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Rapid Differentiation of Cryptic Species within *Cryptococcus gattii* by a Duplex PCR Assay

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***Cryptococcus gattii* consists of four cryptic species, VGI, VGII, VGIII, and VGIV. Herein, a duplex PCR assay using two primer pairs targeting the vacuolar membrane gene and the intergenic spacer region was developed. It successfully distinguished the cryptic species according to the distinct size of the amplicons.**

The basidiomycetous yeast *Cryptococcus gattii* is a primary pathogen that has a predilection for infecting immunocompetent individuals. A wide geographic distribution, other than restriction in tropical and subtropical areas, has been recognized for this species in many studies (1–7). Based on genetic differences, four molecular types, VGI to VGIV, within *C. gattii* have been determined (8). The four molecular types are also considered cryptic species due to sequence divergence and no nuclear recombination events observed between them (1, 9). The cryptic species differ in their biologies, epidemiologies, virulence characteristics, antifungal susceptibilities, and geographic distributions (1–16). Among the four cryptic species, VGII is more concerning because of the outbreak of the *C. gattii* VGII genotype in the Pacific Northwest and Australia, as well as its higher virulence in experimental models, mating efficiency, and decreased susceptibility to antifungal agents (1, 10–14, 16). In addition, VGI accounts for the most cryptococcal cases caused by *C. gattii* around the world and has the widest geographic distribution (1, 2, 7); VGIII and VGIV are more geographically restricted and usually associated with HIV-infected cases (5, 15).

Several molecular tools have been developed to distinguish the four cryptic species (1, 7, 8, 16–21). However, these techniques include time-consuming and laborious PCR-based methods or molecular methods requiring expensive instruments. PCR analyses of differences in intron size or intron loss have been established to differentiate closely related species of *Candida* and the *Cryptococcus neoformans-C. gattii* species complex and have been confirmed to be simple, inexpensive, and reliable methods (22, 23). Recently, genomic studies revealed that intron loss was also a phylogenetic marker for distinct cryptic species within *C. gattii* (24).

In this study, adjacent introns within protein-coding genes were detected and compared between type strains WM276 (VGI) and R265 (VGII) by the LAGAN tool as previously described (22). The putative vacuolar membrane gene was chosen due to its different types of intron loss among the type strains of WM276 (VGI), R265 (VGII), and WM779 (VGIV), as well as WM161 (VGIII), which is identical to R265 (VGII) (Fig. 1). No intron difference among all four cryptic species was found in our study, which corresponded with a recent report (24). Additionally, a VGII-specific primer pair which could specifically yield a 156-bp amplicon for VGII strains by PCR analysis (25) was utilized herein to distinguish VGII from VGIII.

A *C. gattii*-specific primer pair targeting the vacuolar membrane gene, VACF (5′ AGCCCACGGCAAAATAGTG 3′) and VACR (5′ CACGGTCCAAAACTTGATTGTT 3′), was designed. A duplex PCR system using two primer pairs, VACF and VACR and IGSF (5′ CCGAGGCAGGACACACATAC 3′) and IGSR (5′ GGCGGAATACAAATACTACTTACCT 3′), was established. PCR was performed in a final volume of 50 μl containing 50 ng DNA, 1× PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, 0.2 μM of each primer, and 1.5 U of *Taq* polymerase. PCR was conducted in a Bio-Rad thermal cycler at 94°C for 5 min for initial denaturation, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 20 s, with a final extension step at 72°C for 6 min. PCR products were separated on a 2% (wt/vol) agarose gel at 90 V for 2 h. All PCRs were conducted in duplicate. Strains tested included *C. gattii* ($n = 80$), *C. neoformans* ($n = 16$), and other pathogenic yeast species ($n = 42$). The latter were involved to detect the specificity of the primer pairs. Genomic DNA was prepared from each strain as described previously (22). Detailed information regarding the strains tested here is presented in Table S1 in the supplemental material.

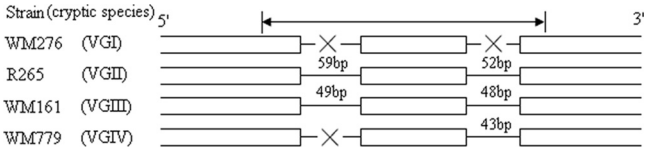

FIG 1 Schematic representation of the amplified regions of the putative vacuolar membrane gene. Open boxes represent exons (exon2, exon3, and exon4, in turn), while lines between exons represent introns. "×" represents intron loss; intron length is indicated by the numeral above the line; the region between the arrows represents the amplified fragment.

Electrophoretic and sequence analysis revealed an expected 166-bp amplicon for *C. gattii* VGI, a combination of 277-bp and

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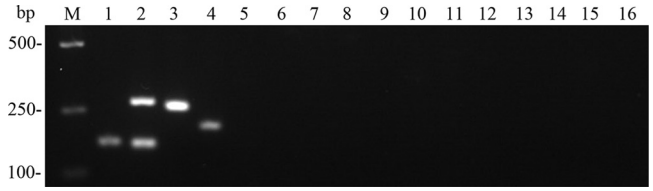

FIG 2 Partial results in agarose gel electrophoresis analysis. Lanes (species and cryptic species are shown in parentheses): M, DL2000 ladder; 1, WM179 (*C. gattii* VGI); 2, WM178 (*C. gattii* VGII); 3, WM161 (*C. gattii* VGIII); 4, WM779 (*C. gattii* VGIV); 5, WM148 (*C. neoformans* VNI); 6, WM626 (*C. neoformans* VNII); 7, WM628 (*C. neoformans* VNIII); 8, WM629 (*C. neoformans* VNIV); 9, Y2090 (*Cryptococcus albidus*); 10, Y2536 (*Cryptococcus laurentii*); 11, ATCC 10231 (*Candida albicans*); 12, ATCC 750 (*Candida tropicalis*); 13, CBS604 (*Candida parapsilosis*); 14, ATCC 90030 (*Candida glabrata*); 15, Y17953 (*Trichosporon asahii*); 16, negative control.

156-bp amplicons for *C. gattii* VGII, a 263-bp amplicon for *C. gattii* VGIII, and a 209-bp amplicon for *C. gattii* VGIV (Fig. 2). As a result, all *C. gattii* strains tested were exactly assigned to the cryptic species level, and none of the other pathogenic yeast species, including the sibling species *C. neoformans*, resulted in an amplification product by using this approach. Moreover, the rarely reported *C. neoformans* × *C. gattii* hybrids may be further characterized by the singleplex PCR as previously described (22).

Differences in intron loss in the protein-coding genes were found not only among species or varieties within the *C. neoformans-C. gattii* species complex but also among the monophylogenetic clusters within *C. gattii* (24, 26). The latter was utilized herein for molecular typing of *C. gattii*. Thus, we report a duplex PCR assay which performed well in distinguishing among the four cryptic species belonging to *C. gattii*.

Nucleotide sequence accession numbers. The sequences of the vacuolar membrane gene fragment of strains WM161 and WM779 were deposited in GenBank under accession numbers KF010296 and KF010297, respectively.

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