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Evolutionary Relationships among Pathogenic *Candida* Species and Relatives

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Small subunit rRNA sequences have been determined for 10 of the most clinically important pathogenic species of the yeast genus *Candida* (including *Torulopsis* [*Candida*] *glabrata* and *Yarrowia* [*Candida*] *lipolytica*) and for *Hansenula polymorpha*. Phylogenetic analyses of these sequences and those of *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* var. *lactis*, and *Aspergillus fumigatus* indicate that *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. viswanathii* form a subgroup within the genus. The remaining significant pathogen, *T. glabrata*, falls into a second, distinct subgroup and is specifically related to *S. cerevisiae* and more distantly related to *C. kefyr* (*psuedotropicalis*) and *K. marxianus* var. *lactis*. The 18S rRNA sequence of *Y. lipolytica* has evolved rapidly in relation to the other *Candida* sequences examined and appears to be only distantly related to them. As anticipated, species of several other genera appear to bear specific relationships to members of the genus *Candida*.

The genus *Candida* comprises an array of yeast species unified mainly by the absence of any sexual form. This method of classification has created a somewhat confusing taxonomy in which most species ultimately are assigned to at least two genera (by sexual and nonsexual forms). Many attempts have been made to define natural relationships among *Candida* species, but these have been generally confounded by the great heterogeneity within the group. The artificiality of the classification scheme has manifested itself in the partial failure of biochemical and genetic tests to define coherent taxa (14). Several members of the genus are associated with significant human disease, including the leading yeast pathogen, *Candida albicans* (24), and thus development of a more meaningful taxonomy for the group is particularly worthwhile.

A few researchers have turned to nucleic acid analyses to resolve relationships within the genus (15, 16, 20, 26), and analysis of DNA sequences in particular has been proposed as an objective method for such assessment (21). The advantages of rRNA sequencing for phylogentic studies have been discussed (7, 33). Use of such macromolecular data allows determination of relationships independent of morphological and physiological criteria, criteria which, among the *Candida* species and relatives in particular, have yielded often confusing and contradictory classification systems. Although rRNA sequences have been widely used to elucidate bacterial evolutionary relationships (33), only a few rRNA sequences of fungal species have been determined (mostly for 5S rRNA [1, 32, 35]; some for 18S rRNA [2, 3, 27]).

We have sequenced cellular rRNAs or cloned 18S genes of 10 of the most commonly isolated pathogenic candidas as well as *Hansenula polymorpha* and *Aspergillus fumigatus*. These sequences, together with those of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* var. *lactis* were used to explore evolutionary relationships amongst the species. Results indicate that *C. albicans*, *Candida tropicalis*, *C. parapsilosis*, and *C. viswanathii* form a highly interrelated group, whereas the sequence of the other significant yeast pathogen, *Torulopsis glabrata*, is associated with a second distinct phylogenetic group in the tree. Analyses confirm earlier observations of the relatively close relationship of *C. albicans* and *S. cerevisiae* (11) and indicate that *Yarrowia lipolytica* is unusual in both its rRNA sequence evolution and its relationship to other members of the genus.

MATERIALS AND METHODS

Source and growth of strains. The fungal strains examined were obtained from the American Type Culture Collection and are listed in Table 1. All strains were cultivated in 100 ml of YM broth (Difco) shaken at room temperature. Cells for analysis were harvested by centrifugation and frozen on dry ice for storage before nucleic acid extraction.

Cellular rRNA isolation and sequencing. (i) Nucleic acid extraction and purification. Cell pellets (0.2 to 0.5 g [wet weight]) from all strains except *C. albicans* and *A. fumigatus* were lysed in a bead beating vial (Biospec Products) containing approximately 400 μl of 0.5-mm acid washed, baked glass beads (Biospec), 750 μl of buffer (50 mM sodium acetate, 10 mM EDTA [pH 5.1]), 50 μl of 20% sodium dodecyl sulfate (SDS), and enough buffer-saturated phenol to fill the tube (approximately 500 μl) by beating for 4 min on a Biospec Products Mini-Beadbeater. Tubes were heated for 10 to 15 min at 60°C to further disrupt cell walls and nucleic acids and then beaten for an additional 2 min. Beads were pelleted by 10 min of centrifugation in a Speed Vac Concentrator (Savant) (note: low-speed centrifugation is required at this step to prevent rupture of tubes). Aqueous phases were reextracted two to three times with 400 μl of buffer-saturated phenol and 100 μl of chloroform-isoamyl alcohol (24:1, wt/vol). Total nucleic acids were precipitated by the addition of 0.1 volume of 3.0 M sodium acetate and 2 volumes of ethanol followed by incubation on dry ice for 10 min. Pellets were dried after centrifugation, suspended in 1.45 ml of

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TABLE 1. Strains of fungi used in this analysis

Species	ATCC[a] no.	GenBank acession no.
Aspergillus fumigatus	36607	M60300 (clone sequence) M60301 (rRNA sequence)
Candida albicans	18804	M60302
Candida guilliermondii	6260	M60304
Candida kefyr (*pseudotropicalis*)	4135	M60303
Candida krusei	6258	M60305
Candida lusitaniae	42720	M60306
Candida parapsilosis	22019	M60307
Candida tropicalis	750	M60308
Candida viswanathii	22891	M60309
Hansenula polymorpha	34438	M60310
Torulopsis (*Candida*) *glabrata*	2001	M60311
Yarrowia (*Candida*) *lipolytica*	18942	M60312

[a] ATCC, American Type Culture Collection.

TMK buffer (10 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 30 mM KCl), and incubated 5 min at room temperature with 20 U of RQ1 DNase (Promega). The samples were mixed with 2.1 ml of 2.01-g/ml cesium trifluoroacetate (Pharmacia) and loaded into a 3-ml Quick Seal tube (Beckman). High-molecular-weight RNA was pelleted by equilibrium density centrifugation (>4 h at 150,000 × *g* at 4°C) in a TLA 100.3 rotor with a Beckman TL100 ultracentrifuge. Pellets were suspended in 200 μl of TMK buffer, precipitated with sodium acetate and ethanol, dried, and suspended in RNase-free water.

(ii) RNA sequencing. Dideoxynucleotide-terminated sequencing with reverse transcriptase was carried out as described by Lane (14a). Use of the synthetic oliodeoxynucleotide primers complementary to conserved 18S rRNA sequences listed in Table 2 allowed determination of approximately 1,650 nucleotides of each rRNA sequence.

18S rDNA cloning and sequencing. Chromosomal DNA was isolated from *C. albicans* spheroplasts by SDS lysis followed by phenol-chloroform extraction as described by Magee et al. (16). Approximately 1 ml of cells of *A. fumigatus* was suspended in 4 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and vortexed in the presence of 1 ml of 0.5-mm glass beads to disrupt the fungal "ball." Then 8 mg each of lysing enzymes L-2265, L-8757, β-glucuronidase (Sigma), and pronase (Calbiochem) were added and mixed, and the suspension was incubated for 30 min at 37°C. The resultant spheroplasts were lysed by the addition of EDTA to 10 mM and SDS to 1% (final concentrations), followed by incubation at 37°C for 30 min. Bulk nucleic acids were isolated from the mixture during two extractions with water-saturated phenol-chloroform-isoamyl alcohol (24:24:1) and precipitated by the addition of ammonium acetate to 2.5 M and 2 volumes of ethanol. The dried nucleic acid pellet was suspended in 500 μl of TMK buffer mixed with 25 μl of 10-mg/ml ethidium bromide and 600 μl of 2.01-g/ml cesium trifluoroactetate (Pharmacia). DNA was banded in the gradient by overnight ultracentrifugation at 130,000 × *g* as described above for rRNA isolation. The resultant DNA band was removed from the tube, precipitated with 2-propanol, suspended in 100 μl of TE buffer, and extracted with water-saturated butanol to remove ethidium bromide. Isopropanol was used to reprecipitate the DNA, and the dried pellet was suspended in TE for amplification. Small subunit rRNA genes were amplified in vitro by the polymerase chain reaction with oligodeoxynucleotide primers complementary to conserved regions of the 5′ and 3′ termini of the genes and containing polylinker sequences to facilitate cloning (19). Amplification products were subsequently cloned into phage M13 and sequenced by dideoxynucleotide chain-termination methods (4). All sequences are available from GenBank and from the authors (see Table 1).

TABLE 2. Summary of small subunit oligodeoxynucleotide reverse sequencing primers used to generate 18S rRNA sequence data[a]

E. coli position no.	Sequence
121-102R	5′-TAATGAGCCATTCGCAG
395-343R	5′-GCCTGCTGCCTTCCTTG
536-519R	5′-GWATTACCGCGGCKGCTG
705-689R	5′-TCCAAGAATTTCACCTC
741-725R	5′-TTGGCAAATGCTTTCGC
926-907R	5′-CCGTCAATTCMTTTRAGTTT
1106-1090R	5′-TTCGTTATCGCAATTAA
1242-1255R	5′-TCAGTGTAGCGCGCGTGC
1406-1392R	5′-ACGGGCGGTGTGTRC
1506-1490R	5′-ACCTTGTTACGACTTTT

[a] W, A or T; M, A or C; R, A or G. Some primer sequences have been published previously (5). Each primer yielded 200 to 300 nucleotides of data; thus many overlapping sequence readings were possible.

Data analysis. 18S rDNA sequences of *S. cerevisiae* (25) and *K. marxianus* var. *lactis* (18) were obtained from the literature. Sequences were manually aligned on the basis of conserved sequence and secondary structural elements (9). Regions of ambiguous sequence alignment were excluded form subsequent analyses to minimize systematic and random errors. A distance-type analysis was used to estimate the average number of mutations accumulated per sequence position between pairs of contemporary organisms (6). Evolutionary trees were inferred as described by Olsen (23). Branching orders were chosen to minimize the weighted mean square difference between the pairwise calculated evolutionary distances and the additive branch lengths for pairs of organisms in the tree (23). The *A. fumigatus* sequence was included to provide an outgroup for the tree algorithm.

RESULTS AND DISCUSSION

Phylogenetic analyses. A matrix of weighted pairwise evolutionary distances and total base differences is given in Table 3. The data from experiment 1 of Table 3 were obtained with approximately 1,240 weighted nucleotide positions, whereas the data from experiment 2 were determined with approximately 1,600 positions. The calculation of similarities for analysis of the total sequence group was constrained by the pronounced divergence of the *Y. lipolytica* sequence, which reduced the set of unambiguously alignable positions. Thus, similarities in this portion of Table 3 are quite high. For the more closely related organisms represented in experiment 2, greater numbers of comparable positions were unambiguously alignable; therefore, greater resolution of sequence relationships was possible in separate analyses of these subgroups. It is important to note that phylogenetic trees inferred from sequence subsets gave topologies for the organisms included here that were the same as those for larger evolutionary trees with all sequences.

The optimized evolutionary tree for the examined sequences is given in Fig. 1. The validity of the branching

TABLE 3. Total pairwise sequence differences and evolutionary distances among fungal sequences examined

Expt and species	Pairwise sequence difference (lightface type) or evolutionary distance (boldface type), avg no. of changes per nucleotide × 100													
	C. albicans	*C. viswanathii*	*C. tropicalis*	*C. parapsilosis*	*C. guilliermondii*	*C. lusitaniae*	*C. krusei*	*H. polymorpha*	*T. glabrata*	*S. cerevisiae*	*C. kefyr*	*K. marxianus* var. *lactis*	*Y. lipolytica*	*A. fumigatus*
Expt 1[a]														
C. albicans		4	4	6	14	49	86	46	38	55	47	47	117	107
C. viswanathii	**0.32**		2	2	14	50	82	44	37	52	46	47	117	109
C. tropicalis	**0.32**	**0.16**		2	12	50	82	43	36	52	44	46	117	110
C. parapsilosis	**0.49**	**0.24**	**0.16**		12	49	83	43	36	52	44	46	116	109
C. guilliermondii	**1.10**	**1.02**	**0.93**	**0.93**		48	84	38	36	48	38	42	111	104
C. lusitaniae	**3.96**	**4.05**	**3.97**	**3.96**	**3.75**		97	70	63	77	65	68	131	112
C. krusei	**6.86**	**6.51**	**6.61**	**6.59**	**6.55**	**7.86**		71	79	90	80	81	158	146
H. polymorpha	**3.61**	**3.45**	**3.36**	**3.36**	**2.98**	**5.68**	**5.52**		36	47	42	42	119	113
T. glabrata	**3.09**	**3.01**	**2.93**	**2.92**	**2.80**	**5.05**	**6.11**	**2.81**		17	18	21	122	110
S. cerevisiae	**4.23**	**4.15**	**4.07**	**4.06**	**3.75**	**6.13**	**7.12**	**3.69**	**1.34**		27	28	132	121
C. kefyr	**3.78**	**3.70**	**3.61**	**3.61**	**3.06**	**5.32**	**6.40**	**3.33**	**1.39**	**2.01**		2	123	111
K. marxianus var. *lactis*	**3.85**	**3.77**	**3.69**	**3.68**	**3.38**	**5.57**	**6.55**	**3.40**	**1.55**	**2.17**	**0.24**		124	111
Y. lipolytica	**9.86**	**9.78**	**9.79**	**9.77**	**9.35**	**11.02**	**13.66**	**9.96**	**10.20**	**11.07**	**10.32**	**10.57**		156
A. fumigatus	**8.96**	**9.05**	**9.15**	**9.14**	**8.63**	**9.34**	**12.29**	**9.51**	**9.10**	**9.96**	**99.21**	**9.28**	**13.37**	
Expt 2[b]														
C. albicans		11	11	16										
C. viswanathii	**0.60**		8	11										
C. tropicalis	**0.70**	**0.41**		13										
C. parapsilosis	**0.95**	**0.67**	**0.76**											
T. glabrata										41	55	58		
S. cerevisiae									**2.44**		55	59		
C. kefyr									**3.31**	**3.18**		5		
K. marxianus var. *lactis*									**3.51**	**3.37**	**0.31**			

[a] Values were obtained using about 1240 unambiguous positions, constrained by the inclusion of the evolutionarily divergent *Y. lipolytica* sequence.
[b] Values obtained using approximately 1600 positions among the organisms listed.

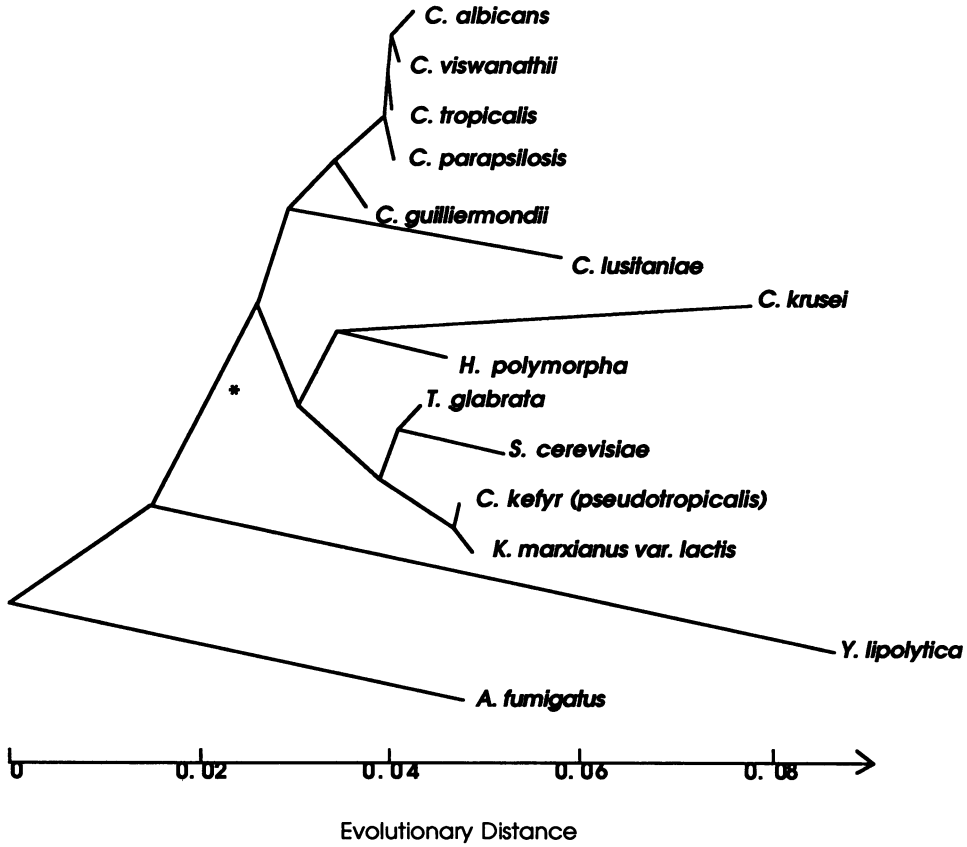

FIG. 1. Evolutionary tree for members of the genus *Candida* and relatives, based on the evolutionary distance data given in Table 3. Evolutionary distance between pairs of organisms is indicated by the sums of the horizontal components of the lengths of the line segments connecting them, as indicated by the scale (average number of changes per nucleotide position).

order of this tree was assessed by the criterion of robustness (5, 23); the topology was generally insensitive to selection of included sequences and nucleotide positions. This is an especially important test for a tree containing sequences with widely differing rates of evolution (as indicated by the large variation in pairwise evolutionary distances between the *C. albicans-C. viswanathii* and *Y. lipolytica-A. fumigatus* sequences) (23). The exception to this robustness was the ambiguous placement of the *C. lusitaniae* sequence, as indicated by the alternative branch point marked with an asterisk in Fig. 1. Although the upper placement appeared in the statistically optimal topology, the lower branching occurred in many near-optimal trees and was dependent upon the organism composition within the tree. Thus, we were not able to resolve the correct placement of the *C. lusitaniae* branch. Inclusion of additional non-*Candida* fungal sequences (unpublished analysis) indicates that the root of this tree is at the base of the *A. fumigatus* segment (Fig. 1).

Natural groupings and relationships. Membership in the genus *Candida* has been said to occur "by default rather than by design" (21), and the phylogenetic positions of representatives from other yeast genera among the *Candida* sequences in the tree reflect this. Non-*Candida* species were chosen for this analysis based on previous indications of specific relationships to members of the genus *Candida*. The resultant phylogeny is monophyletic; all sequences analyzed probably arose from a single common ancestor, and the assumed relationships with other genera are evident.

The subgroup of *C. albicans*, *C. viswanathii*, *C. tropicalis*, and *C. parapsilosis* shows very high levels of intersequence similarities (>99%). Although previous studies of DNA-DNA hybridization found little homology between *C. albicans* and *C. tropicalis* (15), rRNA-DNA studies have suggested a much higher level of similarity (26), a finding reflected in the current tree. Antigenic (10, 29) and nucleic acid base composition (G+C ratios) (15) studies also indicate the high similarity of these two species. A recent study of cellular long-chain fatty acid composition and electrophoretic karyotypes of *Candida* species support the grouping of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. viswanathii*, and *C. lusitaniae* to the exclusion of other species (31), as observed in the rRNA sequence relationships, and further support the preferred branch point of *C. lusitaniae* shown in Fig. 1. Proton magnetic resonance studies also group *C. albicans*, *C. tropicalis*, *C. viswanathii*, *C. guilliermondii*, and *C. lusitaniae* (8). A number of other phenotypic criteria have historically supported the grouping of *C. albicans*, *C. parapsilosis*, and *C. tropicalis* (21), and these species are three of the most commonly associated with candidiasis. *C. guilliermondii* appears related to this group at a fairly high level of sequence similarity, and limited interfertility between this species and *C. albicans* has been observed (17, 28). A previous rRNA-DNA hybridization study grouped *C. guilliermondii*, *C. krusei*, and *C. kefyr* (*pseudotropicalis*) equally with respect to degree of homology with *C. albicans* (26). Evolutionary data indicate that *C. krusei* is most distantly related to *C. albicans*, followed by *C. kefyr* and the more closely related *C. guilliermondii*.

S. cerevisiae and *C. albicans* have been grouped together as ascomycetous yeasts within the family *Saccharomycetaceae* by physiological criteria, and a recent phylogenetic analysis of the 18S rDNA sequence of *C. albicans* supports this relationship (11). The current analyses place *S. cerevisiae* among the *Candida* species examined and suggest that it is quite closely related to *T. glabrata*. *T. glabrata* is considered a significant pathogen along with *C. albicans*, *C. tropicalis*, and *C. parapsilosis* yet does not group among them phylogenetically. There has been considerable debate in the literature over the validity of maintaining *Candida* and *Torulopsis* as distinct genera (12, 14, 21). Our results indicate that *T. glabrata* is more closely related to *S. cerevisiae*, a non-*Candida* yeast, than to any *Candida* species examined.

The close relationship between *C. kefyr* and *K. marxianus* var. *lactis* was expected; *C. kefyr* is the imperfect (asexual) nomenclature for *K. marxianus* var. *marxianus* (12). Thus the observed specific grouping of the two organisms represents a relationship at the variety level. *H. polymorpha* (*Pichia angusta* [13]) was similarly chosen on the basis that several members of the genus *Hansenula* have imperfect forms designated among the candidas (12); the tree reflects this relationship of genera. There are several other genera (*Pichia*, *Metschnikowia*, *Issatchenkia*, etc.) to which perfect forms of *Candida* species are assigned (12), and extensive phylognetic analysis of the non-*Candida* members of these associated genera could help define true taxonomic boundaries.

The sequence of *Y. lipolytica* appears in the present analysis to be quite distantly related to those of the *Candida* species; this is likely due to a rapid evolution of its lineage from the most recent common ancestor of the group (34). Such rapidly evolving sequences tend to branch more deeply in trees than they should (22, 23), and thus *Y. lipolytica* may be more closely related to the candidas than is indicated. Classification of this species within the *Candida* genus has been questioned, and unusually heterogeneous size classes of rDNA operons have been reported within and between strains (30), an unusual character for a yeast species. In addition, a recent review found *Y. lipolytica* to have the highest G+C content (49.6 mol%) of 65 ascomycetous yeasts listed (14). Analysis of sequence data from closer relatives of *Y. lipolytica* may help resolve its true evolutionary affinities.

In an attempt to maximize the alignable sequence positions available for analysis, efforts were made to fold primary sequences into secondary structure helices E21-1 to E21-8 as proposed by Hendriks et al. (11) for *C. albicans*. Comparative analysis of the resultant structures indicates that the primary sequence of helix E21-5 is highly conserved across the group (thus revealing nothing about secondary structure conservation), whereas helix E21-6 is not conserved in sequence or structure. Analysis of covariant base substitutions (9) supports the existence of helices E21-1 and E21-3; however, the sizes of these structures vary widely. The proposed pseudoknot of helices E21-7 and E21-8 was subject to one or two base deletions relative to *C. albicans* in most of the examined sequences.

Organisms were chosen for this analysis on the basis of their importance as agents of disease in humans. The resultant close evolutionary relationships revealed between significant opportunistic pathogens such as *T. glabrata* and generally nonpathogenic species such as *S. cerevisiae* were unexpected. Integrated use of physiological and molecular data in the future may allow construction of a natural taxonomy of the candidas and other genera. This should facilitate understanding of fundamental properties of these organisms and their relationships to disease.

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