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Frequency, Intensity, Species, and Strains of Oral *Candida* Vary as a Function of Host Age

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While the age of the host has been suggested as a determining factor in yeast carriage, no studies in which the genetic relatedness of isolates has been assessed in combination with the frequency and intensity of carriage as a function of host age have been performed in a single geographical locale and over a short time window. Therefore, by using a simple plating protocol to determine the frequency and intensity of carriage, sugar assimilation patterns to determine species, and Southern blot hybridization with the DNA fingerprinting probe Ca3 combined with computer-assisted analysis to determine the genetic relatedness of strains of *Candida albicans*, yeast carriage was analyzed as a function of age. All test individuals lived in the Iowa City, Iowa, locale and, except for some of the 0.5- to 1.5-year-olds, were dentate. The results demonstrate that for this test population, the frequency, average intensity, predominant species, and genetic relatedness of *C. albicans* strains varied as a function of host age. In addition, comparison with oral commensal organisms from the Ann Arbor, Mich., locale confirms the geographical specificity of *C. albicans* strains and the existence of an Iowa City-enriched strain which is most prevalent in elderly individuals.

Candida albicans and several related *Candida* species are opportunistic pathogens which live as benign commensal organisms in the oral cavities of healthy individuals. Usually in response to physiological changes in the host, these yeasts can become pathogenic, resulting in oral candidiasis. Since roughly one-third to one-half of adult individuals do not carry measurable levels of *Candida* spp. in the oral cavity (12, 23), carriage per se may represent a risk factor for subsequent infection. Little is known about the requirements for carriage or the natural barriers against carriage which result in yeast-free healthy individuals. One factor which may play a role in carriage is host age. It was demonstrated in a study by Russell and Lay (13) that the frequency of oral carriage at birth was low, doubled by the time that infants were discharged from the hospital, and increased sharply after 1 month at home. While old age has been suggested as a predisposing condition, it seems likely that an increase in colonization in elderly individuals may be secondary to increased medication and prosthetic tooth replacement (12). However, there has been no study of oral yeast carriage in which the genetic relatedness of strains was assessed as a function of age and which was performed in a single geographical locale and within a short time window.

Using a simple plating protocol to measure the frequency and relative intensity of carriage, sugar assimilation patterns to type the species of isolates, and Southern blot hybridization with a species-specific DNA fingerprinting probe combined with computer-assisted methods to assess the genetic relatedness of *C. albicans* isolates, we have performed an analysis of yeast carriage as a function of age in healthy individuals from the following five age groups: 0.5 to 1.5, 5 to 7, 15 to 18, 30 to 45, and ≥60 years. All sampled individuals had resided in the Iowa City, Iowa, area for either their whole lives or at least 1 year prior to sampling. All samples were collected in a 10-week period, and all test individuals except for a portion of those in the 0.5- to 1.5-year-old group were dentate. The results of the study demonstrate that for this test population, the frequency and intensity of oral yeast carriage, the frequency of oral *C. albicans* carriage in particular, and the genetic relatedness of oral *C. albicans* strains varied as a function of the age of the host.

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MATERIALS AND METHODS

Collection of isolates. A total of 172 volunteer subjects were recruited from the general population of Iowa City. Subjects were confined to five age groups: group A1, 0.5 to 1.5 years; group A2, 5 to 7 years; group A3, 15 to 18 years; group A4, 30 to 45 years; and group A5, ≥60 years. All subjects in group A1 had resided in Iowa City for their entire lives, and all subjects in groups A2 to A5 had resided in that locale for at least the 1-year period prior to sampling. All test individuals were sampled within a 10-week time window. All had unremarkable health histories and were free of signs or symptoms of oral candidiasis or other mucosal disease, as determined by oral examination by an oral pathologist. All individuals in the last four age groups were dentate and had no major removable oral devices.

A sample was collected from each of three oral locales: the buccal mucosa (B), the floor of the mouth (F), and the dorsal surface of the tongue (T). Samples were coded according to age group (A1, A2, A3, A4, and A5), test individual number, sample location (B, F, or T), and, when indicated, clone. Therefore, sample A1-33Fa represents clonal isolate "a" from the floor of the mouth of test individual 33 in group A1 (age 0.5 to 1.5 years).

Each sample was collected by passing a sterile cotton swab (Culturette; Becton Dickinson Microbiology Systems, Cockeysville, Md.) several times across the particular oral surface. Immediately after sampling, each swab was replaced in its sterile containment tube and was moistened with sterile salt solution by crushing the glass ampoule in the tube. The containment tubes were transported within 2 h of sampling from the place of collection to the laboratory. The cotton end of each swab was inserted into 0.5 ml of sterile water in a microcentrifuge tube, the tube was rigorously mixed for 30 s with a laboratory tabletop vortex mixer, and 0.15 ml of the wash was spread onto each of three agar plates containing supplemented Lee's medium (3). This resulted in three plates containing samples from each oral location, totaling nine plates with samples from each subject's oral cavity. The plates were incubated for 7 days at 25°C. The total number of yeast colonies on the three plates for each site was considered the relative intensity of carriage per oral site, and the total number of yeast colonies on the nine plates was considered the relative intensity of oral carriage. Sampling was rigidly performed in an identical fashion for each individual since comparisons between samples involved measurements of relative intensities.

When more than one colony morphology was present on a culture dish, the proportions of the different colony morphologies were scored, and at least one colony exhibiting each morphology was streaked onto an agar slant. To assess the genetic homogeneity of clonal populations, multiple colonies with the same colony morphologies from select primary plates were individually streaked onto agar slants and were stored for subsequent analysis. Cells from each streaked

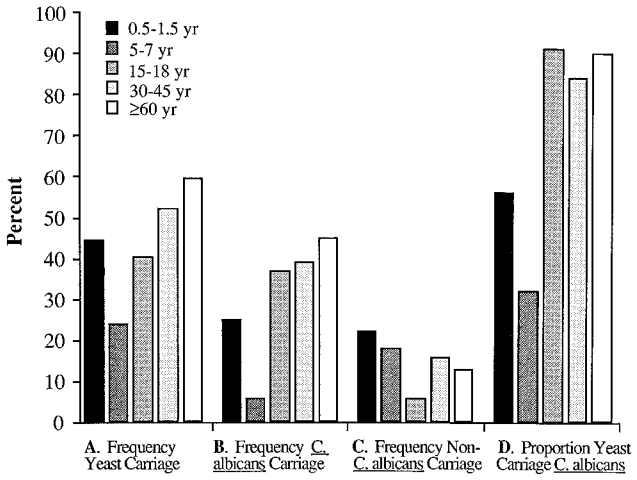

FIG. 1. Histograms of the frequency of carriage of all yeast isolates (A), the frequency of carriage of the species *C. albicans* (B), the frequency of carriage of non-*C. albicans* species (C), and the proportion of yeast carriage which is *C. albicans* (D) as a function of age. The symbols for the age groups are designated in the upper left hand corner.

colony were examined microscopically to verify that they were yeast colonies. Cells from each isolate were then fingerprinted (see next section) with the DNA probe Ca3, which generates a complex pattern of 15 to 25 bands with *Eco*RI-digested DNA of *C. albicans* (18, 24), a marginal pattern of two weak bands with *Eco*RI-digested DNA of *C. tropicalis* (14, 24, 25), and no hybridization with *Eco*RI-digested DNA of the other *Candida* species tested. Cells from each non-*C. albicans* isolate were finally typed for species by sugar determining the assimilation pattern with the automated Vitek YBC system (bioMerieux Vitex, Inc., Hazelwood, Mo.).

Southern blot hybridization with the Ca3 probe. Cells from an individual isolate were removed from a storage slant and were inoculated into 2 ml of YPD medium (2% glucose, 2% Bacto Peptone, 1% yeast extract) in a sterile test tube and were grown overnight at 30°C. DNA was prepared from each clone by the methods of Scherer and Stevens (16). DNA was measured in a Sequoia-Turner 45 fluorometer (Barnstead/Thermolyne, Dubuque, Iowa), digested with the restriction enzyme *Eco*RI, and separated on a 0.8% (wt/vol) agarose gel. *Eco*RI-digested DNA from the test isolates was run in the inner 13 lanes, and *Eco*RI-digested DNA from reference strain 3153A was run in the outer 2 lanes of a 15-lane gel. The patterns of strain 3153A were used to unwarp the gel and to compare the banding patterns with that of a global standard for computer-assisted analysis (18, 22). Gels were stained with ethidium bromide prior to hybridization to verify equal loading of the lanes and were then destained and transferred to a nitrocellulose membrane by capillary transfer (15). The Southern blots were then hybridized with the nick-translated Ca3 probe (1, 9, 14, 18, 22, 24) and exposed to X-OMAT film (Eastman Kodak, Rochester, N.Y.).

Computer-assisted analysis of DNA fingerprints. DNA fingerprints were digitized into the database of the Dendron software program (6, 9, 10, 18, 20, 22) with a Scanjet II cx scanner with the transparency option (Hewlett-Packard, Palo Alto, Calif.). Patterns were unwarped and normalized against a universal standard for cross comparisons. Lanes were automatically scanned, and the bands were identified and automatically assigned categories of 0 (no band), 1, 2, or 3, in increasing order of intensity. The similarity coefficient (S_{AB}) based on band intensities and positions was then computed for every pair of patterns (isolates) by the following formula:

$$S_{AB} = \frac{\sum_{i=1}^{k} (a_i + b_i - |a_i - b_i|)}{\sum_{i=1}^{k} (a_i + b_i)}$$

where a_i and b_i are the intensities of band i in patterns A and B, respectively, and k is the number of bands. Dendrograms based on the S_{AB} values were generated by the unweighted pair group method (21).

For comparison of the Iowa City isolates collected for the present study and oral isolates collected at approximately the same time in Ann Arbor, Mich., for another study (10), dendrograms were generated from the data files of both collections by using the Dendron software program. Comparisons were possible because of the normalization of all gels to a global standard.

Statistical methods. Statistical analysis for group relationships was determined by the chi-square test. The relationships were statistically significant where noted in the text.

RESULTS

Frequency of carriage varies as a function of age. The frequency of yeast carriage for each age group was computed as the percentage of individuals whose samples grew one or more yeast colonies on the nine culture plates, which included three plates containing buccal mucosa samples, three plates containing samples from the floor of the mouth, and three plates containing samples from the surface of the tongue. While 44% of the tested individuals in group A1 were positive, only 24% of tested individuals in group A2 were positive (Fig. 1A). The frequency of carriage increased to 40% in group A3, to 53% in group A4, and to 59% in group A5 (Fig. 1A). The difference in the frequency of yeast carriage between group A1 and group A4 was marginally significant, with a P value of <0.1, but the difference between group A2 and group A5 was significant, with a P value of 0.05.

Intensity of carriage varies as a function of age. The intensity of yeast carriage for each test individual was computed as the combined number of colonies on the nine agar plates containing samples derived from the oral cavity. Of the test subjects carrying yeast isolates, 50% of the oral cavities of the individuals in groups A1 and A2 contained 1 to 15 colonies while only 12 to 22% of the oral cavities of individuals in groups A3, A4, and A5 contained these numbers of colonies. Of the test subjects carrying yeast isolates, 47 and 51% of the oral cavities of individuals in groups A1 and A2, respectively, carried yeast isolates in the 15 to 100 and 101 to 500 intensity ranges combined, while 67, 78, and 64% of individuals in groups A3, A4, and A5, respectively, carried yeast isolates in these combined intensity ranges (Fig. 2). Finally, of the test subjects carrying yeast isolates, only 6 and 0% of the oral cavities of individuals in groups A1 and A2, respectively, contained isolates in the range of >500, while 13, 20, and 14% of the oral cavities of individuals in groups A3, A4, and A5, respectively, contained isolates in this highest intensity range (Fig. 2). These results suggest that the average intensity of carriage and the proportion of individuals with carriage in the highest intensity range were greater for individuals in the three oldest age groups (groups A3, A4, and A5).

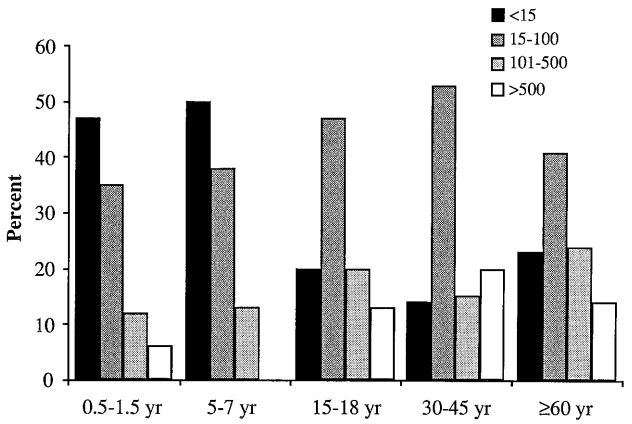

FIG. 2. Histograms of the intensity of carriage in the five age groups. Intensities are separated into <15, 15 to 100, 101 to 500, and >500 colonies per nine dishes including three samples from the back of the tongue, three samples from under the tongue, and three samples from the inner cheek.

TABLE 1. Proportion of yeast species in the entire collection of isolates

Species	Proportion (%)
Candida albicans	69
Candida parapsilosis	10
Candida krusei	4
Candida famata	4
Candida guillermondii	3
Candida zeylanoides	3
Candida rugosa	1
Candida dubliniensis	1
Candida geocandidium	1
Yarrowia lipolytica	1
Trichosporon beigelii	1
Saccharomyces cerevisiae	1

Differences in frequency of yeast carriage between age groups are due primarily to differences in the frequency of *C. albicans* carriage. The species of each yeast isolate was assessed first by DNA fingerprinting with the species-specific probe Ca3, which separates *C. albicans* from non-*C. albicans* species, and then by analyzing the sugar assimilation profile of each non-*C. albicans* isolate, which types the species. For the first two age groups (groups A1 and A2), the frequencies of *C. albicans* carriage were 25 and 6%, respectively, while for the last three age groups, the frequencies were 37, 39, and 45%, respectively (Fig. 1B). The differences between age group A2 and age groups A3, A4, and A5 were significant, with P values of <0.05. The frequency of carriage of non-*C. albicans* species in the five age groups varied between 22 and 6% and appeared to be slightly elevated in age groups A1 and A2, while it was reduced in age group A3 (Fig. 1C). The proportions of yeast isolates which were *C. albicans* in groups A1 and A2 were therefore 55 and 32%, respectively, while the proportions in groups A3, A4, and A5 were 91, 84, and 90%, respectively (Fig. 1D). Conversely, the proportions of yeast isolates which were non-*C. albicans* isolates in age groups A1 and A2 were 44 and 68%, respectively, while the proportions in age groups A3, A4, and A5 were 9, 16, and 10%, respectively (Fig. 1D). These results demonstrate that the differences in yeast carriage between the first two age groups and the last three age groups is due primarily to a dramatic decrease in the frequency of *C. albicans* carriage in the first two groups (Fig. 1B).

Table 1 lists the proportions of *Candida* species among the entire collection of samples (i.e., combined isolates from the five age groups), and in Table 2 the proportion of each species obtained from individuals in each age group is enumerated. In the entire collection, 69% of individuals carrying yeast isolates carried *C. albicans* and 10% carried *C. parapsilosis* (Table 1). The remaining 20% carried *C. krusei*, *C. famata*, *C. guillermondii*, *C. zeylanoides*, *C. rugosa*, *C. dubliniensis*, *C. geocandidium*, *Yarrowia lipolytica*, *Trichosporon beigelii*, *C. lusitaniae*, *Blastoschizomyces capitatus*, or *Saccharomyces cerevisiae* (Table 1A). *C. glabrata* may have been missed because of the poor ability of many strains of this species to grow on supplemented Lee's medium (8). Notably absent were isolates of *C. tropicalis*, which readily grow on supplemented Lee's medium (25). Two individuals in age group A1 and one individual each in age groups A2, A3, and A5 carried multiple species. This represents 3% of all tested individuals and 7% of all individuals with yeast carriage. The combinations were *C. albicans* plus *C. parapsilosis* for test individuals A1-25 and A1-77, *C. parapsilosis* plus *C. zeylanoides* for A2-16, *C. albicans* plus *S. cerevisiae* for A3-49, and *C. albicans* plus *Y. lipolytica* for A5-31. For the last two individuals, differences in colony morphology and a reasonable intensity of carriage allowed us to score the proportion of colonization by each of the strains in a pair. In the case of individual A3-49, the proportion of *C. albicans* to *S. cerevisiae* was 42 to 13, or 76 to 24%, and in the case of individual A5-31, the proportion of *C. albicans* to *Y. lipolytica* was 70 to 412, or 15 to 85%. The intensities and proportions suggest that the levels of carriage of both species in a pair were substantial.

Differences in intensity of carriage between the age groups are due primarily to differences in frequency of *C. albicans* carriage. The individuals in age groups A1 and A2 exhibited both a lower frequency of yeast carriage and a lower average intensity of yeast carriage. The lower frequency of yeast carriage was demonstrated to be due primarily to a lower frequency of carriage of the species *C. albicans*. We therefore tested whether the lower average intensity of carriage in age groups A1 and A2 was also due primarily to a lower proportion of *C. albicans* carriage by computing the proportion of *C. albicans* versus other species in the four carriage intensity ranges of <15, 15 to 100, 101 to 500, and >500 colonies per nine culture dishes containing three oral swab specimens from three sites of the oral cavity of each test individual. The results, presented in Table 3, support the suggestion that the lower intensity of carriage in age groups A1 and A2 is due to the lower frequency of *C. albicans* carriage and further demonstrate that high levels of yeast carriage in healthy individuals (>500 colonies per nine plates) is the exclusive result of *C. albicans* carriage. This is best demonstrated by following the proportion of other species as a function of intensity. The proportion decreased from 60 to 29 to 15 to 0% through the increasing intensity ranges (Table 3). In contrast, the proportion of *C. albicans* increased from 40 to 64 to 69 to 100% through the increasing intensity ranges (Table 3).

Carriage as a function of gender. The proportions of males and females with yeast carriage in age groups A1, A2, A3, and A5 differed by 11, 8, 18, and 4%, respectively (Table 4). How-

TABLE 2. Proportions of yeast species as a function of age assessed by sugar assimilation patterns

Age group	Organism (% of isolates)
A1 (n = 16)	*C. albicans* (56),[a] *C. parapsilosis* (38),[a] *C. guillermondii* (6), *C. krusei* (6), *C. geocandida* (6)
A2 (n = 8)	*C. albicans* (38), *C. parapsilosis* (13),[b] *C. dubliniensis* (13), *C. zeylanoides* (13),[b] *C. krusei* (13), *C. guillermondii* (13), unidentified (6)
A3 (n = 14)	*C. albicans* (93),[c] *C. famata* (7), *S. cerevisiae* (7)[c]
A4 (n = 20)	*C. albicans* (75), *C. parapsilosis* (5), *C. zeylanoides* (5), *T. beigelii* (5), *C. famata* (5), unidentified (5)
A5 (n = 17)	*C. albicans* (76),[d] *C. rugosa* (6), *C. kruseii* (6), *C. famata* (6), *Y. lipolytica* (6),[d] unidentified (6%)

[a] Two individuals in group A1 carried both *C. albicans* and *C. parapsilosis.*
[b] One individual in group A2 carried both *C. parapsilosis* and *C. zeylanoides.*
[c] One individual in group A3 carried both *C. albicans* and *S. cerevisiae.*
[d] One individual in group A5 carried both *C. albicans* and *Y. lipolytica.*

TABLE 3. Intensity of carriage as a function of species[a]

Intensity range	No. of isolates	Proportion (%) of species[b]		
		C. albicans	Mixed *C. albicans*-other species	Other species
<15	20	40	0	60
15–100	33	64	9	27
101–500	13	69	15	15
>500	9	100	0	0

[a] The proportion of species (*C. albicans* versus other species) is presented for each of the four intensity ranges, which represent the number of colonies on the nine dishes containing samples on the three sampling swabs from three sites from each test individual.

[b] For simplicity, proportions were computed for *C. albicans*, mixed carriage of *C. albicans* plus another species, and other species.

ever, the proportions of males and females with yeast carriage in age group A4 differed by 42% (Table 4). While females in age group A4 carried yeast isolates at a frequency of 74% which was outside the range of frequencies for both genders in all other age groups, the males in this group carried yeast isolates at a frequency of 32%, which was within the range of carriage observed in the other age groups (Table 4). The difference in yeast carriage between males and females in age group A4 was marginally significant, with a P value of <0.09. The increase in the frequency of carriage by females in group A4 was due primarily to an increase in the frequency of *C. albicans* carriage.

Commensal populations are primarily clonal but contain subgenotypes. To assess the genetic homogeneity of commensal populations of *C. albicans*, multiple isolates from 34 individuals were cloned directly from the oral cavity and were fingerprinted by Southern blot hybridization with the complex Ca3 probe, which is species specific for *C. albicans* (1, 9, 14, 18). As noted earlier, 5 (7%) of the 75 test individuals with oral yeast carriage carried two different species simultaneously in the oral cavity, and this is considered an underestimate. In the few cases of mixed yeast populations, only the Southern blot hybridization patterns of the *C. albicans* isolates could be compared.

Figure 3A presents examples of the DNA fingerprint patterns obtained with the Ca3 probe. Note that the outer lanes (lanes a and o) of the gel contain the pattern of reference strain 3153A, which was used to unwarp any smiles, frowns, or distortions with the Dendron software program and to normalize to a global standard in the Dendron database so that the pattern of each fingerprint could be compared with that of any other isolate on another gel digitized into the database. In lanes b and c, two isolates from the tongue of test individual A3-80 exhibited identical patterns; in lane d, an isolate from the tongue of test individual A4-2 showed no hybridization to Ca3, suggesting that it is a species other than *C. albicans*; in lanes e, f, and g, oral isolates from three unrelated individuals in group A4 (individuals A4-6, A4-8, and A4-9, respectively) exhibited significantly different (unrelated) patterns; in lanes h and i, isolates from the buccal mucosa and dorsum of the tongue of test individual A4-10, respectively, exhibited highly similar but nonidentical patterns; in lanes k and l, isolates from two individuals (individuals A5-15 and A3-22, respectively) exhibited similar but nonidentical patterns.

A synopsis of the comparative analyses of 34 sets of multiple isolates from individual oral cavities is presented in Table 5, and representative gels of multiple isolates from the same oral cavities of test individuals A5-71 and A5-53 are presented in Fig. 3B and C, respectively. Of the 23 sets of three or more *C. albicans* isolates, 10 consisted of isolates with identical Ca3 patterns, while 13 consisted of isolates with two or more highly related but nonidentical patterns (Table 5). Of the 11 sets of two isolates, 8 sets included identical isolates, 2 sets included isolates with highly related but nonidentical patterns, and 1 (for individual A3-14) included unrelated isolates. Of the combined 148 *C. albicans* isolates among the 33 sets of multiple isolates fingerprinted, 100 (68%) exhibited the major genotype of their set, 47 (32%) exhibited a highly related minor genotype of their set, and 1 (<1%) exhibited an unrelated genotype (Table 5).

Figure 3B presents an example of a set of six isolates from the buccal mucosa of test individual A5-71 which exhibited identical Ca3 hybridization patterns. Figure 3A, lanes b and c, presents a second example of two isolates, respectively, from the surface of the tongue of test individual A3-80 which again exhibited identical Ca3 hybridization patterns. Figure 3C presents an example of five isolates from the buccal mucosa, the floor of the mouth, and the dorsum of the tongue of test individual A5-53 which exhibited minor variations in the positions of high-molecular-weight bands (>9.0 kb). Three patterns were distinguishable, and the patterns differed by one to two band positions. Isolate A5-53Ba exhibited one pattern, isolates A5-53Bb and A5-53Tb exhibited a second pattern, and isolates A5-53F and A5-53Ta exhibited a third pattern (Fig. 3C). Figure 3A, lanes h and i, presents a second example of highly similar but nonidentical fingerprints of two oral isolates from test individual A4-10. The differences in the two patterns include high-molecular-weight bands and a band slightly larger than 5.4 kb. The results of the analysis of multiple isolates of *C. albicans* demonstrate that the majority of commensal populations are clonal. However, 44% of these populations contain minor variants which can be distinguished by changes primarily in the high-molecular-weight bands of the Ca3 Southern blot hybridization pattern. These minor variants were still highly related to the primary *C. albicans* strain in each population, and this is supported by the S_{AB}s computed between the minor and major genotypes of each set of multiple isolates tested. While the average S_{AB} between unrelated strains has been computed to be between 0.69 and 0.72, measures of relatedness have been considered to be 0.92 or higher (6, 9, 10, 17, 18, 23). Except in one case (the isolate from individual A3-14), the average S_{AB}s for the 34 analyzed sets of multiple isolates ranged between 0.93 and 1.0 (Table 4). The mean S_{AB} for the 34 sets of multiple isolates was 0.98 (Table 5).

Age-related clustering of isolates. Having established that individuals primarily carry clonal populations of *C. albicans*, we next examined the relatedness of a representative isolate from each individual carrying *C. albicans* in each age group and then compared isolates between age groups. Comparisons were

TABLE 4. Carriage as a function of age and sex

Age group	No. of individuals	Proportion (%) of test individuals		Frequency (%) of yeast carriage		
		Males	Females	Males	Females	Males plus females
A1	36	50	50	39	50	44
A2	34	68	32	26	18	24
A3	35	46	54	50	32	40
A4	38	50	50	32	74	53
A5	29	31	69	56	60	59
Mean		49	51	38	49	44

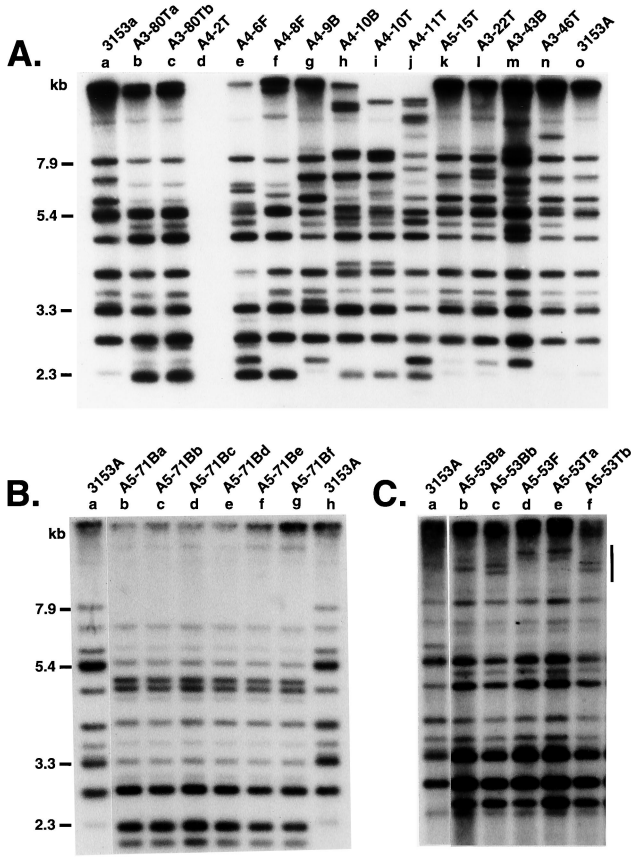

FIG. 3. Examples of fingerprinting gels representing *Eco*RI-digested DNA of the isolate probed with the Ca3 probe. (A) A 15-lane test gel; lanes a and o, control lanes of *Eco*RI-digested DNA of standard strain 3153A. (B) Isolates from the buccal epithelium of test individual A5-71, demonstrating identical fingerprint patterns. (C) Isolates from the three oral locations of individual A5-53, demonstrating highly similar but nonidentical patterns because of variations in the high-molecular-mass bands (indicated by a bar to the right of the gel).

made between isolates by automatically computing the S_{AB} with the Dendron program. Dendrograms were then generated on the basis of the S_{AB}s computed between all possible pairs of isolates (Fig. 4). An S_{AB} threshold of 0.87, halfway between the computed S_{AB} for random strains in the Iowa City locale and the S_{AB} for identical isolates, was used to define clusters in dendrograms. In the present analysis isolates connected in dendrograms by branch points at an S_{AB} of $\geq$0.87 were considered to be clustered. The *C. albicans* isolates from nine individuals in age group A1 had an average S_{AB} of 0.77 $\pm$ 0.08 and formed a single cluster of four isolates, which represented 44% of the isolates from the group (Fig. 4A). The *C. albicans* isolates from two of the individuals in age group A2 had an average S_{AB} of 0.65 (Fig. 4B). Since the frequency of *C. albicans* carriage was so low in this age group (Fig. 1B), clustering could not be analyzed. The *C. albicans* isolates from 13 of the individuals in age group A3 had an average S_{AB} of 0.69 $\pm$ 0.09 and formed four clusters of two isolates each (Fig. 4C). However, two of the four clusters (isolates from individuals A3-80 and A3-79 and from individuals A3-44 and A3-44) represented isolates from pairs of siblings. When one of the two isolates from each of these sibling pairs was removed, only two clusters remained in age group A3, and the remaining four isolates in these clusters represented 36% of the total isolates in the reduced dendrogram. The *C. albicans* isolates from the 15 individuals in age group A4 had an average S_{AB} of 0.74 $\pm$ 0.07 and formed one cluster of four isolates (Fig. 4D). Two isolates in this cluster (isolates from individuals A4-65 and A4-33) were from a cohabiting couple. When one of these two isolates was removed from the cluster, the remaining isolates in the cluster represented 21% of the total isolates in the reduced dendrogram. The 13 *C. albicans* isolates from individuals in age group A5 had an average S_{AB} of 0.75 $\pm$ 0.12 and formed a large cluster of seven isolates. This cluster therefore contained 54% of the total isolates from individuals in this age group, and no reduction was warranted because there were no cohabitants in the cluster. To test whether the isolates in the clusters of isolates from subjects in the individual age groups were related, coclustering was assessed in a common dendrogram generated from the *C. albicans* isolates from individuals in the five age groups (Fig. 5A). In the combined dendrogram, a major cluster formed at a threshold of 0.87, which contained 17 (33%) of the 52 *C. albicans* isolates compared. This major cluster (cluster A) contained all of the isolates in the major clusters identified in the individual dendrograms generated for isolates from individuals in age groups A1, A4, and A5, as well as two weakly related isolates (isolates from individuals A3-38 and A3-22) from individuals in age group A3. An additional cluster (cluster B) which comprised four isolates derived from individuals in three of the age groups was formed. Three ad-

TABLE 5. Homogeneity of commensal populations of *C. albicans*

No. of isolates	Test individual	Oral location (no. of isolates)[a]	No. of patterns (proportions)	Average S_{AB}[b]
12	A1-76	B (1), F (1), T (10)	1	1.00
12	A3-79	B (9), F (1), T (2)	2 (6:6)	0.98 $\pm$ 0.02
12	A5-71	B (10), F (1), T (2)	1	1.00
10	A3-80	F (2), T (8)	2 (9:1)	0.99 $\pm$ 0.02
9	A3-68	B (3), F (3), T (3)	3 (4:3:2)	0.97 $\pm$ 0.02
8	A1-61	B (1), F (1), T (6)	1	1.00
7	A5-53	B (2), F (1), T (4)	4 (2:2:2:1)	0.97 $\pm$ 0.02
5	A3-22	B (2), F (1), T (2)	3 (2:2:1)	0.98 $\pm$ 0.01
5	A3-39	B (2), F (2), T (1)	3 (3:1:1)	0.92 $\pm$ 0.07
5	A4-72	B (2), F (1), T (2)	1	1.00
4	A4-65	B (1), F (1), T (2)	3 (2:1:1)	0.98 $\pm$ 0.01
4	A4-75	B (2), F (1), T (1)	2 (2:2)	0.99 $\pm$ 0.02
3	A4-4	B (1), F (1), T (1)	2 (2:1)	0.99 $\pm$ 0.01
3	A4-6	B (1), F (1), T (1)	2 (2:1)	0.93 $\pm$ 0.06
3	A4-8	F (1), T (2)	2 (2:1)	0.99 $\pm$ 0.01
3	A4-10	B (1), T (2)	2 (2:1)	0.94 $\pm$ 0.05
3	A4-11	B (1), F (1), T (1)	2 (2:1)	0.97 $\pm$ 0.02
3	A4-20	B (1), F (1), T (1)	1	1.00
3	A4-40	B (1), F (1), T (1)	1	1.00
3	A5-29	B (1), F (1), T (1)	1	1.00
3	A5-35	B (1), F (1), T (1)	1	1.00
3	A5-51	F (1), T (2)	1	1.00
3	A5-69	B (1), F (1), T (1)	1	1.00
2	A1-25	B (1), T (1)	1	1.00
2	A1-74	B (1), F (1)	1	1.00
2	A1-77	F (1), T (1)	2	0.97
2	A3-14	B (1), T (1)	2	0.70
2	A3-17	B (1), T (1)	2	0.98
2	A3-38	F (1), T (1)	1	1.00
2	A3-66	F (1), T (1)	1	1.00
2	A4-3	F (1), T (1)	1	1.00
2	A4-9	B (1), T (1)	1	1.00
2	A4-18	B (1), T (1)	1	1.00
2	A5-28	B (1), T (1)	1	1.00

[a] Oral locations: B, buccal epithelium; F, floor of mouth; T, back of tongue.
[b] The mean value (0.98) was computed by dividing the sum of the S_{AB} times the number of isolates for each test individual by the total number of isolates (n = 148).

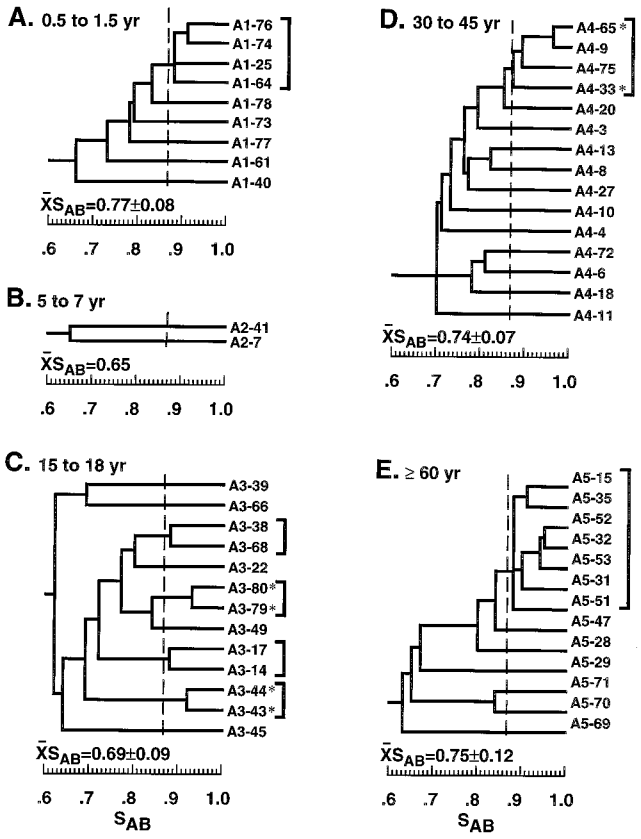

FIG. 4. Dendrograms of *C. albicans* isolates from individuals in the five age groups. An arbitrary threshold of relatedness for defining clusters of 0.87, represented by a vertical dashed line, represents the halfway point between the S_{AB} for unrelatedness and the S_{AB} for identicalness. The mean S_{AB} ± standard deviation is presented in the lower left-hand corner of each dendrogram. Clusters are bracketed to the right of each dendrogram. Starred isolates within a cluster represent those from cohabiting siblings or couples, and in analyses of the proportion of isolates in clusters, one of the two isolates was removed from the computation. This removes two clusters of two isolates each from group A3.

ditional clusters, of two isolates each, also formed in the combined dendrogram. One of these included isolates from siblings (individuals A3-14B and A3-17B).

Geographical specificity of the Iowa City cluster. To test whether the major A and minor B clusters were specific to the geographical locale of Iowa City, a common dendrogram was generated from the 52 strains in this analysis plus 22 oral isolates collected during approximately the same time window in Ann Arbor, Mich. (Fig. 5B). If there were no geographical specificity, 33% of the 22 Ann Arbor isolates (seven isolates) would be expected to penetrate cluster A. In the combined dendrogram, a major cluster which contained 16 of the 17 isolates in the major cluster (cluster A) of the dendrogram in Fig. 4A but only 3 isolates from Ann Arbor was formed. This is less than half the number expected if there was no geographical specificity. While 33% of Iowa City isolates grouped in the major cluster of genetically related strains, only 14% of Ann Arbor isolates fell into this cluster, suggesting that this cluster is enriched for Iowa City isolates. None of the Ann Arbor isolates coclustered with the four Iowa City isolates in cluster B (Fig. 5B). If there were no geographical specificity, we would expect two Ann Arbor isolates in cluster B. The Ann Arbor strains paired into five additional clusters of two isolates each, but there was no mixing of the Ann Arbor isolates with the Iowa City isolates in such small clusters (Fig. 5B). Therefore, while 38% of the Iowa City isolates fell into two clusters of four or more isolates each in the combined dendrogram in Fig. 5B, only 14% of the Ann Arbor isolates penetrated these clusters, supporting the conclusion that strains in these two clusters are enriched in the Iowa City locale.

To assess just how unrelated the Iowa City and Ann Arbor isolates were, we computed the frequency of pairing of isolates from the two locales at S_{AB}s above 0.87. Each isolate which was paired to another isolate or group of isolates with a branch point above 0.87 was scored for the identity of its branch partner(s) (i.e., Iowa City versus Ann Arbor). If pairing were random, each Iowa City isolate would have a probability of 51 of 73 (70%) of pairing with another Iowa City isolate and a probability of 22 of 73 (30%) of pairing with an Ann Arbor isolate. Each Ann Arbor isolate would have a probability of 21 of 73 (29%) of pairing with another Ann Arbor isolate and a probability of 52 of 73 (71%) of pairing with an Iowa City isolate. A total of 93% of the Iowa City isolates paired with Iowa City isolates, compared with a value of 70% if the pairing were random, and 7% paired with Ann Arbor isolates, compared with a value of 30% if the pairing were random. A total of 92% of the Ann Arbor isolates paired with Ann Arbor isolates, compared with a value of 29% if the pairing were random, and 8% paired with Iowa City isolates, compared with

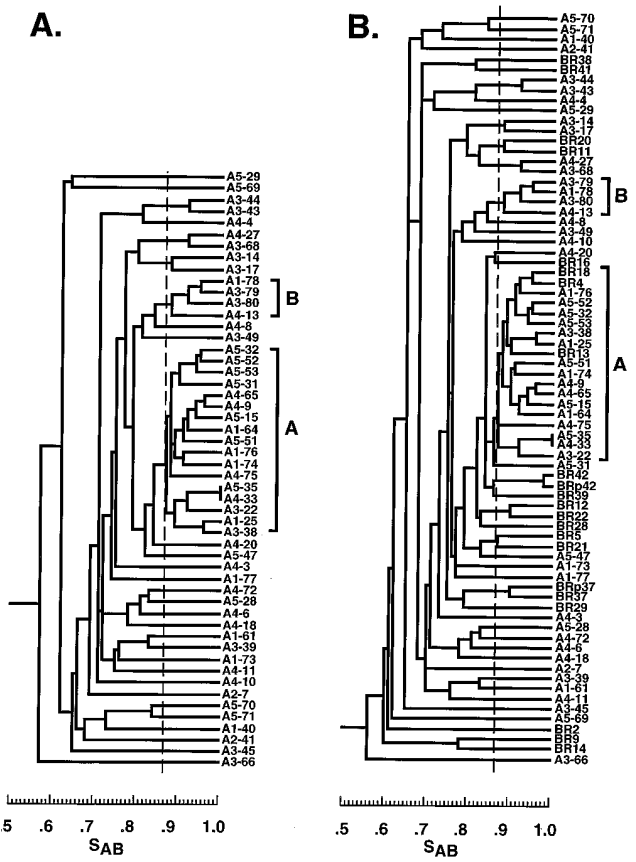

FIG. 5. Dendrogram of the entire collection of isolates from the five age groups (A) and a dendrogram from the entire collection plus 22 oral isolates from a study in Ann Arbor (B). Average S_{AB}s ± standard deviations are presented in the lower left-hand corner of each dendrogram. An arbitrary threshold of 0.87 is noted by a vertical dashed line. Major clusters are bracketed to the right of the dendrograms. Oral isolates from Ann Arbor are named BR plus the isolate number.

a value of 71% if the pairing were random. These results further support the geographical specificities of the isolates.

Relatedness of isolates from cohabiting siblings and couples. In the present study, six pairs of cohabiting siblings, eight pairs of cohabiting adults (couples), and one set of parents and siblings were sampled. In the case of siblings, three pairs included one sibling carrying *C. albicans* and one carrying no culturable yeast isolate, two pairs included siblings carrying *C. albicans* isolates with highly similar but nonidentical fingerprints (S_{AB}s of 0.92 and 0.95, respectively), and one pair included siblings carrying unrelated *C. albicans* isolates (S_{AB} of 0.78). In the case of couples, four included one partner carrying *C. albicans* and one carrying no culturable yeast isolate, one included one partner carrying *C. albicans* and a second carrying a non-*C. albicans* strain, one included one partner carrying *C. albicans* isolates with highly similar but nonidentical fingerprints (S_{AB} of 0.91), and two included partners carrying unrelated *C. albicans* isolates (S_{AB}s of 0.68 and 0.65). In the one case of parents and siblings, the mother and infant both carried *C. parapsilosis*, while the father carried *B. capitatus*. As noted earlier, clustering of isolates from two pairs of related siblings and one pair of related adults was taken into account when assessing the level of clustering in the relevant age groups.

DISCUSSION

Not all healthy individuals carry yeast isolates as commensal organisms in the oral cavity (12, 23). In a review by Odds (12) of 41 studies in which the frequency of carriage was measured in the oral cavities of healthy individuals, the four highest reported frequencies were 71% of schoolchildren in the United Kingdom (11), 69% of a nursing staff in Germany (19), 56% of children in Israel (4), and 54% of infants in the United Kingdom (7, 13). In a study of 52 healthy women in Iowa City, the frequency was 56% (23). It is therefore a reasonable possibility that natural barriers against yeast colonization exist in body fluids and at mucosal surfaces, that these barriers differ between individuals, and that these barriers may vary as a function of age. In a study of infants, Russell and Lay (13) found that only 5.7% of neonates carried *C. albicans* in the oral cavity, and that figure increased to 14.2% at the time of discharge, usually 7 days after birth, and to 82% 4 weeks after birth. The frequency decreased, however (to 50%), at 1 year of age. Although one could suggest that these changes in frequency may be due to physiological changes related to age, the changes in environment (hospital versus home residence) and diet (breast-feeding versus formula milk feeding) may represent important factors. At the opposite end of the age spectrum, Wilkieson et al. (26) found that 88% of elderly individuals in a long-term-care facility either carried or were infected with a *Candida* spp. It is generally assumed that elderly individuals have a higher yeast carriage rate, but again, this may be more a function of denture wear and increased medication than of changes in host physiology.

To directly assess the relationship of carriage and age, we therefore sampled the oral cavities of individuals in five age groups who exhibited no signs of oral disease. Each test individual was examined by an oral pathologist to confirm the healthy state of the individual's oral tissue, and individuals with even questionable symptoms were eliminated from the study. Except for some of the infants in the study, all other test individuals were dentate. Individuals with major orthodontic appliances or dentures were also removed from the study. In addition, because there has been increasing evidence for the geographical specificity of strains (17) and demonstration of microevolution in clonal populations of commensal and infecting strains at the anatomical site of colonization (9), we collected samples from individuals who had lived in Iowa City for at least the 12-month period preceding collection and limited collection to a 10-week time window. Because carriage is usually more intense on the dorsal surface of the tongue but is also found in saliva and on the oral mucosa of the inner cheek (2), we sampled all three sites and combined the number of colonies obtained on culture of samples from these sites to score the relative intensity of carriage. As noted by Cannon et al. (5), the level of carriage does not usually reflect a disease state. The overall frequency of yeast carriage was 40% in the present study, which correlates nicely with the 30 to 50% carriage range noted by Odds (12) in his review of studies of oral yeast carriage. The threshold of sensitivity has been found to be lower for buccal swabbing than for imprint or saliva collection (12), so the overall percentage of individuals carrying yeast isolates in the oral cavity may be slightly higher than that which we have determined in the present study.

We first found that the frequency of oral yeast carriage varied as a function of age. While the frequency of yeast carriage in groups A1, A3, A4, and A5 varied between 40 and 59%, the frequency of yeast carriage in group A2 was 24%, and this difference was significant. The decline in the frequency of carriage in group A2 was due to a decrease in those individuals carrying *C. albicans*. This decrease did not correlate with a decrease in the frequency of carriage of other species. The frequency of carriage of *C. albicans* was also lower in age group A1, although this was counterbalanced by a slight increase in the frequency of carriage of other species. The selective decrease in the frequency of *C. albicans* carriage suggests that if it is due to an increase in a particular barrier to colonization, the barrier must be specific to the species *C. albicans.*

The average intensity of yeast carriage was also lower both in age group A2 and in age group A1. The reduction in carriage intensity in age groups A1 and A2 was most dramatic in the highest intensity range. While in age groups A3 to A5 the proportion of individuals carrying yeast isolates at the highest intensity (>500 colonies per nine plates) ranged between 12 and 20%, the proportion in age group A1 was 5% and the proportion in age group A2 was 0%. Since age groups A1 and A2 also had a lower frequency of *C. albicans* carriage, we tested the possibility that high-intensity carriage in healthy individuals was due primarily to the species *C. albicans*. By pooling the results for the five age groups, we found that the species *C. albicans* was responsible for 100% of carriage in the highest intensity range and 69 to 84% of carriage in the next highest intensity range. In contrast, *C. albicans* accounted for only 40% of carriage in the lowest intensity range, while other species accounted for 60% of carriage. These results demonstrate that, at least in this test group, *C. albicans* is carried on average at higher densities than other yeast species.

We finally tested whether strains of the predominant species *C. albicans* varied as a function of age. There have been indications of strain specificity during carriage and disease. First, it has been demonstrated that healthy women can carry unrelated strains or highly similar but nonidentical strains in the oral cavity and vaginal canal (23), strongly suggesting differences in adaptation to different anatomical locales. Second, a small but significant proportion of women with recurrent vaginitis continue to carry one commensal strain in the oral cavity while suffering recurrent infections in the vulvovaginal region caused by a second, unrelated strain (10, 20). To test for species specificity as a function of age, we took care, as mentioned, to sample individuals in a single geographical locale and in a short time window, and we demonstrated that the comparisons between isolates from different individuals were primarily com-

parisons of clonal populations of *C. albicans*. We have found no indication that a single strain or a set of highly related strains is specific to a particular age group. We have, however, found evidence that a number of highly related isolates form clusters of three or more isolates each in dendrograms individually developed for isolates from individuals in age groups A1, A4, and A5. When a dendrogram is generated for all *C. albicans* isolates from individuals in the five age groups, a single cluster (cluster A) formed. Cluster A contains almost all of these related isolates, and the isolates in cluster A represent 33% of the isolates in the entire collection. Cluster A isolates were most prevalent in group A5, accounting for 54% of all isolates from individuals in that group.

In a prior analysis of isolates from vaginitis patients and their male partners from the Ann Arbor locale, it was demonstrated that the Ann Arbor isolates did not cocluster with Iowa City isolates (17), suggesting a geographical specificity of the strains. To test whether isolates in cluster A were specific to the Iowa City locale, we generated a mixed dendrogram of the oral *C. albicans* isolates from individuals in the five age groups collected in Iowa City and oral *C. albicans* isolates collected at roughly the same time from individuals in Ann Arbor. While all of the Iowa City isolates in cluster A again coclustered, only 14% of the Ann Arbor isolates penetrated cluster A. Therefore, although isolates in cluster A were not absolutely specific for the Iowa City locale, they were enriched in the Iowa City locale and are more predominant in age group A5.

The results of the present study are specific to a healthy test group from a single geographical locale, short time window, and an economically and racially homogeneous population. Therefore, interpretations relate specifically to this population, and any generalizations must be verified by repeating the study in other geographical locales. The results, however, demonstrate age-related variation in the frequency of carriage, the intensity of carriage, the predominant species which are carried, and the predominance of a geographically enriched set of related strains. The results suggest that for this test population, the lowest frequency and intensity of yeast carriage is in age group A2, and this is due primarily to a decrease in the frequency of *C. albicans* carriage. The results also suggest that *C. albicans* carriage is on average more intense than the carriage of other yeast species and, therefore, that *C. albicans* is more adapted than other species as an oral commensal organism. Individuals older than 15 years of age had higher intensities of yeast carriage and higher frequencies of *C. albicans* carriage. However, individuals in age group A5 carried yeast isolates at only slightly higher levels than individuals in age groups A3 and A4. There was also no indication in individuals in group A4 of an increase in the rate of carriage of other yeast species. The results for individuals $\geq$60 years old in the present study are restricted, however, to healthy individuals who are dentate, and therefore probably represent a selected minority, while dentate healthy individuals in the age groups A2 to A4 represent the majority. The present study may have, in fact, selected for older individuals who are unusually youthful, and a horizontal analysis of yeast carriage in dentate and nondentate individuals fitted with dentures is therefore warranted.

The results of a genetic comparison of isolates from Iowa City and Ann Arbor suggest that a major group of related strains, which may have a clonal origin, are enriched in the Iowa City locale and are best established in dentate elderly individuals. However, because our study was performed in a short time window, we still not know how fast strains of *C. albicans* establish themselves in a geographical locale, how long they remain prevalent, how fast they undergo microevolution and therefore diversification, how they move through age groups, whether they function as both commensal organisms and pathogens, and how fast a prevalent strain is pushed out by a new prevalent strain. By developing a database and repeating the study presented here at 3-year intervals with healthy and infected individuals, we hope to answer these questions soon.

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