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Identification and Differentiation of *Candida parapsilosis* Complex Species by Use of Exon-Primed Intron-Crossing PCR

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The *Candida parapsilosis* complex is composed of *Candida parapsilosis sensu stricto*, *Candida orthopsilosis*, *Candida metapsilosis*, and the closely related species *Lodderomyces elongisporus*. An exon-primed intron-crossing PCR assay was developed here to distinguish the members of the species complex on the basis of the distinct sizes of amplicons, and *Candida orthopsilosis* and *Candida metapsilosis* were further discriminated by restriction enzyme analysis.

The *Candida parapsilosis* complex has been reported as the second most common species isolated from patients with invasive candidiasis such as candidemia or superficial candidiasis in many studies (1–5). On the basis of genetic analysis, the *C. parapsilosis* complex consists of three genetically distinct species, namely, *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*, which are phenotypically indistinguishable from each other (6). The three species exhibit differences in epidemiology, virulence, biofilm formation, and antifungal susceptibility (7–17). *C. metapsilosis* has been demonstrated to be the least virulent member of the complex (7, 8). *Lodderomyces elongisporus*, a closely related species, has also been verified as a cause of infection in case reports and epidemiologic studies (2, 18–20). Thus, a four-species complex has been suggested to replace the three-species complex (19). Thus, it is clinically important to distinguish among the members of this species complex.

So far, various molecular approaches, such as PCR-restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA, real-time PCR, PCR with specific primers, matrix-assisted laser desorption ionization-time of flight mass spectrometry, sequencing analysis, and a simple PCR developed recently, were established to differentiate the former three-species complex (6, 21–27). Although turquoise blue colonies on BBL CHROMagar *Candida* medium (Becton, Dickinson and Company, Sparks, MD) or sequence analysis could be used to distinguish *L. elongisporus* from the three-species complex (19), no other molecular tool has been well established. PCR analyses based on intron size differences or intron loss have been used to easily differentiate closely related clinically important yeast species, and exon-primed intron-crossing (EPIC) PCR analyses of intron length polymorphisms were also widely used as a molecular typing tool for multiple eukaryotes (28–31). Herein, an EPIC PCR combined with restriction enzyme analysis was developed to differentiate the present four-species complex and verified to be a simple, inexpensive, and reliable method.

Genomic sequence data for the type strains *C. parapsilosis* CDC 317, *C. orthopsilosis* Co90-125, and *L. elongisporus* YB-4239 (available at http://www.ncbi.nlm.nih.gov/genome) were aligned and analyzed by LAGAN as described previously (28). *C. metapsilosis* was not included in the analysis because of unavailability of the genomic data online until now. The gene for manganese superoxide dismutase (MnSOD), a phylogenetic marker previously used to identify closely related fungal species (32), was chosen because of its marked difference in intron length among *C. parapsilosis* CDC 317, *C. orthopsilosis* Co90-125, and *L. elongisporus* YB-4239, and its homologous sequence in *C. metapsilosis* MCO448 was cloned and sequenced. A slight difference in intron length between C. *metapsilosis* and *C. orthopsilosis* was found, which is consistent with the fact that C. *metapsilosis* is more closely related to *C. orthopsilosis* than to the others (6). We calculated the intron length of one allele of the MnSOD gene as 169 bp for *L. elongisporus* YB-4239, 79 bp for *C. metapsilosis* MCO448, and 64 bp for *C. orthopsilosis* Co 90-125 and established intron loss by *C. parapsilosis* CDC 317 (Fig. 1).

A degenerate primer pair, MnSODF (5′ GCTTTAGTGGACA AATCAATGAYCT 3′) and MnSODR (5′ AGTTGATGTAACCA CCACCRTTG 3′), was designed on the basis of the exon region of the MnSOD gene. PCR was performed in a final volume of 50 μl containing 50 ng of 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 an Eppendorf Mastercycler 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 and a final extension step of 72°C for 6 min. PCR products were separated on a 2.0% (wt/vol) agarose gel at 90 V for 50 min. All PCRs were conducted in duplicate. PCR products with sizes similar to those of *C. metapsilosis* and *C. orthopsilosis* were further discriminated by restriction digestion with FastDigest enzyme StyI with the cutting site located in the exon region of the *C. orthopsilosis* gene but not *C. metapsilosis* (Fig. 1). Restriction di-

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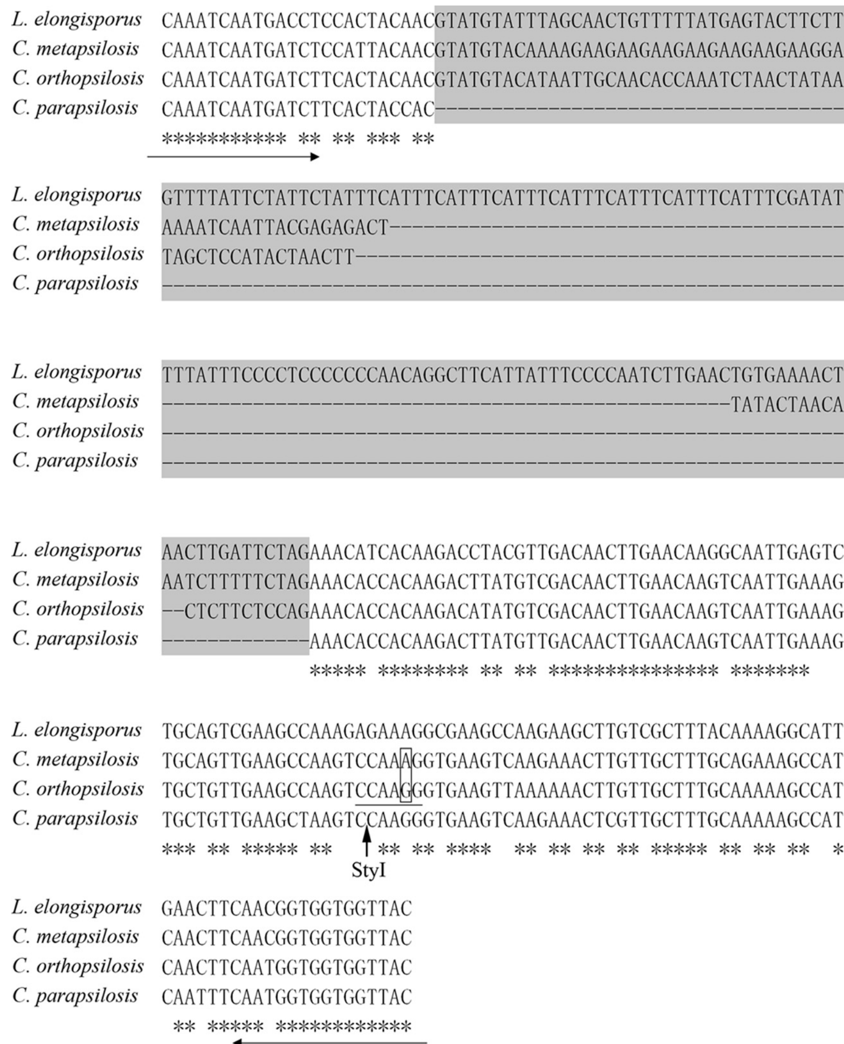

FIG 1 Multiple-sequence alignment of fragments of the MnSOD gene orthologs in type strains. The intron within the gene fragment is on a gray background. Intron loss and size differences are indicated by dashes. The positions of the primers used are indicated by the horizontal arrows. The cutting site of the StyI enzyme is indicated by the vertical arrow. The sequence recognized by StyI is underlined. The nucleotide difference between *C. metapsilosis* and *C. orthopsilosis* in the recognition site is boxed.

gestions were performed according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania), and the reaction mixture was incubated at 37°C for 20 min before separation of the product on a 2.0% (wt/vol) agarose gel at 90 V for 50 min.

A total of 210 strains belonging to the *C. parapsilosis* complex (112 *C. parapsilosis* isolates, 56 *C. metapsilosis* isolates, 30 *C. orthopsilosis* isolates, and 12 *L. elongisporus* isolates) were involved in the assay. Among them, nonreference strains were identified as belonging to one of the four species by sequencing of the internal transcribed spacer region as previously described (28). For detailed information regarding the strains tested, see Table S1 in the supplemental material. Genomic DNA was extracted with the MasterPure Yeast DNA Purification kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions. Additionally, reference strains of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae*, and *C. famata* were chosen to verify the specificity of the primer pair.

As expected, electrophoretic analysis revealed an approximately 340-bp amplicon for *L. elongisporus*, a 250-bp amplicon for *C. metapsilosis*, a 235-bp amplicon for *C. orthopsilosis*, and a 171-bp amplicon for *C. parapsilosis* (Fig. 2). No StyI cutting site was found in any of the amplicons from *C. metapsilosis* isolates, but amplicons from all of the *C. orthopsilosis* isolates were cut into 165- and 70-bp fragments in restriction enzyme analyses (Fig. 2). All of the isolates belonging to the four-species complex involved were correctly identified, and none of the other *Candida* species tested produced an amplification product in this assay.

It was reported that *C. parapsilosis sensu stricto* was the predominant species in the group, followed by *C. orthopsilosis* and *C. metapsilosis*, which together accounted for fewer than 10% of the *C. parapsilosis* complex isolates from infections (16). In addition, *L. elongisporus* was the least frequently encountered species in this group in clinical samples (2, 16). Unlike the PCR-RFLP analyses reported in other studies (6, 21, 23), only strains of *C. orthopsilosis* and *C. metapsilosis* that are difficult to discriminated by agarose gel electrophoresis are required to be further distinguished by

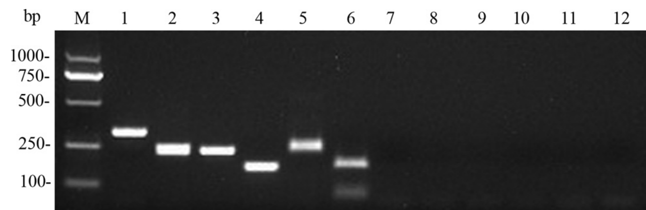

FIG 2 Agarose gel electrophoresis of PCR and enzyme digestion products. Lanes: M, DL2000 ladder; 1, *L. elongisporus* CBS10974; 2, *C. metapsilosis* MCO448; 3, *C. orthopsilosis* Y27733; 4, *C. parapsilosis* ATCC 22019; 5, *C. metapsilosis* MCO448 enzyme digestion product; 6, *C. orthopsilosis* Y27733 enzyme digestion product; 7, *C. albicans* ATCC 10231; 8, *C. tropicalis* ATCC 750; 9, *C. guilliermondii* CBS 2030; 10, *C. glabrata* ATCC 90030; 11, *C. krusei* ATCC 6258; 12, negative control.

rapid restriction enzyme analysis. Most of the strains can be identified without the further restriction enzyme analysis because *C. orthopsilosis* and C. *metapsilosis* are less commonly recovered from clinical specimens. The proportion of *L. elongisporus* isolates in clinical samples may be underestimated because of the unavailability of a suitable molecular tool, and our approach can solve this problem. This approach could be used to distinguish the members of the four-species complex among untyped *Candida* isolates or strains routinely identified as *C. parapsilosis* in a clinical laboratory. Finally, we report here a simple PCR assay with restriction enzyme analysis that performed well in discriminating among the members of the present four-species complex of *C. parapsilosis sensu lato*.

Nucleotide sequence accession number. The sequence of the MnSOD gene fragment of reference strain MCO448 was deposited in GenBank under accession number KF974528.

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REFERENCES

1. **Won EJ, Shin JH, Lee K, Kim MN, Lee HS, Park YJ, Joo MY, Kim SH, Shin MG, Suh SP, Ryang DW.** 2013. Accuracy of species-level identification of yeast isolates from blood cultures from 10 university hospitals in South Korea by use of the matrix-assisted laser desorption ionization-time of flight mass spectrometry-based Vitek MS system. J. Clin. Microbiol. **51:**3063–3065. http://dx.doi.org/10.1128/JCM.00945-13.
2. **Wang H, Xiao M, Chen SC, Kong F, Sun ZY, Liao K, Lu J, Shao HF, Yan Y, Fan H, Hu ZD, Chu YZ, Hu TS, Ni YX, Zou GL, Xu YC.** 2012. *In vitro* susceptibilities of yeast species to fluconazole and voriconazole as determined by the 2010 National China Hospital Invasive Fungal Surveillance Net (CHIF-NET) study. J. Clin. Microbiol. **50:**3952–3959. http://dx.doi.org/10.1128/JCM.01130-12.
3. **Bassetti M, Merelli M, Righi E, Diaz-Martin A, Rosello EM, Luzzati R, Parra A, Trecarichi EM, Sanguinetti M, Posteraro B, Garnacho-Montero J, Sartor A, Rello J, Tumbarello M.** 2013. Epidemiology, species distribution, antifungal susceptibility, and outcome of candidemia across five sites in Italy and Spain. J. Clin. Microbiol. **51:**4167–4172. http://dx.doi.org/10.1128/JCM.01998-13.
4. **Feng X, Ling B, Yang G, Yu X, Ren D, Yao Z.** 2012. Prevalence and distribution profiles of *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* responsible for superficial candidiasis in a Chinese university hospital. Mycopathologia **173:**229–234. http://dx.doi.org/10.1007/s11046-011-9496-5.
5. **Cisterna R, Ezpeleta G, Telleria O.** 2010. Nationwide sentinel surveillance of bloodstream *Candida* infections in 40 tertiary care hospitals in Spain. J. Clin. Microbiol. **48:**4200–4206. http://dx.doi.org/10.1128/JCM.00920-10.
6. **Tavanti A, Davidson AD, Gow NA, Maiden MC, Odds FC.** 2005. *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. J. Clin. Microbiol. **43:**284–292. http://dx.doi.org/10.1128/JCM.43.1.284-292.2005.
7. **Bertini A, De Bernardis F, Hensgens LA, Sandini S, Senesi S, Tavanti A.** 2013. Comparison of *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* adhesive properties and pathogenicity. Int. J. Med. Microbiol. **303:**98–103. http://dx.doi.org/10.1016/j.ijmm.2012.12.006.
8. **Németh T, Toth A, Szenzenstein J, Horvath P, Nosanchuk JD, Grozer Z, Toth R, Papp C, Hamari Z, Vagvolgyi C, Gacser A.** 2013. Characterization of virulence properties in the *C. parapsilosis* sensu lato species. PLoS One **8:**e68704. http://dx.doi.org/10.1371/journal.pone.0068704.
9. **Treviño-Rangel RJ, Gonzalez JG, Gonzalez GM.** 2013. Aspartyl proteinase, phospholipase, esterase and hemolysin activities of clinical isolates of the *Candida parapsilosis* species complex. Med. Mycol. **51:**331–335. http://dx.doi.org/10.3109/13693786.2012.712724.
10. **de Toro M, Torres MJ, Maite R, Aznar J.** 2011. Characterization of *Candida parapsilosis* complex isolates. Clin. Microbiol. Infect. **17:**418–424. http://dx.doi.org/10.1111/j.1469-0691.2010.03302.x.
11. **Cantón E, Espinel-Ingroff A, Peman J, Del CL.** 2010. *In vitro* fungicidal activities of echinocandins against *Candida metapsilosis*, *C. orthopsilosis*, and *C. parapsilosis* evaluated by time-kill studies. Antimicrob. Agents Chemother. **54:**2194–2197. http://dx.doi.org/10.1128/AAC.01538-09.
12. **Romeo O, Delfino D, Costanzo B, Cascio A, Criseo G.** 2012. Molecular characterization of Italian *Candida parapsilosis* isolates reveals the cryptic presence of the newly described species *Candida orthopsilosis* in blood cultures from newborns. Diagn. Microbiol. Infect. Dis. **72:**234–238. http://dx.doi.org/10.1016/j.diagmicrobio.2011.12.002.
13. **Garcia-Effron G, Cantón E, Peman J, Dilger A, Roma E, Perlin DS.** 2012. Epidemiology and echinocandin susceptibility of *Candida parapsilosis* sensu lato species isolated from bloodstream infections at a Spanish university hospital. J. Antimicrob. Chemother. **67:**2739–2748. http://dx.doi.org/10.1093/jac/dks271.
14. **Cantón E, Peman J, Quindos G, Eraso E, Miranda-Zapico I, Alvarez M, Merino P, Campos-Herrero I, Marco F, de la Pedrosa EG, Yague G, Guna R, Rubio C, Miranda C, Pazos C, Velasco D.** 2011. Prospective multicenter study of the epidemiology, molecular identification, and antifungal susceptibility of *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* isolated from patients with candidemia. Antimicrob. Agents Chemother. **55:**5590–5596. http://dx.doi.org/10.1128/AAC.00466-11.
15. **Silva AP, Miranda IM, Lisboa C, Pina-Vaz C, Rodrigues AG.** 2009. Prevalence, distribution, and antifungal susceptibility profiles of *Candida parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* in a tertiary care hospital. J. Clin. Microbiol. **47:**2392–2397. http://dx.doi.org/10.1128/JCM.02379-08.
16. **Lockhart SR, Messer SA, Pfaller MA, Diekema DJ.** 2008. Geographic distribution and antifungal susceptibility of the newly described species *Candida orthopsilosis* and *Candida metapsilosis* in comparison to the closely related species *Candida parapsilosis*. J. Clin. Microbiol. **46:**2659–2664. http://dx.doi.org/10.1128/JCM.00803-08.
17. **Gomez-Lopez A, Alastruey-Izquierdo A, Rodriguez D, Almirante B, Pahissa A, Rodriguez-Tudela JL, Cuenca-Estrella M.** 2008. Prevalence and susceptibility profile of *Candida metapsilosis* and *Candida orthopsilosis*: results from population-based surveillance of candidemia in Spain. Antimicrob. Agents Chemother. **52:**1506–1509. http://dx.doi.org/10.1128/AAC.01595-07.
18. **Daveson KL, Woods ML.** 2012. *Lodderomyces elongisporus* endocarditis in an intravenous drug user: a new entity in fungal endocarditis. J. Med. Microbiol. **61:**1338–1340. http://dx.doi.org/10.1099/jmm.0.047548-0.

19. **Lockhart SR, Messer SA, Pfaller MA, Diekema DJ.** 2008. *Lodderomyces elongisporus* masquerading as *Candida parapsilosis* as a cause of bloodstream infections. J. Clin. Microbiol. **46:**374–376. http://dx.doi.org/10.1128/JCM.01790-07.
20. **Ahmad S, Khan ZU, Johny M, Ashour NM, Al-Tourah WH, Joseph L, Chandy R.** 2013. Isolation of *Lodderomyces elongisporus* from the catheter tip of a fungemia patient in the Middle East. Case Rep. Med. **2013:**560406. http://dx.doi.org/10.1155/2013/560406.
21. **Mirhendi H, Bruun B, Schonheyder HC, Christensen JJ, Fuursted K, Gahrn-Hansen B, Johansen HK, Nielsen L, Knudsen JD, Arendrup MC.** 2010. Molecular screening for *Candida orthopsilosis* and *Candida metapsilosis* among Danish *Candida parapsilosis* group blood culture isolates: proposal of a new RFLP profile for differentiation. J. Med. Microbiol. **59:**414–420. http://dx.doi.org/10.1099/jmm.0.017293-0.
22. **Souza AC, Ferreira RC, Goncalves SS, Quindos G, Eraso E, Bizerra FC, Briones MR, Colombo AL.** 2012. Accurate identification of *Candida parapsilosis* (sensu lato) by use of mitochondrial DNA and real-time PCR. J. Clin. Microbiol. **50:**2310–2314. http://dx.doi.org/10.1128/JCM.00303-12.
23. **Garcia-Effron G, Cantón E, Peman J, Dilger A, Roma E, Perlin DS.** 2011. Assessment of two new molecular methods for identification of *Candida parapsilosis* sensu lato species. J. Clin. Microbiol. **49:**3257–3261. http://dx.doi.org/10.1128/JCM.00508-11.
24. **del Pilar Vercher M, Garcia MJ, Cantón E, Peman J, Gomez GM, Gomez EV, Del CAL.** 2011. Differentiation of *Candida parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* by specific PCR amplification of the RPS0 intron. Int. J. Med. Microbiol. **301:**531–535. http://dx.doi.org/10.1016/j.ijmm.2011.02.001.
25. **Hays C, Duhamel C, Cattoir V, Bonhomme J.** 2011. Rapid and accurate identification of species belonging to the *Candida parapsilosis* complex by real-time PCR and melting curve analysis. J. Med. Microbiol. **60:**477–480. http://dx.doi.org/10.1099/jmm.0.026633-0.
26. **Quiles-Melero I, Garcia-Rodriguez J, Gomez-Lopez A, Mingorance J.** 2012. Evaluation of matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry for identification of *Candida parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*. Eur. J. Clin. Microbiol. Infect. Dis. **31:**67–71. http://dx.doi.org/10.1007/s10096-011-1277-z.
27. **Prandini TH, Theodoro RC, Bruder-Nascimento AC, Scheel CM, Bagagli E.** 2013. Analysis of Inteins in the *Candida parapsilosis* complex for simple and accurate species identification. J. Clin. Microbiol. **51:**2830–2836. http://dx.doi.org/10.1128/JCM.00981-13.
28. **Feng X, Fu X, Ling B, Wang L, Liao W, Yao Z.** 2013. Development of a singleplex PCR assay for rapid identification and differentiation of *Cryptococcus neoformans* var. *grubii*, *Cryptococcus neoformans* var. *neoformans*, *Cryptococcus gattii*, and hybrids. J. Clin. Microbiol. **51:**1920–1923. http://dx.doi.org/10.1128/JCM.00064-13.
29. **Feng X, Fu X, Ling B, Wang L, Liao W, Pan W, Yao Z.** 2013. Rapid differentiation of cryptic species within *Cryptococcus gattii* by a duplex PCR assay. J. Clin. Microbiol. **51:**3110–3112. http://dx.doi.org/10.1128/JCM.01455-13.
30. **Enache-Angoulvant A, Guitard J, Grenouillet F, Martin T, Durrens P, Fairhead C, Hennequin C.** 2011. Rapid discrimination between *Candida glabrata*, *Candida nivariensis*, and *Candida bracarensis* by use of a singleplex PCR. J. Clin. Microbiol. **49:**3375–3379. http://dx.doi.org/10.1128/JCM.00688-11.
31. **Wang X, Zhao X, Zhu J, Wu W.** 2005. Genome-wide investigation of intron length polymorphisms and their potential as molecular markers in rice (*Oryza sativa* L.). DNA Res. **12:**417–427. http://dx.doi.org/10.1093/dnares/dsi019.
32. **Fréalle E, Noel C, Nolard N, Symoens F, Felipe MS, Dei-Cas E, Camus D, Viscogliosi E, Delhaes L.** 2006. Manganese superoxide dismutase based phylogeny of pathogenic fungi. Mol. Phylogenet. Evol. **41:**28–39. http://dx.doi.org/10.1016/j.ympev.2006.05.001.