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Development of Two Molecular Approaches for Differentiation of Clinically Relevant Yeast Species Closely Related to *Candida guilliermondii* and *Candida famata*

Xiaobo Feng,[a] Jingsong Wu,[b] Bo Ling,[a] Xianwei Yang,[a] Wanqing Liao,[c] Weihua Pan,[c] Zhirong Yao[a]

Medical Mycology Laboratory, Department of Dermatology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, People's Republic of China[a]; Department of Laboratory Medicine, Shenzhen People's Hospital, Second Clinical Medical College of Jinan University, Shenzhen, People's Republic of China[b]; Shanghai Key Laboratory of Molecular Medical Mycology, Shanghai Institute of Medical Mycology, Changzheng Hospital, Second Military Medical University, Shanghai, People's Republic of China[c]

The emerging pathogens *Candida palmioleophila*, *Candida fermentati*, and *Debaryomyces nepalensis* are often misidentified as *Candida guilliermondii* or *Candida famata* in the clinical laboratory. Due to the significant differences in antifungal susceptibilities and epidemiologies among these closely related species, a lot of studies have focused on the identification of these emerging yeast species in clinical specimens. Nevertheless, limited tools are currently available for their discrimination. Here, two new molecular approaches were established to distinguish these closely related species. The first approach differentiates these species by use of restriction fragment length polymorphism analysis of partial internal transcribed spacer 2 (ITS2) and large subunit ribosomal DNA with the enzymes BsaHI and XbaI in a double digestion. The second method involves a multiplex PCR based on the intron size differences of *RPL18*, a gene coding for a protein component of the large (60S) ribosomal subunit, and species-specific amplification. These two methods worked well in differentiation of these closely related yeast species and have the potential to serve as effective molecular tools suitable for laboratory diagnoses and epidemiological studies.

Newly emerging species that are closely related to the common *Candida* species pose a challenge to conventional methods performed in the clinical laboratory (1–4). These closely related species actually belong to diverse species complexes, as revealed by sequence and phylogenetic analyses. Recent advances in molecular techniques have allowed differentiation of these species complexes, such as the *Candida albicans* complex composed of *C. albicans*, *Candida dubliniensis*, *Candida africana*, and *Candida stellatoidea* type I, the *Candida parapsilosis* complex composed of *C. parapsilosis*, *Candida orthopsilosis*, *Candida metapsilosis*, and *Lodderomyces elongisporus*, and the *Candida glabrata* complex composed of *C. glabrata*, *Candida nivariensis*, and *Candida bracarensis* (1, 5–17).

Candida guilliermondii (the anamorph of *Pichia guilliermondii*), a species with decreased susceptibility to fluconazole and echinocandins, was reported as a common cause of candidiasis and sometimes even candidemia (18–21). *Candida fermentati* (the anamorph of *Pichia caribbica*), an emerging species that is very closely related to *C. guilliermondii*, has often been misidentified as *C. guilliermondii* using routine identification methods (22–26). Because of lower susceptibility to triazoles, *Candida palmioleophila*, which is often misidentified as *Candida famata* (the anamorph of *Debaryomyces hansenii*) or *C. guilliermondii*, has been emphasized in recent studies (24, 25, 27). Additionally, *Debaryomyces nepalensis* and *Debaryomyces fabryi*, two species that are closely related to *C. famata*, have been isolated from clinical samples, including blood (22, 28, 29), and also are potential pathogens. The above-mentioned yeast species are likely to be confused with one another by conventional identification methods. In particular, isolates confused with *C. famata* were frequently misidentified, and *C. famata* was, in fact, very rare in clinical specimens (22, 24, 25, 27).

Currently, limited tools are available for molecular differentiation of these closely related yeast species. Molecular methods such as PCR-restriction fragment length polymorphism (RFLP), real-time PCR, Luminex techniques, electrophoretic karyotyping, and PCR with type-specific primers have been developed to distinguish some of these species, but other species were not involved in those studies (30–33). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is a promising technique that works well to distinguish yeast species, although a few closely related species (such as *C. fermentati*) may still be misidentified (34–36).

Because the internal transcribed spacer (ITS) regions (ITS1, 5.8S, and ITS2) and the D1 and D2 regions of the large ribosomal subunit were confirmed as the most useful targets for species-level identification of yeasts (37–39), these regions were sequenced for these closely related yeast species, for further RFLP analysis. In addition, PCR analyses based on intron size differences or intron loss were used to easily differentiate closely related yeast species in previous studies (9, 16, 40, 41). Because ribosomal protein-coding genes carry longer introns than nonribosomal protein-coding genes (16, 42), the *RPL18* gene, coding for a protein component of the large (60S) ribosomal subunit, was chosen, and a long intron within this gene was characterized for *D. hansenii* type strain

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Address correspondence to Weihua Pan, panweihua@medmail.com.cn, or Zhirong Yao, dermatology.yao@sohu.com.
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TABLE 1 Primers used in this study

Primer	Sequence (5′ to 3′)	Position	Purpose
ITS1	TCCGTAGGTGAACCTGCGG	18S rDNA	ITS sequencing
ITS4	TCCTCCGCTTATTGATATGC	26S rDNA	ITS sequencing
IGS1F	TGTAAGCAGTAGAGTAGCCTTGTTG	26S rDNA	IGS1 sequencing
IGS1R	AGACCGAGTAGTGTAGTGGGAGAC	5S rDNA	IGS1 sequencing
ITS2F	GATGTATTAGGTTTATCCAACTCGT	ITS2 rDNA	PCR-RFLP assay
26SR	TCATTTCAACCCCAATACCTC	26S rDNA	PCR-RFLP assay
DG5F	GCCCTCCTTCTTAGCTCGTAWGTAT	*RPL18* gene	Multiplex PCR assay
DG5R	GGCAGATGACCTTGTTGAATGG	*RPL18* gene	Multiplex PCR assay
GuF	TGCTATATCTTTGGCTCAGCG	IGS1 rDNA	Multiplex PCR assay
GuR	GTCGTCTAGCATTGGTTTTGACT	IGS1 rDNA	Multiplex PCR assay
PalF	GCGGCGAATTGTTATTTAATACT	ITS1 rDNA	Multiplex PCR assay
PalR	GTGAATGCACTTCTCAGCGTC	ITS2 rDNA	Multiplex PCR assay

CBS767 (GenBank accession no. NC_006048; locus tag DEHA 2F16566g). Differences in intron sizes among these closely related species were analyzed for further multiplex PCR assays. In this study, two PCR-based methods were established to distinguish *C. guilliermondii* complex, *C. famata* complex, and the closely related species *C. palmioleophila*; both of them are simple, inexpensive, and reliable molecular tools.

MATERIALS AND METHODS

Strains and identification. One hundred six strains, including 46 *C. guilliermondii* isolates, 19 *C. famata* isolates, 17 *C. palmioleophila* isolates, 11 *C. fermentati* isolates, 8 *D. nepalensis* isolates, and 5 *D. fabryi* isolates, were included in this study. Among them, 29 were type strains from the CBS and NRRL culture collections and 77 were clinical isolates, of which 27 were kindly provided by other researchers (see Acknowledgments) and 50 were from our own collection. Genomic DNA was extracted with the MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI), according to the manufacturer's instructions. All isolates were identified and confirmed by sequencing of internal transcribed spacers 1 and 2, including the 5.8S ribosomal DNA (rDNA) (ITS) region, using primers ITS1 and ITS4 (Table 1), as described previously (40). In order to distinguish among *D. fabryi*, *D. nepalensis*, and *C. famata*, strains identified as *C. famata* with ITS sequencing were further analyzed by amplification and sequencing of the intergenic spacer 1 (IGS1) region using primes IGS1F and IGS1R (Table 1). Species identification was determined by comparison of the DNA sequences of PCR products with corresponding sequences of the type strains using the BLASTN tool online. Additionally, other common pathogenic *Candida* species, including *C. albicans*, *C. glabrata*, *Candida tropicalis*, *C. parapsilosis*, *Candida krusei*, and *Candida lusitaniae*, were tested as controls.

Sequence and phylogenetic analyses of ITS region. Sequence alignments were conducted using ClustalW2. Phylogenetic analysis based on the ITS sequences was performed by using MEGA 6.05 software to clarify the genetic relationships among these closely related species. A total of 76 selected sequences together with 10 sequences available from GenBank were included in the analysis. A dendrogram was produced by use of neighbor-joining analysis using a Kimura 2-parameter model. Gaps were treated as pairwise deletions. Statistical support for each clade was assessed using bootstrap analysis with 500 replicates. The sequence from *Saccharomyces cerevisiae* type strain ATCC MYA-4900 was used to root the tree.

PCR-RFLP analysis of partial ITS2 and 26S rDNA. Primers specific for partial ITS2 and 26S rDNA, including the D1/D2 region, of the *C. guilliermondii* complex, the *C. famata* complex, and *C. palmioleophila* were designed based on the consensus nucleotide sequences of the ITS2 and 26S rDNA regions, respectively, of the reference strains. PCR was performed in a final volume of 50 μl containing 50 ng DNA, 1× PCR buffer with 2 mM $MgSO_4$, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, 0.2 μM each primer, and 2.5 U of *Taq* polymerase. PCR was performed in a Bio-Rad thermal cycler, with initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 50 s, and final extension at 72°C for 8 min. PCR products were separated on a 1.0% (wt/vol) agarose gel at 140 V for 20 min. Amplicons were doubly digested with FastDigest enzymes BsaHI and XbaI for RFLP analysis. Restriction digestions were performed according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania), and the reaction mixtures were incubated at 37°C for 30 min before separation on a 2.0% (wt/vol) agarose gel at 90 V for 50 min.

Multiplex PCR assay based on *RPL18* gene, ITS, and IGS1 region. The homologous sequences of the *RPL18* gene in the reference strains *C. guilliermondii* Y-324, *C. fermentati* Y-27403, *C. palmioleophila* Y-17323, *D. fabryi* CBS796, and *D. nepalensis* CBS2334 were cloned and sequenced. Then, intron lengths of the *RPL18* gene were calculated as 182 bp for *C. guilliermondii*, 191 bp for *C. fermentati*, 391 bp for *C. palmioleophila*, 472 bp for *C. famata*, 473 bp for *D. fabryi*, and 401 bp for *D. nepalensis*. Specific primers for amplification of the *RPL18* gene of the *C. guilliermondii* complex, the *C. famata* complex, and *C. palmioleophila*, DG5F and DG5R, were designed based on the consensus nucleotide sequences of the *RPL18* gene orthologs of the reference strains. *C. guilliermondii*-specific primers GuF and GuR were designed based on the IGS1 region of rDNA, and the *C. palmioleophila*-specific primer pair of PalF and PalR was designed based on the ITS region of rDNA. A multiplex PCR with the six primers was established and performed in a final volume of 50 μl containing 50 ng DNA, 1× PCR buffer with 2 mM $MgSO_4$, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, 0.2 μM each primer, and 2.5 U of *Taq* polymerase. PCR was performed in a Bio-Rad thermal cycler, with initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 7 min. PCR products were separated on a 2.0% (wt/vol) agarose gel at 100 V for 1 h. All PCRs were conducted in duplicate. The primers used are listed in Table 1.

Nucleotide sequence accession numbers. The sequences of the *RPL18* gene fragments of the reference strains were deposited in GenBank under accession numbers KJ705079 to KJ705083. The ITS and partial 26S rDNA sequences obtained in this study were deposited in GenBank under accession numbers KJ705003 to KJ705078.

RESULTS

Sequence alignment and phylogenetic analysis based on ITS region. The ITS sequences of the *C. guilliermondii* group and the *C. fermentati* group were 99% identical, showing only four distinguishable nucleotide differences. The ITS sequences of the *C. famata* group and the *D. nepalensis* group were 99% identical, showing five distinguishable nucleotide differences. The ITS sequences of the *C. famata* group and the *D. fabryi* group were 99% to 100% identical, showing no discernible nucleotide differences. Phylogenetic analysis showed that *C. famata*, *D. fabryi*, and *D. nepalensis* constitute one cluster, which has a sibling relationship

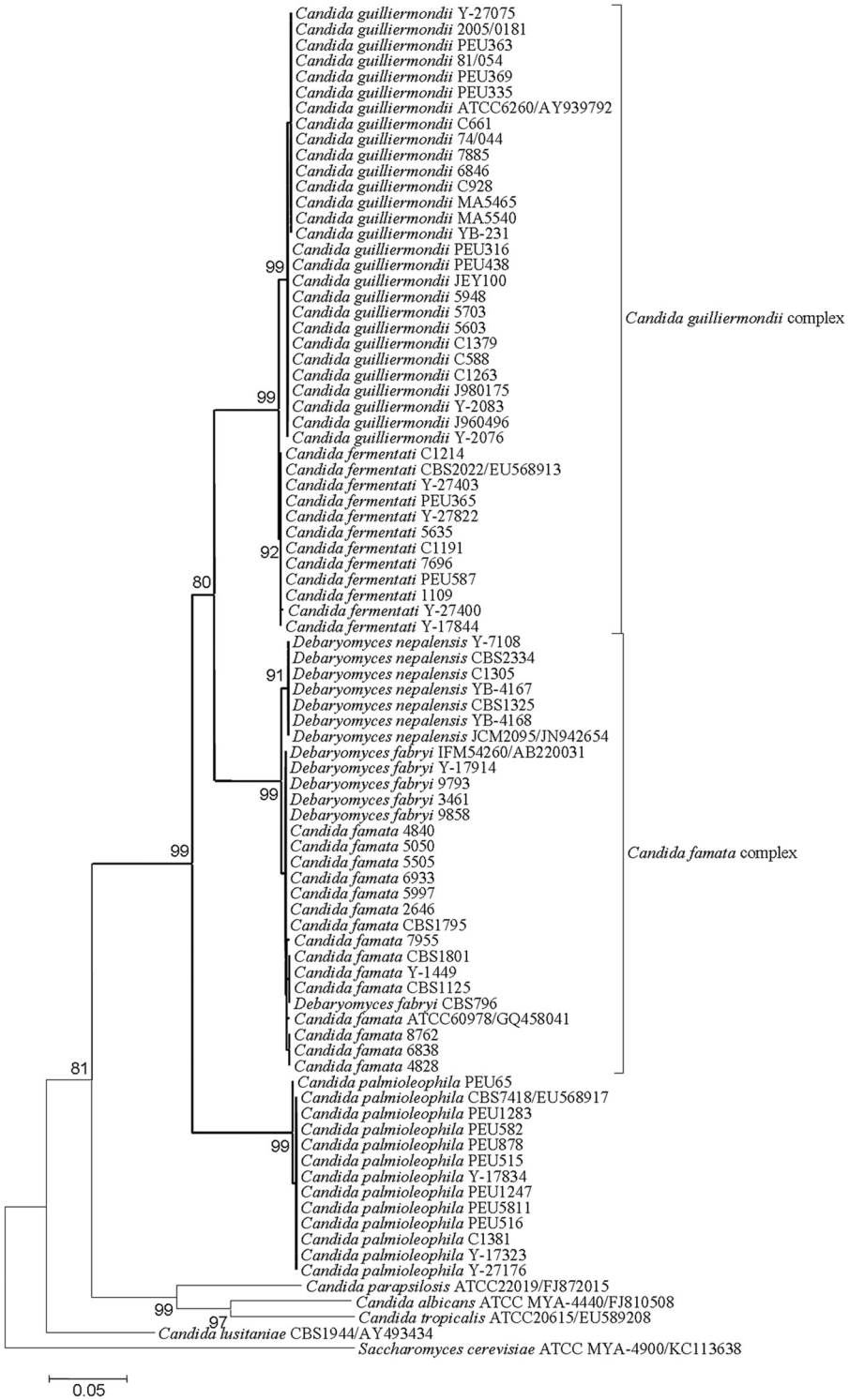

FIG 1 Neighbor-joining tree for *Candida* strains determined through analysis of ITS sequences. The *Saccharomyces cerevisiae* type strain was used as an outgroup. Bootstrap values of >70% are indicated for the main branches. Clusters containing the *C. famata* complex, the *C. guilliermondii* complex, and *C. palmioleophila* are shown in bold type.

with the cluster composed of *C. guilliermondii* and *C. fermentati*. The *C. palmioleophila* clade clustered basal to the aforementioned species with strong bootstrap support (Fig. 1).

Molecular identification using PCR-RFLP analysis. PCR using the primer set ITS2F-26SR yielded about 1.1-kb amplicons for strains of the *C. guilliermondii* complex, the *C. famata* complex, and *C. palmioleophila*, whereas no amplicon was produced from the other *Candida* species tested. In concordance with the *in silico*

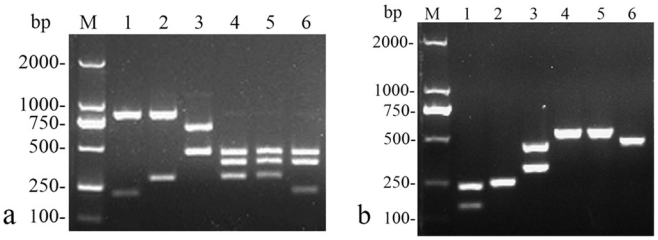

FIG 2 Agarose gel electrophoresis of enzyme-digested products (a) and amplicons from the multiplex PCR assay (b). Lanes M, DL2000 ladder; lanes 1, CBS2030 (*C. guilliermondii*); lanes 2, Y-27403 (*C. fermentati*); lanes 3, Y-17323 (*C. palmioleophila*); lanes 4, CBS1795 (*C. famata*); lanes 5, CBS796 (*D. fabryi*); lanes 6, CBS2334 (*D. nepalensis*). Bands of <100 bp in the RFLP analysis are not shown.

analysis, RFLP patterns of amplification products from the *C. guilliermondii*, *C. fermentati*, *C. palmioleophila*, *C. famata*, *D. fabryi*, and *D. nepalensis* strains showed 3 bands (822 bp, 212 bp, and 76 bp), 2 bands (822 bp and 288 bp), 2 bands (660 bp and 446 bp), 3 bands (447 bp, 375 bp, and 290 bp), 3 bands (447 bp, 375 bp, and 290 bp), and 4 bands (447 bp, 375 bp, 214 bp, and 76 bp), respectively (Fig. 2a). All included isolates belonging to the *C. guilliermondii* complex, the *C. famata* complex, or *C. palmioleophila* were correctly identified by using this analysis.

Molecular determination by multiplex PCR assay. The multiplex PCR assay resulted in amplified products of approximately 235 bp and 151 bp for *C. guilliermondii*, 244 bp for *C. fermentati*, 444 bp and 317 bp for *C. palmioleophila*, 525 bp for *C. famata*, 526 bp for *D. fabryi*, and 454 bp for *D. nepalensis* (Fig. 2b). Negative results were obtained for the other *Candida* species tested. All isolates belonging to the *C. guilliermondii* complex, the *C. famata* complex, or *C. palmioleophila* that were included herein were also successfully identified by use of this assay.

DISCUSSION

Accurate identification of clinical isolates closely related to *C. guilliermondii* or *C. famata* has clinical importance because of the remarkably diverse susceptibility profiles of these species (22–24). Because these genetically related species are difficult to identify by conventional methods, molecular techniques are more suitable for their differentiation (24, 30). ITS sequence analysis has been widely used as the gold standard for yeast species identification and for phylogenetic analysis (24, 25, 38). In this study, *D. nepalensis* exhibits little divergence from *D. hansenii* based on ITS sequences, like the *C. guilliermondii* complex. So, we propose that *D. nepalensis* may be a member of the *C. famata* complex. *D. fabryi* was proposed as a species instead of *D. hansenii* var. *fabryi* in several studies (32, 42). In the present study, however, no distinguishable nucleotide difference within the ITS region was found to distinguish this species from *D. hansenii*. Similarly, the PCR-RFLP and multiplex PCR assays developed here did not distinguish between them and, in the latter analysis, no marked difference in intron lengths within the *RPL18* gene was observed between the two species. According to the above-mentioned molecular evidence, we think that the nomination of *D. fabryi* needs to be reevaluated. *C. guilliermondii* and *C. fermentati* are very closely related species and constitute the *C. guilliermondii* complex; slight intron differences in the *RPL18* gene were observed between them, similar to the observations for another two closely related *Candida* species, i.e., *C. orthopsilosis* and *C. metapsilosis*, published previously (9). Thus, a *C. guilliermondii*-specific primer pair targeting the IGS1 region, which shows extensive sequence divergence between *C. guilliermondii* and *C. fermentati*, was designed to further differentiate *C. guilliermondii* from *C. fermentati* in the multiplex PCR assay (32). Similarly, a *C. palmioleophila*-specific primer pair was designed to distinguish *C. palmioleophila* from the other species, because the ITS sequence of *C. palmioleophila* is highly divergent from those of the *C. guilliermondii* complex and the *C. famata* complex.

In the present study, PCR analysis based on intron length differences combined with species-specific amplification, in which fewer primers were used than in regular multiplex PCR, successfully identified these closely related species. PCR-RFLP analysis based on two hypervariable parts of the rDNA region that are most commonly used for yeast species identification also works well for differentiation. Results from the two molecular assays developed herein are exactly consistent with the results of ITS sequencing, thus providing two rapid reliable methods for laboratory identification of clinically relevant species closely related to *C. guilliermondii* and *C. famata*.

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