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FACULDADE DE ODONTOLOGIA DE PIRACICABA

USO DA ELETROFORESE DE ENZIMAS CONSTITUTIVAS NA CARACTERIZAÇÃO INTERESPECÍFICA DE LEVEDURAS ORAIS

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EDVALDO ANTONIO RIBEIRO ROSA

Tese apresentada ao curso de Pós-Graduação em Odontologia – Área de Biologia e Patologia Buco-Dental, Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas – UNICAMP, para obtenção do título de Doutor em Ciências

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5. Prof. Dr. REGINALDO BRUNO GONCALVES

DEDICATÓRIA

Este trabalho é dedicado à memória daqueles que fizeram da Microbiologia uma ciência aplicada a melhoria da Saúde Pública, e cujos nomes sempre serão evocados onde quer que duas ou mais pessoas reunam-se para discutir os grandes feitos da Humanidade.

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Se não houver frutos,
valeu a beleza das flores.
Se não houver flores,
valeu a sombra das folhas.
Se não houver folhas,
valeu a intenção da semente.
Henfil (1944-1988)

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SUMÁRIO

ABREVIAÇÕES

µg = micrograma;

µL = microlitro;

^{0} **C** = escala Célsius de temperatura;

^{35}**S** = radioisótopo 35 do enxofre;

5S e **18S** = sub-unidades ribossômicas de índice de sedimentação 5 e 18, respectivamente;

CBS = Centraalbureau voor Schimmelcultures, Baarn, Netherlands;

DNA = ácido desoxirribonucléico (do inglês: deoxiribonucleic acid);

***Eco*R1** = endonuclease de restrição para o sítio 5'-G/AATTC-3';

EST = esterase;

g = grama (unidade de massa);

g = gravidade (unidade de força centrífuga);

In = em (do inglês: in)

kDa = kilodalton;

KOH = hidróxido de potássio;

M = molar;

MM = massa molecular;

mRNA = ácido ribonucléico mensageiro (do inglês: messenger ribonucleic acid)

mg = miligrama;

mL = mililitro;

mm = milímetro;

mM = milimolar;

mRNA = ácido ribonucléico mensageiro (do inglês: messager ribonucleic acid);

NTSYS™ = Numerical Taxonomy and Multivariate Analysis System;

PAGE = eletroforese em gel de poliacrilamida (do inglês: polyacrylamide gel electrophoresis);

pH = potencial hidrogeniônico;

RAPD = polimorfismo de DNA amplificado ao acaso (do inglês: random amplified polymorfic DNA);

RNA = ácido ribonucléico (do inglês: ribonucleic acid);

rpm = rotações por minuto

SDS = dodecilssulfato de sódio (do inglês: sodium dodecyl sulfate);

S_{sm} = coeficiente de similaridade cofenética "simple matching";

UPGMA = agrupamento pareado sem peso com signifcado aritmético (do inglês: unweighted pair group method with arithmetic mean);

W = watts;

YPD = meio extrato de levedura-peptona-glucose (do inglês: yeast peptone dextrose).

RESUMO

Na presente tese, buscou-se estabelecer os graus de diversidade existentes entre diferentes espécies de leveduras do gênero *Candida* isoladas da cavidade oral de indivíduos saudáveis por meio do emprego da técnica de eletroforese de enzimas constitutivas. Para tanto, linhagens representativas de diferentes espécies de *Candida* (*C. albicans, C. tropicalis, C. parapsilosis, C. krusei,* e *C. guilliermondii*) e também suas respectivas linhagens-tipo foram crescidas em frascos com meio de cultura líquido, os quais após incubação (37°C, 150rpm, 18 horas) foram centrifugados e seus *pellets* lavados. As massas celulares obtidas foram processadas num disrruptor celular tipo Mini Bead-beater que permitiu a obtenção dos extratos citossólicos brutos, os quais foram aplicados em tiras de papel de filtro e suas proteínas separadas por eletroforese em gel de amido. A atividade enzimática foi acessada para 20 sistemas: álcool desidrogenase (ADH), lactato desidrogenase (LDH), malato desidrogenase (MDH), isocitrato desidrogenase (IDH), glicose-6-fosfato desidrogenase (G6PDH), aspartato desidrogenase (ASD), glucose desidrogenase (GDH), manitol desidrogenase (MADH), sorbitol desidrogenase (SDH), aconitase (ACO), enzima málica (ME), catalase (CAT), superóxido dismutase (SOD), transaminase glutâmico-oxalacética (GOT), α-esterase (EST), β-esterase (EST), leucina aminopeptidase (LAP), glicosil transferase (GTF), peroxidase (PO) e α-amilase (α-AM). A partir dos géis revelados foram feitos os diagramas representativos da mobilidade eletroforética das bandas que permitiram a confecção de fenogramas de similaridade para cada sistema enzimático.

Os sistemas que forneceram bandas perceptíveis permitiram a análise da diversidade das espécies analisadas tanto em termos fenéticos quanto genéticos, que possibilitaram a redação de quatro artigos originais. Nesses originais pode-se observar a diversidade acessada através da análise conjunta de todas enzimas, da análise conjunta de todas as desidrogenases, e da análise genética dos diferentes *loci*, bem como uma comparação da capacidade discriminatória estabelecida entre a técnica de eletroforese de enzimas constitutivas (MLEE) e a técnica de eletroforese de proteínas totais em gel de poliacrilamida (SDS-PAGE).

ABSTRACT

In the present thesis, it was intended to establish the existing diversity grades among different yeast species of *Candida* genus isolated from oral cavities of healthy individuals by means of multilocus enzyme electrophoresis. For this, representative strains of different species of *Candida* (*C. albicans, C. tropicalis, C. parapsilosis, C. krusei,* e *C. guilliermondii*) and their respective type-strains were grown in liquid culture medium bottles, which, after incubation (37°C, 150rpm, 18 hours), were centrifuged and their pellets were washed. The cell masses were processed in a Mini Bead-beater cell disrupter in order to obtain the crude cytosolic extracts, which were separated by starch gel electrophoresis. The enzymatic activity was accessed for 20 systems: alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PDH), aspartate dehydrogenase (ASD), glucose dehydrogenase (GDH), mannitol dehydrogenase (MADH), sorbitol dehydrogenase (SDH), aconitase (ACO), malic enzyme (ME), catalase (CAT), superoxide dismutase (SOD), glutamic-oxalacetate transaminase (GOT), α-esterase (EST), β-esterase (EST), leucine aminopeptidase (LAP), glucosil transferase (GTF), peroxidase (PO) e α-amylase (α-AM). From revealed gels it were built the diagrams representing the electrophoretic banding mobilities that allowed the construction of similarity phenograms for each enzymatic system.

The systems which furnished detectable bands allowed the species' diversity analysis in phenetic and genetic terms, what allowed the writing of four original papers. On these articles one can notice the diversity accessed from the overall analysis of enzymes, from the overall analysis dehydrogenases, and from the genetic analysis of different loci, as good as one comparison of discriminatory capacity determined by multilocus enzyme electrophoresis (MLEE) and whole-cell protein electrophoresis on polycrylamide gel slabs (SDS-PAGE).

INTRODUÇÃO

O gênero *Candida* compreende um extenso grupo de espécies de leveduras que podem ser encontradas em diversos ecossistemas, seja coexistindo de forma saprófita, seja provocando sérias patologias, sobretudo em crianças, idosos e pacientes imunossuprimidos iatrogenicamente ou por imunodeficiências adquiridas, onde passa a atuar como um agente oportunista.

As várias espécies que compõem esse gênero estão distribuídas em diferentes *phyla* (ou divisões), de acordo com suas características sexuais, o que implica numa grande diversidade. Muitas espécies apresentam seus estados anamórficos (imperfeitos) no gênero *Candida* e seus estados teleomórficos nos *phyla* Ascomycota ou Basidiomycota, além daquelas que não apresentam estágios sexuais conhecidos, caso das espécies *C. albicans* e *C. tropicalis*.

A ocorrência dessas leveduras na cavidade oral é descrita desde longa data e seu papel como agente etiológico de diversas patologias da cavidade oral permanece indiscutível até hoje. Contudo, somente mais recentemente esse grupo de microrganismos vem recebendo uma maior atenção por parte dos cirurgiões dentistas, visto que modernos recursos de Biologia Celular e Molecular têm revelado os mecanismos pelos quais ocorre o estabelecimento do quadro mórbido.

Em sentido convergente, muitos outros estudos têm buscado estabelecer a compreensão da biodiversidade dessas leveduras, tanto em termos interespecíficos, como em termos infraespecíficos. Na busca dessa compreensão, o emprego de ferramentas desenvolvidas pela Biologia Molecular tem sido de fundamental importância.

Um dos primeiros marcadores de biodiversidade desenvolvidos, baseia-se no polimorfismo de expressão eletroforética de enzimas constitutivas (do inglês: multilocus enzyme electrophoresis - MLEE) classificadas como isoenzimas ou aloenzimas, em função dos diferentes *loci* codificadores ou da presença de múltiplos alelos em um mesmo *locus*, respectivamente (PRAKASH *et al.*, 1969). Conforme revisão concebida por HÖFLING & ROSA (1999), a MLEE é uma técnica que apresenta a propriedade de separar, em distintos *taxa*, linhagens de espécies estreitamente relacionadas tanto morfologicamente, quanto fisiologicamente, o que vem a justificar o uso dessa técnica em estudos de Sistemática ou mesmo de Epidemiologia. Outras metodologias, levantadas por esses autores, também podem ser empregadas. Porém, para

leveduras, a eletroforese de enzimas constitutivas foi apontada por PUJOL *et al.* (1997a) como sendo um método de maior reprodutibilidade, quando comparado com métodos baseados na reação da polimerase em cadeia (PCR). A opção pelo uso dessa técnica tem outros aspectos positivos como o relativo baixo custo operacional (FERREIRA E GRATTAPAGLIA, 1995) e a alta capacidade discriminatória entre linhagens de uma mesma espécie de *Candida* (LEHMANN *et al.*, 1989a; LEHMANN *et al.*, 1989b; CAUGANT & SANDVEN, 1993; ARNAVIELHE *et al.*, 1996; BOERLIN *et al.*, 1995; BOERLIN *et al.*, 1996; DOEBBELING *et al.*, 1993; LACHER & LEHMANN, 1991; LE GUENNEC *et al.*, 1995; LEHMANN *et al.*, 1991, LEHMANN *et al.*, 1993).

A literatura tem mostrado ao longo dos últimos anos que a eletroforese de enzimas constitutivas apresenta grande poder resolutivo na identificação de tipos clonais de *Candida* spp. além de uma expressiva reprodutibilidade. Entretanto, existe relativamente pouca informação disponível acerca do emprego dessa metodologia como determinante de diversidade interespecífica e como método agrupante de isolados clínicos em seus respectivos *taxa* espécie-específicos.

O pleno entendimento da diversidade infra e interespecífica dessas espécies de importância odontológica pode contribuir de forma significante para a compreensão de diversas lacunas existentes acerca da genética, evolução, patologia e epidemiologia dessas leveduras.

PROPOSIÇÃO

Com base na relativa escassez de informação acerca da possibilidade do emprego da eletroforese de enzimas constitutivas na organização de espécies orais de *Candida* em *clusters* espécie-específicos e buscando contribuir na direção do pleno entendimento da diversidade apresentada por leveduras desse gênero, nos propusemos a conduzir uma série de experimentos onde representantes de cinco espécies de importância clínico-odontológica, a saber *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, e *C. guilliermondii*, fossem caracterizados pela técnica em questão e seus resultados submetidos à análise numérica e à análise da diversidade genética baseada no polimorfismo de múltiplos *loci*.

PUBLICAÇÕES

- **Main techniques employed on molecular epidemiology of *Candida* species.** Höfling, J.F. & Rosa, E.A.R. ***Alpe-Adria Microbiology Journal 8 (1): 5-23, 1999***

- **Grouping oral *Candida* species by multilocus enzyme electrophoresis.** Rosa, E.A.R.; Pereira, C.P.; Rosa, R.T. & Höfling, J.F. ***International Journal of Systematic and Evolutionary Microbiology 50: 1343-1349, 2000***

- **Evaluation of different dehydrogenases potential to recognize *Candida* species commonly isolated from human oral cavities.** Rosa, E.A.R.; Pereira, C.V.; Rosa, R.T. & Höfling, J.F. ***Revista Argentina de Microbiologia 32:123-128, 2000***

- **Analysis of parity between protein-based electrophoretic methods for the characterization of oral *Candida* species.** Rosa, E.A.R.; Rosa, R.T.; Pereira, C.V.; Boriollo, M.F.G. & Höfling, J.F. ***Memórias do Instituto Osvaldo Cruz (in press)*** .

- **Inter and infra-specific genetic variability of oral *Candida* species.** Rosa, E.A.R.; Rosa, R.T.; Pereira, C.V.; Boriollo, M.F.G. & Höfling, J.F. ***Revista Iberoamericana de Micologia (enviado para publicação)***

- **Clonal variability among oral *Candida albicans* assessed by allozyme electrophoresis analysis.** Mata, A.L.; Rosa, R.T.; Rosa, E.A.R.; Gonçalves, R.B. & Höfling, J.F. ***Oral Microbiology and Immunology (in press)***

Main techniques employed on molecular epidemiology of *Candida* species

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INTRODUCTION

The genus *Candida* is a group of yeasts that can be found dispersed in various ecosystems, from tropical forests (1) and Brazilian rivers (2), to a component of the spectrum of yeasts that contaminate German teas (3), besides some species of medical interest (4), that have been showing a relative increase of importance in the establishment of diseases derived from abusive use of immunosuppressive medications, or as opportunist manifestations in patients suffering from congenital or acquired immunodeficiencies (5).

The different species that compose the genus are distributed in different phyla (or divisions), in agreement with sexual characteristics of each one. Thus, the anamorph states of *C. guilliermondii*, *C. guilliermondii* var. *membranaefaciens*, *C. krusei*, *C. sorbosa*, *C. pseudotropicalis*, *C. parapsilosis* and *C. pulcherrima* have their teleomorph states in the phylum Ascomycota, in the species *Yamadazyma guilliermondii*, *Pichia ohmeri*, *Issatchenkia orientalis*, *Issatchenkia occidentalis*, *Kluyveromyces marxianus*, *Lodderomyces elongisporus* and *Metschnikovia pulcherrima*, respectively (6-10). Some species of *Candida* present their teleomorph states in the phylum Basidiomycota, for example, *C. scotii*, *C. capsuligena*, *C. frigida* and *C. gelida*, which are imperfect forms of *Leucosporidium scotii*, *Filobasidium capsuligenum*, *L. frigidum* and *L. gelidum*, respectively (11, 12). There are species that do not present well-known perfect state, such as *C. albicans*, main species of medical interest, and *C. tropicalis* (13). In agreement with Porto (14), there were until that date 81 species of yeasts classified within the genus *Candida*.

The determination of a systematic classification of *Candida* strains was initially developed from previous knowledge concerning the physiology and biochemistry of those yeasts, and it has developed in order to allow the individual characterization for each isolate ("fingerprinting") and favor the understanding of the relationships of phenetic, genetic and phylogenetic features between two or more entities. Initially, the routine of identification and characterization of such yeasts involved - and still today are used - techniques of evaluation of typical cellular structures formation, such as chlamydospores, pseudohyphae and true hyphae (15). Thus, *C. albicans* can be differentiated from other species, because it produces globose terminal chlamydospores, whose walls are thick and generally in great number (16), with abundant pseudomycelia that comes from the non separation of budding blastospores, and in old cultures true mycelia can be found. The last author still adds that *C. stellatoidea* can be differentiated from *C. albicans*, although with certain difficulty, since the former presents scarce chlamydospores production, which, when present, are found in chains of two or three spores. Samaranayake & Macfarlane (17) add that some strains of *C. tropicalis*, can eventually present formation of small pear-shaped terminal chlamydospores. Other species do not produce such structures.

Auxanographic methods, which evaluate the behavior of the specimens that will be identified according to their fermentation capacities and carbohydrate assimilation, were firstly proposed by Wickerman & Burton (18) and also by Wickerman (19). The assimilation tests were later appraised by Fiol (20), who observed the low capacity of sporogenous yeasts speciation - for certain sugars - proposing a reformulation in the way those tests should be conducted. Still in 1975, Land *et al.* (21) demonstrated that the incorporation of dyes in the culture media increased significantly the capacity of speciation of medically important yeasts, and more recently, Sandven (15) validated such methods emphasizing their discriminatory capacity at the species level. Thus, isolates which do not produce chlamydospores can be classified in other species that compose the genus. Another auxanographic method, based on the capacity that some species present of hydrolyzing amides, was proposed by Mira-Gutierrez *et al.* (22), and it allowed the characterization of seven genera and nineteen species of yeasts isolated from clinical material and confirmed by conventional methods of identification.

Under the historical view, during the 1950 and 1960's, the necessity of settling down the degree of infraspecific variability, as a function of the pathological and ecological importance of those yeasts, drove several research groups to develop serological and chemotaxonomic methods that could discriminate different clonal populations of *Candida.* In serology, the publications of great relevance explored the use of double immunodiffusion, agglutination, immunofluorescence and immunoelectrophoresis techniques, using cytoplasmatic proteins or wall polysaccharide. In this investigation field, Tsuchiya *et al.* (23) began the presentation of a series of works that demonstrated the possibility of the employment of thermostable and thermolabile antigens, obtained from cellular wall, in taxonomic application. Hasenclever & Mitchel (24) published an article where they reclassified the species *C. stellatoidea* in the different serotypes of *C. albicans.* In 1973, Axelsen (25) observed that the double-dimensional immunoelectrophoresis could characterize strains of *C. albicans*, starting from the existent variability among 78 antigenic fractions obtained from the cellular extract of that species. Other variations of the immunoelectrophoresis technique, such as crossed immunoelectrophoresis, have been used as taxonomic tools for species of *Candida* (26). The serology continues helping the classification of *C. albicans* strains, through agglutination tests, allowing the separation of the isolates in two serogroups (A and B), collaborating to a better characterization of those microorganisms (27).

CHEMOTAXONOMICAL METHODS

Chemotaxonomical methods try to establish the affinity relationships among different strains through the comparison of chemical compositions of several cellular structures, such as: polysaccharides, proteins, nucleic acids, enzymes, fatty acids, etc. The nuclear magnetic resonance (NMR) of cell wall polysaccharides was widely used for yeasts of several genera with taxonomic purposes: *Nadsonia, Hanseniaspora, Kloeckera* and *Saccharomycodes* (28), *Hansenula* and *Pichia* (29), *Torulopsis, Debaryomyces* and *Metschnikowia* (30-32) and *Candida* (33, 34). The main polysaccharides analyzed are the mannans, whose side chains can present variable concentrations of oligosaccharides with β1-2, β1-3 and β1-6 links, of relative systematic value (35). The technique of magnetic nuclear resonance was used by Shibata *et al.* (36) to differentiate, through the structure of the mannans with β1-2 links, *C. famata* from *C. saitoana*, and *C. guilliermondii* from other species of *Candida* (37). Variations in the proportions of α1-6,

α1-2 and α1-3 links of mannans allowed Kogan *et al.* (38) to establish differences between *C. albicans* serotypes A and B and *C. parapsilosis.*

The constitutional profile of fatty acids with long chain, established with gas-liquid chromatography, allowed the differentiation of several yeast genera (39) and was also used in the study of the perfect and imperfect states of ascomycetes of the genus *Candida* by Viljoen *et al.* (40). Inside the cells, those compounds can be found as triglycerides or free polar fatty acids, such as oleic acid and stearic acid (41). Botha & Kock (42) observed that the analysis of fatty acids with long chain should not be the first choice in presumptive identification processes of yeasts, specially for basidiomycetes. Nevertheless, that technique discriminates different strains of a species and different species of a genus.

Cytochromes are heme-proteins involved in final oxy-reduction processes of the respiratory chain, whose spectrophotometric absorption spectra can be used in systematics of *Candida* (43, 44) and, in yeasts, they can vary from 510 to 605 μm (45). Showing special taxonomic importance, the cytochrome C already had its aminoacid composition determined and its gene sequenced by Freire-Picos *et al.* (46), who evaluated the degrees of homology and the possible phylogenetic relationships existing among *Kluyveromyces lactis, Schwanniomyces occidentalis, Saccharomyces cerevisiae* and *Candida krusei.*

In accordance with Mendonça-Hagler & Hagler (47), the first chemical property of nucleic acids employed as taxonomic criterion was the composition of DNA bases, generally expressed in percentile molar of guanine plus cytosine (mol%G+C), whose value is constant for each microorganism. In the double helix of DNA, between the homologue bases guanine and cytosine, there are three interactions of "hydrogen-bond" type, that are more thermostable than the two "hydrogen-bonds" between adenine and thymine. The greater mol%G+C in a double strand of DNA, the greater temperature is necessary to promote the thermal denaturation. According to Johnson (48), the mol%G+C can be determined by using a thermocontrollable ultraviolet spectrophotometer (260nm), that supplies a sigmoid whose midpoint represents the mean temperature of denaturation of the double strand (called melting temperature = Tm). The higher the value of Tm, the greater the mol%G+C.

Among the early researches involving the differential determination of yeasts based on the different molar proportions of nucleotides, the one conducted by Dupont & Hedrick (49) can be

emphasized, in which the first proportions for the genus *Trichosporon* were established, and the studies of Gheho (50), who determined the mol%G+C values for several species of the genus *Geotrichum*, classifying them in the phyla Ascomycota and Basidiomycota. De Hoog & Gueho (51) evaluated the mol%G+C values of the "type-strains" of some species of the genera *Moniliella, Trichosporonoides* and *Hyalodendron*, establishing variation patterns of those values, for the referred species. Still in 1984, Gueho *et al.* (52) used the technique in the classification of 28 species of *Trichosporon*, what allowed the division of these species in two groups, where the first - that seemed to be related with the phylum Ascomycota - included species with mol%G+C values lower than 50% (34.7-48.8), and the second group, that seemed to be related with basidiomycetes, included those species whose mol%G+C values was greater than 50% (57-64). Similar experiment was conducted by Pappagianis *et al.* (53), who evaluated the applicability of the technique for *Coccidioides*. The main merit of the studies mentioned above was to present the possibility that the technique has to relate species of yeasts with the phyla in which they are classified. Nakase *et al.* (54) used such technique to help the systematic study of ballistosporogenous yeasts. The authors observed the diversity of these yeasts, belonging to two genera (*Ballistosporomyces* and *Kockovaella*), that presented variation from 39 to 68.5% for mol%G+C values in the chromosomal DNA. Hamajima *et al.* (55) used the mol%G+C values determined by the Tm and HPLC (High Performance Liquid Chromatography) techniques, to propose the exclusion of strains of *C. tropicalis* that did not absorb a certain monospecific serum factor, commonly absorbed by cells of *C. tropicalis*. More recently, Gueho *et al.* (56) evaluated the mol%G+C compositions of three species of *Malassezia* that compose the genus, and they proposed the inclusion of other four, increasing the total number of species to seven.

SODIUM DODECILSULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Several researchers (57-60) have been using the electrophoretic analysis of whole-cell proteins (SDS-PAGE) in the taxonomy of fungi. Type, number and frequency of aminoacids determine the size of the protein, its shape and total electric charge, determining its electrophoretic mobility. Small differences in sequences of aminoacids can cause great differences in the mobility of proteins. Shechter *et al.* (61) developed a comparative study on protein electrophoresis of different species of dermatophytes (*Microsporum gypseum, M. kennels,*

Trichophyton mentagrophytes, *T. tonsurans*, *T. rubrum* and *Epidermophyton floccosum*), in which they evaluated the electrophoretic profiles of proteins extracted from mycelial cells, demonstrating that the technique allowed the grouping of the isolates in genera, and the differentiation of the species from the same genus. Few years later, Hall *et al.* (62) conducted taxonomic studies using electrophoresis in polyacrylamide gel for the identification of oomycetes from the genus *Phytophthora*, showing variations among the bands obtained from different species.

Variations in the original electrophoresis technique of whole-cell proteins allowed the study of the infra and inter-specific variabilities among isolates of the genus *Candida*. Lee *et al.* (63) proposed a characterization system for isolates of *C. albicans* based on electrophoretic separation of total proteins, followed by transfer to a nitrocellulose membrane where the proteins were revealed through the combination with conjugated of polyclonal antibodies-alkaline phosphatase, that produce colored complexes (technique denominated "Western blot"). The authors observed the occurrence of 16 different serotypes among 190 isolates. The same isolates were submitted to the agglutination technique developed by Hasenclever & Mitchell (24) and they just produced 2 serotypes groups, demonstrating the great capacity of the electrophoretic procedure in the differentiation of isolates of the same species. Another variation of the technique was developed by Shen *et al.* (64), who introduced the analysis of the profiles of (^{35}S)-methionine radiolabeled proteins, in the differential identification of seven species of *Candida* (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. kefyr*, *C. lipolytica* and *C. lusitaniae*), two species of *Torulopsis* (*T. glabrata* and *T. Candida*), a species of *Trichosporon* (*T. beigelii*) and a species of *Saccharomyces* (*S. cerevisiae*), obtaining extremely satisfactory results.

Seeking for an application for electrophoresis in the understanding of the biodiversity of the intrabuccal environment, Maiden & Tanner (65), working with yeast samples isolated from the oral cavity, employed the polyacrylamide gel electrophoresis for their identifications. They obtained patterns of protein bands whose molecular masses varied from 29 to 116 kDa (kilodaltons), which is enough for the differential analysis of those organisms. Those authors emphasized the high specificity of the technique, added to the fast obtainance of a great number of data with classificatory significance. In a publication from 1991, Vancanneyt *et al.* (60) proposed the use of whole-cell proteins electrophoresis of several genera and species of yeasts

with medical and industrial importance with the purpose of promoting their differentiation and characterization. The authors pointed out the importance and necessity of type-strains inclusion, with the purpose of characterizing the corresponding and correlated groups, in the moment of the construction of similarity phenograms, as well as they established reproducibility criteria for the evaluation driven in different gels.

In 1992, that same group of researchers (66) published another work justifying the application of whole-cell protein electrophoresis in the identification and classification of yeasts. The authors, after polyacrylamide gel electrophoresis (SDS-PAGE), submitted the gels with their respective slots to scanning densitometry with posterior achievement of correlation matrixes and construction of protein phenograms. Their results confirmed the success of the methodology for the characterization of yeasts of genera *Cystofilobasidium, Filobasidium, Filobasidiella, Kondoa, Leucosporidium* and *Rhodosporidium*, with their characteristic fingerprints. Guillamon et al. (67) used SDS-PAGE to establish the affinity relationships, at infraspecific level, for different strains of *Saccharomyces cerevisiae* isolated from fermentative processes of Spanish vinifacteur industry and observed the variability due to artificial selection for human interference, checking - once again - the importance and versatility of that methodology.

In a great number of cases, it is now known that one-dimensional electrophoregrams of whole-cell proteins discriminate, so much as, the information derived from data of DNA-DNA hybridization (68-73). Bacterial strains with 90-100% of homologue DNA sequences generally present protein profiles almost identical, and strains with at least 70% of DNA homology tend to have similar protein profiles. Those observations are, according to Kersters (72), the greatest pillars in which the application of protein electrophoresis in Microbial Systematics are founded. The comparison of electrophoretic profiles is a resource with satisfactory taxonomic resolution, that is applicable to the level of species, subspecies or biotypes.

MULTILOCUS ENZYME ELECTROPHORESIS (MLEE)

Isoenzymes are, according to Dixon & Webb (74), multiple forms of an enzyme occurring in an unique species. This fact occurs due to the presence of several *loci*, coding different versions of an enzyme, or due to the existence of multiple alleles in an single *locus* (75), and in the last case, each enzyme is called alloenzyme or allozyme (76). When studying the expression and enzymatic activity, we can evaluate parameters as homozygosis, heterozysis, variation in the

molecular masses and ionic charge of isoenzymes, that give polymorphic characteristics between two or more alleles or among distant genes that code different molecular forms of the same enzyme. The isoenzymes can be detected through electrophoresis in gel. This technique has been used in taxonomic and epidemiological studies with filamentous fungi (77-80), myxomycetes (81), and in assays with yeasts (82-84).

Lehmann *et al.* (85), working with some species of the genus *Candida* as *C. albicans*, *C. stellatoidea*, *C. tropicalis* and *C. paratropicalis*, isolated from several infectious focuses, studied the polymorphism for 4 enzymatic systems, and they obtained peculiar results for each studied species. In the same year, Lehmann *et al.* (86) made the numerical analysis of those isolates, based on the interpretation of the clusters formed after the electrophoresis and detection of multiple forms of these and other enzymes, grouping the isolates in two larger clusters: A) *C. albicans–C. stellatoidea* I and II, and B) *C. tropicalis–C. paratropicalis*. Caugant & Sandven (87), working with 98 isolates of *C. albicans*, studied 9 enzymatic systems and observed the polymorphism for those enzymes inside of that yeast population. Other researchers also published papers concerning the classification of *Candida* species and yeasts from other genera, through the analysis of multilocus-enzyme electrophoresis (88-102).

RESTRICTION ENDONUCLEASE ANALISYS (REA)

The Total DNA Restriction Pattern Analysis, commonly designated as REA (Restriction Endonucleases Analysis), was firstly used in the characterization of yeasts by Nath & Bollon (103) and for *Candida* species by Scherer & Stevens (104). The REA technique is based on the characteristic that the bacterial restriction endonucleases have of cleaving the double strand of DNA, when they recognize small specific sequences with 4 to 8 base-pairs, denominated palindromes. The polymorphism observed in the REA technique occurs because the genomes of genetically distinct individuals differ in the nucleotide sequence along the DNA (105) and the presence or absence of the palindromes, recognized and cleaved by the restriction enzymes, can vary among the different individuals, generating polymorphism. Differences in DNA sequences can also result from insertions, deletions or other causes (translocations, invertions) that alter the distances between restriction sites. The fragments obtained from the digestion of chromosomal DNA are separated using electrophoresis in agarose or polyacrylamide support (106), generating

genomic fingerprints, once the number and the location of the cleavage sites are specific for each genome (107).

Such tool has been used in taxonomy of *Candida* and other yeasts, either in the analysis of total genomic DNA (108-111), or in specific parts of DNA. Pfaller *et al.* (112) promoted total digestion of *C. albicans* genome with Eco R1 and after electrophoretic separation of the fragments, they observed that isolates obtained from different anatomical sites of the same patient followed a clonal pattern of colonization. Sanchez *et al.* (113) evaluated the nosocomial acquisition of *C. parapsilosis* in patients submitted to bone marrow transplants using that technique. In the following year, two publications (114, 115) also pointed out that REA could help the understanding of the mechanisms of *C. albicans* crossed infection in hospital ambient, demonstrating the relevance of the technique.

In 1989, Su & Meyer (116) proposed the REA technique to analyze the polymorphism of mitochondrial DNA (mtDNA) of *C. parapsilosis*, *C. kefir* and *C. albicans*, and to establish differentiation parameters for those species. Carruba *et al.* (117) pointed out that for *C. parapsilosis*, fragments of mitochondrial DNA supply more information than fragments of total genomic DNA. Gimenez-Jurado *et al.* (118) also used mtDNA polymorphism to analyze the genetic diversity existing within the genus *Metschnikowia*. Morace *et al.* (119) accomplished the digestion of amplicons of the cytochrome P-450 (L1A1) lanosterol-1,4 α-demetylase gene, that contains a highly conserved region of DNA in different species of *Candida*, and submitted the fragments to electrophoresis, obtaining characteristic profiles for each species.

In recent years, amplicons of conserved sequences of DNA, that code 18S ribosomal sub-units, have also been digested by restriction endonucleases (ssu18S rRNA PCR-REA) and electrophoretic migrations of the fragments have been employed in the evaluation of inter and intraspecific polymorphism. Vilgalys & Hester (120) were the first researchers who used such a technique for characterization of *Candida*, *Cryptococcus* and *Trichosporon*. Those authors emphasized the relative simplicity of the method, that does not involve either transfer procedures ("Southern blot"), or hybridization. Hopfer *et al.* (121) used this technique to digest the amplicons of some cryptococci, *Candida* and *Trischoporon*. In 1995, Baleiras-Couto *et al.* (122) submitted *S. cerevisiae*, *C. valida* and *C. lipolytica* to analysis based on RAPD markers and they observed that this last technique supplied less stable results than the polymorphism of restriction

of the gene of ssu 18S rRNA for those rehearsed species. Other ribosomal sub-units and conserved regions were also analyzed in relation to the derived polymorphism of amplification and enzymatic cleavage. Williams *et al.* (123) evaluated the existing variability in amplicons of intergenic spacing areas of rDNA from several species of *Candida* and reported that *C. guilliermondii*, *C. glabrata* and *C. pseudotropicalis* could not be characterized individually, whereas *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. parapsilosis* and *C. krusei* showed different profiles for each species. Nho *et al.* (124) published an article, in which they described an adaptation of the technique for the gene that codes the rRNA 5.8S sub-unit of *C. krusei*, *C. inconspicua* and *C. norvegensis*, and its applicability once due to species-specific bands obtained by those authors.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Another technique, denominated RFLP (Restriction Fragment Length Polymorphism), is also derived from cleavage of the genome of yeasts, and involves the transfer of fragments of DNA to inert membranes of nitrocellulose or nylon, with posterior denaturation of double strand, and hybridization with radiolabeled or chemoluminescent specific probes of DNA, whose radiation or ligth are detected by radiographic films (125, 126).

Two publications reported the efficiency of a specific probe (CkF1,2) for the characterization of *C. krusei*, that allowed the differentiation of that species from others (127), as well as the characterization of different strains of *C. krusei* (128). Roy & Meyer (129) used the RFLP analysis to characterize 3 groups of *C. parapsilosis* isolated from clinical material, and they could quantify the genetic divergence existing among those groups. Faix *et al.* (130) characterized strains of *C. albicans* isolated from events of neonatal candidemia using Eco R1 and Xba 1 restriction enzymes, with posterior hybridization with 27A probe, obtaining profiles that allowed comparison of the degrees of likeness among those strains. Mathaba *et al.* (131) also used 27A probe, that presents moderate frequency of repetitive sequences, to characterize 57 strains of *C. albicans* isolated from oral cavity of 18 patients, and they observed that, in most of the cases, only one clone of the yeast prevailed. Still regarding the employment of markers RFLP in the characterization of oral strains of *C. albicans*, Boerlin *et al.* (90) used Eco R1 and Hinf 1 endonucleases to digest the genome and Ca3 probe to observe the polymorphism in the pattern of restriction from 189 strains of that yeast. Those authors also concluded that only one clone of *C.*

albicans was involved in recurrent episodes of oropharyngeal candidosis. Carmougrand *et al.* (132) analyzed the polymorphism existing in the patterns of restriction of the mtDNA of *C. parapsilosis*, detectable by hybridization with the segments of the sub-units 6 and 8 of ATPase, that allowed the differentiation of several isolates. The construction of an artificial oligonucleotide with radiolabeled repetitive sequences poly d(GT), poly d(CA) and poly (GT), in order to be used as probes, was proposed by Wilkinson *et al.* (133), in 1992, who observed the discriminatory power of such oligonucleotides for different strains of *C. albicans.* In 1993, Niersters *et al.* (134) proposed that the amplification of the domains V4 in the genes of ssu rRNA, with subsequent enzymatic cleavage and hybridization with species-specific probes, could be used in the differential determination of several species of *Candida* with medical interest.

PULSED FIELD GEL ELECTROPHORESIS (PFGE)

PFGE (Pulsed Field Gel Electrophoresis) is a technique that has been providing important taxonomic information. PFGE is based on the limiting size of the separable DNA fragments by conventional electrophoresis in agarose (about 50 kbp), which can be increased by the introduction of pulses, or alterations in the direction of the electric field (135). Pizzirani-Kleiner & Azevedo (136) reported that molecules of DNA longer than 50-60 kbp show electrophoretic mobilities that are independent of their respective molecular masses, that is, all of them migrated together in the gel.

Schwartz & Cantor (137), working with *Saccharomyces cerevisiae*, solved the problem of electrophoretic separation of great segments of DNA. Those researchers observed that, when alternating the direction of electric field, those molecules started to migrate differentially. Applying a variable electric field, the molecules of DNA moved diagonally, in "zigzag", making possible the isolation due to their different molecular masses.

That technique allowed the establishment of electrophoretic karyotypes (EK) of *C. albicans* (138), *C. stellatoidea* and *C. claussenii* (139), *C. krusei* and *C. inconspicua* (140), *C. boidinii* (141), *C. parapsilosis* (142, 143), *C. tropicalis* (144), *C. rugosa* (145), *C. glabrata* (146), *C. lusitaniae* (96), several species of *Saccharomyces* and *Zygossaccharomyces* (147), *Hortaea*, *Filobasidiella* and *Mallassezia* (148), and it was used by Zervos & Vazquez (111) and Vazquez *et al.* (149) to evaluate the possible origins of fungal flora involved in infections.

PFGE can be used in the characterization of chromosome, plasmide, or mitochondrial DNA (mtDNA) structures. In 1993, Fukuhara *et al.* (150) evaluated the lineal conformation of mtDNA of *Pichia pijperi*, *P. jadinii* and *Williopsis mrakii*, that when compared with the circular mtDNA of kindred species, demonstrated high genic homology, suggesting that those two mtDNA forms do not have distant origins, therefore, they show little taxonomic value. PFGE is also an useful technique in the separation of great fragments of fungal chromosomes digested by restriction endonucleases for rare cleavage sites (135). The use of that technique allows the construction of physical maps for several genera of yeasts, because those endonucleases furnish great fragments (151). Those physical maps can provide important information regarding the extension of the genome (152). DNA macrorestriction profiles, obtained from that procedure, allowed Cormican *et al.* (153) to determine that an uniclonal predominance for *C. glabrata* occurred in different anatomical sites of the same individual. In 1994, Branchini *et al.* (154) used that technique to demonstrate the genotypic variability of *C. parapsilosis* isolated catheter and from peripheral sanguineous circulation. Pontieri *et al.* (155) observed the existing relationships among strains of *C. parapsilosis* isolated from blood, vagina and soil. After comparison of the results, the authors concluded that technique allows the obtainance of genomic fingerprints for *C. parapsilosis.* Waggoner-Fountain *et al.* (156) used the polymorphism of macrorestriction fragments separated through PFGE to evaluate the vertical and horizontal transmissions of *Candida* in premature newborns, that were maintained in nosocomial ambient.

METHODS BASED ON THE POLYMERASE CHAIN REACTION (PCR)

The PCR (Polymerase Chain Reaction) is a powerful technique, that involves *in vitro* synthesis of millions of copies of a specific segment of DNA in the presence of Taq DNA polymerase. The technique of PCR is based on the enzymatic amplification and anelling of "primers" (initiators, starting from 5' end) that define the sequences of the double strand DNA to be amplified (157), using sequences of the microorganism DNA strands as template. Those primers are artificially synthesized, so that the nucleotide sequences are complementary to those that flank the area that will be amplified. Those amplifications are conducted in appropriate apparels denominated thermocyclers, that control the temperature of each amplification cycle phase. The amplification products ("amplicons") can be separated by agarose or polyacrylamide gel electrophoresis.

Phylogenetic studies involving amplification by PCR and sequencing of small ribosomal RNA sub-units (srRNA) were conducted by Hendricks *et al.* (158), who evaluated the polymorphism degrees of those regions, for several species of *Candida*, *Kluyveromyces*, *Torulaspora* and *Saccharomyces cerevisiae*, establishing evolutionary distances for such species. Still with evolutionary interest, the cytochrome P450-L1A1 gene was analyzed by Burgener-Kairuz *et al.* (159), who compared the sequences of amplification product of that gene for different species of *Candida*, *Cryptococcus*, *Trischoporon* and *Torulopsis*, establishing degrees of phylogenetic likeness, derived from the polymorphism generated during evolutionary processes. The analysis of direct sequencing of amplicons of *Lodderomyces elongisporus* 18S RNA (ssu rRNA) gene sequences allowed James *et al.* (160) to evaluate the existing interrelations among that and other ascomycete species and ascomycete-like organisms. The results obtained by those authors indicated that *L. elongisporus* should be the teleomorph state of *C. parapsilosis*.

Carlotti *et al.* (161) described the employment of the technique in the characterization of *C. krusei*, with the purpose of accomplishing the identification of isolates of that species. The authors developed two primers that amplify tandemly repetitive and polymorphic sequences (CKRS-1) of non-transcriptionable intergenic regions of rRNA genes of *C. krusei*. That group of researchers (162) also compared their results with other results derived from hybridization with CkF 1,2 probe, and concluded that the polymorphism detectable in amplification of CKRS-1 allows the determination of the fingerprints of different *C. krusei* isolates. Nishikawa *et al.* (163) developed primers for species-specific sequences of ribosomal sub-units, that allow the differentiation of isolates of the *Debaryomyces hansenii/Candida famata* complex from other isolates of *C. guilliermondii*.

In 1995, Walsh *et al.* (164) proposed that one variation of the original PCR technique, the analysis of the simple strand conformational polymorphism (SSCP-PCR), could be useful in identification and characterization of pathogen-opportunist fungi and yeasts. However, those authors did not discriminate isolates of *C. albicans*, *C. tropicalis* and *C. parapsilosis* as differentiated entities, when the amplicons of small fragments of 18S rRNA gene were employed

Many other groups of researchers published papers in which the PCR technique was used as an auxiliary tool in the identification and/or characterization of isolates of *Candida*, *C. krusei* (165), *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei* (166), *C. albicans* (167) and *C.*

albicans, *C. glabrata*, *C. tropicalis* and *C. krusei* (134) and other yeasts related with that genus, like *Saccharomyces* (168, 169) or *Metschnikowia* (170).

ARBITRARY PRIMED PCR (AP-PCR)

Among the different applications of PCR, the technique of AP-PCR (Arbitrary Primed PCR), which determines the RAPD markers (Random Amplified Polymorphism of DNA), is the most used method in systematic or epidemic studies. In that technique only one primer is used, in an arbitrary way, while in the technique of classic PCR, two primers that code a known aim sequence are used (171). The appearance of electrophoretic bands allows the observation of the molecular nature of the polymorphism of the RAPD *loci*. Williams *et al.* (172) reported that experimental evidences have been showing that differences of just a pair of bases (punctual mutations) are enough to cause the non-complement of the primer with the template strand, what do not allow the amplification of the segment. Other polymorphism sources can include deletions or inserts in the connection sites of the primer, that increase the distances to be copied by the Taq polymerase. By this way, the genetic polymorphism detected through RAPD markers has a binary nature and the amplified segment may be present or absent. In 1997, San Milan *et al.* (10) demonstrated that several clinical isolates presumptively identified as *C. guilliermondii*, in fact presented great affinity with *C. fermentati*, for RAPD markers. Those authors pointed out that AP-PCR technique allows more precise discriminations in the characterization of those yeasts at species level.

The capacity of strains characterization from the same species of *Candida* through RAPD markers was demonstrated by Holmberg & Feroze (173), who obtained different electrophoretic banding profiles for amplicons of several strains of *C. albicans*. However, in another characterization study with nineteen oral isolates of *C. albicans*, Howell *et al.* (174) showed that AP-PCR technique supplied three different types for RAPD markers, against other five types obtained by REA technique, with *Eco* R1 and *Hinf* I enzymes. The genomic heterogeneity of *C. parapsilosis*, commonly accessed through the electrokaryotyping by PFGE (117), is also possible of being accomplished by AP-PCR, with similar results, as indicated by Lott *et al.* (175). Several species of *Kluyveromyces*, many of them classified as teleomorph states of several species of *Candida*, were differentiated based on RAPD markers, by Molnar *et al.* (176), who checked the

discriminatory capacity of AP-PCR technique for differentiation of species. Yeasts of other genera, like *Phaffia* (177), have also been analyzed through that analytical resource.

PERSPECTIVES

Several revision works on the employment of molecular tools as analytical auxiliary in systematic, taxonomic, evolutionary and epidemic surveys involving microorganisms - and especially, yeasts - have been published and some of them were described here. In spite of the high resolutive capacity of those techniques in the characterization inter and/or infra-specific, the accumulated experience points for the necessity of using more than a technique in the characterization of genera, species or strains (5, 35, 48, 112, 178-186). In 1995, Bart-Delabesse *et al.* (187) accomplished an epidemic survey in a burn care unit, where they used the techniques of AP-PCR, RFLP and electrokaryotyping by PFGE to understand the mechanism of cross-infection in patients with candidemia, and concluded that the combination of at least two different methods should be used in the characterization of strains of *Candida*, confirming such statements.

In an interval of some decades since the first investigations with taxonomic emphasis, in which the previous knowledge concerning the physiology, biochemistry and genetics of yeasts were used, the great progress provided by molecular characterization of species and strains of *Candida*, through the described techniques, is evident. Although new techniques will still be developed, the knowledge already accumulated is of fundamental importance.

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Grouping oral *Candida* species by multilocus enzyme electrophoresis

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Running title: Oral *Candida* species grouped by MLEE.

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

Summary

Multilocus enzyme electrophoresis (MLEE) and numerical taxonomic methods were carried out in order to establish relatedness degrees among five *Candida* species commonly isolated from oral cavity of humans. Of twenty enzymatic systems assayed five had not shown any enzymatic activity (ASDH, MADH, SDH, GTF, and α-AM). The obtained data revealed that some of these enzymes are capable of distinguish strains of different species, but most of them could not organize all strains in their respective species-specific clusters. Numerical classifications based on MLEE polymorphism must be regarded for surveys involving just one *Candida* species.

Introduction

Candida, mainly the species *C. albicans*, remain the most common fungi found in the oral cavity of humans, and in the recent years have been receiving more attention because of their involvement in a numberless cases of opportunist oral infections in patients with AIDS and those having immunosupresive medication. Of epidemiological interest, characterization procedures that furnish molecular fingerprints have been applied in order to establish possible relationships among *Candida* isolates that could have some oral relevance (McCullough *et al.*, 1996). The multilocus enzyme electrophoresis (MLEE) is a resource that has been used in studies involving a

large number of microorganisms (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 works pointed out the capacity that some enzymatic systems have in discriminate less related strains or even species from the other genera. The proposition of this paper is to evaluate the parity existing among some enzymatic systems for fingerprinting five *Candida* species (*C. albicans, C. tropicalis, C. krusei, C. parapsilosis,* and *C. guilliermondii*) isolated from healthy subjects saliva.

Material and methods

***Candida* strains:** Representative strains of different *Candida* species isolated from oral cavity 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 Microbiology and Immunology Laboratory, Dentistry College of São José dos Campos: *Candida albicans* (97a, F72, E37, 17b, CBS562^T), *Candida guilliermondii* (FCF405, FCF152, CBS566^T), *Candida parapsilosis* (21c, 7a,CBS604^T), *Candida krusei* (1M90, 4c, CBS573^T), *Candida tropicalis* (1b, FCF430, CBS94^T). All strains (excluding the type strains) were isolated from the oral cavities of healthy human beings. In this work, we added the *Saccharomyces cerevisiae* type-strain (CBS1171^T) as an extra-generic organism.

Cells cultivation and enzyme extraction: All the strains were grown in 50mL of YPD medium (2% dextrose, 2% peptone, 1% yeast extract), on a shaker table at 150rpm and 30C, overnight. The cells were harvested by centrifugation of total culture medium volume at 2,000 *g* for 3 minutes and the pellets were washed 4 times with cold sterile water for ensure completely culture medium traces or extra-cellular metabolites remotion (Woontner & Jaehning, 1990). The last washed pellets were transferred to microcentrifuge tubes of 2mL, and were added equal amounts of acid washed glass beads and 200µL of cold sterile water. The tubes were adapted in a Mini-Bead Beater cell disrupter (Biospec), where the cell lysis have been done at 4600rpm., 4 times of 30 seconds, with intervals of 5 minutes, when the samples were conditioned in an ice bath. After cell disruption, the microcentrifuge tubes were centrifuged at 10,000 *g* for 2 minutes, and the

supernatants were applied on Whatman 3 filter paper wicks of 5x12mm. These wicks were maintained at –70C.

Starch gel electrophoresis: The electrophoreses were carried out according to Val *et al.* (1981), 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 x 120 x 10mm), and left on the bench, at room temperature until complete solidification. They were cut longitudinally at 2.5cm from one border. The 2.5cm segments were separated and the wicks were applied on the cut. Wicks with 0.2% bromphenol blue were applied in both extremities of the cuts to indicate migration. After jointed the parts, cotton cloth bridges were done connecting the gels to electrode tanks with Tris-citrate buffer pH 8.0 (Selander *et al.*, 1986; Caugant & Sandven, 1993). Electrophoreses were carried out at 4C and 130V until the migration markers move at least 80mm from application point. At this time, the electrophoreses were terminated and the gels were sliced in their high in slices into 1.2mm thickness.

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

Computing numerical data: Dendrograms were generated for the different enzymatic systems by using the same measurement of relatedness, the Simple Matching (S_{SM}) association coefficient (Sokal & Michener, 1958; Sneath & Sokal, 1973), based on band positions computed with the NTSYS software package, version 1.70 (Applied Biostatistics, Inc.) This S_{SM} measures the

proportion of bands with the same and the different Rm values in patterns of two OTUs (Operational Taxonomic Unit), **k** and **j**, by the formula: $S_{SM} = E/E + b + c$, where E is the positive combination of bands (present or 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**. For the present study, a S_{SM} of 1.00 represents identically matches (i.e., all the bands in the patterns of OTUs **k** and **j** match), a S_{SM} of 0.00 represents no matches, and S_{SM} values ranging from 0.01 to 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 arithmetic average (UPGMA) clustering method (Rohlf, 1963; Sneath & Sokal, 1973).

Results

The one-dimensional electrophoreses of protein extracts of 12 *Candida* strains, their respective type strains, and the *Saccharomyces cerevisiae* type strain, showed that among twenty assayed enzymes five had not shown any enzymatic activity (ASDH, MADH, SDH, GTF, and α-AM). The remaining systems furnished electrophoretic bands that allowed the construction of fifteen individual dendrograms showed in Fig. 1 (a, b). *Candida albicans* strains CBS562^T, 97a, F72, and E37 were clustered in dendrograms A (ADH), B (ME), E (IDH), F (LDH), H (ACO), and K (CAT). *Candida albicans* strain 17b showed atypical patterns of MLEE 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 dendrogram derived from IDH system. Dendrogram G (MDH) shows a grouping of such clinical strains of *C. guilliermondii* with the inclusion of *C. albicans* strain E37 and the type strain of *C. guilliermondii* clustered with three *C. albicans* strains (CBS562, 97a, and F72). For *C. krusei*, IDH system could group two strains, CBS573^T and 1M90. Catalase could group strains CBS.573 and 4.c, and PER 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 (CBS94^T) and the clinical isolate FCF430 were the specimens that revealed the highest relationship (S_{SM} = 1.00) for four enzymatic systems (IDH, LDH, α-EST, and β-EST) and for ADH, ME, and MDH, grouped with $S_{SM} > 0.85$. 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 these

four last trees, associated with strains of other species. The *S. cerevisiae* type strain CBS1171^T combined with different species of *Candida* according to the different enzymatic systems.

The results of the individual MLEE analyses were pooled for each strain, and a non-rooted relatedness dendrogram of the 18 analyzed strains based on the similarity calculated by Simple Matching coefficient of association, were built (Fig. 2). The nine phenons (clusters) were established by the perpendicular line that represents the average value for S_{SM} of all OTUs, and it is 0.841.

Phenon I contains the strains CBS152^T, 97a, F72, and E37 (*C. albicans*), grouped with a $S_{SM} \geq 0.898$. Phenon II has two strains of *C. guilliermondii* (FCF152 and FCF405) and one *C. tropicalis* (1b) as components, grouped with $S_{SM} \geq 0.847$. Phenon III just contains the type strain CBS1171^T of *S. cerevisiae*. Phenon IV contains the three strains of *C. parapsilosis* (CBS604^T, 21c, and 7a) and the atypical *C. albicans* strain 17b, with $S_{SM} \geq 0.845$. Phenon V is a cluster composed of a unique *C. krusei* strain (4c). Phenon VI contains the type-strain CBS566^T of *C. guilliermondii*. Phenon VII is composed of two strains of *C. tropicalis* (CBS94^T, and FCF430) grouped with $S_{SM} = 0.917$. Phenon VIII has the strain 1M90 of *C. krusei*. Phenon IX contains the type strain of *C. krusei* (CBS573^T).

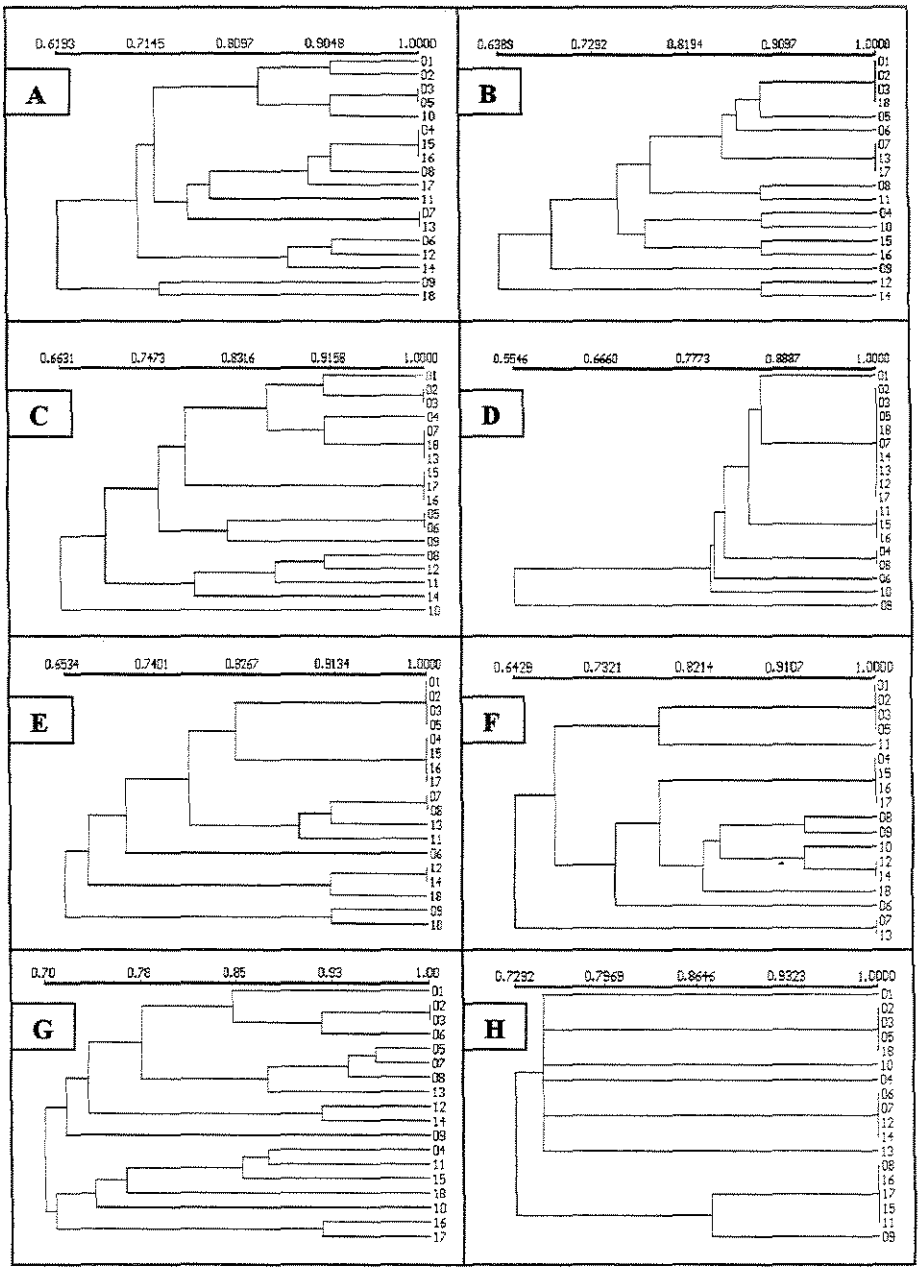

Fig. 1a: Dendrograms showing the relatedness levels among different *Candida* species, based on their respective MLEE patterns and statistical treatment with Simple Matching association coefficient (S_{SM}) and UPGMA clustering method: A) ADH; B) ME; C) G6PDH; D) GDH; E) IDH; F) LDH; G) MDH; H) ACO. Strains (OTUs): 01) CBS562^T (*C. albicans*); 02) 97a (*C. albicans*); 3) F72 (*C. albicans*); 4) 17b (*C. albicans*); 5) E37 (*C. albicans*); 6) CBS566^T (*C. guilliermondii*); 7) FCF152 (*C. guilliermondii*); 8) FCF405 (*C. guilliermondii*); 9) CBS573^T (*C. krusei*); 10) 1M90 (*C. krusei*); 11) 4c (*C. krusei*); 12) CBS94^T (*C. tropicalis*); 13) 1b (*C. tropicalis*); 14) FCF430 (*C. tropicalis*); 15) CBS604^T (*C. parapsilosis*); 16) 21c (*C. parapsilosis*); 17) 7a (*C. parapsilosis*); 18) CBS1171^T (*S. cerevisiae*).

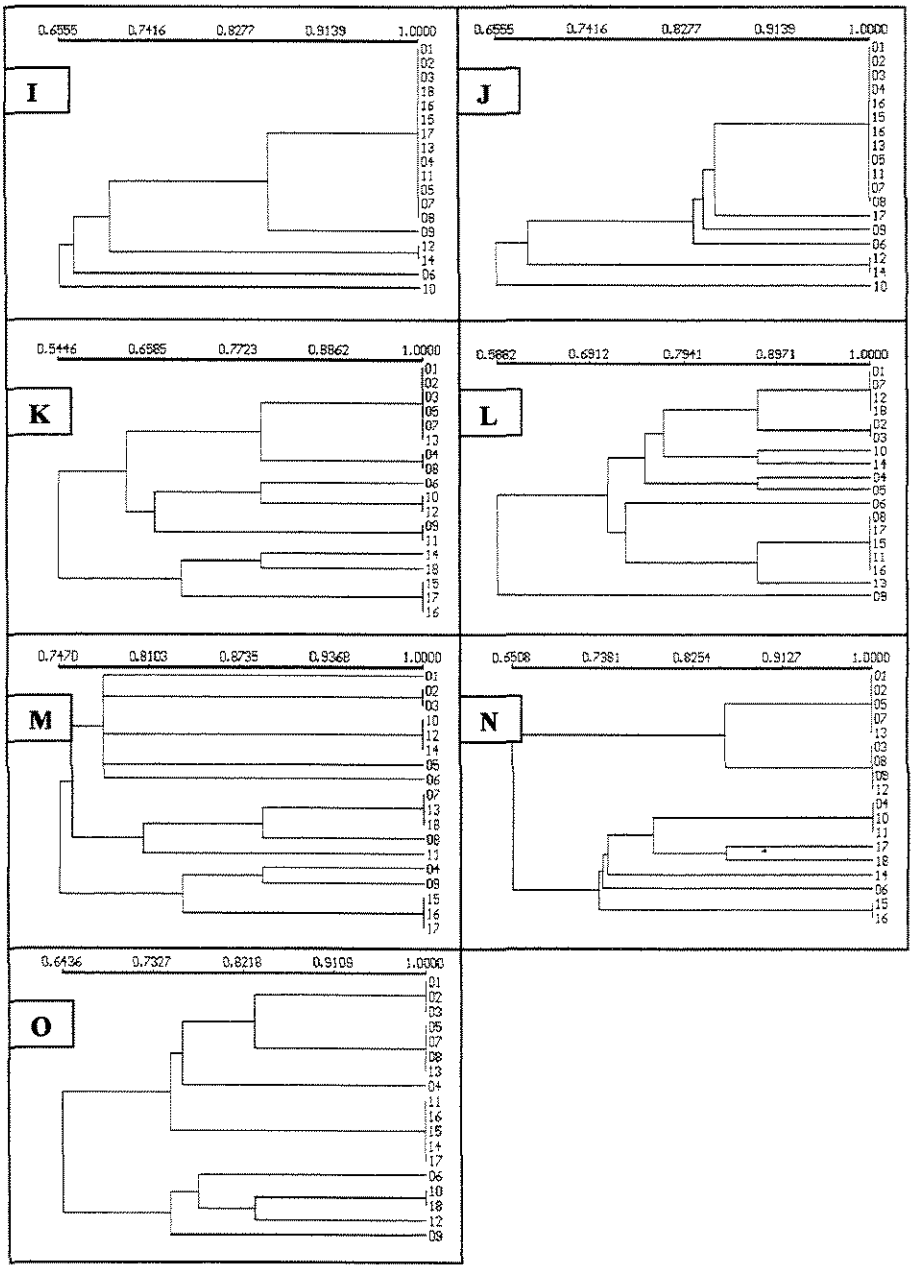

Fig. 1b: Dendograms showing the relatedness levels among different *Candida* species, based on their respective MLEE patterns and statistical treatment with Simple Matching association coefficient (S_{SM}) and UPGMA clustering method: I) α-EST; J) β-EST; K) CAT; L) GOT; M) LAP; N) PER; O) SOD.
Strains (OTUs): 01) CBS562^T (*C. albicans*); 02) 97a (*C. albicans*); 3) F72 (*C. albicans*); 4) 17b (*C. albicans*); 5) E37 (*C. albicans*); 6) CBS566^T (*C. guilliermondii*); 7) FCF152 (*C. guilliermondii*); 8) FCF405 (*C. guilliermondii*); 9) CBS573^T (*C. krusei*); 10) 1M90 (*C. krusei*); 11) 4c (*C. krusei*); 12) CBS94^T (*C. tropicalis*); 13) 1b (*C. tropicalis*); 14) FCF430 (*C. tropicalis*); 15) CBS604^T (*C. parapsilosis*); 16) 21c (*C. parapsilosis*); 17) 7a (*C. parapsilosis*); 18) CBS1171^T (*S. cerevisiae*).

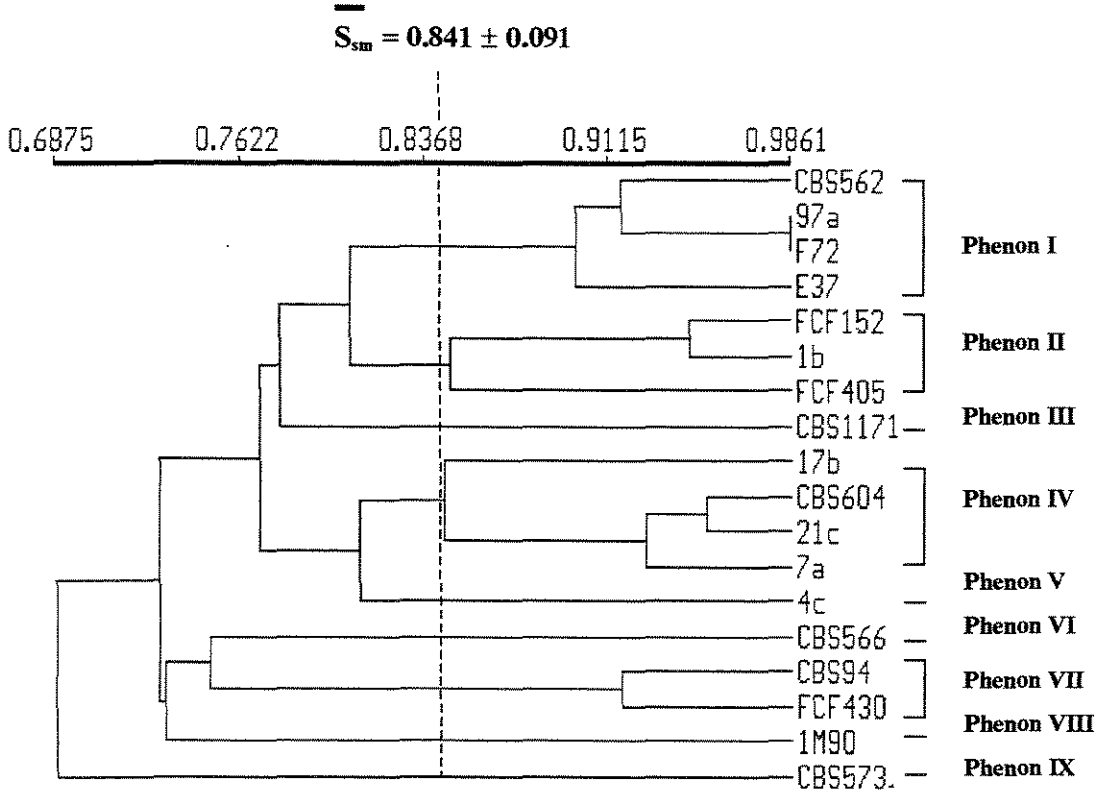

Fig. 02. Non-rooted relatedness dendrogram of oral *Candida* species grouped by the sum of all enzymatic patterns, S_{SM} coefficient and UPGMA algorithm.

Discussion

Yeasts of genus *Candida* form a heterogeneous group that has species whose teleomorph states are stayed in the Ascomycota or those that do not have perfect state defined yet. In oral cavity, several species of *Candida* can be isolated, justifying the necessity of understanding their ecological involvement. Proceedings 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, transferases, beside others, comprehend the most interesting enzymes applicable to MLEE technique, due to their relative stability, specificity for substrate, and occurrence in living organisms (Dixon & Webb, 1979; Selander *et al.*, 1986; Gabriel & Gersten, 1992).

In Fig. 1 (a, b) it can be observed that the enzymatic system that could group the major part of strains in their respective species clusters was IDH. Such fact had already been pointed out by Lehmann *et al.* (1989a) that also added 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 bands detection protocols, we could not obtain such band patterns, even when other protocols were tested. In other hand, the systems that furnished the worst grouping were GDH, α-esterase, and β-esterase, maybe due to the non-formation of bands in many strains. *Candida parapsilosis* strains could be grouped together with S_{SM} = 1.000, in species-specific or composite clusters, for most of non-dehydrogenases (ACO, CAT, GOT, LAP, and SOD), showing be the species whose strains are the most related, even being isolated from different individuals. Same behavior was detected for *C. albicans* strains CBS562^T, 97a, and F72, in different enzymatic systems.

Non-rooted dendrogram presented in Fig. 2 shows the sum of all partial dendrograms in figure 01, where a compensation of one each other was expected. Clusters were formed from limiting line derived from the average of all OTU's similarity with 0.841 and standard deviation of 0.091. Some strains clustered together either with others of their own species (species-specific clusters) as phenons I (*C. albicans*) and VII (*C. tropicalis*) or of different species (composite clusters) as phenons II, and IV.

According with this figure, MLEE technique could grouped most of *C. albicans* strains in a single phenon, with exception for 17b strain that showed be the less related. This fact was also observed in previous assays involving SDS-PAGE of whole cell proteins for these strains (Höfling *et al,* 1999). The 17b strains was reidentified in order to determine whether or not it is a *C. albicans* isolate. The phenotypic characteristics that included the use of CHROMagar *Candida* medium that differentiates *C. albicans* from the recently described *C. dubliniensis* (Schoofs *et al.*, 1997) ensured that such isolate was a *C. albicans* strain. The analyzed enzymes also grouped all strains of *C. parapsilosis* with strain 17b of *C. albicans*. Such aspect of composite cluster generating from MLEE was already observed by other authors as following. 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 MLEE technique and obtained a dendrogram in which occurred the inclusion of isolates from certain species inner taxa of other species or even of other genera. These authors pointed out that this fact may occurs when only a few isolates of each species are included in the surveys. Boerlin *et al.* (1995) used 16 enzymatic systems for characterizing 21genetically atypical strains of chlamydospore-forming and germ tube-positive *C .albicans* recovered from human immunodeficiency virus-positive drug users, and demonstrated that some of these strains were grouped in different clusters, showing high diversity on allelic composition.

Extensive enzyme heterogeneity among strains of *Candida* or other yeast genera had already been observed by other groups of researchers that pointed out that it may occur increasing the possibility of dividing such specimens in various groups or clusters (Lehmann *et al.*, 1989a; Lehmann *et al.*, 1989b, Lehmann *et al.*, 1993; Caugant & Sandven, 1993; Naumov *et al.*, 1997). Lehmann *et al.* (1991) related the phenomenon of isoenzymatic patterns changing of *C. albicans* during its conservation in laboratories, what 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 few strains of *C. tropicalis, C. glabrata* and *C. krusei* in the survey obtaining characteristic species-specific clusters. However, the fact that just few specimens of these species were added could have influenced the organization of such clusters.

In order to ensure whether or not UPGMA algorithm well fits the assemblance between two OTUs in the dendrogram construction, a product-moment correlation coefficient was computed between the elements S_{JK} of the original similarity matrix S and cophenetic values C_{JK} of the matrix C derived from the dendrogram. This cophenetic 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 value $r_{CS} = 0.932$, that is comprehended between 0.60 and 0.95 (Sneath & Sokal, 1973) or higher than 0.90 (Sokal & Rohlf, 1970), values considered as good, corroborating by this way, with the finds of Farris (1969), that pointed out the fact that 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, fermentation

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and assimilation of sugars, obtaining the same results. This evidence discards the possibility of bad previous identification of the strains.

Based on the presented observations, we could propose that the grouping of *Candida* species by mean MLEE patterns from the assayed enzymes followed by numerical taxonomy statistical treatment is not efficient when involving few isolates from more than one species, regarding such resource for surveys conduced with a single species of *Candida*, for what, the MLEE technique had already proved be a method useful for systematic or epidemiological ends.

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EVALUATION OF DIFFERENT DEHYDROGENASES POTENTIAL TO RECOGNIZE *Candida* SPECIES COMMONLY ISOLATED FROM HUMAN ORAL CAVITIES.

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Running head: Parity among dehydrogenases of *Candida* spp

ABSTRACT

Electrophoresis of some dehydrogenases were carried out in order to establish relatedness degrees among five *Candida* species commonly isolated from oral cavity of humans by numerical taxonomy methods. The obtained data revealed that some of dehydrogenases are capable of distinguishing strains of different species, but most of these enzymes could not organize all strains in their respective clusters. Numerical classifications based on dehydrogenases polymorphism must be regarded for surveys involving just one species of such yeast genus, where this resource had already shown be useful.

Key words: *Candida* species, dehydrogenases patterns, cluster analysis.

RESUMEN

Se investigaron las relaciones entre algunas espécies de *Candida* aisló de la cavidad oral de humanos. Dehidrogenases de estas amuestras fueron evaluadas a través del empreo de tecnicas de electroforesis. Aunque nuestros resultados han mostrados que algunas de las dehidrogenases son capables de distinguir entre las diferentes espécies, la mayoria de estas enzimas no fueron de valor por el agrupamiento de estas amuestras en sus respectivos grupos. Deben considerarse clasificaciones numéricas basadas en el polimorfismo de dehidrogenases para estudios con simplemente una especie de tal género de levadura, donde sabemos que los recursos son útiles, ya se habia mostrado.

Palabras claves: Especies de *Candida*, padrón del dehidrogenases, análisis del grupos.

INTRODUCTION

The yeasts from genus *Candida* remain the most common eukaryotic microorganisms found in human oral cavity and, in recent years, have been receiving more attention since they are involved in numerous cases of opportunistic oral infections, mainly in patients with AIDS and iatrogenic immunosuppressions (19). Certain species, such as *C. albicans* and *C. tropicalis*, are easily recovered from saliva, even in healthy carriers (5, 7).

With epidemiological interest characterization procedures, which furnish molecular fingerprints, have been used in order to establish possible relationships among *Candida* isolates that could have some oral relevance (15). Such techniques can be done with cellular substances from variable nature, but the most impressive researches have been conducted with molecules that can show sufficient individual polymorphism and chemical stability for application in molecular epidemiological surveys, as deoxyribonucleic acid (18) and proteins (8). One technique which evaluates protein polymorphism is the multilocus enzyme electrophoresis (MLEE) that has been used in studies where single/multiple species (11) or the presence of single/multiple clones (21, 22) colonization were investigated.

The proposition of this study is to evaluate the capacity of some dehydrogenases in establishing the fingerprinting of five *Candida* species (*C. albicans, C. tropicalis, C. krusei, C. parapsilosis,* and *C. guilliermondii*) isolated from the saliva of healthy subjects.

MATERIALS AND METHODS

***Candida* strains** - Representative strains of different *Candida* species isolated from oral cavity and identified by biochemical and physiological tests were obtained from the Microbiology and Immunology Laboratory, Dentistry College of São José dos Campos: *C. albicans* (97.a, F.72, E.37, 17.b, CBS.562^T), *C. guilliermondii* (FCF.405, FCF.152, CBS.566^T), *C. parapsilosis* (21.c, 7.a,CBS.604^T), *C. krusei* (1M.90, 4.c, CBS.573^T), *C. tropicalis* (1.b, FCF.430, CBS.94^T). The superscripted T in CBS strains implicate that they are the respective type-strains of such species and in this work we added the *Saccharomyces cerevisiae* type-strain (CBS.1171^T) as an extrageneric organism.

Cell cultivation and enzyme extraction - All the strains were grown in Erlenmeyer flasks with 50mL of YPD medium (2% dextrose, 2% peptone, 1% yeast extract), at 30°C, overnight, in a shaker table under 150 rpm of agitation. The cells were harvested by centrifugation of total culture medium volume at 2000 *g* for 3 min and the pellets were washed 4 times with cold sterile water to ensure complete removal of culture medium traces or extra-cellular metabolites (32). The washed pellets were transferred to microcentrifuge tubes of 2 mL, and equal amounts of acid-washed glass beads and 200 µL of cold sterile water were added. The tubes were adapted in a Mini-Bead Beater cell disrupter (Biospec, Inc.), where the cell lysis was conducted at 4600 rpm, 4 times of 30 s, with 5-min intervals, then the samples were conditioned in an ice bath. After cell disruption, the microcentrifuge tubes were centrifuged at 10 000 *g* for 2 min, and the supernatants were applied on Whatman 3 filter paper wicks of 5x12mm. These wicks were maintained at –70°C.

Starch gel electrophoresis - The electrophoresis was carried out according to Val *et al.* (31), by solubilizing hydrolyzed corn starch (Penetrose 30, from Refinações de Milho Brasil Ltda) up to a final concentration of 13% in 1:30 pH 8.0 Tris-citrate buffer, with vigorous agitation on a Bunsen burner. The formed gels were poured in perplex casting moulds (200x120x10 mm), and let over bench at room temperature until complete solidification, when they were cut on their longitudinal dimensions at 2.5 cm from one border. The smaller parts were separated and the wicks were applied on the cut. Wicks with 0.2% bromphenol blue were applied in both extremities of the cuts for migrating indication. After jointed the parts, cotton cloth bridges were placed connecting the gels to electrode tanks containing pH 8.0 Tris-citrate buffer

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(3, 24). Electrophoresis was carried out at 4°C and 130 V until the migration markers ran through at least 80 mm from wick's inserting point. At this time, the electrophoresis was interrupted and the gels were sliced in their high in slices with 1.2 mm thickness.

Band revelation - The gel slices were submitted to some protocols to reveal the dehydrogenase active bands, according to Selander et al. (24), Alfenas et al. (1), and Ballve et al. (2). Enzymatic systems assayed were: alcohol dehydrogenase (ADH - E.C. 1.1.1.1), lactate dehydrogenase (LDH - E.C. 1.1.1.27), malate dehydrogenase (MDH - E.C. 1.1.1.37), isocitrate dehydrogenase (IDH - E.C. 1.1.1.42), glucose-6-phosphate dehydrogenase (G6PDH - E.C. 1.1.1.49), aspartate dehydrogenase (ASDH – E.C. 1.4.3.x), glucose dehydrogenase (GDH – E.C. 1.1.1.47), mannitol dehydrogenase (MADH – E.C. 1.1.1.67), sorbitol dehydrogenase (SDH – E.C. 1.1.1.14), malic enzyme (ME – E.C. 1.1.1.40). Table 1 shows the protocol for each enzymatic reaction.

Table 1. Staining systems for dehydrogenases [a]

Dehydrogenases[b]	Substrate (amt)	Buffer amt (mL)	Salt (amt)	Coenzyme[b]
ADH	Ethanol (3mL)	0.2M Tris-HCl pH 8.0 (50)		NAD
ASDH	Aspartic acid (50mg)	Phosphate pH 7.0 (50mL)[c]		NAD
GDH	D-glucose (1g)	0.2M Tris-HCl pH 8.0 (100)		NAD
G6PDH	Glucose 6-phosphate dissodium (100mg)	0.2M Tris-HCl pH 8.0 (50)	0.1M $MgCl_2$ (1mL)	NADP
IDH	1.0M isocitric acid (2mL)	0.2M Tris-HCl pH 8.0 (50)	0.1M $MgCl_2$ (1mL)	NADP
LDH	Lactic acid 85% (10mL)	0.1M Tris-HCl pH 7.5 (100)	0.1M $MgCl_2$ (1mL)	NAD
MDH	2.0M malic acid[d] (6mL)	0.2M Tris-HCl pH 8.0 (40)		NAD
ME	2.0M malic acid[d] (6mL)	0.2M Tris-HCl pH 8.0 (40)	0.1M $MgCl_2$ (1mL)	NADP
MADH	Mannitol (100mg)	0.1M Tris-HCl pH 8.5 (100)		NADP
SDH	Sorbitol (500mg)	0.05M Tris-HCl pH 8.0 (50)		NAD

[a] All systems must be stained in the dark at 37°C. The time is variable between 20 and 60 minutes.
[b] To stain dehydrogenases, dissolve substrates in suitable buffer, then add 1.0 mL of dimethylthiazol tetrazolium (MTT) solution (1.25 g in 100 mL of water) and 0.5 mL of phenazine methosulfate (PMS) solution (1 g in 100 mL of water) and either 2.0 mL of NAD solution (1 g of NAD-free acid in 100 mL of water) or 1 ml of NADP solution (1 g of dissodium NADP in 100 mL of water), as indicated. Keep solutions refrigerated and in the dark.
[c] Sodium phosphate (pH 7.0) buffer: mix equal parts of 27.6 g of $NaH_2PO_4.H_2O$ in 1 liter of water and 53.6 g of $Na_2HPO_4.7H_2O$ in 1 liter of water, then dilute the mixture 1:25 with water.
[d] Malic acid solution: 268 g of DL-malic acid and 160 g of NaOH in 1 liter of water. *Caution: potentially explosive reaction.*

Computing numerical data - Dendrograms were generated for the different enzymatic systems by using the same measurement of relatedness, the Simple Matching (S_{SM}) association coefficient (17, 26, 27), based on band positions computed with the NTSYS software package, version 1.70 (Applied Biostatistics, Inc.) This S_{SM} measures the proportion of bands with the same and the different Rm values in patterns of two OTUs (Operational Taxonomic Unit), **k** and **j**, by the formula: $S_{SM} = E/E + b + c$, where E is the positive combination of bands (present or 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**. For the present study, a S_{SM} of 1.00 represents identically matches (i.e., all the bands in the patterns of OTUs **k** and **j** match), a S_{SM} of 0.00 represents no matches, and S_{SM} values ranging from 0.01 to 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 arithmetic average (UPGMA) clustering method (17, 23, 26).

RESULTS

The one-dimensional electrophoresis of 12 *Candida* strains extracts, their respective type-strains, and *S. cerevisiae* type-strain showed that, among ten assayed dehydrogenases, three did not show any enzymatic activity (ASDH, MADH, and SDH). The remaining systems furnished electrophoretic bands that allowed the construction of seven individual dendrograms shown in figures 1, 2, and 3. *Candida albicans* strains CBS.562^T, 97.a, F.72, and E.37 were grouped in pure or compost clusters in dendrograms A (ADH), G (ME), D (IDH), and E (LDH). *C. albicans* strain 17.b showed atypical patterns of MLEE for all assayed enzymes, grouping with strains of other *Candida* species in a non-repetitive way. Pure cluster of *C. guilliermondii* was only detected in a dendrogram derived from IDH (D) system, even though grouping just the two clinical strains FCF.152 and FCF.405. Dendrogram F (MDH) shows a grouping of such strains with the inclusion of *C. albicans* strain E.37. For *C. krusei*, just the IDH (D) system could group two strains, CBS.573^T and 1M.90. This species showed to be the least able for clustering of the species used in this survey. Among *C. tropicalis* strains, the type-strain (CBS.94^T) and the clinical isolate FCF.430 were the specimens that revealed the highest relationship ($S_{SM} = 1.00$) for two enzymatic systems [IDH (D) and LDH (E)] and for ADH (A), ME (G), and MDH (F), grouped with $S_{SM} > 0.85$. All three strains of *C. parapsilosis* appeared together in a single cluster

for the dendrograms of G6PDH (C), IDH (D), and LDH (F), although in these two last trees, they were associated with the atypical *C. albicans* strain 17.b. The *S. cerevisiae* type-strain CBS.1171^T combined with different species of *Candida* according to the different enzymatic systems.

The results of the individual MLEE analyses were pooled and a non-rooted relatedness dendrogram of the 18 analyzed strains based on the similarity calculated by Simple Matching coefficient of association was built (Fig. 4). The phenons (clusters) were established by the perpendicular line that represents the average value for S_{SM} of all OTUs, and it is 0.8357 ± 0.0905.

Phenon I contains the strains CBS.562^T, 97.a, F.72, and E.37 (*C. albicans*), grouped with a $S_{SM} \geq 0.897$. Phenon II has a unique strain of *C. guilliermondii* (CBS.566^T) as a component. Phenon III contains the strain 1M.90 of *C. krusei*. Phenon IV contains the *S. cerevisiae* type-strain CBS.1171^T. Phenon V is an impure cluster composed by three strains of *C. parapsilosis* (CBS.604, 21.c, and 7.a) with the inclusion of a *C. albicans* strain (17.b), and a value for $S_{SM} \geq 0.875$. Phenon VI contains the strain FCF.152 of *C. guilliermondii* and the strain 1.b of *C. tropicalis* grouped with $S_{SM} = 0.929$. Phenon VII is composed by a unique strain of *C. guilliermondii* (FCF.405). Phenon VIII has the strain 4.c of *C. krusei*. Phenon IX contains two strains of *C. tropicalis* (CBS.94^T, and FCF.430) grouped with $S_{SM} = 0.905$. Phenon X has a unique strain of *C. krusei* (CBS.573^T).

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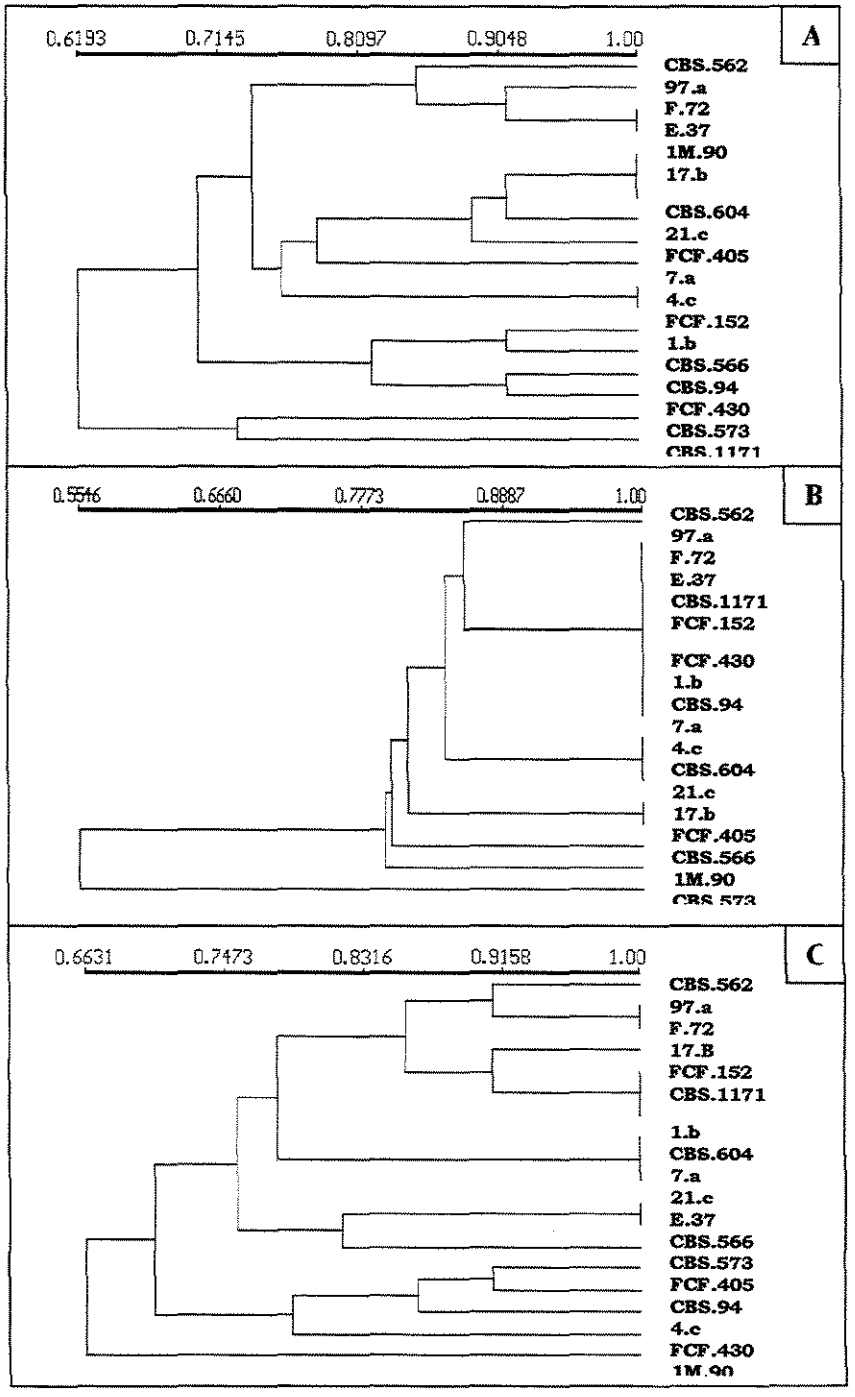

Fig. 1: Dendrograms (A to C) showing the relatedness levels among different *Candida* species, based on their respective dehydrogenases MLEE patterns and statistical treatment with Simple Matching association coefficient (S_{SM}) and UPGMA clustering method: A) ADH; B) GDH; C) G6PDH.

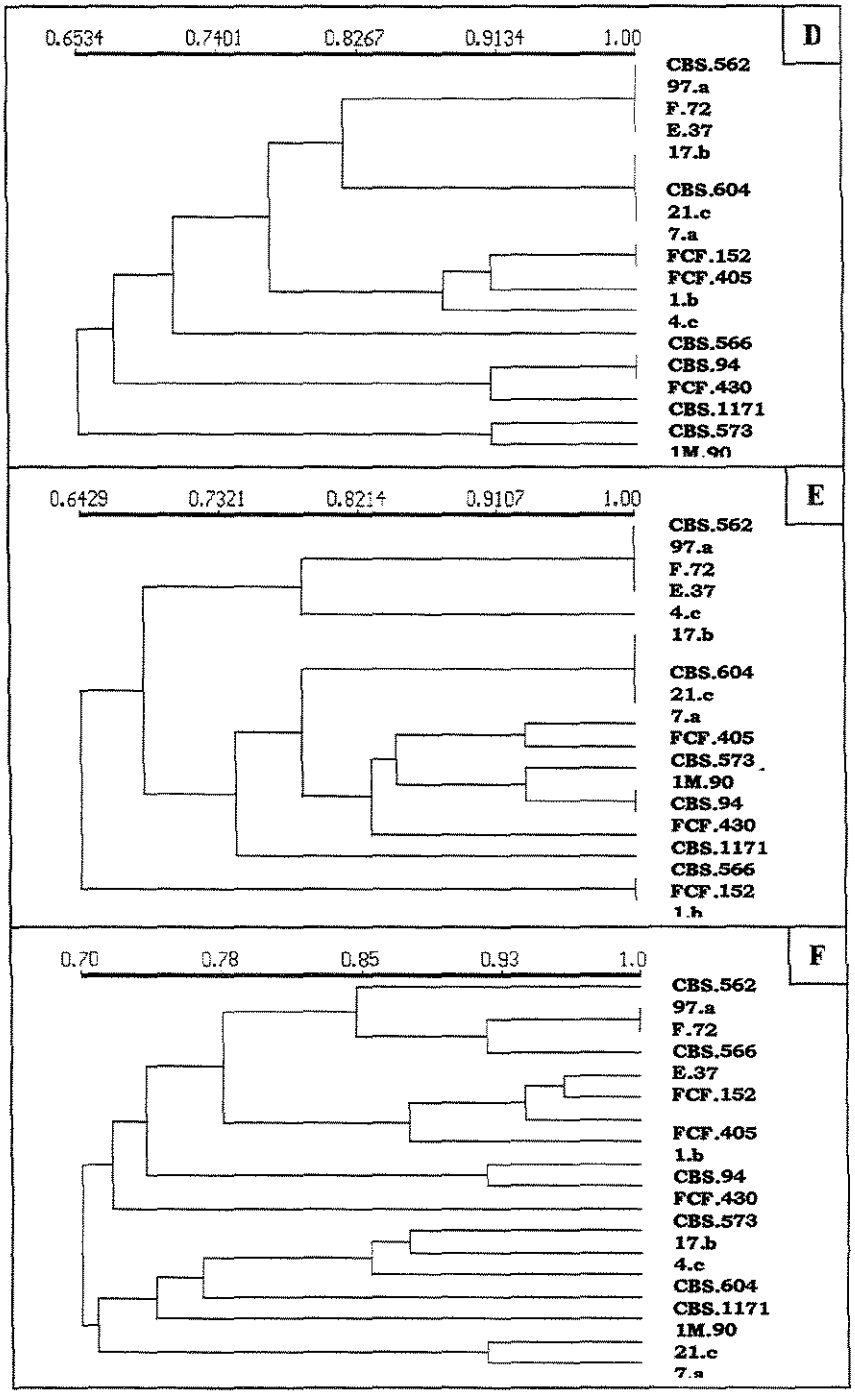

Fig. 2: Dendrograms (D to F) showing the relatedness levels among different *Candida* species, based on their respective dehydrogenases MLEE patterns and statistical treatment with Simple Matching association coefficient (S_{SM}) and UPGMA clustering method: D) IDH; E) LDH; F) MDH.

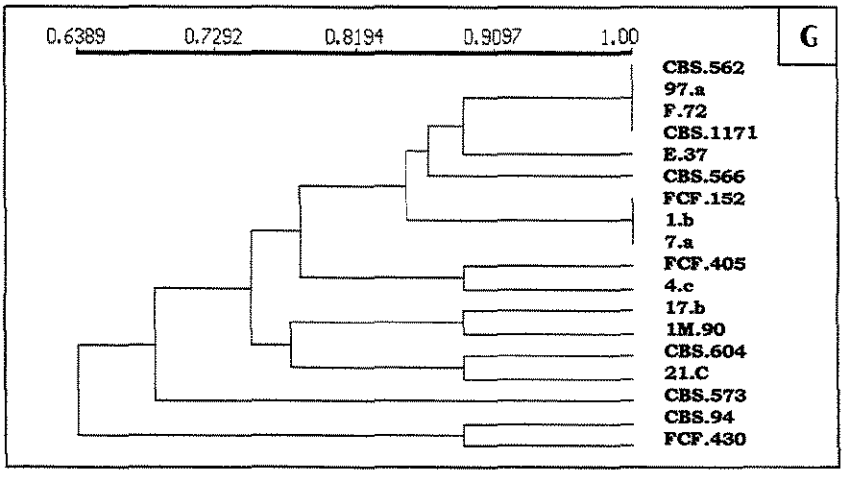

Fig. 3: Dendrogram G (ME) showing the relatedness levels among different *Candida* species, based on their respective dehydrogenases MLEE patterns and statistical treatment with Simple Matching association coefficient (S_{SM}) and UPGMA clustering method.

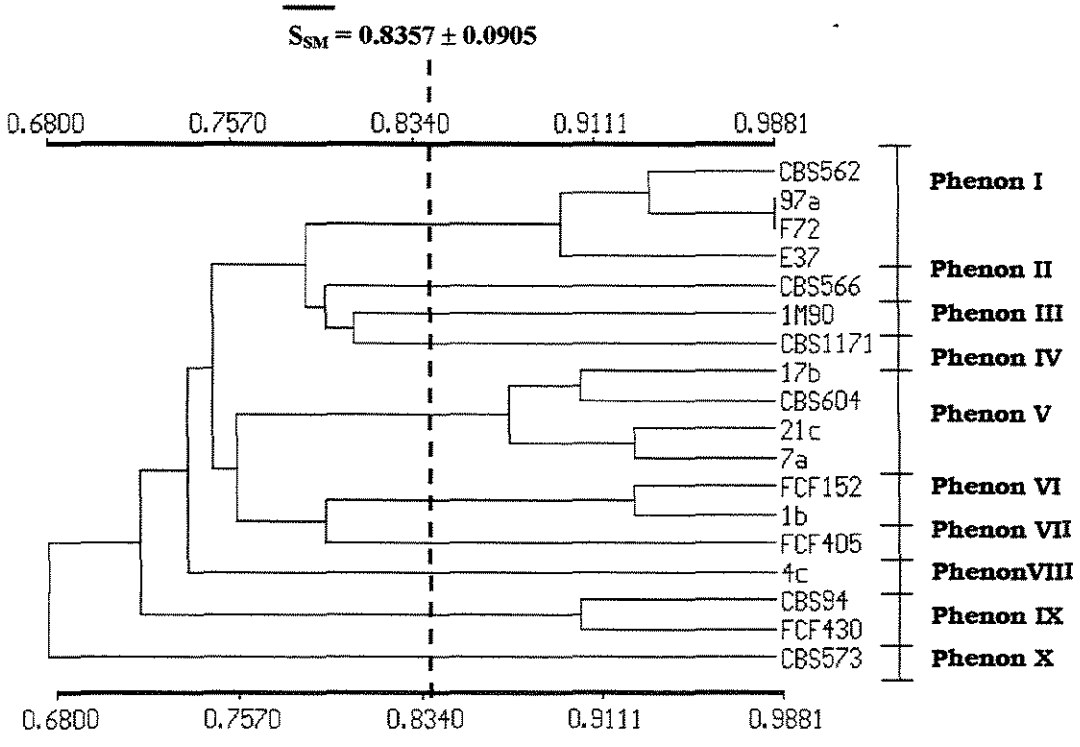

Fig. 4. Nonrooted relatedness dendrogram of *Candida* species grouped by the sum of all dehydrogenase systems and UPGMA algorithm.

DISCUSSION

Genus *Candida* includes a heterogeneous group of yeasts that has species whose teleomorph states are classified in different phyla as Basidiomycota, Ascomycota, or those that do not have perfect state defined yet (20). Proceedings involving the evaluation of MLEE patterns in *Candida* have supplied some useful information in epidemiological surveys, genetic and physiological studies, and have shown the applicability of such technique for the most varied purposes when involving these yeasts. Among the wide range of enzyme classes, the dehydrogenases, a sub-group of oxi-reductases, show the highest specificity for their substrates (4), and probably the lowest capacity for generating unspecific bands, that could act as either electrophoretic or stain artifacts.

In figures 1, 2, and 3 it can be observed that the enzymatic system which could group the major part of strains in their respective species clusters was the IDH. Such fact has already been pointed out by Lehmann et al. (11) who also added that IDH and SDH solely distinguish species and do not have any value in biotyping *Candida* isolates. In the present investigation, we could not obtain such band patterns for different repetitions of SDH band detection protocols (data not shown). On the other hand, the system that furnished the worst grouping was GDH, due to the lack of formation of bands in many strains.

As we have used different species of *Candida*, some of them anamorph of certain Ascomycota presenting diverse allelic organization with few loci sharing among the OTUs, we could not confidently assign genotypes to all loci patterns, and the method employed to calculate similarities between OTUs, generated by MLEE, was the treatment of such bands as phenotypic characters as proposed by Meloni et al. (16). Simple Matching coefficient was applied in the construction of all dendrograms according to the proposition of Sneath & Sokal (26).

The nonrooted dendrogram presented in Fig. 4 shows the sum of all partial dendrograms in figures 1, 2 and 3, where the compensation of each other was expected. Clusters were formed from limiting line derived from the average of all OTU's similarity with 0.8357 and standard deviation of 0.0905. Some strains formed taxons of their own species (pure clusters) as phenons I and IX, of different species (impure clusters) as phenons V and VI, and taxons composed from one strain solely.

According to this figure, dehydrogenases could group most of *C. albicans* strains in a single phenon, with the exception of 17.b strain, that showed to be the least related. This fact was also observed in previous assays involving SDS-PAGE of whole cell proteins for these strains (9). These enzymes also grouped all strains of *C. parapsilosis* with strain 17.b of *C. albicans*. Such aspect of impure cluster generated from MLEE was already observed by other authors as following. Stout & Shaw (30) obtained the same behavior working with certain species of *Mucor*, which were grouped based on MLEE polymorphism. Smith et al. (25), characterizing groups of different genera and species of English "stock beer" yeasts *Brettanomyces* and *Dekkera*, obtained a dendrogram in which some specimens 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 (10) developed a classification system for dermatophytes based on MLEE comparisons and obtained a dendrogram in which occurred the inclusion of isolates from certain species inner taxons of other species or even genera. These last authors supported that this fact occurs when only a few isolates of each species are studied.

Other groups of researchers have observed that extensive enzyme heterogeneity among strains from *Candida* or other yeast species may occur, increasing the possibility of dividing such specimens in various groups or clusters (3, 11, 12, 14, 17). Lehmann et al. (13) related the phenomenon of change in isoenzymatic patterns of *C. albicans* during its conservation in laboratories, what could increase the apparent polymorphism. Pujol et al. (20) found atypical strains of *C. albicans* in AIDS patients, showing diverse allelic polymorphism. The same investigators included few strains of *C. tropicalis, C. glabrata* and *C. krusei* in the survey obtaining characteristic species-specific clusters. However, the fact that these species were added with few specimens could have influenced the cluster's organization.

In order to ensure whether or not UPGMA algorithm fits well the assemblance between two OTUs in the dendrogram construction, a product-moment correlation coefficient was computed between the elements S_{JK} of the original similarity matrix S and cophenetic values C_{JK} of the matrix C derived from the dendrogram. This cophenetic correlation coefficient is a measure of the agreement between similarity values implied by the dendrogram and those of the original similarity matrix (28). This coefficient has a value of $r_{CS} = 0.89$, that is comprehended between 0.60 and 0.95 (23) or 0.74 and 0.90 (29), values considered as good, corroborating by

this way, with the findings of Farris (6), who pointed out the fact that UPGMA algorithm will always maximize r_{CS} values.

In this work other similarity coefficients were tested (data not shown) in order to observe if this event was repetitive, as Jaccard (S_J), Dice (S_D) and the correlation coefficient based on product-moment of Pearson (r), and this interference behavior was conserved on them. Also, all the strains employed were previously and independently classified up to species level in two different laboratories by means of chlamydospore and germ tube formation, fermentation and assimilation of sugars, obtaining the same results. This evidence discards the possibility of bad identification of the strains.

Based on the present observations, we could propose that the grouping of *Candida* species based on MLEE for dehydrogenases is not efficient when involving few isolates from more than one species, regarding such technique for surveys conducted with a single species of *Candida*, for what, the MLEE resource has already proven to be a powerful systematical tool.

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Analysis of Parity Between Protein-Based Electrophoretic Methods for the Characterization of Oral *Candida* Species

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Electrophoretic studies of multilocus-enzymes (MLEE) and whole-cell protein (SDS-PAGE) were carried out in order to evaluate the parity between different methods for the characterization of five *Candida* species commonly isolated from oral cavity of humans by numerical taxonomy methods. The obtained data revealed that sodium dodecyl sulfate polyacrylamide gel electrophoresis is more efficient in grouping strains in their respective species while MLEE has much limited resolution in organizing all strains in their respective species-specific clusters. MLEE technique must be regarded for surveys in which just one species of *Candida* is involved.

Key words: polyacrylamide gel electrophoresis - multilocus enzyme electrophoresis - *Candida* - numerical analysis.

The yeasts pertaining to the genus *Candida* are found dispersed in different epitelial areas of the body, including oral mucosa. In recent years, they have been given more attention due to their involvement in a increasing number of cases of opportunist oral infections in patients with Aids and those having immunosuppresive medication. Of epidemiological interest, characterization procedures based on molecular fingerprints have been applied in order to

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establish possible relationships among *Candida* isolates involved in oral infections (McCullough et al. 1996).

Different types of electrophoretic techniques have been used for the characterization or typing of *Candida* including electrophoretic separation of chromosomes (Asakura et al. 1991, Monod et al. 1990), DNA fragments (Scherer & Stevens 1987), multilocus-enzymes (Lehmann et al. 1989a, Pujol et al. 1993, Reynes et al. 1996), and whole-cell proteins (Shen et al. 1988, Vancanneyt et al. 1991, 1992, Höfling et al. 1998). The two latter methods have been used successfully for yeast characterization. The resulting electrophoretic profiles can be plotted into a binary data matrix that, with computer-assisted support, produces comparative results expressed as similarity or cophenetic correlation matrices or dendrograms (Kersters 1985).

In this experiment, we compare multilocus-enzyme electrophoresis (MLEE) and polyacrylamide gel electrophoresis (SDS-PAGE) for their ability to discriminate five *Candida* species isolated from saliva of healthy subjects.

MATERIALS AND METHODS

Candida strains - Representative strains of different *Candida* species isolated from human oral cavity and identified by biochemical and physiological tests were obtained from the Microbiology and Immunology Laboratory, Dentistry College of São José dos Campos: *C. albicans* (97.a, F.72, E.37, 17.b, CBS.562^T), *C. guilliermondii* (FCF.405, FCF.152, CBS.566^T), *C. parapsilosis* (21.c, 7.a,CBS.604^T), *C. krusei* (1M.90, 4.c, CBS.573^T), *C. tropicalis* (1.b, FCF.430, CBS.94^T). The superscript T in CBS strains indicates that they are the respective type-strains for each species. *Saccharomyces cerevisiae* type-strain (CBS.1171^T) was included as an extra-generic organism (Costas et al. 1989).

Cell cultivation and whole-cell protein extraction - All strains were grown in 50 ml of Yeast Peptone Dextrose medium (2% dextrose, 2% peptone, 1% yeast extract) in a shaker table under 150 rpm, at 30°C, overnight. The cells were harvested by centrifugation at 2,000 g for 3 min and the pellets were washed four times with cold sterile water in order to remove either culture medium traces or extra-cellular metabolites (Woontner & Jaehning 1990). The last washed pellets were transferred to 2ml microcentrifuge tubes and acid-washed glass beads (v/v) plus 200 µl of

cold sterile water were added. Cells were lysed using a Mini-Bead Beater cell disrupter (Biospec) at 4600 r.p.m., repeating four times of 30 sec at 5-min intervals, and placed in an ice bath. After cell disruption, the microcentrifuge tubes were centrifuged at 10,000 g for 2 min, and the supernatant's protein concentration were determined according to Bradford (1976) and adjusted to 80 μg/ml (Ames 1974). The MLEE supernatants were applied on Whatman 3 filter paper wicks of 5x12 mm (Selander et al. 1986), and for SDS-PAGE technique equal volumes of supernatant and loading buffer of Bruneau and Guinet (1989) (5mM Tris, 2.5% 2-mercaptoethanol, 1.5% SDS, 0.025% bromophenol blue) were combined and heated in a boiling water bath for 10 min.

MLEE and specific-enzyme staining - The electrophoreses were carried out using hydrolyzed corn starch Penetrose 30 (Refinações de Milho Brasil) up to a final concentration of 13% (Val et al. 1981) in 1:30 pH 8.0 Tris-citrate buffer (Selander et al. 1986, Caugant and Sandven 1993). Electrophoreses were carried out at 4°C and 130 V until the bromphenol blue migration markers had run at least 80 mm from application point. At this time, the electrophoresis was interrupted and the gels were sliced with 1.2 mm thickness. The gel slices were revealed for enzyme active band detection, according to Selander et al. (1986) protocols. Enzymatic systems assayed were: alcohol dehydrogenase (ADH - E.C. 1.1.1.1), lactate dehydrogenase (LDH - E.C. 1.1.1.27), malate dehydrogenase (MDH - E.C. 1.1.1.37), isocitrate dehydrogenase (IDH - E.C. 1.1.1.42), glucose-6-phosphate dehydrogenase (G6PDH - E.C. 1.1.1.49), aspartate dehydrogenase (ASDH - E.C. 1.4.3.x), glucose dehydrogenase (GDH - E.C. 1.1.1.47), mannitol dehydrogenase (MADH - E.C. 1.1.1.67), sorbitol dehydrogenase (SDH - E.C. 1.1.1.14), malic enzyme (ME - E.C. 1.1.1.40), aconitase (ACO - E.C. 4.2.1.3), catalase (CAT - E.C. 1.11.1.6), superoxide dismutase (SOD - E.C. 1.15.1.1), glutamate-oxalacetate transaminase (GOT - E.C. 2.6.1.1), α-esterase (EST - E.C. 3.1.1.1), β-esterase (EST - E.C. 3.1.1.1), leucine aminopeptidase (LAP - E.C. 3.4.1.1), glucosil transferase (GTF - E.C. 2.4.1.11), peroxidase (PO - E.C. 1.11.1.7) e α-amylase (α-AM - E.C. 3.2.1.1).

SDS-PAGE protein analysis - SDS-PAGE protein profiles were obtained after electrophoresis of 50μl of protein solution in polyacrylamide slab gel with sodium dodecylsulfate (SDS) in a discontinuous buffer system (Laemmli 1970) with 4.5% stacking gel and 12.5% running gel. The electrophoresis was conduced at 125 volts in a cold chamber and the gels were stained with

Coomassie blue G-250 0.25%. After destaining, the gels were scanned and the profiles of each lane transferred to a densitometry interface in the SigmaGel software (Jandel software) where the exact position of the protein peaks were determined.

Computing numerical data - Dendrograms for the different MLEE systems and SDS-PAGE were generated by using the simple matching (S_{SM}) association coefficient (Sokal and Michener 1958, Sneath & Sokal 1973, Naumov et al 1997), based on band positions calculated by the NTSYS software package, version 1.70 (Applied Biostatistics, Inc.). For the present study, a S_{SM} of 1.00 represents identical matches (i.e., all the bands match), a S_{SM} of 0.00 represents no matches, and increasing intermediate values represent increasing proportions of matched bands. Dendrograms, represented by non-rooted trees, based on S_{SM} values were generated by the unweighted pair-group arithmetic average (UPGMA) clustering method (Rohlf 1963, Sneath and Sokal 1973, Naumov et al. 1997).

RESULTS

The application of UPGMA clustering produced two similarity dendrograms shown in Figs 1 and 2, in which several clusters (phenons) could be distinguished. These clusters may be defined by their average similarity values (S_{SM}).

Phenons generated by SDS-PAGE

Phenon I: there is the *S. cerevisiae* type-strain CBS.1171^T

Phenon II: there are three strains of *C. krusei*, with $S_{SM} \geq 0.872$.

Phenon III: there are three strains of *C. tropicalis*, with $S_{SM} \geq 0.897$

Phenon IV: there are three strains of *C. guilliermondii*, with $S_{SM} \geq 0.823$

Phenon V: there are three strains of *C. parapsilosis*, with $S_{SM} \geq 0.833$

Phenon VI: there are five strains of *C. albicans*, with $S_{SM} \geq 0.833$

Interspecific comparison by SDS-PAGE - Among all the species, *C. albicans* (phenon VI) was the most frequently isolated species and its cluster could be grouped to others with $S_{SM} = 0.513$.

C. krusei (phenon II) showed some similarity with *S. cerevisiae* CBS 1171 with $S_{SM} = 0.692$, and both could be isolated from others with $S_{SM} = 0.597$.

C. guilliermondii (phenon IV) and *C. parapsilosis* (cluster V) showed a value of $S_{SM} = 0.7749$, and these two clusters could be grouped with *C. tropicalis* (phenon III) with $S_{SM} = 0.655$.

Reproducibility of SDS-PAGE patterns - The protein profiles of analyzed strains on different gels were reproducible after three repetitions of each electrophoretic running. Protein extracts of *S. cerevisiae* (CBS 1171) and molecular mass markers were applied in all gels providing mean values $S_{SM} = 0.853$ and 1.000, respectively.

Enzymatic systems - The one-dimensional electrophoreses of protein extracts from 12 *Candida* strains, their respective type-strains, and *S. cerevisiae* type-strain, showed that among twenty assayed enzymes, five did not show any enzymatic activity (ASDH, MADH, SDH, GTF, and α-AM).

Phenons generated by MLEE

Phenon I: there are four strains of *C. albicans* (CBS.152^T, 97.a, F.72, and E.37) with $S_{SM} \geq 0.898$

Phenon II: there are two strains of *C. guilliermondii* (FCF.152 and FCF.405) and one *C. tropicalis* (1.b), with $S_{SM} \geq 0.847$

Phenon III: there is the *S. cerevisiae* type-strain CBS.1171^T

Phenon IV: there are three strains of *C. parapsilosis* (CBS.604^T, 21.c, and 7.a) and one *C. albicans* strain (17.b), with $S_{SM} \geq 0.845$

Phenon V: there is a *C. krusei* strain (4.c)

Phenon VI: there is the *C. guilliermondii* type-strain CBS.566^T

Phenon VII: there are two strains of *C. tropicalis* (CBS.94^T, and FCF.430), with $S_{SM} = 0.917$

Phenon VIII: there is the strain 1M.90 of *C. krusei*

Phenon IX: there is the *C. krusei* type-strain (CBS.573^T)

Interspecific comparison by MLEE - Excluding phenon I, composed only by *C. albicans*, and those in which only one strain were detected (phenons III, V, VI, VIII, and IX), all other clusters had an impure composition with more than one species component.

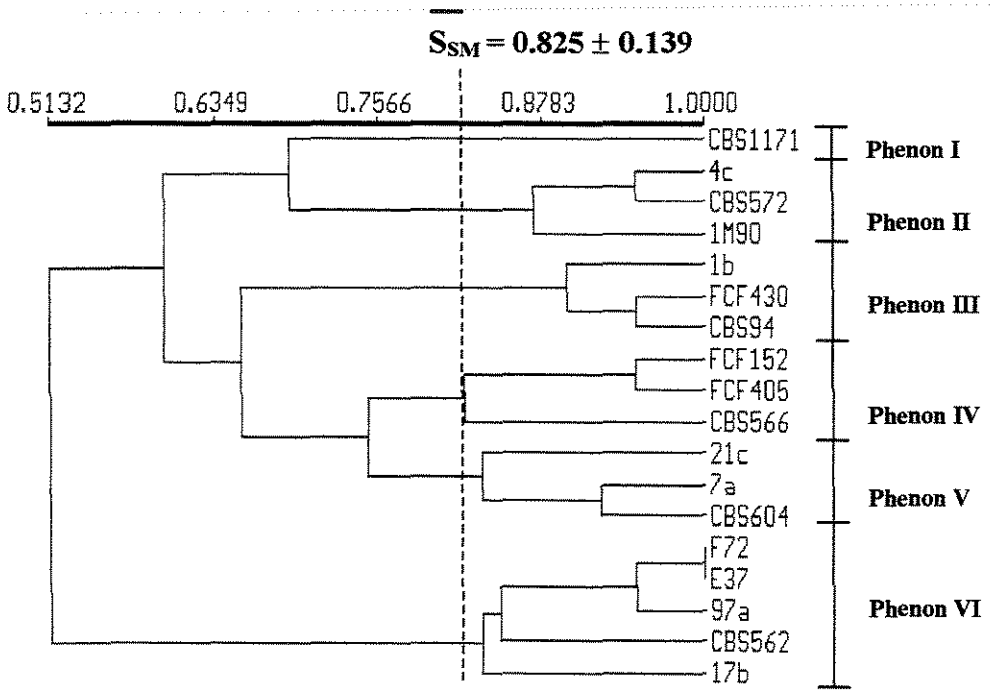

Fig.1: non-rooted dendrogram of similarity among *Candida* strains grouped by simple matching associative coefficient and UPGMA algorithm from sodium dodecyl sulfate polyacrylamide gel electrophoresis profiles.

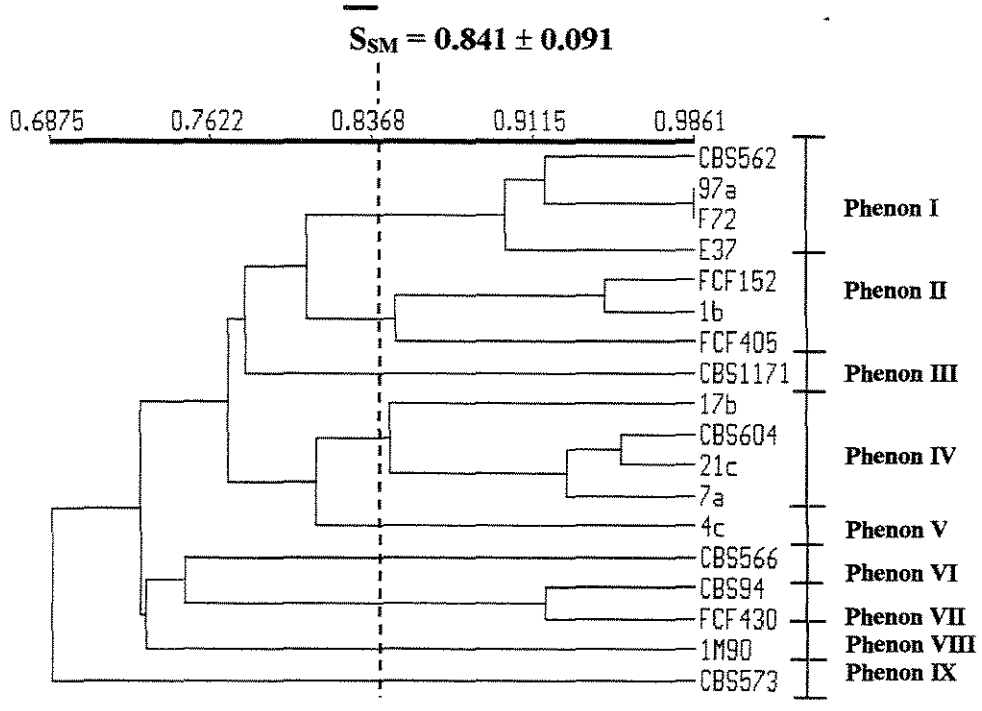

Fig. 2: non-rooted dendrogram of similarity among *Candida* strains grouped by simple matching associative coefficient and UPGMA algorithm from multilocus enzyme electrophoresis profiles.

DISCUSSION

The analysis of electrophoretic profiles of proteins and multilocus-enzymes has allowed the identification, classification of numerous strains, species and genera of yeasts (Baptist and Kurtzman 1976, Okunishi et al. 1979, Yamazaki and Komagata 1981, Maiden and Tanner 1991, Vancanneyt et al. 1991, 1992).

The reproducibility of electrophoretic profiles on different slab SDS-PAGE gels was evaluated by the inclusion of molecular mass markers, besides protein extract of a organism from a non-correlated genus (Costas et al. 1989, Bruneau and Guinet 1989) and gave similarity correlation values $S_{SM} = 0.853$ for three repetitions of *S. cerevisiae* and $S_{SM} = 1.000$ for three repetitions of molecular mass markers. These values are in agreement with the minimum acceptable proposed by Sneath and Johnson (1972) that was 0.800. The data obtained from grouping of *Candida* strains based on their electrophoretic profiles showed high level of agreement with the inter-specific classification established by conventional methods. Moreover, the isolates of each species showed identical or very similar profiles when compared. This fact suggests that these protein profiles obtained by SDS-PAGE are relatively stable taxonomic characteristics.

As shown in Fig. 1, the use of type-strains allowed the identification of clusters at the species level, since the *Candida* isolates were grouped with their respective type-strains. With regard to cluster compositions, the SDS-PAGE technique allowed the organization of all isolates in distinct clusters, with similarity coefficients $S_{SM} \geq 0.833$ for *C. albicans*, $S_{SM} \geq 0.833$ for *C. parapsilosis*, $S_{SM} \geq 0.823$ for *C. guilliermondii*, $S_{SM} \geq 0.897$ for *C. tropicalis*, and $S_{SM} \geq 0.872$ for *C. krusei*.

Shechter et al. (1972), using non-denatured acid and basic protein electrophoresis and association coefficient of Jaccard (S_J), that excludes negative matches, obtained a phenogram in which the species *C. albicans*, *C. krusei* and *C. parapsilosis* combined among them with 40% of similarity. The species *C. guilliermondii* clustered to this group with 32% and *C. parapsilosis* was the last one to group, with approximately 25% of similarity. This behavior, different from that found in our research, is due to the fact that non-denatured proteins migrate through the gel according to their molecular mass, structural conformation and net charge. In contrast, SDS denatured proteins migrate according to molecular mass only. As molecular mass is more

conserved than net charge, electrophoretic profiles based on this criterion should, in theory, detect better taxonomic relationships (Kersters 1985).

The systematic proximity between *C. krusei* and *S. cerevisiae* (S_{SM} = 0.692) assessed by SDS-PAGE technique was also observed by Barns et al. (1991) in their analyses based on phylogenetic analysis of 18S ribosomal sub-units RNA genes. Hendricks et al. (1989) support that *Candida* and *Saccharomyces* should have a close phylogenetic relationship, detectable by 18S rRNA sequence analysis.

According to Fig. 2, the MLEE technique grouped most *C. albicans* strains into a single phenon, except for 17.b strain that was shown to be the less related. These enzymes were able to group all strains of *C. parapsilosis* with strain 17.b of *C. albicans*. Such aspect of multispecific cluster generated from MLEE was already observed by Smith et al. (1990), that 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 and Noble (1982) established electrophoretic comparisons among species of dermatophytes based on MLEE technique showing the inclusion of isolates from certain species inner taxa of other species or even of other genera. These authors pointed out that this fact may occur when only a few isolates of each species are included in the surveys. Boerlin et al. (1995) used 16 enzymatic systems for characterizing 21 genetically atypical strains of chlamydospore-forming and germ tube-positive *C. albicans* recovered from human immunodeficiency virus-positive drug users, and demonstrated that some of these strains were grouped in different clusters, showing high diversity on allelic composition.

Extensive enzyme heterogeneity among *Candida* or other yeast genera had already been observed by other groups of researchers that pointed out that it may occur increasing the possibility of distributing such specimens in various groups or clusters (Lehmann et al. 1989a, 1989b, Caugant & Sandven 1993, Naumov et al. 1997). Lehmann et al. (1991) related the phenomenon of isoenzymatic patterns changing of *C. albicans* during its conservation in laboratories, what could increase the apparent polymorphism. Pujol et al. (1997) found atypical strains of *C. albicans* in Aids patients, showing diverse allelic polymorphism.

When comparing the results assessed by SDS-PAGE and MLEE, it can easily be seen that the first one is more useful for grouping isolates in their respective species, maybe due to the

expression of species-specific bands while the second one perhaps better explores the variability at a sub-specific level, being useful for analyses of genetic polymorphism among strains of a certain *Candida* species.

In order to ensure whether or not the UPGMA algorithm assesses resemblance between two OTUs in the dendrogram constructions, a product-moment correlation coefficient was computed between the elements S_{JK} of the original similarity matrix S and cophenetic values C_{JK} of the matrix C derived from the dendrogram. The cophenetic correlation coefficient is a measure of the agreement between similarity values implied by the dendrogram and those of the original similarity matrix (Sokal and Rohlf 1962). These coefficient had values $r_{CS} = 0.928$ for SDS-PAGE and $r_{CS} = 0.932$ for MLEE, that range between 0.60 and 0.95 (Sneath and Sokal 1973) or higher than 0.90 (Sokal and Rohlf 1970), considered acceptable, corroborating by this way, with the finds of Farris (1969), that pointed out the fact that UPGMA algorithm always maximizes r_{CS} values.

The protein profile analysis by SDS-PAGE improves the knowledge about the taxonomic relationships among oral yeasts. This method shows good reproducibility and allows collection of useful information for numerical analysis. This methodology brings relevant information in systematic evaluation of related species. We propose that the grouping of *Candida* species by MLEE patterns from the assayed enzymes is not efficient when only based on a few isolates from more than one species, regarding such resource for surveys conduced with a single species of *Candida*, for what, the MLEE technique had already proved to be a useful method for systematic or epidemiological purposes.

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INTER AND INFRA-SPECIFIC GENETIC VARIABILITY OF ORAL *Candida* SPECIES

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Running title: Genetic variability of oral *Candida*

ABSTRACT

In this research, strains of five different *Candida* species (*C. albicans, C. guilliermondii, C. tropicalis, C. krusei*, and *C. parapsilosis*) isolated from healthy human oral cavities as well as their respective type-strains were used in order to establish the genetic diversity existing among the different species and within a certain species, by the analysis of their electrophoretic alloenzyme patterns. These profiles were analyzed for their band positions in the gels, what allowed to group the strains of a same species in species-specific clusters and to treat them as conspecific populations. A total of thirteen enzymatic loci were obtained (ACO, ADH1, ADH2, CAT, G6PDH, GDH, GOT, IDH1, IDH2, LAP, LDH, PER, and SOD). The allelic frequencies (p) and the heterozygosity (*h*) for all the thirteen loci were determined and diversity index formulas. The G_{ST} index is the estimated proportion of genetic diversity that was applied in order to establish inter and infra populational diversity, what, for our results, indicated that 37.75% of total genetic diversity was attributable to differences among the species and the remaining 62.25% was attributable to differences within these populations. An Euclidian distance dendrogram for the different conspecific populations was built, showing that *C. guilliermondii* grouped first with *C. tropicalis* that formed a expanded cluster with *C. albicans*. This cluster combined later with another one composed by *C. parapsilosis* and *C. krusei*. Comparing our results to the others that were obtained by different molecular techniques, we have observed that the clustering hierarchies follow different paths of organization, varying according to the methodology employed.

KEY WORDS: *Candida* spp, genetic variability, MLEE

RESUMEN

En esta investigación, lineages de cinco especies de *Candida* (*C. albicans, C. guilliermondii, C. tropicalis, C. krusei*, y *C. parapsilosis*) aisladas de las cavidades orales humanas de personas saludables así como sus respectivos lineages-tipo fueron usados para establecer la diversidad genética que existe entre las diferentes especies y dentro de una miesma especie, por el análisis de sus perfiles de aloenzimas. Estos perfiles se analizaron según suas posiciones en los geles, lo que permitió agruparse las lineages de una misma especie en gupos especie-específicos y tratarlos como poblaciones conspecificas. Un total de trece loci enzimáticos

fue obtenido (ACO, ADH1, ADH2, CAT, G6PDH, GDH, GOT, IDH1, IDH2, LAP, LDH, PER, y SOD). Las frecuencias allelicas (p) y la heterocigosidad (h) para todos los trece loci fueram determinados per fórmulas de índice de diversidad. El índice de G_{ST} es la proporción estimada de diversidad genética que fue aplicada para establecer los grados de diversidad inter y infrapoblacional, que para nuestros resultados, indicó que 37.75% de diversidad genética total eran atribuibles a las diferencias entre las especies y 62.25% era atribuible a las diferencias dentro de estas poblaciones. Un dendrograma de distancias Euclidianas para las poblaciones conspecificas fue construido y muestra que *C. guilliermondii* se agrupó primero con C. *tropicalis* que formó un grupo extendido *con C. albicans*. Este grupo combinó después con otro compuesto por *C. parapsilosis* y *C. krusei*. Comparando nuestros resultados a los otros que fueron obtenidos por técnicas moleculares diferentes, nosotros hemos observado que las jerarquías de agrupamento siguen caminos diferentes de organización y varían según la metodología empleada.
PALABLAS-CLAVE: *Candida albicans,* diversidad genética, MLEE

INTRODUCTION

The fungi of *Candida* genus are the most commonly eukaryotic microorganism found on human oral cavity, standing or not involved with oral diseases [18]. Several workers have compared the different species of *Candida* in order to determine their evolutionary relationships [1] and systematic implications [11]. These articles and others [20, 23, 6, 3, 12, 2, 25,26] also pointed out the applicability of molecular techniques based on protein or nucleic acids for the identification of yeast species and establishment of similarities or correlation among clinical or environmental isolates of a certain species of *Candida* what can be employed in epidemiological or ecological surveys. Among the molecular techniques based on protein polymorphism, the multilocus enzyme electrophoresis (MLEE) is a resource that can be applied in studies involving either characterization of isolates or assignment of yeast genotypes.

The MLEE allowed LEHMANN *et al.* [13] to characterize *C. albicans*, *C. stellatoidea*, *C. tropicalis* and *C. paratropicalis*, isolated from several infectious focuses. LEHMANN *et al.* [14] made the numerical analysis of the same isolates grouping them in two larger clusters: A) *C. albicans–C. stellatoidea* I and II, and B) *C. tropicalis–C. paratropicalis.* CAUGANT & SANDVEN [4], working with 98 isolates of *C. albicans* could observe the enzymatic

polymorphism on that yeast population. Other researchers also published papers concerning to the classification of *Candida* species and of yeasts from another genera through the analysis of multilocus-enzyme electrophoresis [15, 16, 27, 6, 12, 2, 28, 25, 26].

In this research, collections of isolates belonging to the same species were jointed together and treated as conspecific populations and the diversity and genetic distance among them were established.

MATERIAL AND METHODS

***Candida* strains:** Representative strains of different *Candida* species isolated from human oral cavity and identified by biochemical and physiological tests, were obtained from Microbiology and Immunology Laboratory, Dentistry College of São José dos Campos: *C. albicans* (97.a, F.72, E.37, 17.b, CBS.562^T), *C. guilliermondii* (FCF.405, FCF.152, CBS.566^T), *C. parapsilosis* (21.c, 7.a,CBS.604^T), *C. krusei* (1M.90, 4.c, CBS.573^T), *C. tropicalis* (1.b, FCF.430, CBS.94^T). The over-scripted capital letters T in CBS strains implicate that them are the respective type-strains of such species.

Cells cultivation and enzyme extraction: All the strains were grown in 50mL of YPD medium (2% dextrose, 2% peptone, 1% yeast extract) in a shaker table under 150rpm, at 30°C, overnight. The cells were harvested by centrifugation at 2000x*g* for 3 minutes and the pellets were washed 4 times with cold sterile water in order to avoid neither culture medium traces nor extra-cellular metabolites [42]. The last washed pellets were transferred to microcentrifuge tubes of 2mL, and added of acid-washed glass beads (v/v) plus 200µL of cold sterile water. The tubes were adapted in a Mini-Bead Beater cell disrupter (BIOSPEC, Inc.), where the cell lysis were processed at 4600rpm, 4 times of 30 seconds, with intervals of 5 minutes, when the samples were conditioned in an ice bath. After cell disruption, the microcentrifuge tubes were centrifuged at 10000x*g* for 2 minutes, and the supernatants were applied on Whatman 3 filter paper wicks of 5x12mm and kept at –70°C [31].

Starch gel electrophoresis: The electrophoreses were carried out using hydrolyzed corn starch Penetrose 30 (Refinações de Milho Brasil) up to final concentration of 13% [39] in 1:30 pH8.0 Tris-citrate buffer, with vigorous agitation on a Bunsen burner. The formed gels were poured in perplex casting moulds (200x120x10mm), and let over bench at room temperature until

complete solidification, when they were cut on their longitudinal dimensions at 2.5cm from one border. The smaller parts were separated and the wicks were applied on the cut. Wicks with 0.2% bromophenol blue were applied in both extremities of the cuts for migrating indication. After jointed the parts, cotton cloth bridges were done connecting the gels to electrode tanks with pH8.0 Tris-citrate buffer [31, 4]. Electrophoreses were carried out at 4°C and 130V until the migration markers run through at least 80mm from application point. At this time, the electrophoreses were interrupted and the gels were sliced with 1.2mm thickness.

Bands revelation: The gel slices were revealed for enzyme active bands detection, according to SELANDER *et al.* [31] protocols. Enzymatic systems assayed were alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PDH), glucose dehydrogenase (GDH), aconitase (ACO), catalase (CAT), superoxide dismutase (SOD), glutamate-oxalacetate transaminase (GOT), leucine aminopeptidase (LAP), peroxidase (PER). Values of relative mobility (Rm) for each band were determined dividing the migrating band values by the bromophenol blue dye distal line.

Diversity and genetic distance determination: Isolates belonging to the same species were jointed together and treated as conspecific populations. Allelic frequencies for all loci were determined and applied in formulas of diversity index [23] where H_T is the total diversity of a certain species for one locus, H_S is the diversity component within the populations, D_{ST} is the diversity component between two populations, and G_{ST} is the estimated proportion of genetic diversity attributed to the diversity component between two populations. The genetic distances among different *Candida* species were assessed by ROGERS' distance [29] with allelic frequencies, by the formula:

$$D = \frac{1}{L}\sum_{j=1}^{L}\sqrt{\frac{1}{2}\sum_{i=1}^{Aj}(P_{ijx} - P_{ijy})^2},$$

where $\mathbf{P_{ix}}$ and $\mathbf{P_{iy}}$ are the frequencies of allele **i** at the locus **j** in the populations **X** and **Y** respectively, **L** is the number of examined loci, and **A** is the number of alleles at the locus **j**. A distance matrix and a distance dendrogram were built following the protocols of DIAS [5].

RESULTS

A total of thirteen enzyme loci could be assessed after gel revelations and they were organized in the Table 01. The loci denominated as null were those where none electrophoretic band could be assigned.

Table 01: Genotypes of *Candida* species assessed by alloenzyme electrophoresis

	enzyme loci												
Strains	ACO	ADH1	ADH2	CAT	G6PDH	GDH	GOT	IDH1	IDH2	LAP	LDH	PER	SOD
CBS.562	aa	bc	cc	aa	bc	aa	cc	aa	null	aa	ac	ab	ab
97.a	bb	bb	cc	aa	cc	null	bc	aa	null	aa	ac	ab	ab
F.72	bb	bb	null	aa	cc	null	bc	aa	null	aa	ac	aa	ab
17.b	cc	null	ac	ab	cc	cc	bb	null	aa	cc	bc	cc	ab
E.37	bb	bb	null	aa	cc	null	bc	aa	null	bb	ac	ab	bb
CBS.566	bb	cc	null	bb	cc	bb	bb	bb	cc	cc	ab	aa	cc
FCF.152	bb	cc	null	aa	cc	null	cc	bb	bb	bb	ac	ab	bb
FCF.405	cc	cc	ac	ab	cc	cc	bb	bb	bb	bb	bc	aa	bb
CBS.573	cc	ac	null	bb	bb	ac	aa	aa	cc	cc	bb	aa	bb
1M.90	cc	bc	null	cc	aa	bb	bb	aa	cc	bb	bc	cc	null
4.c	cc	null	bc	bb	cc	cc	bb	null	bb	cc	bc	cc	ab
CBS.94	bb	cc	bb	cc	cc	null	cc	bc	null	bb	bb	ab	aa
1.b	bb	cc	null	aa	bc	null	bc	bb	null	bb	ac	ab	bb
FCF.430	bb	cc	ac	cc	cc	null	bc	bc	null	bb	bb	aa	ab
CBS.604	cc	null	ab	bb	cc	cc	bb	null	aa	cc	bc	bb	ab
21.c	cc	null	ab	bb	cc	cc	bb	null	aa	cc	bc	bb	ab
7.a	cc	null	bb	bb	cc	null	bb	null	aa	cc	bc	null	ab

The genotypes allowed the determination of allelic frequencies and heterozygosis for each loci in all species (Table 02). From these data, H_T, H_S, D_{ST}, G_{ST} values could be calculated (Table 03). The mean G_{ST} value for all species was 0.3775, what implies in 37.75% of total genetic variability attributable to differences among the five species, and 62.25% of total genetic variability attributable to differences within such populations.

The genetic distances (D) among the *Candida* species were assessed by ROGERS' distance method, what generated the dendrogram showed in Figure 01. In such dendrogram it can be observed that *C. guilliermondii* grouped first to *C. tropicalis* and these two species formed a expanded cluster with *C. albicans.* Other cluster was formed by the grouping of *C. parapsilosis* and *C. krusei.*

Table 02: Allelic frequency and heterozygosis of some *Candida* isoenzymes

	C.albicans				*C. guilliermondii*				*C. krusei*				*C. tropicalis*				*C. parapsilosis*			
loci	p(a)	p(b)	p(c)	H	p(a)	p(b)	p(c)	H	p(a)	p(b)	p(c)	H	p(a)	p(b)	p(c)	H	p(a)	p(b)	p(c)	H
ACO	0.20	0.60	0.20	0.56	0.00	0.66	0.33	0.45	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00
ADH1	0.00	0.87	0.12	0.23	0.00	0.00	1.00	0.00	0.25	0.25	0.50	0.62	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00
ADH2	0.16	0.00	0.83	0.28	0.50	0.00	0.50	0.50	0.00	0.50	0.50	0.50	0.25	0.50	0.25	0.62	0.33	0.66	0.00	0.45
CAT	0.90	0.10	0.00	0.18	0.50	0.50	0.00	0.50	0.00	0.66	0.33	0.45	0.33	0.00	0.66	0.45	0.00	1.00	0.00	0.00
G6PDH	0.00	0.10	0.90	0.18	0.00	0.00	1.00	0.00	0.33	0.33	0.33	0.67	0.00	0.16	0.83	0.28	0.00	0.00	1.00	0.00
GDH	0.50	0.00	0.50	0.50	0.00	0.50	0.50	0.50	0.16	0.33	0.50	0.61	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00
GOT	0.00	0.50	0.50	0.50	0.00	0.66	0.33	0.45	0.33	0.66	0.00	0.45	0.00	0.33	0.66	0.45	0.00	1.00	0.00	0.00
IDH1	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.66	0.33	0.45	0.00	0.00	0.00	1.00
IDH2	1.00	0.00	0.00	0.00	0.00	0.66	0.33	0.45	0.00	0.33	0.66	0.45	0.00	0.00	0.00	1.00	1.00	0.00	0.00	0.00
LAP	0.60	0.20	0.20	0.56	0.00	0.66	0.33	0.45	0.00	0.33	0.66	0.45	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00
LDH	0.40	0.10	0.50	0.58	0.33	0.33	0.33	0.67	0.00	0.66	0.33	0.45	0.16	0.66	0.16	0.51	0.00	0.50	0.50	0.50
PER	0.50	0.30	0.20	0.62	0.83	0.16	0.00	0.28	0.33	0.00	0.66	0.45	0.66	0.33	0.00	0.45	0.00	1.00	0.00	0.00
SOD	0.40	0.60	0.00	0.48	0.00	0.66	0.33	0.45	0.25	0.75	0.00	0.37	0.50	0.50	0.00	0.50	0.50	0.50	0.00	0.50

Table 03: H_T, H_S, D_{ST} and G_{ST} values of diversity

	H_T	H_S	D_{ST}	G_{ST}
ACO	0.5553	0.2450	0.3103	0.5588
ADH1	0.6810	0.3555	0.3256	0.4780
ADH2	0.6418	0.4514	0.1904	0.2967
CAT	0.6318	0.3018	0.3301	0.5224
G6PDH	0.3069	0.2224	0.0846	0.2755
GDH	0.6978	0.5205	0.1773	0.2541
GOT	0.5152	0.3879	0.1273	0.2470
IDH1	0.6893	0.2568	0.4326	0.6275
IDH2	0.7175	0.3371	0.3804	0.5302
LAP	0.6327	0.3253	0.3074	0.4858
LDH	0.6468	0.5486	0.0982	0.1518
PER	0.6267	0.3934	0.2333	0.3723
SOD	0.5201	0.4641	0.0560	0.1076
			Gst (average) =	0.3775

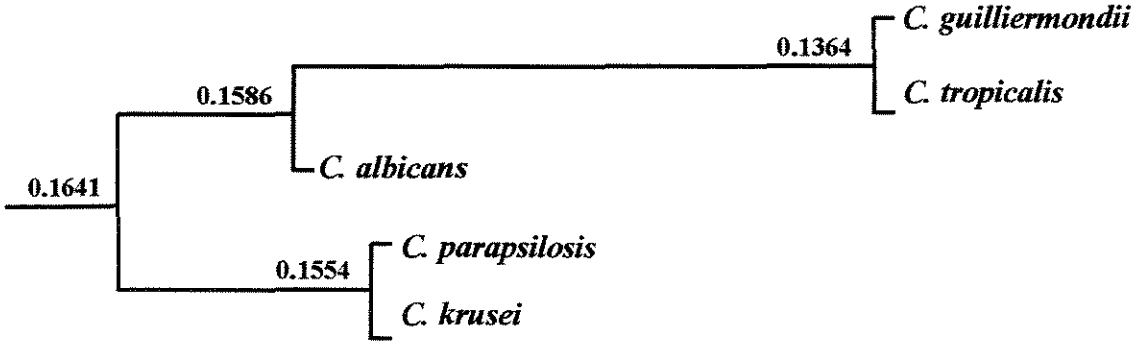

Figure 01: Dendrogram of genetic (Euclidian) distances based on the overall of allelic frequencies, assessed by multilocus enzyme electrophoresis.

DISCUSSION

Several research groups have been published papers about *Candida* population genetics and phylogenetic relationship among its species [34, 35; 9, 13, 14, 17, 15, 1, 11]. Multilocus enzyme electrophoresis is a resource that have been employed on studies involving either characterization of organisms [43, 19, 20, 10] or genetic structure of populations of microorganisms [32, 33, 3, 21, 22].

The genotypes presented on Table 01 show a great number of null loci due to the absence of enzymatic bands on the respective Rm values. In most of cases, this fact occurred in a randomly manner showing be a strain-specific characteristic, but in a small number of cases this absence of bands happened in all members of a certain species, in a species-specific way. The G_{ST} mean value (Table 03) obtained from all enzyme loci is the ratio of genetic diversity of NEI [23], which for our results indicates that 37.75% of total genetic variability is attributable to differences among the species and 62.25% to differences within the different species. This relative low mean value for inter-specific polymorphism had probably occurred due to the fact that most of these species has unknown sexual reaction, that limits the possibility of speciation. HAMRICK [7], working with plants, noticed some phenomena in which sexual barriers induce to a less genetic differentiation.

The relative bigger mean value for conspecific polymorphism found in our experiments, perhaps is resulting from cryptic speciation [38], diploid genome [24, 40, 30], heterozygosity by either aneuploidy or gene duplication [30, 27], mitotic recombination [41], and phenotypic instability [36]. LEHMANN *et al.* [13] pointed out that their results, after MLEE analysis, indicated that extensive heterogeneity exists in strains of *C. albicans*.

The genetic relationships among different species of *Candida* were established using the ROGERS' distance [29], due to the fact that Euclidian distance (more commonly employed) has minimum value as zero (when populations have the same allelic frequencies) and maximum as two (when populations "fix" different alleles), we applied the formula of ROGERS [29] that corrects the D values distribution letting them at the interval $0.0 \leq D \leq 1.0$ [5]. Figure 01 shows a dendrogram with ROGERS' distances values for the different conspecific populations, where *C. guilliermondii* was grouped with *C. tropicalis* followed by *C. albicans*. In other cluster, *C. parapsilosis* and *C. krusei* formed a separated group. Using the same species and different

techniques, different authors obtained dendrograms whose clustering ordination were very dissimilar. SHECHTER *et al.* [34] separated acidic and basic proteins by disc polyacrylamide gel electrophoresis and after numerical analysis with Jaccard's similarity coefficient obtained a dendrogram with the following grouping sequence: *C. albicans* – *C. stellatoidea*, *C. pseudotropicalis*, *C. krusei*, *C. parapsilosis*, *C. guilliermondii*, and *C. tropicalis*. In 1991, BARNS *et al.* [1] established the evolutionary relationships among several pathogenic *Candida* species and other yeasts by the polymorphism of small subunit rRNA sequences, their results showed that *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. viswanathii* form a subgroup within the genus while *C. guilliermondii*, *C. lusitaniae*, *C. kefir*, *C. glabrata*, and *C. krusei* form clusters in other cladogram branchs. More recently, HÖFLING *et al.* [11] obtained, by SDS-PAGE and Pearson product-moment correlation coefficient, a dendrogram in with the following clustering sequence: *C guilliermondii*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. albicans*. We could deduce from the comparison of these different results and ours, that hierarchical schemes of species grouping vary according to molecular marker and coefficient employed.

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Clonal variability among oral *Candida albicans* assessed by allozyme electrophoresis analysis

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ABSTRACT

A total of forty-nine *Candida albicans* strains were isolated from eleven healthy children's saliva in Piracicaba, Brazil, and were analyzed according to their alloenzymatic patterns. Among eight loci assayed, seven were polymorphic and allowed to determine allelic and genotype frequencies, in order to establish the genetic variables for this fungal population. Some children showed just one genetic type whereas other harbored two or more clones of such yeast, in a multiclonal manner of colonization by *Candida albicans.*

Key-words: *Candida albicans*, oral colonization, clonality patterns.

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INTRODUCTION

Among the eukaryotic microorganisms colonizing the oral mucosa, *Candida albicans* (Robin) Berkhout (1923) is the most commonly found (1, 5), being or not associated to oral pathologies (9). It is known that during human immunodeficiency virus infection one or more than one genetic type of such yeast can be isolated from oropharynx of a certain patient (11, 15, 16, 17). Some authors have described the occurrence of multiple *Candida albicans* and other *Candida* non-*albicans* strains colonizing the same individual or the same anatomical region, other than the oral cavity (6, 10, 19, 22).

MERZ et al. (12) observed that electrophoretic karyotyping of *Candida albicans* clinical isolates assessed by orthogonal-field-alternation gel electrophoresis (OFAGE), for a certain subject, were likely to have identical electrophoretic patterns, even though this resource can be used to designate a strain for epidemiologic studies. According to BOERLIN et al. (3), neither of typing methods based on restriction fragment length polymorphism analysis with DNA probes, electrophoretic karyotyping, nor random amplification of polymorphic DNA are recognized as a standard for the delineation of genomic groups, and none has been clearly demonstrated to correlate with classical methods used in taxonomy and phylogeny reconstruction.

The lack of information about uni/multiclonal pattern of oral *Candida albicans* colonization in healthy children drove us to the development of this experimental work. We employed the MLEE technique in order to determine how genetically variable this yeast is, in such population.

MATERIAL AND METHODS

***Candida albicans* samples:** A group of eleven children (8-10 year-old) belonging to five different socioeconomic categories was taken for the presenting study. All of them presented systemic health, assessed by a prior physician examination, DMFT index lesser than 5, and no suggestive indicia of active candidosis, althought, it was possible to isolate *Candida* cells from their saliva. None of the subjects was taking antibiotics or any other medication at the time of the saliva collection. Non-stimulated whole-saliva was sampled, maintained in ice (maximum 60 minutes), diluted in sterile saline solution to 10^{-1}, dispersed over Sabouraud-Chloramphenycol Agar plates (100μL) and let at 30°C. After 48 hours, ten characteristic *Candida* colonies were

chosen from each plate, according to their colony differences (19). A total of forty-nine (mean = 4.45±1.21 colonies/subject) chlamydospore positive, germ-tube forming and *C. albicans* characteristic fermenting/assimilating sugar strains were obtained from the 110 selected colonies. The remaining 61 colonies were identified as other *Candida* spp and were not included in this study.

Cell cultivation and protein extraction: All the strains were grown in 50mL of YPD medium (2% dextrose, 2% peptone, 1% yeast extract), at 30°C, overnight, in a shaker table under 150rpm of orbital rotation. The cells were harvested by centrifugation of total culture medium volume at 2000*g* for 3 min and the pellets were washed 4 times with cold sterile water to ensure complete removal of culture medium traces or extra-cellular metabolites (23). The last washed pellets were transferred to 2mL microcentrifuge tubes, and equal amounts of acid-washed glass beads and 200µL of cold sterile water were added. The tubes were adapted in a Mini-Bead Beater cell disrupter (Biospec, Inc.), where the cell lysis was conducted at 4600rpm, 4 times of 30s, with 5 minutes intervals, when the samples were conditioned in an ice bath. After cell disruption, the microcentrifuge tubes were centrifuged at 10000*g* for 2min, and the supernatants were applied on Whatman 3 filter paper wicks of 5x12mm. These wicks were maintained at –70°C until use.

Starch gel electrophoresis: The electrophoreses were carried out, using a hydrolyzed corn starch (21) up to a final concentration of 13% in 1:30 pH8.0 Tris-citrate buffer, with vigorous agitation on a Bunsen burner. The formed gels were poured in perplex casting moulds (200x120x10 mm), and let over bench at room temperature until complete solidification, when they were cut on their longitudinal dimensions at 2.5cm from one border. The smaller parts were separated and the wicks were applied on the cut. Wicks with 0.2% bromophenol blue were applied in both extremities of the cuts for migrating indication. After jointed the parts, cotton cloth bridges were placed connecting the gels to electrode tanks with pH8.0 Tris-citrate buffer (4, 18). Electrophoreses were carried out at 4°C and 130V until the migration markers run through at least 80 mm from application point. At this time, the electrophoreses were interrupted and the gels were sliced on their high in slices with 1.2mm thickness.

Specific enzyme staining: The gel slices were submitted to some protocols to reveal the dehydrogenase active bands, according to SELANDER et al. (18). Enzymatic systems assayed were: alcohol dehydrogenase (ADH - E.C. 1.1.1.1), malate dehydrogenase (MDH - E.C.

1.1.1.37), glucose-6-phosphate dehydrogenase (G6PDH - E.C. 1.1.1.49), leucine aminopeptidase (LAP - E.C. 3.4.1.1), and peroxidase (PO – E.C. 1.11.1.7). the bands on the gels were numbered in order of decreasing mobility, and the corresponding alleles were numbered by using the same nomenclature. Lack of demonstrable activity for an enzyme was scored as two null alleles at the corresponding gene locus (3). Each unique combination of alleles over the 8 enzyme loci examined was considered as an electromorph type (ET).

Allele and genotype frequencies and heterozygosity determination: Allelic frequencies for each enzyme (p) locus were calculated (2), where p1, p2, p3, and p4 derive from the times when such allele appears divided by the sum of all alleles at that locus. Observed and expected genotype frequencies were determined according to CAUGANT & SANDVEN (4). Heterozygosity (h) for these loci was determined as proposed by NEI (13) following the formula $h = 1- \Sigma x_i^2$, where x_i is the frequency of each allele in a certain locus.

RESULTS

For the collection of 49 saliva isolates sampled on this survey, 7 (87.5%) of the 8 enzyme loci (PO, LAP, ADH2, MDH1, MDH2, MDH3, and G6PDH) were polymorphic for two or more alleles. ADH1 locus was monomorphic with only one allele prevailing. Heterozygotes at the LAP and PO loci showed two bands (monomeric enzyme) while G6PDH, MDH1, MDH2, and MDH3 loci showed 3 bands (dimeric enzyme). Both ADH1 and ADH2 loci only showed homozygous behavior. The assigned genotypes of all strains involved in this survey were scored (3) and expressed in table 01.

According to these genotypes, its possible to observe that individuals I, VII, VIII, IX, X and XI showed just one clone occurring on saliva, while the other patients showed two or more distinct genetic variants, as follows: III and VI (2 clones), IV and V (4 clones), and II (6 clones).

Table 01. Assigned genotypes of 49 *C. albicans* isolates

Patient	Strains	Enzyme loci							
		PO	LAP	ADH1	ADH2	MDH1	MDH2	MDH3	G6PDH
I	A5	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
	A9	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
	A11	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
	A14	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
	A19	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
II	A40	2/2	1/1	1/1	2/2	2/3	2/2	2/3	2/2
	A41	1/1	3/3	1/1	1/1	2/3	2/2	2/3	2/2
	A42	1/2	3/3	1/1	1/1	2/3	2/2	1/2	2/2
	A43	1/2	1/2	1/1	2/2	2/3	2/2	2/3	2/2
	A44	-	2/2	1/1	2/2	2/3	2/2	2/3	2/2
	A45	1/2	2/2	1/1	1/1	2/3	2/2	2/2	2/2
	A46	1/2	2/2	1/1	1/1	2/3	2/2	2/2	2/2
III	B3	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
	B4	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
	B7	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
	B8	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
	B9	1/2	1/2	1/1	-	1/1	1/1	1/1	2/2
IV	C10	1/2	1/2	1/1	-	1/2	-	1/2	2/2
	C13	1/2	1/1	1/1	-	1/1	2/2	1/1	2/3
	C15	1/1	1/1	1/1	-	1/1	1/1	1/1	2/3
	C43	1/2	1/1	1/1	-	1/1	2/2	1/1	3/3
	C48	1/2	1/1	1/1	-	1/1	2/2	1/1	3/3
V	C34	1/2	1/1	1/1	-	1/1	2/2	1/1	2/3
	C35	1/2	1/1	1/1	-	1/1	1/1	1/1	2/3
	C36	-	1/1	1/1	-	1/1	1/1	1/1	2/3
	C38	1/2	1/1	1/1	-	1/1	1/1	1/1	2/3
	C41	2/2	1/1	1/1	-	1/1	1/1	1/1	2/3
VI	D28	1/2	1/1	1/1	-	1/1	1/1	1/1	1/1
	D29	1/2	1/1	1/1	-	1/1	1/1	1/1	1/1
	D30	1/2	1/1	1/1	-	1/1	1/1	1/1	1/1
	D31	1/1	1/1	1/1	2/2	-	1/2	2/3	2/2
VII	D2	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
	D32	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
	D33	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
	D34	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
	D35	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
VIII	D12	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
	D15	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
	D16	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
	D37	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
IX	E2	1/2	2/2	1/1	-	2/2	2/2	1/3	3/3
	E32	1/2	2/2	1/1	-	2/2	2/2	1/3	3/3
	E33	1/2	2/2	1/1	-	2/2	2/2	1/3	3/3
X	E43	1/2	1/1	1/1	-	2/2	1/1	2/2	2/2
	E45	1/2	1/1	1/1	-	2/2	1/1	2/2	2/2
	E46	1/2	1/1	1/1	-	2/2	1/1	2/2	2/2
XI	E20	1/2	1/1	1/1	-	2/2	1/1	1/3	2/2
	E54	1/2	1/1	1/1	-	2/2	1/1	1/3	2/2
	E55	1/2	1/1	1/1	-	2/2	1/1	1/3	2/2

(-) null allele (absence of any enzymatic band)

Table 02 shows the allelic frequencies and heterozygosity for the different assayed loci, ranged between 0.421 and 0.582. The mean heterozygosity found among polymorphic loci was 0.527.

Table 02. Allelic frequencies and heterozygozity in enzyme loci

loci	p(1)	p(2)	P(3)	p(4)	H
PO	0.415	0.585	0	0	0.485
LAP	0.622	0.155	0.040	0.183	0.555
ADH1	1.000	0	0	0	0
ADH2	0.500	0.500	0	0	0.500
MDH1	0.469	0.459	0.072	0	0.573
MDH2	0.698	0.302	0	0	0.421
MDH3	0.523	0.362	0.115	0	0.582
G6PDH	0.246	0.581	0.173	0	0.573
					h (avg) for polymorphic loci = 0.527

Table 03. Assigned genotypes of 21 *C. albicans* ETs.

ET	N° of	Enzyme loci							
	isolates	PO	LAP	ADH1	ADH2	MDH1	MDH2	MDH3	G6PDH
1	9	1/2	1/1	1/1	-	1/1	1/1	1/1	2/2
2	1	1/2	1/2	1/1	-	1/1	1/1	1/1	2/2
3	1	1/2	1/2	1/1	-	1/2	-	1/2	2/2
4	2	1/2	1/1	1/1	-	1/1	2/2	1/1	- 2/3
5	1	1/1	1/1	1/1	-	1/1	1/1	1/1	2/3
6	2	1/2	1/1	1/1	-	1/1	2/2	1/1	3/3
7	2	1/2	1/1	1/1	-	1/1	1/1	1/1	2/3
8	1	-	1/1	1/1	-	1/1	1/1	1/1	2/3
9	1	2/2	1/1	1/1	-	1/1	1/1	1/1	2/3
10	3	1/2	1/1	1/1	-	1/1	1/1	1/1	1/1
11	1	1/1	1/1	1/1	2/2	-	1/2	2/3	2/2
12	9	2/2	4/4	1/1	-	2/2	1/1	2/2	1/1
13	3	1/2	2/2	1/1	-	2/2	2/2	1/3	3/3
14	3	1/2	1/1	1/1	-	2/2	1/1	2/2	2/2
15	3	1/2	1/1	1/1	-	2/2	1/1	1/3	2/2
16	1	2/2	1/1	1/1	2/2	2/3	2/2	2/3	2/2
17	1	1/1	3/3	1/1	1/1	2/3	2/2	2/3	2/2
18	1	1/2	3/3	1/1	1/1	2/3	2/2	1/2	2/2
19	1	1/2	1/2	1/1	2/2	2/3	2/2	2/3	2/2
20	1	-	2/2	1/1	2/2	2/3	2/2	2/3	2/2
21	2	1/2	2/2	1/1	1/1	2/3	2/2	2/3	2/2

(-) null allele (absence of any enzymatic band)

Table 04. Observed and expected genotype frequencies in *C. albicans* isolates and in ETs.

Enzyme locus	Genotype	Frequency in 49 isolates		Frequency in 21 ETs	
		Observed	Expected*	Observed	Expected*
PO	1/1	0.061	0.158	0.143	0.204
	2/2	0.224	0.315	0.143	0.204
	1/2	0.653	0.446	0.619	0.408
LAP	1/1	0.591	0.387	0.571	0.412
	2/2	0.122	0.023	0.143	0.045
	3/3	0.041	<0.001	0.095	0.009
	4/4	0.183	0.033	0.047	0.002
	1/2	0.061	0.190	0.143	0.274
	1/3	0	0.050	0	0.122
	1/4	0	0.228	0	0.060
	2/3	0	0.012	0	0.040
	2/4	0	0.056	0	0.020
	3/4	0	0.014	0	0.009
ADH1	1/1	1.000	1.000	1.000	1.000
ADH2	1/1	0.081	0.006	0.143	0.163
	2/2	0.081	0.006	0.143	0.163
	1/2	0	0.013	0	0.040
MDH1	1/1	0.449	0.210	0.428	0.204
	2/2	0.367	0.201	0.190	0.127
	3/3	0	0.005	0	0.020
	1/2	0.020	0.412	0.047	0.322
	1/3	0	0.065	0	0.129
	2/3	0.149	0.063	0.285	0.102
MDH2	1/1	0.673	0.467	0.476	0.250
	2/2	0.285	0.087	0.428	0.204
	1/2	0.020	0.403	0.047	0.452
MDH3	1/1	0.449	0.281	0.428	0.274
	2/2	0.285	0.120	0.095	0.068
	3/3	0	0.012	0	0.036
	1/2	0.041	0.367	0.095	0.274
	1/3	0.122	0.118	0.095	0.198
	2/3	0.102	0.077	0.285	0.099
G6PDH	1/1	0.245	0.060	0.095	0.009
	2/2	0.510	0.337	0.571	0.444
	3/3	0.102	0.026	0.095	0.046
	1/2	0	0.284	0	0.126
	1/3	0	0.080	0	0.040
	2/3	0.143	0.189	0.238	0.285

*Expected genotype frequencies are calculated as the square of the allele frequency for homozygote genotypes and twice the product of the allele frequencies for the heterozygotes (Hardy-Weinberg test).

Observed and expected genotype frequencies for each locus were drawn on table 04 that shows both frequencies either in isolates and in ETs. No significant deviation from expected

values was seen when applied de Hardy-Weinberg test for the frequencies of single-locus genotypes, neither in isolates nor in ET's frequencies.

Throughout the samples, twenty-one different eletromorph types (ET) were observed, occurring the same ones even in distinct individuals. Table 03 shows these ET's composition and the number of isolates scored among them.

DISCUSSION

The observation of many heterozygous loci comes to the agreement with the notion that *C. albicans* is a diploid organism, as early proposed (4, 14). PUJOL et al. (16) also pointed out this phenomenon and concluded that this heterozygous band pattern was not due to either aneuploidy or gene duplication.

The literature data relating different biotypes of *C. albicans* occurring in a same oral-healthy individual are not properly consistent, whereas in other with oral candidosis are more abundant. REYNES et al. (17) showed that among 70 *C. albicans* isolates from 7 human immunodeficiency virus-infected patients with oral candidosis, 2 to 3 different ETs were found. LE GUENNEC et al. (7) showed that HIV type 1-positive adults with oropharyngeal manifestations of candidosis and were under azole therapy, presented more than one single yeast strain colonizing their mucosa, in most of the cases. In their survey, the authors observed from one to six different strains of *C. albicans*, occurring throughout the antifungal schedule (ranging from 8 to 33 months), in a manner that a resistance-increasing selective succession occurred.

In our study, five children (45.45%) showed an uniclonal pattern of colonization, although individuals VII and VIII have carried the same ET, and individuals X and XI just have diverged at MDH3 locus. This occurrence is, at least in part, due to the fact that in both cases the children were relatives (brothers).

Subjects II, IV, and V showed high degree of genetic variability with 4-6 ETs that did not show apparent relatedness. No conclusive explanations could be attributed to this phenomenon because of the lack of more information about the hygienic and feeding habits of such populations. Subjects I (one clone) and III (2 clones) showed the prevalence of a same ET (ET 1, table 3). The observed variability occurring on LAP locus, that generated the second clonal type on individual III, could be due to an occasional recombination, a hypothesis that was not

excluded by PUJOL et al. (16) for *C. albicans* populations, even do they follow a clonal manner of propagation. On our point of view, this possibility is very improbable once we could not obtain any other allele "2" for that locus in that individual.

The expected heterozygosity values (*h*) for several loci were not correlated with the sum of observed heterozygotes in this population because, even do, no significant deviation from expected Hardy-Weinberg equilibrium values was obtained for any polymorphic locus (neither for isolates nor ETs), the relative higher proportion of homozygotes on the overall loci (81.26%) drove to the non-appearance of a enough quantity of heterozygotes. Such fact diverges from NEI's (13) observation, in which the estimation of heterozygosis per locus is equal to the expected proportion of heterozygotes for a diploid organism population under Hardy-Weinberg equilibrium. PUJOL et al. (16) related that geographical isolation may be associated with different allelic frequencies in different populations, even if each separate population is panmictic. Such strains obtained from different local sources, when combined may generate an apparent departure from Hardy-Weinberg expectations, particularly with a deficit of heterozygotes, the Wahlung effect (20). This effect could be corroborated with our results, once assayed strains were obtained from individuals of different geographical subdivisions (letters A to E preceding each strain label imply 5 distinct areas in Piracicaba, Brazil) with different particularities, as socioeconomic background, access to oral care units, etc.

Artificial enzyme variability derived from long-term laboratory stocking hypothesis, called "domestication" (8), was excluded because the assayed strains were recently isolated from saliva.

Finally, we could conclude that healthy children may carry more than one genetic type of *C. albicans* in their oral cavities in a multiclonal manner of colonization. Complementary studies involving their families and neighborhood, hygiene and nutritional habits must be done, in order to establish the sources of that multicolonization pattern, in such childish populations.

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DISCUSSÃO

O emprego da MLEE associada aos recursos de análise numérica propiciou a redação de dois artigos originais onde isolados clínicos de diferentes espécies de *Candida* foram analisados quanto a sua capacidade de agrupamento monoespecífico (ROSA *et al.*, 1999; ROSA *et al.*, 2000). Em ROSA *et al.* (1999), o polimorfismo enzimático de sete desidrogenases (álcool desidrogenase – ADH, glucose desidrogenase – GDH, glucose-6-fosfato desidrogenase – G6PDH, isocitrato desidrogenase – IDH, lactato desidrogenase – LDH, malato desidrogenase – MDH, e enzima málica – EM) permitiu a construção de dendrogramas independentes cuja capacidade discriminatória não foi satisfatória, excetuando-se a isocitrato desidrogenase que, conforme já apontado por LEHMANN *et al.* (1989a), talvez seja a desidrogenase que permita o melhor agrupamento conspecífico para *Candida* spp. Contudo, quando foi realizada a confecção do dendrograma baseado na somatória de todas as desidrogenases, pôde-se obter *clusters* espécie-específicos para *C. albicans* e *C. parapsilosis*, inclusive com a inclusão de suas respectivas linhagens-tipo. O uso das desidrogenases como marcador molecular pode, ao menos em parte, ser justificado pelo fato de que dentre as mais diversas classes de enzimas, essas oxi-redutases apresentam a mais alta especificidade por seus substratos (DIXON & WEBB, 1979) e provavelmente apresentem uma menor probabilidade em gerar bandas não-específicas.

Já em ROSA *et al.* (2000), outras classes de enzimas foram adicionadas (aconitase –ACO, alfa esterase - αEST, beta esterase - βEST, catalase – CAT, glutamato oxalalato transaminase – GOT, leucina aminopeptidase – LAP, peroxidase – PO, e superóxido dismutase – SOD) àquelas anteriormente citadas, e a espécie *C. parapsilosis* foi a melhor agrupada (com valores de similaridade iguais a 1,00), formando *clusters* monoespecíficos e compostos. Algumas linhagens de *C. albicans* (CBS562, 97a, e F72), de forma análoga aos resultados obtidos por ROSA *et al.* (1999), também formaram *clusters* monoespecíficos, reforçando a posição de que as espécies *C. albicans* e *C. parapsilosis* são aquelas que melhor agrupam suas linhagens, através da análise de enzimas constitutivas individuais. Quando da construção do dendrograma geral envolvendo todas as 15 enzimas, observou-se que o *cluster* de *C. albicans* passou a incorporar a linhagem E37, e ocorreu a formação de novos *clusters*, porém compostos ou que não contemplavam todas as

linhagens ensaiadas (*phena* II e VII). Esse comportamento pode, em parte, ser explicado pelo fato de que em alguns gêneros fúngicos as enzimas constitutivas fornecem bandas que funcionam como verdadeiros *fingerprint*, caracterizando o indivíduo muito mais que a espécie. Essa propriedade levou SMITH *et al.* (1990) a obterem que alguns isolados de *Brettanomyces* e *Dekkera* podiam serem agrupados em *clusters* multiespecíficos, como ainda, apresentavam relativos baixos valores de similaridade quando da formação de *clusters* monoespecíficos. Ainda, JONES & NOBLE (1982) apontam para o fato de que a análise de MLEE gera *clusters* compostos por isolados de diferentes espécies e mesmo de diferentes gêneros de fungos dermatófitos.

Nossos resultados passam a apresentar um maior entendimento quando analisamos os resultados obtidos por ROSA *et al.* (enviado para publicação) a partir da análise da diversidade genética infraespecífica acessada pela observação de polimorfismo alélico. Nesse trabalho, os isolados de uma mesma espécie foram juntados e tratados como populações conspecíficas e as freqüências alélicas para todos os *loci* gênicos foram determinadas e aplicadas em fórmulas de índices de diversidade (NEI, 1973). Os resultados obtidos revelaram que 37,75% da variabilidade genética total era atribuível às diferenças entre as espécies, ao passo que os 62,25% de diversidade restantes expressavam a variabilidade contida dentro das espécies. Diversas causas podem estar contribuindo para esse deslocamento em direção da diversidade intraespecífica, tais como especiação críptica (TIBAYRENC *et al.*, 1991), diploidia do genoma na levedura (PUJOL *et al.*, 1993; SCHERER & MAGEE, 1990; WHELAN & KWON-CHUNG, 1988), heterozigose por aneuploidia ou por duplicação gênica (PUJOL *et al.*, 1993; SCHERER & MAGEE, 1990), ou recombinação mitótica (WHELAN *et al.*, 1980). No caso específico de *C. albicans*, LEHMANN *et al.* (1989b) já havia anteriormente descrito sua extensa heterogeneidade para enzimas constitutivas. Essas observações conduzem a inferência de que a análise de marcadores MLEE aplicada às *Candida* spp. é bastante influenciada pelo polimorfismo intraespecífico, de grande importância quando se desejam determinações de grande poder resolutivo, caso de levantamentos epidemiológicos.

A capacidade de promoção de agrupamentos monoespecíficos pela MLEE foi comparada a aquela promovida pela técnica de eletroforese de proteínas totais em gel de poliacrilamida (SDS-PAGE), no artigo de ROSA *et al.* (in press). Nesse artigo, foram comparados os

dendrogramas originados a partir das duas técnicas cujos resultados foram analisados numericamente, e enquanto a MLEE forneceu um gráfico contendo nove *phena*, alguns deles compostos e outros contendo somente uma linhagem, a SDS-PAGE permitiu o enquadramento de todas as linhagens de uma dada espécie em seu respectivo *cluster* espécie-específico. Essa propriedade de agrupamento monoespecífico de isolados de *Candida* spp. pela SDS-PAGE já havia sido observada, mesmo quando variando critérios na análise numérica (HÖFLING *et al.*, 1999). Esses resultados apontam para a possibilidade de que a expressão de bandas espécie-específicas mais conservadas na SDS-PAGE podem influenciar na composição dos *clusters* mantendo-os monoespecíficos, ao passo que a MLEE, muito provavelmente, explore melhor a variabilidade em um nível subespecífico. Posto isso, podemos presumir que estudos envolvendo mais de uma espécie de *Candida*, tais como aqueles de implicação na Sistemática, seriam melhor avaliados empregando-se a eletroforese de proteínas totais, enquanto que levantamentos de ordem epidemiológica, onde se desejam acompanhar a ocorrência de clones de importância clínica, seriam melhor conduzidos empregando-se a eletroforese de enzimas constitutivas.

Nessa direção, MATA *et al.* (in press) empregaram a eletroforese de enzimas constitutivas para avaliar a ocorrência de diferentes tipos genéticos de *C. albicans* na saliva de escolares da região de Piracicaba, SP, oriundos de diversas classes socioeconômicas. Foram empregados cinco sistemas enzimáticos que geraram oito *loci*, sendo que sete deles (PO, LAP, ADH2, MDH1, MDH2, MDH3, e G6PDH) foram polimórficos para dois ou mais alelos. Numa amostragem com onze crianças, cinco delas (45,45%) apresentavam infecção uniclonal pela levedura, e três outras apresentavam entre 4 e 6 clones de *C. albicans* na saliva. A técnica possibilitou ainda detectar a ocorrência de dois clones muito relacionados provenientes das amostras de dois irmãos e a existência de um mesmo tipo genético ocorrendo em dois indivíduos que estudavam numa mesma escola. A análise dos diferentes *loci* revelou baixa diversidade alélica entre os clones, decorrente da baixa proporção de heterozigotos – fenômeno de Wahlung (TIBAYRENC *et al.*, 1991) – que pode estar associado ao fato de que os clones de *C. albicans* dessas crianças eram isolados entre si por elementos geográficos (PUJOL *et al.*, 1993) e por diferenças na situação socioeconômica de suas famílias, além do fato de que essa espécie não apresenta estágios sexuais conhecidos.

Em ROSA *et al.* (1999), ROSA *et al.* (2000), ROSA *et al.* (in press), como critérios para a análise numérica foi empregado o coeficiente de associação *Simple Matching* (S_{SM}) para se determinar os graus de similaridade e como método de agrupamento, o algoritmo UPGMA (NAUMOV *et al.*, 1997). O coeficiente *Simple Matching* foi eleito dentre vários outros testados (*Jaccard*, *Dice*, e *Pearson*) devido ao fato de que o mesmo fornecia os maiores valores de similaridade para repetições de uma mesma linhagem. Para se determinar qual algoritmo melhor respondesse à formação dos *clusters*, vários foram avaliados (*single linkage*, *complete linkage*, *neighbor joining*) e o UPGMA foi aquele que apresentou os maiores valores de coeficiente de correlação cofenética (r_{cs}) entre a matriz de associação gerada pelo *Simple Matching* e a matriz cofenética gerada pelo algoritmo. Esses achados estão em concordância com os de FARRIS (1969) que preconiza o uso do UPGMA, pois o mesmo tende sempre a maximizar os valores r_{cs}.

CONCLUSÕES

1. A eletroforese de enzimas constitutivas de diferentes classes, seguida da análise numérica, apresentou baixa eficiência como recurso de agrupamento monoespecífico de isolados orais de *Candida* spp.;
2. A eletroforese de desidrogenases, seguida da análise numérica, apresentou baixa eficiência como recurso de agrupamento monoespecífico de isolados orais de *Candida* spp.;
3. A eletroforese de proteínas totais, seguida da análise numérica, apresentou relativa alta eficiência como recurso de agrupamento monoespecífico de isolados orais de *Candida* spp., quando comparado com os resultados obtidos na eletroforese de enzimas constitutivas;
4. A eletroforese de enzimas constitutivas é uma técnica com alta capacidade resolutiva na identificação e caracterização de diferentes tipos genéticos dentro de uma determinada espécie de *Candida* oral;
5. Isolados de diferentes *Candida* spp. apresentam maior diversidade intraespecífica que interespecífica, quando acessadas através da eletroforese de enzimas constitutivas, seguida de análise do polimorfismo de múltiplos *loci*;
6. A eletroforese de enzimas constitutivas apresenta como principais méritos, além da alta capacidade resolutiva infraespecífica e da robustez na reprodutibilidade, a possibilidade de condução subseqüente de análise numérica baseada em critérios andansonianos, ou de análise de diversidade genética baseada no polimorfismo de múltiplos *loci*.

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