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Genetic relatedness of oral yeasts within and between patients with marginal periodontitis and subjects with oral health

Xiaobo Song[1,2], Emenike R. K. Eribe[1], Jinglu Sun[1], B. Frode Hansen[2], Ingar Olsen[1]
[1]Department of Oral Biology and [2]Department of Periodontology, Faculty of Dentistry, University of Oslo, Oslo, Norway

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Background: Yeasts are found in periodontal pockets at a frequency of 15–21%. However, the genetic relatedness of oral yeasts within and between patients with marginal periodontitis is not clear.

Objectives: Assay genetic relatedness of oral yeasts from marginal periodontitis patients and oral health subjects, as well as genetic relatedness of yeasts from different oral sites in these two groups of participants.

Material and methods: Yeast isolates were collected from 23 marginal periodontitis patients and 19 oral health subjects. Random amplified polymorphic DNA (RAPD) fingerprinting and the Dendron computer-assisted program for gel analyses were applied for estimation of genetic relatedness of yeasts.

Results: The similarity coefficient (S_{AB}) of the marginal periodontitis group ranged from 0.49 to 1.00 with an average of 0.64 ± 0.11, whereas the S_{AB} of the oral health group ranged from 0.62 to 1.00 with an average of 0.72 ± 0.07. Three genetic clusters and 73 genotypes were obtained from the marginal periodontitis group, whereas three genetic clusters and 55 genotypes were found in the oral health group. In the pooled dendrogram, 57% of the yeast isolates and the type strain of *Candida albicans* fell in a major cluster V. There were no significant differences between the frequencies of clusters from the different oral sites within the two participant groups.

Conclusions: Genetically heterogeneous yeasts were found in the oral cavities of marginal periodontitis patients and oral health subjects. Similar genetic clustering patterns were obtained from the yeasts of the two groups, with cluster V being most predominant. Yeasts of the marginal periodontitis group were more genetically diverse than yeasts of the oral health group, and some yeasts of the marginal periodontitis group exhibited unique genetic patterns. There was no clear association between yeast genetic clusters and oral sites in the two participant groups.

Xiaobo Song, Department of Oral Biology, Dental Faculty, University of Oslo, P. B. 1052 Blindern, N-0316 Oslo, Norway
Tel: +47 22840350
Fax: +47 22840302
e-mail: xiaobos@odont.uio.no

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Yeasts are regarded as opportunistic commensals in the human mouth. Mucosal surfaces are the primary oral body reservoirs for these microorganisms (1–3). Yeasts can also be found in dental plaque (3, 4). Increasing evidence shows that *Candida* can be associated with periodontal destruction in immunodeficient patients (5–7); however, it is unclear whether subgingival *Candida* strains participate in the pathogenesis of periodontal disease in general.

Recently, the development of molecular methods based on differences in the DNA of yeasts has opened for investigations into the pathogenesis and epidemiology of yeast infections. A number of studies have demonstrated that commensal yeasts dominate in oral candidosis and vaginitis in immunocompromised patients (8, 9), whereas controversial evidence shows that genetically homogeneous, hypervirulent strains of *C. albicans* are involved in disease (6, 10). There is little information on genotype characterization of yeasts in periodontal pockets and other oral sites of patients with marginal periodontitis.

The aims of the present study were to assay (i) the genetic similarity of oral yeasts within and between marginal periodontitis patients and oral health subjects, and (ii) genetic similarity of yeasts from different oral sites of marginal periodontitis patients and oral health subjects.

Materials and methods

Participants

Patient group — Seventy patients (35 males and 35 females) with marginal periodontitis were chosen from the patients who came for periodontal treatment at the Faculty of Dentistry, University of Oslo. The criterion for patient inclusion was at least two teeth with periodontal pockets deeper than 5 mm (3). The exclusion criteria were full or partial maxillar or mandibular dentures, oral mucosal lesions, usage of antibiotics, corticosteroids, immunosuppressive agents, cytotoxic chemotherapy, and irradiation therapy in the last 3 months, pregnancy, endocrine disorders, malignancies, immunodeficiencies, and malnutrition. The age of the patients varied from 14 to 84 years (51.2 ± 12.8 years).

Healthy group — Forty-five individuals (19 males, 26 females) were chosen from the people attending dental examination and staff members of the Faculty of Dentistry, University of Oslo. The inclusion criterion for healthy subjects was no oral infection. The exclusion criteria were the same as for the marginal periodontitis patients. The age of the healthy subjects varied from 16 to 74 (32.9 ± 14.1) years.

Yeast sampling, cultivation and isolation

Yeasts were sampled from periodontal pockets with depths ≥ 5 mm of marginal periodontitis patients and from gingival sulci of oral health subjects, as well as from the buccal and palatal mucosa of each participant. Pooled subgingival plaque samples were collected by inserting two to five sterile paper points (Roeko, Munich, Germany) into two to four of the deepest pockets for 15 s after removing supragingival plaque. Palatal and buccal mucosa samples were taken by streaking the sites with sterile cotton-tipped swabs (Selefatrade, Spanga, Sweden). The samples were streaked directly onto Sabouraud dextrose agar plates at the chair-side. The plates were incubated aerobically at 37°C for 3 days. Colonies exhibiting distinct morphologies from each oral site were picked, subcultured, transferred to liquid Todd Hewitt with dimethylsulfoxide, and stored at −80°C until further use. Sample collection, cultivation and isolation were standardized and performed by one examiner (XS).

Type strains

Nine type strains of yeasts, i.e. *Candida albicans* var. *albicans* CBS 562, *Candida dubliniensis* CBS 7987, *Candida parapsilosis* var. *parapsilosis* CBS 604, *Candida tropicalis* var. *tropicalis* CBS 94, *Candida glabrata* CBS 138, *Candida krusei* var. *krusei* (*Issatchenkia orientalis*) CBS 573, *Candida inconspicua* CBS 180, *Candida norvegensis* (*Pichia norvegensis*) CBS 1922, and *Saccharomyces cerevisiae* CBS 1171 were used in the present study as controls. The strains were purchased directly from the Centraalbureau Voor Schimmelcultures (CBS), Delft, the Netherlands.

Yeast DNA preparation

Yeasts were seeded from frozen stocks onto Sabouraud dextrose agar plates and recultured at 30°C for 24 h. After plating, yeast cells were incubated in 1 ml SPPZ buffer (1 M sorbitol, 50 mM potassium phosphate, 1 mg Zymolase 20T) and 3 µl 0.1% β-mercaptoethanol (Sigma, St Louis, MO, USA) at 37°C for 90 min. The pellets were then incubated in 500 µl GES (60% guanidine thiocyanate, 0.1 M EDTA pH 8, 0.5% lauroylsarcosine) for 30 min at room temperature, then in 100 µl of 5 M potassium acetate (Sigma) for 30 min on ice, and afterwards washed with 500 µl chloroform : pentanol (24:1 v/v) (Sigma). After centrifugation, the upper aqueous phase was transferred to a new Eppendorf tube. An equal volume of cold ethanol was added. After centrifugation and drying, DNA was treated with RNase (Sigma) in 100 µl TE (10mM Tris-Cl + 1 mMEDTA pH8) buffer at 37°C for 30 min, and washed with 200 µl isopropanol. DNA was precipitated by centrifugation, dried, and dissolved in 100 µl of TE buffer. DNA concentrations (A_{260}/A_{280} ratios) were determined with a spectrophotometer (MBA 2000; Perkin-Elmer, Boston, MA, USA).

Random amplified polymorphic DNA fingerprinting

Polymerase chain reaction (PCR) cocktails contained approximately 50 ng yeast DNA as template, PCR buffer (20 mM Tris/HCl, pH 8.4, 50 mM KCl), 200 µM dNTPs, 25 mM $MgCl_2$, 10 pM primer and 1.5 U *Taq* polymerase (Applied Biosystem, Foster City, CA, USA). The primer GC10/1 (5′-CGGTGCGACG-3′) (Invitrogen, Carlsbad, CA, USA) was selected from two primers, GC10/1 and M13, tested in pilot experiments because they were more discriminative than alternatives

tested. Amplification was performed in a GeneAmp PCR system 2700 (Applied Biosystem) with initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 30°C for 1 min, extension at 72°C for 2 min, and finally extension at 72°C for 10 min.

The PCR products were electrophoresed in agarose gels (1.5%) in TBE buffer (89 mM Tris/HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.0) at 150 V for 70 min. Gels were stained with ethidium bromide and the bands were visualized with UV light (302 nm). The images were captured and transferred to a computer by a Kodak DC 120 digital camera (Eastman Kodak Company, New Haven, CT, USA). The experiment was repeated twice involving cultivation, extraction and random amplified polymorphic DNA (RAPD).

Dendron computer-assisted gel analyses

DNA fingerprinting patterns of the yeast isolates on gels were analyzed with the Dendron computer-assisted program version 2.4 (Solltech Inc. Oakadale, IA, USA). A similarity coefficient (S_{AB}) that compares the band positions and intensities was used for analyses of genetic relationships among strains. Coefficients ranging from 0 to 1 reflect increasing similarity that encompasses both an increasing proportion of common bands and intensities. Once S_{AB} values had been computed between all pairs of the collection of isolates, a dendrogram was generated based on S_{AB} by the unweighted pair-group method using arithmetic average (UPGMA) (11). The S_{AB} for the pair of strains, A and B, was calculated by the formula of the L2 method (Dendron User's Manual). In the present study, an average S_{AB} was chosen as a threshold for similar strains clustering, as Pujol *et al.* (12) demonstrated a strong congruence in clustering between RAPD analyses, multilocus enzyme electrophoresis (MLEE) and analyses with Ca3 by using an average S_{AB}.

Statistical analyses

The chi-squared test was used to analyse the relationship between the oral sites and genetic clusters of the marginal periodontitis and oral health group, with a significance level of $p < 0.05$.

Results

Random amplified polymorphic DNA fingerprints of yeast isolates with primer GC10/1

RAPD fingerprints were obtained from 103 yeast isolates of 23 marginal periodontitis patients, 60 isolates of 19 oral health subjects and nine species type strains using the primer GC10/1. The fingerprints of each strain contained one to seven bands ranging from 154 to 1033 kb. The RAPD patterns of the *C. albicans* isolates exhibited an identical principal band at 453 kb from both subject groups, whereas *C. dubliniensis* showed an identical principal band at about 330 kb (Figs 1a and b). Similar patterns were observed with the respective species type strains used for comparison (Fig. 1c).

Genetic relatedness of oral yeasts from marginal periodontitis patients and oral health subjects

The dendrograms were generated from the RAPD fingerprints of 103 yeast isolates from 23 marginal periodontitis patients and 60 isolates from 19 oral health individuals (Figs 2 and 3). Fingerprints of pooled isolates and type strains are shown in Fig. 4.

In the dendrogram of the marginal periodontitis group (Fig. 2), the S_{AB} ranged from 0.49 to 1 with an average of 0.64 ± 0.11. Three genetic clusters (i, ii and iii) were obtained at the threshold of an S_{AB} of 0.64, comprising 73 genotypes, 99 yeast isolates (96%) and all the 23 marginal periodontitis patients. Cluster i and iii were predominant, accounting for 42 isolates (41%) from 14 patients (61%) and 44 (43%) isolates from 12 patients (52%), respectively.

In the dendrogram of the oral health group (Fig. 3), the S_{AB} ranged from 0.62 to 1 with an average of 0.72 ± 0.07. Three genetic clusters (j, jj and jjj) were obtained at the threshold of an S_{AB} of 0.72, comprising 55 genotypes, 58 yeast isolates

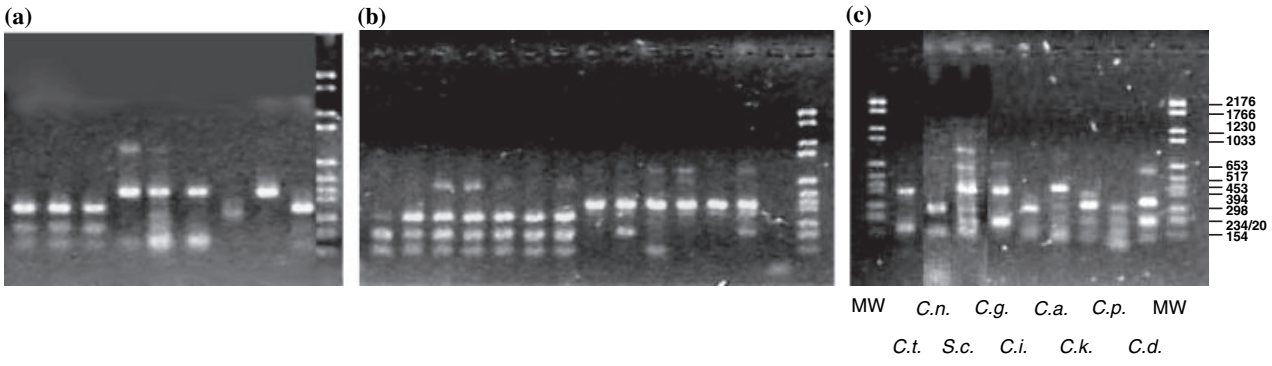

Fig. 1. Random amplified polymorphic DNA (RAPD) fingerprints of yeast isolates from patients with marginal periodontitis (a), subjects with oral health (b) and yeast type strains (c). MW: molecular weight marker VI; *C. n.*: *Candida norvegensis* CBS 1922; *C. g.*: *Candida glabrata* CBS 138; *C. a.*: *Candida albicans* var. *albicans* CBS 562; *C. p.*: *Candida parapsilosis* var. *parapsilosis* CBS 604; *C. t.*: *Candida tropicalis* var. *tropicalis* CBS 94; *S. c.*: *Saccharomyces cerevisiae* CBS 1171; *C. i.*: *Candida inconspicua* CBS 180; *C. k.*: *Candida krusei* var. *krusei* CBS 573; *C. d.*: *Candida dubliniensis* CBS 7987 (see Material and methods for alternative names).

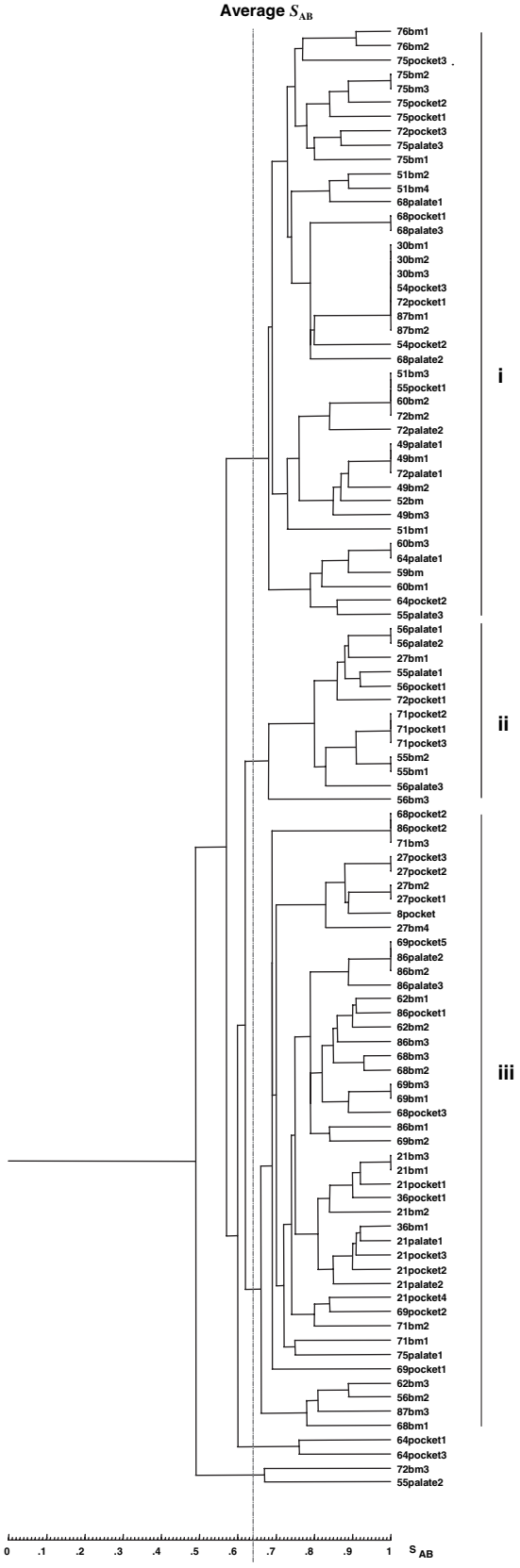

Fig. 2. Dendrogram of oral yeasts of patients with marginal periodontitis. The isolates in the dendrogram are designated as follows: patient number, oral site, and numbers of different colonies. bm: buccal mucosa. i, ii and iii represent genetic clusters.

(97%) and all the 19 oral health individuals. Cluster jjj was most predominant, accounting for 39 isolates (65%) from all the 19 oral health individuals.

In the pooled dendrogram of oral yeasts of marginal periodontitis patients and oral health subjects, and yeast type strains (Fig. 4), the S_{AB} ranged from 0.50 to 1 with an average of 0.66 ± 0.09. Five genetic clusters (I–V) were generated at the threshold of an S_{AB} of 0.66, comprising 128 genotypes, 159 isolates (98%) and all the 42 yeast-positive individuals. Cluster V was the most predominant cluster, accounting for 93 isolates from 34 individuals. Cluster V was also the most predominant cluster of the isolates (40% of marginal periodontitis isolates and 87% of oral health isolates) and the participants (70% of the marginal periodontitis patients and 95% of the oral health individuals). Cluster I consisted of yeast isolates exclusively from one oral health individual, whereas clusters II, III and IV contained isolates exclusively from the marginal periodontitis patients except one (157bm2) from an oral health individual. The type strain CBS 562 of *C. albicans* fell in cluster V.

Genetic relatedness of yeast isolates from different oral sites

Figure 5 shows the frequency of individuals in each genetic cluster based on oral sites. In the marginal periodontitis group, clusters II, III and V were from the buccal mucosa, periodontal pockets and palate. Cluster V was predominant in the buccal mucosa and palate, but gave way to cluster III in periodontal pockets. Cluster IV was exclusively from the buccal mucosa. In the oral health group, cluster V was prominent in the buccal mucosa, gingival sulcus and palate at a frequency of 79%, 47% and 63%, respectively. There was no significant difference in the frequencies of clusters from different oral sites within the two groups. However, the frequency of cluster V in the marginal periodontitis group was significantly lower than in the oral health group from the subgingival areas and buccal mucosa ($\chi^2 = 5.84$ $p = 0.025$; $\chi^2 = 5.43$ $p = 0.025$, respectively).

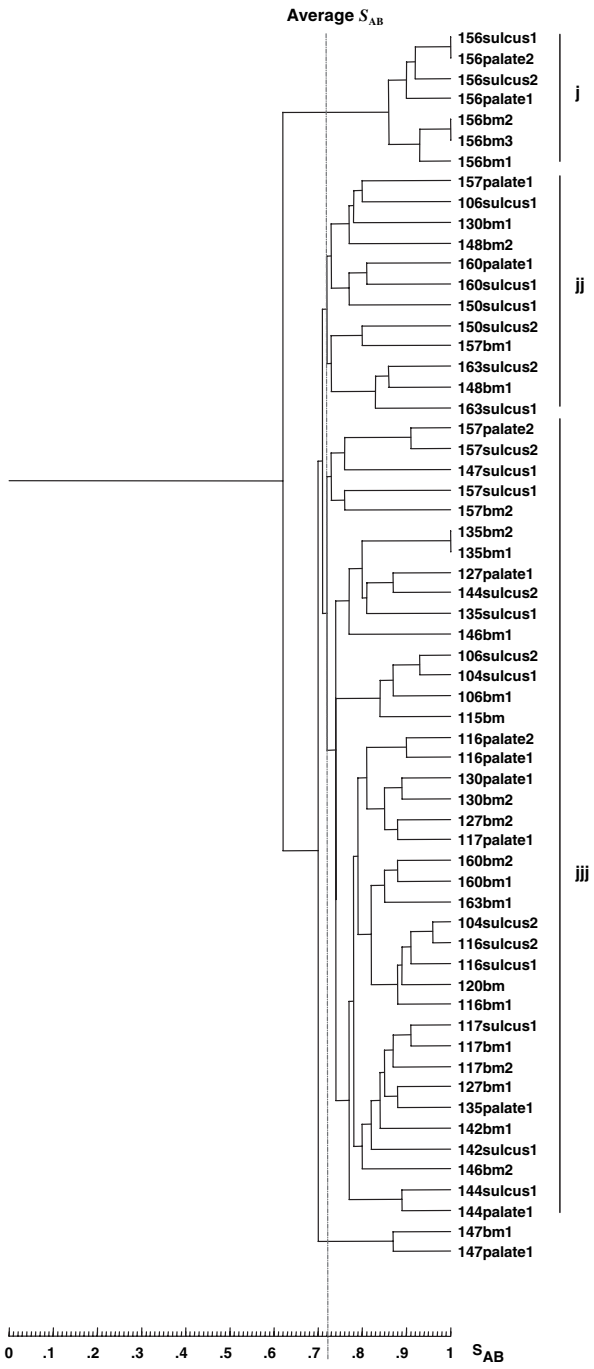

Fig. 3. Dendrogram of oral yeasts of subjects with oral health. The isolates in the dendrogram are designated as follows: patient number, oral site, and numbers of different colonies. bm: buccal mucosa. j, jj and jjj represent genetic clusters.

Discussion

In recent years, several molecular typing methods have been used to characterize yeast isolates and delineate strain relatedness (12–14). RAPD has become quite common for all infectious fungi and has been successfully applied to assess the genetic relatedness of *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, *C. lusitania*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *Candida famata*, *Candida rugosa*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* (13). Even though RAPD has been demonstrated as an efficient method for yeast DNA fingerprinting, it has a reported problem of reproducibility, especially for low-intensity bands (13, 15). The experience from repeated tests in the present study was that standardizing methodology contributed to reproducible RAPD fingerprints, such as the ratio of template and primer, concentration of magnesium, temperatures of the amplification reaction, concentration of the agarose gel, and the voltage.

Genetic similarity of oral yeasts of the marginal periodontitis and oral health groups

Multiple genotypes, average S_{AB} values and genetic clusters derived from the yeasts of the marginal periodontitis patients and oral health subjects suggested a heterogeneous yeast population in both groups. Yeasts of the marginal periodontitis group were more genetically diverse than yeasts of the oral health group because of its larger S_{AB} range, lower average S_{AB} value and three unique genetic clusters in the pooled dendrogram. Yeasts of the same marginal periodontitis group also showed higher diversity in colony morphology and biotype than yeasts of the oral health group (3). This implies some association between yeast phenotype and genetic attributes in health and disease. On the contrary, Xu *et al.* (16) found a greater genotypic diversity in the non-clinical population of *C. albicans* than that derived from clinical specimens regardless of HIV status. In the pooled dendrogram, 86% of the yeasts from 95% of the oral health individuals mixed with 40% of marginal periodontitis yeasts from 70% of the marginal periodontitis patients in cluster V. This indicated that a large portion of the marginal periodontitis yeasts shared a moderate genetic relatedness with dominant oral health yeasts. Hellstein *et al.* (17) and Pizzo *et al.* (10) also observed that genetically identical yeasts appeared in both healthy and diseased individuals. The three exclusive marginal periodontitis clusters in the pooled dengrogram showed that yeasts with

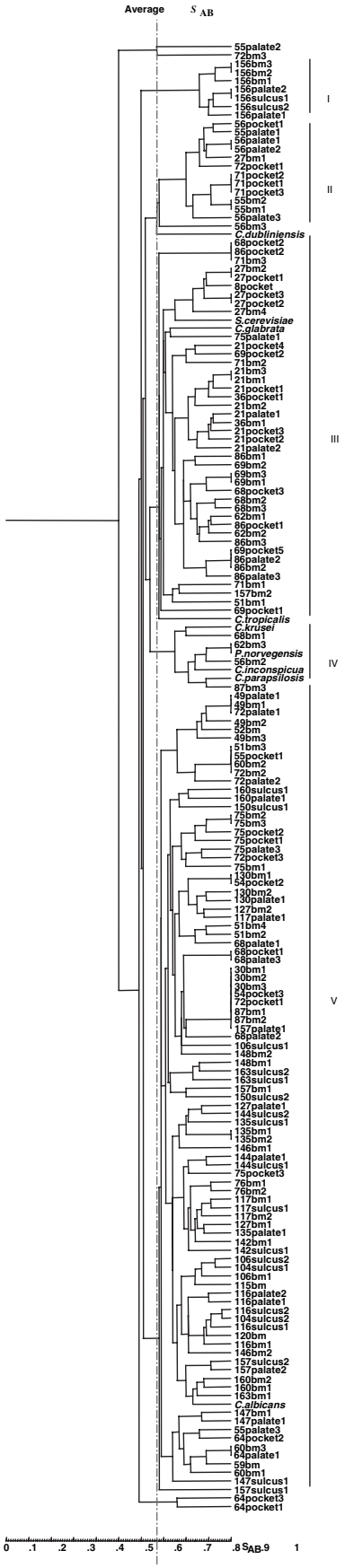

Fig. 4. Dendrogram of pooled oral yeasts of marginal periodontitis patients and oral health subjects, and yeast type strains. bm: buccal mucosa (see legend for Fig. 1 for species names). The isolates in the dendrogram are named as follows: patient number, oral site, and numbers of different colonies. I, II, III, IV and V represent genetic clusters.

unique genetic patterns occurred in the marginal periodontitis oral cavities. This finding agrees with previous studies (18, 19) revealing modifications in clonal profiles both phenotypically and genotypically during disease progression in symptomatic oral candidosis and asymptomatic carriage in HIV disease. The explanation for the unique genetic clusters might be that genetic variations, such as gene conversion, mitotic recombination and chromosomal translations, occurred in the marginal periodontitis yeasts to make them more adaptive and virulent in disease (20–22). Despite the high genetic diversity of yeasts in the marginal periodontitis group, the dendrograms of the two groups exhibited similar genetic clustering patterns that consisted of three genetic clusters at the respective average S_{AB}. Schmid *et al.* (23) and Lockhart *et al.* (24) found a genetically more homogeneous group of *Candida* strains in AIDS patients,

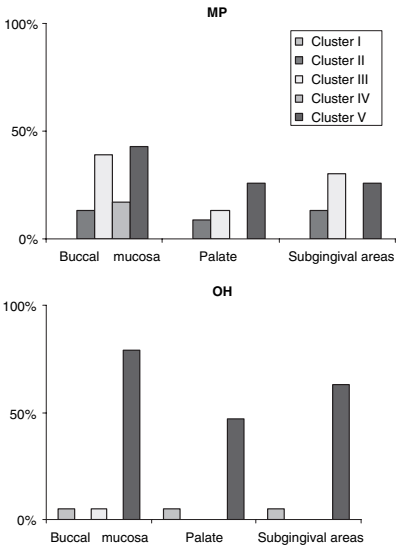

Fig. 5. Frequency (%) of individuals with marginal periodontitis (MP) and oral health (OH) in genetic clusters according to different oral sites.

and hypothesized that commensal yeasts were replaced by genetic homogenous strains in AIDS, whereas Lupetti *et al.* (25) detected more different karyotypes of *C. albicans* strains from HIV-infected patients compared with those from healthy individuals.

Genetic relatedness of oral yeast isolates from different oral sites

The present study showed no obvious association between genetic clusters and oral sites in the two participant groups. Cluster V was regarded as a genetic cluster of commensals because it was predominant in all the oral sites of the oral health individuals. Cluster V was also found mostly in the buccal mucosa and the palate of the marginal periodontitis patients. However, the frequency of cluster V of the marginal periodontitis group was significantly lower than that of the oral health group in the subgingival areas and in the buccal mucosa. Xu *et al.* (26) and Pizzo *et al.* (10) also recovered identical yeast patterns from different anatomical sites regardless of host condition. In contrast, Ruiz-Diez *et al.* (27) and Lamster *et al.* (6) found that subgingival *Candida* could be distinguished from *Candida* from the tongue and buccal mucosa in HIV patients and postulated that such distinction was due to a reduced Th1 lymphocyte response in the subgingival environment, which might also occur in the subgingival area of marginal periodontitis patients.

Conclusions

Genetically heterogeneous yeasts were found in the oral cavities of marginal periodontitis patients and oral health subjects. Similar genetic clustering patterns were obtained from the yeast isolates of both groups. Cluster V was the most predominant cluster in which 86% of oral health yeasts and 40% of marginal periodontitis yeasts fell. However, yeasts of the marginal periodontitis group appeared more genetically diverse than yeasts of the oral health group. Three genetic clusters of the marginal periodontitis group indicated that yeasts with

unique genetic attributes harboured marginal periodontitis oral cavities. There was no obvious association between genetic clusters and oral sites of the two participant groups, whereas the frequency of cluster V of the marginal periodontitis group was lower than that of the oral health group in subgingival areas and buccal mucosa.

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