Phylogenetic analysis of five medically important *Candida* species as deduced on the basis of small ribosomal subunit RNA sequences

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The classification of species belonging to the genus *Candida* Berkhout is problematic. Therefore, we have determined the small ribosomal subunit RNA (srRNA) sequences of the type strains of three human pathogenic *Candida* species; *Candida krusei*, *C. lusitaniae* and *C. tropicalis*. The srRNA sequences were aligned with published eukaryotic srRNA sequences and evolutionary trees were inferred using a matrix optimization method. An evolutionary tree comprising all available eukaryotic srRNA sequences, including two other pathogenic *Candida* species, *C. albicans* and *C. glabrata*, showed that the yeasts diverge rather late in the course of eukaryote evolution, namely at the same depth as green plants, ciliates and some smaller taxa. The cluster of the higher fungi consists of 10 ascomycetes and ascomycete-like species with the first branches leading to *Neurospora crassa*, *Pneumocystis carinii*, *Candida lusitaniae* and *C. krusei*, in that order. Next there is a dichotomous divergence leading to a group consisting of *Torulaspora delbrueckii*, *Saccharomyces cerevisiae*, *C. glabrata* and *Kluyveromyces lactis* and a smaller group comprising *C. tropicalis* and *C. albicans*. The divergence pattern obtained on the basis of srRNA sequence data is also compared to various other chemotaxonomic data.

Introduction

Since the classification of higher fungi, Ascomycotina and Basidiomycotina, is based mainly on the morphology of the sexual asco- or basidiospores, other classification criteria have to be used for the Deuteromycotina. The classification of the genus *Candida* Berkhout, based on the morphology of asexual structures (hyphae, pseudohyphae and sympoduloconidia), as well as various biochemical properties, resulted in an artificial grouping. After the discovery by van der Walt & Hopsu-Havu (1976) of the cell wall reaction to diazonium blue B, the genus *Candida* Berkhout as presented by Meyer *et al.* (1984) was shown to be heterogeneous and composed of ascomycete- as well as basidiomycete-related yeasts. Recently, the genus *Candida* Berkhout has been re-defined, mainly on the basis of cell wall properties

(Weijman *et al.*, 1988), and now comprises only yeasts with an ascomycete affiliation. The yeasts with a basidiomycete relationship have been placed in the genera *Rhodotorula* and *Cryptococcus*. In spite of this new classification the genus *Candida* still exhibits some heterogeneity due to the affiliation of some species to different teleomorphic ascomycete genera. A historical review of the classification of the genus *Candida* is given by Viljoen & Kock (1989a).

The genus *Candida* (sensu Weijman *et al.*, 1988) includes several human pathogens. Infections caused by *C. albicans* and *C. tropicalis* occur most frequently (Ahearn, 1978). In addition, *C. krusei*, *C. lusitaniae* and *C. glabrata* have been described as opportunistic pathogens (Ahearn, 1978; Blinkhorn *et al.*, 1989).

For *C. albicans*, *C. tropicalis* and *C. glabrata* no teleomorphic counterparts have been described. In contrast, *C. krusei* and *C. lusitaniae* correspond respectively to *Issatchenkia orientalis* and *Clavispora lusitaniae*, two ascosporeogenous yeasts (Kreger-van Rij, 1984). These anamorph/teleomorph relationships are proven by several chemotaxonomic data (Viljoen *et al.*, 1989, and references cited there).

Abbreviation: srRNA, small ribosomal subunit RNA

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers M55526 (*C. lusitaniae*), M55527 (*C. tropicalis*) and M55528 (*C. krusei*).

The ascosporeogenous affiliation for *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. lusitaniae* and *C. krusei* is supported by a negative diazonium blue B staining reaction (Van der Walt & Hopsu-Havu, 1976; Hagler & Ahearn, 1981) and a negative urease test (Hagler & Ahearn, 1981).

Coenzyme Q analysis (the number of isoprene units per ubiquinone molecule) revealed that *C. albicans* and *C. tropicalis* are characterized by a Q-9 system. *C. lusitaniae* and *C. krusei* have a Q-8 and Q-7 system, respectively, while *C. glabrata* is characterized by a Q-6 system (Yamada & Kondo, 1972; Yamada *et al.*, 1976). Other studies have shown a high similarity between *C. albicans* and *C. tropicalis*, e.g. fatty acids analysis (Viljoen & Kock, 1989b; Viljoen *et al.*, 1989), proton magnetic resonance studies of mannans (Spencer & Gorin, 1969), antigenic comparison of enzymes (Bruneau *et al.*, 1985), an analysis based on 79 phenotypic characters (Odds *et al.*, 1990) and protein electrophoresis patterns (Vancanneyt *et al.*, 1991). Furthermore, *C. albicans*, *C. lusitaniae*, *C. tropicalis* and *C. krusei* are placed in the same group because of the presence of linoleic and linolenic acids in their fatty acid profile. *C. glabrata* is differentiated from these four species by the absence of linoleic and linolenic acid (Viljoen & Kock, 1989b).

In order to obtain more information on the evolutionary positions of five pathogenic *Candida* species and to enlarge our knowledge of the scope of the heterogeneous genus *Candida*, the small ribosomal subunit RNA (srRNA) gene sequences of the type strains of the species *C. tropicalis*, *C. lusitaniae* and *C. krusei* were determined and aligned with those from other organisms (Neefs *et al.*, 1990) including *C. albicans* (MUCL 20800, type strain) (Hendriks *et al.*, 1989) and *C. glabrata* (CBS 138, type strain) (Wong & Clark-Walker, 1990). As mentioned above, the five *Candida* species are well characterized for various phenotypic markers, viz. fatty acid profiles, coenzyme Q system and diazonium blue reaction, which enables us to compare our results with analyses based on other criteria. Only the type strain of each *Candida* species was investigated because the intraspecific homogeneity of these taxa was demonstrated by Vancanneyt *et al.* (1991). Starting from the alignment of the srRNA sequences, evolutionary trees were constructed using a matrix method.

Methods

Yeast strains. The *Candida* strains studied are all type strains and are listed in Table 1. The yeasts were grown in 2% (w/v) agar containing 2% (w/v) glucose, 1% (w/v) peptone, 0.5% (w/v) yeast extract and 3% (w/v) CaCO₃ at 28 °C. Cells were collected and washed twice with 0.01 M-phosphate (KH₂PO₄/Na₂HPO₄) buffer pH 7.0 and stored at -70 °C before use.

Table 1. Yeast type strains used in this study

Yeast	MUCL no.*	CBS no.†	Restriction enzyme fragment used‡
<i>Candida lusitaniae</i>	MUCL 29855§	CBS 4413§	<i>Pst</i> I (9.0 kb)
<i>Candida krusei</i>	MUCL 29849§	CBS 573§	<i>Pst</i> I (6.7 kb)
<i>Candida tropicalis</i>	MUCL 30002	CBS 2310	<i>Hind</i> III (9.0 kb)

* MUCL, Mycothèque de l'Université Catholique, Louvain-la-Neuve, Belgium.

† Corresponding CBS (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) number.

‡ Restriction fragment containing the srRNA gene which was used for sequencing listed together with its length in basepairs.

§ Type strain.

|| Type strain of *Candida vulgaris* Berkhout, original name of the type species of the genus and synonym of *Candida tropicalis*.

Isolation of genomic DNA. High molecular mass DNA (> 50 kb) was isolated by grinding 2 g of yeast cells in a mortar in the presence of 1 g alumina and liquid nitrogen. The crushed cells were then suspended in 50 mM-Tris, 10 mM-MgCl₂, 50 mM-NaCl, 1%, w/v, SDS, adjusted with HCl to pH 7.4. Phenol extraction and nucleic acids precipitations were done as described in Sambrook *et al.* (1989).

Cloning and sequencing. Genomic yeast DNA was digested with a set of hexamer-recognizing restriction enzymes. The DNA fragments thus generated were separated on a 0.8% (w/v) agarose gel (1.2 V cm⁻¹, 16 h) and blotted (Southern, 1975) on a Hybond N plus membrane (Amersham). The membrane was hybridized with an srDNA probe (Hendriks *et al.*, 1989) which was radioactively labelled by nick-translation (Rigby *et al.*, 1977) using an Amersham nick-translation kit. Hybridization was carried out according to the manufacturer's instructions. Subsequent autoradiography allowed us to identify and select restriction enzymes generating DNA fragments that comprise the entire srRNA gene for each species (Table 1), and which were used for the cloning step. For this purpose, the plasmid vector pUC18 (Yanisch-Perron *et al.*, 1985) was digested with a compatible restriction enzyme and ligated (Sambrook *et al.*, 1989) to the genomic yeast restriction enzyme fragments. *Escherichia coli* DH5- α cells (BRL) were transformed (Hanahan, 1983) with the plasmids and grown on a suitable agar broth (Sambrook *et al.*, 1989). In order to detect srRNA gene-containing recombinants a colony hybridization was performed according to Dale & Greenaway (1984). These recombinant plasmids were amplified and DNA was extracted using the boiling method (Holmes & Quigley, 1981) and used as template for the dideoxy sequencing method (Sanger *et al.*, 1977). For this purpose 16 primers complementary to the coding and non-coding strands of evolutionarily conserved areas of the srRNA gene were used as described by Hendriks *et al.* (1989). The polymerase used was Sequenase (USB, Cleveland, Ohio, USA) and the reaction conditions were as described by the manufacturer.

Sequence alignment and construction of evolutionary trees. The srRNA sequences of the three *Candida* species were aligned with other complete srRNA sequences (Neefs *et al.*, 1990). The alignment, which comprises the srRNA sequences of 64 eukaryotic species, is obtainable from the authors in computer-readable form.

For the inference of evolutionary trees by distance matrix methods, dissimilarity values must be computed for all pairwise comparisons of aligned nucleotide sequences. Dissimilarity (*D*) between each pair of

sequences A and B is defined as (Van de Peer *et al.*, 1990, Hendriks *et al.*, 1988)

$$D_{AB} = -\frac{3}{4} \ln \left[1 - \frac{4}{3} \left(\frac{S}{I+S} \right) \right] \left[1 - \frac{G}{T} \right] + \frac{G}{T}$$

where *I* is the number of identical nucleotides, *S* is the number of positions showing a substitution and *G* is the number of gaps in one sequence with respect to the other. *T* is the sum of *I*, *S* and *G*. The first term of the equation accounts for substitutions and comprises a correction factor for multiple mutations per site (Jukes & Cantor, 1969; Kimura & Ohta, 1972). The second term accounts for deletions and insertions and comprises no correction. Adjacent gaps are treated as a single gap.

The resulting dissimilarity matrix, which in general is non-additive, serves as the input for an algorithm developed by De Soete (1983), which computes the additive matrix that optimally approximates the input matrix according to a least squares criterion. Next, the program computes the unique tree defined by this additive matrix.

Either all alignment positions or parts of the alignment, corresponding to evolutionarily conserved areas of the molecule, were taken into account to construct evolutionary trees.

Results and Discussion

Primary structure and secondary structure of the srRNA

The sequences of the srRNA of *C. krusei*, *C. lusitaniae* and *C. tropicalis* are shown in the form of an alignment in Fig. 1. The location of the 5'- and 3'-termini were derived by comparison with other srRNA sequences.

In Fig. 2 the primary structure of *C. krusei* srRNA is folded into a general secondary structure model for eukaryotic srRNAs (Neefs *et al.*, 1990). The helix numbering system is according to the latter paper.

Phylogenetic implications

The alignment (Neefs *et al.*, 1990), which already comprised the srRNA sequences of the fungi *Neurospora crassa*, *Pneumocystis carinii* and *Saccharomyces cerevisiae*, was completed with the srRNA sequences of *C. albicans* (Hendriks *et al.*, 1989), *C. glabrata* (Wong & Clark-Walker, 1990), *Kluyveromyces lactis* (Maleszka & Clark-Walker, 1990) and *Torulaspora delbrueckii* (Hendriks *et al.*, 1990), and with the srRNA sequences of the yeasts examined in the present work. Fig. 3 shows an outline of an evolutionary tree inferred by a matrix optimization method (De Soete, 1983) and comprising 53 srRNA sequences of eukaryotes. Of the 12 *Tetrahymena* species for which the srRNA sequence is known, only *T. thermophila* was included in the analysis, which reduces the number of eukaryotic species from 64 to 53. The tree is based on a limited part of the total eukaryotic alignment in which the sequence can be aligned unambiguously. These areas are boxed in Fig. 2 and correspond to more conserved sections of the srRNA molecule. In order to root the tree, an archaeobacterial

sequence was included. In the eukaryotic part of the tree in Fig. 3, several early branching protist lineages can be discerned, viz. the diplomonad *Giardia lamblia*, the microsporidian *Vairimorpha necatrix*, kinetoplastids plus *Euglena gracilis*, the amoeba *Naegleria gruberi* and the slime moulds *Physarum polycephalum* and *Dictyostelium discoideum*. Next there is a nearly simultaneous branching into several clusters, viz. metazoa, sporozoa, ciliates plus the dinoflagellate *Prorocentrum micans*, a cluster consisting of two chromophytes and an oomycete, higher fungi and the green plants. The overall picture is very similar to srRNA trees published previously (e.g. Gunderson *et al.*, 1987; Clark & Cross, 1988; Hendriks *et al.*, 1988; Nairn & Ferl, 1988; Rausch *et al.*, 1989; Sogin *et al.*, 1989).

Because srRNA molecules occur universally in all organisms, are not subjected to lateral gene transfer and are slowly ticking molecular clocks, they are virtually the only instruments that allow the evolutionary study of a group as complex and diverse as the eukaryotes on a molecular basis. Comparison with analyses based on other molecular clocks or phenotypic markers is rather difficult because such studies are often limited to a subtaxon of the eukaryotes. However, Ragan (1989) has compared the srRNA trees with biochemical pathway data. In some cases the srRNA tree topology is congruent with the pathway data, while in other cases it is not. Also, when a tree constructed on the basis of 137 ultrastructural, developmental and biochemical characters (Lipscomb, 1989) is compared to the srRNA tree, the same major lines of descent can be discerned in both trees but their branching order differs.

As can be seen from the srRNA tree in Fig. 3 the higher fungi (ascomycetes and ascomycete-like yeasts) diverge rather late in the course of eukaryote evolution. They seem to originate at nearly the same depth as green plants, ciliates and the chromophytes/oomycete cluster.

In order to increase the resolution of the evolutionary distances between the yeast species included in the fungal subcluster, a new tree, comprising only the 10 higher fungi, was constructed. This subtree, depicted in Fig. 4, was computed from a dissimilarity matrix including all alignment positions, using the same algorithm as for the construction of the tree in Fig. 3, and taking *Homo sapiens* as an outgroup species. Within the fungi the deepest branches lead, in order, to *N. crassa*, *P. carinii*, *C. lusitaniae* and *C. krusei*. Next a branch consisting of *C. albicans* and *C. tropicalis* splits off. Finally, there is a dichotomous divergence leading to *C. glabrata* plus *K. lactis* on the one hand and to *T. delbrueckii* plus *S. cerevisiae* on the other hand.

A comparison of the tree (Fig. 4) with the type of ubiquinone system present seems to be most valuable

C. t.	UAUCUGGUUGAUCUGCCAGUAGUCAUAUGCUUGUCUCAAAGAUAAGCCAUGCAUGUCUAAGUAUAAGCA--UUUAUCAGUGAAACUGCGAAUGGCUCA	98
C. k.	UAUCUGGUUGAUCUGCCAGUAGUCAUAUGCUUGUCUCAAAGAUAAGCCAUGCAUGUCUAAGUAUAAGCA--UUUAUCAGUGAAACUGCGAAUGGCUCA	98
C. l.	UAUCUGGUUGAUCUGCCAGUAGUCAUAUGCUUGUCUCAAAGAUAAGCCAUGCAUGUCUAAGUAUAAGCAUUUAUCAGUGAAACUGCGAAUGGCUCA	100
C. l.	UUAAAUCAGUUAUCGUUUUAUUUGAUGUACCUUGUCUAAUUGGCAUACGCCUGGCAAUUCUAGAGCUAAUACAUGCGUCCAGCCGAC--UCUGGAAGGG	197
C. k.	UUAAAUCAGUUAUCGUUUUAUUUGAUGUACCUUGUCUAAUUGGCAUACGCCUGGCAAUUCUAGAGCUAAUACAUGCGUCCAGCCGAC--UCUGGAAGGG	196
C. t.	UUAAAUCAGUUAUCGUUUUAUUUGAUGUACCUUGUCUAAUUGGCAUACGCCUGGCAAUUCUAGAGCUAAUACAUGCGUCCAGCCGAC--UCUGGAAGGG	199
C. l.	CUGUAUUUAUAGUAUUUUUAUUA--CAUCCG-----UGAUGAUUCAUAUAUAACUUGUCGAAUCGCGAGGCCUCGAGCCGCGAUGGUUCAUUCAAA	288
C. k.	-UGUAUUUAUAGUAUUUUUAUUA--CAUCCG-----UGAUGAUUCAUAUAUAACUUGUCGAAUCGCGAGGCCUCGAGCCGCGAUGGUUCAUUCAAA	294
C. t.	AUGUAUUUAUAGUAUUUUUAUUA--CAUCCG-----UGAUGAUUCAUAUAUAACUUGUCGAAUCGCGAGGCCUCGAGCCGCGAUGGUUCAUUCAAA	299
C. l.	UUUCUGCCCUAUCACCUUUCGUAUGGUAGGAUAGAGGCCUACCAUGGUUUCAACGGGUAAACGGGGAUUAGGGUUCGGUUCGGAGAGGGAGCCUGAGAAA	388
C. k.	UUUCUGCCCUAUCACCUUUCGUAUGGUAGGAUAGAGGCCUACCAUGGUUUCAACGGGUAAACGGGGAUUAGGGUUCGGUUCGGAGAGGGAGCCUGAGAAA	394
C. t.	UUUCUGCCCUAUCACCUUUCGUAUGGUAGGAUAGAGGCCUACCAUGGUUUCAACGGGUAAACGGGGAUUAGGGUUCGGUUCGGAGAGGGAGCCUGAGAAA	399
C. l.	CGGCUACCAUCCAAGGAAGGCAGCAGCGCGCAAAUUAACCAUCCCGACACGGGGAUGGUAUGACAAUAAUAAACGAUACAGGGCCUUCUGGGUCU	488
C. k.	CGGCUACCAUCCAAGGAAGGCAGCAGCGCGCAAAUUAACCAUCCCGACACGGGGAUGGUAUGACAAUAAUAAACGAUACAGGGCCUUCUGGGUCU	491
C. t.	CGGCUACCAUCCAAGGAAGGCAGCAGCGCGCAAAUUAACCAUCCCGACACGGGGAUGGUAUGACAAUAAUAAACGAUACAGGGCCUUCUGGGUCU	499
C. l.	UGUAAUUGGAAUGAGUACAAUGUAAAUACCUUAACGAGGAACAAUUGGAGGGCAAGUCUGGUGCCAGCAGCGCGGUAUUCCAGCUCCAAAGCGUAUA	588
C. k.	UGUAAUUGGAAUGAGUACAAUGUAAAUACCUUAACGAGGAACAAUUGGAGGGCAAGUCUGGUGCCAGCAGCGCGGUAUUCCAGCUCCAAAGCGUAUA	591
C. t.	UGUAAUUGGAAUGAGUACAAUGUAAAUACCUUAACGAGGAACAAUUGGAGGGCAAGUCUGGUGCCAGCAGCGCGGUAUUCCAGCUCCAAAGCGUAUA	599
C. l.	UUAAAGUUGUUGCAGUUAAAAGCUCGUAGUUGAACCUUGGAGGCG--CCGUGCCGUC--CGCUUA--GGCGAGCAC--UGGAGGCGGCG--CCUCUU--C	678
C. k.	UUAAAGUUGUUGCAGUUAAAAGCUCGUAGUUGAACCUUGGAGGCG--CCGUGCCGUC--CGCUUA--GGCGAGCAC--UGGAGGCGGCG--CCUCUU--C	686
C. t.	UUAAAGUUGUUGCAGUUAAAAGCUCGUAGUUGAACCUUGGAGGCG--CCGUGCCGUC--CGCUUA--GGCGAGCAC--UGGAGGCGGCG--CCUCUU--C	698
C. l.	CUCCUCCUUCUAGCAAUAGAGGAGGACUGUUAUUGAGUAAUUGAGAGUGUCAAAGCAGGCGCACCGCUUGAAUCUGUAGCAUGGAAUAAUAGAAU	778
C. k.	CUCCUCCUUCUAGCAAUAGAGGAGGACUGUUAUUGAGUAAUUGAGAGUGUCAAAGCAGGCGCACCGCUUGAAUCUGUAGCAUGGAAUAAUAGAAU	784
C. t.	CUCCUCCUUCUAGCAAUAGAGGAGGACUGUUAUUGAGUAAUUGAGAGUGUCAAAGCAGGCGCACCGCUUGAAUCUGUAGCAUGGAAUAAUAGAAU	796
C. l.	AGGACGC--AUGGUUCUAUUUGUUGGUUUUAGGACCAUCGUAUUGAUAUAGGGACGGUCGGGGGCAUCAGUAUUCAGUUGUCAGAGGUAAAUUCUU	877
C. k.	AGGACGC--AUGGUUCUAUUUGUUGGUUUUAGGACCAUCGUAUUGAUAUAGGGACGGUCGGGGGCAUCAGUAUUCAGUUGUCAGAGGUAAAUUCUU	883
C. t.	AGGACGUUAUGGUUCUAUUUGUUGGUUUUAGGACCAUCGUAUUGAUAUAGGGACGGUCGGGGGCAUCAGUAUUCAGUUGUCAGAGGUAAAUUCUU	896
C. l.	GGAUUUAUCGAAACUAACUACUUGCGAAAGCAUUUGCCAAAGGACGUUUUCAUUAUCAAAGCAAGAAUUAAGGGGAUCGAAAGUAUGACAUACCGUCGU	977
C. k.	GGAUUUAUCGAAACUAACUACUUGCGAAAGCAUUUGCCAAAGGACGUUUUCAUUAUCAAAGCAAGAAUUAAGGGGAUCGAAAGUAUGACAUACCGUCGU	983
C. t.	GGAUUUAUCGAAACUAACUACUUGCGAAAGCAUUUGCCAAAGGACGUUUUCAUUAUCAAAGCAAGAAUUAAGGGGAUCGAAAGUAUGACAUACCGUCGU	996
C. l.	AGUCUUAACCAUAAACUAUUGCCGACUAGGGAUCGGGCGGCGUUCAUUUAGUGACGCGCUCCGACCUUACGAGAAUCAAAGUCUUUGGUUCUGGGGGG	1077
C. k.	AGUCUUAACCAUAAACUAUUGCCGACUAGGGAUCGGGCGGCGUUCAUUU--G--CCCAUCUGGACCUUACGAGAAUCAAAGUUUUUUGGUUCUGGGGGG	1079
C. t.	AGUCUUAACCAUAAACUAUUGCCGACUAGGGAUCGGGCGGCGUUCAUUUUAUUGACGCAAUCCGACCUUACGAGAAUCAAAGUCUUUGGUUCUGGGGGG	1096
C. l.	AGUAGGUGCGAAGGCGUAAACUUAAGGAAUUGACGGAAGGGCACCAACAGGAGUGGAGCCUCCGGCUUAUUUUGACUUAACACCGGGAAACUACCCAG	1177
C. k.	AGUAGGUGCGAAGGCGUAAACUUAAGGAAUUGACGGAAGGGCACCAACAGGAGUGGAGCCUCCGGCUUAUUUUGACUUAACACCGGGAAACUACCCAG	1179
C. t.	AGUAGGUGCGAAGGCGUAAACUUAAGGAAUUGACGGAAGGGCACCAACAGGAGUGGAGCCUCCGGCUUAUUUUGACUUAACACCGGGAAACUACCCAG	1196
C. l.	GUCCAGACACAAUAGGAUUGACAGAUUGAGAGCUUUUCUUGAUUUUGUGGGUGGUGGUGCAUGGCCGUUUUAUGUUGGUGGAGUGAUUUUGUCUGCUUA	1277
C. k.	GUCCAGACACAAUAGGAUUGACAGAUUGAGAGCUUUUCUUGAUUUUGUGGGUGGUGGUGCAUGGCCGUUUUAUGUUGGUGGAGUGAUUUUGUCUGCUUA	1278
C. t.	GUCCAGACACAAUAGGAUUGACAGAUUGAGAGCUUUUCUUGAUUUUGUGGGUGGUGGUGCAUGGCCGUUUUAUGUUGGUGGAGUGAUUUUGUCUGCUUA	1296
C. l.	AUUGCGAUAAACGAACGAGACCUUAACCU--CUAAAUAGCGCGCGUAGCCUUGUGCGG--CGCGCGCUUUUAAGGGGAUAUUGACUUAAGUGCAUGGAA	1375
C. k.	AUUGCGAUAAACGAACGAGACCUUAACCUUGCUAAAUAGGGCGUGCGAGCAUCUGCGCGG--GUGCUUUUAUGAGGGGUAUUGGGUAUCAAACCAUGGAA	1376
C. t.	AUUGCGAUAAACGAACGAGACCUUAACCUUAUAAUAGUGCGUAGCAUUUGCGGUUAUGUCACUUCUUAAGGGGAUAUCGAUUUUAAGUGCAUGGAA	1394
C. l.	GUUUGAGGCAUAACAGGUUGUGAUGCCCUUAGAGCUUUGGGCCGACCGCGCGUACACUGACGGAGCCAGCGAG--UUGACCUUUGGCCGAGAGGUCU	1473
C. k.	GUUUGAGGCAUAACAGGUUGUGAUGCCCUUAGAGCUUUGGGCCGACCGCGCGUACACUGACGGAGCCAGCAAGU--CCAAACCUUGGUCGAGAGGCC	1475
C. t.	GUUUGAGGCAUAACAGGUUGUGAUGCCCUUAGAGCUUUGGGCCGACCGCGCGUACACUGACGGAGCCAGCGAGUAUAAACCUUGGCCGAGAGGCUU	1494
C. l.	GGGAAAUUCUGGGAAACUCGCGUGGUGGUGGGGAUAGAGCAUUGCAAUUAUUGUCUUCUACAGAGGAAUCCUAGUAAGCGCAAGUCAUCAGCUUGCGUUG	1573
C. k.	GGGAAAUUCUGGAAACUCGCGUGGUGGUGGGGAUAGAGCAUUGCAAUUAUUGUCUUCUACAGAGGAAUCCUAGUAAGCGCAAGUCAUCAGCUUGCGUUG	1575
C. t.	GGGAAAUUCUGGAAACUCGCGUGGUGGUGGGGAUAGAGCAUUGCAAUUAUUGUCUUCUACAGAGGAAUCCUAGUAAGCGCAAGUCAUCAGCUUGCGUUG	1594
C. l.	AUUACGUCCUUGCCUUUGUACACACCGCCCGUGCUACUACCGAUUGAAUUGGUUAGUGAGGCGUCCGGAUUUGUCUAAGCCGAGG--CA--CCUUGGC	1669
C. k.	AUUACGUCCUUGCCUUUGUACACACCGCCCGUGCUACUACCGAUUGAAUUGGUUAGUGAGGCGUCCGGAUUUGGUUAGUGAGGCGUCCGGAUUUGGUU	1674
C. t.	AUUACGUCCUUGCCUUUGUACACACCGCCCGUGCUACUACCGAUUGAAUUGGUUAGUGAGGCGUCCGGAUUUGGUUAGUGAGGCGUCCGGAUUUGGUU	1692
C. l.	GGUGA--CGGAGAGCGUGGUAACCUUGGUCAUUUAGAGGAAGUAAAGUCGUAAACAGGUUUCCGUAGGUAGAACUCCGGAAGGAUUAUUA	1759
C. k.	A--UGGGGCGGAGAUUCUAGUCAAACUUGGUCAUUUAGAGGUCGUAAAGUCGUAAACAGGUUUCCGUAGGUAGAACUCCGGAAGGAUUAUUA	1765
C. t.	UCUGGAAACGAGAGCUAGUCAAACUUGGUCAUUUAGAGGAAGUAAAGUCGUAAACAGGUUUCCGUAGGCGAACUCCGGAAGGAUUAUUA	1784

Fig. 1. Alignment of the srRNA sequences of *C. tropicalis* (C.t.), *C. krusei* (C.k.) and *C. lusitanae* (C.l.).

Fig. 2. Secondary structure model of the srRNA of *C. krusei*. The helix numbering in area E21-*n* is not continuous because helices E21-2 and E21-4, which are found only in the srRNAs of a limited number of species, are missing in *C. krusei* as well as in the two other *Candida* species, *C. tropicalis* and *C. lusitanae*. The boxed area indicates more conserved parts of the molecule which can be aligned unambiguously among eukaryotes. The secondary structure models of *C. tropicalis* and *C. lusitanae* srRNAs are very similar to that of *C. krusei*.

Fig. 3. Simplified version of an evolutionary tree based on 1263 unambiguously aligned positions (comprising the boxed area of Fig. 2) of srRNA sequences of 52 eukaryotes. Clusters are represented as isosceles triangles with a height equal to the mean distance from the deepest branching point to the species forming the cluster, and a base proportional to the number of species. Taxon designations are placed to the right of the corresponding cluster followed by the number of organisms included in the cluster. The dissimilarity between two groups is obtained by summing the lengths of the connecting branches along the horizontal axis, using the scale at the top. Branches originating at levels differing less than 0.0025 in dissimilarity are drawn as diverging simultaneously. The root was placed such as to equalize the distance to the outgroup species (the archaeobacterium *Halobacterium halobium*) and the average distance to all eukaryotes.

because the coenzyme Q number appears to be conserved in most sporogenous and some asporogenous yeast genera. The type of ubiquinone would thus seem to be a significant generic parameter and ranges between Q-6 and Q-10 among the ascospore-forming genera. Genera with the same number may differ markedly in other properties (Kreger-van Rij, 1984). Fig. 4 shows that four yeast species with a Q-6 system, *T. delbrueckii*, *S. cerevisiae*, *C. glabrata* and *K. lactis* (Yamada & Kondo, 1972; Yamada *et al.*, 1976), group together. Both species

possessing the Q-9 system, *C. albicans* and *C. tropicalis*, represent a separate cluster. *C. krusei* and *C. lusitaniae* are more distantly related in our tree and are characterized by a Q-7 and Q-8 system, respectively (Yamada & Kondo, 1972). We conclude that, at least for this subset of yeast species, the srRNA sequences and the ubiquinone system yield congruent results.

We also compared the srRNA sequence similarity with other chemotaxonomic markers which were analysed using alternative treeing methods often based

Fig. 4. Evolutionary tree comprising 10 fungal species. All alignment positions are used to construct the tree by a matrix optimization method. Placement of the root was as in Fig. 3, using *Homo sapiens* as the outgroup species.

on different subsets of species. The close relationship between *C. albicans* and *C. tropicalis* (Fig. 4) is also seen on the basis of phenotypic and biochemical criteria (Spencer & Gorin, 1969; Bruneau *et al.*, 1985; Viljoen & Kock, 1989a,b; Odds *et al.*, 1990; Viljoen *et al.*, 1989; Vancanneyt *et al.*, 1991). Furthermore, a close evolutionary relationship between *S. cerevisiae* and *C. glabrata*, as shown in Fig. 4, is confirmed by a combined analysis of long-chain fatty acid profiles, cell differentiation and carbon source utilization (Viljoen *et al.*, 1988). In the tree in Fig. 4, *S. cerevisiae* and *T. delbrueckii* seem to be very closely related. However, on the basis of electrophoretic whole-cell protein patterns (Vancanneyt *et al.*, 1991) and mol% G + C values (Kreger-van Rij, 1984) the latter relationship is not detected. Based on protein electrophoresis patterns (Vancanneyt *et al.*, 1991), the divergence order of *C. albicans*, *C. tropicalis*, *C. krusei* and *C. lusitanae* is analogous to that depicted in the tree in Fig. 4. In contrast, a combined analysis of fatty acid profiles and electrophoretic karyotype yielded other results with *C. albicans*, *C. tropicalis* and *C. lusitanae* forming part of the same subgroup while *C. krusei* belongs to another subgroup (Viljoen & Kock, 1989b).

In conclusion, inconsistencies can be detected when one compares evolutionary trees based on different chemotaxonomic criteria and on srRNA sequence

analysis. These discrepancies can be due to the fact that different sets of organisms and/or other treeing algorithms have been used. Alternatively, the discrepancies between the trees can also be a consequence of the fact that some of the chemotaxonomic markers used may not be suitable for a study of the relationships among species which are not very closely related. Fatty acid profiles, electrophoretic karyotype analysis and protein electrophoresis profiles have proven their usefulness for the determination of the teleomorph/anamorph relationship between species and/or for the inference of the relationship between species (or strains) which are evolutionarily close, as is the case for *C. albicans* and *C. tropicalis*. However, the type of ubiquinone system and the srRNA analysis may be more suitable for the study of less related organisms.

Analysis of srRNA sequences has already been successfully applied to studies on the evolution of bacteria (Woese, 1987) and protists (Gunderson *et al.*, 1987; Sogin *et al.*, 1989). The results presented in this work, though still limited, also show the potential of srRNA analysis for the deduction of evolutionary relationships among yeast species. To define the status of the majority of *Candida* species, many more sequences of ascomycetes and ascomycete-like yeasts should be determined.

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