10      | LPW23241 5'-CAAGTGCTGCAGGTCTA-3' | qPCR |
|              | LPW23242 5'-TATATCCACCCCTAGGCAGG-3' | qPCR |
| Cytchrome P450| LPW23438 5'-GAGGCGATGTCATCTCCAGG-3' | qPCR |
|              | LPW23439 5'-GAGGCGGAACGCGATATACA-3' | qPCR |
| Conserved    | LPW23499 5'-TGTCGGGACATGGTGTAT-3' | qPCR |
| Hypothetical protein | LPW23500 5'-CATTTTTCCTGATGCAGGCGG-3' | qPCR |

doi:10.1371/journal.pntd.0002398.t001

construct the pSilent-M1 plasmid as previously described [10]. Briefly, the internal fragments (sense and antisense) were amplified with primers shown in Table 1 and cloned into the XhoI-HindIII and BglII-RsrII sites of pSilent-1 plasmid, resulting in pSilent-M1. The wild type P. marneffei strain PM1 was transformed with linearized pSilent-M1, using 200 μg/ml hygromycin for selection.

Quantitative real-time RT-PCR

Total RNA was extracted using Ribopure-Yeast (Ambion). Reverse transcription was performed using the SuperScript III kit (Invitrogen). Real-time RT-PCR assays were performed as described previously with modifications [36], using primers shown in Table 1. Results from actin were used for normalization. cDNA was amplified in an ABI 7900HT Fast Real-Time PCR System (Life Technologies) in 20-μl reaction mixtures containing FastStart DNA Master SYBR Green I Mix reagent kit (Roche, Basel, Switzerland), using the standard qPCR conditions (40 cycles of 95°C for 15 s, followed by 60°C for 1 min) and dissociation curve in the control software of SDS 2.4 (Life Technologies). Statistical analyses of qRT-PCR data were performed using Student's t-test (SPSS version 19).

Nucleotide sequence accession number

The nucleotide sequences of the dcl-1, dcl-2 and qde-2 genes of P. marneffei have been deposited in GenBank under accession no. KC686608, KC686609 and KC686610 respectively. The Illumina small RNA sequences have been deposited in SRA NCBI database under accession no. SRX306604.

Results

Identification of P. marneffei small RNAs by deep sequencing

To examine small RNA species in the two growth phases of P. marneffei, cDNA libraries of small RNAs ≤60 nt extracted from mold and yeast cultures respectively were sequenced using the Illumina/Solexa Genome Analyzer IIx. The total number of both raw and filtered reads from mycelial and yeast phase was similar (Table 2). However, small RNAs were more abundant in mycelial than yeast phase of P. marneffei. We obtained a total of 3,155,063 and 270,782 high-quality, small RNA sequences of size 17–30 nt from mycelial and yeast phases respectively that perfectly match the P. marneffei genome. Among these, 362,805 and 56,543 unique small RNA sequences were identified from mycelial and yeast phases respectively that perfectly match the P. marneffei genome. Among these, 362,805 and 56,543 unique small RNA sequences were identified from mycelial and yeast phases respectively that perfectly match the P. marneffei genome. The nucleotide sequences of the gene targets were described previously with modifications [36], using primers shown in Table 1. Results from actin were used for normalization. cDNA was amplified in an ABI 7900HT Fast Real-Time PCR System (Life Technologies) in 20-μl reaction mixtures containing FastStart DNA Master SYBR Green I Mix reagent kit (Roche, Basel, Switzerland), using the standard qPCR conditions (40 cycles of 95°C for 15 s, followed by 60°C for 1 min) and dissociation curve in the control software of SDS 2.4 (Life Technologies). Statistical analyses of qRT-PCR data were performed using Student's t-test (SPSS version 19).

Potential miRNAs in P. marneffei

Based on the distinguishing feature of known plant and animal miRNAs, 24 miRNA candidates, with flanking sequences forming hairpin secondary structures and at least five reads, were identified (Table 3). Their size distribution was shown in Fig. 2C, with a peak at 21 nt. There was also strong preference for U at their 5' termini (67%, 16 of the 24 miRNA candidates) (Fig. 2D). These include 17 potential miRNAs (2,502 reads) in mycelial phase and seven potential miRNAs (232 reads) in yeast phase respectively (Table 3).

Identification and sequence analysis of dcl-1, dcl-2 and qde-2 genes

Using the respective homologues of N. crassa for BLAST search of P. marneffei strain PM1 draft genome sequence, two dcl genes, dcl-1 and dcl-2, encoding putative Dicer-like proteins and a gene, qde-2, encoding a putative Argonaute-like protein were identified (Fig. 3A). Dicer and Argonaute proteins are known to be involved in the biogenesis of miRNAs in animals and plants [56,57,58]. The dcl-1 gene is 5,383 bp in length, comprising 15 introns with total length of 889 bp. The resultant mRNA encodes 1,497 amino acid residues with a predicted molecular mass of 170.31 kDa. The dcl-2 gene is 4,636 bp in length, comprising six introns with total length of 340 bp. The resultant mRNA encodes 1,431 amino acids with a predicted mass of 161.15 kDa. These putative proteins possessed 42% and 32% amino acid identity to the DCL-1 and DCL-2 of N. crassa respectively. Both predicted proteins contain all four domains characteristic of the Dicer family. Two RNAse III domains are present in the C-terminal region, and a DEAD-box ATP binding domain is present in the N-terminal region. In between there are RNA helicase and double stranded RNA binding domains. The qde-2 gene is 3,199 bp in length, comprising three introns with total length of 160 bp. The resultant mRNA encodes 1,012 amino acid residues with a predicted molecular mass of 111.75 kDa. The predicted QDE-2 protein possessed 35% amino acid identity to the QDE-2 of N. crassa. It contains two characteristic domains of the argonaute family, PAZ and Piwi domains, and the DUF1785 domain conserved in many argonaute proteins. The domain organization of DCL-1, DCL-2 and QDE-2 of P. marneffei is similar to that of the corresponding homologues in N. crassa [59].

Our previous study based on mitochondrial genome sequence has shown that P. marneffei is phylogenetically more closely related to those of filamentous fungi, including Aspergillus species, than...
Figure 1. Deletion of (A) dcl-1, (B) dcl-2, (C) qde-2, (D) dcl-1/dcl-2 in *P. marneffei* by homologous recombination. Plasmids pAN7-1 and pAN8-1 were used to construct the knockout plasmids of pAN7-dcl-1, pAN7-dcl-2, pAN7-qde-2 and pAN8-dcl-1 respectively.

doi:10.1371/journal.pntd.0002398.g001
Phylogenetic analysis of both ITS, another important marker for fungal identification and phylogeny, and \textit{dcl-1} gene showed that the corresponding sequences in \textit{P. marneffei} were most closely related to \textit{Talaromyces stipitatus} (a teleomorph of \textit{Penicillium emmonsii}), \textit{Penicillium chrysogenum} and \textit{Aspergillus} spp. (Fig. 3). In contrast, phylogenetic analysis of \textit{dcl-2} and \textit{qde-2} genes showed a different evolutionary topology. The \textit{dcl-2} of \textit{P. marneffei} and its homologue in \textit{T. stipitatus} are more closely related to those of the thermal dimorphic pathogenic fungi, \textit{Histoplasma capsulatum}, \textit{Blastomyces dermatitidis}, \textit{Paracoccidioides brasiliensis} and \textit{Coccidioides immitis} than to \textit{P. chrysogenum} and \textit{Aspergillus} spp., suggesting the co-evolution of \textit{dcl-2} among the thermal dimorphic fungi. On the other hand, \textit{qde-2} of \textit{P. marneffei} is most closely related to its homologues in other thermal dimorphic fungi than to that in \textit{T. stipitatus}, \textit{P. chrysogenum} and \textit{Aspergillus} spp.

Differential mRNA expression of \textit{dcl-1}, \textit{dcl-2} and \textit{qde-2} in mycelial and yeast phase

The mRNA expression level of \textit{dcl-1} in yeast phase was significantly higher than mycelial phase by 25 folds ($P<0.001$ by

|                  | Total reads | Unique reads |
|------------------|-------------|--------------|
| **Mycelial**     |             |              |
| Raw reads        | 39,809,400  |              |
| Filtered reads   | 27,914,677  |              |
| Adaptors or rRNA reads | 11,435,372  |              |
| Small RNA reads (17–30 nt) | 6,910,710  | 1,077,964     |
| Small RNA reads (17–30 nt) mapped to \textit{P. marneffei} genome | 3,155,063  | 362,805       |
| **Yeast**        |             |              |
| Raw reads        | 36,999,600  |              |
| Filtered reads   | 28,424,899  |              |
| Adaptors or rRNA reads | 15,670,222  |              |
| Small RNA reads (17–30 nt) | 768,705   | 165,545       |
| Small RNA reads (17–30 nt) mapped to \textit{P. marneffei} genome | 270,782   | 56,543        |

Table 2. Analysis of total and small RNA sequences in mycelial and yeast phase of \textit{P. marneffei}. 

doi:10.1371/journal.pntd.0002398.t002
student t test). In contrast, the mRNA expression levels of dcl-2 and qde-2 were higher in mycelial phase than in yeast phase by 7, and 2 folds respectively (P<0.001 by Student’s t-test) (Fig. 4).

### Dicer-dependent biogenesis of miRNA in *P. marneffei*

Northern blot analyses showed the production of miRNAs from two of the predicted miRNA loci, PM-miR-M1 and PM-miR-M2, both from mycelial phase of *P. marneffei*, with their predicted miRNA precursor (pre-miRNA) structures shown in Fig. 5. Their predicted precursors were approximately 70-nt and 91-nt in size overhang in miRNA arm, with total of 1482 small RNAs sequenced from *P. marneffei* type strain PM1. To assess the role of *dcl-1* and *dcl-2* in the biogenesis of PM-miR-M1 and PM-miR-M2, *dcl-1KO* and *dcl-2KO* mutants were generated using homologous recombination. All deletion mutants exhibited similar growth rates and phenotypic characteristics to wild-type strain in both mycelial and yeast phases, although the *dcl-2KO* mutant exhibited poor sporulation and reduced red pigment production compared to wild-type strain upon transition from yeast to mycelial phase on sabouraud agar (data not shown).  

However, Northern blot and small RNA sequencing results indicating mycelial-specific expression (Fig. 5A) with little or no expression in yeast phase (data not shown) of wild-type strain PM1. To assess the role of *dcl-1*, *dcl-2* and *qde-2* in the biogenesis of PM-miR-M1 and PM-miR-M2, *dcl-1KO*, *dcl-2KO*, *dcl-1KO/dcl-2KO* and *qde-2KO* mutants were generated using homologous recombination. All deletion mutants exhibited similar growth rates and phenotypic characteristics to wild-type strain in both mycelial and yeast phases. The other 22 loci were considered miRNA candidates (named PM-miR-MC1…MC17 for miRNA candidates in mycelial phase and PM-miR-YC1…YC7 for those in yeast phase). These novel miRNAs or miRNA candidates showed no sequence similarity to known miRNAs miRBase as of March 2013.

To study the expression profile of PM-miR-M1 and PM-miR-M2 in mycelial and yeast phases, Northern blot analyses of small RNAs were performed, which cross-validated the Illumina sequencing results indicating mycelial-specific expression (Fig. 5A) with little or no expression in yeast phase (data not shown) of wild-type strain PM1. To assess the role of *dcl-1*, *dcl-2* and *qde-2* in the biogenesis of PM-miR-M1 and PM-miR-M2, *dcl-1KO*, *dcl-2KO*, *dcl-1KO/dcl-2KO* and *qde-2KO* mutants were generated using homologous recombination. All deletion mutants exhibited similar growth rates and phenotypic characteristics to wild-type strain in both mycelial and yeast phases. The other 22 loci were considered miRNA candidates (named PM-miR-MC1…MC17 for miRNA candidates in mycelial phase and PM-miR-YC1…YC7 for those in yeast phase). These novel miRNAs or miRNA candidates showed no sequence similarity to known miRNAs miRBase as of March 2013.

Produced from a Dicer-like enzyme (Fig. 5) [62]. Since loci which produce mature miRNAs and miRNA* sequences are considered miRNA loci, the two loci are tentatively named as *P. marneffei* miR-1 (PM-miR-1) and PM-miR-2. The locus, PM-miR-1, was situated within the coding region of a hypothetical protein, whereas PM-miR-2 was situated in the opposite strand of a pogo transposable element within a repeat region in the *P. marneffei* genome.

### Table 3. Potential miRNA candidates in mycelial and yeast phase of *P. marneffei*.

| miRNA     | Sequence (5'–3')          | Length (nt) | Reads  |
|-----------|---------------------------|-------------|--------|
| PM-miR-M1 | GAGAAAGCCCUUAUGAUUGCAC   | 21          | 1482   |
| PM-miR-M1*| UGACUCGGAAGGCCCUUA       | 18          | 1      |
| PM-miR-M2 | GUCCUAUAGAAUAGCCAGUC     | 20          | 10     |
| PM-miR-M2*| AUUUCAGUCCUAAAGCCUU      | 21          | 1      |
| PM-miR-M3 | UGUAUCAAAAGGGCUAUC       | 20          | 351    |
| PM-miR-M4 | UCAAGUCAACCCCUAACCUC     | 18          | 198    |
| PM-miR-M5 | UUGCUAUAAGGAAGCCUAGCA    | 22          | 127    |
| PM-miR-M6 | AACGUAUAUUAAUCUGAUAACA   | 24          | 101    |
| PM-miR-M7 | UAGGAUAGAGAUUAGAGUU      | 20          | 97     |
| PM-miR-M8 | UUUUCAGAGUCUGCAAGGCU     | 21          | 44     |
| PM-miR-M9 | UUGCGUGGUGGGUGAAUUG      | 19          | 22     |
| PM-miR-M10| UCGACUGGCUCACUGAUAGCC    | 21          | 14     |
| PM-miR-M11| UGUAUGAGAUCCUUGUUGGA     | 20          | 12     |
| PM-miR-M12| UGUUCAUGACAGUCUGUAGA     | 21          | 9      |
| PM-miR-M13| UGCCACUGCAUCAUUGGUGG     | 20          | 8      |
| PM-miR-M14| UAGAGCGUCAUAGUUAAGG      | 21          | 8      |
| PM-miR-M15|UUCCAGUGAGUUAAUCAC        | 20          | 8      |
| PM-miR-M16| CAUAAGGUGCAGAUCUGCC      | 21          | 6      |
| PM-miR-M17| UGGGGAGCGGAUGGUGGAGG     | 21          | 5      |
| PM-miR-YC1| UGCCAGUAGUAGUAGCA        | 19          | 76     |
| PM-miR-YC2| CAGGGGUGAGAAGACCC         | 17          | 47     |
| PM-miR-YC3| CCGGCUUCAAAAGGGCUAGAC    | 22          | 44     |
| PM-miR-YC4| UUGCAUAUGAAGAGCGAGCA     | 22          | 30     |
| PM-miR-YC5| UUUCUGUCAUCCUUGAGAGU     | 21          | 19     |
| PM-miR-YC6| UUUCGUGGUGCGAGUCAUU      | 21          | 8      |
| PM-miR-YC7| CCUCAGAUCUGGCGUAGC       | 22          | 8      |

*doi:10.1371/journal.pntd.0002398.t003*
Figure 3. Sequence analysis of dcl-1, dcl-2 and qde-2 genes in *P. marneffei*. (A) Predicted domains of Dicer and QDE-2 proteins in *P. marneffei* strain PM1. Black bars represent the full protein sequence. The boxes represent the identified domains, each with its starting and stopping amino acid. Both DCL-1 and DCL-2 of *P. marneffei* contain a DEAD box, a helicase C domain (hel C), a double stranded RNA binding domain (dsRBD), and two RNase III domains (RNase IIIa and RNase IIIb). QDE-2 contains a PAZ domain, a Piwi domain and a DUF1785 domains. Phylogenetic tree showing the relationship of predicted protein sequences of (B) dcl-1, (C) dcl-2, (D) qde-2 and (E) ITS of *P. marneffei* to homologues in other fungi constructed by maximum-likelihood method with *Homo sapiens* (DCL-1, DCL-2 and QDE-2) and *Ustilago maydis* (ITS) as the root. The thermal dimorphic pathogenic...
Northern blot analysis of PM-miR-M1 in wild-type and deletion mutants showed that a band corresponding to the mature miRNA product with approximate size of 21 nt was present in wild-type strain, \(dcl-1^{KO}\) and \(qde-2^{KO}\) mutants, but absent in \(dcl-2^{KO}\) and \(dclDKO\) mutants (Fig. 5A). Moreover, a band with approximate size of 70 nt, which matches the size of the predicted precursor of miRNA (pre-miRNA) of PM-miR-M1, was present in \(dcl-2^{KO}\) and \(dclDKO\) mutants but not in wild-type strain, \(dcl-1^{KO}\) or \(qde-2^{KO}\) mutants. In addition, a band of approximately 30 nt is also seen in \(dcl-2^{KO}\) and \(dclDKO\) mutants but not in wild-type strain, \(dcl-1^{KO}\) or \(qde-2^{KO}\) mutants, which may represent an intermediate product of the precursor. This suggested that DCL-2 protein is required for the biogenesis of mature miRNA from PM-miR-M1 and that the band at about 70 nt is likely the pre-miRNA. In the \(dcl-1^{KO}\) and \(qde-2^{KO}\) mutants, the levels of mature miRNA were similar to that of wild-type, indicating that DCL-1 and QDE-2 are not required for miRNA production from PM-miR-M1.

For PM-miR-M2, the band corresponding to its mature miRNA product, with approximate size of 20 nt, was also present in wild-type strain, \(dcl-1^{KO}\) and \(qde-2^{KO}\) mutants, but was absent in \(dcl-2^{KO}\) and \(dclDKO\) mutants (Fig. 5A). This suggested that DCL-2 protein is also required for the biogenesis of mature miRNA from PM-miR-M2. In the \(dcl-1^{KO}\) and \(qde-2^{KO}\) mutants, the levels of mature miRNA were similar to that of wild-type, indicating that DCL-1 and QDE-2 are not required for miRNA production from PM-miR-M2.

### Predicted miRNA targets in P. marneffei

Among the 24 potential miRNA candidates identified in the present study, 21 were predicted to have potential targets while three have no predicted targets (Supplementary Table S1). One of the candidates, PM-miR-MC17, was predicted to have up to 353 potential targets. These miRNAs candidates with predicted targets bind either perfectly or imperfectly complementary sequences. However, both PM-miR-M1 and PM-miR-M2 were predicted to bind complementary sequences of their targets imperfectly, similar to miRNAs in animals and the filamentous fungus, \(N. crassa\) [30]. The predicted targets of PM-miR-M1 include a putative Ran-binding protein RanBP10, a putative benzoate 4-monooxygenase cytochrome P450 and a conserved hypothetical protein. RanBP10 is a cytoplasmic guanine nucleotide exchange factor that modulates noncentrosomal microtubules involved in mitosis, while cytochrome P450 catalyses diverse reactions in fungal primary and secondary metabolism, and xenobiotic detoxification. As for PM-miR-M2, 20 potential targets were predicted, which include 13 transposon or transposable elements and seven conserved hypothetical proteins.

### Regulation of target gene expression by PM-miR-M1

To test for potential regulation of target gene expression by these miRNAs, we generated a knockdown strain of PM-miR-M1 gene and measured the mRNA expression levels of the three predicted target genes. The knockdown strain, PM-miR-M1\(^{KD}\), only exhibited 8% transcription level of PM-miR-M1 gene in mycelial phase compared to wild type strain PM1 (Fig. 6A). The mRNA expression levels of the three predicted targets, putative RanBP10, putative benzoate 4-monooxygenase cytochrome P450 and a conserved hypothetical protein, were upregulated in PM-miR-M1\(^{KD}\) by 1.9 (Fig. 6B), 1.7 (Fig. 6C) and 3.8 folds (Fig. 6D).
respectively compared to wild type strain PM1 ($P < 0.05$ by student t test).

**Discussion**

This is the first report of milRNAs in a human thermal dimorphic pathogenic fungus and their differential expression in mycelial and yeast phases. RNAi proteins such as Dicer and Argonaute have been identified in many fungi, such as the model filamentous fungus *N. crassa* [63] and fission yeast *Schizosaccharomyces pombe* [64]. Although RNAi proteins were lost in the famous budding yeast *Saccharomyces cerevisiae*, the closely related species *Saccharomyces castellii* encoded a defected but functional Dicer-like homolog [65]. However, till 2005, no endogenous miRNAs have been reported in fungi but only reports of antisense transcripts encoded in the genome of *C. neoformans* [66]. No plant or animal like miRNAs was found in *Aspergillus* species by computational analysis of six *Aspergillus* genomes (*Aspergillus nidulans, Aspergillus oryzae, Aspergillus fumigatus, Aspergillus terreus, Aspergillus clavatus, and Neoartomyces fischeri*) [67]. It was therefore uncertain whether fungi have microRNAs until the recent discovery of miRNAs in the filamentous fungi, *N. crassa, S. sclerotiorum* and *M. anisopliae*, as well as the human pathogenic yeast, *C. neoformans* [30–33]. Nevertheless, the presence of miRNAs in human pathogenic filamentous and dimorphic fungi was largely unknown. We have previously shown that target gene expression can be specifically knocked down by an RNAi-based method in *P. marneffei* [36,40]. Moreover, we found that two *dcl* genes encoding putative dicer-like proteins and a *qde-2* gene encoding a putative Argonaute-like protein, QDE-2, can be identified in *P. marneffei* strain PM1 draft genome, which are known to play key roles in the biogenesis of miRNAs and siRNAs [68]. Since miRNAs are important gene regulatory molecules in multicellular organisms, we hypothesized that *P. marneffei* possesses functional RNAi machinery and may encode miRNAs, which may be involved in the regulation of thermal dimorphism. In this study, using high throughput sequencing of small RNAs extracted from mycelial and yeast cultures of *P. marneffei*, we showed that small RNAs are more abundantly expressed in mycelial than yeast phase by 10 folds. The sequencing result is also in line with the more abundant small RNAs (approximately 20–24 nt) observed in mycelial than yeast phase upon Sybr Gold stained 12% denaturing polyacylamide gel electrophoresis (data not shown). After exclusion of other non-coding RNAs, a total of 2,734 reads were identified as potential miRNA candidates including 17 candidates in mycelial phase and seven in yeast phase, suggesting that miRNAs are differentially

![Figure 5. miRNA biogenesis mechanism for PM-milR-M1 and PM-milR-M2 in *P. marneffei*.](https://doi.org/10.1371/journal.pntd.0002398.g005)
MicroRNA-Like RNAs in Penicillium marneffei

expressed in the two growth phases and may be more abundant in mycelial than yeast phase of P. marneffei. Two milRNAs, PM-miR-M1 and PM-miR-M2, both expressed in mycelial phase, were confirmed by Northern blot analyses. They share similar characteristics to miRNAs in animals and plants, being dependent on a Dicer-like protein for production and arisen from highly specific stem-loop RNA precursors. PM-miR-M1 was also shown to regulate the mRNA expression of its predicted target genes. The present results supported that dimorphic fungi may encode milRNAs which are likely conserved regulators of gene expression in diverse eukaryotes including fungi [18].

DCL-2 is likely a conserved protein involved in milRNA biogenesis among thermal dimorphic fungi. Dicer is a member of RNAse III family of nucleases and is responsible for miRNA processing in animals and plants [18]. While dicer-like proteins are known to be important for RNAi silencing in various fungi [36,40,69,70], its role in milRNAs in fungi has been less well studied. A recent study on N. crassa has revealed diverse pathways in the generation of milRNAs and Dicer-independent small interfering RNAs (disiRNAs) [30]. In this study, the production of PM-miR-M1 and PM-miR-M2, as well as the pre-miRNA of PM-miR-M1, was dependent on the presence of DCL-2 but not DCL-1 or QDE-2 in P. marneffei. The pre-miRNA of PM-miR-M2 was not obvious upon Northern blot analyses, which may be due to degradation into small RNAs because of instability. No identifiable homologues of PM-miR-M1 and PM-miR-M2 could be in animals and plants, which supported the independent evolution of milRNAs in fungi [30,33]. On the other hand, homologues of their precursors can be identified in T. stipitatus (data not shown). Nevertheless, it remains to be determined if such miRNA homologues are also expressed and processed in the same way. Interestingly, in contrast to ITS and del-1 sequences which were both phylogenetically most closely related to the homologues in T. stipitatus, P. chrysogenum and Aspergillus spp., the del-2 gene of P. marneffei is more closely related to the homologues in other geographically restricted thermal dimorphic fungi than to P. chrysogenum and Aspergillus spp.. This suggested that the del-2 gene may have co-evolved among the thermal dimorphic fungi and serve similar function. Since these thermal dimorphic fungi are different from other fungi by their ability to cause systemic mycosis

Figure 6. Regulation of target gene expression by PM-miR-M1. Relative mRNA expression of (A) PM-miR-M1 gene, (B) RanBP10, (C) benzoate 4-monooxygenase cytochrome P450 and (D) a conserved hypothetical protein in mycelial phase of wild type strain PM1 and knockdown strain PM-miR-M1KD by qRT-PCR. Results were obtained from three independent experimental replicates.
doi:10.1371/journal.pntd.0002398.g006
as intracellular yeasts and survive in natural environments as molds, it would be interesting to explore the potential role of DCL-2 in fungal dimorphism as well as virulence. In *N. crassa*, at least four different mechanisms that involved a combination of factors were identified for the production of miRNAs. In fact, apart from direct and QDE-2, homologues of QDE-2 interacting protein (QIP) and mitochondrial ribosomal protein L3 (MRPL5), which were also involved in biogenesis of some miRNAs in *N. crassa* [30], can also be found in the *P. marneffei* genome, with 27–49% amino acid identities (data not shown). Further studies are required to explore for possible role of these proteins in miRNA biogenesis in *P. marneffei*.

In contrast to miRNAs from animals and plants which are known to play different functions from multicellular development to stress response, the potential function(s) of miRNAs in fungi remain to be determined. Some miRNAs in plants and animals are known to exhibit temporal or tissue-specific expression patterns [18,71,72]. As for fungi, a recent study showed that some miRNAs are differentially expressed in sclerotial development of *S. sclerotiorum* [31]. In *C. neoformans*, miRNAs were shown to cause transgene silencing via the canonical RNAi pathway and proposed to play a role in regulating transposons and pseudogene expression [33]. In this study, we showed the mRNA expression level of *dcl-2* was higher in mycelial than yeast phase, suggesting that DCL-2 may function predominantly in the mycelial phase. This, in turn, may explain why *PM-miR-M1* and *PM-miR-M2* were only expressed in mycelial but not yeast form of *P. marneffei*. Therefore, it is likely that *PM-miR-M1* and *PM-miR-M2* are only produced from DCL-2 and serve important function during mycelial phase. A number of potential targets were predicted for both *PM-miR-M1* and *PM-miR-M2*. For example, the predicted targets of *PM-miR-M1* include *RanBP10* and cytchrome P450, while transposon or transposable elements were the predominant predicted targets of *PM-miR-M2*. The targets of *PM-miR-M1* were also confirmed to be upregulated at the RNA level in the knockdown strain, *PM-miR-M1* [30], supporting the mRNA cleavage function of miRNAs. These results suggested that miRNAs in *P. marneffei* may regulate cell division, metabolism as well as transposons, although further studies are required to investigate their biological function. Nevertheless, the present study demonstrated the potential role of differential post-transcriptional control in different growth phases of thermal dimorphic fungi, which may provide new insights into the mechanism governing thermal dimorphism.

### Supporting Information

#### Table S1 Predicted targets of miRNAs in *P. marneffei*.

| Target         | Function          |
|----------------|-------------------|
| *RanBP10*      | Transcription     |
| Cytochrome P450 | Cell cycle        |
| *PM-miR-M1*    | Transposon        |
| *PM-miR-M2*    | Stress response   |

### Acknowledgments

We thank Dr P.J. Punt for providing us with the pAN7-1 and pAN8-1 plasmids.

### Author Contributions

Conceived and designed the experiments: SKPL WNC SL PCYW KYY. Performed the experiments: WNC AYPW NZ JB. Analyzed the data: SKPL WNC AYPW JB SL. Contributed reagents/materials/analysis tools: SKPL SL PCYW KYY. Wrote the paper: SKPL WNC PCYW KYY.

### References

1. Hauch PR, Teng LJ, Hung CC, Hu JH, Yang PC, et al. (2000) Molecular evidence for strain dissemination of *Penicillium marneffei*: an emerging pathogen in Taiwan. J Infect Dis 181: 1706–1712.
2. Supparatpinyo K, Khamwan C, Baosoung V, Nelson KE, Sirisanthana T (1994) Disseminated *Penicillium marneffei* infection in southeast Asia. Lanceet 344: 110–113.
3. Huang YT, Hung CC, Liao CH, Sun HY, Chang SC, et al. (2007) Detection of *Penicillium marneffei* infection and aspergillosis among patients infected with human immunodeficiency virus. J Clin Microbiol 45: 2530–2532.
4. Yuen KY, Wong SS, Tsang DN, Chau PY (1994) Serodiagnosis of *Penicillium marneffei* infection. Lancet 344: 444–445.
5. Samson RA, Yilmaz N, Houbenaken J, Spierebrugge H, Seifert KA, et al. (2011) Phylogeny and nomenclature of the genus *Talassomyces* and taxa accommodated in *Penicillium subgenus Biverticillium*. Stud Mycol 70: 159–183.
6. Chariyalertaks S, Vanittanakom P, Nelson KE, Sirisanthana T, Vanitakom N (1996) *Rhizomyces simonatus* and *Cannomyces ladus*, new natural animal hosts of *Penicillium marneffei*. J Med Vet Mycol 34: 105–110.
7. Deng ZL, Yuan M, Ajello L (1986) Human penicilliosis marneffei and its relation to the bamboo rat (*Rhizomyces simonatus*). J Med Vet Mycol 24: 383–389.
8. Deng ZL, Connor DH (1995) Progressive disseminated penicilliosis caused by *Penicillium marneffei*. Report of eight cases and differentiation of the causative organism from *Penicillium marneffei* and *Candida tropicalis*. J Med Vet Mycol 34: 105–110.
9. Lo CY, Chan DT, Yuen KY, Li FK, Cheng KP (1995) *Penicillium marneffei* infection in a patient with SLE. Lupus 4: 229–231.
10. Wang JL, Hung CC, Chang SC, Chueh SC, La MK (2003) Disseminted *Penicillium marneffei* infection in a renal-plantasent recipient successfully treated with liposomal amphotericin B. Transplantation 76: 1136–1137.
11. Vanittanakom N, Jr Cooper CR, Fisher MC, Sirisanthana T (2006) *Penicillium marneffei* mixed fungaemia in a patient with Waldenstrom's macroglobulinaemia. Eur J Clin Microbiol Infect Dis 25: 132–135.
12. Woon PC, Lau SK, Lau CH, Chong KT, Hui WT, et al. (2005) *Penicillium marneffei* fungaemia in an agalgenic bone marrow transplant recipient. Bone Marrow Transplant 35: 831–833.
13. Boyce KJ, Andrianopoulos A (2013) Morphogenetic circuitry regulating growth and development in the dimorphic pathogen *Penicillium marneffei*. Eukaryot Cell 12: 154–160.
14. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.
15. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. Cell 115: 767–778.
16. Li X, Carthew RW (2005) A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the *Drosophila* eye. Cell 123: 1267–1277.
17. Xiao C, Rajewsky K (2009) MicroRNA control in the immune system: basic principles. Cell 138: 28–30.
18. Bartel DP (2004) MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57: 19–53.
19. Navarro L, Dunoyer P, Fay J, Arnold B, Dhammasri N, et al. (2006) A plant mirRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312: 436–439.
20. Baulcombe D (2004) RNA silencing in plants. Nature 403: 901–906.
21. Griffith-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34(Database issue): D140–D144.
22. Lee HC, Li L, Gu W, Xue Z, Crosthwaite SK, et al. (2010) Diverse pathways generate microRNA-like RNAs and Dicer-independent small interfering RNAs in *C. elegans*. Cell 143: 797–809.
23. Xiao C, Rajewsky K (2009) MicroRNA control in the immune system: basic principles. Cell 138: 28–30.
24. Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57: 19–53.
25. Navarro L, Dunoyer P, Fay J, Arnold B, Dhammasri N, et al. (2006) A plant mirRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312: 436–439.
26. Baulcombe D (2004) RNA silencing in plants. Nature 403: 935–936.
27. Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell 75: 843–854.
28. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, et al. (2000) The 21-nucleotide 46–27 RNA regulates developmental timing in *C. elegans*. Nature 403: 491–496.
29. Grünfeld-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34(Database issue): D140–D144.
31. Zhou J, Yu Y, Xie J, Li B, Jiang D, et al. (2012) Identification of microRNA-like RNAs in a plant pathogenic fungus *Sclerotinia sclerotiorum* by high-throughput sequencing. Mol Genet Genomics 287: 275–282.

32. Zhou Q, Wang Z, Zhang J, Meng H, Huang B (2012) Genome-wide identification and profiling of microRNA-like RNAs from *Methanobrevibacter smithii* during development. Fungal Biol 116: 1156–1162.

33. Jiang N, Yang Y, Jianbo G, Pan J, Zhi X (2012) Identification and functional demonstration of miRNAs in the fungus *Cryptococcus neoformans*. PLoS One 7: e25724.

34. Woo PC, Zhen H, Cai JJ, Yu J, Lau SK, et al. (2003) The mitochondrial genome of the thermophilic fungus *Penicillium marneffei* is more closely related to those of molds than yeasts. FEBS Lett 535: 469–477.

35. Woo PC, Chong KT, Tse H, Cai JJ, Lau CC, et al. (2006) Genomic and experimental evidence for a potential sexual cycle in the pathogenic thermophilic fungus *Penicillium marneffei*. FEBS Lett 580: 3409–3416.

36. Woo PC, Tam EW, Chong KT, Cai JJ, Tung ET, et al. (2010) High diversity of polyketide synthase genes and the melanin biosynthesis gene cluster in *Penicillium marneffei*. FEBS J 277: 3750–3758.

37. Yuen KY, Pascal G, Wong SS, Glaser P, Woo PC, et al. (2003) Exploring the *Penicillium marneffei* genome. Arch Microbiol 179: 339–353.

38. Woo PC, Lam CW, Tam EW, Leung CK, Wong SS, et al. (2012) First discovery of two polyketide synthase genes for mitochondrially encoded yellow pigment biosynthesis and implications in virulence of the pathogenic fungus *Penicillium marneffei*. PLoS Negl Trop Dis 6: e1871.

39. Jiang N, Yang Y, Janbon G, Pan J, Zhu X (2012) Identification and functional demonstration of microRNAs in the fungus *Penicillium marneffei* genome. Arch Microbiol 179: 339–353.

40. Zhou Q, Wang Z, Zhang J, Meng H, Huang B (2012) Genome-wide identification and profiling of microRNA-like RNAs from *Methanobrevibacter smithii* during development. Fungal Biol 116: 1156–1162.

41. Henk DA, Shahar-Golan R, Devi KR, Boyce KJ, Zhan N, et al. (2012) Clonality based multilocus sequence system for typing the pathogenic fungus *Penicillium marneffei*. Arch Microbiol 179: 339–353.

42. Hafner M, Landgraf P, Ludwig J, Rice A, Ojo T, et al. (2008) Identification of conserved secondary structures in *Penicillium marneffei*. FEBS J 275: 3750–3758.

43. Fulci V, Macino G (2007) Quelling: post-transcriptional gene silencing guided by small RNAs in the basidiomycetous yeast and human pathogen *Neurospora crassa*. FEMS Microbiol Lett 262: 407–415.

44. McGuire AM, Galagan JE (2008) Conserved Secondary Structures in *Neurospora crassa*. FEMS Microbiol Lett 262: 407–415.

45. Drinnenberg IA, Weinberg DE, Xie KT, Mower JP, Wolfe KH, et al. (2009) Gene silencing in budding yeast. SCIENCE 325: 764–768.

46. Loftus BJ, Fung E, Roncaglia P, Rowley D, Amedeo P, et al. (2005) The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. Science 307: 1321–1324.

47. Carthew RW, Sontheimer EJ (2009) Origins and Mechanisms of miRNAs and siRNAs. Cell 136: 642–653.

48. Segers GC, Zhang X, Deng F, Sun Q, Nuss DL (2007) Evidence that RNA silencing functions as an antiviral defense mechanism in fungi. Proc Natl Acad Sci U S A 104: 12902–12906.

49. Kadotani N, Nakayashiki H, Tosa Y, Mayama S (2004) One of the two Dicer-family RNase III enzymes is required for RNA silencing in the basidiomycete fungus *Neurospora crassa*. Genes Dev 18: 2353–2356.

50. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10: R25.

51. Friedlander MR, Chen W, Adamidi C, Maaskola J, Einzinger R, et al. (2008) Discovering microRNAs from deep sequencing data using miDeep. Nat Biotechnol 26: 407–415.

52. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731–2739.

53. Prasad M, Vouloumanos AJ, Xu Z, Wang X, Chiang YH, et al. (2009) RNAi in budding yeast. SCIENCE 325: 764–768.

54. Lofts BJ, Fung E, Roncaglia P, Rowley D, Amedeo P, et al. (2005) The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. SCIENCE 307: 1321–1324.

55. McGuire AM, Galagan JE (2008) Conserved Secondary Structures in *Aspergillus*. PLoS ONE 3(7): e2812.

56. Carthew RW, Sontheimer EJ (2009) Origins and Mechanisms of miRNAs and siRNAs. Cell 136: 642–653.

57. Segers GC, Zhang X, Deng F, Sun Q, Nuss DL (2007) Evidence that RNA silencing functions as an antiviral defense mechanism in fungi. Proc Natl Acad Sci U S A 104: 12902–12906.

58. Kadotani N, Nakayashiki H, Tosa Y, Mayama S (2004) One of the two Dicer-like proteins in the filamentous fungus *Magnaporthe oryzae* genome is responsible for hairpin RNA-triggered RNA silencing and related small interfering RNA accumulation. J Biol Chem 279: 4467–4474.

59. Rehmeier M, Steffen P, Hochmann M, Giegerich R (2004) Fast and effective prediction of microRNA/target duplexes. RNA 10: 1507–1517.

60. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 127: 723–730.

61. Nakayashiki H, Hanada S, Nguyen BQ, Kadotani N, Tosa Y, et al. (2005) RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet Biol 42: 273–283.

62. Smitya G, Moson S, Santos DM, Jing R, Fevereiro MP, et al. (2008) High-throughput sequencing of Medicago truncatula short RNAs identifies eight new miRNA families. BMC Genomics 9:593.

63. Rathjen T, Pais H, Sweetman D, Moulton V, Munsterberg A, et al. (2009) High-throughput sequencing of microRNAs in chicken somites. FEBS Lett 583:1422–1426.

64. Mukherjee K, Campos H, Kolaczkowski B (2013) Evolution of animal and plant dicers: early parallel duplications and recurrent adaptation of antiviral RNA silencing in plants. Mol Biol Evol 30: 5237–5246.

65. van Mierlo JT, Bronkhorst AW, Overeul GD, Sadaanand SA, Ektrom JO, et al. (2012) Convergent evolution of argonaute-2 slicer antagonism in two distinct insect RNA viruses. PLoS Pathog 8: e1002872.

66. Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CA (1987) Transformation of *Neurospora crassa* with transforming DNA. Mol Cell Biol 4: 3407–3425.

67. Sigova A, Rhind N, Zamore PD (2004) A single Argonaute protein mediates both transcriptional and posttranscriptional silencing in *Arabidopsis thaliana*. Genes Dev 20: 3407–3425.

68. Fulci V, Macino G (2007) Quelling: post-transcriptional gene silencing guided by small RNAs in *Neurospora crassa*. Curr Opin Microbiol 10: 199–203.

69. Segers GC, Zhang X, Deng F, Sun Q, Nuss DL (2007) Evidence that RNA silencing functions as an antiviral defense mechanism in fungi. Proc Natl Acad Sci U S A 104: 12902–12906.

70. Kadotani N, Nakayashiki H, Tosa Y, Mayama S (2004) One of the two Dicer-like proteins in the filamentous fungus *Magnaporthe oryzae* genome is responsible for hairpin RNA-triggered RNA silencing and related small interfering RNA accumulation. J Biol Chem 279: 4467–4474.

71. Mc Guire AM, Galagan JE (2008) Conserved Secondary Structures in *Aspergillus*. PLoS ONE 3(7): e2812.