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Oral yeast flora and its ITS sequence diversity among a large cohort of medical students in Hainan, China

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Abstract The most prevalent fungal infection of humans is candidiasis which is caused by species of *Candida* that are typical members of the commensal microbial flora of the oral mucosa and other body surfaces. Since species of *Candida* differ in virulence properties and susceptibilities to anti-fungal drugs, understanding the human commensal yeast flora will have a significant impact on designing treatment and prevention strategies against yeast infections. However, although there is a global interest in *Candida* species, the global distributions of *Candida* species remain largely unknown, especially among healthy hosts. Here we report the oral yeast flora from the surveys of over 1,000 medical students in China. Our results showed that this population had a yeast carriage rate (4.5%) much lower than other population samples reported previously from Mainland China (40–70%). In addition, *C. albicans* was isolated at a much higher frequency than those from other Chinese samples, with a frequency (80.9%) more similar to those in developed regions such as North America. The oral yeast carriage rates and yeast species compositions were similar between male and female students and between the hosts borne and raised on Hainan Island and those borne and raised on Mainland China. Furthermore, the sequence variation at the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster was analyzed for strains of the dominant species, *C. albicans*. Our analysis identified 14 ITS types among the 41 Hainan isolates of *C. albicans*. However, only four of the 14 ITS types were identical to those in reference strains from Europe and North America. Taken together, our analyses suggest that the oral yeast flora among host populations in China is highly heterogeneous and that there is a high ITS sequence diversity in the Hainan population of *C. albicans*.

Keywords Yeast carriage rate · Yeast diversity · *Candida albicans* · ITS · Genetic diversity · Oral hygiene

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Introduction

Due to the increasing number of immunocompromised hosts, infections caused by opportunistic pathogens have become a serious problem worldwide. Species of *Candida* and other yeasts are among the most important opportunistic pathogens. These opportunistic pathogens are typical components of the normal commensal flora and they can be

commonly found on the oral mucosa and other body surfaces. However, when the host immune system is compromised by a variety of external or internal forces such as during chemotherapy, organ transplantation, or infections by HIV, these commensal yeasts may penetrate the mucosal surfaces and invade and grow on typically sterile body sites and cause disease. Some of the infections can be fatal. Since yeast infections are often caused by endogenous species and strains, and because species of *Candida* and other commensal yeasts differ in their pathogenicity, susceptibility to antifungal drugs, and other clinically important phenotypes [1–4], it is important to investigate the normal commensal flora of hosts at the population level. Such understandings could significantly increase our ability to design targeted prevention and treatment strategies.

Although opportunistic yeast infections are a global problem and there is a global interest in these causal agents, we know relatively little of the global distribution and their associated ecological characteristics of *Candida* and other opportunistic yeast pathogens. Most data on the distributions of pathogenic yeast species have come from patient populations in Europe and North America. These studies have reported yeast carriage rates of about 20–40% in typical population samples [e.g., 1, 2, 5]. In these studies, *C. albicans* has been found to be the dominant species, accounting for up to about 90% of all isolated yeasts. In samples obtained from patients in developing countries such as those in Africa, the yeast carriage rates were similar to or slightly higher than those reported in developed countries [e.g., 6]. Interestingly, the diversities of yeast species were typically higher in developing countries and that the frequency of *C. albicans* was usually much lower than those found in developed countries [e.g., 6]. The reasons for differences in yeast species composition and diversity remain unknown.

Several recent surveys identified that the distributions of opportunistic yeast pathogens from healthy hosts in North America were similar to those in patient populations in North America and most other Western countries in both the yeast carriage rate and the yeast species distribution, with *C. albicans* as the dominant species [5, 7, 8]. In addition, different groups of healthy hosts in Hong Kong had yeast carriage rates and yeast species compositions highly similar to those in North America and Europe [9–11]. In contrast, a comparable survey of healthy hosts in several regions in Mainland China identified a significantly higher rate of yeast carriage (66.9%) than those reported in other regions [8]. Furthermore, *C. albicans* was ranked only the fourth most prevalent, accounting for only 9.4% of all isolated yeast strains [8].

The Mainland Chinese samples examined by Xu and Mitchell [8] were from two rural and three urban communities that had existed for at least 200 years. Several hypotheses were proposed to explain the significant differences in oral yeast flora between China and elsewhere. The most prominent was the oral hygiene hypothesis. According to this hypothesis, the high-yeast carriage rate and the high-oral yeast species diversity were the result of inadequate oral hygiene for many of the hosts examined in those Chinese communities [8].

The objective of this study was to gain further understanding of the commensal yeast flora of healthy hosts in China. Specifically, we were interested in examining whether sex, the place of birth and growth, and oral hygiene practices had any influence on oral yeast carriage rate and/or on oral yeast species composition in China at the population level. To achieve these goals, we chose a large homogeneous group of hosts—the entering medical students in a university. Selecting such a homogeneous group for our study eliminated the potential influences of confounding factors in our survey. These confounding factors include age and dietary habits, both of which have been suggested to influence the oral yeast flora [5, 10, 12]. We sampled and analyzed a total of 1,039 first-year, hygiene conscious, medical students with regard to their oral yeast flora. To examine the genetic diversity of these yeasts, we analyzed the sequence variation at the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster among strains of the most prevalent yeast species isolated here: *C. albicans* (see survey results below). Furthermore, the ITS sequence variation in the Hainan *C. albicans* population was compared to those in 16 reference strains from North America and Europe. We found significant sequence diversity with 14 ITS types in the Hainan *C. albicans* population. These results were discussed in the context of those reported in earlier studies.

Materials and methods

The surveyed host population

All first-year students in 2005 entering Hainan Medical College were recruited for this study. The college is located in Haikou, the capital city of the tropical island province of Hainan. These students were borne and raised in 26 out of the 28 provinces in China, but had come to attend Hainan Medical College recently. They ranged between 18 and 21 years old and all lived in the same student's residence complex and ate at the same university cafeteria. As reported by themselves, these students typically brushed their teeth 2–3 times per day, since at least from high-school years.

A total of 1,039 students were surveyed, including 470 male students and 569 female students. Of these 1,039 students, 454 were borne and raised in Hainan province (230 male and 224 female) and most never visited anywhere outside of Hainan. The remaining 585 were borne and raised in 25 Mainland Chinese provinces and regions. Most Mainland students never stepped outside of their own provinces and the trip to Hainan Medical College in Haikou was their first major journey outside the communities, where they were borne and raised. These students had come from the following provinces (number of students in parenthesis): Hainan (454 students), Guangdong (34 students), Guangxi (32), Yunnan (17), Fujian (20), Jiangxi (56), Hunan (44), Guizhou (32), Zhejiang (17), Jiangsu (17), Anhui (30), Hubei (30), Chongqing (17), Sichuan (30), Shandong (16), Henan (30), Shaanxi (17), Gansu (7), Qinghai (6), Xinjiang (2), Hebei (45), Shanxi (30), Liaoning (20), Jilin (20), Helongjiang (9), and Inner Mongolia (7). The 585 students borne and raised outside of Hainan included 240 male students and 345 female students. Almost all students arrived at Hainan Medical College at the beginning of September 2005. All samples were taken from mid-September to mid-October in 2005.

Yeast sampling and identification

During the time of sampling, no individual had an apparent yeast infection, and none had taken any antifungal medication in the weeks before samples were obtained. Sterile swabs were used to sample the upper and lower outer gingiva of each person. After sampling, the tip of each swab was immediately severed and submerged in a sterile cryogenic tube for selective yeast growth, storage, and transport. Each tube contained 0.5 ml of sterile enrichment YEPD broth composed of 2% (weight/volume) yeast extract, 1% Bacto-peptone (BD), 2% dextrose, 18% glycerol, and the antibiotic chloramphenicol (50 μg/ml). As was shown previously, this medium afforded excellent recovery of medical yeasts [8]. After 2–3 days of incubation to enrich yeast population in each sample, subcultures of the medium suspension were streaked onto Sabouraud glucose agar medium for isolation of yeasts. All yeast colonies were then restreaked onto CHROMAgar medium. The CHROMAgar medium allows direct putative identification of *Candida albicans* (green color) and *Candida tropicalis* (blue color). Other yeast species may also exhibit different colors and colony morphologies [13]. For each swab sample, yeast colonies with distinct color and colony morphology on CHROMAgar were further subcultured onto the YEPD agar medium (YEPD broth containing 2% agar). Cells from these selected colonies on YEPD medium were then subjected to genomic DNA extraction using a protocol described in Xu et al. [14]. The internal transcribed spacer (ITS1 and ITS2) regions for each of the isolates were amplified using the fungal universal primers ITS1 and ITS4 [15]. ITS sequencing was conducted at the Mobix laboratory at McMaster University in Ontario, Canada. These ITS sequences were then compared with sequences from the GenBank database for species identification. All strains were unambiguously identified to the species level.

Analysis of yeast isolation rates

To compare the potential differences in yeast carriage rates and yeast species compositions among groups of hosts, we used the Chi-square contingency table test. Comparisons were made based on the sex of the hosts (male vs. female) and/or geographic areas where the students were borne and raised (Hainan Island vs. Mainland China). When the expected numbers were smaller than five for certain categories, the smallest categories were combined in the test as one group [16].

ITS sequence variation in the *C. albicans* population

Our survey identified that *C. albicans* was the dominant species (see Results below), accounting

for about 80% of all isolated yeasts. Therefore, we proceeded to investigate the potential sequence variation within the Hainan population of *C. albicans* and compare their diversity with those from reference strains to other parts of the world. The Hainan sample of *C. albicans* included 41 strains. Three out of the 41 strains were from one host (strains B35-1, B35-2, and B35-3), while two other strains were from another host (strains M37 and M37-2). All other 36 strains were from a different host. The reference sample included 16 strains representing all five major clades of *C. albicans* as-identified previously by Ca3 probing in Southern hybridization [17]. Drs. David Soll and Claude Pujol of the University of Iowa generously provided us these reference strains. The ITS sequences from the reference strains were obtained as described above. The ITS types and their relationships among all 57 ITS sequences were analyzed together using the computer program PAUP 4.10b10 [18].

For phylogenetic analysis of the ITS sequences, we used the maximum parsimony (MP) approach. All variable nucleotide sites were included for genotype determination and for phylogenetic inferences. MP trees were obtained using heuristic searches and the tree-bisection reconnection (TBR) branch swapping with 100 starting trees obtained by a random sequential addition of taxa. Bootstrap support for individual branches was obtained using 1,000 repeats. Mid-point rooting was used for the presentation of the phylogenetic tree.

Results

Oral yeast carriage rate

Our survey identified that among the 1,039 medical students sampled, 47 carried yeasts in their oral cavities, representing an overall oral yeast carriage rate of 4.52%. Each of these 47 students was found to have only one species of oral yeast in our survey. The combined oral yeast carriage rate for male students was 3.83%, slightly lower than that for the female student sample (5.10%). However, these two rates were statistically not different from each other (Chi-square value = 1.426, df = 1, $P = 0.1966$). Similarly, we found no statistically significant difference in oral yeast carriage rates between those borne and raised on the tropical island of Hainan and those borne and raised in other provinces on Mainland China (Chi-square value = 1.513, df = 1, $P = 0.2352$). The summary results on yeast carriage rates are presented in Table 1. No significant difference in oral yeast carriage rates was found among students from different provinces on the Mainland (data not shown).

Based on the obtained ITS sequences, all 47 yeast isolates were unambiguously identified to the species level. Our identification showed that the dominant species in this collection was *C. albicans*, with a prevalence of 80.9% (38/47). The second most prevalent was *Candida parapsilosis*, with a prevalence of 10.6% (5/47). Three other species were also found in our survey: *Pichia anomala* (2 strains), *C. tropicalis* (1 strain) and *Trichosporon asteroides*

Table 1 Summary of oral yeast flora among samples of medical students in Hainan, China

Place of birth and growth	Sex	No. of hosts	No. with yeasts(%)	No. of each species isolated				
				Ca(%)	Cp	Ct	Pa	Ta
Hainan	Male	230	9(3.91)	8(88.9)	1	0	0	0
	Female	224	15(6.69)	13(86.7)	0	0	1	1
	Total	454	24(5.29)	21(87.5)	1	0	1	1
Mainland	Male	240	9(3.75)	7(77.8)	0	1	1	0
	Female	345	14(4.06)	10(71.4)	4	0	0	0
	Total	585	23(3.93)	17(73.9)	4	1	1	0
Combined	Male	470	18(3.83)	15(83.3)	1	1	1	0
	Female	569	29(5.10)	23(79.3)	4	0	1	1
	Total	1039	47(4.52)	38(80.9)	5	1	2	1

Note. Ca, *Candida albicans*; Cp, *Candida parapsilosis*; Ct, *Candida tropicalis*; Pa, *Pichia anomala*; Ta, *Trichosporon asteroides*

(1 strain). Overall, we found no statistically significant difference between male and female students in their oral yeast species composition, either within Hainan, or within the Mainland group, or for the total sample (Chi-square value = 0.01, df = 1, $P = 0.996$, for the total samples). Similarly, we found no difference in yeast species composition between those borne and raised in Hainan and those borne and raised elsewhere in Mainland China for each of the two individual sexes as well as for the combined sample (Chi-square value = 1.4229, df = 1, $P = 0.4043$, for the total samples).

Sequence variation at the ITS region in the Hainan sample of *C. albicans*

Among the 536 sequenced nucleotides around the ITS region of the nuclear rRNA gene cluster, 515 were constant and 21 were variable among the 57 *C. albicans* isolates. These 21 variable sites contained 14 transitional substitutions and 7 transversional ones. One of the three sites with a transversional mutation had a third allele, a single base deletion. Taken together, the transition to transversion ratio within the nuclear ITS DNA fragment was 2 (i.e., 14/7).

Through BLAST searches, all 41 Hainan *C. albicans* isolates analyzed here had ITS sequences with greater than 98% nucleotide identity to that of the sequenced model laboratory strain SC5314 of *C. albicans*. In contrast, these ITS sequences all had less than 94% nucleotide identity to the full-length ITS sequence from the closely related species *Candida dubliniensis*. Thus, our ITS sequences confirmed that these 41 isolates belonged to *C. albicans*. Similarly, all 16 reference strains also had ITS sequences with greater than 98% nucleotide identity to strain SC5314.

The phylogenetic relationships among the 57 strains as reflected by ITS sequence typing are presented in Fig. 1. Among the 16 reference strains, we identified a total of six ITS types. However, the ITS types did not completely correspond to their clade identification based on Ca3 probing in Southern hybridization (Fig. 1). As can be seen in Fig. 1, the four strains in clade I, three strains in clade II, and three strains in clade III all had identical ITS types distinct from each other. However, strains within clades E and S were not clustered together as were for strains in clades I, II and III. Of the three strains in clade E, one (strain P057096 from Brazil) had an ITS type identical to that of clade II while the other two strains (strains P75010 from Belgium and P80021 from Italy) had an identical but different ITS type from strain P057096 (Fig. 1). Furthermore, all three clade S strains had ITS sequences different from each other: strain P52084 from Canada had an identical ITS type as that of the three clade II strains while strains P22078 from England and P75071 from Italy each had an ITS type distinctly different from representative strains in other clades (Fig. 1).

Using the ITS types among the above 16 strains as references, we now proceed to describe the ITS sequence variation in the Hainan *C. albicans* sample. Among the 41 isolates from Hainan, we identified 14 ITS sequence types (Fig. 1). Of the two hosts from which multiple isolates were analyzed, strains from the same host were found to have identical ITS sequences. In total, four of the 14 ITS types were identical to those in the reference strains (i.e. ITS types for clade I, clade II, two of the three clade E strains, and one of the clade S strains). The remaining 10 types that included 21 strains were different from those in the reference strains. The uniqueness of the Hainan ITS types were further confirmed by BLAST analyses against *C. albicans* ITS sequences in the GenBank. Among the 41 strains, only 10 were found to have 100% sequence identity to the published, full-length *C. albicans* ITS sequences in the GenBank (there are about 30 such sequences in the GenBank). Furthermore, all 10 were the same as that of the four clade I strains, including strain SC5134 (Fig. 1). Out the remaining 31 strains, a total of 13 ITS types were identified and each of the 13 types had a nucleotide sequence differed by 1 to 6 bases from the published full-length ITS sequences in the GenBank. The ITS results thus suggested that the analyzed strains here contained a high level novel variation in the nuclear genome.

The high-level sequence diversity in this sample of *C. albicans* was not structured based on the birthplace or the sex of the hosts. Specifically, all seven ITS types shared by multiple strains included those from hosts of both sexes and borne in different regions in China, including Hainan (Fig. 1). In particular, strains from hosts borne in Hainan had ITS types very similar to those from hosts borne and raised on Mainland China.

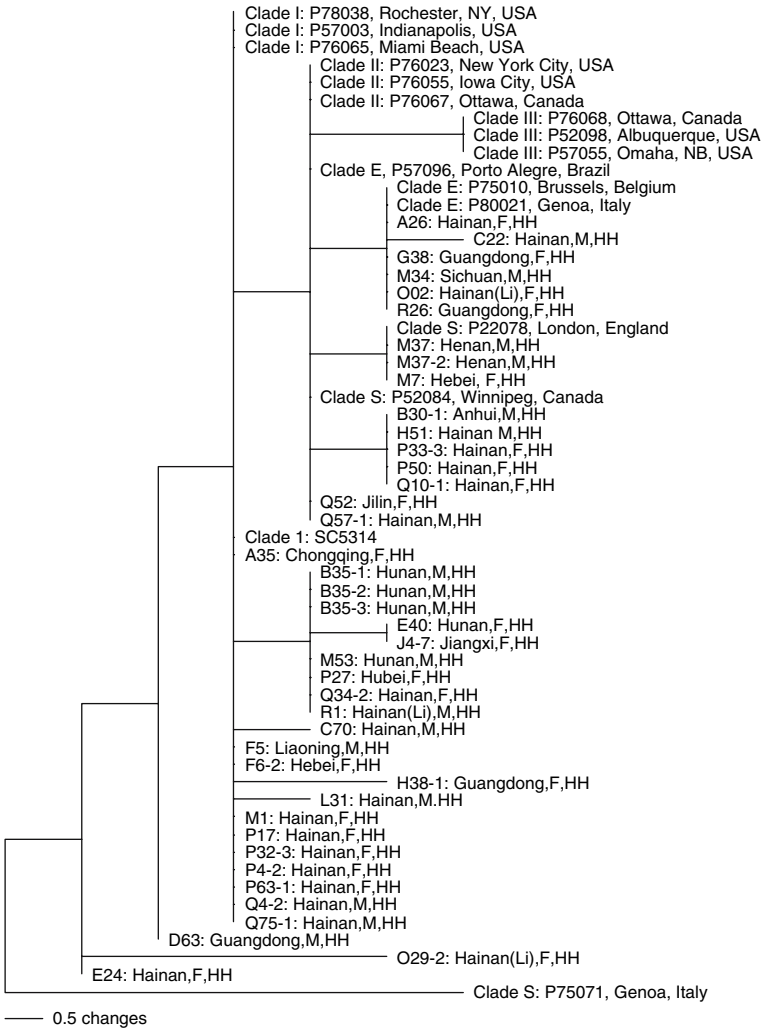

Fig. 1 One of 92 maximum parsimonious trees derived from the sequences of the nuclear internal transcribed spacer (ITS) regions. Strain label includes strain identification number, the province or municipality where their hosts were borne and raised, sex of the host (F, female; M, male), and the health status of individual hosts (HH, healthy hosts). The total tree length was 26 with a consistency index of 0.808 and a retention index of 0.924. As comparisons, the ITS sequences from 16 reference strains were also included. Mid-point rooting was used. The scale bar represents 0.5 nucleotide substitution and the branch lengths are proportional to the amount of sequence divergence

Discussion

To our knowledge, this study included one of the largest numbers of healthy hosts in a survey of commensal oral yeasts. Most studies published so far included less than 300 hosts, less than a third of the sampled hosts here. Interestingly, we found that the surveyed medical students had a very low-oral yeast carriage at 4.52%, a rate significantly lower than those reported in other healthy, as well as patient groups, both in China as well as in other parts of the world (12–80% of carriage rates depending on the sampled hosts) [1, 5–12]. We observed no significant difference in oral yeast carriage rates between male and female students. In addition, despite the large differences in climate and diet among the places where the hosts were borne and raised, the places of birth and growth seemed to have little influence on host's yeast carriage rate and yeast species composition in this population. Our analysis identified that *C. albicans* was the dominant yeast species in our sample, accounting for 80.9% of all isolates. Interestingly, this observed species composition in our sample was more similar to those reported in developed countries/regions in the West than to those reported for several healthy communities in China [1, 2, 7, 8, 14].

There may be several possibilities for the significant difference in oral yeast carriage rates between the current population sample and the samples examined earlier in China. The first is that the low rate of oral yeast carriage in this study might be due to differences

in our study methods. However, we believe this explanation is highly unlikely. Though the species identification systems were different (ITS sequencing in this study vs. API20 yeast identification system in others), the sampling protocols for this study and for that of Xu and Mitchell [8] were identical. In addition, using the same sampling protocol, we found yeast carriage rates in several communities in North America to be similar to those reported by others using different sampling methods [e.g., 5, 7]. Finally, the yeast species identification was the last step in the survey. As a result, it should have no impact on estimates of yeast carriage rates based on data obtained earlier in the survey.

The second possibility for the low rate of yeast carriage is that the age structure and group behavior of the hosts in this study were different from those in the early study from Mainland China by Xu and Mitchell [8]. The earlier study included people from 17 to 75 years old while all surveyed individuals in this study were between 18 and 21 years old. To test the possibility of age structure-related influences, we pooled the 18–21 year olds from the earlier study [8] and found that 5 out of the 16 individuals in this age group carried yeasts in their oral cavities. While this rate (31.2%) was still higher than that reported here, it was indeed lower than those in other age groups in the earlier study. Though the sample size for this age group in the earlier study was small (only 16 individuals), the difference in yeast carriage rates among the different age groups is consistent with results from previous studies and suggests that there might be some intrinsic physiological [e.g., 19] or behavioral differences among them that could have contributed to the observed differences.

One significant behavioral difference between the medical students examined here and the general community populations sampled earlier was their overall oral hygiene practices. The medical students sampled here brushed their teeth 2–3 times a day while many of the hosts sampled earlier in the general communities did very little brushing. For example, most individuals (including all five 18–21 year olds) in the two communities from rural Jiangxi province had no habit of toothbrushing and those two communities were found to have the highest oral yeast carriage rates [8]. While indirect, our inference was consistent with the hypothesis that oral hygiene practices likely played a significant role in oral yeast carriage rates among host groups in China.

Aside from a lower-oral yeast carriage rate, the yeast species diversity observed here was also lower than those observed in other communities in China (Simpson's diversity index = 0.35 in this study vs. 0.67–0.86 reported in the other study)[8, 14]. Interestingly, our observed species diversity here was very similar to those observed in North America (range 0.11–0.59) and other developed regions, including Hong Kong, perhaps the most westernized place in China at present. In addition, as was with those in developed regions where commensal yeasts were generally dominated by *C. albicans*, oral yeasts from the medical students examined here were predominantly *C. albicans*.

The high ratio of *C. albicans* in our sample seemed to suggest that a shift of oral yeast species composition might be occurring in China in the young generation. While this might be true, further inspections suggest that the overall prevalence of *C. albicans* in the total sample of 1,039 medical students (36/1,039, or 3.4%) was in fact not significantly different from that reported from typical Chinese communities (15/239, or 6.3% in ref. 8) (Chi-square value = 3.41, df = 1, $P = 0.068$). Both of these rates were much lower than those reported in North America and most other developed regions, including Hong Kong [9–11]. Therefore, our results suggest that the prevalence of *C. albicans* in the college student sample might not be due to the replacement of other yeast species by *C. albicans*. Instead, the oral hygiene and/or other factors among the medical students have probably helped eliminating non-*C. albicans* yeasts from their oral cavities. In addition, it seems that *C. albicans* might not be affected as much as other yeasts by medical students' oral hygiene practices or other factors. In North America, Europe as well as Hong Kong, good oral hygiene has been practiced for a long time and *C. albicans* is the dominant oral yeast species in these regions, regardless of the ethnical background of the hosts [8–11]. As appropriate oral hygiene practices are increasingly emphasized for the general population in China (like the surveyed medical students), it is possible that the oral yeast flora among people in China may become progressively dominated by *C. albicans*, similar to those in developed countries and regions.

Our analyses identified a large number of ITS types in the Hainan *C. albicans* population not found

in either the reference sample or the GenBank. This result suggests that the Hainan population of *C. albicans* is highly diverse and heterogeneous. In addition, our ITS sequence analysis did not identify any apparent pattern of ITS sequence variation based on either the birthplace or the sex of the hosts. Our results suggest that the *C. albicans* populations in Hainan have likely experienced significant gene flow and clonal dispersal in recent years with those in Mainland China as well as outside of China. This hypothesis is consistent with the human demographics in Hainan. Specifically, a large number of tourists and migrants have come to Hainan Island from Mainland China and elsewhere each year over the last two decades. Our observation of significant gene flow and clonal dispersal is also consistent with other studies on geographic populations of *C. albicans* [e.g., 17, 20]. At present, the impact of gene flow and clonal dispersal on local yeast infections remains largely unknown.

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