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PCR Fingerprinting of *Candida albicans* Associated with Chronic Hyperplastic Candidosis and Other Oral Conditions

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The purpose of this study was to genotype strains of *Candida albicans* to determine whether specific types were associated with chronic hyperplastic candidosis (CHC). A total of 67 candidal isolates from CHC patients ($n = 17$) and from patients with other oral conditions ($n = 21$) were genotyped by PCR fingerprinting employing two interrepeat primer combinations (1245 and 1246 primers or 1251 primer) and a single minisatellite-specific M13 primer. The most suitable primer for fingerprint analysis was found to be primer 1251, yielding well-resolved banding patterns. For the 67 isolates tested, PCR fingerprinting delineated 25 (1245 and 1246 primers), 27 (1251 primer), and 25 (M13 primer) profiles. The majority of *C. albicans* isolates from multiple sites within the mouth produced identical profiles (six out of nine subjects examined). For patients for whom a series of longitudinal isolates was available, strain persistence for up to 7 years was evident for five out of eight individuals, despite episodes of antifungal therapy. Computer-assisted comparison of the interrepeat PCR fingerprints identified seven distinct profiles that were shared among isolates from different individuals. However, no association was evident among isolates of *C. albicans* from specific clinical conditions. Eight isolates that were initially identified as *C. albicans* but having atypical PCR profiles were later confirmed as *Candida dubliniensis*. In conclusion, the genotypic data do not indicate clonal restriction of *C. albicans* with respect to CHC. Furthermore, these results have demonstrated that in the majority of individuals, colonizing populations of *C. albicans* are clonal in nature and exhibit strain persistence.

The opportunistic pathogen *Candida albicans* is responsible for a range of human infections (candidoses). While systemic infection by the organism can occur, particularly in the immunocompromised individual, superficial forms of infection affecting the oral and vaginal mucosa are most prevalent (21). Oral candidosis most frequently presents as removable pseudomembranes or areas of erythema, particularly under dentures. Chronic hyperplastic candidosis (CHC) is a rarer oral infection associated with the invasion of candidal hyphae into the surface epithelium (2). Clinically, CHC appears as a speckled or homogeneous white patch that cannot be removed by gentle scraping of the mucosa, thereby distinguishing it from pseudomembranous and erythematous forms of candidosis. A biopsy of the white patch is required for a definitive diagnosis of CHC and histopathological examination typically reveals a hyperplastic keratinized epithelium that is heavily infiltrated with neutrophils and invaded by candidal hyphae.

The importance of CHC is its reported association with the development of malignancy at the lesional site (2). The role of candida in this dysplastic change remains unclear. It has been suggested that the presence of candida represents a secondary infection within a preexisting altered epithelium. However, clinical resolution of CHC and a reduction in the extent of epithelial dysplasia following systemic antifungal therapy have supported a direct role for candida (11). In addition, a correlation between histologically confirmed fungal invasion and epithelial dysplasia in oral mucosal lesions has been reported (1). Furthermore, the ability of certain *C. albicans* strains to promote neoplastic changes (22) and to produce carcinogenic nitrosamines from saliva (9) has highlighted the potential role of candida in malignant transformation.

There is evidence to suggest that particular strains of *C. albicans* are associated with specific forms of oral candidosis. Examples include biotype differences between isolates from CHC and normal mucosa and phenotypic variation between isolates recovered from speckled and homogeneous forms of CHC (10, 25). However, a recent biotyping investigation performed at Cardiff Dental School failed to identify such clonal restriction of 35 *C. albicans* isolates from CHC (43). The main problem of biotyping *C. albicans* is that a large number of epidemiologically unrelated strains frequently have identical biotypes, with a single profile predominating for the majority of isolates (36).

Genotyping methods based on nucleic acid analysis potentially offer greater discrimination and reproducibility for strain delineation than phenotyping (18). PCR-based strategies have evolved as some of the most useful methods for candidal genotyping. Repetitive DNA sequences exist that display extensive allelic variation, including enterobacterial repetitive intergenic consensus (ERIC) sequences (39) and telomeric DNA (26) in prokaryotes and eukaryotes, respectively. Consensus primers to these elements have been designed to enable PCR amplification of polymorphic loci between neighboring repetitive elements. This procedure, termed interrepeat PCR (IR PCR), generates unambiguous DNA fingerprints that enable differentiation of *C. albicans* strains (37). A similar PCR technique exists in which a single primer specific to the core sequence of

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phage M13 minisatellite DNA is employed to amplify hypervariable genomic DNA sequences (38).

The major advantage of PCR-based typing is that it requires little starting material, has universal application, and is rapid and simple to perform. The discriminatory power of two IR PCR assays has also been found to be comparable to that of the more established complex techniques of total genomic DNA digestion, electrophoretic karyotyping, and Southern hybridization with *C. albicans*-specific probes (3, 37).

Despite the high frequency of commensal carriage of *C. albicans* and its prominence as a major fungal pathogen, little is known concerning its genetic homogeneity, evolution, and persistence during commensalism and infection. The development of genotypic techniques has allowed the assessment of strain relatedness at the genetic level and facilitated epidemiological analysis (35). DNA fingerprinting has been applied to investigate strain persistence during chronic or recurrent infection or relapse following antifungal therapy, microevolution in infecting populations, and the origin of candidosis. Attempts have also been made to identify strain types associated with particular disease conditions, anatomical sites, or geographic locales. These issues underscore the need for fingerprinting methods which provide measures of genetic distance between strains and which are amenable to computer-assisted methods for generating large databases for comparative and retrospective analyses (29).

The present study describes the use of PCR fingerprinting to determine the genetic relatedness among isolates of *C. albicans* in a retrospective analysis of the epidemiology of CHC and other forms of oral candidosis. Three molecular typing assays involving IR PCR ($n = 2$) and M13 PCR fingerprinting were evaluated and the genotypes of *C. albicans* isolates from CHC and non-CHC infections were compared in an effort to determine whether clonal restriction occurs in CHC. In addition, the analysis of sequential isolates was applied longitudinally to monitor *C. albicans* infection and colonization in a group of individuals and the type of isolates at multiple sites within an individual.

MATERIALS AND METHODS

Isolates and patients. Table 1 provides details of the 67 *C. albicans* isolates from the 36 patients used in the study (CHC only, 15 patients; CHC and non-CHC, 2 patients; non-CHC only, 19 patients). CHC lesions were confirmed by biopsy for 15 patients. Recovery of candida from the oral cavity involved the use of an imprint culture on Sabouraud dextrose agar and a differential agar (Pagano-Levin or Chromagar Candida) when defined clinical lesions were evident or an oral rinse in other situations. Isolates were identified based on germ-tube formation and carbohydrate assimilation using the API-20C system (bioMérieux, Basingstoke, United Kingdom) as previously described (41).

Histological investigation. When available, formalin-fixed incision biopsy sections were stained with hematoxylin-eosin and evaluated for the presence of epithelial dysplasia. Sections were also stained by the periodic acid-Schiff technique and evaluated for the presence of candida.

DNA isolation. All biochemicals were purchased from Sigma (Poole, United Kingdom) unless otherwise stated. A modification of a method used to extract *C. albicans* DNA (27) was followed, with the omission of diethyl pyrocarbonate in the spheroplast lysis buffer. Isolates were cultured overnight at 37°C in yeast nitrogen base (Difco, East Molesey, United Kingdom) supplemented with 0.5% (wt/vol) glucose. A 1.5-ml portion of this culture was centrifuged (15,000 rpm for 10 min; Microspin 12; Dupont UK Ltd., Stevenage, United Kingdom) and the cell pellet was resuspended in 1 ml of 1 M sorbitol. Following centrifugation and removal of the supernatant, a 1-ml volume of 1 M sorbitol in 50 mM potassium phosphate buffer (pH 7.5) with 0.1% (vol/vol) 2-mercaptoethanol and 0.2 mg of lyticase per ml was added. The cell lysis solution was then incubated at 30°C for 30 min and the resulting spheroplasts were harvested by centrifugation. The spheroplasts were resuspended in 0.5 ml of 50 mM sodium EDTA buffer (pH 8.5) containing 2 mg of sodium dodecyl sulfate per ml and the preparation was incubated at 70°C for 30 min. A 50-μl portion of 5 M potassium acetate was added to the aqueous layer and the mixture was incubated on ice for 30 min. The supernatant was decanted into 1 ml of absolute ethanol and clarified by centrifugation. The resulting DNA precipitate was washed twice with 200 μl of 70% ethanol and dried. A 100-μl portion of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 10 μg of RNase A per ml was then added to the precipitate. Following a 15-min incubation at 37°C, 200 μl of isopropyl alcohol was added, the mixture was centrifuged, and the resulting DNA precipitate was dried. The DNA was resuspended in TE buffer. DNA was quantified by diluting in sterile water and by measuring the absorbance at 260 nm.

IR PCR genotyping. IR PCR fingerprinting analysis was performed using two sets of interrepeat primers consisting of a prokaryotic primer pair, 1245 and 1246 (37), and a single eukaryotic repeat primer, 1251 (37). Reaction mixtures (50 μl) consisted of 10 mM Tris-HCl (pH 9.0), 50 mM potassium chloride, 0.1% Triton X-100, 2.5 mM magnesium chloride, 0.2 mM (each) dNTPs (Roche Diagnostics Ltd., Lewes, United Kingdom), 1.25 U of *Taq* polymerase (Promega, Southampton, United Kingdom), and 0.5 μM (each) primers 1245 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) and 1246 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) or primer 1251 (5′-TGG GTG TGT GGG TGT GTG GGT GTG-3′) (Life Technologies Ltd., Paisley, United Kingdom). A 5-μl volume of the candidal genomic DNA preparation (approximately 250 ng) in TE buffer was used as the template.

All PCR amplifications were carried out in a PCR Express thermal cycler (Hybaid Ltd., Ashford, United Kingdom). For the prokaryotic repeat primers 1245 and 1246, a program of 35 cycles for 1 min at 94°C, 1 min at 25°C, and 2 min at 74°C, preceded by a 5-min denaturation at 94°C and followed by a final extension cycle at 74°C for 10 min, was used. The eukaryotic repeat primer 1251 required a program consisting of 40 cycles for 1 min at 94°C, 2 min at 52°C, and 3 min at 74°C. Repeating the experiments (DNA extraction and PCR) assessed reproducibility.

M13 PCR fingerprinting. An oligonucleotide derived from the core sequence of the wild-type phage M13 was used as a single primer for M13 PCR fingerprinting (30). Amplification reactions (50 μl) consisted of 5 μl of candidal DNA extract, 10 mM Tris-HCl (pH 9.0), 50 mM potassium chloride, 0.1% Triton X-100, 1.5 mM magnesium chloride, 0.2 mM (each) dNTPs (Roche Diagnostics Ltd.), 3 mM magnesium acetate, 2.5 U of *Taq* polymerase (Promega), and 30 ng of M13 primer (5′-GAG GGT GGC GGT TCT-3′; Life Technologies Ltd.). Thirty-five cycles of 20 s at 94°C, 1 min at 50°C, and 20 s at 72°C were performed, followed by a single extension cycle at 72°C for 6 min. Reproducibility was assessed as described previously for IR PCR.

Agarose gel electrophoresis. A 20-μl portion of each PCR product was added to 4 μl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% Ficoll type 400; Pharmacia Biotech, St. Albans, United Kingdom) and 2 μl of an ethidium bromide solution (500 μg/ml). Sample mixtures were loaded into a 0.75% agarose (Bioline, London, United Kingdom) and 0.75% NuSieve (Flowgen, Ashby de la Zouch, United Kingdom) gel. In order to estimate the size of the PCR products and to perform gel normalization, molecular weight markers consisting of a 100-bp DNA ladder (Life Technologies Ltd.) for primers 1251 and M13 and a 50-bp DNA ladder (Promega) for primers 1245 and 1246 were electrophoresed concurrently with the PCR products at eight-lane intervals according to the manufacturers' instructions. The agarose gel was placed in an electrophoresis apparatus (Flowgen) containing 0.5× Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3) and a potential of 150 V was applied using an LKB 2197 DC transformer (Pharmacia LKB, Uppsala, Sweden) for 5 h at 4°C.

PCR profile analysis. The DNA bands were visualized under UV illumination and the gel image was captured using a GelDoc 1000 system with Molecular Analyst software version 1.0 (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom). The banding profiles for each isolate were compared visually. In addition, IR PCR fingerprints were analyzed by Gelcompar software, version 1.3 (Applied Maths, Kortrijk, Belgium). The gels were normalized in order to avoid inter- and intragel variation and aligned by associating bands of the internal molecular weight standards on each gel with the stored reference positions.

The similarities of the banding profiles of two given isolates were scored using the Dice coefficient of similarity (4). Matrices of similarity coefficients between all possible pairs of strains were calculated and cluster analysis was performed by the unweighted pair group method with arithmetic averages (32). Dendrograms were constructed to reflect the similarities among the strains in the matrix. The optimization feature was enabled which allowed small global shifts of up to 5.0% in patterns that were not perfectly aligned. A tolerance in the band position of 1.5% was applied during the comparison of PCR fingerprinting patterns.

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TABLE 1. Clinical and genotypic data for 67 *C. albicans* isolates used in the present study

Isolate	Date isolated (day.mo.yr)	Patient no.	Infection, location	Severity of dysplasia[c]	Hyphal invasion[d]	Genotype (I/II/III)[e]
576/93	5.10.93	1[a]	CHC, left buccal mucosa	Mild	None	A/a/1
705/93	13.12.93	1	CHC, left buccal mucosa			B/b/2
211/00	13.4.00	2	CHC, right commissure	ND	ND	C/c/3
1190/97	5.11.97	3	CHC, right buccal mucosa	Moderate	+	D/d/1
848/99	12.10.99	4	CHC, tongue	None	±	E/e/4
121/94	9.3.94	5	CHC, tongue	Moderate	++	F/f/5
324LA/94	9.6.94	6[a,b]	CHC, left commissure	Moderate	+	G/g/6
324RA/94	9.6.94	6	CHC, right commissure			G/g/6
324P/94	9.6.94	6	CHC, palate			G/g/6
332T1/97*[f]	4.4.97	6	CHC, tongue	Severe	+	H/h/7
332L1/97*	4.4.97	6	CHC, left commissure			H/h/7
332R1/97*	4.4.97	6	CHC, right commissure			H/h/7
965L/97*	12.9.97	6	CHC, left buccal mucosa			H/h/7
965R/97	12.9.97	6	CHC, right buccal mucosa			G/g/6
490/99*	25.6.99	6	CHC, commissures			H/h/7
455rgh/94	28.7.94	7[b]	CHC, tongue	None	++	I/i/8
455sm/94	28.7.94	7	CHC, tongue			I/i/8
456P/94	28.7.94	7	CHC, palate			I/i/8
458R/94	29.7.94	8	CHC, right buccal mucosa	Mild	+	J/j/9
674/93	23.11.93	9	CHC, tongue	Moderate	±	K/k/10
688/93	1.12.93	10	CHC, right buccal mucosa	Mild	+	L/l/11
376R/98	5.6.98	11[b]	CHC, right buccal mucosa	Mild	+	M/m/12
376L/98	5.6.98	11	CHC, left buccal mucosa			M/m/12
870/99	22.10.99	12	CHC, tongue	ND	ND	N/n/13
600R/93	15.10.93	13[a]	CHC, tongue	None	++	L/l/5
319a/94	1.6.94	13	CHC, tongue			L/l/5
775(1)/99	21.9.99	13	CHC, tongue			L/l/5
153(1)/00	20.3.00	13	CHC, tongue			L/l/5
680/98	16.10.98	14[a]	CHC, palate	Mild	++	A/a/1
80/99	5.2.99	14	CHC, palate			A/a/1
552/99	9.7.99	14	CHC, palate			A/a/1
PTR/94	1994	15[b]	CHC, right buccal mucosa	Moderate	++	L/l/5
134a/94	15.3.94	16[a,b]	CHC, left buccal mucosa	Mild	+	O/o/14
135BM1/94	15.3.94	16	CHC, left buccal mucosa			O/o/14
135BM2/94	15.3.94	16	CHC, left buccal mucosa			O/o/14
135MX1/94	15.3.94	16	CHC, maxilla			O/o/13
135MX2/94	15.3.94	16	CHC, maxilla			O/o/14
655R/93	12.11.93	17	CHC, right buccal mucosa	None	±	N/n/15
640/99	7.8.99	18	Keratosis, tongue	Moderate	+	L/l/11
196D/94	12.4.94	19[b]	Keratosis, denture	Mild	None	L/p/11
196P/94	12.4.94	19	Keratosis, palate			P/q/16
819/99	1.10.99	20	Keratosis, sublingual	None	None	L/l/5
79(1)/99	5.2.99	21	Keratosis, sublingual	None	None	L/r/10
372RB/94	24.6.94	22[b]	Keratosis, right buccal mucosa	None	None	Q/s/17
372F/94	24.6.94	22	Keratosis, palate			F/f/18
372LB/94	24.6.94	22	Keratosis, left buccal mucosa			F/f/18
59a/94	8.2.94	23	Keratosis, right buccal mucosa	None	None	R/t/5
460(1)/98*	21.7.98	24	Keratosis, right buccal mucosa	None	None	H/h/7
PTL/94	1994	15	Keratosis, left buccal mucosa	None	None	L/l/11
408/99	26.5.99	25	SCC, tongue	NA	None	S/u/19
32/00	21.1.00	26	SCC, flap	NA	None	D/d/1
34/00	24.1.00	16	Previous SCC, soft palate	NA	None	O/o/14
689/99	24.8.99	27	White patch, tongue	ND	ND	T/v/20
243/00	28.4.00	28	Lichen planus, right buccal mucosa	Mild	++	K/k/10
679/98	16.10.98	29[a,b]	White patch, right buccal mucosa	ND	ND	L/l/5
209L(1)/99*	26.3.99	29	White patch, left buccal mucosa			U/w/21
209R/99*	26.3.99	29	White patch, right buccal mucosa			U/w/21
626/93	4.11.93	30[a,b]	Polyp, right buccal mucosa	None	None	A/a/1
91R/94	22.2.94	30	Angular cheilitis, right commissure	ND	ND	A/a/1
91L/94	22.2.94	30	Angular cheilitis, left commissure			A/a/1

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TABLE 1—*Continued*

Isolate	Date isolated (day.mo.yr)	Patient no.	Infection, location	Severity of dysplasia[c]	Hyphal invasion[d]	Genotype (I/II/III)[e]
827/99	5.10.99	31	White patch, tongue	ND	ND	V/x/22
718/99	3.9.99	32	White patch, tongue	ND	ND	D/d/1
WK1/93	1993	33[a]	Healthy buccal mucosa	ND	ND	N/n/15
WK2/00	2000	33	Healthy buccal mucosa			N/n/15
DW1/93	1993	34	Healthy buccal mucosa	ND	ND	W/y/23
PB1/93	1993	35	Healthy buccal mucosa	ND	ND	X/z/24
LR1/93	1993	36	Healthy buccal mucosa	ND	ND	Y/a′/25

[a] Individual from whom sequential isolates were available.
[b] Individual from whom isolates from multiple oral sites were available.
[c] ND, not determined; NA, not applicable.
[d] +, high; ++, moderate; ±, low.
[e] DNA composite type: I, primer combination 1245–1246; II, primer 1251; III, primer M13.
[f] *, atypical *C. albicans* profile.
[g] SCC, squamous cell carcinoma.

RESULTS

All 67 isolates of *C. albicans* examined in this study were amenable to typing by both IR PCR- and M13-mediated genotyping. Replicate IR PCR analysis produced identical PCR profiles in all instances. However, variations were observed in the intensities of faintly staining M13 PCR products between reactions. Identical banding profiles were obtained over a 10- to 100-fold range of DNA template concentrations for all three PCRs (data not shown).

The resolution of the banding patterns varied according to the primer choice. Examples of the patterns obtained by the prokaryotic consensus repeat motifs and eukaryotic repeat-like primers are presented in Fig. 1 and 2. Primer combination 1245-1246 generated a profile of unevenly staining polymorphic bands (mean of six bands) in the 100- to 800-bp region for each strain. The single primer 1251 rendered discrete evenly staining banding patterns, with a mean of eight bands per isolate ranging in size from 400 to 1,300 bp. The similarity of IR PCR patterns with primer 1251 among the isolates of *C. albicans* examined was more obvious than with 1245-1246 primer pairing. The M13 primer generated the lowest number of amplification products, typically consisting of several closely packed faint bands in the 800- to 1,200-bp region, occasionally with a predominant single 600-bp band (Fig. 3).

For the 67 isolates examined, visual analysis allowed the categorization of the profiles into 25 (1245 and 1246 primers),

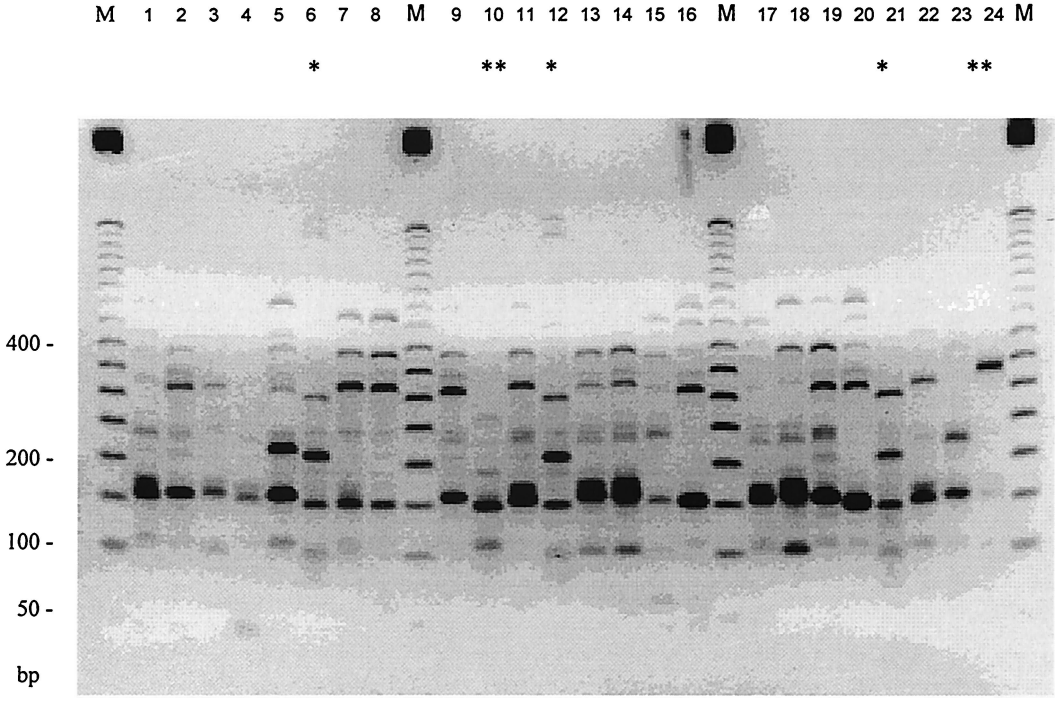

FIG. 1. Representative IR PCR profiles of *Candida* isolates amplified with the primer pair 1245-1246. A negative image is shown. Lanes M, 50-bp ladder, with sizes indicated on the left; lanes 1 to 24, *Candida* isolates 372LB/94, 600R/93, 134a/94, 718/99, 456P/94, 332L1/97*, 376R/98, 376L/98, 775(1)/99, 79(2)/99**, 79(1)/99, 332R1/97*, 552/99, 680/98, 458R/94, 965R/97, 408/99, 91L/94, 243/00, 324LA/94, 209L(1)/99*, 674/93, 59/94, and 45/99**. *, atypical *C. albicans* profile; **, non-*C. albicans*.

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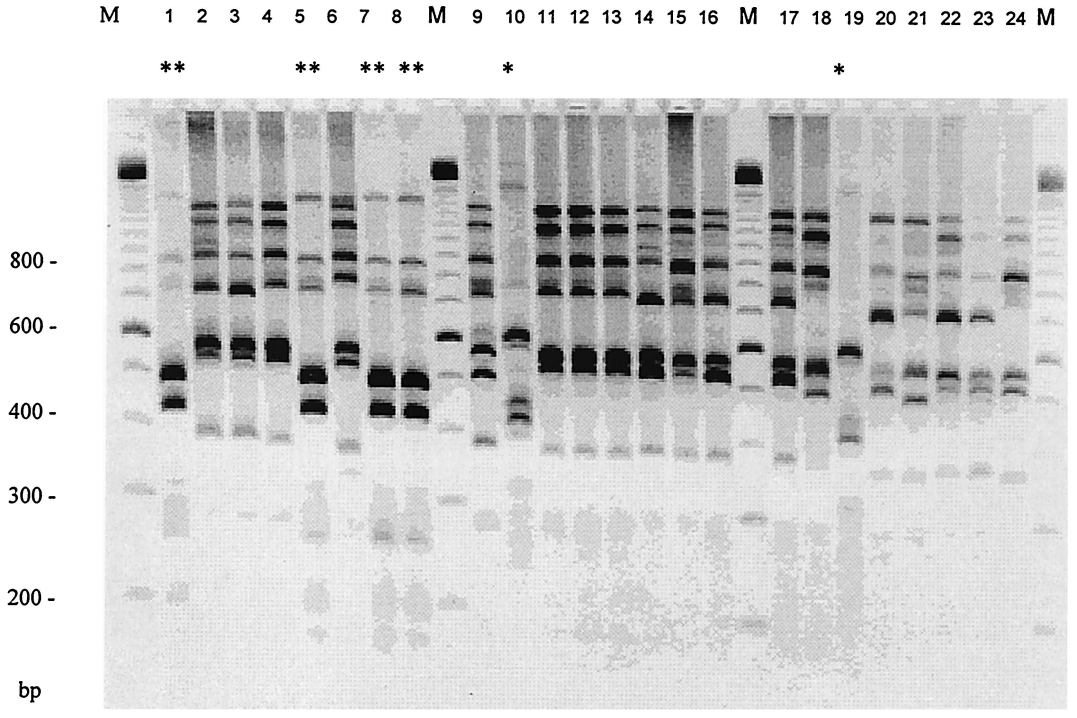

FIG. 2. Representative IR PCR profiles of *Candida* isolates amplified with the 1251 primer. A negative image is shown. Lanes M, 100-bp ladder, with sizes indicated on the left; lanes 1 to 24, *Candida* isolates 153(2)/00**, 153(1)/00, 319b/94, 135BM1/94, 775(2)/99**, 1190/97, 122rgh/94**, 122/94**, 848/99, 332T1/97*, 135BM2/94, 135MX2/94, 135MX1/94, 372RB/94, 870/99, WK1/93, WK2/00, 689/99, 460(1)/98*, DW1/93, 80/99, PTR/94, 688/94, and 211/00. *, atypical *C. albicans* profile; **, non-*C. albicans*.

27 (1251 primer), and 25 (M13 primer) types. The banding patterns obtained were encoded with letters "A" to "Y" (1245 and 1246 primers) and "a" to "a′" (1251 primer) and with numbers "1" to "25" (M13 primer). The results of all the PCR typing assays are collated in Table 1. Generally, there was excellent agreement between the identical profiles identified by the IR PCR techniques. The slight disagreement was caused by the 1251 primer further differentiating strains 196D/94 (L/p) and 79(1)/99 (L/r), which were assigned to an identical genotype by 1245-1246 PCR. Less agreement was found between the identical profiles delineated by M13 PCR and IR PCR. Strain associations derived only from IR PCR were considered further in this study because of the reduced correlation and reproducibility evident for the M13 PCR assay.

For six out of nine individuals from whom isolates were available from multiple oral sites, the same IR PCR pattern was observed for the isolates (Table 1). The remaining three subjects carried two distinct strain types within the oral cavity. Two or more sequential isolates of *C. albicans* were obtained from eight subjects up to 7 years apart, allowing the longitudinal analysis of oral candidal colonization (Table 1). In five of these individuals, strain persistence was demonstrated. One of the three subjects exhibiting strain replacement (patient 6) was colonized with two different strain types that alternated throughout each episode of CHC infection during a 5-year observation period.

Dendrograms based on IR PCR analysis are presented in Fig. 4A (1245-1246 PCR) and Fig. 4B (1251 PCR). Both IR PCR assays identified seven genotypes shared among strains from unrelated hosts at 100% similarity. The clustering capacities of IR PCR methods demonstrated moderate levels of correlation between the assays. On superficial inspection, a closely related IR PCR profile predominated among the majority of the clinical isolates of *C. albicans*. The remainder of the isolates exhibited significantly more variation from the common profile to be placed outside the main group.

Individuals with a specific disease condition were not found to harbor a distinct genotype of *C. albicans* isolates. A comparative examination of the 67 isolates failed to corroborate any obvious differences in the genotypic variability in the CHC and non-CHC patient groups. This was further supported by the fact that in each of the dendrograms, clusters included isolates from both clinical categories. In addition, no subgrouping of the isolates occurred according to the degree of dysplasia or hyphal invasion as assessed from the biopsies of the lesions (data not shown).

DISCUSSION

This study was concerned with the genotypic and comparative analysis of 67 *C. albicans* isolates obtained over a period of 7 years from 36 patients. The study was performed to help clarify the conflicting reports of an association of particular *C. albicans* biotypes with CHC (25, 43). Three PCR fingerprinting methods involving IR PCR (primers 1245 and 1246 and primer 1251) and M13 PCR-mediated genotyping were used to assess the genetic relatedness of *C. albicans* isolates from CHC and non-CHC patient populations.

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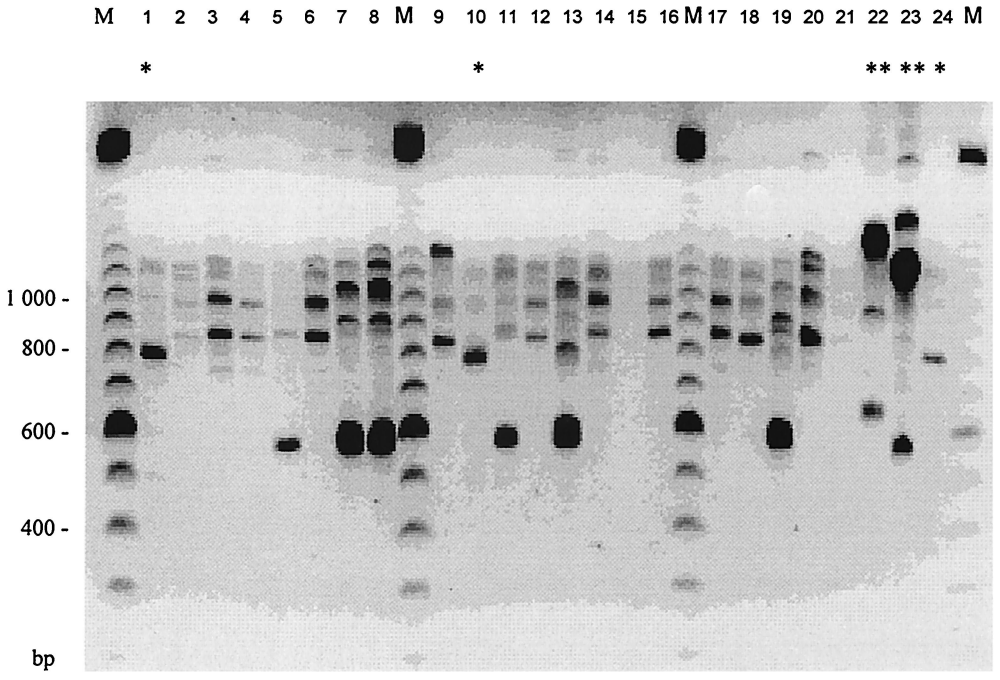

FIG. 3. Representative M13 PCR profiles of *Candida* isolates amplified with the M13 core sequence. A negative image is shown. Lanes M, 100-bp ladder, with sizes indicated on the left; lanes 1 to 24, *Candida* isolates 332T1/97*, 135BM2/94, 135MX2/94, 135MX1/94, 372RB/94, 870/99, WK1/93, WK2/00, 689/99, 460(1)/98*, DW1/93, 80/99, PTR/94, 688/94, 211/00, 455rgh/94, 455sm/94, PB1/93, 372F/94, 91R/94, 196D/94, 684/93**, 458L/94**, and 965L/97*. *, atypical *C. albicans* profile; **, non-*C. albicans*.

Primer 1251 was found to be the most effective primer for the detection of DNA polymorphism between strains of *C. albicans*. Overall, the IR PCR methods were more reproducible than M13 PCR. Variations in the intensities of fragments amplified with the M13 primer, particularly with the low-intensity bands, compromised the reproducibility of the method, as noted previously (34). These low-intensity bands may result from minor variations in the PCR conditions or inefficient amplification due to mismatch between the primer and the target sequence (12, 13). The most appreciable difference between the methods used in this study was the ease of interpreting the PCR banding profiles. The 1251 profiles generated were unambiguous due to the high resolution of the amplified bands and hence were more amenable to computer-assisted analysis. The more complex and uneven patterns generated by ERIC (1245/6) and M13 PCR were found to be less suitable for visual comparison and required considerably more effort in introducing the fingerprinting data into the database. Consequently, the analyses of strain association were restricted to the profiles obtained by IR PCR.

In the present study, one or two individual genotypes colonized any given patient within the oral cavity. The majority of the patients sampled at multiple sites within the mouth (six out of nine) were colonized by a single genotype of *C. albicans*. The finding of a limited number of genotypes within a body location has been interpreted as being indicative of a clonal origin for these strains of commensal and infecting isolates of *C. albicans* (8, 16).

The occurrence of strain persistence in five out of eight individuals is consistent with other epidemiological studies of recurrent candidoses (33). In the present study, the same strain of *C. albicans* was isolated from a CHC lesion on the tongue over a 7-year period in patient 13. Similarly, patient 16, originally diagnosed with CHC in 1994, was found to be colonized by an identical strain of *C. albicans* 6 years later following treatment for oral squamous cell carcinoma. Furthermore, patient 33 was shown to be colonized with the same strain of *C. albicans* when sampled over a 7-year interval from a healthy buccal mucosa. The stability of colonizing populations of *C. albicans* within the mouth highlights the successful adaptation of the organism as a commensal within the oral niche.

Strain replacement has been documented in a minority of cases, due to acquisition of either a new strain of *C. albicans* or a different *Candida* species. This could arise through an initial colonization by multiple strains or by recolonization from an exogenous source (31). Furthermore, reported cases of strain replacement may in part be explained by small sampling numbers (15). In the present study, three patients exhibited strain replacement. Subject 1 possessed two distinct genotypes of *C. albicans* isolated 2 months apart from a CHC lesion on the buccal mucosa. Subject 29 clinically presented with a white patch on the buccal mucosa and from this site two distinct strains were recovered over a period of 6 months. The remaining individual, subject 6, a CHC patient, was found to be colonized by two strain types in the commissure region of the buccal mucosa over 5 years.

It has been postulated that strain diversity may contribute to treatment failure of CHC lesions (25). Strain differentiation by biotyping has indicated the presence of a more homogeneous composition of the oral mycoflora in patients with reversible candidal lesions than in patients with nonreversible lesions. It has been speculated that variations in the genotype could stra-

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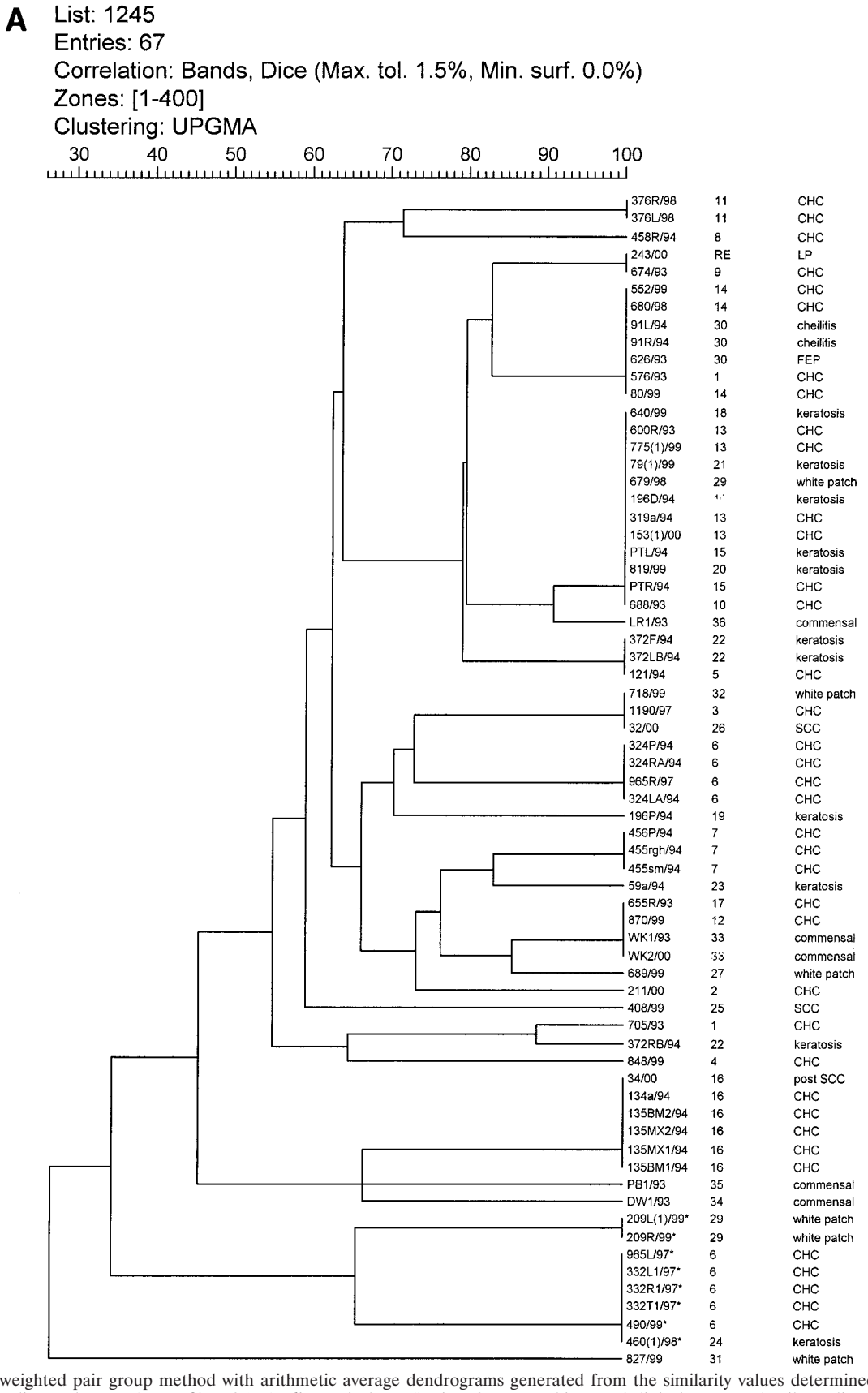

FIG. 4. Unweighted pair group method with arithmetic average dendrograms generated from the similarity values determined by the Dice coefficient according to the IR PCR profiles of 67 *C. albicans* isolates. Strain reference, subject, and clinical category details are listed on the right. LP, lichen planus; SCC, squamous cell carcinoma; FEP, fibroepithelial polyp. (A) Primer combination 1245-1246. (B) Primer 1251. *, atypical profile of *C. albicans*.

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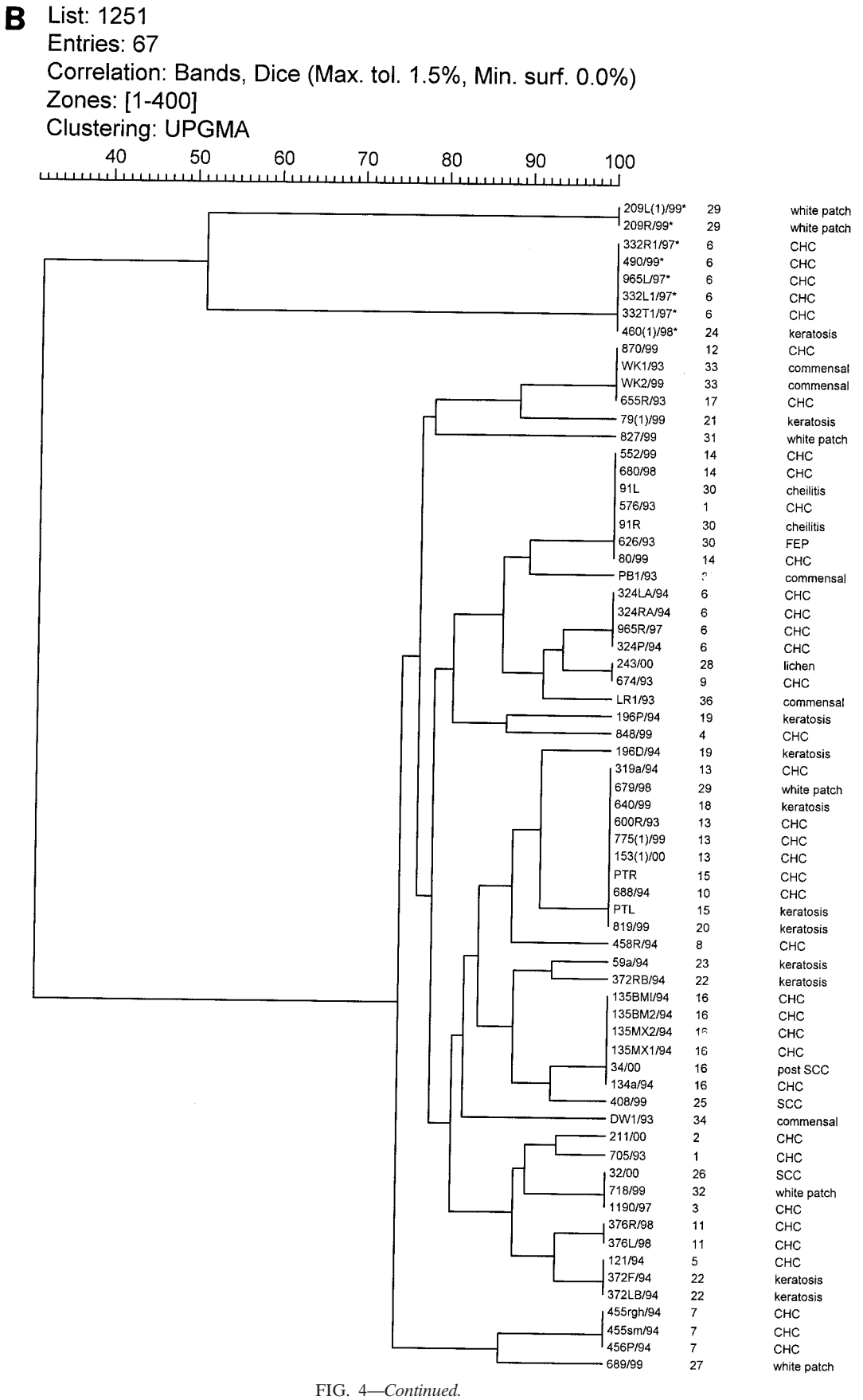

FIG. 4—*Continued.*

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tegically enhance survival of an organism in the human oral cavity and trigger the induction of genes to change from a commensal to an invasive phenotype in response to the local environment (40). Five of the 17 CHC patients included in this study were found to be colonized by *Candida* species other than *C. albicans* (data not shown). Further investigation is required to assess the contribution of these species to CHC progression.

The finding of strain persistence in CHC patients despite the provision of antifungal therapy suggests that the clinical recurrence of the condition may be due to failure of the antifungal therapy to eradicate the colonizing strain. Although topical antifungal therapy has been shown to effectively reduce lesion sizes and resolve clinical symptoms in approximately a third of cases, the prevalence of yeast colonization remains persistently high (14). Groups have expressed caution in using prophylactic antifungals, mindful of the selection and emergence of drug-resistant strains (24). Further studies are required to determine whether the long-term use of antifungals in CHC patients is beneficial. Perhaps the eradication of yeast from the oral cavity is an unrealistic proposition, and instead, efforts should be concentrated on addressing the host factors that predispose to candida infection, in particular smoking.

In the present study, seven genotypes were shared among 35 apparently epidemiologically unrelated patients. Previous molecular epidemiology studies have demonstrated that different persons can harbor the same or very similar genotypes of *C. albicans* (44). Explanations for the close relatedness of patient samples include possible strain transmission and geographical specificity of *C. albicans* colonizing populations (23). Epidemiological studies using repetitive probes have found that in a given patient population a single cluster of genetically related *C. albicans* isolates usually predominates, acting as an etiologic agent in all forms of candidosis, regardless of patient type or infection (28). The widespread nature of these endemic strains may result from natural selection favoring the colonization of a specific genotype of *C. albicans*. The considerable genetic similarity among geographically diverse populations of *C. albicans* isolates has been taken as evidence consistent with a predominantly clonal population structure (5, 6).

Although shared genotypes existed among the patients in the present study, approximately one-half of unrelated individuals (17 out of 36) harbored *C. albicans* strains with unique profiles. The finding of significant strain diversity, with the majority of patients being colonized or infected with their own distinct strain of *C. albicans*, has lent support to the concept of endogenous infection (15).

There was a general agreement between the dendrograms, with the partition of two visually atypical profiles (H/h/7 and U/w/21) from the major clusters of closely related isolates. Subsequent determination of the identity of these atypical *C. albicans* isolates by the API 32C system established that these eight strains were *Candida dubliniensis*. The presence of *C. dubliniensis* isolates within the strain collection was initially overlooked due to reliance on the API 20C system, which is unable to distinguish *C. dubliniensis* isolates from *C. albicans* isolates, for identification (7). Previous workers have utilized the variation in IR PCR profiles to distinguish between the closely related isolates of *C. dubliniensis* and *C. albicans* (19, 20).

The present report appears to be the first that describes a possible association of *C. dubliniensis* with CHC. The finding of *C. dubliniensis* isolates within three individuals, patients 6 (CHC), 24 (keratosis), and 29 (undesignated white patch), raises questions concerning the true prevalence of *C. dubliniensis* associated with candidoses. Further studies to recover candidal DNA from the formalin-fixed sections would be required to confirm the identification of the species invading the oral epithelium of subject 6 (42).

Analysis of the genotypes from the CHC and non-CHC individuals revealed no clonal restriction among the strains. The genetic similarity of individual isolates from the different disease groups was shown to be comparable, with no correlation between the genotypic affiliations and the clinical condition of the patient. It would appear that the CHC patient population is not preferentially infected with a distinct group of *C. albicans* strains. This is in agreement with earlier reports that failed to find a link between a particular isolate genotype and the underlying disease of the respective patient (15, 44). The lack of clonal restriction lends support to the theory that host factors play a significant role in the etiology of oral candidosis, with strain differences perhaps being less important (17).

In conclusion, the present study has confirmed the value of IR PCR-mediated genotyping of *C. albicans* isolates for epidemiological analysis. Of these approaches, the use of primer 1251 was found to generate the most reproducible fingerprint profiles. Results of the study indicate that clonal populations of *C. albicans* are widely distributed and tend to persist within an individual despite antifungal therapy. However, no specific strain types were found to be associated with CHC.

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