

Clonal Dissemination of Antifungal-Resistant *Candida haemulonii*, China

Xinfei Chen, Xinmiao Jia, Jian Bing, Han Zhang, Nan Hong, Yun Liu, Haiyang Xi, Weiping Wang, Zhiyong Liu, Qiangqiang Zhang, Li Li, Mei Kang, Yuling Xiao, Bin Yang, Yulan Lin, Hui Xu, Xin Fan, Jingjing Huang, Jie Gong, Juan Xu, Xiuli Xie, Wenhang Yang, Ge Zhang, Jingjia Zhang, Wei Kang, He Wang, Xin Hou, Meng Xiao,¹ Yingchun Xu¹

Candida haemulonii, a relative of *C. auris*, frequently shows antifungal resistance and is transmissible. However, molecular tools for genotyping and investigating outbreaks are not yet established. We performed genome-based population analysis on 94 *C. haemulonii* strains, including 58 isolates from China and 36 other published strains. Phylogenetic analysis revealed that *C. haemulonii* can be divided into 4 clades. Clade 1 comprised strains from China and other global strains; clades 2–4 contained only isolates from China, were more recently evolved, and showed higher antifungal resistance. Four regional epidemic clusters (A, B, C, and D) were identified in China, each comprising ≥ 5 cases (largest intracluster pairwise single-nucleotide polymorphism differences < 50 bp). Cluster A was identified in 2 hospitals located in the same city, suggesting potential intracity transmissions. Cluster D was resistant to 3 classes of antifungals. The emergence of more resistant phylogenetic clades and regional dissemination of antifungal-resistant *C. haemulonii* warrants further monitoring.

The first case of human infection caused by the yeast *Candida haemulonii* was reported in 1984 (1). Recent research has indicated that the previously recognized *C. haemulonii* species is actually a species complex comprising 4 phylogenetically closely

related species, *C. haemulonii*, *C. duobushaemulonii*, *C. pseudohaemulonii*, and *C. vulturina* (1,2). The emerging, highly problematic pathogen *C. auris*, which is also a closely related species of the *C. haemulonii* complex, was first reported in Japan in 2009; it has attracted widespread attention worldwide owing to its multidrug resistance and capacity to cause nosocomial outbreaks (3–5). Because the overall prevalence of *C. haemulonii* sensu stricto remains low worldwide, less attention has been paid to this species. Like *C. auris*, *C. haemulonii* exhibits notable resistance to various classes of antifungal agents, including azoles and amphotericin B (6–8), and some reports have described nosocomial outbreaks caused by *C. haemulonii* (9). However, although *C. haemulonii* s.s. has been discovered in a broad range of wild environmental and animal sources (10–15), it has not been isolated from a hospital environment.

Molecular methods play important roles in clinical mycology, including laboratory diagnostics, taxonomic investigations, phylogenetic analysis, and confirmation of outbreaks (16). Previous studies on the *C. haemulonii* complex have applied methods such as sequencing of the rDNA internal transcribed spacer (ITS) region, amplified fragment-length polymorphism, and random amplified polymorphic DNA; however, the

Author affiliations: Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China (X. Chen, X. Jia, H. Zhang, J. Huang, X. Xie, W. Yang, G. Zhang, J. Zhang, W. Kang, M. Xiao, Y. Xu); State Key Laboratory of Complex Severe and Rare Diseases and Beijing Key Laboratory for Mechanisms Research and Precision Diagnosis of Invasive Fungal Diseases, Beijing (X. Chen, X. Jia, H. Zhang, J. Huang, X. Xie, W. Yang, G. Zhang, J. Zhang, W. Kang, M. Xiao, Y. Xu); Fudan University, Shanghai, China (J. Bing, Q. Zhang, L. Li); Nanjing University School of Medicine, Nanjing, China (N. Hong, H. Xi, W. Wang); Changhai Hospital, Shanghai (Y. Liu); Southwest Hospital Affiliated the Third Military Medical University, Chongqing,

China (Z. Liu); Sichuan University, Chengdu, China (M. Kang, Y. Xiao); The First Affiliated Hospital of Fujian Medical University, Fuzhou, China (B. Yang, Y. Lin); The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China (H. Xu); Capital Medical University, Beijing (X. Fan); Chinese Center for Disease Control and Prevention, Beijing (J. Gong, J. Xu); Dynamiker Sub-Center of Beijing Key Laboratory for Mechanisms Research and Precision Diagnosis of Invasive Fungal Disease, Tianjin, China (H. Wang); Peking University Third Hospital, Beijing (X. Hou)

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¹These senior authors contributed equally to this article.

discriminatory powers of those methods are limited and only capable of assigning isolates to the species level (2). Whole-genome sequencing (WGS) provides a high-resolution alternative. In fact, WGS-based genomic analysis has assisted in tracing the phylogenetic evolution and dissemination of *C. auris* globally (17), confirming nosocomial transmission of *C. auris* in healthcare facilities (5,18,19), and analyzing potential antifungal resistance mechanisms (20–22).

The global phylogeny of *C. haemulonii* remains uncharacterized. The China Hospital Invasive Fungal Surveillance Net (CHIF-NET) program identified several regional clustered cases ($n \geq 5$) in China caused by *C. haemulonii*; however, the overall prevalence of this species remained low (0.8%) (23). We performed WGS-based analysis of 94 *C. haemulonii* strains, 58 isolates collected from 23 hospitals by the CHIF-NET study over 8 years in China and 36 previously published international strain genomes (24). The primary goal of our study was to illustrate the phylogenetic character of this species worldwide and determine the population relatedness of regional cluster cases in China. In addition, we sought to predict major antifungal resistance mechanisms using bioinformatic analysis.

Our study was approved by the Human Research Ethics Committee of the Peking Union Medical College Hospital (protocol S-263).

Materials and Methods

We examined 58 nonduplicated clinical *C. haemulonii* isolates collected from 23 hospitals distributed across 15 provinces in China during August 2009–July 2017 (Figure 1). Of those strains, 31 had been previously reported (7). We also included publicly available genomic data for 36 international *C. haemulonii* strains, obtained from the National Center for Biotechnology Information Sequence Read Archive.

Of the strains from China, 69% (40/58) were isolated from the blood and 13.8% (8/58) from the cerebrospinal fluid. The remaining strains were isolated from venous catheters (8.6%, 5/58), secretions (3.4%, 2/58), tissue fluid (1.7%, 1/58), ascitic fluid (1.7%, 1/58), and drainage (1.7%, 1/58) (Appendix 1 Table 1, <https://wwwnc.cdc.gov/EID/article/29/3/22-1082-App1.xlsx>). Samples came from from patients in medical wards (53.4%, 31/58), surgical wards (22.4%, 13/58), intensive care units (22.4%, 13/58), and emergency departments (1.7%, 1/58).

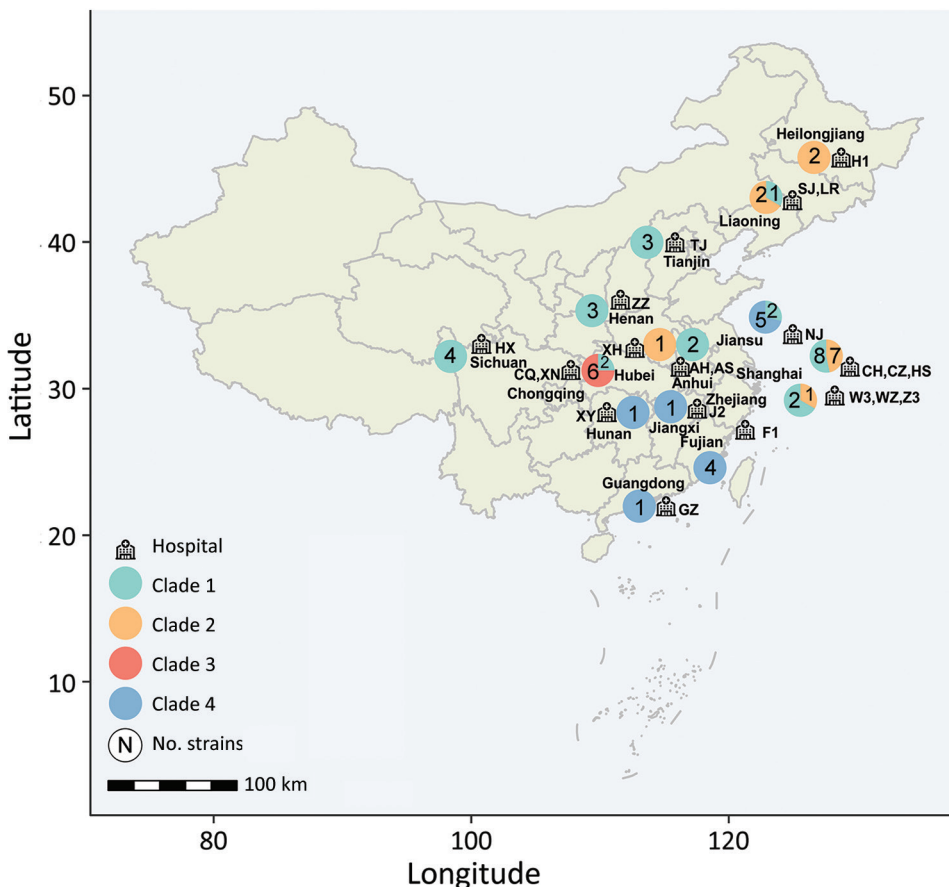


Figure 1. Regional distribution of 58 invasive infections caused by *C. haemulonii* in China during 2010–2017, collected from the China Hospital Invasive Fungal Surveillance Net study. Province names are listed, and hospital locations are marked by icons; the abbreviation codes of hospitals are listed next to each location. The pie charts adjacent to the province names indicate the number of isolates collected; phylogenetic clades are labeled in different colors.

The international strains were isolated from 3 continents: 18 from South America (Venezuela, $n = 7$; Colombia, $n = 11$), 17 from North America (United States, $n = 13$; Panama, $n = 4$), and 1 from Asia (Israel, $n = 1$). Of the strains, 94.4% (34/36) were from humans (blood, wounds, bone bronchial wash, foot, vaginal secretion, catheter, urine, or peritoneal fluid), 2.8% (1/36) from animals (fish), and 2.8% (1/36) with no source information (Appendix 1 Table 1).

We identified all strains by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and ITS sequencing (Appendix 2, <https://wwwnc.cdc.gov/EID/article/29/3/22-1082-App2.pdf>). We evaluated in vitro susceptibility and performed WGS to explore the molecular features of the isolates. Raw genome reads are available from the National Center for Biotechnology Information (BioProject no. PRJNA827237).

Results

Collection of Isolates

We identified all strains as *C. haemulonii* by using Autotof MS 1000 (Autobio Diagnostics Co., Ltd; <https://en.autobio.com.cn>) and Vitek MS (bioMérieux; <https://www.biomerieux-usa.com/>). The phylogenetic tree based on rDNA ITS region sequences revealed that CHIF-NET strains clustered with *C. haemulonii* CBS5149^T rather than other species within the *C. haemulonii* species complex.

C. haemulonii Genome Highly Conserved

We performed single-nucleotide polymorphism (SNP) calling for all 94 isolates. Although derived from vast international geographic regions, we found *C. haemulonii* genomes to be highly conserved. We found 6,807 SNPs among the 94 *C. haemulonii* genomes, which was a considerably smaller number than that first reported for *C. auris* (119,188 SNPs) (4). The pairwise SNP differences among all international strains ranged from 6 to 553 (median 269). SNP differences between Chinese and international isolates ranged from 4 to 653 (median 333), and pairwise SNP differences between different Chinese strains ranged from 6 to 581 (median 297).

Four Phylogenetic Clades Identified Worldwide

Fast hierarchical Bayesian analysis of population structure revealed that all strains could be divided into 4 major clades, and principal components analysis results clearly supported the presence of these 4 groups (Figure 2; Appendix 2 Figure 1). We classified 63 isolates (67%) as clade 1, 13 (13.8%) as clade 2, 6

(6.4%) as clade 3, and 12 (12.8%) as clade 4 (Appendix 1 Table 1). From the phylogenetic tree, we observed that clade 1 strains were widely distributed across vast geographic regions (Figure 1). In comparison, all isolates in clades 2, 3, and 4 were exclusively from China (clade 2, $n = 13$; clade 3, $n = 6$; clade 4, $n = 12$), and those 3 branches are suggested to have evolved from clade 1 in the phylogenetic tree. Of note, analysis of the mating-type locus showed that all 94 isolates were *MATa*.

Regional Clustered Cases Associated with Spread of Specific Clones

We observed several clustered regional cases. To investigate potential clonal spreads or outbreaks, we first concentrated on any hospital with ≥ 5 cases of *C. haemulonii* infections that occurred during the surveillance period. We found that the maximum pairwise SNP differences for isolates within the same clade from the same hospital were all < 50 (33 SNPs for clade 1 in hospital HS, 28 for clade 2 in hospital CH, 34 for clade 3 in hospital XN, and 45 for clade 4 in hospital NJ). Except for isolates of clade 3 that were identified in only 1 hospital, the above differences were considerably less than the average intra-clade pairwise SNP differences of all isolates within the same clade, which were 301 SNPs for clade 1, 131 for clade 2, and 160 for clade 4. We therefore used a criterion of ≤ 50 SNPs for defining clonal clusters in our primary analysis. On the basis of those criteria, we identified 4 obvious clusters.

We discovered cluster A, initially, in hospital CH in East China; 6 cases accounted for 85.7% (6/7) of the *C. haemulonii* infection cases found in that institution. Cluster A isolates belonged to clade 2, and SNP differences between any 2 cluster A strains ranged from 10 to 28 bp (median 21). Three strains were isolated from the surgical ward, 2 strains from the medical department, and 1 strain from the intensive care unit. Five strains were isolated from blood and 1 strain from cerebrospinal fluid. The remaining non-cluster A isolates from that hospital belonged to clade 1, which differed from the cluster A strains, ranging from 349 to 399 bp (median 398). Of note, 1 strain isolated from another hospital (hospital CZ, also located in hospital CH's city) fell into cluster A (paired SNPs 13 to 22 versus CH cluster A strains), suggesting intra-city transmission of *C. haemulonii* from August 2016 through April 2017 (Figure 3).

Cluster B, belonging to clade 1, was detected in hospital HS in East China, comprising 6 cases, and the inter-cluster pairwise SNP differences ranged from 6 to 33 bp (median 20). Although hospitals HS, CH,

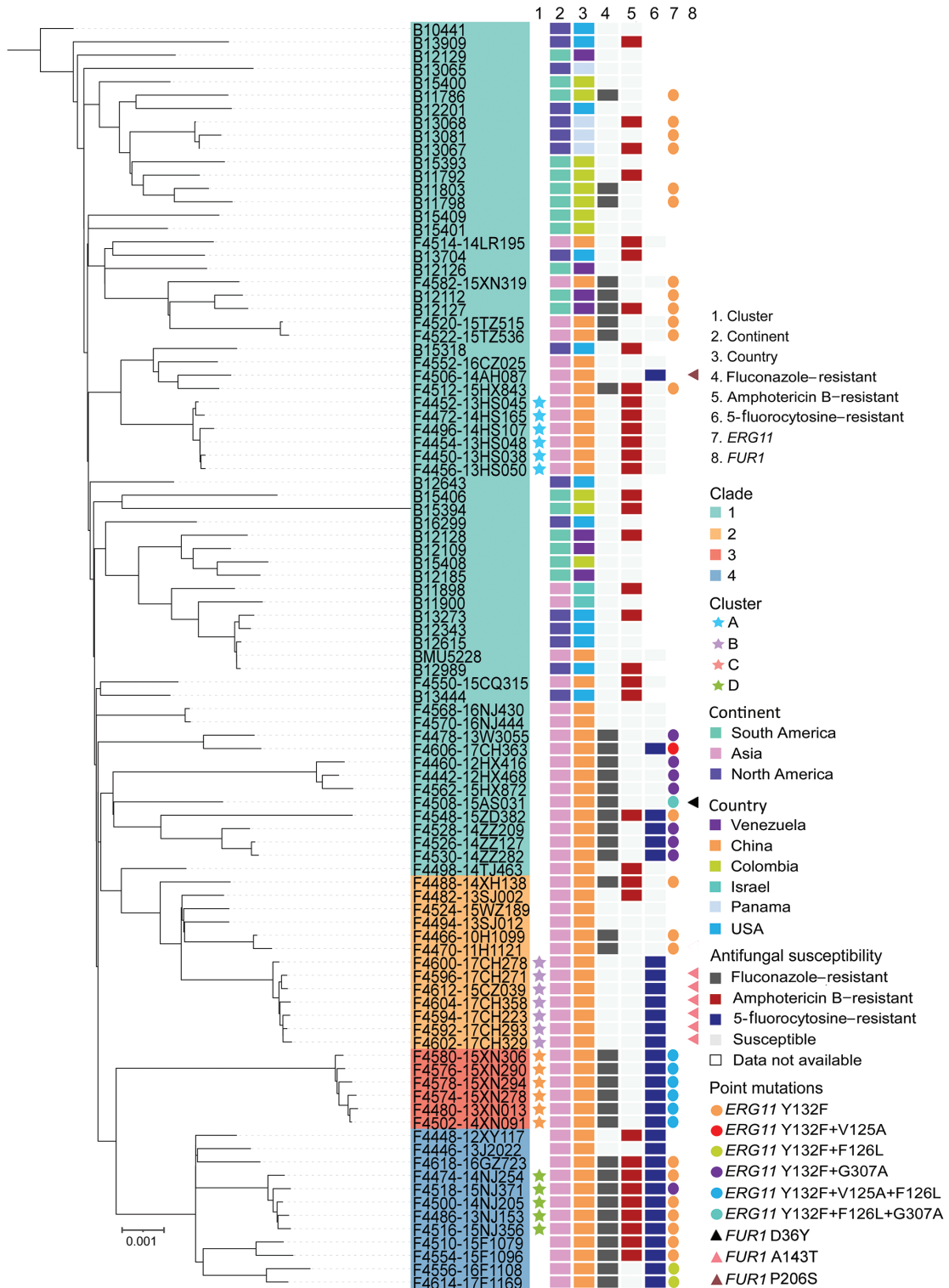


Figure 2. Maximum-likelihood phylogenetic tree constructed based on whole-genome single-nucleotide polymorphisms and phylogenetic clades in a study of antifungal-resistant *Candida haemulonii* in China. Information is labeled for each strain: geographic origin, antifungal susceptibilities for representative drugs of different classes (fluconazole, amphotericin B, and 5-fluorocytosine), and key amino acid substitutions related to antifungal resistance that were observed in genes encoding lanosterol 14- α -demethylase (*ERG11*) and uracil phosphoribosyltransferase (*FUR1*). The tree was rooted to strain B10441 (CBS5149), which is the most ancient *C. haemulonii* strain, identified in 1962 (from *Haemulon sciurus*). All remaining strains were isolated after 2010.

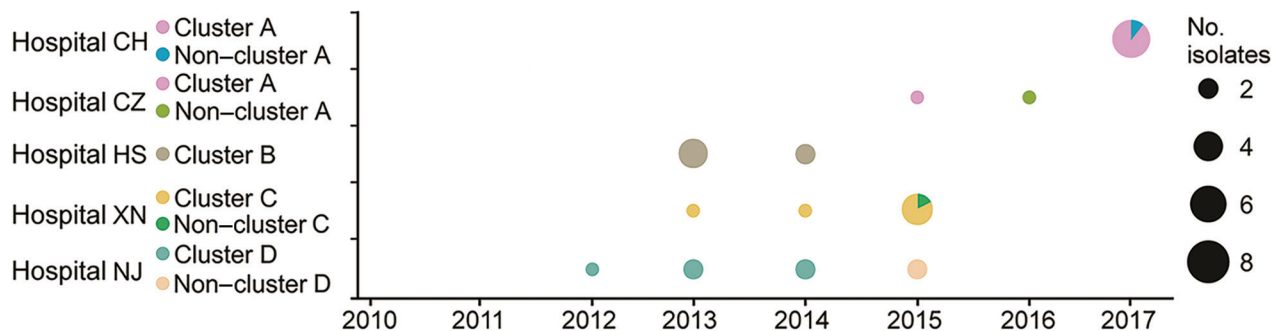


Figure 3. Distribution of 4 regionally disseminated *Candida haemulonii* clusters (clusters A, B, C, and D) in 5 hospitals in China. Isolates not belonging to the 4 major clusters were summarized as noncluster strains. Pie charts indicate number of isolates (indicated by size of pie) and distribution of clusters (distinguished by color).

and CZ were in the same city, cluster B diverged from cluster A (>258 bp differences). Four cluster B isolates were collected from the infectious department ward, 1 isolate was collected from the emergency department, and 1 isolate was collected from the intensive care unit. All strains were isolated from cerebrospinal fluid during October 2013–August 2014 (Figure 3).

Cluster C was detected in hospital XN in Southwest China and belonged to clade 3. Of the 7 strains isolated from hospital XN, 85.7% (6/7) were attributed to cluster C. The remaining isolate was from clade 3 but differed from other cluster C strains by 451–463 bp. Within cluster C strains, pairwise SNP differences ranged from 11 to 34 bp (median 28). All cluster C strains from hospital XN were isolated from the hepatobiliary ward; 3 of those strains were cultured from blood and catheter samples. The timeline for the isolation of cluster C isolates in hospital XN was >2 years (September 2013–October 2015) (Figure 3).

Cluster D isolates belonging to clade 4 were detected from hospital NJ in East China and comprised 5 cases. The number of SNPs in cluster D ranged from 19 to 45 bp (median 37). All 6 cluster D strains were isolated from the general surgery wards. As with cluster C strains, cluster D strains persisted in hospital NJ for >2 years (September 2013–November 2015) (Figure 3). Of interest, 2 additional non-cluster D strains from that hospital belonged to clade 1. Both strains were isolated from the nephrology ward (August 2016–November 2016), and pairwise SNP differences between the 2 strains were only 11, suggesting another potential nosocomial transmission.

In summary, 4 clonal dissemination events of *C. haemulonii* were identified in China. Moreover, evidence suggests the occurrence of an intra-city clonal spread caused by a multidrug-resistant clone.

Notable Antifungal Resistance in *C. haemulonii*

Among the 94 isolates studied, only an international strain from Venezuela was reported to be resistant to caspofungin (MIC = 16 µg/mL) (24). All other strains remained susceptible to echinocandins. Of the isolates, 40.4% (38/94) were resistant to fluconazole and 21.2% (20/94) were resistant to voriconazole, including 24.5% (23/94) that were cross-resistant to the 2 azoles. The resistance rate in China was 56.9% (33/58) for fluconazole and 34.5% (20/58) for voriconazole, both rates higher than the rates among international strains, which were 13.9% (5/36) for fluconazole and 0% (0/36) for voriconazole. In comparison, only 9.6% (9/94) of the isolates were resistant to itraconazole, and only 1 isolate had a minimum inhibitory concentration ≥ 1 µg/mL for posaconazole. Nearly half (44.7%, 42/94) of the isolates were resistant to amphotericin B. Although data for 5-fluorocytosine resistance were not available for the 36 international strains, more than half (53.4%) of the 58 strains from China were 5-fluorocytosine resistant. Moreover, in China, 25.8% (15/58) of the isolates were multidrug resistant, including 15.5% (9/58) that were resistant to 3 classes of antifungal agents.

Antifungal resistance was associated with the clonal background of the strains. For instance, fluconazole resistance rates were above 80% for clade 3 (100%) and clade 4 (83.3%) strains, whereas only 30.2% of clade 1 and 46.2% of clade 2 isolates were fluconazole resistant (Appendix 1 Table 2). In addition, China clade 1 isolates exhibited a higher fluconazole resistance rate (51.9%) than the international strains (13.9%). The amphotericin B resistance rate of strains in clade 1 (49.2%) and in clade 4 (58.3%) were higher compared with other clades (<20%). The 5-fluorocytosine resistance rate was 100% in clades 3 and 4. Strains resistant to 3 classes of antifungals were mainly

distributed in clade 4 (66.7%), including all cluster D isolates (Appendix 1 Table 2).

Potential Resistance Mechanisms of *C. haemulonii*

We used the genome of strain BMU5228 as a wild-type sequence to annotate gene mutations in 25 known important antifungal resistance genes (Appendix 1 Table 3). Among the 33 fluconazole-resistant strains in China, 100% harbored the Y132F substitution in Erg11p (Table). We also found the Y132F substitution in 11.1% (4/36) of the international strains, and 2 of them were fluconazole resistant. We found 54.5% (18/33) of fluconazole-resistant strains in China harbored ≥ 1 of the substitutions V125A, F126L, and G307A (Table; Appendix 1 Table 3). We screened other genes reported to cause azole resistance and found that 6 cluster C strains had the substitution M589L in Tac1Bp, the transcriptional regulator of the efflux pump Cdr1. Our analysis of the distribution of copy number variations revealed that 13 (22.4%) strains in China had >1 copy of the *ERG11* gene, and those strains were all resistant to fluconazole (Appendix 2 Figure 2). For strains with >1 copy of *ERG11*, 6 strains were from clade 3 and 7 strains were from other clades (Appendix 2 Figure 2). Isolates with >1 copy of *ERG11* had significantly higher MICs against fluconazole than did isolates with 1 copy ($p < 0.05$ by Mann-Whitney test). For 5-fluorocytosine, of the 31 resistant isolates, 25.8% (8/31) had noteworthy mutations in the *FUR1* gene, including 7 strains carrying the substitution A143T (all of which were cluster C) and 1 strain carried the substitution P206S. Although 42.6% (40/94) of the strains were resistant to amphotericin B, we observed no mutations of note in the previously described resistance-related genes, including *ERG2*, *ERG3*, or *ERG6* (Appendix 1 Table 3).

Discussion

In recent years, the number of human infections caused by emerging pathogens has increased gradually (24). Among those pathogens, *C. haemulonii* and its closely related species *C. auris*, belonging to the family Metschnikowiaceae, have received great public attention because of their notable trends of antifungal resistance and capacity to cause nosocomial transmission (5,9,25).

Genome-based phylogenetic studies of *C. auris* have revealed that 5 distinct clades (I, II, III, IV, and V) are distributed in East Asia, South Asia, South Africa, and South America (17,26), whereas the population structure of *C. haemulonii* has not been previously defined. In this study, we found that *C. haemulonii* can also be divided into 4 phylogenetic clades. Root-

ed by the most ancient *C. haemulonii* strain B10441 (CBS5149) that was isolated in 1962, strains from clades 2–4 emerged more recently, with isolates identified exclusively in China and antifungal resistance observed more notably compared with clade 1. We found that 46.2% of clade 2 isolates were fluconazole resistant versus 30.2% in clade 1, all clade 3 isolates were cross-resistant to fluconazole and 5-fluorocytosine, and 66.7% of clade 4 isolates were resistant to 3 classes of antifungals.

Although *C. haemulonii* can be divided into 4 clades, the total number of SNPs identified in the 13.31 Mb whole genome of *C. haemulonii* was only 6,807 ($< 0.005\%$), which was considerably less than that in *C. auris*, which has a similar genome size ($> 210,000$ SNPs in a 12.4 Mb genome) (27–29). When we compared the most ancient *C. haemulonii* strains identified to date (strain ID no. CBS5149, isolated from *Haemulon sciurus* fish in 1962) with the other strains in our study, the maximum genome sequence difference was only 384 bp. Those factors indicate that the genome of *C. haemulonii* is highly conserved. In some *Candida* species, mating can lead to an increase in genetic diversity, and opposite mating types have been observed in *C. auris* (17,29). However, all *C. haemulonii* isolates identified were of the same mating type (type α), and a sexual cycle has not been observed in this species (2), which is a possible reason for the conservation of the species' genome found in previous studies (24,29).

Although the global prevalence of *C. haemulonii* remains low, nosocomial outbreaks have been reported (9,24). Nosocomial transmission of *C. haemulonii* was first reported in Kuwait in 2006 (9). Because bloodstream infection caused by *C. haemulonii* was rare at the time of the report, the outbreaks were determined by successive isolations of *C. haemulonii* with identical phenotypic characteristics made in the

Table. Distribution of noteworthy Erg11p substitutions among 4 clades of *Candida haemulonii* strains studied as part of an investigation of antifungal-resistant *C. haemulonii*, China

Clade/ geographic origin	Erg11p substitutions				No. isolates
	Y132F	V125A	F126L	G307A	
Clade 1					
International	Y	N	N	N	8
China	Y	N	N	N	5
	Y	N	N	Y	7
	Y	Y	N	N	1
	Y	N	Y	Y	1
Clade 2					
China	Y	N	N	N	3
Clade 3					
China	Y	Y	Y	N	6
Clade 4					
China	Y	N	N	N	7
	Y	N	Y	N	2
	Y	N	N	Y	1

same ward (a neonatal intensive care unit) within a short period of time (3 months), but the outbreaks were not verified by molecular typing. Such molecular methods as ITS sequence typing, random amplified polymorphic DNA analysis, amplified fragment length polymorphism analysis, and, more recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry have been applied in *C. haemulonii* studies (2,30); however, all of those methods have limitations in discriminatory power. Considering the low genetic diversity of *C. haemulonii*, traditional molecular typing assays are not suitable for providing solid evidence for the dissemination of *C. haemulonii*.

WGS-based analysis provides a high-resolution alternative for confirming bacterial and fungal outbreak transmission (16,31,32). Even for species with low genetic diversity, such as *Saprochaete clavata*, this approach can clearly distinguish between sporadic cases and epidemic outbreaks (SNPs <400) (33). In this study, we proposed a pairwise SNP difference of ≤50 bp as a criterion for determining clonal cluster cases in *C. haemulonii*, and identified 4 regional clusters that met the criterion. In the absence of medical record evidence, we could not determine whether the case clusters were outbreaks. A previous study by Chow et al. used a 12-bp SNP difference as a cutoff value for interpreting *C. auris* outbreaks (25). We suggest that an equally strict pairwise difference might be needed to characterize *C. haemulonii* outbreak events; however, this hypothesis requires further investigation.

C. auris has a potent ability to colonize humans and persist in the hospital environment, and biofilm formation is considered the main contributor (34). *C. haemulonii* can form dense biofilms (35,36), which are thought to enhance its capacity to cause regional dissemination and nosocomial transmission. The epidemic cluster events of *C. haemulonii* identified in China had further implications. Several studies have reported that *C. haemulonii* has a low susceptibility to triazoles and amphotericin B (9,37,38). Our study further revealed that antifungal resistance was more obvious among *C. haemulonii* strains from China than among those from other geographic regions (24). Moreover, the 4 regional clusters we identified were all caused by antifungal-resistant clones: cluster A was caused by a clone resistant to amphotericin B, cluster B by a clone resistant to 5-fluorocytosine, cluster C by a clone cross-resistant to fluconazole and 5-fluorocytosine, and cluster D by a clone resistant to 3 classes of antifungals. Cluster A was recovered from 2 hospitals located in the same city,

suggesting interfacility transmission. Gade et al. reported that 2 strains of *C. haemulonii* isolated from different healthcare facilities in Valencia, Venezuela, were closely related (with only 32 SNPs) (24). As with the closely related species *C. auris*, which presents a serious global health threat (5,25), the emergence of *C. haemulonii* clones with high rates of both transmission and antifungal resistance should be taken as a warning.

A potential limitation of this study is that only a limited number of publicly available genomes were available for *C. haemulonii*, and they were derived from systematic epidemiology surveillances. These isolates, therefore, may not represent real-world *C. haemulonii* distributions globally. To this end, further genomic-based studies need to be conducted with more isolates from different geographic regions.

In conclusion, we studied a total of 94 *C. haemulonii* genomes, including 36 international strains (38.2%) from the National Center for Biotechnology Information Sequence Read Archive database and 58 strains (61.7%) from 23 hospitals in China. We observed 4 phylogenetic clades, 3 of which were identified exclusively in China and exhibited higher antifungal resistance. All fluconazole-resistant strains carried the Y132F substitution in Erg11p. WGS confirmed that the 4 regional cluster cases were caused by specific clones. We additionally noted a potential interfacility transmission within the same city and the spread of multidrug-resistant clones. As with its close relative *C. auris*, *C. haemulonii* should be recognized as a potential threat to global health, and further monitoring and stewardship steps to limit excessive antifungal usage are warranted.

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About the Author

Dr. Chen is a doctoral student in clinical laboratory diagnostics at the Peking Union Medical College Hospital whose research interests are genomic epidemiology of clinical *Candida* isolates.

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Address for correspondence: Yingchun Xu and Meng Xiao, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Department of Laboratory Medicine, No.1 Shuaifuyuan Wangfujing Dongcheng District, Beijing, 100730 China; email: xycpumch@139.com and cjtcxiaomeng@aliyun.com

Clonal Dissemination of Antifungal-Resistant *Candida haemulonii*, China

Appendix 2

Materials and Methods

Identification

All strains were identified by Autof MS 1000 (Autobio, Zhengzhou, China) and Vitek MS (bioMérieux, Marcy l'Étoile, France) and by sequencing of the rDNA internal transcribed spacer (ITS) region (ABI 3730XL, Thermo Fisher Scientific, Cleveland, OH, USA). The ITS sequences of *Candida haemulonii* CBS 5149^T, *C. duobushaemulonii* CBS 7798^T, and *C. pseudohaemulonii* CBS 10004^T were used as references.

Antifungal susceptibility testing

The in vitro susceptibility of the strains to nine antifungal drugs (fluconazole, voriconazole, itraconazole, posaconazole, anidulafungin, micafungin, caspofungin, 5-fluorocytosine, and amphotericin B) was determined using the commercial chromogenic susceptibility plate Sensititer YeastOne (Thermo Fisher Scientific). MIC values were interpreted according to the CLSI document M27-S3 (1). In addition, MIC ≥ 4 $\mu\text{g/mL}$ was used for interpreting the “resistance” of amphotericin B (2). Multidrug resistance was defined as resistance to at least two classes of antifungal agents.

Whole-genome sequencing (WGS)

Genomic DNA was extracted using the Fungi Genomic DNA Extraction Kit (Solarbio Science & Technology, Beijing, China), according to the manufacturer's recommended

protocols. Library construction and genomic sequencing were performed by Novogene Co. (Beijing, China) according to the manufacturer's protocols. Sequencing libraries were generated using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Each sample was then sequenced using the Illumina NovaSeq6000 platform with 2×150 -bp reads and $100 \times$ minimum coverage. Raw genome reads are available at the National Centre for Biotechnology Information (NCBI) under the BioProject accession number PRJNA827237.

Accessing publicly available *C. haemulonii* genomes

We searched the NCBI Sequence Read Archive (SRA) database and acquired all *C. haemulonii* genomes available for download till June 15, 2022. All these genomes were in raw reads data format, with average sequencing depth of $>50\times$. Detailed information for these strains could be found in Appendix 2 Table 4.

SNP analysis

Variant calling was performed for all strains of *C. haemulonii* (3). A threshold of 0.01 with (Phred score of 20) was used for trimming the raw Illumina sequencing reads. Quality control was performed for sequencing data using the fastx_toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/download.html). Subsequently, paired-end reads were mapped to the *C. haemulonii* BMU5228 reference genome (GenBank assembly accession number: GCA_011426285.1) using BWA 0.7.17. SNPs were analyzed using SAMtools 0.1.18 (4–6). The filtering criteria for SNPs were the same as that reported previously (7). Gene prediction and annotation of the reference genome were performed using Augustus 3.3.2 (8) and eggNOG 5.0 (9), respectively.

Phylogenetic and population structure genetic analyses

A phylogenetic tree of whole-genome SNPs was constructed using the SNPs of all strains studied, based on the maximum likelihood method with 1000 ultrafast bootstrap approximation

replicates on the IQ-TREE web server (10). We placed the root of the tree to strain B10441 (CBS5149), which was the most ancient *C. haemulonii* strains identified to date in 1962 (all remaining strains were isolated after 2010). An interactive phylogenetic tree was generated using iTOL v5 (11). Fastbaps v1.0.1 (12) and YMAP V1.0 (13) were used for clade typing and copy number variation analysis. The aligned fasta file was used as input for a principal component analysis (PCA) of genetic covariance using the function `fasta2genlight` in the R package `adeget` 1.3–1 (14). Moreover, the mating types (MTs) of the strains were further determined as previously described (3). In general, for each strain, the coverage depth of their whole genome was calculated using SAMtools from aligned binary alignment map (BAM) file (6). Subsequently, by mapping to the corresponding reference genomes, normalized average read depth at MT locus was obtained, and the MT of the strains was further determined.

Karyotype analysis

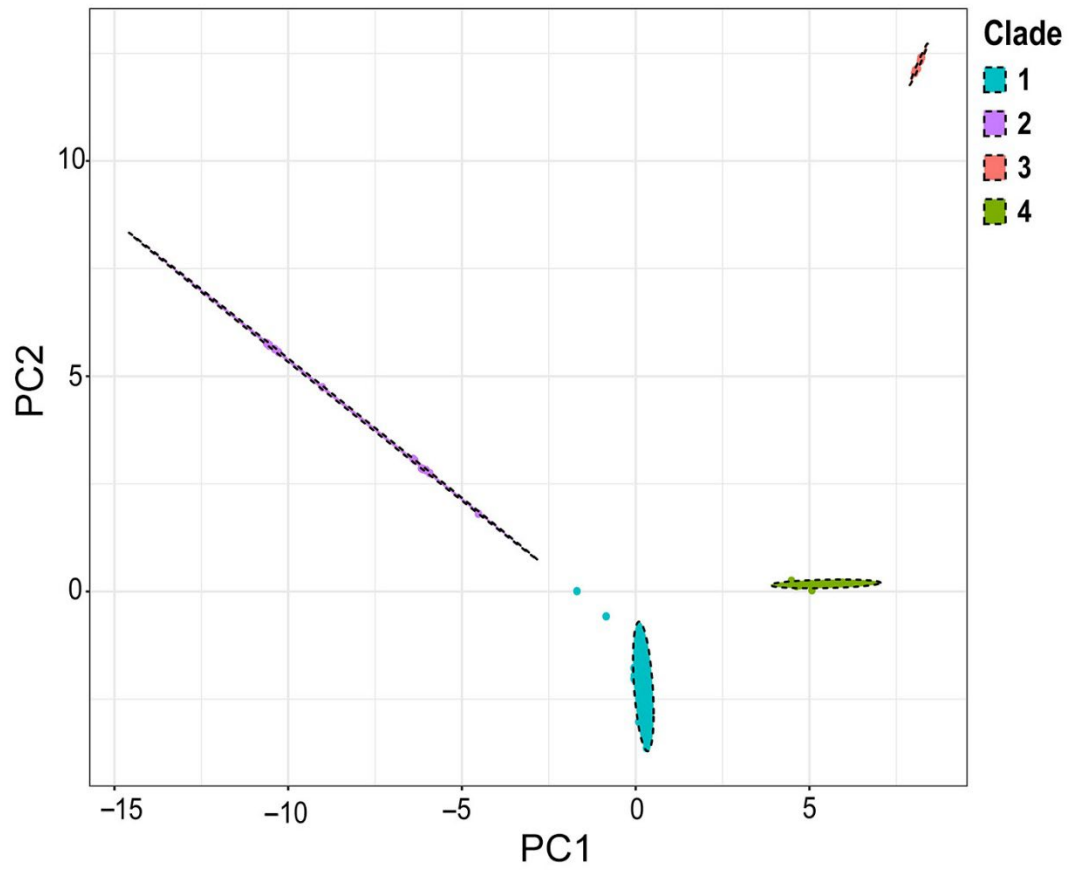
Karyotypes of 13 isolates with notable aneuploidy or gene CNVs were visualized using the YMAP V1.0 software. The scaffold copy number is shown in a \log_2 ratio relative to that of the haploid B11899 reference strain (GenBank assembly accession: GCA_002926055.1) on the y-axis, with 1 copy at the midline clipped to show a maximum of 2 copies. The x-axis indicates the positions of reads on each scaffold mapped to the genome of strain B11899. A vertical red line is used to show the position of the *ERG11* gene (Appendix 1 Figure 2).

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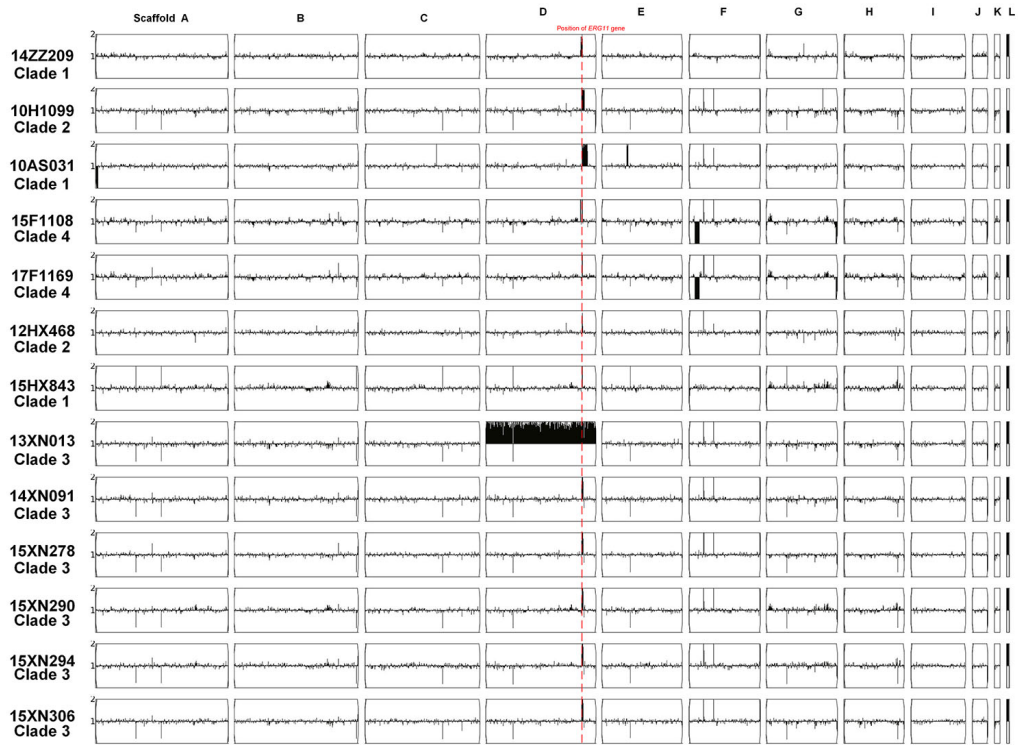
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Appendix 2 Figure 1. Population structure differentiation of 4 *Candida haemulonii* clades demonstrated by principal component analysis (PCA).



Appendix 2 Figure 2. Copy number variations (CNVs) identified in 13 *Candida haemulonii* isolates.