

Multilocus sequence typing for *Candida albicans* strains from the intestinal tract of patients with cirrhosis

J. ZHU¹, J.-F. CAO², T. ZHANG³, T.-Y. LI⁴, X.-W. LI⁵, W. SHEN¹

¹Department of Gastroenterology and Hepatology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China

²Department of Gastroenterology and Hepatology, The Affiliated Hospital of Guizhou Medical University, Guizhou, China

³Department of Gastroenterology and Hepatology, The First People Hospital of Guiyang, Guizhou, China

⁴Pediatric Department of Respiration, The Children Hospital of Guiyang, Guizhou, China

⁵Department of Gastroenterology and Hepatology, Guiyang Hospital of Guizhou Aviation Industry Group, Guiyang, China

Abstract. – OBJECTIVE: Multilocus sequence typing (MLST) was applied to investigate the genetic diversity of *Candida albicans* in the intestinal tract of cirrhosis patients.

PATIENTS AND METHODS: We used CHROM agar *Candida* medium to obtain 105 *Candida* sp. isolates from fecal samples (276 subjects), including 63 isolates from the cirrhosis group (141 subjects) and 42 isolates from the healthy control group (135 subjects).

RESULTS: Among the 105 *Candida* strains isolated, 60 strains were identified as *Candida albicans*. Patients with cirrhosis had significantly higher rates of colonization by *Candida* sp. (44.68% vs. 31.11%, $p < 0.05$) and *C. albicans* (27.66% vs. 15.56%, $p < 0.05$) relative to healthy controls. In the cirrhosis group, the rate of colonization further increased with disease progression and antibiotic treatment ($p < 0.01$). Sixty *C. albicans* isolates were analyzed by MLST. Fifty diploid sequence types (DST) were observed, and 26 new DSTs and 3 novel alleles were found. The majority of isolates were distributed among three clades, clade 8 (31.67%), clade 14 (15.00%) and clade 18 (21.67%). Among 39 strains from the cirrhosis group, 16 strains (41.02%) belonged to clade 8, while only 3 strains (14.29%) from healthy group belonged to clade 8 ($p < 0.05$). In addition, concatenated sequences of the 7 housekeeping gene fragments were analyzed for all the different DSTs in clade 8 to evaluate the loss of heterozygosity (LOH), which indicates *C. albicans* microvariation in the gut of cirrhosis patients.

CONCLUSIONS: This study suggests that cirrhosis disease progression and antibiotic treatment is associated with increased colonization

by *Candida* sp. and *C. albicans*. We are the first to provide MLST-based genotype profiles for *C. albicans* Guizhou China, and to identify clade 8 as the potential main clade of *C. albicans* colonization in the gut of cirrhosis patients.

Key Words:

Candida albicans, Multilocus sequence typing (MLST), Intestinal tract, Cirrhosis.

Introduction

Hepatic cirrhosis is the end-phase of all chronic hepatic disease which has various etiologies and is divided into initial stage and advanced stage cirrhosis, with the latter including decompensated cirrhosis and acute-on-chronic liver failure^{1,2}. Cirrhosis-associated immune dysfunction (CAID) refers to systemic inflammation and immunodeficiency, and even immunoparalysis, which may be present during cirrhosis. Along with immune dysfunction, the risk of infection (both bacterial and fungal) increases gradually in patients with cirrhosis³⁻⁶. While the majority of research has focused on the bacterial pathogens of the gastrointestinal tract in cirrhotic patients, minimal attention has been given to the gut fungal microbiota-mycobiota, especially their genetic dynamics. Several studies⁷⁻¹¹ have suggested that the intestinal mycobiome may act as a potent source of infection in immunodeficient hosts. The dimorphic, symbiotic

yeast *C. albicans* is a normal constituent of the human digestive tract. However, *C. albicans* is also an opportunistic pathogen that can produce high morbidity and mortality among immunocompromised patients^{12,13}. Effective therapy against candidiasis requires characterization of its population structure as well as strain- and species-level identification using genotyping methods as *Candida* species show species-specific differences drug susceptibility patterns^{14,15}. Moreover, molecular typing can provide local molecular epidemiological information, trace the source of transmission, and be employed on species phylogenetic analysis.

Numerous molecular biological techniques have been utilized for genotyping *C. albicans*, but many of these may be unavailable in most laboratories. A rapid, accurate, reproducible and economical strain typing system is required for use in clinical laboratories. The MLST system for typing *C. albicans* is an effective, reproducible and easy-to-use genotyping approach, which examines the profiles of seven housekeeping genes (AAT1a + AAC1 + ADP1 + MPIb + SYA1 + VPS13 + ZWF1b), the results of which can be shared online in the *C. albicans* MLST database (<http://pubmlst.org/calbicans>). Compared to other molecular typing methods, MLST has the best discriminatory power (99.7%) for *C. albicans*¹⁶. Currently, based on MLST, *C. albicans* strains can be separated into over 3000 DSTs and 18 clades from various geographical locations¹⁷. *C. albicans* population structure apparently varies by geographical location¹⁸, with specific genotypes or clades often found in patients from the same geographical region^{19,20}. Strains may differ in terms of drug-resistance, virulence, and pathogenesis depending on their genotype or clade. For instance, a considerable proportion of isolates in clade 1 exhibit resistance to flucytosine and terbinafine¹⁵, and a study²¹ suggested that the bloodstream infectious isolates of MLST clade 18 have higher levels of farnesol secretion than isolates of other clades.

However, there have been no reports on the population traits of *C. albicans* isolates from cirrhosis patients from Guizhou in China. We conducted MLST to investigate the population structure and genetic diversity of 60 *C. albicans* strains isolated from feces, including 39 strains from patients with cirrhosis and 21 strains from healthy individuals, to evaluate the geographic characteristics of *C. albi-*

cans clinical strains obtained from Guizhou in China and to determine whether *C. albicans* strains from patients with cirrhosis belong to a specific genotype or clade.

Patients and Methods

Sample and Data Collection

All participants enrolled in this investigation signed the informed consent. The study was approved by the Ethics Committee of Guiyang Hospital in Guizhou Aviation Industry Group (Grant No.: 2015-037). One hundred and forty-one patients were determined as having cirrhosis (cirrhosis group) by medical history, clinical manifestations, liver function test results, and imageological diagnosis. The degree of cirrhosis was evaluated by Child-Pugh scoring as A (mild, n = 26), B (moderate, n = 64) or C (severe, n = 51). In addition, 135 healthy volunteers were recruited to this work as the control group. Participants' relevant data were recorded, including gender, nationality, residential address, admission date, ward number, etiology, and antibiotic use history. In total 276 fecal samples were collected from these subjects.

Culture and Identification

Each fecal sample was immediately inoculated onto a Sabouraud dextrose agar plate containing antibiotics and then cultured at 37°C for 48 h. *Candida* sp. was initially identified by colony color on CHROMagar (CHROM agar Microbiology, Paris, France) plates, with green indicating *C. albicans*, blue *Candida tropicalis*, pink with fuzz *Candida krusei*, amaranth with moist *Candida glabrata* and white or other colors for other *Candida* sp.

DNA Extraction and Amplification of Housekeeping Genes

Total cellular *C. albicans* DNA was extracted using a Gen Toru Kun kit (TaKaRa Shuzo Co. Ltd, Otsu, Shiga, Japan) as recommended by the manufacturer. One microliter of extracted DNA was used to amplify each of the 7 housekeeping genes using the TaKaRa Ex Taq PCR amplification kit (TaKaRa Shuzo, Otsu, Shiga, Japan). The primers used have previously been described by Bounoux et al¹⁶. The reactions were performed in a final reaction mixture (50 µL) containing genomic DNA template 2 µL, 10 pmol 2 µL of each primer (forward and reverse primers), 2 ×

Es Taq Master Mix 25 µL, distillation-distillation water 19 µL. The amplification reactions were performed with the following cycling parameters: 93°C for 5 min, 30 cycles of denaturation for 30 s at 93°C, annealing for 60 s at 56°C, extension for 1 min at 72°C, and a final extension at 72°C for 4 min.

Sequencing of PCR

Amplification Products

PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Both strands of the PCR products were then sequenced directly on an ABI prism 3100 DNA sequencer using a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems Japan Co. Ltd., Tokyo, Japan) as recommended by the kit manufacturer.

Diploid Sequence Types (DST)

Determination

As described previously^{16,22}, isolates' sequence types (ST) were determined based on seven different housekeeping genes. MLST data for all *C. albicans* strains were determined based on the combined ST patterns obtained from the seven housekeeping genes, and DSTs were defined by comparison with the *C. albicans* MLST database (<http://pubmlst.org/calbicans>). New alleles, new STs and/or new DSTs were founded if no matching sequence and relative information had previously been submitted to the *C. albicans* MLST database, following which new accession numbers were acquired.

Molecular Phylogenetic Trees Analysis

DNA sequences were aligned using GENE-TYX genetic information processing software (Software Development Co., Ltd., Tokyo, Japan). Sequences were used to construct a dendrogram using the unweighted pair-group method with arithmetic mean (UPGMA) method and MEGA 7.0 software.

ABC Genotyping

ABC genotypes of *C. albicans* isolates were determined using PCR primers that span the site of the transposable intron of the 25S rDNA sequence, as described by McCullough et al²³.

Statistical Analysis

Chi-squared tests were conducted in Statistical Product and Service Solutions (SPSS) version 19.0 software (IBM, Armonk, NY, USA) to compare *Candida* sp and *C. albicans* intestinal colonization rates by the degree of cirrhosis, different etiologies as well as antibiotic treatment, and to analyze differences in clade prevalence of *C. albicans* between the cirrhosis group and the control group. $p < 0.05$ was considered statistically significant.

Results

Comparison of *Candida* sp. in Fecal Samples From Cirrhosis vs. Control Groups

In the cirrhosis group, 63 fecal samples (44.68%) had detectable *Candida* sp. by CHROMagar (including 39 strains of *C. albicans*, 5 strains of *C. tropicalis*, 6 strains of *C. parapsilosis*, 6 strains of *C. krusei* and 7 strains of other *Candida* sp.). Among 135 healthy individuals in the control group, there were 42 (31.11%) *Candida* sp. carriers (including 21 strains of *C. albicans*, 3 strains of *C. tropicalis*, 4 strains of *C. parapsilosis*, 2 strains of *C. krusei* and 12 strains of other *Candida* sp.). Compared with the healthy control group, the cirrhosis group had significantly more carriers of *Candida* sp. and *C. albicans* ($p < 0.05$, Table I).

Comparison of *Candida* sp. in Fecal Samples Within the Cirrhosis Patient Group

Within the cirrhosis group, *Candida* sp. and *C. albicans* colonization rates increased significant-

Table I. Comparison of colonization rate of *Candida* spp or *C. albicans* between the cirrhosis and control groups.

Groups	No. of subjects	<i>Candida</i> spp positivity, n (%)	χ^2	p	<i>C. albicans</i> positivity, n (%)	χ^2	p
Cirrhosis group	141	63 (44.68%)	5.39	0.02*	39 (27.66%)	5.94	0.015*
Control group	135	42 (31.11%)			21 (15.56%)		

* $p < 0.05$ indicates a significant difference between groups.

Table II. Relationship of Child–Pugh classification and positive rate of *Candida* spp or *C. albicans* in the cirrhosis group.

Child-pugh classification	No. of subjects	<i>Candida</i> spp positivity, n (%)	χ^2	<i>p</i>	<i>C. albicans</i> positivity, n (%)	χ^2	<i>p</i>
A	26	6 (23.08%)	13.87	< 0.001	4 (15.38%)	9.99	0.002
B	64	24 (37.50%)			12 (18.75%)		
C	51	33 (64.71%)			23 (45.10%)		

ly with the degree of cirrhosis ($p < 0.01$, Table II) as well as with antibiotic treatment ($p < 0.01$, Table III). However, there were no significant differences in *Candida* sp. or *C. albicans* colonization rates between different cirrhosis etiologies ($p > 0.05$, Table IV).

C. albicans DST and Phylogenetic Analysis

In total, sixty strains of *C. albicans* were detected, 39 from the cirrhosis group and 21 from the control group. Among the 60 strains, 50 DSTs were identified by MLST, including 26 new DSTs. The 26 new DSTs included 18 genotypes from the cirrhosis group and 8 genotypes from the healthy control group. Three new alleles were identified in ADP1, AAT1a, and MPIb. All sequence information of new alleles and DSTs were submitted to the *C. albicans* MLST database, and accession numbers were granted for each. Forty-six DSTs were identified in only one isolate each (31 DSTs in cirrhosis group and 15 DSTs in healthy group); 4 DSTs were detected in multiple isolates: DST1931 was found in 6 isolates (5 from 5 cirrhosis patients and 1 from a healthy individual), DST1220 in 3 isolates (2 from 2 cirrhosis patients and 1 from a healthy individual), DST1097 in 3 isolates (1 from a cirrhosis patient and 2 from 2 healthy individuals) and DST307 in 2 isolates from 2 healthy individuals. Although isolates from 4 patients shared DST 1931 and isolates from 2 patients shared DST 1220, these patients did not stay in the same wards or have the same admission date. So we can exclude the nosocomial transmission.

The 50 DSTs found in our study, together with 1011 DSTs from the *C. albicans* MLST database were divided into twelve clades (Figure 1), and a phylogenetic tree of the 50 DSTs was constructed using MEGA 7.0 software, as presented in Figure 2. Clade 8 was the most common clade, containing 19 of the 60 *C. albicans* isolates (31.67%), followed by clade 18 (21.67%) and clade 14 (15.00%). Clade 8 contained significantly more isolates from the cirrhosis group (16/39) compared to the control group (3/21) ($\chi^2 = 4.510$, $p = 0.034 < 0.05$). To further investigate genetic microvariation between all DSTs in clade 8, the concatenated sequences of the 7 housekeeping gene sequences profiles were compared for these DSTs. We found that all DSTs presented different extent of LOH at different alleles (Data not shown).

ABC Typing of *C. albicans* Samples

Among a total of 60 isolates, 37 isolates (61.67%) were identified as type A, 15 isolates (25%) as type B and 8 isolates (13.33%) as type C (data not showed). Type A was the dominant type in the cirrhosis group (27/39, 69.23%) as well as the control group (10/21, 47.61%), with no statistical difference in the proportion of type A isolates between the two groups ($\chi^2 = 2.697$, $p = 0.101 > 0.05$). Among the three major clades (clade 8, clade 14, and clade 18), several isolates in clade 8 and all in clade 18 were type A, while all 9 isolates in clade 14 were type B. These isolates with the same DST (DST 1931, DST 307, DST 1220, DST 1097) had identical ABC typing.

Table III. Relationship of recent antibiotic treatment and the positivity rate of *Candida* spp or *C. albicans* in the cirrhosis group.

Recent antibiotic treatment	No. of patients	<i>Candida</i> spp positivity, n (%)	χ^2	<i>p</i>	<i>C. albicans</i> positivity, n (%)	χ^2	<i>p</i>
Yes	51	36 (70.59%)	21.7	< 0.001	21 (41.18%)	7.3	0.007
No	90	27 (30.00%)			18 (20.00%)		

Table IV. Relationship of the cause of cirrhosis and the positivity rate of *Candida* spp or *C. albicans* in the cirrhosis group.

Cirrhosis cause	No. of patients	<i>Candida</i> spp positivity, n (%)	χ^2	<i>p</i>	<i>C. albicans</i> positivity, n (%)	χ^2	<i>p</i>
Virus	62	26 (41.94%)	2.68	0.44	19 (30.65%)	2.18	0.54
Alcohol	37	17 (45.95%)			9 (24.32%)		
Mixed factors*	26	10 (38.46%)			5 (19.23%)		
Other factor	16	10 (62.50%)			6 (37.50%)		

*Mixed factors refer to ≥ 2 factors.

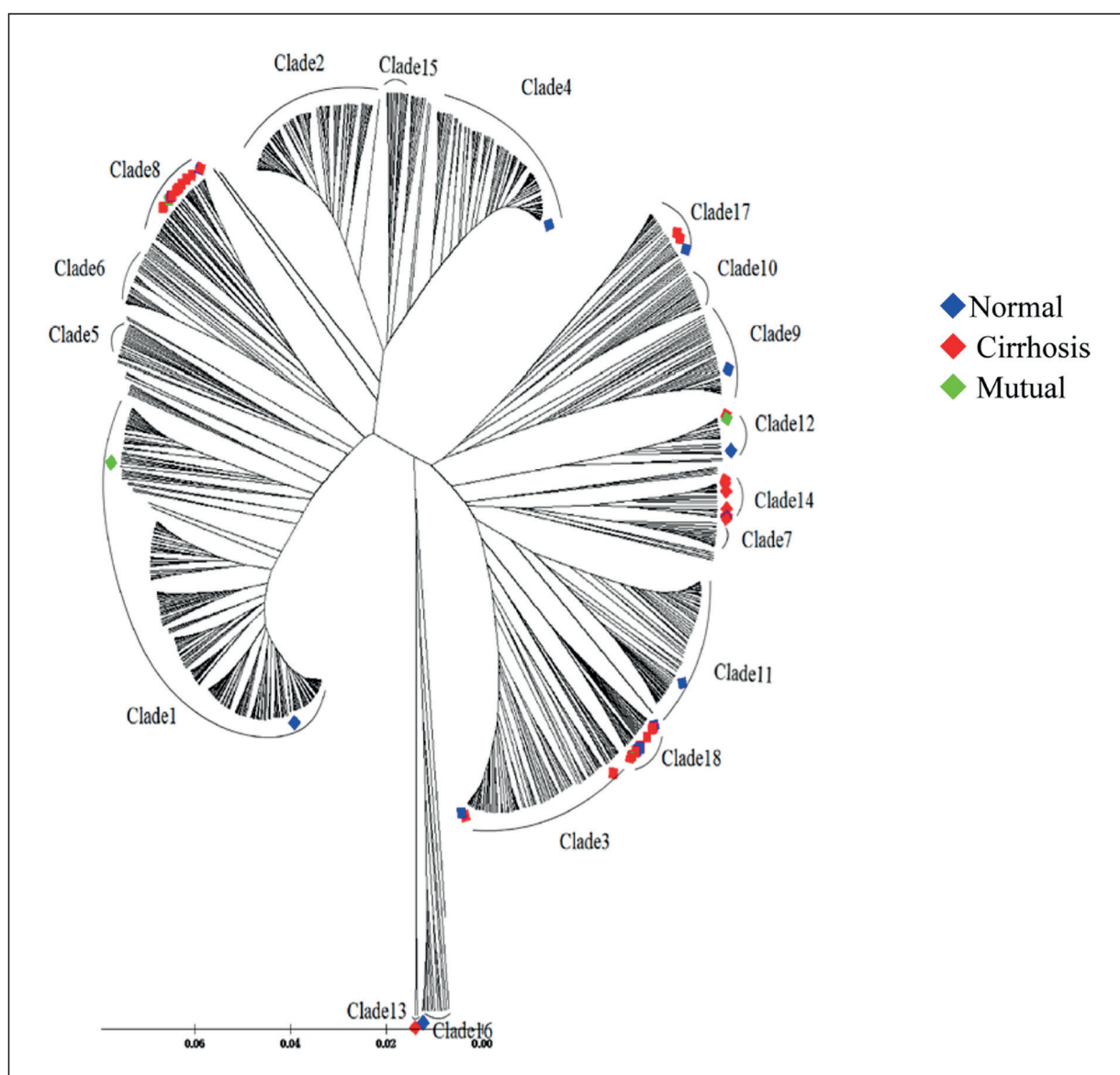


Figure 1. Dendrogram constructed by UPGMA analysis of p-distances (0.04) based on concatenated sequences of the seven housekeeping genes from 50 DSTs identified in this study and 1011 isolates from the MLST database. The isolates from this study were distributed among 12 clades, namely clades 1, 3, 4, 8, 9, 11, 12, 13, 14, 16, 17, and 18. DSTs were marked as blue, red and green dots if they came from healthy controls, cirrhosis patients, or were shared by both groups, respectively.

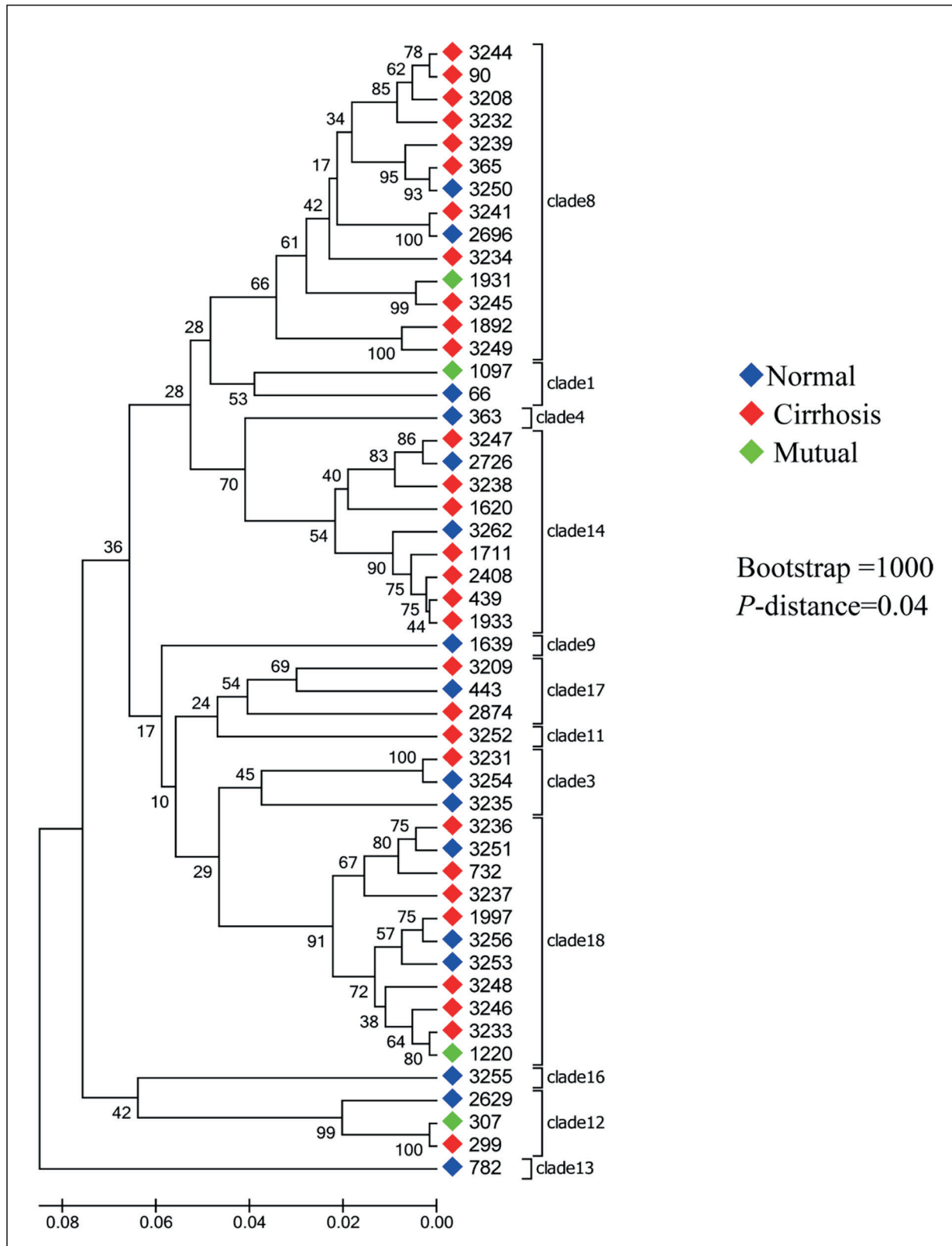


Figure 2. Dendrogram constructed by UPGMA analysis of p-distances (0.04) based on the concatenated sequence of the seven housekeeping genes from 50 DSTs identified in this study. Labeled red: DSTs from cirrhosis samples, blue: DSTs from healthy controls and green: found in both groups.

Discussion

CAID and disturbances in gut microbiota are the main causes of increased infection, including fungal infection during the course of cirrhosis progression. In a study on medical intensive care unit patients, those with cirrhosis fungal colonization had the higher mortality rates²⁴. In our work, *C. albicans* was the most common species among *Candida* sp. isolates recovered from stools, and cirrhosis patients had significantly higher colonization rates of *Candida* sp. and *C. albicans* compared to healthy controls. Further, intestinal colonization of *Candida* sp. and *C. albicans* was positively associated with the severity of cirrhosis and antibiotic treatment, which is in agreement with the literature⁷, and suggests that gastrointestinal environmental changes in patients with cirrhosis contribute to fungal colonization.

MLST was utilized to analyze the genetic diversity of 60 *C. albicans* isolates, including 39 isolates from cirrhosis patients and 21 isolates from healthy individuals. In total, 50 DSTs were identified including 26 novel DSTs and 3 alleles were defined, which illustrates the molecular diversity of *C. albicans* colonizing the gut. According to the literature, isolates in clade 14 and clade 18 are mainly distributed in East Asia^{17,19,25}. In this study, only 4 isolates belonged to clade 1, which is the largest clade worldwide²⁰, while the majority of isolates were classified into clades 8, 14, and 18 (31.67%, 15%, and 21.67%, respectively), suggesting that these three clades have geographical specificity. In addition, clade 2, 5, 6, 7, 10, and 15 were not found. To investigate the population structural differences in *C. albicans* between the cirrhosis group and the healthy control group, we applied statistical analysis to evaluate the three major clades of *C. albicans*: clade 8 was the main clade of strains recovered from cirrhosis patients. In previous researches^{26,27}, isolates in clade 8 were primarily recovered from South America and appeared frequently on samples from wildlife. Although isolates belonging to clade 8 also emerged in China, the clade served as a minor clade^{14,19,25}. In our study, clade 8 was the largest clade among cirrhosis patients, suggesting that strains belonging to this clade may be adapted to survive in the intestinal environment of cirrhosis patients.

Gong et al¹⁹ found that DST 1593 was the dominant isolate in the digestive tract of dyspeptic patients, suggesting that disease susceptibility

could be associated with a specific genotype. However, we did not identify such a single dominant *C. albicans* genotype associated with cirrhosis patients. DST 1931 in clade 8 was present in 6 strains, 5 isolated from cirrhosis patient and only one from healthy individuals (12.82% vs. 4.76%), but we were unable to conclusively establish the specificity of DST 1931 for cirrhosis patients due to the small sample size.

Sampaio et al²⁸ showed that environmental changes in the host contribute to genetic variants of *C. albicans* isolates. LOH is recognized as an important mechanism leading to microvariation of *C. albicans*²⁹. Although we did not obtain longitudinal samples to test the evolution of isolates over time, we analyzed differences in the concatenated sequences of all DSTs in clade 8 to investigate genetic microvariation of *C. albicans* strains. We found all DSTs isolates in clade 8 had varying degrees of homozygosity at different variant loci. Although speculative, the genetic microvariation seen here may have resulted from LOH events. A large number of DSTs isolates in clade 8 were recovered from cirrhosis patients, which may suggest that the LOH events in these isolates are associated with changes in the gut environment in cirrhosis patients. Indeed, adverse environmental factors such as antifungal agents, ultraviolet light or oxidative stress frequently promote LOH events³⁰. During the course of cirrhosis, the gut environmental system undergoes evident changes, such as changes in gut microbiota, host immune system, and host metabolites^{3,31,32}. We, therefore, speculate that gut environmental changes could contribute to LOH events of *C. albicans*.

ABC typing of *C. albicans* isolates was conducted based on the presence/absence of the transposable intron in the 25S rDNA sequence encoding RNA²³. In our investigation, isolates with the same MLST DST also had identical ABC types. There was no significant difference in ABC type distribution between cirrhosis patients and healthy controls. The majority of isolates belonged to type A (37/60, 61.67%), illustrating its prevalence in the Guizhou region. We combined MLST clades with ABC types to analyze the A/B/C type distribution in the three major clades. Among the three major clades (18, 8, and 14), all isolates in clade 18 and all isolates in clade 8 except DST 3232 and DST 2696 were typed as A, while the two DST isolates (No. H7 and N9, data not showed) were type C; all isolates in clade 14 were type B. According to Odds et

al²⁶, the majority of *C. albicans* isolates in clade 8 and clade 14 are type A and B, respectively, which is in agreement with our findings.

Conclusions

We showed that *Candida* sp. and in particular *C. albicans* colonization in the gut is increased in cirrhosis patients compared to healthy individuals, and this colonization further increases with disease progression as well as with antibiotic treatment in cirrhosis patients. *C. albicans* MLST clade 8, clade 14, and clade 18 were the dominant clades identified in our study, which may be specific to the Guizhou region of China, while *C. albicans* MLST clade 8 was the dominant clade among cirrhosis patients. In particular, DST 1931 from clade 8 was more prevalent among cirrhosis patients, but further validation with a larger sample size is required to confirm this finding. Importantly, *C. albicans* isolates from cirrhosis patients displayed LOH events more frequently, which may be caused by environmental stress in the gut. These results provide useful information for future studies since *Candida* species show species-specific differences in drug susceptibility patterns. Additionally, molecular typing provides molecular epidemiological information, which is useful to trace the source of transmission.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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