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Molecular Mechanisms of Fluconazole Resistance in *Candida parapsilosis* Isolates from a U.S. Surveillance System

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***Candida parapsilosis* is the second or third most common cause of candidemia in many countries. The Infectious Diseases Society of America recommends fluconazole as the primary therapy for *C. parapsilosis* candidemia. Although the rate of fluconazole resistance among *C. parapsilosis* isolates is low in most U.S. institutions, the resistance rate can be as high as 7.5%. This study was designed to assess the mechanisms of fluconazole resistance in 706 incident bloodstream isolates from U.S. hospitals. We sequenced the *ERG11* and *MRR1* genes of 122 *C. parapsilosis* isolates with resistant (30 isolates; 4.2%), susceptible dose-dependent (37 isolates; 5.2%), and susceptible (55 isolates) fluconazole MIC values and used real-time PCR of RNA from 17 isolates to investigate the regulation of *MDR1*. By comparing these isolates to fully fluconazole-susceptible isolates, we detected at least two mechanisms of fluconazole resistance: an amino acid substitution in the 14-α-demethylase gene *ERG11* and overexpression of the efflux pump *MDR1*, possibly due to point mutations in the *MRR1* transcription factor that regulates *MDR1*. The *ERG11* single nucleotide polymorphism (SNP) was found in 57% of the fluconazole-resistant isolates and in no susceptible isolates. The *MRR1* SNPs were more difficult to characterize, as not all resulted in overexpression of *MDR1* and not all *MDR1* overexpression was associated with an SNP in *MRR1*. Further work to characterize the *MRR1* SNPs and search for overexpression of other efflux pumps is needed.**

Candida species in general and *Candida parapsilosis* in particular are opportunistic pathogens frequently responsible for hospital-acquired infections (1–3). While the burden of *C. parapsilosis* varies geographically and by patient population, *C. parapsilosis* is responsible for about 12 to 17% of cases of candidemia in the United States (4–6) and is identified in many studies to be the second or third most common cause of candidemia both in the United States and internationally, with the average mortality rate being 29% (range, 4% to 45%) (7). *C. parapsilosis* is particularly notable for the risk that it poses to neonates, among whom it is estimated to be responsible for 34% of all cases of candidemia in the United States and 33% internationally, with the average crude mortality rate being 10% (8).

The Infectious Diseases Society of America recommends fluconazole as the primary therapy for *C. parapsilosis* candidemia (9). The majority of clinical isolates of *C. parapsilosis* are susceptible to fluconazole; the rates of resistance in the United States from two surveillance studies range regionally from 0 to 7.5% (4, 5), though at least one hospital has reported higher rates of resistance (10). One small study of invasive fungal infections in liver transplant patients conducted antifungal susceptibility testing on 6 of 16 *C. parapsilosis* isolates and found that all were resistant to fluconazole according to current CLSI breakpoints. The authors noted that this coincided with a hospital-wide peak in the incidence of fluconazole-resistant *C. parapsilosis*, which later subsided (10).

Despite the potentially rising incidence of *C. parapsilosis* (11) and the threat that fluconazole resistance could pose in a clonally expanding population, little is known about the molecular mechanisms of *C. parapsilosis* fluconazole resistance. Fluconazole prevents fungal cell growth by inhibiting 14-α-demethylase, which is responsible for the production of an ergosterol precursor and is encoded by the gene *ERG11*. *C. albicans*, whose resistance mechanisms are well characterized, evades the effects of fluconazole in four known ways: (i) the upregulation of drug efflux pumps, primarily *CDR1*, *CDR2*, and *MDR1*, which transport fluconazole out of the cell; (ii) mutational changes to 14-α-demethylase that reduce its affinity to fluconazole; (iii) upregulation of *ERG11* to dilute fluconazole binding; and (iv) other alterations to the cell's sterol pathway (12).

To date, there has been only a single study on the fluconazole resistance mechanisms of *C. parapsilosis* (13). In that study, which used isolates with *in vitro*-induced resistance, the authors found that *MDR1*, a drug efflux pump, was upregulated 19-fold in an isolate with induced fluconazole resistance compared to the level of regulation of its susceptible parent. This corresponded to a point mutation in the *MRR1* gene, a transcription factor for *MDR1*. The authors therefore hypothesized that fluconazole resistance in *C. parapsilosis* was achieved through a gain-of-function mutation in *MRR1* that upregulated *MDR1* and removed fluconazole to an extent sufficient to prevent effective buildup within the cell. However, it was not clear whether the results were generalizable to resistant isolates from patients.

Using isolates collected during population-based U.S. surveillance of candidemia, we focused on two potential mechanisms of resistance: an *MRR1* gain-of-function mutation and alterations to *ERG11*. To determine if either of these mechanisms was present in clinical isolates, we sequenced the *ERG11* and *MRR1* genes of 122

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TABLE 1 Primers for amplification and sequencing[a]

Gene (purpose)	Primer name	Sequence	Annealing temp (°C)	Extension time (min)
ERG11 (PCR and sequencing)	ERG11 F1F	TAG TGG GAT CGG TGG ATC TT	60	1
	ERG11 F2R	CTT TAT CTA AAT CAG CAT ACA ATT GAG		
	ERG11 F3F	TCT AGA TCC TTA TTA GGA GAA GCA ATG	60	1
	ERG11 F4R	ACT GAC TCC TGC CCT CAG ATT		
ERG11 (sequencing only)	ERG11 F1R	ATG ATG TTG TAA ATG AAA GGA GCA		
	ERG11 F2F	TTA GCC CTT CAT GGG TAC AAC T		
	ERG11 F3R	TAC TTT GTG TTT GGC ACA ACC		
	ERG11 F4F	AAA AGT TGT TTC TCC CTT GGT TG		
MRR1 (PCR and sequencing)	MRR1 F1F	CTG TAT GGA GAG TGA GAT TTT AGG TT	60	1.25
	MRR1 F3R	TCC TTG GTT ACC TCA TTG CTC		
	MRR1 F4F	ATG GAG ACC ATT AAT TTT TTT GAC A	60	1.25
	MRR1 F6R	GAA TGA CTT CAT TGA AAT GTA ATG CT		
	MRR1 F7F	AAG AAA ATT CTT AGC TTA ACT GGA	53	1.25
	MRR1 F9R	AGA AAA TCT AAT TGG TAA AGA AGA AAG GA		
MRR1 (sequencing only)	MRR1 F1R	TAA AAC CTT CTT CGT CAT AAC AAC A		
	MRR1 F2F	ACC TCA AAC GAA TGA AAT AAA GGA		
	MRR1 F2R	ATA ACA GAG GTT GAA TCG TTG GC		
	MRR1 F3F	CTA ATT CGT TGC TTGA GAT CAA AA		
	MRR1 F4R	CCA ATG CCA AGT CTA GTC TTT TCT		
	MRR1 F5F	TAG AAT AAG AAG GAC TCT TCC AAG C		
	MRR1 F5R	CCA AGA TGA TTC TTT CTC TTA TCT GTT		
	MRR1 F6F	GCA AGT TTG CCT TTG ATT CAA		
	MRR1 F7R	GAA TGA CTC TTT GTC AAT TTC CA		
	MRR1 F8F	CTA CAG ATT AAA ATC TCA GCC TGA CC		
	MRR1 F8R	CTG CGA GAT GCC GTA GTT C		
	MRR1 F9F	TCC ACT CCG ACT AGT GAT ACA TC		

[a] The source of all primer sequences was this study.

C. parapsilosis patient isolates with resistant, susceptible dose-dependent (SDD), and susceptible fluconazole MIC values. Upon finding alterations in the *MRR1* sequences of 23 isolates, we conducted real-time PCR (RT-PCR) to determine whether any of these corresponded to an upregulation of *MDR1*. Additionally, we performed microsatellite analysis to determine whether isolates with shared mutations came from a shared lineage. Our results suggest that *ERG11* mutations are a frequent cause of fluconazole resistance in *C. parapsilosis*.

MATERIALS AND METHODS

Isolates and susceptibility testing. Isolates were selected from a pool of *C. parapsilosis* isolates collected as part of population-based candidemia surveillance in the metropolitan Atlanta, GA, area (from March 2008 to May 2013, $n = 397$), Baltimore City and County, MD (from June 2008 to May 2013, $n = 262$), Knox County, TN (from January 2011 to May 2013, $n = 19$), and the metropolitan Portland, OR, area (from January 2011 to May 2013, $n = 28$) (4, 11). All isolates were *C. parapsilosis sensu stricto*; no *C. orthopsilosis* or *C. metapsilosis* isolates were included in the study. Isolates were stored frozen at −70°C until needed. Susceptibility testing was performed as previously described for this collection (4). The final isolates were chosen either by having a nonsusceptible fluconazole MIC (MIC ≥ 4 μg/ml) or randomly from those with a susceptible fluconazole MIC distribution.

Sequencing of *ERG11* and *MRR1*. DNA was prepared using an UltraClean microbial DNA isolation kit (MO BIO Laboratories, Carlsbad, CA). Amplification of *ERG11* and *MRR1* was performed using the Roche master mix, as described by the manufacturer (Roche Diagnostics, Indianapolis, IN). Annealing temperatures and the extension time varied by primer (Table 1). PCR products were treated with the ExoSAP-IT reagent (Affymetrix, Santa Clara, CA) per the manufacturer's instruction and sequenced with sequencing primers (Table 1) using a BigDye Terminator kit (Applied Biosystems, Foster City, CA). The sequences were analyzed using Sequencher (version 5.1) software (Gene Codes Corporation, Ann Arbor, MI) and compared to the *C. parapsilosis* isolate ATCC 22019 wild-type *ERG11* sequence and *C. parapsilosis* isolate CDC317 wild-type *MRR1* sequence, respectively, using the Clustal W program. Mann-Whitney and Pearson chi-square tests were performed using SPSS software (IBM, Armonk, NY).

Microsatellite amplification and analysis. The microsatellite loci amplified were those described by Reiss and coworkers (14). The amplification mix consisted of 13.3 μl water, 2 μl 10× PCR buffer (Roche), 0.2 mM deoxynucleoside triphosphate mix (Roche), 1 μl dimethyl sulfoxide, 0.6 U *Taq* DNA polymerase (Roche), 0.2 pM forward and reverse primers (14), and 2 μl DNA per reaction mixture. PCR was performed using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) using the following conditions: 4 min denaturation at 96°C; 30 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C; and a final 30-min extension at 72°C. Amplified sequences were sized using an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) and compared to the GeneScan 500 6-carboxytetramethylrhodamine size standard (Applied Biosystems) in the 35- to 500-nucleotide range. Results were read using PeakScanner (version 2.0) software (Applied Biosystems, Foster City, CA) and analyzed using Microsatellite Analyser software (15) to determine Nei's chord distances (16). An unweighted-pair group method using average linkages (UPGMA) tree was constructed from the resulting distance matrix using the PHYLIP Neighbor executable (version 3.6) program (University of Washington, Seattle, WA) and edited using Geneious (version 6.1.6) software (Biomatters, Auckland, New Zealand).

TABLE 2 Primers and probes for qRT-PCR[a]

Gene	Primer name	Sequence	5′ label	3′ label	Efficiency (%)	Dynamic range (in quantification cycles)
MDR1	MDR1-F-2	CCC TTG TCG TTG GCA TTA			94.5	21.57–32.89
	MDR1-R-2	GCC TTC CTA GCA AGC AAT GTA				
	MDR1 probe 2	AGC TGG CTG GAG ATG GTG	FAM	BHQ1		
ACT1	ACT1-F-2	CGA ACG TGG TTA CGG TTT CTC CAC TA			81.3	18.56–33.58
	ACT1-R-2	ACT TGA CCA TCT GGC AAT TCG TAT				
	ACT1 probe	TGC TTT GGA CTT TGA ACA AGA AAT GCA AAC CTC AT	HEX	BHQ1		
TUB4	TUB4-F-A	CGG TGG CAC CAT TCA ACA			83.2	21.21–36.37
	TUB4-R-A	CAT CTG ACA ATT CCA AAA ACA TGT C				
	TUB4 probe A	CCA GTC GCA CCA CAA CTA CAT CAA CGA G	HEX	BHQ1		

[a] FAM, 6-carbocyfluorescein; BHQ1, black hole quencher 1; HEX, 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein.

Quantitative real-time PCR. Four milliliters of Sabouraud dextrose broth was inoculated with *C. parapsilosis* to a concentration of 7×10^4 to 25×10^4 cells/ml and incubated in a rotary shaker at 37°C for approximately 18 h. Concentrations were checked by use of a hemocytometer within 2 h of harvesting to ensure a maximum final concentration of 1.0×10^8 cells/ml, indicative of semilogarithmic growth. RNA extraction was performed using a RiboPure yeast kit (Ambion, Austen, TX) according to the manufacturer's instructions. RNA integrity was checked visually by nondenaturing gel electrophoresis and quantitated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). As quantitative real-time PCR (qRT-PCR) controls without reverse transcriptase showed evidence of genomic DNA contamination, some RNAs were subjected to DNase digestion followed by RNA cleanup with an RNeasy minikit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions, or by repeating the RiboPure yeast kit DNase protocol using twice the recommended volume of DNase I and incubating for twice the recommended time, after which all were treated with a Turbo DNA-free kit (Invitrogen, Carlsbad, CA), according to the manufacturer's rigorous DNase treatment procedure, and cleaned up with the RNeasy minikit as described above. A lack of DNA contamination was confirmed by reverse transcriptase-free quantitative PCR with primers TUB4-F-A and TUB4-R-A and TUB4 probe A (Table 2). The absence of interfering mutations in the primer-probe region of each gene was confirmed by sequencing using the primers and conditions described in Table 3.

qRT-PCR was run on a CFx-96 real-time PCR detection system (Bio-Rad, Hercules, CA) using a QuantiTect multiplex RT-PCR kit (Qiagen) in a 20-μl reaction volume, according to the manufacturer's instructions, with the following exceptions: the *MDR1* primers and *ACT1* probe were used at a final concentration of 0.3 μM. Triplicate reactions were run in singleplex using the primers and probes listed in Table 2. Primers and probes were designed using LightCycler probe design (version 2.0) software (Roche) and Primer Express (version 2.0) software (ABI, Foster City, CA). Sample replicates with standard deviations above 0.35 were repeated. The constitutively expressed *ACT1* and *TUB4* genes were used as a reference for normalizing the relative gene expression levels. Normalized gene expression analysis was performed using CFx manager software (Bio-Rad), which also performed interrun normalization using a common calibrator sample.

TABLE 3 Primers for sequencing of qRT-PCR genes[a]

Gene	Primer name	Sequence
MDR1 (partial)	MDR1seq F	CTG GGT TTT GTA TCC TTA GAT TCC T
	MDR1seq R	AAG CGC CTC GAC CAA AAT
ACT1 (partial)	ACT1seq F	TTC AGG TGA TGG TGT CAC TCA
	ACT1seq R	AGT CAC ACT TCA TGA TAG AGT TGA AAG
TUB4 (partial)	TUB4seq F	CTA CTT CGT TTC AAG GCA CAA AC
	TUB4seq R	TTG TAC GTG CTT GAA CTT TCA AA

[a] The source of all primer sequences was this study, and for all primers the annealing temperature was 55°C and the extension time was 30 s.

RESULTS

Identification of resistant and SDD isolates. A total of 706 isolates of *C. parapsilosis* from 80 hospitals were tested for fluconazole susceptibility. There were 30 isolates that were resistant (MIC ≥ 8 μg/ml) and 37 isolates that were susceptible dose-dependent (SDD) (MIC = 4 μg/ml) to fluconazole. The majority of fluconazole-resistant or -nonsusceptible isolates were collected from patients in Atlanta hospitals (70.0% of resistant isolates, 64.9% of SDD isolates), followed by Baltimore (26.7% of resistant isolates, 27.0% of SDD isolates), Portland (0.0% of resistant isolates, 8.1% of SDD isolates), and Knox County (3.3% of resistant isolates, 0.0% of SDD isolates). The proportions of resistance and dose-dependent susceptibility were 5.3% and 6.1%, respectively, for the Atlanta study area and 3.0% and 3.8%, respectively, for the Baltimore study area.

Sequencing of *ERG11*. The *ERG11* genes of 122 isolates (30 resistant isolates, 37 susceptible dose-dependent isolates, and 55 randomly chosen susceptible isolates) were amplified and sequenced. By comparison to the sequence of the wild-type *C. parapsilosis* isolate ATCC 22019, five different single nucleotide polymorphisms (SNPs) in 54 isolates were identified. One of the SNPs, A395T (here called SNP 1; amino acid substitution, Y132F), was present in 17 of 30 resistant isolates (56.7%; heterozygous in 1 isolate, homozygous in 16 isolates) but none of the SDD or susceptible isolates. These isolates, listed in Table 4, were found in five different hospitals but primarily concentrated in three, with 71% of the isolates occurring in two hospitals in Atlanta and 18% occurring in one hospital in Baltimore. Two other SNPs, C−111T in the 5′ untranslated region and G1193T (R398I), were found together in 36 isolates (6 resistant, 13 SDD, and 17 susceptible isolates). Finally, two SDD isolates had one SNP each, T533C (M178T) and A847T (N283Y), respectively, the latter of which was heterozygous. The geometric mean MIC values of isolates homozygous for and without SNP 1 were 14.7 μg/ml and 1.92 μg/ml, respectively. The difference between the two groups was statistically significant ($P < 0.0005$; two-tailed, Mann Whitney U test value = 1,604.500). Isolates containing *ERG11* SNP 1 ac-

TABLE 4 MICs of isolates with *ERG11* SNP 1[a]

Isolate	Hospital[b]	MIC (μg/ml)[c]	
		Fluconazole	Voriconazole
CAS08-0490	ATL05	16 (R)	1 (R)
CAS08-0796	ATL05	8 (R)	0.5 (I)
CAS09-0912	ATL05	8 (R)	0.25 (I)
CAS09-0959	BAL09	32 (R)	1 (R)
CAS09-1107	ATL05	8 (R)	0.5 (I)
CAS09-1291	ATL05	16 (R)	1 (R)
CAS09-1321	BAL09	64 (R)	1 (R)
CAS09-1504	BAL09	32 (R)	2 (R)
CAS09-1783	ATL05	16 (R)	0.25 (I)
CAS10-1966	ATL05	8 (R)	0.25 (I)
CAS10-2364	ATL10	16 (R)	0.25 (I)
CAS10-2602	ATL10	8 (R)	0.5 (I)
CAS11-3037	ATL17	16 (R)	0.25 (I)
CAS11-3324	ATL10	16 (R)	1 (R)
CAS11-3362	ATL05	8 (R)	0.125 (S)
CAS12-3954	ATL10	16 (R)	0.25 (I)
CAS12-3992	ATL14	8 (R)	0.125 (S)

[a] SNP 1 is the A395T substitution (amino acid substitution, Y132F).
[b] ATL, an Atlanta-area hospital; BAL, a Baltimore-area hospital; KNX, a Knoxville-area hospital; POR, a Portland-area hospital.
[c] R, resistant; I, intermediate; S, susceptible.

counted for 67% of resistant isolates in Atlanta and 38% in Baltimore.

Sequencing of *MRR1*. The *MRR1* genes of the same 122 isolates described above were sequenced. Comparison against the *MRR1* sequence of wild-type *C. parapsilosis* identified 23 (18.9%) isolates with SNPs (Table 5). These included nine different nonsynonymous SNPs (including one nonsense mutation), a synonymous SNP, a 5′ untranslated region SNP, and a 5′ untranslated region insertion. Of the six SNPs that occurred in multiple isolates, none occurred exclusively in resistant isolates, although two *MRR1* polymorphisms, G−53A and C1856T (A619V), occurred only in resistant and SDD isolates. Three *MRR1* polymorphisms, G2575A (A859T), −102_−101insT (where −101insT indicates insertion of a T nucleotide at position −101), and G2337T (L779F), occurred in one resistant isolate each, and another nonsynonymous SNP, G1436A (R478K), occurred in one SDD isolate. At least one *MRR1* polymorphism was present in 12.7% of susceptible isolates ($n = 7$), 16.2% of SDD isolates ($n = 6$), and 33.3% of resistant isolates ($n = 10$). The proportions of susceptible and resistant isolates with and without *MRR1* SNPs were significantly different ($P = 0.023$). These polymorphisms were not concentrated in any particular hospitals.

Resistance to voriconazole. Of all 706 *C. parapsilosis* isolates with MIC data, 6 were resistant (MIC ≥ 1 μg/ml) to voriconazole. All six of the voriconazole-resistant isolates were also resistant to fluconazole and contained *ERG11* SNP 1. The distributions of voriconazole MIC values of isolates with homozygous SNP 1 and without SNP 1 (listed in Table 4) differed significantly ($P < 0.0005$). The geometric mean MICs for the two groups were 0.48 μg/ml and 0.04 μg/ml, respectively.

qRT-PCR of *MDR1*. To determine whether any of the SNPs identified in *MRR1* were correlated with the upregulation of *MDR1*, qRT-PCR quantification of *MDR1* RNA was conducted on all isolates with one of the six *MRR1* nonsynonymous SNPs that were present only in resistant or SDD isolates, as well as in

TABLE 5 Isolates with *MRR1* polymorphisms

Nucleotide (amino acid) polymorphism	Isolate	Hospital	Fluconazole MIC[a] (μg/ml)	Note
G−53A	CAS08-0060	ATL12	16 (R)	
	CAS08-0419	ATL05	16 (R)	
	CAS10-2578	ATL14	4 (SDD)	
C1856T (A619V)	CAS09-1299	ATL01	8 (R)	Heterozygous
	CAS09-1761	ATL01	4 (SDD)	Heterozygous
G1214A (R405K)	CAS10-1852	BAL05	8 (R)	
	CAS09-1025	ATL05	4 (SDD)	
	CAS13-4604	ATL14	0.5 (S)	
G531T (K177N)	CAS10-1830	BAL06	8 (R)	
	CAS12-4406	KNX01	8 (R)	
	CAS08-0029	ATL01	0.5 (S)	
	CAS10-2116	BAL06	0.5 (S)	
	CAS12-4166	BAL09	2 (S)	
C3157T (Q1053X)	CAS10-1830	BAL06	8 (R)	Heterozygous
	CAS08-0029	ATL01	0.5 (S)	Heterozygous
	CAS10-2116	BAL06	0.5 (S)	Heterozygous
A1844G (D615G)	CAS11-3108	ATL03	8 (R)	Heterozygous
	CAS09-0941	BAL01	4 (SDD)	Heterozygous
	CAS10-1841	BAL09	4 (SDD)	Heterozygous
	CAS09-1196	ATL03	0.25 (S)	
	CAS10-2702	BAL02	0.5 (S)	
G2575A (A859T)	CAS08-0339	BAL02	8 (R)	Heterozygous
C744T (no change)	CAS09-1025	ATL05	4 (SDD)	Heterozygous
C1139A (P380H)	CAS12-4003	POR01	1 (S)	Heterozygous
−102_−101insT	CAS12-4480	BAL04	8 (R)	
G2337T (L779F)	CAS12-4342	ATL03	32 (R)	
G1436A (R478K)	CAS13-4861	ATL05	4 (SDD)	Heterozygous

[a] R, resistant; SDD, susceptible dose dependent; S, susceptible.

eight isolates without an *MRR1* SNP (four resistant, two SDD, and two susceptible isolates). The efficiencies and ranges of detection of each primer set are reported in Table 2. The coefficients of variation of reference genes *ACT1* and *TUB4* were 0.146 and 0.174, respectively, and their M value was 0.455, indicating that the genes were sufficiently stable (17).

Relative gene expression analysis results are presented in Fig. 1. Compared to the averaged expression of susceptible control isolates, RNA from nine isolates showed at least a 5-fold upregulation in *MDR1* expression. Six of these isolates had an *MRR1* SNP, and three did not. These included both isolates with C1856T (A619V) and each of the four nonsusceptible isolates with a unique nonsynonymous SNP, G2575A (A859T), −102_−101insT, G2337T (L779F), and G1436A (R478K). The isolates containing L779F or R478K exhibited particularly high levels of *MDR1* expression, with 72.9-fold and 35.7-fold increases, respectively. Of the four resistant isolates without *MRR1* SNPs included as controls, one had increased expression, as did both of the control SDD isolates without *MRR1* SNPs.

Microsatellite analysis. To understand whether resistance or shared SNPs were a function of shared ancestry, microsatellite analysis was conducted on all isolates with a polymorphism in either *ERG11* or *MRR1*, all additional resistant isolates, and all

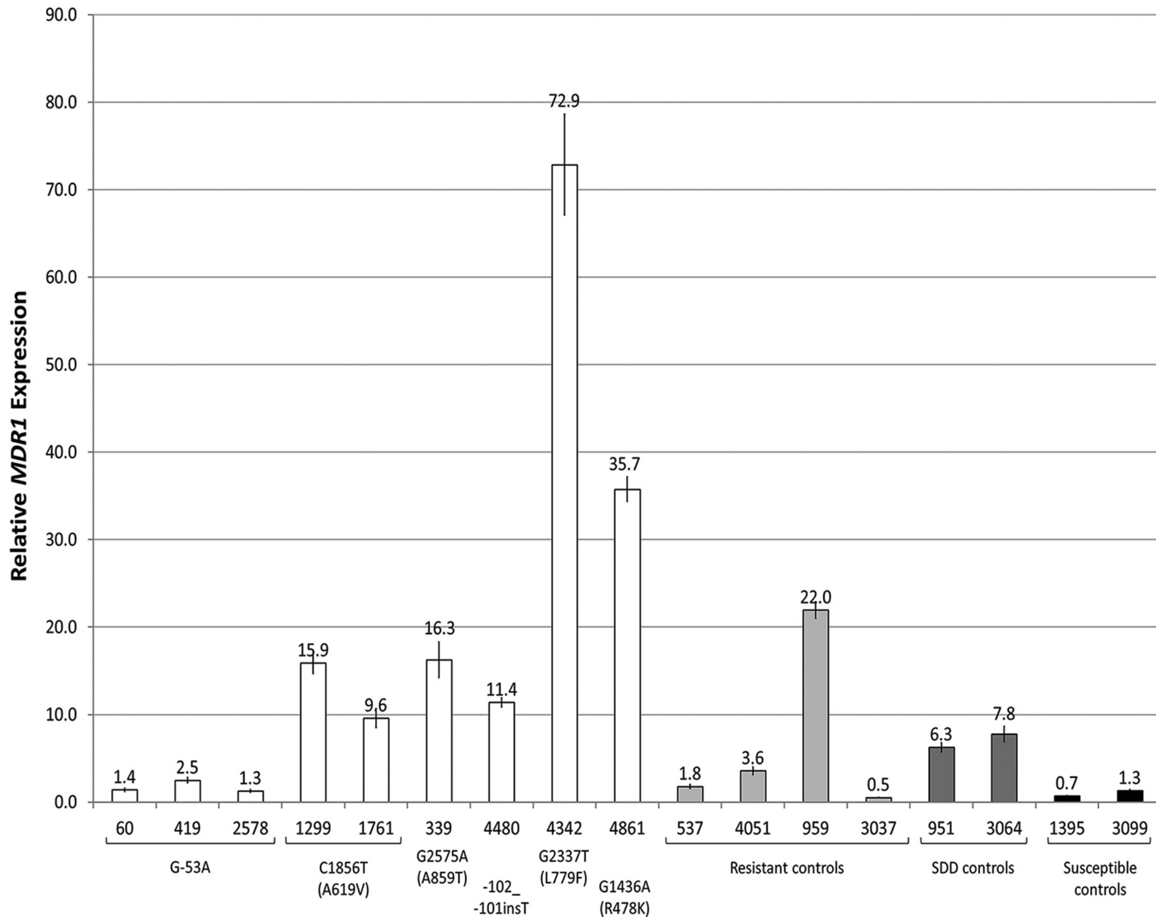

FIG 1 Relative *MDR1* expression analysis from qRT-PCR of isolates with *MRR1* SNPs exclusive to fluconazole-nonsusceptible isolates. *MDR1* values were normalized to each isolate's level of *ACT1* and *TUB4* expression, and the average for two susceptible controls was used as the control value and defined as 1-fold expression. Error bars represent 1 standard error of the mean. Bars are grouped by the isolate's shared *MRR1* SNP, which is indicated beneath each group or individual by base change and, when applicable, amino acid change (in parentheses). Control isolates without *MRR1* SNPs are grouped and shaded by resistance level.

isolates from the three hospitals with multiple resistant isolates (hospitals ATL05, ATL10, and BAL09), for a total of 92 isolates: 30 resistant, 26 SDD, and 36 susceptible isolates. Two of these, one SDD isolate and one susceptible isolate, returned triploid results for one locus, which could not be analyzed using our methodology, and were therefore excluded. The 90 remaining isolates produced 81 unique genotypes, including 3 instances of clonal pairs and 3 instances of clonal sets of three. All but seven of the isolates were distributed in four clades (Fig. 2). Isolates with *ERG11* SNP 1 showed a tight cluster, with all but 1 isolate (of 17) occurring in clade 3. For the most part, clustering was not a function of geography or institution, with isolates from the same hospitals being disbursed across the tree. There were, however, two notable clusters of isolates with *ERG11* SNP 1 within clade 3, one consisting of six isolates from hospital ATL05 collected over an 18-month period and the other consisting of three identical isolates from hospital BAL09 collected over an 8-month period.

DISCUSSION

Although fluconazole is the drug of choice for the treatment of *C. parapsilosis*, prior to this study we knew almost nothing about the mechanisms of *C. parapsilosis* patient isolate resistance to fluconazole. We addressed this problem in three ways. The first was the detection of mutations in *ERG11*, the target of fluconazole. The second was detection of mutations in *MRR1*, a gene that regulates a major fluconazole efflux pump. The third method was the detection of overexpression of *MDR1*, a major fluconazole efflux pump in *C. parapsilosis*.

The sole *ERG11* SNP that was found exclusively in fluconazole-resistant isolates, SNP 1, may be responsible for a sizeable portion of *C. parapsilosis* fluconazole resistance. The strong association between this SNP and fluconazole resistance in this study is bolstered by the fact that this SNP was reported in *C. albicans* by Perea et al. (18), who found it to confer fluconazole resistance when the *C. albicans ERG11* gene containing this SNP was transformed into otherwise susceptible *Saccharomyces cerevisiae* isolates. In another study, it was also found to decrease the susceptibility of *C. albicans* to voriconazole, mirroring the significantly increased voriconazole MICs that we found in *C. parapsilosis* isolates with *ERG11* SNP 1 (19). The same SNP has subsequently been identified in other studies of fluconazole resistance in *C. albicans* and *C. tropicalis* (20, 21). A different substitution at the same amino acid in *C. albicans* Erg11p has been demonstrated to diminish the protein's ability to bind to fluconazole without affecting its enzymatic ac-

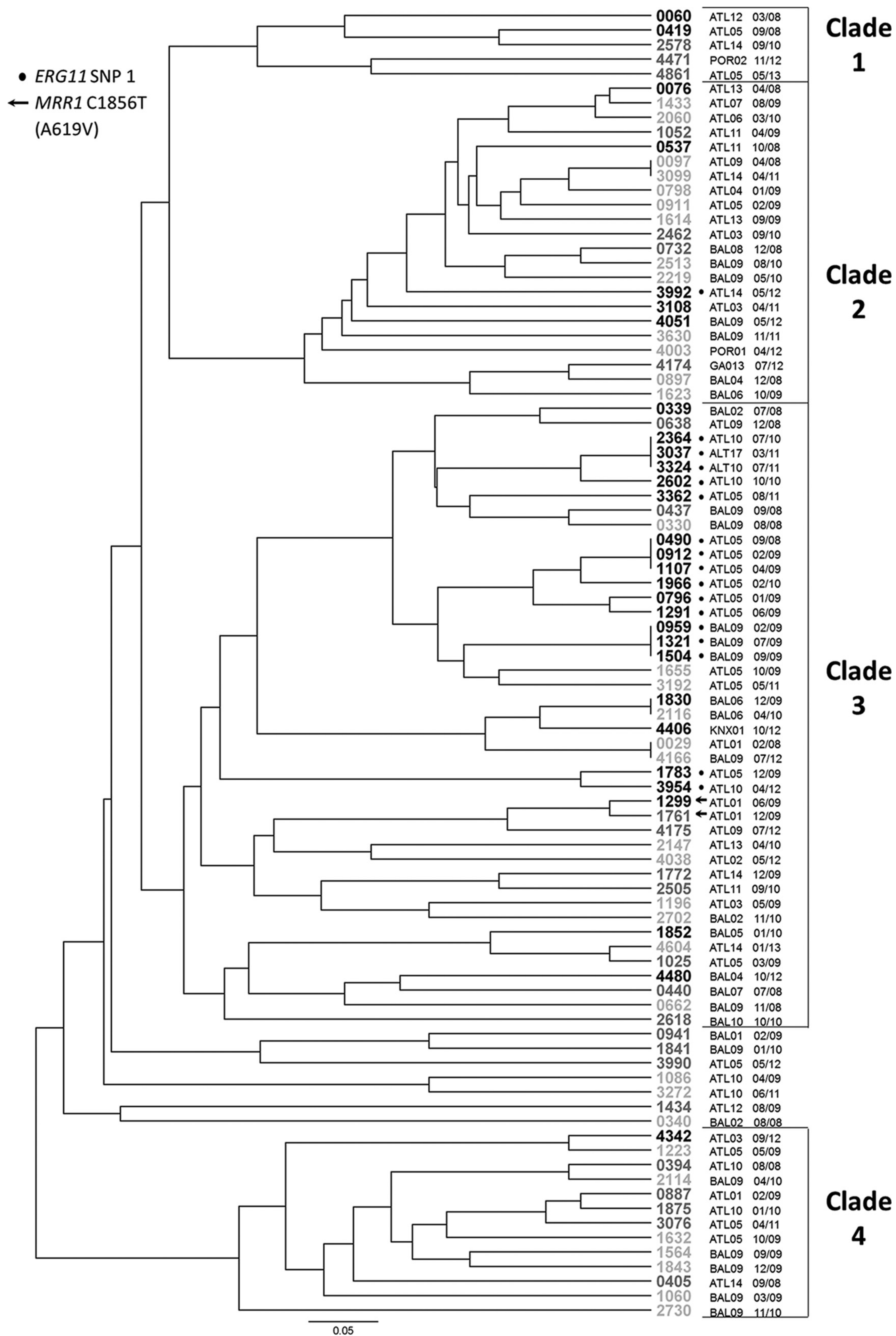

FIG 2 Results of microsatellite analysis presented as a UPGMA tree. Isolate names are given at the end of each branch, with the font color indicating the fluconazole susceptibility level: black letters, resistant isolates; dark gray letters, susceptible dose-dependent isolates; light gray letters, susceptible isolates. Hospital codes are given to the right of each isolate name, with ATL indicating an Atlanta-area hospital, BAL indicating a Baltimore-area hospital, KNX indicating a Knoxville-area hospital, and POR indicating a Portland-area hospital. The month and year of the isolate's collection are given to the right of the hospital code. Isolates with *ERG11* SNP 1 are indicated with a black circle next to the isolate name. Those with C1856T (A619V), the only *MRR1* SNP to occur exclusively in multiple nonsusceptible isolates with *MDR1* expression elevated more than 5-fold, are indicated with an arrow.

tivity (22), and modeling of the same substitution as SNP 1 in *C. tropicalis* Erg11p has suggested that it would produce the same result (21). The MIC values of the isolates with *ERG11* SNP 1, shown in Table 1, also validate the new species-specific breakpoints for *C. parapsilosis* and fluconazole (23, 24). These data confirm that isolates with known mutations in genes involved in fluconazole resistance have MIC values well below the previous resistance values of 64 μg/ml but above the current limit for susceptibility of 4 μg/ml.

The sequencing of transcription factor *MRR1* and qRT-PCR quantification of *MDR1* expression revealed five polymorphisms that were present exclusively in a resistant or SDD isolate or isolates with upregulated *MDR1* expression: amino acid substitutions A619V, A859T, L779F, and R478K and promoter insertion −101insT. None of these can be definitively said to cause either the *MDR1* upregulation or the reduced fluconazole susceptibility, as *MDR1* upregulation was also found in three isolates without an *MRR1* SNP, indicating the existence of *MDR1* upregulation mechanisms beyond alterations in *MRR1*. Nonetheless, they represent the first set of potentially *MDR1*-upregulating mutations in clinical isolates of *C. parapsilosis*, and their precise activities should be further explored.

Of the five polymorphisms, only A619V occurred in multiple isolates, one resistant isolate with 16-fold-increased *MDR1* levels and one SDD isolate with 10-fold-increased *MDR1* levels, suggesting that the SNP may have moderate gain-of-function activity. Aligning the protein sequences of wild-type *C. albicans* and *C. parapsilosis* MRR1p revealed that one of the unique SNPs (A859T) is located at the amino acid equivalent to that of a *C. albicans* SNP (A880E) that has been demonstrated to increase *MDR1* expression (25). This SNP lies within a hot spot ranging from *C. albicans* amino acids 873 to 896 (*C. parapsilosis* amino acids 852 to 875), within which seven demonstrated or putative *C. albicans* gain-of-function mutations and one *C. dubliniensis* mutation have been found (25–28). The alignment also showed that L779F, the SNP present in an isolate with 73-fold *MDR1* upregulation, is located only 3 amino acids away from an amino acid equivalent to the position of *C. albicans* N803D, another SNP shown to cause *MDR1* upregulation (25). The notably high expression, combined with the isolate's particularly elevated MIC, 32 μg/ml, suggests that if it can be linked to an *MRR1* gain of function, L779F may be a particularly potent resistance mechanism.

Interestingly, we found resistant isolates to be significantly more likely to contain an *MRR1* mutation than susceptible isolates. This disparity became especially apparent when resistant isolates that contained *ERG11* SNP 1 (and that therefore already had a putative mechanism of resistance), none of which contained an *MRR1* polymorphism, were excluded. Of the resistant isolates without *ERG11* SNP1, 76.9% contained at least one *MRR1* polymorphism, whereas only 12.7% of susceptible isolates contained at least one *MRR1* polymorphism. Even after excluding isolates with the five potentially *MDR1* overexpression-linked SNPs, 66.7% of resistant isolates without *ERG11* SNP 1 contained an *MRR1* SNP. The persistent disproportionate presence of *MRR1* mutations in resistant isolates suggests that they may play a role in *C. parapsilosis* fluconazole resistance wider than that which can be demonstrated in this research. By discounting SNPs that were present exclusively in isolates with reduced susceptibility and *MDR1* upregulation, it is possible that SNPs that may be selectively upregulating *MDR1* in conjunction with some other, unidentified mechanism were overlooked. Research has also indicated that in *C. albicans*, *MRR1* can increase the expression of many genes beyond *MDR1*, and hyperactive *MRR1* reduces the susceptibility of isolates even in *MDR1* knockouts (29). Therefore, some SNPs that were shown not to cause *MDR1* overexpression could still potentially reduce fluconazole susceptibility through the regulation of other genes.

Microsatellite analysis revealed that most of the SNPs identified in *ERG11* and *MRR1* were found in isolates that tended to be closely related and concentrated in a small number of hospitals. This was particularly noticeable for *ERG11* SNP 1, which was, with one exception, exclusively present in one large clade and in three groups of three isolates that appeared to be clonally related by the methods employed in this study. Three small clusters consisted of fluconazole-resistant isolates from the same hospital, suggesting the persistence of a strain within a hospital or within the general geographic area. This result may also imply that our results may apply only to our small catchment area and may not be generalizable to other areas of the United States or to other countries. Interestingly, in resistant isolates without *ERG11* SNP 1, no hospital specificity was detected, suggesting perhaps that *ERG11* SNP 1 or other associated factors in the clonal isolates may enable those strains to be particularly resilient.

There are several limitations to this study. The first is that we did not try to detect *MDR1* overexpression in the presence of fluconazole induction. It is possible that the presence of fluconazole could be a trigger for *MDR1* overexpression, and the lack of overexpression of *MDR1* for some of the Mrr1p mutations may reflect this limitation. Another limitation is that we have data only for *in vitro* resistance. It is not clear whether this would have translated to treatment failure in each case. Finally, we did not perform any transformation experiments to see if the mutations that we describe could confer resistance to a susceptible isolate.

Here we described the first mutations in clinical isolates of *C. parapsilosis* that confer fluconazole resistance. More alarmingly, we showed that the most prevalent mutation, *ERG11* SNP 1, is present in small clonal clusters and may show a propensity to persist in particular hospitals or communities. With its ability to remain on the hands of health care workers and its perceived current increase in abundance in U.S. hospitals, further surveillance for *C. parapsilosis* isolates harboring these mutations is warranted.

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