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Development of a Singleplex PCR Assay for Rapid Identification and Differentiation of *Cryptococcus neoformans* var. *grubii*, *Cryptococcus neoformans* var. *neoformans*, *Cryptococcus gattii*, and Hybrids

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A singleplex PCR assay using a single primer pair targeting the putative sugar transporter gene was developed here to distinguish *Cryptococcus neoformans* var. *grubii*, *Cryptococcus neoformans* var. *neoformans*, and *Cryptococcus gattii* according to the distinct size of the amplicon. The interspecies and intravarietal hybrids were also characterized on the basis of distinct combined profiles of amplicons. This PCR assay is a rapid, simple, and reliable approach suitable for laboratory diagnoses and large-scale epidemiologic studies.

The *Cryptococcus neoformans*/*Cryptococcus gattii* species complex is the causative agent of cryptococcosis and has been classified into two sibling species, namely, *C. neoformans* and *C. gattii*. *C. neoformans* is an opportunistic pathogen and causes infections mainly in immunocompromised individuals. This species contains two varieties, *Cryptococcus neoformans* var. *grubii* and *C. neoformans* var. *neoformans*, which correspond to serotypes A and D, respectively. Contrary to *C. neoformans*, *C. gattii* is a primary pathogen and often causes infections in immunocompetent individuals. Recent surveys indicated that this pathogen is more widely distributed than previously thought, especially since the *C. gattii* outbreak occurred in the Pacific Northwest (1). Phylogenetic analyses and molecular typing studies have identified eight major genotypes within the *C. neoformans*/*C. gattii* species complex (2). The relationship of the major genotypes to species, varieties, and serotypes are as follows: VNI and VNII, *C. neoformans* var. *grubii*, serotype A; VNIII, AD hybrid, serotype AD; VNIV, *C. neoformans* var. *neoformans*, serotype D; and VGI, VGII, VGIII, and VGIV, *C. gattii*, serotypes B and C (2, 3). Additionally, a geographically restricted genotype, VNB, belonging to *C. neoformans* var. *grubii* from Botswana has been reported (4).

Of note, differences in epidemiology, pathogenicity, biology, clinical features, and drug susceptibility have been reported to be associated with species or varieties in the *C. neoformans*/*C. gattii* species complex (2, 5, 6). Besides the intravarietal AD hybrid, a few novel interspecies hybrid isolates between *C. neoformans* and *C. gattii* have been characterized, and specific pathogenic attributes may be related to the strains (7). Thus, a rapid method for identification of the species, varieties, and hybrids within the pathogen will play an important role in laboratory diagnosis and epidemiologic investigations.

Several molecular approaches, such as multiplex PCR, PCR-restriction fragment length polymorphism (RFLP) analysis, hyperbranched rolling circle amplification (HRCA), loop-mediated isothermal DNA amplification, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), have been developed to identify species, varieties, and hybrids (2, 8–12). Overall, these methods, while reliable, usually require expensive instruments or complicated methods. Singleplex PCR was reported to differentiate the closely related species of *Candida* spp. according to the distinct sizes of amplicons, which are based mainly on intron loss or differences in intron size of the group I intron or the nuclear pre-mRNA intron (13–15). Introns are widely used as phylogenetic markers in evolutionary analysis. It is reported that intron loss significantly predominates over gain in the evolution of the *Cryptococcus* clade, and short introns (~60 bp) were found to predominate in the genome of the *C. neoformans*/*C. gattii* species complex (16, 17). However, molecular typing based on difference of introns for this clinically important pathogen has never been reported up to now. Here, the intron loss and intron size differences were analyzed for differentiation of the species complex.

The genomic sequence alignment tool LAGAN (18) was used to analyze the intron loss and intron size differences among members of the species complex. The genome sequence and gene structure of *C. neoformans* var. *grubii* strain H99 and *C. gattii* strain R265 (available at http://www.broadinstitute.org/), and *C. neoformans* var. *neoformans* strain JEC21 and *C. gattii* strain WM276 (available at http://www.ncbi.nlm.nih.gov/genome/) were aligned and analyzed by this tool. Similarly, loss of short introns rather than intron size differences within longer introns between the species or varieties was detected in our study. Ultimately, two adjacent introns within the putative sugar transporter (*STR1*) gene were selected due to their different types of intron loss among the species and varieties. Sequence alignments of orthologs among the major genotypes were conducted using ClustalW2 and demonstrated that differences in size were the result of intron loss (Fig. 1).

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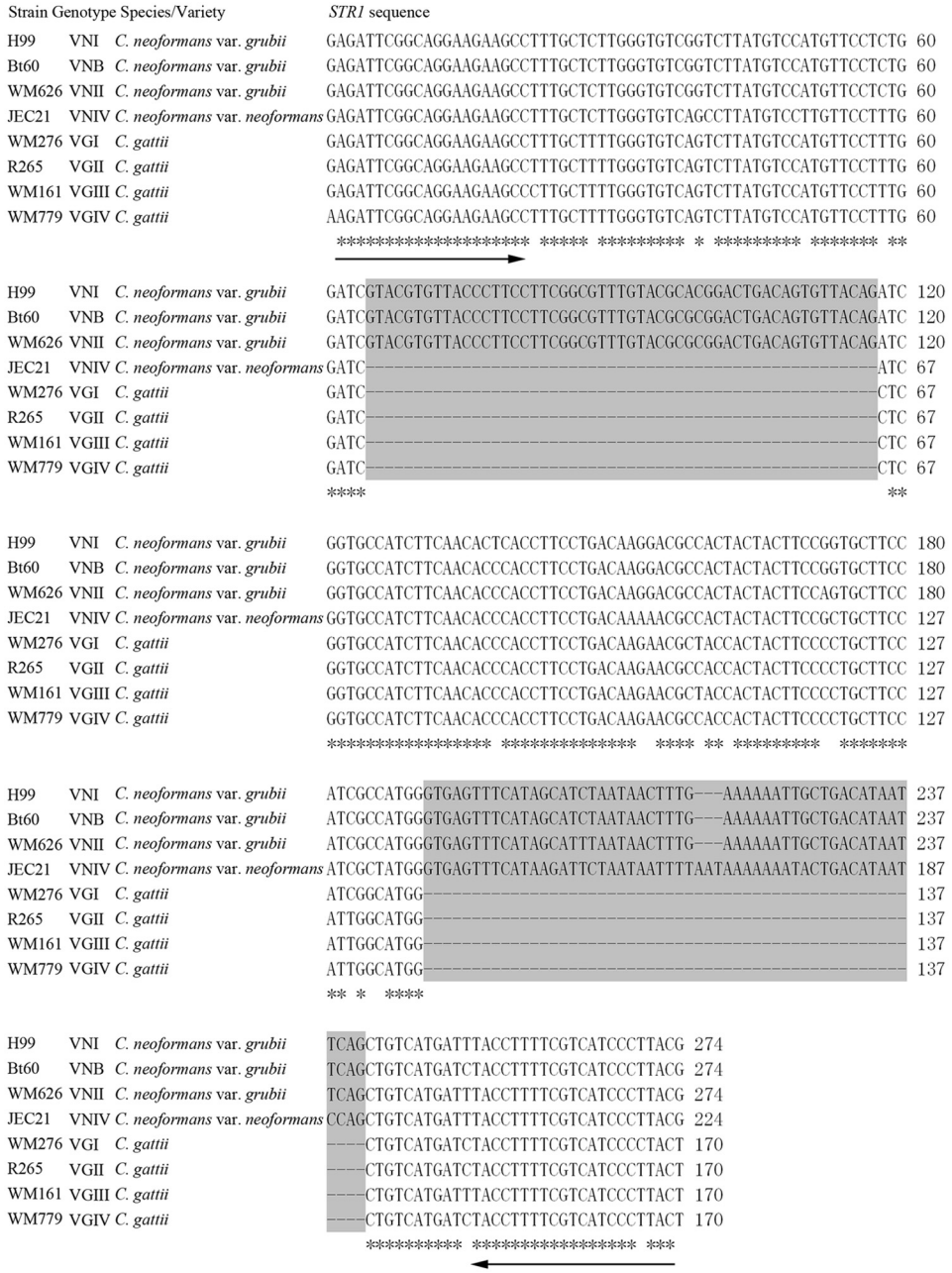

FIG 1 Multiple sequence alignment of fragments of the *STR1* gene orthologs in type strains. Primers STR1F and STR1R are underlined. Two adjacent introns within the gene fragment are marked by a gray background. Gaps representing intron loss are indicated by dashes.

Primers targeted to the *STR1* gene, STR1F (5′ GAGATTCGG CAGGAAGAAGC 3′) and STR1R (5′ CGTAAGGGATGACGAA AAGGTA 3′), were designed based on consensus nucleotide sequences of the exon region of the reference strains H99 (VNI), WM626 (VNII), Bt60 (VNB), JEC21 (VNIV), WM276 (VGI), R265 (VGII), WM161 (VGIII), WM779 (VGIV). 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, a 0.2 μM concentration 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, 55°C for 30 s, and 72°C for 30 s with a final extension step at 72°C for 6 min. PCR products were separated on a 1.6% (wt/vol) agarose gel at 100 V for 1 h.

This PCR assay was applied to our strain collection for identification. Genomic DNA was prepared from each strain as described previously (19). A total of 255 isolates belonging to the *C. neoformans*/*C. gattii* species complex were reference strains and those characterized by using conventional methods and PCR fingerprinting and PCR-RFLP analysis of the *GEF1* gene, as previously reported (2, 9). The strains included *C. neoformans* var. *grubii* ($n = 166$), *C. neoformans* var. *neoformans* ($n = 14$), *C. gattii* ($n = 56$), AD hybrid ($n = 17$), AB

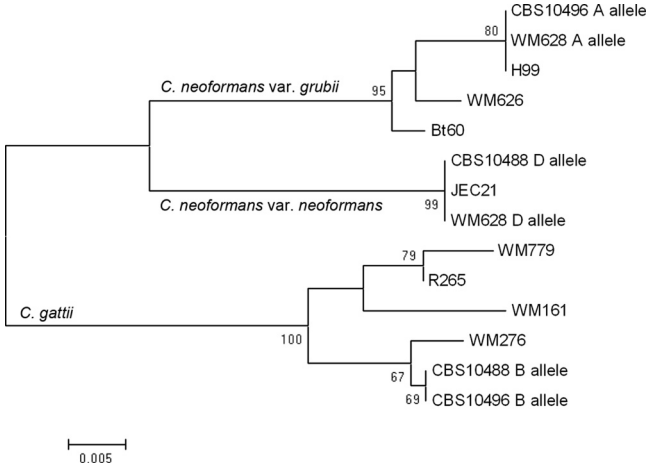

FIG 2 Neighbor-joining tree for type strains, including hybrid strains obtained by analysis of 170-bp fragments of the *STR1* gene. Bootstrap values (500 replicates) are indicated for the main branches. Intron is excluded from the phylogenetic analysis.

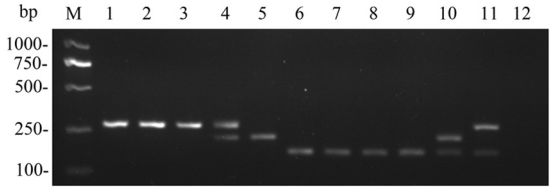

FIG 3 Agarose gel electrophoresis of PCR products. Species, variety and major genotype are depicted in brackets. Lanes: M, DL2000 ladder; 1, WM148 (*C. neoformans* var. *grubii* VNI); 2, Bt60 (*C. neoformans* var. *grubii* VNB); 3, WM626 (*C. neoformans* var. *grubii* VNII); 4, WM628 (*C. neoformans* AD hybrid VNIII); 5, WM629 (*C. neoformans* var. *neoformans* VNIV); 6, WM179 (*C. gattii* VGI); 7, WM178 (*C. gattii* VGII); 8, WM161 (*C. gattii* VGIII); 9, WM779 (*C. gattii* VGIV); 10, CBS10488 (*C. neoformans*×*C. gattii* BD hybrid); 11, CBS10496 (*C. neoformans* × *C. gattii* AB hybrid); 12, negative control.

hybrid ($n = 1$), and BD hybrid ($n = 1$). Another 42 pathogenic yeast isolates, which were either reference strains or strains that had been characterized by sequencing of the internal transcribed spacer (ITS) region as previously described (20), were chosen to detect the specificity of the primer pair of STR1F-STR1R. Detailed information regarding the strains used here is presented in Table S1 in the supplemental material. In addition, amplified PCR products of the three hybrid strains WM628 (AD), CBS10496 (AB), and CBS10488 (BD) were cloned and sequenced to determine their hybrid identities. The sequences obtained in this study and those from GenBank were used for phylogenetic analysis performed by using MEGA version 4.0 software (available at http://www.megasoftware.net/). A dendrogram was produced by neighbor-joining analysis using sequences alignment with the Kimura 2-parameter method. Introns were excluded from the phylogenetic analysis. Statistical support for each clade was assessed using bootstrap analysis with 500 replicates (Fig. 2).

No amplicon was produced when PCR was performed with the other pathogenic yeasts tested. As expected, the singleplex PCR amplified one fragment for *C. neoformans* var. *grubii* (274 bp), *C. neoformans* var. *neoformans* (224 bp), and *C. gattii* (170 bp) and two fragments for the AD hybrid (274 and 224 bp), AB hybrid (274 bp and 170 bp) and BD hybrid (224 and 170 bp) (Fig. 3). All amplicon sizes were confirmed by sequencing of the corresponding representative strains. By PCR analysis, 253 of 255 (99.2%) isolates of the *C. neoformans*/*C. gattii* species complex, including the two novel AB and BD hybrids, were correctly assigned to the relevant species, varieties, or hybrids. Nevertheless, 2 of 17 (11.8%) AD hybrid strains (IUM92-6198 and CBS132) produced only one amplicon corresponding to *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*, respectively, by the PCR analysis, which was in correspondence with other reports using single or a few target genes (3, 10, 11). This result may be due to genomic instability in AD hybrid strains with loss of heterozygosity for the allele of *STR1* gene, which may be resolved by molecular tools designed for use at the whole-genome level, such as amplified fragment length polymorphism (AFLP) and PCR fingerprinting (2, 7, 19). Phylogenetic analysis revealed that strains representing the three hybrid types had two distinct alleles clustered with the corresponding species, varieties, and genotypes.

Our study demonstrates that differences in intron loss in protein-coding genes may be used as a reliable genetic marker for molecular typing of pathogenic fungi. Thus, we report here a simple PCR assay with a single primer pair, which performed well in discrimination between members of the *C. neoformans*/*C. gattii* species complex. The simple method and the rapidity and reliability of this technique make it a useful tool for laboratory diagnoses and large-scale epidemiologic studies.

Nucleotide sequence accession numbers. The sequences of the *STR1* gene fragment of reference strains Bt60, WM626, WM161, and WM779 were deposited under the accession numbers KC429573 to KC429576.

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