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Distribution and hydrolytic enzyme characteristics of *Candida albicans* strains isolated from diabetic patients and their non-diabetic consorts

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Introduction: The aim of this study was to investigate the oral colonization profile of *Candida albicans* strains isolated from diabetic patients and their non-diabetic consorts. In addition hydrolytic enzyme activity of these isolates was analysed.
Methods: The genetic diversity of *C. albicans* oral isolates from 52 couples was established using isoenzyme marker and cluster analysis. Hydrolytic enzyme characteristics, namely secreted aspartyl proteinases (SAPs) and phospholipases (PLs) were also analysed.
Results: Simultaneous colonization by *C. albicans* was observed in the consorts of 12 couples (23.1%). Patterns of monoclonal and polyclonal oral colonization by *C. albicans* strains were identified and the coexistence of identical or highly related strains was observed in both members of eight couples. The genetic diversity observed in the total yeast population revealed four large, genetically distinct groups (A to D) and the coexistence of strains in couples or consorts conjugally unrelated. SAP and PL activity was observed in the majority of *C. albicans* isolates without any association to particular strain, strain clusters (highly related isolates), or clinical characteristics of the consorts (diabetic, non-diabetic, and gender).
Conclusion: Possible sources of transmission and oral propagation of groups (clusters) of strains of *C. albicans* can occur between diabetic and non-diabetic consorts. A conjugal genotypic identity exists in most *C. albicans*-positive couples, that is, both consorts share identical or highly related strains; however, this identity is not couple-specific as seen by the coexistence of clusters in couples and unrelated consorts.

Key words: *Candida albicans*; diabetes mellitus; multilocus enzyme electrophoresis; oral distribution; secreted aspartyl proteinases, phospholipases

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Candida albicans (Ascomycota; Saccharomycetales) and related species are opportunistic pathogens that cause infections ranging from simple mucocutaneous disorders to invasive diseases involving multiple organs. The frequency of such infections has increased worldwide as the result of a multiplicity of predisposing factors which include procedures that disrupt the integrity of the mucocutaneous barrier, qualitative and quantitative neutrophil dysfunction, cell-mediated immunodeficiency, extreme age, and metabolic disorders such as, diabetes mellitus (38, 46, 48). Diabetic patients exhibit high susceptibility to *Candida* species, mainly *C. albicans* (17, 23, 57, 62, 63), and other fungal infections (4, 60). However, not all

diabetic patients present fungal infections or any signs or symptoms associated with such infections (9). On the other hand, *Candida* species can be found commensally in the microbiota of human cavities (rectal, oral, vaginal, urethral, nasal, and aural) and on skin (50). Their presence in the microbiota of healthy people remains unexplained. However, nutritional factors, interactions with bacterial microbiota, and the presence of salivary antibodies have been suggested as influences in the incidence of these yeasts (55).

Interest in acquiring a better understanding of the pathogenesis, epidemiology, genetics, and evolution of *C. albicans* infections has led to the development of much research that has used various methods of protein and DNA fingerprinting. Despite extensive laboratory work, multilocus enzyme electrophoresis (MLEE) has been shown to be an excellent genotypic fingerprinting method for fungal infections, simultaneously it provides information at all resolution levels (i.e. identification of the same strain in independent isolates, identification of microevolutionary changes in a strain – highly related but not identical isolates, clustering of moderately related isolates, and identification of completely unrelated isolates) (53). This information has been used in epidemiological tracing, which allows a better understanding of epidemic development, and represents a valuable complement to the current methods of molecular typing. In addition, MLEE has been considered a typing method with high discriminatory power and reproducibility (6, 7, 18, 41).

In this study, we evaluated the oral colonization profile of *Candida* species, mainly *C. albicans*, in both diabetes mellitus patients under clinical control and their respective non-diabetic consorts. The genetic relationship and distribution of *C. albicans* strains, and some *Candida dubliniensis* strains, found exclusively in one male diabetic patient, were determined by isoenzyme markers and cluster analyses. Finally, the hydrolytic enzyme characteristics of these yeast isolates were evaluated by tests for secreted aspartyl proteinase (SAP) and phospholipase (PL) in specific microbiological culture media.

Materials and methods

Subjects

Two couples (two women and two men) and 50 subjects (15 women and 35 men) with a diagnosis of clinical diabetes mellitus, registered at the Family Health Program [Programa de Saúde da Família (PSF), Ministry of Health, http://www.saude.gov.br], of the municipality of Limeira, State of São Paulo, Brazil, and their respective, clinically non-diabetic consorts (15 men and 35 women) were selected (30–79 years of age, mean 60 ± 10 years). Clinical symptoms of oral candidosis were absent for all subjects. Subjects with the following conditions were included in the study: over 18 years of age, not having taken antifungal medication or antibiotics within the 10 weeks before sample collection, non-pregnant women and non-xerostomic subjects (i.e. patients should produce 3–5 ml of non-stimulated saliva within 15 mins) (19). This investigation was carried out in accordance with Resolution no. 196/1996 of the Conselho Nacional de Saúde (http://conselho.saude.gov.br/comissao/conep/resolucao.html) and approved by the FOP/UNICAMP Committee of Ethics in Research (protocol no. 123/2005).

Oral samples

The subjects were given 50 ml polypropylene tubes containing 10 m sterile phosphate-buffered saline (PBS) (10 ml NaCl; 0.1 M NaH_2PO_4, pH 7.2) and asked to wash their mouths for 1 min in the presence of a physician. The tubes were then centrifuged at 1700 ***g*** (REVAN Centrífuga Ciclo I, Marconi Equipamentos para Laboratórios Ltda,Piracicaba, SP, Brazil) for 10 min, and the sediments were resuspended in sterile PBS (volume similar to that of salivary secretions). The sediments were then transferred to 1.5 ml Eppendorf tubes and agitated by vortex for 30 s (47). Aliquots (50 μl) from each sample were inoculated onto plates containing chromogenic medium CHROMagar Candida™ (Probac do Brasil Produtos Bacteriológicos Ltda, São Paulo, SP, Brazil), and aerobically incubated at 37°C for 48 h (19, 36).

Identification of *C. albicans*

The preliminary identification of *C. albicans* and *C. dubliniensis* oral isolates was effected based on the green color of the colonies on the chromogenic media. The number of colorimetrically distinct colony-forming units (high index >350 CFU/ml <low index) (19) and the *Candida* species diversity were determined (53). Between 10 and 25 green colonies per sample were randomly selected and subcultured on Sabouraud dextrose neutral inclined agar (Emmons' modification: 2% glucose; 1% peptone. 2% agar; pH 6.8–7.0) at 37°C for 24–48 h to obtain pure cultures (22). *C. albicans* identification was confirmed with two tests: (i) colony growth on Sabouraud dextrose agar medium (Emmons' modification) at 42°C and/or 45°C for 24–48 h (39); and (ii) absence of abundant chlamydospore production on the casein agar medium after 24°C for 48 h (32). As the result of the presence of light and dark green colonies on the CHROMagar™ Candida medium, initially suggestive of *C. albicans* and *C. dubliniensis*, respectively (21, 42, 43), it was considered insufficient to identify these two species in primary cultures (59). Based on oral colonization patterns by multiple *C. albicans* strains observed in various immunocompetent and immunosuppressed patients, the genotypes of several *C. albicans* oral isolates ($n = 5$) per subject were assessed by isoenzymatic markers (53).

Enzyme extraction

Yeast cultures were grown in flasks containing 50 ml YEPD medium [1% (weight/volume; W/V) yeast extract, 2% (W/V) peptone and 2% (W/V) D-glucose] at 37°C for 18 h, under constant agitation at 150 r.p.m. (Incubator with Orbital Agitation Platform – Shaker model 430, Nova Ética, Vargem Grande Paulista, São Paulo, Brazil). After growth, cells were centrifuged at 3000 ***g*** for 5 min and washed twice in 0.9% (W/V) NaCl, submitting each wash to the same centrifugal force. Pellets (∼ 500 μl) were transferred to 2-ml microtubes (Biospec Products, Inc., Bartlesville, OK) containing cold, distilled water (approximately 8°C) and glass beads (1 : 1 : 1). These mixtures were kept on ice (4°C) for 5 min and afterwards were agitated four times in a BeadBeater machine (Biospec Products, Inc.) at 4200 r.p.m. for 30 s, at 1-min intervals. Cell fragments were centrifuged at 5000 ***g*** at 4°C for 5 min. The resulting upper aqueous phase was applied to Whatman n3 filter papers (wicks), 12 × 5 mm in size, and maintained at −70°C until use (7).

Multilocus Enzyme Electrophoresis (MLEE)

Enzymes were separated in 13% starch gels (Penetrose 30®; Refinações de Milho Brasil Ltda, Garanhuns, Brazil), with dimensions of 200 × 120 × 10 mm. Wicks were then immediately soaked in 5 μl (0.02% W/V) bromophenol-blue solution and then applied perpendicularly to a gel cut longitudinally (20 mm). Electrophoresis was performed in a horizontal and continuous system, at 130 V at 4°C over-

Table 1. Systems and solutions utilized for multilocus enzyme electrophoresis analysis from metabolic enzymes of *Candida albicans*

Enzyme EC number	Name	Symbol	Compound for staining substrate	Buffer	Salt	Coenzyme	Dye catalyser
1.1.1.1.	Alcohol dehydrogenase	ADH	Ethanol (3 ml) Isopropanol (2 ml)	200 mM Tris–HCl pH 8.0 (50 ml)[1]		NAD 1% (2 ml)	PMS 1% (500 μl) MTT 1.25% (1 ml)
1.1.1.14.	Sorbitol dehydrogenase	SDH	Sorbitol (250 mg)	Tris–HCl 50 mM pH 8.0 (50 ml)[2]		NAD 1% (2 ml)	PMS 1% (500 μl) MTT 1.25% (1 ml)
1.1.1.17.	Mannitol-1-phosphate dehydrogenase	M1P	Mannitol-1-phosphate (5 mg)	Tris–HCl 100 mM pH 8.5 (50 ml)[3]		NAD 1% (2 ml)	PMS 1% (500 μl) MTT 1.25% (1 ml)
1.1.1.37.	Malate dehydrogenase	MDH	2 M Malic acid (6 ml)[4]	Tris–HCl 200 mM pH 8.0 (40 ml)[1]		NAD 1% (2 ml)	PMS 1% (500 μl) MTT 1.25% (1 ml)
1.1.1.42.	Isocitrate dehydrogenase	IDH	1 M Isocitric acid (2 ml)[5]	Tris–HCl 200 mM pH 8.0 (40 ml)[1]	100 mM $MgCl_2$ (1 ml)[6]	NADP 1% (1 ml)	PMS 1% (500 μl) MTT 1.25% (1 ml)
1.1.1.47.	Glucose dehydrogenase	GDH	D-glucose (500 mg)	Tris–HCl 200 mM pH 8.0 (50 ml)[1]		NAD 1% (2 ml)	PMS 1% (500 μl) MTT 1.25% (1 ml)
1.1.1.49.	Glucose-6-phosphate dehydrogenase	G6PDH	Glucose-6-phosphate disodium salt (100 mg)	Tris–HCl 200 mM pH 8.0 (50 ml)[1]	100 mM $MgCl_2$ (1 ml)[6]	NADP 1% (1 ml)	PMS 1% (500 μl) MTT 1.25% (1 ml)
1.4.3.x.	Aspartate dehydrogenase	ASD	Aspartic acid (50 mg)	Sodium phosphate pH 7.0 (50 ml)[7]		NAD 1% (2 ml)	PMS 1% (500 μl) MTT 1.25% (1 ml)
1.11.1.6.	Catalase[8]	CAT					
1.11.1.7.	Peroxidase	PO	H_2O_2 3% (1 ml)	100 mM Sodium acetate pH 4.5 (50 ml)[9]			*o*-dianisidine 2HCl (16 mg)
3.4.11.1.	Leucine aminopeptidase	LAP	L-leucine β-naphthylamide HCl (30 mg)	100 mM Potassium phosphate pH 5.5 (50 ml)[10]	100 mM $MgCl_2$ (1 ml)[6]		Black K (30 mg)

Electrode buffer: Tris–citrate pH 8.0 [83.2 g $C_4H_{11}NO_3$ (Tris), 33.09 g $C_6H_8O_7$. H_2O (Citric acid), 1 litre H_2O]; Gel buffer: Electrode buffer diluted 1 : 29. [1]24.2 g $C_4H_{11}NO_3$ (Tris), 1 litre H_2O (pH adjusted with HCl); [2]6.05 g $C_4H_{11}NO_3$ (Tris), 1 litre H_2O (pH adjusted with HCl); [3]12.1 g $C_4H_{11}NO_3$ (Tris), 1 litre H_2O (pH adjusted with HCl); [4]26.8 g of $C_4H_6O_5$ (DL-malic acid) and 16 g NaOH in 0.1 l H_2O (caution: potentially explosive reaction); [5]29.41 g $C_6H_5O_7Na_3$. $2H_2O$ (DL-isocitric acid) in 0.1 l H_2O; [6]2.03 g $MgCl_2$. 6HCl (Magnesium chloride) in 0.1 l H_2O; [7]mix equal parts of 27.6 g NaH_2PO_4. H_2O (sodium phosphate monobasic monohydrate) in 1 litre H_2O and 53.6 g Na_2HPO_4. $7H_2O$ (sodium phosphate dibasic heptahydrate) in 1 litre H_2O, then dilute the mixture 1 : 25 with H_2O; [8]incubate gel slice for 30 min at 0°C in 50 ml of 0.1 M sodium phosphate pH 7.0 buffer, then pour off solution, and immerse it in 50 ml of 1.5% potassium iodide solution (KI) for 2 min, then, rinse gel slice with water, and immerse it in 50 ml 0.03% hydrogen peroxide (H_2O_2) solution. Mix gently and remove stain solution when white zones appear on dark-blue background; [9]13.61 g $C_2H_3O_2Na$. $3H_2O$ (sodium acetate), 1 litre H_2O; [10] 13.61 g KH_2PO_4 (potassium phosphate), 1 litre of H_2O.

night (bromophenol-blue migration equivalent to 80 mm). To ensure reproducible results, the *C. albicans* CBS-562 enzymes (Centralbureau voor Schimmelcultures, Delft, the Netherlands) were included in each gel. After the electrophoretic run, the gel was put on an acrylic base and sliced into 1.5-mm sections with the aid of rulers and n15 nylon thread. The sections were carefully placed inside white porcelain containers and submitted to a staining process using previously described methods for 11 enzymes (14 enzyme loci) (2, 7). The enzymatic activities determined were: alcohol dehydrogenase (ADH; EC 1.1.1.1), sorbitol dehydrogenase (SDH; EC 1.1.1.14), mannitol-1-phosphate dehydrogenase (M1P; EC 1.1.1.17), malate dehydrogenase (MDH; EC 1.1.1.37), isocitrate dehydrogenase (IDH; EC 1.1.1.42), glucose dehydrogenase (GDH; EC 1.1.1.47), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), aspartate dehydrogenase (ASD; EC 1.4.3.x), catalase (CAT; EC 1.11.1.5), peroxidase (PO; EC 1.11.1.7), and leucine aminopeptidase (LAP; EC 3.4.1.1) (Table 1). Enzymatic expressions of M1P and MDH showed two and three genetically interpretative loci (M1p-1 and M1p-2; Mdh-1, Mdh-2, and Mdh-3).

Genetic interpretation of the MLEE patterns

Pattern interpretation was performed following the general rules commonly accepted in the deduction of the allelic composition and of the genotype of diploid organisms. The bands on the gels were alphabetically classified in order of decreasing mobility, and the corresponding alleles were alphabetically classified using the same nomenclature. Lack of demonstrable activity for an enzyme was scored as two null alleles at the corresponding gene locus. Each unique combination of alleles over the 14 enzyme loci examined results in an electrophoretic type (ET) – subtype or strain (2, 7, 37).

Cluster analyses

The statistic of Nei (35) was used to estimate the genetic distance among the isolates of *C. albicans*: $d_{ij} = -In(I)$, where I is the normalized identity of genes between two populations (range from 0 to infinity), a measure of genetic distance based on the identity of genes (frequency of alleles for all loci, including monomorphic loci) among populations. This genetic distance measures the accumulated allele differences per locus, and it can also be

estimated from amino acid sequences of proteins even for a distantly related species. As a consequence, if enough data are available, genetic distance between any pair of organisms can be measured in terms of d_{ij}. In addition, this measure is applicable to any kind of organism regardless of ploidy or mating scheme. Its interpretation in terms of enzyme loci infers that, on average, 0 to an infinite number of allelic substitutions are detected (by electrophoresis) in every 100 loci, from a common ancestral strain (2, 7, 35).

Trees with two-dimensional classifications (dendrograms) based on matrices d_{ij}, were generated by the SAHN grouping method (Sequential, Agglomerative, Hierarchic, Nonoverlapping Clustering Methods), UPGMA algorithm (Unweighted Pair-Group Method Using an Arithmetic Average) (51). Average threshold values were established in the dendrograms to identify clusters of identical and highly related isolates (7, 51, 53).

The Pearson product–moment correlation coefficient (range from −1 to +1),

$$r_{jk} = \frac{\sum_{i=1}^{n}(X_{ij} - \overline{X}_j)(X_{ik} - \overline{X}_k)}{\sqrt{\sum_{i=1}^{n}(X_{ij} - \overline{X}_j)^2 \sum_{i=1}^{n}(X_{ik} - \overline{X}_k)^2}}$$

[where X_{ij} stands for the character state value of character i in the operational taxonomy unit (OTU) j, $\overline{X_j}$ is the mean of all state values for OTU j, and n is the number of characters sampled], was used as a measure of the agreement between the genetic distance values implied by the UPGMA dendrograms and those of the original genetic distance matrices (d_{ij}). Such agreements were interpreted as follows: $0.9 \le r$, very good fit; $0.8 \le r < 0.9$, good fit; $0.7 \le r < 0.8$, poor fit; $r < 0.7$, very poor fit. All of these analyses were obtained using the program NTSYS-pc 2.1 (7, 44, 51).

Enzymatic activity

Enzymatic activities of SAPs) and PLs were tested to verify the characteristics of virulence *in vitro* of *C. albicans* (8, 20, 24, 34, 40, 63, 65). Oral isolates were cultured in Sabouraud dextrose agar (SDA) for 24 h at 37°C and adjusted to a concentration of 10^7 CFU/ml in sterile saline (absorbance 1.2 at 560 nm). Four different isolates per plate (90 × 10 mm) were inoculated separately in test medium for SAPs (bovine serum albumin fraction V 1% W/V, yeast nitrogen base without ammonium sulfate and amino acids 0.15% W/V, glucose 2% W/V, and agar 2% in distilled water) and test medium for PLs (peptone 1% W/V, glucose 3% W/V, NaCl 5.7% W/V, $CaCl_2$ 0.06% W/V, agar 2% W/V, and egg yolk 5% V/V in distilled water). The plates were incubated at 37°C for 72 h (SAPs) or 48 h (PLs) and all the tests were carried out in duplicate. The enzymatic activity (Pz) was determined from the formation of a halo around the yeast colony, according to previously described methods (40): $Pz = dc/(dc + zp)$, where dc corresponds to colony diameter (mm) and zp to external diameter of the precipitation zone (mm). The results were interpreted as follows: (i)$Pz = 1$, absence of enzyme activity (index 0); (ii) $1 > Pz \ge 0.64$, positive enzyme activity (index 1); and (iii) $Pz < 0.64$, strongly positive enzyme activity (index 2).

Results

Oral colonization by *C. albicans* and related species

Fifty-two couples containing clinically diabetic and non-diabetic consorts without clinical symptoms of oral candidosis were analysed for oral colonization by *C. albicans* and other *Candida* species. Preliminary (isolation and identification in CHROMagar Candida™ chromogenic medium) and confirmatory (colony growth at 42–45°C and absence of abundant chlamydospore production on casein agar medium) assays for *C. albicans* showed a high prevalence of this yeast (Table 2). *C. dubliniensis* was found to be present in one male diabetic consort only. Species of *Candida krusei*, *Candida tropicalis*, and *Candida* spp. were only preliminarily identified by chromogenic medium, therefore the use of genotypic or phenotypic methods for speciation was necessary.

Simultaneous oral colonization by *C. albicans* was observed in both members of 12 couples (23.1%). Of these, five couples showed colonization exclusively by *C. albicans* (subjects $11^{\text{Non-diabetic}}$ and 12^{Diabetic}, $13^{\text{Non-diabetic}}$ and 14^{Diabetic}, $15^{\text{Non-diabetic}}$ and 16^{Diabetic}, 17^{Diabetic}, and 18^{Diabetic} with indexes <350 CFU, and consorts 19^{Diabetic} and 20^{Diabetic} with indexes >350 CFU). In two couples, diabetic subjects showed colonization exclusively by *C. albicans* (subjects 1 and 3 with indexes >350 CFU) and their respective non-diabetic consorts showed multicolonization by *Candida* species (*C. albicans*, *C. tropicalis*, and *Candida* spp. in subject 2 with index >350 CFU; *C. albicans* and *Candida* spp. in subject 4 with index >350 CFU). In three couples, diabetic consorts showed multicolonization by *Candida* species (*C. albicans* and *Candida* spp. in subject 5 with index >350 CFU; *C. albicans*, *C. tropicalis*, and *C. krusei* in subject 7 with index >350 CFU; *C. albicans* and *C. tropicalis* in subject 10 with index <350 CFU) and their respective non-diabetic consorts showed colonization exclusively by *C. albicans* (subjects 6 and 9 with indexes >350 CFU, and subject 8 with index <350 CFU). Finally, two couples showed multicolonization by *Candida* species (*C. albicans* and *C. krusei* in both subjects 21^{Diabetic} and 22^{Diabetic} with indexes >350 CFU; subjects $23^{\text{Non-diabetic}}$ and 24^{Diabetic}, being *C. albicans*, *C. krusei*, and *Candida* spp. in subject 23 with index

Table 2. Oral occurrence of *Candida albicans* and *Candida* spp. among clinically diabetic subjects (15 women and 35 men) and their respective non-diabetic consorts (15 men and 35 women), and among clinically diabetic consorts (two women and two men), without clinical symptoms of oral candidosis

	Oral colonization in both consorts					Oral colonization in one consort						Couples Total	
				∑ Couples						∑ Couples			
	♀D/♂N-D	♀N-D/♂D	♀D/♂D	*n*	%	♀D	♂N-D	♀N-D	♂D	*n*	%	*n*	%
C. albicans associated or not with *Candida* spp.	4	6	2	12	23.1	6	–	9	9	24	46.2	36	69.2
Candida non-*albicans*	2	1	–	3*	5.8	2	–	5	2	9	17.3	12	23.1
Absence of colonization by *Candida* spp.	2	2	–	4	7.7	–	–	–	–	0	0.0	4	7.7
∑	8	9	2	19	36.5	8	0	14	11	33	63.5	52	100.0

D, diabetic; N-D, non-diabetic. * The absence of *C. albicans* colonization occurred in only one of the consorts of those couples.

<350 CFU and *C. albicans* and *C. dubliniensis* in subject 24 with index <350 CFU).

Non-simultaneous oral colonization, between consorts, by *C. albicans*, whether in association with other species of the same genus or not (i.e. preliminarily *C. tropicalis*, *C. krusei*, and *Candida* spp.), was observed in 33 couples (63.4%), that is, 19 diabetic and 14 non-diabetic consorts were colonized. *Candida* non-*albicans* species were found simultaneously in both members of three couples (5.7%) and the absence of colonization by *Candida* species was found in four couples (7.7%).

Genetic interpretation of MLEE patterns

The enzyme profiles of the *C. albicans* samples (n = 119), *C. albicans* type-strain CBS-562, and *C. dubliniensis* samples (n = 6) were reproducible on different gels after three repetitions of each electrophoretic run. The genetic interpretation of MLEE patterns showed the following characteristics (Table 3): 14 (100%) enzymatic loci were polymorphic (that is, for each enzyme locus the frequency of the most common allele was <99%) for two, three, four and five alleles (two alleles:

Table 3. Allelic profiles of 52 electrophoretic types (ET; strains) of *Candida albicans* and *Candida dubliniensis* isolated from the oral cavity of clinically diabetic subjects (four women and six men) and their respective, non-diabetic consorts (six men and four women), and from two clinically diabetic couples

Subject/ Couples	ET	Number of isolates	Alleles of 14 enzymatic loci*													
			Adh	*Asd*	*Cat*	*G6pdh*	*Gdh*	*Idh*	*Lap*	*M1p-1*	*M1p-2*	*Mdh-1*	*Mdh-2*	*Mdh-3*	*Po*	*Sdh*
	TS		bd	cc	bb	bb	aa	ac	cc	–	bb	–	aa	aa	bb	ac
♀$_{D}^{1}$	1	5	dd	ac	bb	bb	aa	ac	cc	–	bb	–	cc	ac	bb	cc
♂$_{N\text{-}D}^{2}$	2	5	dd	cc	bb	bb	aa	aa	cc	–	bb	bb	aa	ac	bc	ac
♀$_{D}^{3}$	3	5	aa	cc	dd	bb	aa	aa	bb	–	bb	aa	aa	aa	–	ac
♂$_{N\text{-}D}^{4}$	4	3	aa	bb	dd	–	aa	aa	bb	–	ad	aa	aa	aa	bc	aa
	5	2	aa	bb	bb	–	aa	aa	bb	–	cc	–	–	–	bb	–
♀$_{D}^{5}$	6	1	bd	cc	bb	bb	aa	–	cc	–	bb	–	aa	aa	bb	aa
	7	4	bd	cc	bb	bb	aa	aa	cc	–	bb	–	aa	aa	bb	aa
♂$_{N\text{-}D}^{6}$	8	1	bd	cc	ab	bb	aa	aa	cc	–	bb	–	aa	–	bb	aa
	9	1	–	–	bb	aa	aa	aa	cc	–	–	–	aa	–	bb	aa
	10	1	dd	–	bb	bb	aa	aa	cc	–	–	–	aa	–	bb	aa
	11	1	cd	cc	bb	bb	aa	–	cc	–	bb	–	aa	–	bb	aa
	12	1	cd	–	bb	bb	aa	–	cc	–	–	–	aa	–	bb	aa
♀$_{D}^{7}$	13	1	dd	ac	–	bb	aa	–	cc	–	be	aa	aa	ac	bb	ac
	14	4	dd	ac	–	bb	aa	aa	cc	–	be	aa	aa	ac	bb	ac
♂$_{N\text{-}D}^{8}$	15	2	cc	cc	–	bb	aa	aa	cc	–	bb	aa	aa	ac	bd	ac
	16	3	dd	cc	–	bb	aa	aa	cc	–	bb	aa	aa	ac	bd	ac
♀$_{N\text{-}D}^{9}$	17	4	dd	cc	bb	bb	aa	aa	cc	–	bb	–	aa	ad	bb	ac
	18	1	ee	dd	bb	bb	bb	bb	cc	–	cc	–	aa	ad	bb	ac
♂$_{D}^{10}$	19	5	dd	cc	–	bb	aa	–	cc	–	bb	aa	aa	cc	bb	ac
♂$_{D}^{12}$	20	2	dd	cc	cc	bb	aa	aa	cc	–	–	–	bb	–	bc	ac
	21	1	dd	cc	cc	bb	aa	aa	cc	–	–	–	bb	–	bb	ac
	22	2	dd	cc	cc	bb	aa	aa	cc	–	–	–	aa	dd	bb	ac
♀$_{N\text{-}D}^{11}$	23	5	cc	cc	dd	bb	aa	–	dd	–	dd	ab	–	aa	aa	bb
♂$_{D}^{14}$	24	5	ab	–	bb	bb	aa	–	bb	–	bb	–	ab	aa	–	ac
♀$_{N\text{-}D}^{13}$	25	1	bb	–	bc	ab	–	aa	bb	–	bb	–	ab	aa	–	ac
	26	1	ab	–	bc	bb	aa	aa	bb	ab	bb	–	aa	–	–	–
	27	1	ab	–	bb	bb	–	–	bb	–	bb	–	ab	aa	–	ac
	28	1	ab	–	bb	bb	–	–	bb	ab	bb	–	aa	aa	–	ac
	29	1	ab	–	bb	cc	aa	–	bb	–	cc	–	aa	aa	–	bd
♂$_{D}^{16}$	30	2	bd	cc	bb	bb	aa	–	cc	–	bb	–	aa	–	bb	ac
	31	2	dd	cc	bb	aa	aa	–	cc	–	bb	–	aa	–	bb	aa
	32	1	dd	cc	bc	aa	aa	aa	cc	–	bb	–	aa	–	bb	aa
♀$_{N\text{-}D}^{15}$	33	5	bd	cc	bb	aa	aa	–	cc	–	bb	–	aa	–	bb	aa
♂$_{D}^{18}$	34	1	dd	cc	cc	bb	aa	aa	cc	–	bb	–	aa	ad	bb	ac
	35	2	dd	cc	cc	bb	aa	aa	cc	–	bb	–	aa	dd	bb	ac
	36	2	cd	cc	cc	bb	aa	aa	cc	–	bb	–	aa	dd	bb	ac
♀$_{N\text{-}D}^{17}$	37	2	dd	cc	bb	bb	aa	–	cc	–	bb	–	aa	ad	bb	ac
	38	1	dd	cc	bb	bb	aa	–	cc	–	bb	–	aa	dd	bb	ac
	39	2	dd	cc	bb	bb	aa	–	cc	–	bb	aa	aa	dd	bb	ac
♀$_{D}^{19}$/♂$_{D}^{20}$	40	10	dd	cc	bb	bb	aa	aa	cc	–	bb	–	aa	aa	bd	ac
♂$_{D}^{22}$	41	4	dd	cc	bb	bb	aa	aa	cc	–	bb	–	aa	ac	bd	ac
	42	1	ce	be	bb	aa	bb	–	ad	–	dd	–	bb	bb	ad	ee
♀$_{D}^{21}$	43	5	dd	cc	bb	bb	aa	aa	cc	–	bb	–	aa	ad	bd	ac
♂$_{D}^{24}$	44	4^{CaCd}	aa	cc	dd	bb	aa	aa	bb	–	bb	–	aa	aa	bb	ac
	45	1^{Cd}	aa	cc	dd	bb	aa	aa	bb	–	bb	–	aa	aa	bb	–
	46	1^{Cd}	aa	cc	dd	bb	aa	aa	bb	–	–	–	aa	aa	–	ac
	47	2^{Cd}	aa	cc	dd	bb	aa	aa	bb	–	bb	–	aa	–	bb	ac
	48	1	aa	cc	–	bb	aa	aa	bb	–	–	–	–	–	bb	ac
	49	1	aa	cc	dd	bb	aa	aa	bb	–	–	–	aa	aa	bb	–
♀$_{N\text{-}D}^{23}$	50	2	aa	cc	dd	bb	aa	aa	bb	–	bb	–	aa	aa	bc	ac
	51	2	aa	cc	dd	bb	aa	aa	bb	–	–	–	aa	aa	bb	ac
	52	1	aa	cc	dd	bb	aa	aa	bb	–	bb	–	aa	aa	–	–

* Heterozygotes represented by: ab, ac, ad, bc, bd, be, cd, and ce; –, null allele; TS, *Candida albicans* type strain CBS-562; *Ca*, *Candida albicans*; *Cd*, *Candida dubliniensis*; $_{D}$, diabetic; $_{N\text{-}D}$, non-diabetic.

Gdh, *M1p-1*, and *Mdh*; three alleles: *G6pdh*, *Idh*, and *Mdh*; four alleles: *Asd*, *Cat*, *Lap*, *Mdh-3*, and *Po*; five alleles: *Adh*, *M1p-2*, and *Sdh*). The average number of alleles per locus/average number of alleles per polymorphic locus was equal to 3.57. The combination of the existing alleles in 14 enzymatic loci showed 52 electrophoretic types (41.6% of the total isolates). Heterozygotes revealed two and three enzymatic bands (two bands: *Adh*, *Asd*, *Cat*, *G6pdh*, *Idh*, *Lap*, *M1p-1*, *M1p-2*, *Mdh-1*, *Mdh-3*, *Po*, and *Sdh*; three bands: *Mdh-2*). Among the homozygotes, two alleles were observed in the *Gdh*, *Idh*, *Mdh-1*, and *Po* loci, three alleles in the *Asd*, *Cat*, *G6pdh*, *Lap*, *M1p-2*, and *Mdh-2* loci, four alleles in the *Mdh-3* and *Sdh* loci, and five alleles in the *Adh* locus.

In addition, the determination of *C. albicans* strains (electrophoretic types – ETs) by the MLEE method and genetic interpretation revealed monoclonal and polyclonal patterns of oral colonization in couples or consorts (Table 3 and Fig. 1). Only one couple (couple 10: subjects 19 and 20), seven clinically diabetic consorts (three men and four women) and three non-diabetic consorts (one man and two women) showed this monoclonal pattern while 11 couples, seven clinically diabetic consorts (five men and two women) and six non-diabetic consorts (three men and three women) showed the polyclonal pattern. Some *C. dubliniensis* strains exclusively isolated from one clinically diabetic man showed a polyclonal pattern of oral colonization.

Cluster analyses

The genetic diversity of oral isolates of *C. albicans* from 12 couples (i.e. intra-couple analysis), represented by clinically diabetic and non-diabetic consorts (couples 1 to 9 and 12) or by both clinically diabetic consorts (couples 10 and 11) was analysed using Nei analysis (35) and UPGMA dendrograms (Fig. 1). Taking into consideration the average genetic distance ($d_{ij} = 0.074$) obtained from the total population of yeast, these analyses also permitted the verification of the coexistence (threshold: $0.074 \geq d_{ij} \geq 0$ – identical or highly related isolates) or non-coexistence (thresholds: $0.164 \geq d_{ij} > 0.074$ – moderately related isolates, $d_{ij} \geq 0.164$ – unrelated isolates) of strains between the consorts and inferred oral transmission between them or possible microevolutionary alterations. Hence, the results showed coexistence in eight (66.7%) of the 12 couples with one (couples 4, 7, 8, 9, 10, and 11) or two (couples 3 and 12) clusters of isolates/strains. Couples 4, 8, 9, and 10 showed only one cluster comprising all isolates/strains ($0.074 \geq d_{ij} \geq 0$) while couples 7 and 11 showed a cluster containing the majority of isolates/strains ($0.074 \geq d_{ij} \geq 0$) with the remaining minority being moderately related or non-related.

The two clusters existing in couples 3 and 12 appeared to be moderately related; however, in both clusters of couple 12 and in only one cluster of couple 3 there was coexistence of isolates/strains between consorts. In four couples (33.3%) there was no coexistence of strains with two (couples 1 and 5) or three (couples 2 and 6) clusters observed. The genetic diversity of the total yeast population ranged from 0 to 0.3521 (Fig. 2), that is, on average, the population of isolates contained from 0 to 35.2 allelic substitutions for each 100 loci, starting from a common ancestor ($\overline{d_{ij}} = 0.074 \pm 0.090$). Four large groups (A through D) and some genetically unrelated strains (9.6%, 4.8% of the isolates) ($d_{ij} > 0.164$) were identified. Group A comprised the greater number of clusters (68.8% of the isolates or 57.7% of the ETs) featuring: (i) the coexistence of strains ($0.074 \geq d_{ij} \geq 0$) in couples or consorts unrelated (Cluster 1), (ii) the exclusive existence of strains in only one clinically diabetic, male consort (Cluster 2), (iii) the exclusive existence of strains in a couple (Cluster 3), and (iv) the coexistence of strains in non-diabetic and diabetic consorts unrelated (Cluster 4).

Group C (17.6% of the isolates or 19.2% of the ETs) comprised clusters 6 and 7 featuring the coexistence of strains in a couple (this included the *C. dubliniensis* strains) and in a clinically diabetic subject unrelated. Groups B (1.6% of the isolates or 3.8% of the ETs) and D (7.2% of the isolates or 96% of the ETs) comprised a single cluster each, featuring respectively, the exclusive existence of strains in a non-diabetic consort (Cluster 5) and the existence of strains in a single couple (cluster 8), which are distantly related to the larger groups of strains.

Virulence characteristics

A total of 114 *C. albicans* isolates (95.8% of these isolates) and six *C. dubliniensis* isolates (100% of these isolates) showed positive activities for SAPs. Indexes 1 and 2 were found in 29 (24.4%) and 85 (71.4%) *C. albicans* isolates, respectively, and in one (16.7%) and five (83.3%) *C. dubliniensis* isolates, respectively. *Pz* values ranged from 0.33 to 1 (average 0.58 ± 0.14). Positive activities for PLs were observed in 108 *C. albicans* isolates (90.7%) and four *C. dubliniensis* isolates (66.7%). Indexes 1 and 2 occurred in 50 (42%) and 58 (48.7%) *C. albicans* isolates, respectively, and two (33.3%) and two (33.3%) *C. dubliniensis* isolates, respectively. *Pz* values varied from 0.41 to 1 (average 0.67 ± 0.14). No correlation was found between positive activities for SAPs or PLs and genetic characteristics of the opportunist pathogen (i.e. strains/ETs and clusters of highly related strains/ETs) or clinical characteristics of the host (i.e. diabetes mellitus, non-diabetes mellitus, and gender) (Table 4).

Discussion

The oral rinse sampling method was chosen as the most appropriate and sensitive technique for evaluating overall oral yeast carriage compared with imprint culture, swab, or saliva sampling (47). Using this method we observed a profile of simultaneous oral colonization by *C. albicans* between both members of 12 couples (23.1%). A high prevalence of this species was found in all diabetic and non-diabetic consorts; however, five couples showed colonization exclusively by *C. albicans* while two couples showed multicolonization by *Candida* species (*C. albicans*, *C. dubliniensis*, and presumptive *C. krusei* identification). In five couples one of the members was monocolonized by *C. albicans* whereas the other was multicolonized (*C. albicans* and presumptive *C. tropicalis*, *C. krusei*, and *Candida* spp. identified). In addition, the density of this mono- or multicolonization was found to be variable (>350 or <350 CFU/ml) in diabetic consorts and non-diabetic consorts, although clinical symptoms of oral candidosis were non-existent. This fact supports the hypothesis that an abundance of organisms in saliva does not necessarily correlate with clinical candidosis. The frequency of *C. albicans* simultaneous isolation in mouthwash or stool specimens has been shown to be relatively low between family members (that is, 12 out of 33 families or 28 out of 71 subjects), and, furthermore, not all family members are shown to be positive for yeast colonization (that is, culture-positive families had two, three, or four positive members each) (31). Various factors could predispose them to colonization and oral infection by *Candida* yeasts, such as impaired salivary gland function, antibiotic therapy, dentures, carbohydrate-

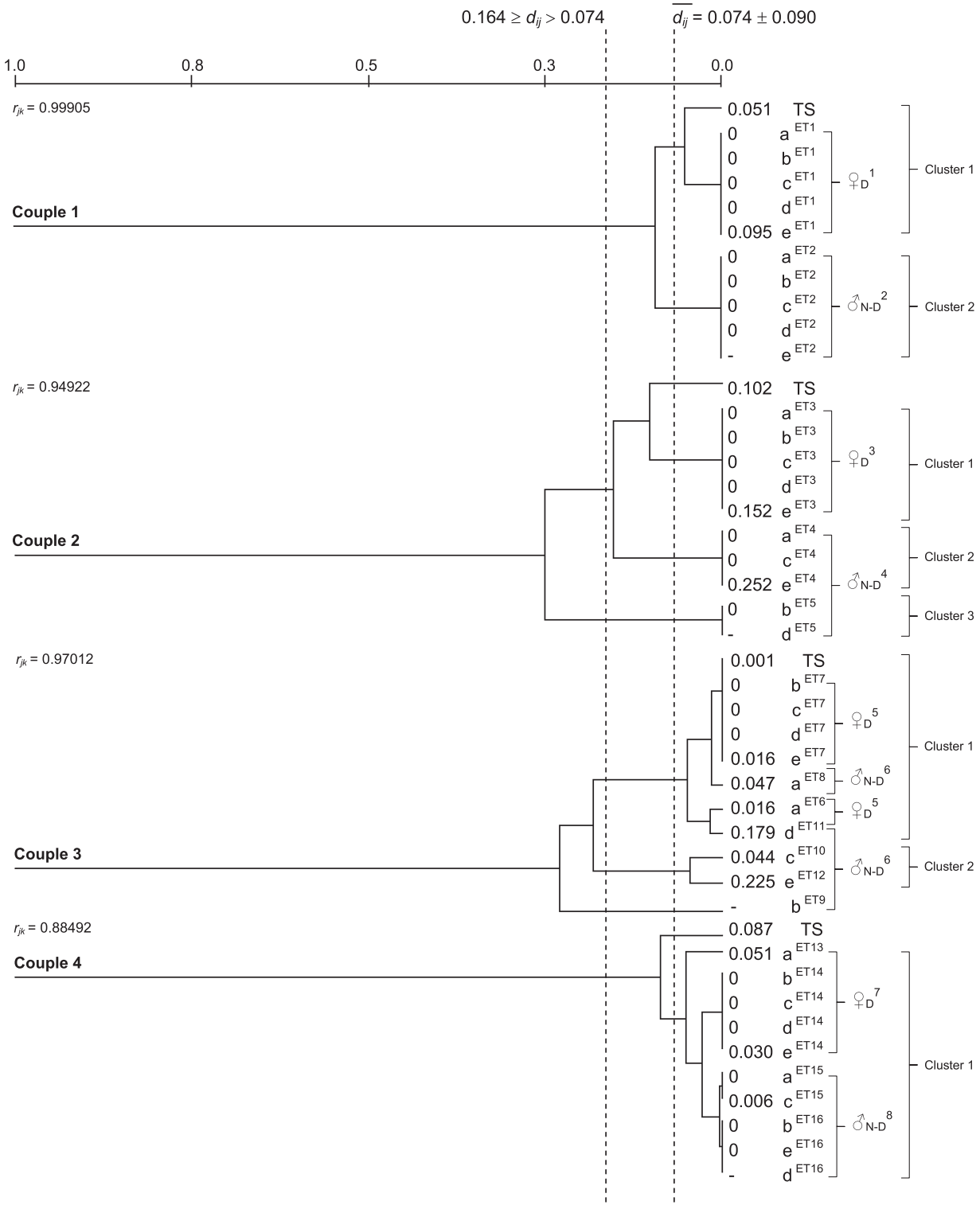

Fig. 1. Genetic diversity of oral isolates of *Candida albicans*, or *C. albicans* and *Candida dubliniensis*, between couples with clinically diabetic and non-diabetic consorts (10 couples) or exclusively diabetic consorts (two couples). UPGMA dendrograms ($1 \leq r_{jk} \leq 0.83793$ – very good fit) generated from matrices of genetic distance d_{ij} (35). Threshold: $0.074 \geq d_{ij} \geq 0$ (isolates highly related or identical); $0.164 \geq d_{ij} > 0.074$ (isolates moderately related). The letters *a* to *j* correspond to oral isolates (see Table 4) and TS, ^{Cd}, $_{D}$ and $_{N\text{-}D}$ correspond to *C. albicans* type-strain (CBS562), *C. dubliniensis* isolates, diabetic subject, and non-diabetic subject, respectively.

rich diets, smoking, diabetes, Cushing syndrome, immunosuppressive conditions such as human immunodeficiency virus (HIV) infection, malignancies, and nutritional deficiencies. Therefore, conditions such as Sjögren syndrome, radiotherapy of the head and neck, or drugs that reduce salivary secretions could lead to an increased risk of oral candidosis (1).

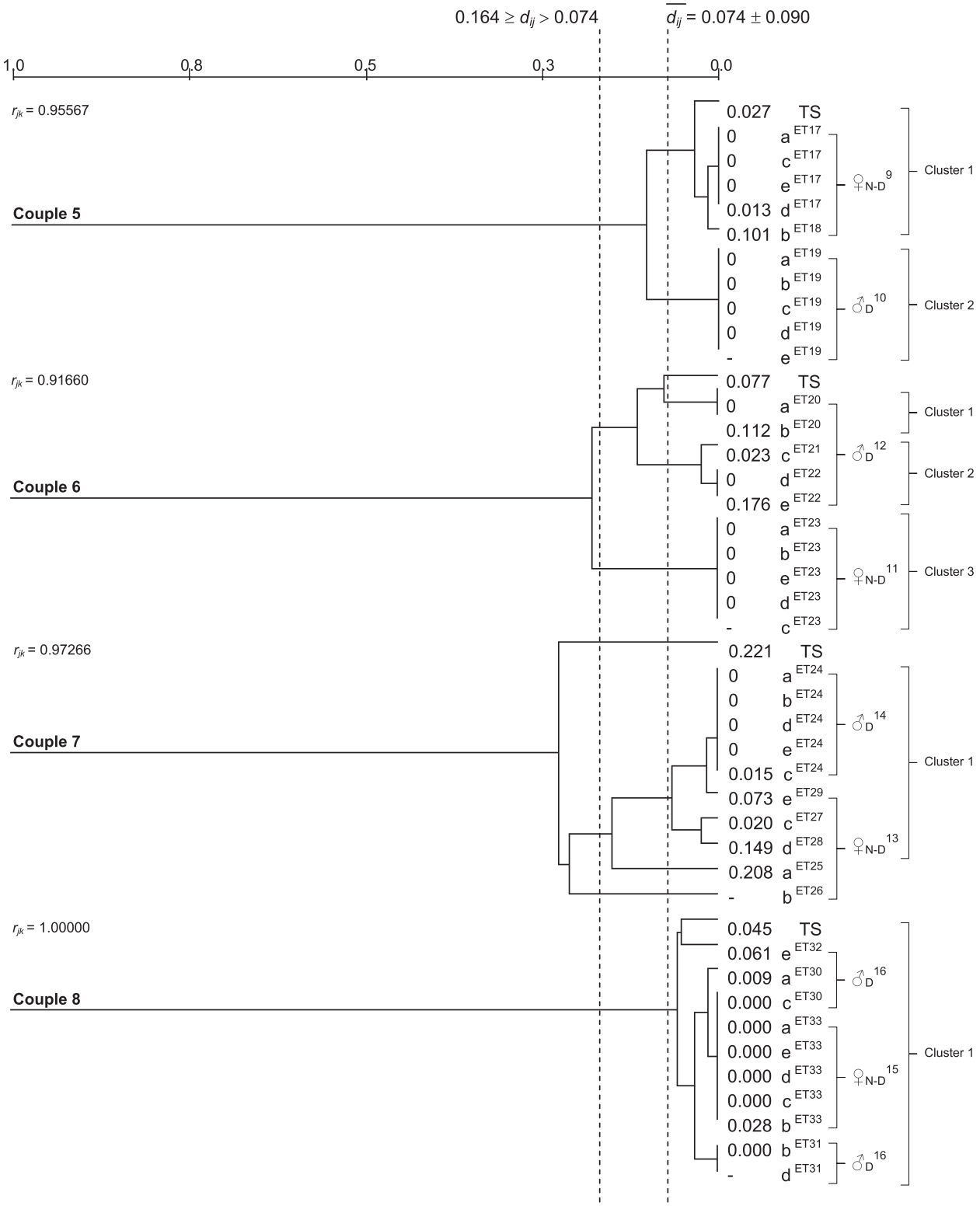

Fig. 1. (Continued)

A recent literature review presented rates of oral carriage of *Candida* in diabetic patients between 18 and 80%, and pointed towards the fact that the wearing of dentures and poor glycaemic control are critical factors that promote oral yeast colonization in these patients (54). *C. albicans* is the most commonly recovered species in more than 80% of *Candida*-positive diabetic patients, and its association with other species of this genus, such as *C. tropicalis*, *C. krusei*, *Candida parapsilosis*, *C. dubliniensis*, *Candida glabrata*, and *Candida rugosa* (13, 14, 58, 64) has been reported. In addition, although

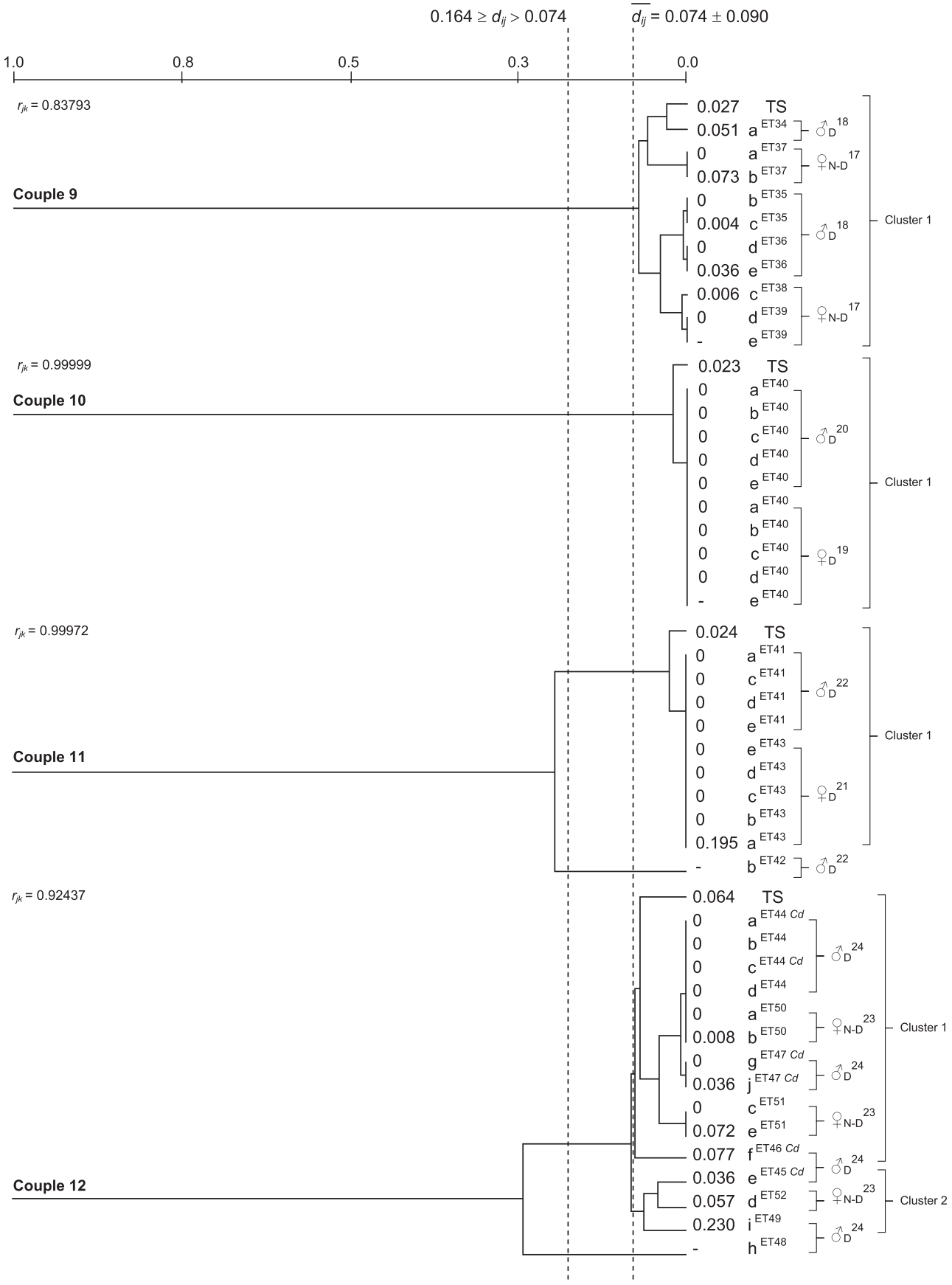

Fig. 1. (Continued)

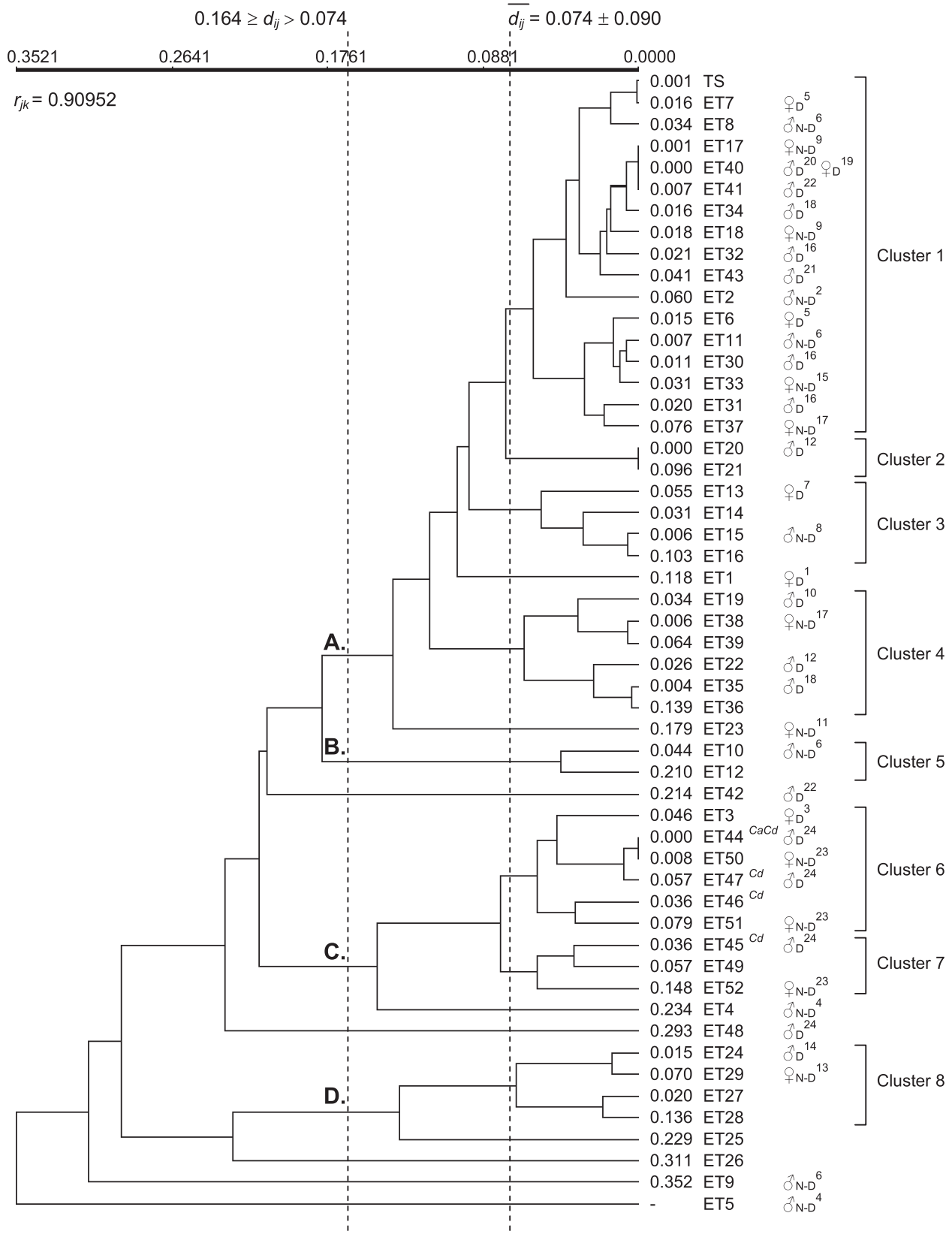

Fig. 2. Genetic diversity of *Candida albicans* and *Candida dubliniensis* strains isolated from oral cavity of clinically diabetic and non-diabetic subjects familially related and unrelated. UPGMA dendrogram (r_{jk} = 0.90952 – very good fit) generated from matrix of genetic distance d_{ij} (35). Threshold: 0.074 ≥ d_{ij} ≥ 0 (isolates highly related or identical); 0.164 ≥ d_{ij} > 0.074 (isolates moderately related). TS, ^{CaCd}, ^{Cd}, $_{D}$ and $_{N\text{-}D}$ correspond to *C. albicans* type-strain (CBS562), oral isolates of *C. albicans* and *C. dubliniensis* identified as same strain (ET – electrophoretic type), oral isolates of *C. dubliniensis*, diabetic subject, and non-diabetic subject, respectively.

Table 4. Enzymatic activity indexes (*Pz*) of aspartyl proteinases (SAPs) and phospholipases (PLs) of *Candida albicans* and *Candida dubliniensis* strains (electrophoretic types; ETs) isolated from the oral cavity of clinically diabetic subjects (four women and six men) and their respective non-diabetic consorts (six women and four men), and from two clinically diabetic couples

Subjects/ Couples	Isolate	Strains	*Pz**		Subjects/ Couples	Isolate	Strains	*Pz*		Subjects/ Couples	Isolate	Strains	*Pz*	
			PLs	Saps				PLs	Saps				PLs	Saps
♀$_{D}^{1}$	a	ET1	2	2	♀$_{N\text{-}D}^{9}$	a	ET17	1	1	♀$_{N\text{-}D}^{17}$	a	ET37	2	2
	b	ET1	2	2		b	ET18	1	2		b	ET37	2	2
	c	ET1	1	2		c	ET17	1	2		c	ET38	2	2
	d	ET1	1	1		d	ET17	1	2		d	ET39	2	2
	e	ET1	2	2		e	ET17	2	2		e	ET39	1	2
♂$_{N\text{-}D}^{2}$	a	ET2	2	2	♂$_{D}^{10}$	a	ET19	2	1	♂$_{D}^{18}$	a	ET34	2	2
	b	ET2	2	1		b	ET19	2	2		b	ET35	0	2
	c	ET2	1	2		c	ET19	2	1		c	ET35	2	1
	d	ET2	2	2		d	ET19	2	1		d	ET36	2	2
	e	ET2	1	2		e	ET19	2	2		e	ET36	2	2
♀$_{D}^{3}$	a	ET3	1	2	♀$_{N\text{-}D}^{11}$	a	ET23	1	1	♀$_{D}^{19}$	a	ET40	2	2
	b	ET3	1	1		b	ET23	1	1		b	ET40	2	2
	c	ET3	2	1		c	ET23	1	1		c	ET40	2	2
	d	ET3	1	1		d	ET23	1	1		d	ET40	2	2
	e	ET3	2	2		e	ET23	1	1		e	ET40	1	2
♂$_{N\text{-}D}^{4}$	a	ET4	0	2	♂$_{D}^{12}$	a	ET20	1	2	♂$_{D}^{20}$	a	ET40	1	2
	b	ET5	0	2		b	ET20	1	2		b	ET40	2	2
	c	ET4	0	2		c	ET21	1	2		c	ET40	2	1
	d	ET5	0	2		d	ET22	1	2		d	ET40	2	1
	e	ET4	2	2		e	ET22	1	2		e	ET40	2	1
♀$_{D}^{5}$	a	ET6	2	1	♀$_{N\text{-}D}^{13}$	a	ET25	2	2	♀$_{D}^{21}$	a	ET43	2	2
	b	ET7	2	2		b	ET26	2	1		b	ET43	2	2
	c	ET7	1	2		c	ET27	1	2		c	ET43	2	2
	d	ET7	1	2		d	ET28	2	2		d	ET43	2	2
	e	ET7	1	2		e	ET29	1	2		e	ET43	1	2
♂$_{N\text{-}D}^{6}$	a	ET8	1	2	♂$_{D}^{14}$	a	ET24	1	2	♂$_{D}^{22}$	a	ET41	2	2
	b	ET9	1	2		b	ET24	1	1		b	ET42	0	2
	c	ET10	1	2		c	ET24	2	1		c	ET41	2	1
	d	ET11	1	2		d	ET24	1	1		d	ET41	2	2
	e	ET12	1	1		e	ET24	2	2		e	ET41	2	2
♀$_{D}^{7}$	a	ET13	2	2	♀$_{N\text{-}D}^{15}$	a	ET33	2	2	♀$_{N\text{-}D}^{23}$	a	ET50	1	0
	b	ET14	1	2		b	ET33	2	2		b	ET50	1	0
	c	ET14	1	2		c	ET33	2	2		c	ET51	1	0
	d	ET14	1	2		d	ET33	2	2		d	ET52	1	0
	e	ET14	2	2		e	ET33	1	2		e	ET51	1	0
♂$_{N\text{-}D}^{8}$	a	ET15	1	2	♂$_{D}^{16}$	a	ET30	1	2	♂$_{D}^{24}$	a^{Cd}	ET44	2	2
	b	ET16	0	2		b	ET31	2	2		b	ET44	2	2
	c	ET15	0	2		c	ET30	2	1		c^{Cd}	ET44	1	1
	d	ET16	0	2		d	ET31	2	1		d	ET44	1	1
	e	ET16	0	2		e	ET32	2	1		e^{Cd}	ET45	2	2
											f^{Cd}	ET456	1	2
											g^{Cd}	ET47	0	2
											h	ET48	0	2
											i	ET49	2	2
											j^{Cd}	ET47	0	2

*Index 0 means absence of enzymatic activity ($Pz = 1$), index 1 means positive enzymatic activity ($1 > Pz \geq 0.64$) and index 2 means strongly positive enzymatic activity ($Pz < 0.64$). ^{Cd}, $_{D}$ and $_{N\text{-}D}$ corresponding to *C. dubliniensis* isolates, diabetic subject, and non-diabetic subject, respectively.

ambiguity exists in the literature concerning *Candida* density in diabetic patients and control subjects (non-diabetic) [i.e. a significantly higher candidal density in diabetic patients than in control subjects (11, 57) or lower rates of yeast carriage in the oral cavities of diabetic patients than in control subjects (3, 27)], there is a significant proportion of patients without clinical symptoms of oral candidosis associated with densities of yeasts above 100 CFU/ml.

The use of the MLEE method was highly reproducible and its genetic interpretation was consistent with the diploid nature of *C. albicans* (49). Patterns of monoclonal and polyclonal oral colonization by *C. albicans* were identified in one and 11 couples, respectively. These patterns could also be observed independent of clinical condition (diabetic or non-diabetic) or gender (male or female) of the consorts. In addition, some *C. dubliniensis* strains exclusively isolated from one clinically diabetic male showed a polyclonal pattern of oral colonization. A total of 52 strains (ETs) (41.6% of the total isolates) were identified in the population of oral yeasts (119 *C. albicans* isolates and six *C. dubliniensis* isolates). Using the same methodology, research into the genetic diversity of *C. albicans* from immunosuppressed and immunocompetent patients showed similar results (6, 7, 10, 25, 29, 41, 62). The cluster analysis using statistic d_{ij} (35) and UPGMA dendrograms successfully explained the relationships between the oral strains (12, 51, 52), according to the Pearson product–moment correlation coefficient values (r_{jk}) [(i.e. high concordance between the d_{ij} (genetic distance matrices) and C_{jk} elements (correlation matrices derived from UPGMA dendrograms)].

These analyses revealed the coexistence of identical or highly related strains, distributed as one or more clusters, in the oral cavity of both members of eight couples (66.7%) suggesting possible

sources of transmission and oral spread of *C. albicans*. Considering that highly related strains come from a common ancestor, whose descendants have undergone microevolutions and adaptations (53), our results also suggest a conjugal genotypic identity [i.e. both members share identical (couple 10) or highly related (couples 3, 4, 7, 8, 9, 11, and 12) strains]. However, groups of isolates or oral strains that are moderately or distantly genetically related can occur at a lower frequency in one or both members of certain couples, suggesting the existence of strains better adapted to oral niches exclusive to one consort or an absence of oral transmission, respectively. Family distribution of *C. albicans* strains from oral and fecal samples have previously been analysed between family members using three DNA fingerprinting assays (31). An intrafamilial genotypic identity (i.e. members of a family sharing the same strain), with two or more members colonized by the same strain (genotype) was observed in culture-positive families (12 of 33 families analysed). In addition, each family carried a distinct strain and no two families shared the same strains (i.e. absence of interfamilial identical strains). However, distinct strains between husbands and wives also constituted the intrafamilial colonization pattern (31). In cases in which *C. albicans* isolates were obtained from the male partner, one or more of these isolates were either identical or highly similar to the vulvovaginal isolates from the female patient. It was suggested that in the majority of cases of both patients with single episodes of infection and patients with recurrent infections, the healthy male partners carry the same general strain responsible for the vaginal infection. Furthermore, in patients with recurrent vulvovaginal infections, different substrains of the established clone dominate in an apparently random fashion termed 'substrain shuffling'. However, the commensal substrain of the male can also differ from the infecting substrain of the female (26). In other research, the transmission of an isogenic *C. albicans* strain within an HIV-infected family, as well as the existence of highly related, azole-resistant *C. albicans* strains among family members, were demonstrated, in a 5-year prospective study, using three molecular methods (inter-repeat polymerase chain reaction, Southern blot hybridization with a *C. albicans* repetitive element 2 probe, and electrophoretic karyotyping). Such findings also reveal that transmission from symptomatic to asymptomatic family members is possible and perhaps represents a previously underappreciated factor in families with HIV infection, among whom more than one family member is at risk of serious and chronic complications of oropharyngeal candidosis (33).

The genetic diversity observed in the total yeast population revealed four larger groups (A through D) and some strains that were distantly genetically related. Of these, one group hosted a higher number of clusters and yeasts (68.8% of the isolates or 57.7% of the ETs) characterizing the coexistence of strains in couples or consorts not conjugally related and, therefore, suggesting that a determined conjugal genotypic identity is not exclusive to a couple that has a single clinically diabetic member (i.e. in the majority of the population of couples the conjugal genotypic identity is not couple specific). However, the exclusive existence of strains in a specific couple may occur less frequently and in smaller genetic groups (i.e. couple-specific conjugal genotypic identity). The genetic relatedness of oral isolates of *C. albicans* from two geographically different patient groups (London, UK and Parma, Italy) affected by diabetes mellitus and from non-diabetic healthy subjects was also examined using polymerase chain reaction fingerprinting (28). Subsequent phylogenetic analysis revealed that there were significant differences between *C. albicans* isolates indicative of the distinct ecological niches that occur in the oral cavities of these patient cohorts. The most diverse group comprised the isolates from the diabetic patients in the UK, possibly reflecting the antifungal treatment that these patients had received (i.e. isolates from UK patients had greater resistance to azole agents and a trend toward greater resistance to amphotericin B than those from diabetic patients from Parma) and the patient cohort from which these strains were isolated (i.e. patients in the UK affected by longstanding diabetes mellitus and with more diabetic complications, including neuropathies and retinopathies, than patients from Italy) (28).

Proteolytic or lipolytic exoenzymes seem to play an important role in pathogenicity (20, 63). Researchers have suggested that enzymatic secretion from *C. albicans* oral isolates, especially SAPs (30) and PLs (15, 63) is determinant not only for pathogenic potential but also for commensal colonization of such yeasts (56). In the present study, aspartyl proteinase and phospholipase exoenzymatic activities were observed in 95.8% (high and low indexes) and 90.7% (high and low indexes) of *C. albicans* oral isolates, and in 100 and 66.7% of the *C. dubliniensis* isolates, respectively. No association was observed between exoenzymatic activities and strains (ETs), strain clusters/highly related isolates, or even, subject characteristics (diabetic, non-diabetic, and gender). The absence of association between a particular genotype or cluster of strains of *C. albicans* and the type, disease progression, and degree of control of diabetes mellitus (28), the production of exoenzymes, and the oral cavity sites (periodontal pocket, gingival sulcus, and oral mucosa) has been observed (5). In addition, a few studies found that enzymatic secretions varied for each isolate from different strains and origins (30, 63). Literature reports that from 30 to 100% of *C. albicans* oral isolates produce PLs, with variable indexes of enzymatic activity (16, 30, 40, 61). These variations could be the result of factors such as the source of isolates, the high phenotypic variability of a certain isolate, or even variations in the methodology. However, it was suggested that only the presence or absence of enzymatic activity is a precise criterion for *C. albicans* biotyping (61).

Finally, some oral *C. dubliniensis* strains exclusively isolated from one clinically diabetic male were highly related (ET45, ET46, and ET47) or identical (ET44) to some *C. albicans* strains from his consort (Fig. 1) or from non-related consorts (Fig. 2). This suggests the use of the MLEE method for molecular epidemiological and genetic intraspecific findings rather than the application for differentiation and complementary identification of *C. dubliniensis* (45), although there is a need to confirm this hypothesis using a larger number of isolates of both species.

We reported the simultaneous oral colonization by potentially virulent (*in vitro*) *C. albicans* to be highly prevalent between both members (diabetic and non-diabetic consorts) of 12 couples (23.1%) without clinical symptoms of oral candidosis. Isoenzyme markers revealed patterns of monoclonal and polyclonal oral colonization independent of clinical condition (diabetic or non-diabetic) and gender (male or female) of the consorts or hydrolytic enzyme characteristics (SAPs and PLs) of the pathogen. A conjugal genotypic identity, i.e. both members share identical or highly related strains, as well as possible sources of transmission and oral spread of *C. albicans* between both members of eight couples were also suggested. However in the majority of couples this conjugal genotypic identity is not

couple specific, as indicated by the coexistence of identical or highly related strains in couples and unrelated consorts.

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References

1. Akpan A, Morgan R. Oral candidosis. Postgrad Med J 2002: **78**: 455–459.
2. Alfenas AC. Eletroforese de isoenzimas e proteínas afins: fundamentos e aplicações em plantas e microrganismos. Viçosa, Minas Gerais, Brazil: Editora UFV, 1998.
3. Al-Karaawi ZM, Manfredi M, Waugh ACW et al. Molecular characterization of *Candida* spp. Isolated from the oral cavities of patients from diverse clinical settings. Oral Microbiol Immunol 2002: **17**: 44–49.
4. Andriole VT. The 1998 Garrod lecture. Current and future antifungal therapy: new targets for antifungal agents. J Antimicrob Chemother 1999: **44**: 151–162.
5. Barros LM, Boriollo MFG, Alves ACBA, Klein MI, Gonçalves RB, Höfling JF. Genetic diversity and exoenzyme activities of *Candida albicans* and *Candida dubliniensis* isolated from the oral cavity of Brazilian periodontal patients. Arch Oral Biol 2008: **53**(12): 1172–1178.
6. Boerlin P. Applications of multilocus enzyme electrophoresis in medical microbiology. J Microbiol Methods 1997: **28**: 221–231.
7. Boriollo MFG, Rosa EAR, Gonçalves RB, Höfling JF. Parity among interpretation methods of MLEE patterns and disparity among clustering methods in epidemiological typing of *Candida albicans*. J Microbiol Methods 2006: **64**(3): 346–365.
8. Candido RC, Azevedo RV, Komesu MC. Enzyme typing of species of the genus *Candida* isolated from the oral cavity. Rev Soc Bras Med Trop 2000: **33**(5): 437–442.
9. Cannon RD, Holmes AR, Mason AB, Monk BC. Oral *Candida*: clearance, colonization, or candidosis? J Dent Res 1995: **74**: 1152–1161.
10. Caugant DA, Sandven P. Epidemiological analysis of *Candida albicans* strains by multilocus enzyme electrophoresis. J Clin Microbiol 1993: **31**: 215–220.
11. Dorocka-Bobkowska B, Budtz-Jorgensen E, Wloch S. Non-insulin-dependent diabetes mellitus as a risk factor for denture stomatitis. J Oral Pathol Med 1996: **25**: 411–415.
12. Farris JS. On the cophenetic correlation coefficient. Syst Zool 1969: **18**: 279–285.
13. Fisher BM, Lamey PJ, Samaranayake LP, MacFarlane TW, Frier BM. Carriage of *Candida* species in the oral cavity in diabetic patients: relationship to glycaemic control. J Oral Pathol 1987: **16**: 282–284.
14. Fongsmut T, Deerochanawong C, Prachyabrued W. Intra oral *Candida* in Thai diabetes patients. J Med Assoc Thai 1998: **81**: 449–453.
15. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 2000: **13**(1): 122–143.
16. Hannula J, Saarela M, Dogan B et al. Comparison of virulence factors of oral *Candida dubliniensis* and *Candida albicans* isolates in healthy people and patients with chronic candidosis. Oral Microbiol Immunol 2000: **15**(4): 238–244.
17. Hill LV, Tan MH, Pereira LH, Embil JA. Association of oral candidosis with diabetic control. J Clin Pathol 1989: **42**: 502–505.
18. Hunter PR. A critical review of typing methods for *Candida albicans* and their applications. Crit Rev Microbiol 1991: **17**: 417–434.
19. Jurevic RJ, Bai M, Chadwick RB, White TC, Dale BA. Single-Nucleotide Polymorphisms (SNPs) in human *β*-defensin 1: high-throughput SNP assays and association with *Candida* carriage in type I diabetics and nondiabetic controls. J Clin Microbiol 2003: **41**: 90–96.
20. Kaminishi H, Miyaguchi H, Tamaki T et al. Degradation of humoral host defense by *Candida albicans* proteinase. Infect Immun 1995: **63**(3): 984–988.
21. Kirkpatrick WR, Revankar SG, McAtee RK et al. Detection of *Candida dubliniensis* in oropharyngeal samples from human immunodeficiency virus-infected patients in North America by primary CHROMagar *Candida* screening and susceptibility testing of isolates. J Clin Microbiol 1998: **36**: 3007–3012.
22. Kwon-Chung KJ, Bennett JE. Medical mycology. Philadelphia, (PA): Lea and Febiger, 1992.
23. Lamey PJ, Darwaza A, Fisher BM, Samaranayake LP, Macfarlane TW, Frier BM. Secretor status, candidal carriage and candidal infection in patients with diabetes mellitus. J Oral Pathol 1988: **17**: 354–357.
24. Lane T, Garcia JR. Phospholipase production in morphological variants of *Candida albicans*. Mycoses 1991: **6**: 217–220.
25. Le Guennec R, Reynes J, Mallie M, Pujol C, Janbon F, Bastide J-M. Fluconazole- and intraconazole-resistant *Candida albicans* strain from AIDS patients: multilocus enzyme electrophoresis analysis and antifungal susceptibilities. J Clin Microbiol 1995: **33**: 2732–2737.
26. Lockhart S, Reed B, Soll DR. Most frequent scenario for recurrent *Candida* vaginitis is strains maintenance with ''substrain shuffling'': demonstration by sequential DNA fingerprinting with probes Ca3, C1, and CARE2. J Clin Microbiol 1996: **34**: 767–777.
27. Manfredi M, McCullough MJ, Al-Karaawi ZM, Hurel SJ, Porter SR. The isolation, identification and molecular analysis of *Candida* spp. isolated from the oral cavities of patients with diabetes mellitus. Oral Microbiol Immunol 2002: **17**: 181–185.
28. Manfredi M, McCullough MJ, Al-Karaawi ZM, Vescovi P, Porter SR. Analysis of the strain relatedness of oral *Candida albicans* in patients with diabetes mellitus using polymerase chain reaction-fingerprinting. Oral Microbiol Immunol 2006: **21**: 353–359.
29. Mata AL, Rosa RT, Rosa EAR, Gonçalves RB, Höfling JF. Clonal variability among oral *Candida albicans* assessed by allozyme electrophoresis analysis. Oral Microbiol Immunol 2000: **15**: 350–354.
30. McMullan-Vogel CG, Jude HD, Ollert MW, Vogel CW. Serotype distribution and secretory acid proteinase activity of *Candida albicans* isolated from the oral mucosa of patients with denture stomatitis. Oral Microbiol Immunol 1999: **14**(3): 183–189.
31. Mehta SK, Stevens DA, Mishra SK, Feroze F, Pierson DL. Distribution of *Candida albicans* genotypes among family members. Diagn Microbiol Infect Dis 1999: **34**: 19–25.
32. Mosca CO, Moragues MD, Liovo J, Al Mosaid A, Coleman DC, Ponto J. Casein agar: a useful medium for differentiating *Candida dubliniensis* from *Candida albicans*. J Clin Microbiol 2003: **41**: 1259–1262.
33. Müller F-MC, Kasai M, Francesconi A et al. Transmission of an azole-resistant isogenic strain of *Candida albicans* among human immunodeficiency virus-infected family members with oropharyngeal candidosis. J Clin Microbiol 1999: **37**(10): 3405–3408.
34. Na B-K, Lee S-I, Kim S-O et al. Purification and characterization of extracellular aspartic proteinase of *Candida albicans*. J Microbiol 1997: **35**: 109–116.
35. Nei M. Genetic distances between populations. Am Naturalist 1972: **106**: 283–292.
36. Odds FC, Bernaerts R. CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J Clin Microbiol 1994: **32**: 1923–1929.
37. Pasteur N, Pasteur G, Bonbomme F, Catalan J, Britton-Davidian J. Manuel technique de génétique par électrophorèse dês proteins. Technique et documentation. Paris, France: Lavoisier, 1987.
38. Pfaller MA, Diekema DJ. Epidemiology of invasive candidosis: a persistent public health problem. Clin Microbiol Rev 2007: **20**(1): 133–163.
39. Pinjon E, Sullivan D, Salkin I, Shanley D, Coleman D. Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. J Clin Microbiol 1998: **36**: 2093–2095.
40. Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia 1982: **20**(1): 7–14.
41. Pujol C, Joly S, Lockhart SR, Noel S, Tibayrenc M, Soll DR. Parity among the randomly amplified polymorphic DNA method, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive DNA probe Ca3 for fingerprinting *Candida albicans*. J Clin Microbiol 1997: **35**: 2348–2358.
42. Revankar SG, Dib OP, Kirkpatrick WR et al. Clinical evaluation and microbiology of oropharyngeal infection due to fluconazole resistant *Candida* in human immunodeficiency virus-infected patients. Clin Infect Dis 1997: **26**: 960–963.

43. Rex JH, Rinaldi MG, Pfaller MA. Resistance of *Candida* species to fluconazole. Antimicrob Agents Chemother 1995: **39**: 1–8.
44. Rohlf FJ. NTSYS-pc numerical taxonomy and multivariate analysis system. New York, NY: Exeter Software Publishing, 1988.
45. Rosa EA, Rosa RT, Boriollo MF, Bernardo WL, Höfling JF. Oral *Candida albicans* and *Candida dubliniensis* differentiation by multilocus enzyme electrophoresis and sodium dodecylsulphate-polyacrylamide gel electrophoresis. Rev Argent Microbiol 2003: **35**(1): 24–28.
46. Samaranayake LP, MacFarlane TW, Lamey PJ, Ferguson MM. A comparison of oral rinse and imprint sampling techniques for the detection of yeast, coliform and *Staphylococcus aureus* carriage in the oral cavity. J Oral Pathol 1986: **15**: 386–388.
47. Samaranayake LP. Oral candidosis: an old disease in new guises. Dent Update 1990: **17**: 36–38.
48. Samaranayake YH, Samaranayake LP, Pow EH, Beena VT, Yeung KW. Antifungal effects of lysozyme and lactoferrin against genetically similar, sequential *Candida albicans* isolates from a human immunodeficiency virus-infected southern Chinese cohort. J Clin Microbiol 2001: **39**: 3296–3302.
49. Scherer S, Magee PT. Genetics of *Candida albicans*. Microbiol Rev 1990: **54**: 226–241.
50. Segal E, Baum GL. Pathogenic yeasts and yeast infections. Boca Raton, FL: CRC Press, Inc., 1994.
51. Sneath PHA, Sokal RR. Numerical taxonomy. San Francisco, CA: W. H. Freeman and Company, 1973.
52. Sokal RR, Rohlf FJ. The intelligent ignoramus, an experiment in numerical taxonomy. Taxon 1970: **19**: 305–319.
53. Soll DR. The ins and outs of DNA fingerprinting the infectious fungi. Clin Microbiol Rev 2000: **13**: 322–370.
54. Soysa NS, Samaranayake LP, Ellepola ANB. Diabetes mellitus as a contributory factor in oral candidosis. Diabetic Med 2005: **23**: 455–459.
55. Stenderup A. Oral mycology. Acta Odontol Scand 1990: **48**(1): 3–10.
56. Sugita T, Kurosaka S, Yajitate M, Sato H, Nishikawa A. Extracellular proteinase and phospholipase activity of three genotypic strains of a human pathogenic yeast, *Candida albicans*. Microbiol Immunol 2002: **46**(12): 881–883.
57. Tapper-Jones LM, Aldred MJ, Walker DM, Hayes TM. Candidal infections and populations of *Candida albicans* in mouths of diabetics. J Clin Pathol 1981: **34**: 706–711.
58. Tekeli A, Dolapci I, Emral R, Cesur S. *Candida* carriage and *Candida dubliniensis* in oropharyngeal samples of type-1 diabetes-mellitus patients. Mycoses 2004: **47**: 315–318.
59. Tintelnot K, Haase G, Seibold M et al. Evaluation of phenotypic markers for selection and identification of *Candida dubliniensis*. J Clin Microbiol 2000: **38**: 1599–1608.
60. Ueta E, Tanida T, Yoneda K, Yamamoto T, Osaki T. Increase of *Candida* cell virulence by anticancer drugs and irradiation. Oral Microbiol Immunol 2001: **16**: 243–249.
61. Williamson MI, Samaranayake LP, MacFarlane TW. Phospholipase activity as a criterion for biotyping *Candida albicans*. J Med Vet Mycol 1986: **24**(5): 415–417.
62. Willis AM, Coulter WA, Fulton CR, Hayes JR, Bell PM, Lamey PJ. Oral candidal carriage and infection in insulin-treated diabetic patients. Diabet Med 1999: **16**: 675–679.
63. Willis AM, Coulter WA, Fulton CR, Hayes JR, Bell PM, Lamey PJ. The influence of antifungal drugs on virulence properties of *Candida albicans* in patients with diabetes mellitus. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001: **91**: 317–321.
64. Willis AM, Coulter WA, Sullivan DJ et al. Isolation of *C. dubliniensis* from insulin-using diabetes mellitus patients. J Oral Pathol Med 2000: **29**: 86–90.
65. Zaugg C, Borg-Von Zepelin M, Reichard U, Sanglard D, Monod M. Secreted aspartic proteinase family of *Candida tropicalis*. Infect Immun 2001: **69**(1): 405–412.