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Grouping oral *Candida* species by multilocus enzyme electrophoresis

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Multilocus enzyme electrophoresis (MLEE) and numerical taxonomic methods were used to establish the degrees of relatedness among five *Candida* species commonly isolated from humans oral cavities. Of twenty enzymic systems assayed, five showed no enzymic activity (aspartate dehydrogenase, mannitol dehydrogenase, sorbitol dehydrogenase, glucosyl transferase and α-amylase). The obtained data revealed that some of these enzymes are capable of distinguishing strains of different species, but most of them could not organize all strains in their respective species-specific clusters. Numerical classification based on MLEE polymorphism must be regarded for surveys involving just one *Candida* species.

Keywords: oral *Candida* spp., cluster analysis, MLEE

INTRODUCTION

Candida species, particularly *Candida albicans*, remain the most common fungi found in the oral cavity of humans and, in recent years, have received attention because of their involvement in a number of cases of opportunist oral infections in patients with AIDS and those having immunosuppressive medication. Of epidemiological interest, characterization procedures that produce molecular fingerprints have been used to establish possible relationships among *Candida* isolates that could have some oral relevance (McCullough *et al*., 1996). Multilocus enzyme electrophoresis (MLEE) is a resource that has been used in studies involving a large number of micro-organisms (Selander & Levin, 1980; Soltis *et al*., 1980; Okunishi *et al*., 1979) including *Candida* species (Lehmann *et al*., 1989a; Pujol *et al*., 1993; Reynes *et al*., 1996). These studies have highlighted the usefulness of some enzymic systems in discriminating less related strains or even species from other genera. The purpose of the present study is to evaluate the parity existing among some enzymic systems for fingerprinting five *Candida* species (*C. albicans*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis* and *Candida guilliermondii*) isolated from the saliva of healthy human subjects.

Abbreviations: ACO, aconitase; ADH, alcohol dehydrogenase; α-AM, α-amylase; ASDH, aspartate dehydrogenase; CAT, catalase; α-EST, α-esterase; β-EST, β-esterase; G6PDH, glucose-6-phosphate dehydrogenase; GDH, glucose dehydrogenase; GOT, glutamate-oxaloacetate transaminase; GTF, glucosyl transferase; IDH, isocitrate dehydrogenase; LAP, leucine aminopeptidase; LDH, lactate dehydrogenase; MADH, mannitol dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; MLEE, multilocus enzyme electrophoresis; OTU, operational taxonomic unit; PO, peroxidase; SDH, sorbitol dehydrogenase; SOD, superoxide dismutase; UPGMA, unweighted pair group method with arithmetic averages.

METHODS

***Candida* strains.** Representative strains of different *Candida* species isolated from oral cavities and identified by colony characteristics on CHROMagar *Candida* differential medium (Anson & Allen, 1997; Bernal *et al*., 1996; San Milan *et al*., 1996), chlamydospore and germ tube formation, and by sugar fermentation and assimilation (Sandven, 1990), were obtained from the Microbiology and Immunology Laboratory, Dentistry College of São José dos Campos: *C. albicans* (97a, F72, E37, 17b, CBS 562^T); *C. guilliermondii* (FCF405, FCF152, CBS 566^T); *C. parapsilosis* (21c, 7a, CBS 604^T); *C. krusei* (1M90, 4c, CBS 573^T); and *C. tropicalis* (1b, FCF430, CBS 94^T). All strains (excluding the type strains) were isolated from the oral cavities of healthy subjects. In this work, the type strain of *Saccharomyces cerevisiae* (CBS 1171^T) was used as an extra-generic organism.

Cell cultivation and enzyme extraction. All the strains were grown overnight in 50 ml YPD medium (2% dextrose, 2% peptone, 1% yeast extract), on a shaker table at 150 r.p.m. and 30 °C. Cells were harvested by centrifugation of total culture medium volume at 2000 ***g*** for 3 min and pellets were washed four times with cold sterile water to ensure complete removal of traces of culture medium and extracellular metabolites (Woontner & Jaehning, 1990). The washed pellets were transferred to 2 ml microcentrifuge tubes and equal amounts of acid-washed glass beads and 200 μl cold

sterile water were added. The tubes were placed in a Mini-Bead Beater cell disrupter (Biospec) and cell lysis was carried out at 4600 r.p.m. for four 30 s bursts, at intervals of 5 min, in which the samples were conditioned in an ice bath. After cell disruption, the microcentrifuge tubes were centrifuged at 10000 ***g*** for 2 min, and the supernatants were applied to Whatman 3 filter paper wicks of 5×12 mm. These wicks were maintained at -70 °C.

Starch gel electrophoresis. Electrophoresis was carried out according to Val *et al.* (1981); gels were formed by solubilizing hydrolysed corn starch Penetrose 30 (Refinações de Milho Brasil, São Paulo) at a final concentration of 13% in diluted 1:30 Tris-citrate buffer pH 8·0 and vigorously agitated heating over a Bunsen burner. The formed gels were poured in perplex casting moulds ($200 \times 120 \times 10$ mm) and left on the bench, at room temperature, until complete solidification. They were cut longitudinally 2·5 cm from one border. The 2·5 cm segments were separated and the wicks were applied on the cut. Wicks with 0·2% bromophenol blue were applied to both extremities of the cuts to indicate migration. After joining the parts, cotton cloth bridges were made to connect the gels to electrode tanks containing Tris-citrate buffer pH 8·0 (Selander *et al.*, 1986; Caugant & Sandven, 1993). Electrophoresis was carried out at 4 °C and 130 V until the migration markers had moved at least 80 mm from the application point. At this time, electrophoresis was terminated and the gels, placed horizontally, were sliced horizontally into 1·2 mm thickness using a small device in which a thin fishing cord sliced the gels over an elevatory table.

Band revelation. The gel slices were stained to reveal the active enzyme bands, according to Selander *et al.* (1986) protocols. Enzymic systems assayed were: alcohol dehydrogenase (ADH; EC 1.1.1.1); lactate dehydrogenase (LDH; EC 1.1.1.27); malate dehydrogenase (MDH; EC 1.1.1.37); isocitrate dehydrogenase (IDH; EC 1.1.1.42); glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49); aspartate dehydrogenase (ASDH; EC 1.4.3.x); glucose dehydrogenase (GDH; EC 1.1.1.47); mannitol dehydrogenase (MADH; EC 1.1.1.67); sorbitol dehydrogenase (SDH; EC 1.1.1.14); malic enzyme (ME; EC 1.1.1.40); aconitase (ACO; EC 4.2.1.3); catalase (CAT; EC 1.11.1.6); superoxide dismutase (SOD; EC 1.15.1.1); glutamate-oxaloacetate transaminase (GOT; EC 2.6.1.1); α-esterase (EST; EC 3.1.1.1); β-esterase (EST; EC 3.1.1.1); leucine aminopeptidase (LAP; EC 3.4.1.1); glucosyl transferase (GTF; EC 2.4.1.11); peroxidase (PO; EC 1.11.1.7); and α-amylase (α-AM; EC 3.2.1.1).

Computing numerical data. Dendrograms were generated for the different enzymic systems by using the same measurement of relatedness, the Simple Matching association coefficient (S_{SM}; Sokal & Michener, 1958; Sneath & Sokal, 1973), based on band positions computed with the NTSYS software package, version 1.70 (Applied Biostatistics) S_{SM} measures the proportion of bands with the same and the different relative mobility values in patterns of two operational taxonomic units (OTU), **k** and **j**, by the formula: $S_{SM} = E/(E+b+c)$, where E is the positive combination of bands (present and absent) shared by OTUs **k** and **j**, b is the number of bands unique to OTU **k**, and c is the number of bands unique to OTU **j**. In the present study, an S_{SM} value of 1·00 represents identical matches (i.e. all the bands in the patterns of OTUs **k** and **j** match), an S_{SM} value of 0·00 represents no matches, and S_{SM} values of 0·01–0·99 represent increasing proportions of matched bands. Dendrograms, represented by non-rooted trees, based on S_{SM} values were generated by the unweighted pair group method with arithmetic means (UPGMA; Rohlf, 1963; Sneath & Sokal, 1973).

RESULTS

The one-dimensional electrophoreses of protein extracts of 12 *Candida* strains, their respective type strains and the *S. cerevisiae* type strain, showed that, of twenty assayed enzymes, five (ASDH, MADH, SDH, GTF and α-AM) did not show any enzymic activity. The remaining systems provided electrophoretic bands that enabled the fifteen individual dendrograms shown in Fig. 1 to be constructed. *C. albicans* strains CBS 562^T, 97a, F72, and E37 clustered in dendrograms A (ADH), B (ME), E (IDH), F (LDH), H (ACO) and K (CAT). *C. albicans* strain 17b showed atypical MLEE patterns for all assayed enzymes, grouping with strains of other *Candida* species in a non-repetitive way. The two clinical strains of *C. guilliermondii* (FCF152 and FCF405) only clustered together in the dendrogram derived from the IDH system. Dendrogram G (MDH) showed grouping of these clinical strains of *C. guilliermondii* with the inclusion of *C. albicans* strain E37; the type strain of *C. guilliermondii* clustered with three *C. albicans* strains (CBS 562^T, 97a, and F72). For *C. krusei*, two strains, CBS 573^T and 1M90, were grouped by the IDH system. The CAT system could group strains CBS 573^T and 4c, and the PO system formed a composite cluster with the inclusion of the atypical *C. albicans* strain 17b between *C. krusei* strains 1M90 and 4c. Among *C. tropicalis* strains, the type strain (CBS 94^T) and the clinical isolate FCF430 were the specimens that revealed the closest relationship ($S_{SM} = 1{\cdot}00$) for four enzymic systems (IDH, LDH, α-EST and β-EST); for the ADH, ME and MDH systems, these strains grouped with $S_{SM} > 0{\cdot}85$. All three strains of *C. parapsilosis* appeared together in a single cluster for the dendrograms of G6PDH, IDH, LDH, ACO, CAT, GOT, LAP and SOD although in the four latter trees, they were associated with strains of other species. The *S. cerevisiae* type strain CBS 1171^T combined with different species of *Candida* depending on the enzymic system.

The results of the individual MLEE analyses were pooled for each strain and a non-rooted relatedness dendrogram of the 18 analysed strains, based on the similarity calculated by S_{SM} values, was constructed (Fig. 2). Nine phenons (clusters) were established by the perpendicular line (dashed) that represents the average value for S_{SM} of all OTUs (i.e. 0·841).

Phenon I contained the strains CBS 562^T, 97a, F72 and E37 (*C. albicans*), grouped with $S_{SM} \geqslant 0{\cdot}898$. Phenon II contained two strains of *C. guilliermondii* (FCF152 and FCF405) and one of *C. tropicalis* (1b) as components, grouped with $S_{SM} \geqslant 0{\cdot}847$. Phenon III just contained CBS 1171^T, the type strain of *S. cerevisiae*. Phenon IV contained the three strains of *C. parapsilosis* (CBS 604^T, 21c and 7a) and the atypical *C. albicans* strain 17b, with $S_{SM} \geqslant 0{\cdot}845$. Phenon V is a cluster composed of a unique *C. krusei* strain (4c). Phenon VI

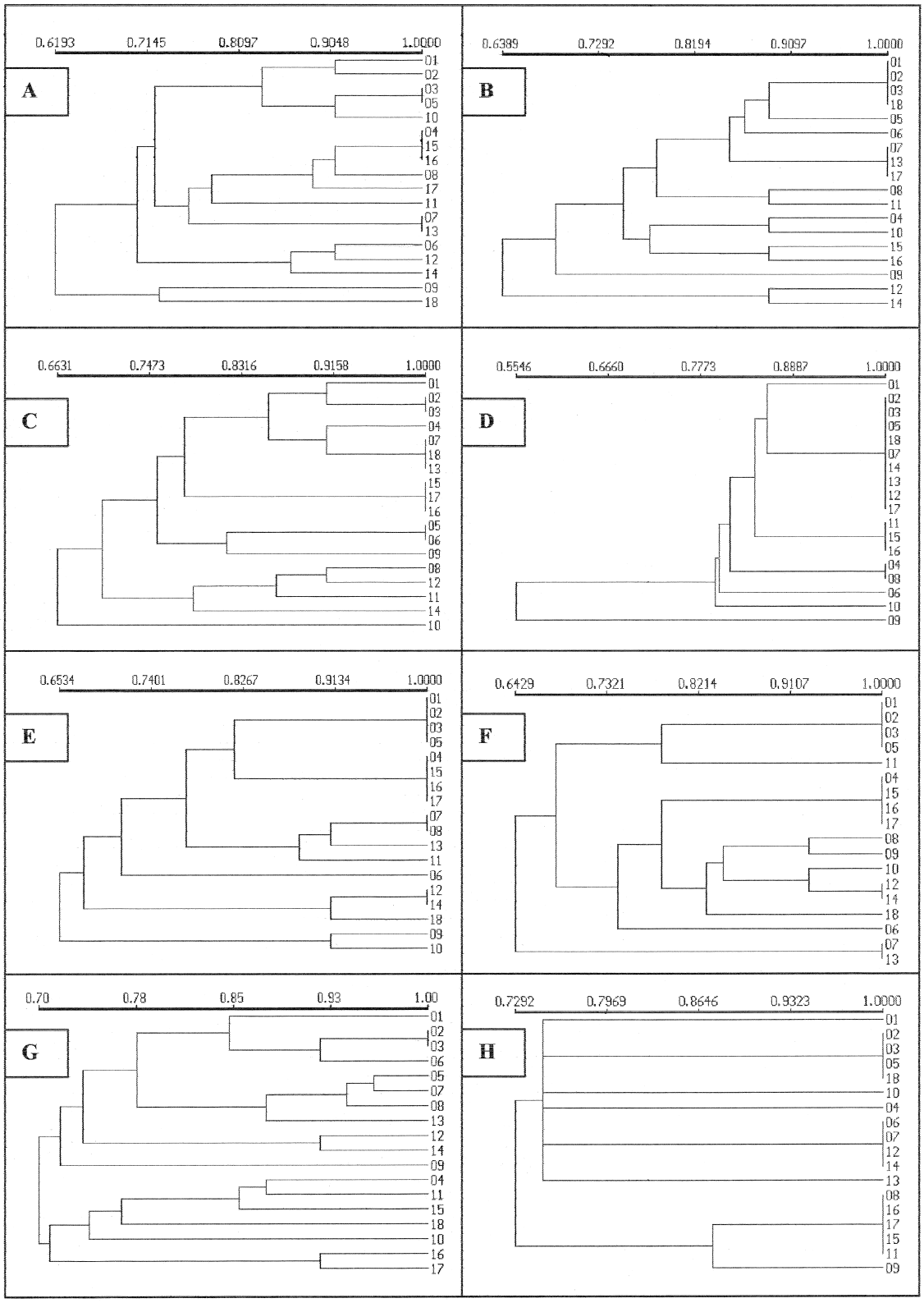

Fig. 1. For legend see page 1346.

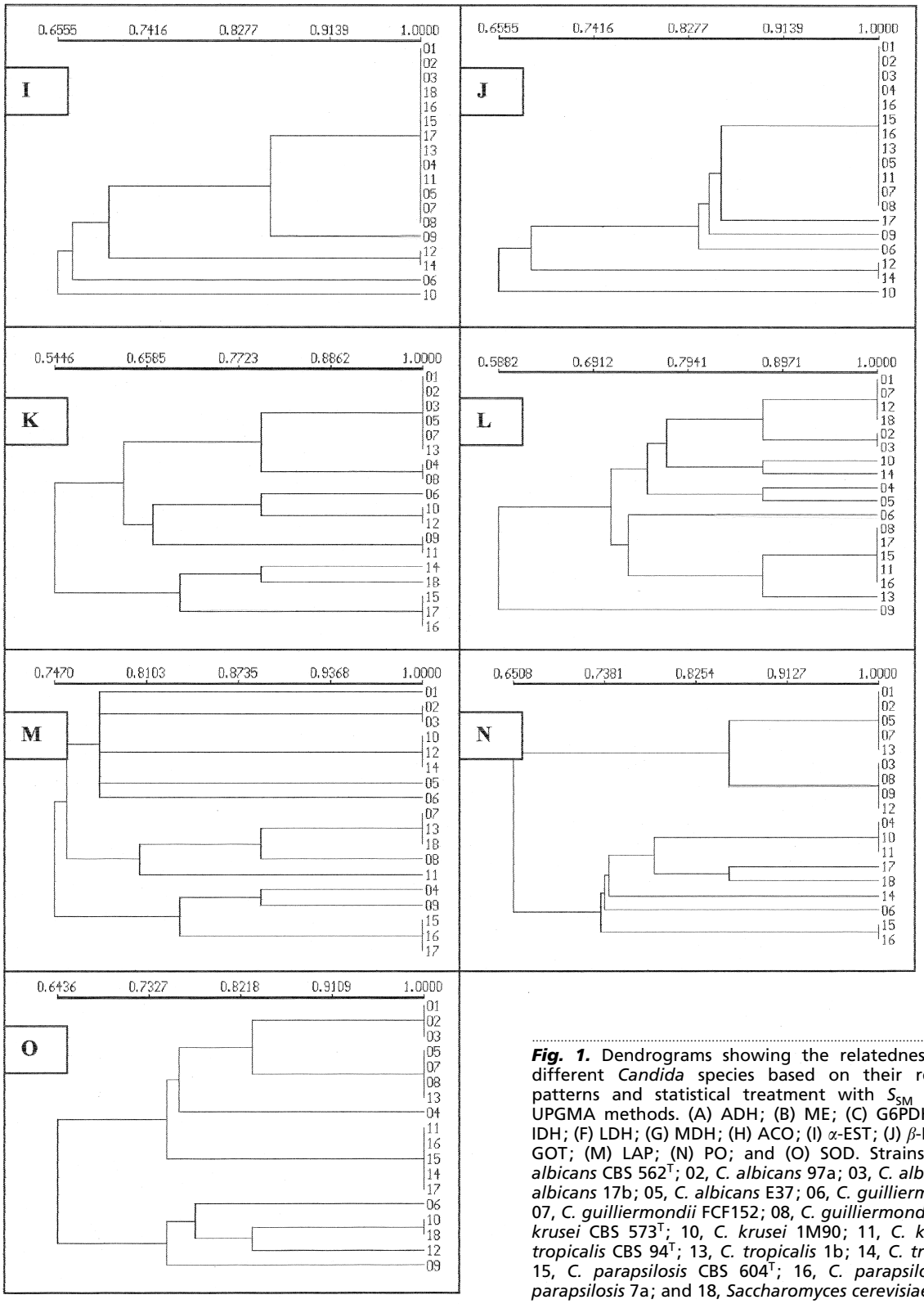

Fig. 1. Dendrograms showing the relatedness levels among different *Candida* species based on their respective MLEE patterns and statistical treatment with S_{SM} coefficient and UPGMA methods. (A) ADH; (B) ME; (C) G6PDH; (D) GDH; (E) IDH; (F) LDH; (G) MDH; (H) ACO; (I) α-EST; (J) β-EST; (K) CAT; (L) GOT; (M) LAP; (N) PO; and (O) SOD. Strains (OTUs): 01, *C. albicans* CBS 562^T; 02, *C. albicans* 97a; 03, *C. albicans* F72; 04, *C. albicans* 17b; 05, *C. albicans* E37; 06, *C. guilliermondii* CBS 566^T; 07, *C. guilliermondii* FCF152; 08, *C. guilliermondii* FCF405; 09, *C. krusei* CBS 573^T; 10, *C. krusei* 1M90; 11, *C. krusei* 4c; 12, *C. tropicalis* CBS 94^T; 13, *C. tropicalis* 1b; 14, *C. tropicalis* FCF430; 15, *C. parapsilosis* CBS 604^T; 16, *C. parapsilosis* 21c; 17, *C. parapsilosis* 7a; and 18, *Saccharomyces cerevisiae* CBS 1171^T.

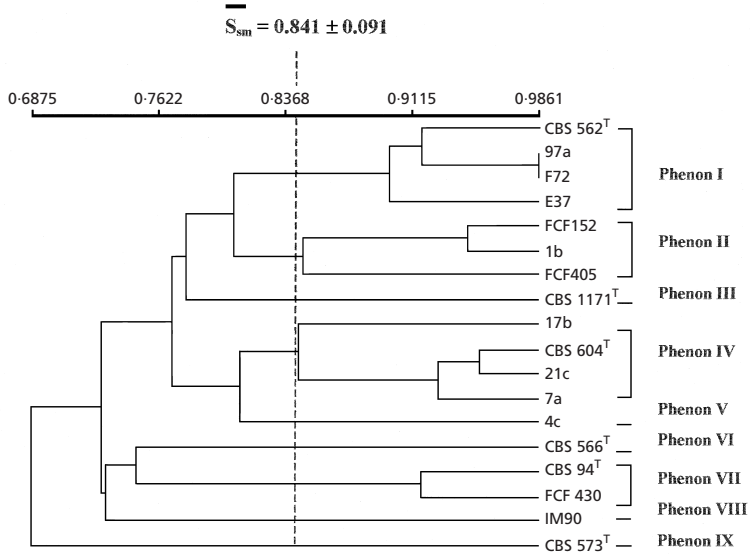

Fig. 2. Non-rooted relatedness dendrogram of oral *Candida* species grouped by the sum of all enzymic patterns, S_{SM} coefficient and UPGMA algorithm.

contained CBS 566^T, the type strain of *C. guilliermondii*. Phenon VII is composed of two strains of *C. tropicalis* (CBS 94^T and FCF430) grouped with $S_{SM} = 0{\cdot}917$. Phenon VIII has strain 1M90 of *C. krusei*. Phenon IX contained the type strain of *C. krusei* (CBS 573^T).

DISCUSSION

Yeasts of the genus *Candida* form a heterogeneous group containing species whose teleomorph states are in the Ascomycota or those that do not have a defined perfect state. Several species of *Candida* can be isolated from the oral cavity, justifying the necessity for understanding their ecological involvement. Reports based on MLEE patterns of *Candida* have supplied useful information in oral epidemiological surveys (Reynes *et al*., 1996; Pujol *et al*., 1993). Among the wide range of enzyme classes, the dehydrogenases, hydrolases and transferases, as well as some others, are the most interesting enzymes applicable to the MLEE technique, due to their relative stability, substrate specificity and occurrence in living organisms (Dixon & Webb, 1979; Selander *et al*., 1986; Gabriel & Gersten, 1992).

In Fig. 1, the enzymic system that could group the majority of strains in their respective species clusters was IDH. Such a fact has already been pointed out by Lehmann *et al*. (1989a), who also noted that IDH and SDH solely distinguish species and do not have any value in biotyping *Candida* isolates. In the present investigation, for different repetitions of SDH band detection protocols, such band patterns could not be obtained, even when other protocols were tested. However, the systems that gave the worst groupings were GDH, α-EST and β-EST, possibly due to the non-formation of bands in many strains. *C. parapsilosis* strains could be grouped together with S_{SM} values of 1·000, in species-specific or composite clusters, in most of the non-dehydrogenases (ACO, CAT, GOT, LAP and SOD), showing this to be the species whose strains are the most related, even being isolated from different individuals. The same behaviour was detected for *C. albicans* strains CBS 562^T, 97a and F72, in different enzymic systems.

The non-rooted dendrogram presented in Fig. 2 shows the sum of all partial dendrograms in Fig. 1. Clusters were formed from the limiting line derived from the average of all OTU similarities of $S_{SM} = 0{\cdot}841$ (SD of 0·091). Some strains clustered together either with others of their own species (species-specific clusters) as in phenons I (*C. albicans*) and VII (*C. tropicalis*) or with different species (composite clusters) as phenons II and IV.

According to Fig. 2, the MLEE technique could group most *C. albicans* strains in a single phenon, with exception of strain 17b which was shown to be less related. This fact was also observed in previous assays involving SDS-PAGE of whole-cell proteins for these strains (Höfling *et al*., 1999). Strain 17b was re-identified in order to determine whether or not it is a *C. albicans* isolate. The phenotypic characteristics, including the use of CHROMagar *Candida* medium which differentiates *C. albicans* from the recently described *Candida dubliniensis* (Schoofs *et al*., 1997), confirmed that this isolate was indeed a *C. albicans* strain. The analysed enzymes also grouped all strains of *C. parapsilosis* with *C. albicans* strain 17b. This aspect of composite cluster generated by MLEE has been previously observed by other authors. Smith *et al*. (1990), characterizing different species of *Brettanomyces* and *Dekkera*, obtained a phenogram in which some strains could not be grouped with high similarity values in their respective species-specific clusters and with interference of some strains in other clusters. Jones & Noble (1982) established electrophoretic

comparisons among species of dermatophytes based on the MLEE technique and obtained a dendrogram in which isolates from certain species were included in the inner taxa of other species or even of other genera. These authors pointed out that this may occur when only a few isolates of each species are included in the surveys. Boerlin *et al*. (1995) used 16 enzymic systems for characterizing 21 genetically atypical strains of chlamydospore-forming, germ-tube-positive *C. albicans* recovered from human immunodeficiency virus-positive drug users, and demonstrated that some of these strains grouped in different clusters, showing high diversity in allelic composition.

Extensive enzyme heterogeneity among strains of *Candida* or other yeast genera has already been observed by other groups of researchers who pointed out that it may increase the possibility of dividing such specimens in various groups or clusters (Lehmann *et al*., 1989a, b, 1993; Caugant & Sandven, 1993; Naumov *et al*., 1997). Lehmann *et al*. (1991) related the phenomenon of isoenzymic patterns of *C. albicans* changing during its conservation in laboratories, which could increase the apparent polymorphism. Pujol *et al*. (1997) found atypical strains of *C. albicans* in AIDS patients, showing diverse allelic polymorphism. The same investigators included some strains of *C. tropicalis*, *Candida glabrata* and *C. krusei* in the survey, obtaining characteristic species-specific clusters. However, the fact that just a few specimens of these species were added could have influenced the organization of such clusters.

In order to ensure that the UPGMA algorithm fits the assemblance between two OTUs in the dendrogram construction well, a product-moment correlation coefficient (r_{CS}) was computed between the elements S_{JK} of the original similarity matrix S and co-phenetic values C_{JK} of the matrix C derived from the dendrogram. This co-phenetic correlation coefficient is a measure of the agreement between similarity values implied by the dendrogram and those of the original similarity matrix (Sokal & Rohlf, 1962). This coefficient has a value of 0·932, which is considered good according to Sneath & Sokal (1973), i.e. it is between 0·60 and 0·95, and Sokal & Rohlf (1970), i.e. it is higher than 0·90; this value supports the results of Farris (1969), which pointed out that the UPGMA algorithm will always maximize r_{CS} values.

All the strains employed here were previously and independently classified up to species level in two different laboratories by mean chlamydospore and germ tube formation, and fermentation and assimilation of sugars; the same results were obtained. This evidence discards the possibility of poor identification of the strains in previous studies.

Based on the presented observations, it is proposed that the grouping of *Candida* species by mean MLEE patterns from assayed enzymes followed by numerical taxonomy statistical treatment is not efficient when involving few isolates from more than one species. Such a resource should be utilized for surveys conducted with a single species of *Candida*, in which the MLEE technique has already proved to be a method useful for systematic or epidemiological purposes.

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