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Analysis of Inteins in the *Candida parapsilosis* Complex for Simple and Accurate Species Identification

Tâmara Heloísa Rocha Prandini,^a Raquel Cordeiro Theodoro,^b Ariane C. M. O. Bruder-Nascimento,^a Christina M. Scheel,^c Eduardo Bagagli^a

Department of Microbiology and Immunology, Institute of Biosciences, UNESP, Botucatu, Brazil^a; Universidade Federal do Rio Grande do Norte, Campus de Natal (UFRN), Rio Grande do Norte, Brazil^b; Centers for Disease Control and Prevention, Atlanta, Georgia, USA^c

Inteins are coding sequences that are transcribed and translated with flanking sequences and then are excised by an autocatalytic process. There are two types of inteins in fungi, mini-inteins and full-length inteins, both of which present a splicing domain containing well-conserved amino acid sequences. Full-length inteins also present a homing endonuclease domain that makes the intein a mobile genetic element. These parasitic genetic elements are located in highly conserved genes and may allow for the differentiation of closely related species of the *Candida parapsilosis* (psilosis) complex. The correct identification of the three psilosis complex species *C. parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis* is very important in the clinical setting for improving antifungal therapy and patient care. In this work, we analyzed inteins that are present in the vacuolar ATPase gene VMA and in the threonyl-tRNA synthetase gene *ThrRS* in 85 strains of the *Candida* psilosis complex (46 *C. parapsilosis*, 17 *C. metapsilosis*, and 22 *C. orthopsilosis*). Here, we describe an accessible and accurate technique based on a single PCR that is able to differentiate the psilosis complex based on the VMA intein. Although the *ThrRS* intein does not distinguish the three species of the psilosis complex by PCR product size, it can differentiate them by sequencing and phylogenetic analysis. Furthermore, this intein is unusually present as both mini- and full-length forms in *C. orthopsilosis*. Additional population studies should be performed to address whether this represents a common intraspecific variability or the presence of subspecies within *C. orthopsilosis*.

Candida parapsilosis is one of the most common non-*albicans* *Candida* species that cause human infections. In some regions in Latin America and Spain, *C. parapsilosis* occurs at the same or even a higher frequency than does *Candida albicans*, particularly in bloodstream infections in young children and premature neonates (1–6). *C. parapsilosis* is considered a normal or transient inhabitant of the skin and is found on the hands of health care workers who install central venous catheters and other medical devices, thus suggesting a nosocomial route of transmission (7–10).

Recently, *C. parapsilosis* was reclassified into 3 species: *C. parapsilosis* (sensu stricto), *Candida orthopsilosis*, and *Candida metapsilosis* (11). However, species definition within the *C. parapsilosis* (psilosis) group may be more complex than was initially thought. Prior studies have identified heterogeneity among *C. orthopsilosis* isolates (12–14), and they have also revealed three polymorphic sites in the internal transcribed spacer (ITS)-5.8S rRNA region of 13 isolates identified as *C. orthopsilosis* that appeared to separate the species into two genotypes. This conclusion was supported by a mating type locus (MTL) analysis, which placed *C. orthopsilosis* into one of two groups, type 1 or type 2 (14).

Although the prevalences, distributions, drug susceptibilities, and biofilm productions of these species remain unclear, there are several studies that demonstrate differences among them (12, 15–22). For instance, the recently published genome of *C. orthopsilosis* shows a reduction in the gene families associated with pathogenesis compared with that of *C. parapsilosis* (23). This is in agreement with the finding that few infections are caused by *C. orthopsilosis* and *C. metapsilosis*, which also suggests that these species are less virulent than *C. parapsilosis*. Even so, *C. orthopsilosis* and *C. parapsilosis* are able to cause similar degrees of tissue damage, whereas *C. metapsilosis* is less virulent (24), more susceptible to host responses, and less efficiently phagocytosed than other spe-

cies of the psilosis complex (25). These findings indicate that correct species identification may have a therapeutic impact.

Several molecular techniques have been used to distinguish psilosis species, including PCR amplification of the secondary alcohol dehydrogenase-encoding gene (*SADH*), followed by digestion with the restriction enzyme *BanI* (11, 17), analysis of randomly amplified polymorphic DNA (RAPD) (12, 26–28), restriction fragment length polymorphism (RFLP) (28, 29), quantitative PCR (qPCR) (30), qPCR followed by high-resolution melt analysis (HRM) (31), nucleotide sequencing analysis (32), pyrosequencing (33), matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (34, 35), and micro-satellite analysis (36). Despite the vast array of techniques described, there is still a need for a simple, rapid, and low-cost method to differentiate these species in molecular diagnostic laboratories in order to monitor the incidence of infection.

Inteins, known as parasitic genetic elements, may prove to be a promising resource for differentiating related species (37, 38) since they are located in highly conserved genes. Inteins are intervening sequences that are transcribed and translated with flanking host protein sequences and then are self-excised by protein splic-

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Address correspondence to Eduardo Bagagli, bagagli@ibb.unesp.br.

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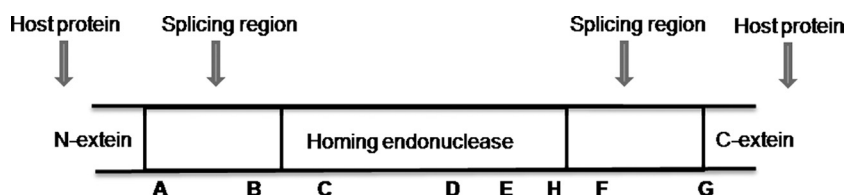


FIG 1 Representation of a full-length intein identified with the nomenclature of Perler et al. (40). Motifs A, B, F, and G are important for self-splicing. Motifs C, D, E, and H are associated with the homing process.

ing; the flanking protein sequences (exteins) are joined by a peptide bond to form the functional protein (39–41).

There are two types of inteins in fungi, the mini-inteins and the full-length inteins, both of which present a splicing domain containing four blocks of relatively well-conserved amino acid sequences (blocks A, B, F, and G) (Fig. 1). In addition, full-length inteins present blocks C, D, E, and H of a homing endonuclease (HE) domain, with the potential to make the intein a mobile genetic element; this results in the occupation of empty alleles and duplication of the parasitic genetic element (42–44).

Primers designed for the flanking conserved gene regions will PCR amplify and discriminate three genotype types: full-length intein, mini-intein, and absence of intein. In addition, the full-length inteins are expected to have more sequence variation (including size variations due to indels) in the endonuclease domain than in the splicing domain due to a more relaxed selection, mainly when the homing endonuclease is no longer active (37).

In the present work, we evaluate the distribution and phylogeny of two inteins in a representative number of isolates from the psilosis group, the vacuolar ATPase membrane (VMA) extein and the threonyl-tRNA synthetase extein (ThrRS) inteins. The data indicate that distribution and variation among VMA inteins within the psilosis complex may allow for species discrimination using a relatively low-cost PCR approach. In addition, we have also found the existence of two idiomorphic inteins, ThrRS-A (a mini-intein) and ThrRS-B (a full-length intein), at the same insertion site of distinct organisms of *C. orthopsilosis* species, which may indicate heterogeneity among *C. orthopsilosis* isolates, different varieties, or even different species.

MATERIALS AND METHODS

Isolates. A total of 85 strains were used in this study (see Table S1 in the supplemental material). Thirty-four strains (16 from *C. metapsilosis* and 18 from *C. orthopsilosis*) were obtained from the Centers for Disease Control and Prevention (CDC) (Atlanta, GA), 48 strains (45 from *C. parapsilosis* and three from *C. orthopsilosis*) were taken from patients of the Hospital das Clínicas (HC), Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu, São Paulo state, Brazil, and three reference strains (from *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*) were obtained from the American Type Culture Collection (ATCC). The identities of all isolates were confirmed by ITS1-5.8S-ITS2 rRNA gene sequencing.

Culture and DNA extraction. The *Candida* isolates were cultured on brain heart infusion agar (BHI) at 35°C for 24 to 48 h. DNA was isolated using the DNeasy blood and tissue kit (Qiagen, Valencia, CA), with slight modifications to the manufacturer's instructions. For each isolate, two to three colonies of *Candida* spp. were picked from the BHI slants and placed in 5-ml polypropylene tubes containing 800 µl Qiagen ATL buffer and 60 U of proteinase K. The mycelia were homogenized using the Omni TH mixer (Omni International, Kennesaw, GA) at low speed for 30 s and then high speed for 30 s, using a clean probe between each isolate. Homoge-

nates were capped and incubated at 55°C for 1 h with frequent vortexing and then were cooled to room temperature. RNase A (Sigma-Aldrich Corp., St. Louis, MO) was then added to obtain a final concentration of 1 mg/ml and the mixture was incubated for 5 min at room temperature (RT), followed by the addition of 900 µl Qiagen buffer AL and vortexing. Homogenates were incubated at 70°C for 10 min and then were transferred to 1.7-ml microcentrifuge tubes and centrifuged at 10,000 × g for 10 min. Clear supernatants (1 ml each) were transferred to clean microcentrifuge tubes, into which a 50% volume of ethanol (Sigma-Aldrich Corp.) was added. The suspensions were vortexed and transferred to Qiagen DNeasy columns, and the manufacturer's instructions were followed throughout the remainder of the procedure. DNA was eluted in 200 µl of 10 mM Tris HCl (pH 8.0) and maintained at –20°C.

Alternatively, some isolates were cultured on Sabouraud dextrose agar (SDA) slants at 35°C and the DNA was extracted by initial cell disruption with glass beads (425 to 600 µm, acid washed) (Sigma, St. Louis, MO) in a solution of 1 M sorbitol and 125 mM EDTA (45).

Amplification and sequencing of the VMA and ThrRS inteins. The primer design involved a preliminary search in the *Candida* Genome Database for the sequences of the VMA and ThrRS genes. *C. orthopsilosis* and *C. parapsilosis* sequences were aligned in MEGA version 5.0, and two degenerate primer pairs were designed in the flanking exteins, one for amplification of the VMA intein (TP1fwd, 5'-ACTGCTGATTAYCCATT GTTG, and TP2rev, 5'-AGATTGAWGCTTCTCTKGCAG-3') and the other for amplification of the ThrRS intein (TP3fwd, 5'-GAARGARGCT GCTGAAAGAG, and TP4rev, 5'-TCTTGTGGAAACGACGAAC-3'). The expected PCR fragment length of the VMA intein and partial extein from *C. orthopsilosis* was 1,909 bp, while the *C. parapsilosis* VMA fragment length was an expected 319 bp, since the intein is not present (23, 46). The expected PCR fragment length for the ThrRS intein from *C. parapsilosis* was 988 bp (46) versus 1,756 in *C. orthopsilosis* (*Candida* Genome Database). For the species *C. metapsilosis*, there was no previous information about the presence or length of either intein.

Each PCR used a reaction volume of 25 µl and contained 23 µl of 1× PCR buffer (200 mM Tris-HCl [pH 8.4], 1.5 mM MgCl₂, 50 mM KCl), 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate (dNTP), 0.4 mM each

TABLE 1 PCR fragment length, type, and size of the inteins VMA and ThrRS in *Candida* species from the psilosis complex

<i>Candida</i> species	Size of intein (bp)		Type of intein		PCR fragment length (bp) ^a	
	VMA	ThrRS	VMA	ThrRS	VMA	ThrRS
<i>C. parapsilosis</i>	Absent	549	Absent	Mini	319	988
<i>C. metapsilosis</i>	1,362	516	Full-length	Mini	1,681	955
<i>C. orthopsilosis</i> A (mini-intein)	1,590	540	Full-length	Full-length	1,909	979
<i>C. orthopsilosis</i> B (full-intein)	1,590	1,317	Full-length	Mini	1,909	1,756

^a The PCR products include the intein plus the 5' and 3' portions of the host gene (extein), except for the CpaVMA, whose amplicon consists only of the extein portion.

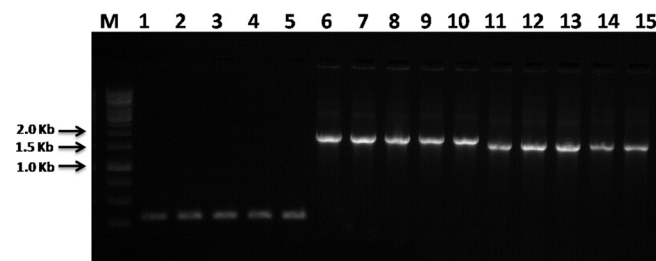


FIG 2 Agarose gel electrophoresis of PCR carried out with TP1 and TP2 primers for amplification of VMA inteins from *C. parapsilosis* (lanes 1 to 5), *C. orthopsilosis* (lanes 6 to 10), and *C. metapsilosis* (lanes 11 to 15). Lane M, 1-kb DNA ladder (Promega).

primer, 1 unit of *Taq* polymerase (Invitrogen), and 2 μ l *Candida* DNA. Thermal cycling conditions were 95°C for 5 min followed by 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The PCR products were identified by 1% agarose gel electrophoresis stained with ethidium bromide.

Amplicons (10 μ l) were purified enzymatically with 4 μ l of Exo-SAP-IT (GE Healthcare) for 15 min at 37°C followed by 15 min at 80°C to inactivate the enzyme. The samples were subsequently submitted to a sequencing reaction and capillary electrophoresis in the ABI3500 DNA analyzer (Applied Biosystems) at the Laboratory of Molecular Diagnosis in the Department of Microbiology and Immunology, Instituto de Biociências (IBB)-UNESP. The DNA samples used for the PCR and sequencing reactions are listed in Table S1 in the supplemental material. A total of 85 strains were used for the VMA and 49 for the ThrRS PCRs. The ThrRS intein was sequenced from 49 strains and the VMA intein from three strains.

Sequence analysis of VMA. The sequences were aligned using the Clustal W algorithm implemented in MEGA v5.0 software (47) to compare the degree of conservation of the splicing and homing endonuclease domains. Full-length VMA inteins were compared to the VMA intein from *Saccharomyces cerevisiae* (GenBank accession no. Q874G3) in order to observe the presence or absence of the two aspartic acid residues (Asp-218 and Asp-326) that are involved in the activity of the homing endonuclease (48, 49).

Phylogenetic analysis of ThrRS intein. Sequences were aligned in MEGA v5.0 (47), after which phylogenetic analyses based on the splicing domain of *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis* ThrRS inteins (CpaThrRS for *C. parapsilosis*, CmeThrRS for *C. metapsilosis*, CorThrRS-A for *C. orthopsilosis* mini-intein, and CorThrRS-B for *C. orthopsilosis* full-length intein) were performed by the maximum likelihood (ML) method using the website software version of PhyML (50). The Whelan and Goldman (WAG) model (51) was used as the distance model according to MEGA v5.0. The transition-to-transversion (Ti/Tv) ratio, gamma shape parameter, and proportion of nonvariant sites were estimated by the maximum likelihood method from a neighbor-joining tree (BIONJ algorithm). Bootstrap resampling (52) was applied to assess support for individual nodes, using 1,000 replicates with random additions and tree bisection and reconnection (TBR) branch swapping. The ThrRS intein sequence from *Candida tropicalis* (CtrThrRS, GenBank accession no. XP002550936) was used as the outgroup.

Polymorphism analysis of ITS (ITS1-5.8S-ITS2) sequence from *C. orthopsilosis*. The ITS sequences (ITS1-5.8S-ITS2 rRNA gene) from the 14 isolates of *C. orthopsilosis* were aligned by Mega 5 in order to detect the two types of *C. orthopsilosis* (type 1 and type 2) that were already proposed (14) and to evaluate whether the ITS polymorphism of the isolates is correlated with the two groups of CorThrRS inteins, one being a mini-intein and the other one a full-length intein (CorThrRS-A and CorThrRS-B, respectively). The ITS sequencing, with the universal fungal primers ITS4 and ITS5 (53), was performed by the CDC group using the

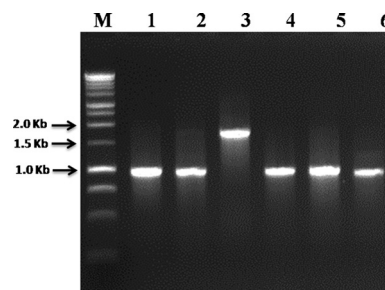


FIG 3 Agarose gel electrophoresis of the PCR carried out with TP3 and TP4 primers for amplification of ThrRS inteins from *C. parapsilosis* (lane 1), *C. orthopsilosis* (lanes 2, 4, and 5, strains with ThrRS-A, and lane 3, strain with ThrRS-B intein), and *C. metapsilosis* (lane 6). Lane M, 1-kb DNA ladder (Promega).

BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Inc.) in a 3730 DNA analyzer (Applied Biosystems, Inc.).

Nucleotide sequence accession numbers. The ThrRS intein, VMA intein, and ITS1-5.8S-ITS2 rRNA gene sequence accession numbers used (which are also listed in Table S1 in the supplemental material) are as follows: for the *C. parapsilosis* ThrRS inteins, KC989746 through KC989748, JQ670685, and JQ670688 through JQ670693; for the *C. orthopsilosis* ThrRS inteins, KC989749 through KC989770; for the *C. metapsilosis* ThrRS inteins, KC989771 through KC989786 and JQ670686; for the *C. orthopsilosis* VMA inteins, KC989788 and KC989789; for the *C. metapsilosis* VMA intein, KC989787; and for the ITS1-5.8S-ITS2 rRNA gene, KC990354 through KC990357.

RESULTS

Analysis of VMA intein in the psilosis complex. The VMA intein is absent in *C. parapsilosis* isolates, while it is present as a full-length intein in all *C. orthopsilosis* and *C. metapsilosis* isolates. The expected PCR fragment length of 319 bp that contains only the extein regions of VMA was observed in all *C. parapsilosis* isolates evaluated, whereas the fragment lengths for *C. orthopsilosis* and *C. metapsilosis* were 1,909 bp and 1,681 bp, respectively, due to the presence of the inteins (Table 1 and Fig. 2). In Fig. 2, the representative gel electrophoresis demonstrates the possibility of distinguishing the three species of the psilosis complex by comparing the PCR fragment length generated by the same pairs of degenerated primers that anneal in the extein regions of VMA.

All amino acid residues involved in protein splicing were found in CorVMA and CmeVMA inteins, with the exception of the Thr-x-x-His motif in block B (46), which is absent (see Fig. S1 in the supplemental material). In addition, the highly conserved blocks A, B, F, and G indicate that the splicing function operates in both CorVMA and CmeVMA. Compared to the VMA intein from *Saccharomyces cerevisiae*, SceVMA (GenBank accession no. Q874G3), we found that the two aspartic acid residues (Asp-218 and Asp-326) that are critical to the activity of the homing endonuclease (48, 49) are conserved in CorVMA. In CmeVMA, the first aspartate was changed to asparagine, while the second one is conserved (see Fig. S1 in the supplemental material).

Analysis of ThrRS intein in the psilosis complex. The degenerate primers used in PCR were designed in the N and C extein terminals of *C. parapsilosis* and *C. orthopsilosis* ThrRS and detected the presence of mini-inteins, herein named CpaThrRS (549 bp), CmeThrRS (516 bp), and CorThrRS-A (540 bp). These mini-inteins were located at the same insertion site in all isolates of *C. parapsilosis* and *C. metapsilosis* and in 13 of the 22 isolates of *C.*

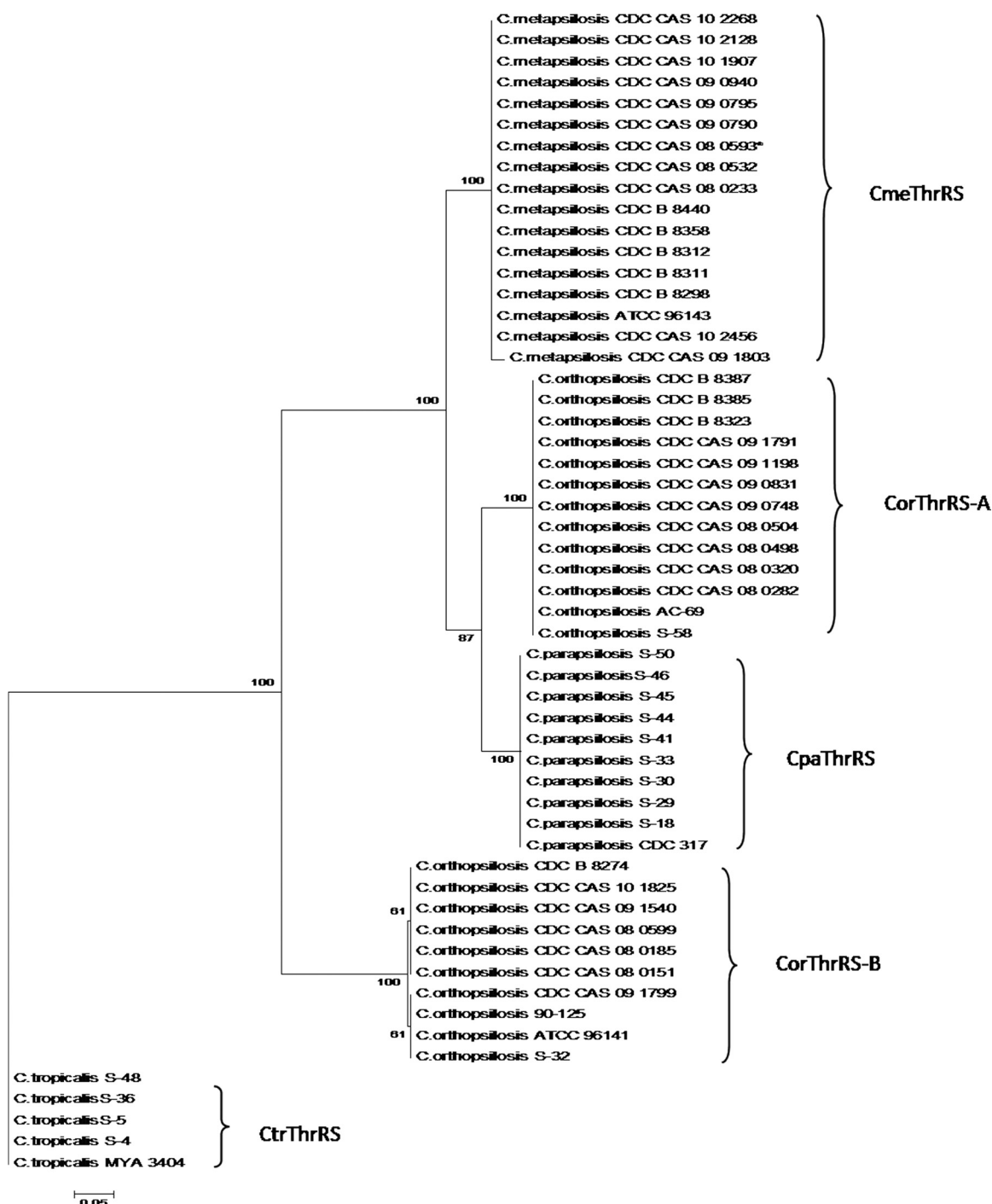


FIG 4 Molecular phylogenetic analysis by maximum likelihood method of amino acid sequences from the splicing domain of the ThrRS inteins. Maximum likelihood (ML) phylogenetic analyses were performed using the website software version of PhyML (50).

orthopsilosis. In the other 9 *C. orthopsilosis* isolates, a full-length intein (CorThrRS-B; 1,317 bp) was observed (Table 1).

The splicing domains were highly conserved in all CpaThrRS, CmeThrRS, CorThrRS-A, and CorThrRS-B inteins (see Fig. S2 in the supplemental material). All of them presented with cysteine (C) as the first amino acid and asparagine (N) the last (essential for the splicing mechanism), and contained the Thr-x-x-His motif in block B. The full-length intein CorThrRS-B also presented an ad-

dition of 3 amino acids (G-R-G and G-K-G, depending on the strain) before block B in the splicing domain, and its HE domain contained both of the critical aspartates, suggesting that it may be active (see Fig. S3 in the supplemental material). The mini-inteins of the three species (CpaThrRS, CmeThrRS, and CorThrRS-A) presented similar amplified fragment sizes (Table 1) that were indistinguishable in agarose (Fig. 3), yet their deduced amino acid sequences differed greatly (see Fig. S2 in the supplemental material).

TABLE 2 Polymorphism analysis of the *C. orthopsilosis* intein ThrRS in relation to the ITS (ITS1-5.8S-ITS2) sequence

Intein type in <i>Candida orthopsilosis</i>	Strain	Presence of indicated polymorphism in ITS sequence					
		Polymorphism 1		Polymorphism 2		Polymorphism 3	
		Type 1	Type 2	Type 1	Type 2	Type 1	Type 2
A (mini-intein)	CAS08-0282	+		+		+	
	CAS08-0320	+		+		+	
	CAS08-0498	+		+		+	
	CAS08-0504		+		+		+
	CAS09-0748	+			+	+	
	CAS09-0831		+		+		+
	CAS09-1198		+		+		+
	CAS09-1791		+		+		+
B (full-length intein)	CAS08-0151	+		+		+	
	CAS08-0185	+		+		+	
	CAS08-0599	+		+		+	
	CAS08-1540	+		+		+	
	CAS09-1799	+		+		+	
	CAS10-1825	+		+		+	

Phylogenetic analysis of the splicing domain regions of all ThrRS inteins produced a tree topology that distinguished the three species of the psilosis complex, mainly when considering the three mini-inteins (CpaThrRS, CmeThrRS, and CorThrRS-A). The mini- and the full-length ThrRS inteins of *C. orthopsilosis* (CorThrRS-A and CorThrRS-B) do not group together, while the intein CorThrRS-A is closely related to CmeThrRS and CpaThrRS, and the intein CorThrRS-B is genetically more distant from the clade that encompasses CmeThrRS, CpaThrRS, and CorThrRS-A (Fig. 4). The ITS sequences of 14 *C. orthopsilosis* isolates, 8 presenting the mini-intein CorThrRS-A and 6 presenting the full-length intein CorThrRS-B, indicate that there is no correlation with the polymorphism pattern types 1 and 2 that were already proposed (14) (Table 2).

DISCUSSION

The differentiation of the psilosis complex into *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* species is important for epidemiological purposes and also for changes in resistance to antifungal therapy, since *C. orthopsilosis* and *C. metapsilosis* are more susceptible to some currently used antifungals (16, 17, 19) and may have a lower capacity for biofilm formation (19, 21).

Ever since *C. parapsilosis* sensu lato was divided into a complex of three species, several authors have proposed different molecular identification techniques that are lengthy or costly, since they require further processing after DNA amplification. In contrast, we were able to differentiate species of the psilosis complex by means of a single PCR assay using degenerate primers that are complementary to the highly conserved VMA gene sites that flank the intein. We found that *C. metapsilosis* presents a VMA intein smaller than the one observed in *C. orthopsilosis*, while *C. parapsilosis* lacks an intein in VMA. This pattern of VMA intein distribution was systematically observed in all of the study isolates, indicating that this approach might be useful to unequivocally distinguish the three species of the psilosis complex. The identification of species using VMA intein sizing (Fig. 1) is an attractive tool for routine use in a clinical laboratory because it is accurate, rapid, and less expensive than other methods that have been previously proposed.

The amino acid residues involved in protein splicing were present in all of the VMA and ThrRS intein sequences analyzed, indicating that the splicing function is operating in both inteins, which was expected since both genes are considered essential for cell function. The Thr-x-x-His motif in block B that is known to assist in the N-terminal transesterification reaction leading to intein splicing (54, 55) is absent in CorVMA and CmeVMA (see Fig. S1 in the supplemental material). However, any residue that can form similar hydrogen bonds can replace these conserved residues without compromising splicing activity (46).

The presence of the essential aspartates Asp218 and Asp 326 in the inteins CorVMA and CorThrRS-B suggests that their HE domains may be active, while the absence of the first aspartate, as well as the degeneration observed in the HE domain of the CmeVMA intein, leads us to speculate that it is not functional, as is the case in most of the yeast VMA inteins (48). This is in agreement with the homing cycle proposed by Burt and Koufopanou (56) for these parasitic genetic elements that present a typical “rise and fall” cycle in the population structure of the affected species: once most of the alleles in a population are occupied by intein invasion, due to the homing process, there is no further constrained selection of a functional endonuclease, which might degenerate and become nonfunctional, so that empty sites may reemerge.

The idiomorphic occurrence of a mini- (CorThrRS-A) and a full-length intein (CorThrRS-B) at the same insertion site of *C. orthopsilosis* had not been observed previously. This interesting observation may indicate intraspecific genetic variation among *C. orthopsilosis* isolates, different varieties, or even different species. The polymorphism analysis of ITS (ITS1-5.8S-ITS2) sequences of the isolates harboring either CorThrRS-A or CorThrRS-B does not corroborate the division into 2 types (types 1 and 2) of *C. orthopsilosis* (14) (Table 2). However, the presence of polymorphisms in the ITS region does not always agree with other molecular markers. For instance, in another study using RAPD and ITS sequencing, the authors (27) divided *C. orthopsilosis* isolates into two groups (P2 and P3). Although the P3 group presents an identical ITS sequence to those of type 2 isolates, there are differences between the P2 group and type 1 isolates. Indeed, despite having been indicated as fungal barcoding (57), analysis of the ITS region

does not appear to be suitable for discrimination between very closely related groups. This was observed, for example, among the cryptic species denominated S1, PS2, and PS3 from the *Paracoccidioides brasiliensis* species complex (58).

Although the phylogeny of the ThrRS inteins clearly distinguished other *Candida* species from the psilosis complex, it did not corroborate the species phylogeny proposed using the ITS region of rRNA (11) or by the analysis of 1,334 partial gene sequences (23); this indicates that *C. metapsilosis* and *C. orthopsilosis* share a more recent common ancestor and comprise the sister clade of *C. parapsilosis*. Furthermore, in our analysis, the intein CorThrRS-B does not group with the CorThrRS-A intein of *C. orthopsilosis*, which might reflect the occurrence of independent intein invasions through two possible scenarios. In the first scenario, the ancestor of *C. orthopsilosis* might have had its ThrRS gene invaded by an intein (ThrRS-A) that followed the homing cycle rules proposed by Burt and Koufopanou (56). The intein may have eventually become fixed in most of the population, leading to the degeneration of its HE domain (which might explain the current mini-intein structure). With the HE degenerated and therefore not functional, empty sites may have increased in the *C. orthopsilosis* population and been reoccupied by another intein, ThrRS-B. In the second scenario, the same site of ThrRS gene might have been occupied at the same moment by two different inteins, ThrRS-A in some populations and ThrRS-B in others. However, the two inteins are not present in the same phase of the homing cycle, because while ThrRS-A is a mini-intein without an HE, ThrRS-B is a full-length intein, probably with a functional HE, which may be an indication that this intein is in the “invasion phase” of the homing cycle and that empty sites might still exist in *C. orthopsilosis* species.

The finding of two types of allelic inteins, mini- and full-length, in a single population is unusual and might provide useful data for future epidemiological and population studies. The discovery of the biological meaning of this observation, whether it represents only an intraspecific polymorphism or two reproductively isolated groups (cryptic species), is still pending and requires additional population studies, especially those that employ sequences from many loci, such as the multilocus sequence typing (MLST), which has been largely used for phylogenetic recognition in many fungal species (59–61). In addition, future studies will also be important to determine whether the two-intein pattern has clinical relevance.

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