Multilocus Sequence Typing Reveals both Shared and Unique Genotypes of *Cryptococcus neoformans* in Jiangxi Province, China

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Cryptococcosis is a globally distributed infectious fungal disease. However, much remains unknown about its molecular epidemiology in many parts of the world. In this study, we analyzed 86 clinical *Cryptococcus neoformans* isolates from 14 regions in Jiangxi Province in south central China. Each isolate was from a different patient and 35 of the 86 (40.7%) patients were infected with HIV. All strains belonged to serotype A and mating type α (MATα). Genotyping based on DNA sequences at seven nuclear loci revealed eight sequence types (STs) among the 86 isolates, including two novel STs that have not been reported from other parts of the world. ST5 was the dominant genotype and our comparative analyses showed that these genotypes in Jiangxi likely originated by dispersal from other regions within and outside of China and/or mutations from another genotype within Jiangxi. Though none of the isolates was resistant to the five tested antifungal drugs (flucytosine, amphotericin B, fluconazole, itraconazole, and voriconazole), obvious differences in their minimum inhibitory concentrations were observed, even among isolates of the same ST. Our results suggest that continuous monitoring should be conducted to understand the changing dynamics of *C. neoformans* in this and other regions.
lar weights ranging from 2 to 20 kDa. To identify whether the isolates belonged to either the \textit{C. neoformans} species complex or the \textit{C. gattii} species complex, we plated all isolates on L-canavanine-glycine-bromothymol blue agar\textsuperscript{15}, followed by sequencing of all isolates at the \textit{URA5} gene. The sequences were then compared with those of five strains representing the known molecular types of \textit{C. neoformans}: WM148 (serotype A, VNI), WM 626 (serotype A, VNII), Bt63 (serotype A, Botswana), WM 628 (serotype D, VNIII), and WM629 (serotype AD, VNIV) as well as those in the GenBank species complex database (http://mlst.mycologylab.com) to obtain sequence type (ST) numbers.

**MLST Analysis.** Aside from obtaining the \textit{SOD1} gene sequence for each of the isolates for species identification, we also obtained the sequences at six other genes as suggested by the ISHAM consensus MLST scheme\textsuperscript{11}. Briefly, these six DNA fragments were located in the following genes: \textit{CAP59}, \textit{GPD1}, \textit{IGS1}, \textit{LAC1}, \textit{PLB1}, and \textit{URA5}. Primers and PCR conditions followed that described in Hiremath et al.\textsuperscript{18}. All sequences were submitted to the National Center for Biotechnology Information (NCBI) database to acquire GenBank accession numbers and the \textit{C. neoformans}/\textit{C. gattii} species complex database (http://mlst.mycologylab.com) to obtain sequence type (ST) numbers.

**Phylogenetic Analysis.** Phylogenetic analysis of the concatenated sequences of seven MLST loci was performed using MEGA version 7.0 software\textsuperscript{19}. A phylogenetic tree was produced by the Neighbor-Joining algorithm using alignments of the concatenated sequences at the seven gene loci from our isolates and two reference strains H99 and WM148.

**Comparison with Other Geographic Populations from China.** To investigate the potential genetic differences between the Jiangxi population of \textit{C. neoformans} and those from other geographic regions in China,

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| Isolate | Location | Sex | Age | Specimen | Underlying condition | ST |
|---------|----------|-----|-----|----------|----------------------|----|
| CN9     | Dexing   | F   | 39  | CSF      | HIV(+)               | 5  |
| CN6     | Dexing   | M   | 49  | CSF      | Immunocompetent      | 5  |
| CN1     | Fengcheng| F   | 74  | Blood    | Systemic lupus erythematosus | 5  |
| CN8     | Fuzhou   | F   | 62  | Blood    | Anca - associated vasculitis | 5  |
| CN51    | Fuzhou   | F   | 63  | CSF      | Chronic hepatitis    | 5  |
| CN12    | Fuzhou   | M   | 37  | Blood    | HIV(+)               | 5  |
| CN56    | Fuzhou   | M   | 31  | Blood    | HIV(+)               | 5  |
| CN2     | Fuzhou   | M   | 33  | CSF      | HIV(+)               | 5  |
| CN26    | Fuzhou   | M   | 28  | CSF      | Immunocompetent      | 139 |
| CN69    | Fuzhou   | M   | 48  | CSF      | Nephrotic syndrome   | 5  |
| CN45    | Fuzhou   | M   | 47  | Blood    | Unknown              | 5  |
| CN76    | Fuzhou   | M   | 68  | CSF      | Chronic hepatitis    | 359 |
| CN28    | Ganzhou  | M   | 18  | Blood    | Immunocompetent      | 5  |
| CN41    | Ganzhou  | M   | 64  | CSF      | Malignant lymphoma   | 31  |
| CN63    | Ganzhou  | F   | 50  | CSF      | Unknown              | 5  |
| CN88    | Ganzhou  | F   | 66  | CSF      | Emphysema            | 5  |
| CN92    | Ganzhou  | M   | 57  | CSF      | Chronic hepatitis    | 5  |
| CN37    | Gaoan    | F   | 43  | CSF      | Unknown              | 5  |
| CN14    | Jian     | F   | 48  | CSF      | HIV(+)               | 5  |
| CN49    | Jian     | M   | 44  | Blood    | HIV(+)               | 5  |
| CN61    | Jian     | M   | 22  | CSF      | HIV(+)               | 5  |
| CN21    | Jian     | M   | 43  | Sputum   | Unknown              | 319 |
| CN50    | Jian     | F   | 36  | CSF      | Unknown              | 5  |
| CN53    | Jian     | F   | 61  | Blood    | Unknown              | 5  |
| CN73    | Jian     | F   | 28  | CSF      | Kidney transplant    | 5  |
| CN74    | Jian     | M   | 67  | CSF      | Tuberculosis         | 5  |
| CN79    | Jian     | F   | 33  | CSF      | HIV(+)               | 5  |
| CN94    | Jian     | F   | 51  | Blood    | SLE                  | 359 |
| CN36    | Jujiang  | F   | 67  | CSF      | Diabetes mellitus    | 5  |
| CN31    | Jujiang  | M   | 39  | Blood    | HIV(+)               | 5  |
| CN48    | Jujiang  | F   | 69  | CSF      | HIV(+)               | 5  |
| CN65    | Jujiang  | M   | 37  | Blood    | HIV(+)               | 5  |
| CN68    | Jujiang  | M   | 49  | CSF      | Immunocompetent      | 5  |
| CN64    | Jujiang  | M   | 65  | CSF      | Unknown              | 5  |
| CN86    | Jujiang  | M   | 25  | CSF      | HIV(+)               | 5  |
| CN87    | Jujiang  | M   | 62  | CSF      | Myasthenia gravis    | 5  |
| CN27    | Leping   | M   | 53  | CSF      | Chronic hepatitis    | 5  |
| CN19    | Leping   | M   | 42  | CSF      | HIV(+)               | 5  |
| CN44    | Leping   | M   | 38  | CSF      | HIV(+)               | 5  |
| CN5     | Leping   | M   | 42  | CSF      | Immunocompetent      | 186 |
| CN10    | Leping   | M   | 44  | Blood    | Malignant lymphoma   | 5  |
| CN34    | Nanchang | M   | 38  | CSF      | Brain trauma         | 5  |
| CN7     | Nanchang | M   | 70  | Blood    | Chronic hepatitis    | 5  |
| CN11    | Nanchang | M   | 23  | Blood    | HIV(+)               | 5  |
| CN22    | Nanchang | M   | 49  | CSF      | HIV(+)               | 5  |
| CN23    | Nanchang | M   | 33  | CSF      | HIV(+)               | 5  |
| CN35    | Nanchang | M   | 40  | CSF      | HIV(+)               | 5  |
| CN58    | Nanchang | M   | 26  | CSF      | HIV(+) + tuberculosis | 5  |
| CN18    | Nanchang | M   | 76  | CSF      | Immunocompetent      | 5  |
| CN24    | Nanchang | M   | 20  | CSF      | Immunocompetent      | 5  |
| CN13    | Nanchang | M   | 52  | CSF      | Kidney transplant    | 359 |
| CN39    | Nanchang | F   | 33  | CSF      | Systemic lupus erythematosus | 5  |
| CN16    | Nanchang | M   | 38  | Marrow   | Tuberculosis         | 5  |
| CN57    | Nanchang | F   | 74  | CSF      | Unknown              | 5  |
| CN71    | Nanchang | M   | 41  | CSF      | HIV(+)               | 5  |
| CN81    | Nanchang | M   | 48  | CSF      | Malignant tumor      | 5  |
| CN85    | Nanchang | M   | 79  | CSF      | Chronic steroid usage | 359 |

Continued
we extracted all the published genotype information for all the Chinese isolates at the seven sequenced loci from the Cryptococcus MLST database (http://mlst.mycologylab.com). These Chinese populations were then analyzed using the GenAlEx software (version 6.5) 20. Two analyses were performed. In the first, the overall genetic variation was partitioned into within and between geographic populations through AMOVA. In the second, the genetic differences between all pairwise geographic populations were analyzed. All regional populations with a sample size of greater than five isolates were included in the above analyses. Statistical significance of the observed genetic differences was determined by 1000 permutations using the GenAlEx software 20.

Antifungal Susceptibility Testing. The in vitro antifungal susceptibility testing of all 86 isolates of amphotericin B (AMB), flucytosine (5FC), fluconazole (FLU), voriconazole (VOR), itraconazole (ITR) was performed using the ATB™ FUNGUS-3 kit (BioMerieux, Marcy L’Etoile, France). The minimal inhibitory concentrations (MIC) were determined following instructions provided by the User’s Manual. Candida krusei ATCC6258 and Candida parapsilosis ATCC22019 were used as reference quality controls. The obtained MIC values were compared to those recommended breakpoints to determine whether the strains were susceptible or resistant to specific antifungal drugs. The MIC breakpoints for fluconazole and flucytosine were \( \geq 16 \mu g/ml \) and \( \geq 32 \mu g/ml \) respectively as suggested based on the User’s Manual of ATB™ FUNGUS-3. For amphotericin B, we followed the resistance breakpoint of \( \geq 2 \mu g/ml \) as suggested by CLSI document M27-A3 21 and Nguyen et al.22. At present, there are no consensus interpretive breakpoints of ITR and VOR based on the ATB™ FUNGUS-3 system for C. neoformans. Here we follow previous studies and used a MIC \( \geq 1 \mu g/ml \) as the resistance breakpoint for both ITR and VOR 23,24.

Data availability statement. All the data described in this manuscript are presented in the paper (for genotype information and MIC values of all 86 isolates) as well as deposited in the publicly accessible database (http://mlst.mycologylab.com) for all nucleotide sequences.

Statements on study approvals. We confirm that all methods used in this study were carried out in accordance with relevant guidelines and regulations. In addition, all experimental protocols were approved by Nanchang University and that informed consent was obtained from all subjects for the Cryptococcus neoformans isolates analyzed in this study.
Results

Demographic Data of the Clinical Isolates. In total, 86 clinical isolates of *C. neoformans* were obtained from patients in 14 cities/counties distributed across Jiangxi Province (Table 1). Each of these isolates was from a different patient. Of the 86 isolates, 60 originated from male patients and 26 from female patients. The age distribution of these 86 cases ranged from 4 to 79 years, and the numbers from each age group were as follows: four (<20 years), 11 (21–30 years), 17 (31–40 years), 22 (41–50 years), 11 (51–60 years), 17 (61–70 years), and four (>70 years). A majority of these isolates were obtained from cerebrospinal fluid (n = 65; 75.5%), followed by blood (n = 18; 20.9%), and one each from bone marrow, sputum and hydrothorax (n = 1 each; 1.2%). Of these 86 isolates, 35 (40.7%) were from HIV-positive individuals, 40 from HIV-negative hosts, and eleven from individuals of unknown disease status. The majority (31) of the 40 HIV-negative hosts (31/86 total hosts, 36.0%) had deficient or suppressed immune systems associated with cancer or liver disease treatments or chronic steroid usage. Only nine hosts (10.5%) had no known risk factors for cryptococcosis (Table 1).

Serotype, Mating Types, and MLST Results. All 86 clinical isolates were identified as *C. neoformans* serotype A, molecular type VNI, and mating type α. MLST analysis divided the 86 isolates into eight sequence types (STs), including 73 isolates of ST5 (84.9%). Of the remaining 7 STs, two (ST186 and ST359) were represented by four isolates each while the remaining five (ST31, ST32, ST139, ST226, and ST319) were represented by one isolate each. The multilocus sequence types of all 86 strains are presented in Table 1. The 73 isolates with the ST5 multilocus genotype came from all sampled regions in Jiangxi Province. Similarly, the remaining two STs, ST186 and ST359, represented by four isolates each were also distributed broadly, in three regions each. The remaining five isolates each with a different multilocus genotype came from different regions of Jiangxi, including Shangrao (ST32) in the northeast; Fuzhou (ST139) and Ji’an (ST319) in the center; Yichun (ST226) in the west; and Ganzhou (ST31) in the south.

The allelic assignments of our individual gene sequences in the MLST database for each of the eight multilocus sequence types are presented in Table 2. Table 2 also shows the distributions of the individual alleles in the Jiangxi population among all the known sequence types in the MLST database. In total, 18 alleles were found at the seven loci in Jiangxi. Two loci, *SOD1* and *URA5* were monomorphic in Jiangxi and their alleles (#1 at both loci) were distributed broadly in many other STs within and outside of China. The remaining five loci were polymorphic, with allele numbers ranging from two (CAP59) to six (GPD1). At each of the remaining five loci, the Jiangxi population shared alleles with a diversity of known STs from other geographic regions (Tables 2 and 3). Among these 18 alleles at the seven loci, none was specific to Jiangxi and all have been found elsewhere (Tables 2 and 3).

Among the eight multilocus STs in our Jiangxi population of *C. neoformans*, six (ST5, ST31, ST32, ST139, ST186, and ST359) have been reported previously from other geographic areas (Table 3). The remaining two genotypes (ST226 and ST319) have only been found in our study population. The geographic distributions of these eight STs are shown in Table 3. Of the six shared STs between Jiangxi and other regions, three (ST5, ST31, and ST32) have been found in multiple continents/countries. For example, ST5 has been reported from the US, Europe, Brazil, South Africa, and several countries in eastern and southeastern Asia. The high prevalence of ST5 in the Jiangxi population is consistent with what has been reported previously from other parts of China and China’s neighbouring countries, such as Korea, Japan, and Vietnam. The remaining three STs (ST139, ST186, and ST359) were geographically unique, had been reported so far only from Africa, Shanghai and Sichuan Province in China, respectively.

Phylogenetic Analysis. To further reveal the relationships among the isolates and genotypes, we conducted a Neighbour-joining analysis of the concatenated gene sequences at the seven MLST loci (Fig. 1). Here, only one representative strain of each of the eight sequence types was included in this analysis to allow better visualization. Two reference strains H99 and WM148, both of the VNI molecular type group, were also included (Fig. 1). Our analysis showed that ST5, ST186, and ST359 were genetically very similar, differ from each other by one to a few

| ST  | CAP59 | GPD1 | IGS1 | LAC1 | PLB1 | SOD1 | URA5 | VN |
|-----|-------|------|------|------|------|------|------|----|
| ST5 | 1     | 1    | 1    | 1    | 1    | 1    | 1    |    |
| ST31| 1     | 1    | 10   | 3    | 2    | 1    | 1    | 1  |
| ST32| 1     | 1    | 10   | 3    | 4    | 1    | 1    | 1  |
| ST139| 1   | 6    | 22   | 18   | 4    | 1    | 1    | 1  |
| ST186| 1   | 26   | 1    | 5    | 2    | 1    | 1    | 1  |
| ST226| 7    | 3    | 1    | 5    | 2    | 1    | 1    | 1  |
| ST319| 1   | 23   | 10   | 18   | 4    | 1    | 1    | 1  |
| ST359| 1   | 25   | 1    | 5    | 2    | 1    | 1    | 1  |

Table 2. Allelic assignments of the eight multilocus sequence types found in this study. 1*CAP59*: Allele #1 has been found in 139 known STs; allele #7 in 55 STs. 2*GPD1*: Allele #1 has been found in 106 known STs; allele #3 in 32 STs; allele #6 in 6 STs; allele #23 in 20 STs; allele #25 in one ST (i.e. ST359); and allele #26 in one ST (i.e. ST139). 3*IGS1*: Allele #1 has been found in 124 known STs; allele #10 in 33 STs; allele #22 in one ST (ST139). 4*LAC1*: Allele #3 has been found in 65 known STs; allele #5 in 31 STs; and allele #18 in 39 STs. 5*PLB1*: Allele #2 has been found in 68 known STs; allele #4 in 71 STs. 6*SOD1*: Allele #1 has been found in 183 STs. 7*URA5*: Allele #1 has been found in 91 STs. 8*VN*: These eight STs found in Jiangxi are among 487 total STs in the VNI molecular type in the MLST database accessed on September 16, 2017.
nucleotides at one (GPD1) of the seven sequenced loci (Fig. 1 and Table 2). Similarly, ST31 and ST32 were very close to each other, differed from each other by a few nucleotides at the PLB1 locus. Overall, these five STs formed a tight cluster with each other. In contrast, the other three STs (ST139, ST226, and ST319) were more distantly related to each other and to those five STs described above based on the concatenated gene sequences at these seven loci.

**Relationships Among Geographic Populations of C. neoformans in China.** The multilocus genotypes of all isolates from China in the Cryptococcus MLST database were retrieved. A total of 385 isolates from

| ST    | Geographic location | Percentage of the population |
|-------|---------------------|------------------------------|
| ST5   | China               |                              |
|       | Beijing             | 30.4% (34/112)              |
|       | Shanghai            | 72.7% (16/22)               |
|       | Sichuan Province    | 89.5% (119/133)             |
|       | Guangdong Province  | 87.1% (27/31)               |
|       | Henan Province      | 93.3% (14/15)               |
|       | Heilongjiang Province| 76.0% (19/25)              |
|       | Liaoning Province   | 83.3% (10/12)               |
|       | Jiangxi Province    | 84.9% (73/86)               |
|       | Hong Kong           | 85.7% (12/14)               |
|       | Japan               | 63.4% (51/81)               |
|       | Thailand            | 13.8% (41/297)              |
|       | South Korea         | 56.2% (9/16)                |
|       | Vietnam             | 47.8% (65/136)              |
|       | United States of America| 28.7% (58/202)           |
|       | Europe              | 11.3% (8/71)                |
|       | Brazil              | 2.1% (3/144)                |
|       | South Africa        | 12% (28/230)                |
| ST31  | China               |                              |
|       | Beijing             | 49.1% (55/112)              |
|       | Hebei Province      | 8.6% (3/35)                 |
|       | Henan Province      | 6.7% (1/15)                 |
|       | Heilongjiang Province| 4.0% (1/25)                |
|       | Liaoning Province   | 8.3% (1/12)                 |
|       | Sichuan Province    | 7.5% (10/133)               |
|       | Jiangxi Province    | 1.2% (1/86)                 |
|       | Japan               | 1.2% (1/83)                 |
|       | Thailand            | 2.0% (6/297)                |
|       | India               | 11.5% (7/61)                |
|       | South Africa        | 0.4% (1/230)                |
|       | Brazil              | 2.1% (3/144)                |
| ST32  | China               |                              |
|       | Beijing             | 4.1% (5/123)                |
|       | Hebei Province      | 8.6% (3/35)                 |
|       | Henan Province      | 6.7% (1/15)                 |
|       | Heilongjiang Province| 4.0% (1/25)                |
|       | Liaoning Province   | 8.3% (1/12)                 |
|       | Sichuan Province    | 7.5% (10/133)               |
|       | Jiangxi Province    | 1.2% (1/86)                 |
|       | Japan               | 1.2% (1/83)                 |
|       | United States of America| 0.5% (1/202)             |
|       | South Africa        | 8.3% (16/230)               |
|       | Europe              | 1.4% (1/71)                 |
|       | Brazil              | 2.1% (3/144)                |
|       | Vietnam             | 5.1% (7/136)                |
|       | Thailand            | 0.3% (1/297)                |
|       | Jiangxi Province    | 1.2% (1/86)                 |
| ST186 | Shanghai            | 4.5% (1/22)                 |
|       | Jiangxi Province    | 4.7% (4/86)                 |
| ST139 | Africa              | Unknown frequency          |
|       | Jiangxi Province    | 1.2% (1/86)                 |
| ST359 | Zhejiang Province   | 9.1% (1/11)                 |
|       | Hebei Province      | 2.9% (1/35)                 |
|       | Jiangxi Province    | 4.7% (4/86)                 |
| ST319 | Novel, Jiangxi Province| 1.2% (1/86)              |
| ST226 | Novel, Jiangxi Province| 1.2% (1/86)              |

Table 3. Summary distributions of the eight sequence types identified in Jiangxi Province in other parts of the world.
27 provinces/municipalities in China have been deposited in the database, including the 86 isolates from Jiangxi Province in our study. Among the 27 geographic populations, 12 had isolates of less than five each (most of these 12 populations had only 1–2 isolates each!) and these populations were excluded from our population genetic comparisons. The remaining 15 populations included a total of 364 isolates (Table 4). Our analyses revealed that overall, geographic separation contributed significantly to the total observed genetic variations of the Chinese population of *C. neoformans*. Specifically, AMOVA result showed that about 65% of the observed genetic variation were due to geographic separation while 35% was found within geographic populations (*P* < 0.001). Among the seven loci, five (GPD1, IGS1, LAC1, SOD1, and URA1) showed significant geographic differentiations while the remaining two (CAP59 and PLB1) showed no significant differentiations (detailed data not shown). Our further analyses identified that the observed genetic differentiations were mostly due to the genetic uniqueness of the population from Beijing (Table 4). Of the remaining 91(14 × 13/2) pairwise comparisons, only the Jiangxi-Sichuan populations showed statistically significant genetic differentiation (Table 4).

**Antifungal Susceptibility.** The antifungal drug susceptibility results are presented in Table 4. Our comparisons with recommended resistance breakpoints for these drugs indicated that all 86 cryptococcal isolates were susceptible to 5FC, AMB, FCA, ITR, and VRC. Even though no drug resistant cryptococcal isolates were found among these 86 isolates, there are several noteworthy features. First, variations in MIC values were found for all five tested drugs, with as high as 4-fold differences for itraconazole and 8-fold differences for fluconazole and voriconazole. Second, the differences in MICs were not associated with sequence types. For example, strains in ST5 had a range of MIC values similar to those observed in the overall population. Similarly, the MICs of other seven STs were within the range shown by strains of ST5. Third, strains with relatively high MIC values were broadly distributed. For example, five strains with fluconazole MICs of 8 µg/ml were found in four cities/counties (Shangrao, Leping, Yichun, and Nanchang) (Tables 1 and 5). Fourth, there were significant positive correlations in MIC values among the three triazole drugs in the Jiangxi population of *C. neoformans*. Specifically, the Pearson's correlation coefficients were 0.781, 0.598, and 0.686 respectively for FCA vs. ITR, FCA vs. VRC, and ITR vs. VRC (*p* values all smaller than 0.001). Finally, despite not being called drug resistant, among the 86 isolates, one (CN29, ST5) showed consistently high MIC values for all five drugs.

**Discussions**

In this study, we analyzed the genotypes and drug susceptibility profiles of 86 isolates obtained from across Jiangxi Province in China. Our analyses identified eight multilocus sequence types, with five of which represented by only one isolate each. Of the eight STs found in our sample from Jiangxi, six have been reported from other geographic regions while two were novel, identified so far only in Jiangxi Province. The dominant sequence type in Jiangxi, ST5, is a broadly distributed genotype and has been commonly found in other parts of China as well as in the Far East. These eight genotypes show several types of allelic and phylogenetic relationships. Our antifungal drug susceptibility test results showed that none of the 86 strains were resistant to the five tested antifungal drugs. However, some of the strains showed relatively high MIC values. Below we discuss the relevance of our results to earlier studies and the potential implications of these results to the management of cryptococcosis in Jiangxi Province.

Although a considerable amount of information exists on the epidemiology and molecular typing of *C. neoformans* strains in China, there is very little data on cryptococcosis from Jiangxi Province. Studies from the Chinese Mainland, Taiwan, and Hong Kong indicated that the prevalence of cryptococcosis in HIV/AIDS patients ranged from 12.9% to 24.7%, which is significantly lower than that of many other regions in the world. While the total
Table 4. Evidence for genetic differentiation among geographic populations of C. neoformans in China. The pairwise population F_{ST} values are shown below diagonal. The probability of the observed F_{ST} values being statistically significant is shown above diagonal, with a P value of < 0.05 rejecting the null hypothesis that the two compared populations are genetically similar to each other. The abbreviations on the top row are the same as those in the left column. Numbers in parenthesis of the top row are the numbers of isolates from each of the geographic populations.

| BJ (112) | JX (86) | GD (31) | SH (22) | SC (19) | HK (15) | HEB (13) | ZJ (11) | JS (8) | SD (8) | HUB (7) | LN (7) | AH (6) | SX (5) |
|----------|---------|---------|---------|---------|---------|----------|---------|-------|-------|---------|-------|--------|-------|
|          | 0.001   | 0.001   | 0.001   | 0.001   | 0.001   | 0.001    | 0.001   | 0.001 | 0.001 | 0.001   | 0.001 | 0.001  | 0.002 |
| Jiangxi (JX) | 0.426   | 0.379   | 0.223   | 0.012   | 0.334   | 0.118   | 0.176   | 0.259 | 0.356 | 0.355   | 0.344 | 0.331  | 0.324 |
| Guangdong (GD) | 0.382   | 0.000   | 0.422   | 0.059   | 0.559   | 0.101   | 0.429   | 0.310 | 0.243 | 0.313   | 0.266 | 0.251  | 0.182 |
| Shanghai (SH)  | 0.289   | 0.009   | 0.000   | 0.273   | 0.390   | 0.383   | 0.304   | 0.327 | 0.354 | 0.310   | 0.346 | 0.363  | 0.263 |
| Sichuan (SC)   | 0.195   | 0.093   | 0.081   | 0.000   | 0.360   | 0.347   | 0.196   | 0.270 | 0.292 | 0.127   | 0.369 | 0.139  | 0.516 |
| Henan (HN)     | 0.337   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000 | 0.000 | 0.000   | 0.026 | 0.051  | 0.007 |
| Hongkong (HK)  | 0.323   | 0.031   | 0.041   | 0.005   | 0.026   | 0.000   | 0.503   | 0.489 | 0.507 | 0.284   | 0.377 | 0.407  | 0.603 |
| Hebei (HEB)    | 0.369   | 0.019   | 0.002   | 0.008   | 0.069   | 0.000   | 0.007   | 0.466 | 0.397 | 0.399   | 0.321 | 0.333  | 0.282 |
| Zhejiang (ZJ)  | 0.379   | 0.000   | 0.000   | 0.007   | 0.085   | 0.000   | 0.015   | 0.000 | 0.421 | 0.711   | 0.487 | 0.636  | 0.573 |
| Jiangsu (JS)   | 0.380   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000   | 1.000 | 0.485 | 0.478   | 0.440 | 0.400  | 0.011 |
| Shandong (SD)  | 0.381   | 0.000   | 0.000   | 0.011   | 0.083   | 0.000   | 0.019   | 0.000 | 0.066 | 0.000   | 0.720 | 0.738  | 0.692 |
| Hunan (HUB)    | 0.356   | 0.000   | 0.000   | 0.000   | 0.045   | 0.000   | 0.000   | 0.000 | 0.000 | 0.020   | 0.001 | 1.000  | 0.422 |
| Liaoning (LN)  | 0.377   | 0.000   | 0.000   | 0.006   | 0.074   | 0.000   | 0.000   | 0.000 | 0.013 | 0.020   | 0.001 | 0.728  | 0.423 |
| Anhui (AH)     | 0.313   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000   | 0.026   | 0.051 | 0.007 | 0.000   | 0.002 | 0.473  | 0.002 |
| Shanxi (SX)    | 0.358   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000   | 0.000 | 0.000 | 0.000   | 0.000 | 0.000  | 0.000 |

The number of HIV-positive patients is not known in Jiangxi Province, HIV-positive patients account for over 40% of the sources of our strains in this study. In contrast, the percentage of isolates from individuals without obvious predisposing risk factors was significantly lower (9/86, 10.5%) than those reported before from other parts of China, but more similar to those from regions outside of China. In Jiangxi Province, cryptococcal infection was more commonly found in middle-aged people, the main group with HIV infections in our samples, than in other age groups. Furthermore, unlike previous studies that found no prominent gender bias in the incidence of cryptococcosis in China, our data showed that the male–female gender ratio was 2.3:1. The ratio in Jiangxi is similar to those reported from Brazil and Europe, in which the male to female gender ratio was about 2.9:1.

Similar to observations from other parts of China and other Asian countries, such as Korea, Japan and Thailand, our data showed that the 86 clinical isolates of C. neoformans from Jiangxi Province had relatively limited amount of genetic variation. All isolates were of the same mating type and the same genotype group VNI. The genotype group VNI is globally the dominant lineage of C. neoformans responsible for cryptococcosis. A previous study by Fang et al. indicated that serotype A, molecular type VNI, and MATα strains of C. neoformans predominate HIV-negative patients in China. Our study suggests this genotype group also predominate the HIV-infected patients in Jiangxi.

To date, seventeen STs of C. neoformans var. grubii have been identified in China. They include ST5, ST31, ST38, ST53, ST57, ST63, ST93, ST186, ST191, ST194, ST195, ST295, ST296, ST359, and ST360 in Mainland China, while ST4 and ST6 are found in Hong Kong. In this study, eight STs (i.e. ST5, ST31, ST32, ST139, ST186, ST226, ST319, and ST359) were founded in Jiangxi Province and only four of these eight STs overlap with those reported from other parts of China. This result suggests that there is likely abundant unique genetic diversity of C. neoformans in Jiangxi Province. Among the shared STs between Jiangxi and outside of Jiangxi, the majority belonged to ST5, the most common ST in all East Asian countries where epidemiology data are available, including China, Japan, and South Korea. Interestingly, two other STs, ST31 and ST32, found in Jiangxi were also broadly distributed. According to Khayhan et al., ST139 has so far been found only in Africa. However, its relative frequency in Africa is not known. The geographic distribution patterns of these six shared STs found in Jiangxi suggest that both long- and short- distance dispersals are common in C. neoformans. Consistent with this hypothesis, aside from the Beijing population, we found limited evidence for genetic differentiation between most pairs of geographic populations of C. neoformans in China. At present, the reason(s) for the genetic distinctiveness of the Beijing population is not known. However, as suggested previously, a diversity of factors such as wind, animals such as pigeons, and anthropogenic activities could have contributed to the dispersals of genotypes between Jiangxi Province and other regions both within and outside of China.

In this study, all 86 cryptococcal isolates were susceptible to 5FC, AMB, FCA, ITR, and VRC. Our results suggest that the standard initial therapy for cryptococcosis, AMB combined with 5FC, should still work for patients in Jiangxi Province. However, variations in MICs were observed among the isolates. For each of the drugs, there were isolates showing high MIC values. At present, there was no apparent relationship between MIC to any of the drugs and geographic origins and/or strain genotypes. We would like to note that some of the strains showed high MIC values to multiple drugs. Our results thus call for close monitoring of drug susceptibilities of cryptococcal strains in Jiangxi Province.

In conclusion, our study revealed both shared and divergent genotypes and patterns of cryptococcal epidemiology between Jiangxi Province and other parts of China. Specifically, in both Jiangxi and other parts of China,
| Isolate | Location | ST | SFC | AMB | FCA | ITR | VRC |
|---------|----------|----|-----|-----|-----|-----|-----|
| CN6     | Dexing   | 5  | <4  | <0.5| 1   | 0.125| 0.06|
| CN9     | Dexing   | 5  | <4  | <0.5| 2   | 0.125| 0.06|
| CN1     | Fengcheng| 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN12    | Fuzhou   | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN2     | Fuzhou   | 5  | <4  | <0.5| 4   | 0.25 | 0.25|
| CN26    | Fuzhou   | 139| <4  | <0.5| 2   | 0.25 | 0.125|
| CN45    | Fuzhou   | 5  | <4  | <0.5| 4   | 0.25 | 0.25|
| CN51    | Fuzhou   | 5  | <4  | <0.5| 2   | 0.25 | 0.25|
| CN56    | Fuzhou   | 5  | <4  | <0.5| 4   | 0.25 | 0.25|
| CN69    | Fuzhou   | 5  | <4  | <0.5| 2   | 0.125| 0.06|
| CN8     | Fuzhou   | 5  | <4  | <0.5| 2   | 0.125| 0.125|
| CN76    | Fuzhou   | 359| <4  | <0.5| 1   | 0.125| 0.06|
| CN28    | Ganzhou  | 5  | <4  | <0.5| 2   | 0.125| 0.25|
| CN41    | Ganzhou  | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN63    | Ganzhou  | 5  | <4  | <0.5| 2   | 0.25 | 0.25|
| CN88    | Ganzhou  | 5  | <4  | <0.5| 2   | 0.125| 0.25|
| CN92    | Ganzhou  | 5  | <4  | <0.5| 1   | 0.125| 0.125|
| CN37    | Gaoan    | 5  | <4  | <0.5| 2   | 0.125| 0.25|
| CN14    | Ji'an    | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN21    | Ji'an    | 319| <4  | <0.5| 4   | 0.125| 0.25|
| CN49    | Ji'an    | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN50    | Ji'an    | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN53    | Ji'an    | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN61    | Ji'an    | 5  | <4  | <0.5| 2   | 0.25 | 0.25|
| CN73    | Ji'an    | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN74    | Ji'an    | 5  | <4  | <0.5| 2   | 0.125| 0.125|
| CN79    | Ji'an    | 5  | <4  | <0.5| 1   | 0.125| 0.06|
| CN94    | Ji'an    | 359| <4  | <0.5| 2   | 0.125| 0.06|
| CN31    | Jiujiang | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN36    | Jiujiang | 5  | <4  | <0.5| 4   | 0.25 | 0.25|
| CN48    | Jiujiang | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN64    | Jiujiang | 5  | <4  | <0.5| 2   | 0.125| 0.125|
| CN65    | Jiujiang | 5  | <4  | <0.5| 2   | 0.25 | 0.25|
| CN68    | Jiujiang | 5  | <4  | <0.5| 4   | 0.25 | 0.25|
| CN86    | Jiujiang | 5  | <4  | <0.5| 1   | 0.125| 0.125|
| CN87    | Jiujiang | 5  | <4  | <0.5| 4   | 0.125| 0.25|
| CN10    | Leping   | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN19    | Leping   | 5  | <4  | <0.5| 8   | 0.5  | 0.25|
| CN27    | Leping   | 5  | 4   | 0.5 | 2   | 0.25 | 0.06|
| CN3     | Leping   | 186| <4  | <0.5| 2   | 0.25 | 0.125|
| CN44    | Leping   | 5  | <4  | <0.5| 2   | 0.125| 0.06|
| CN11    | Nanchang | 5  | <4  | 1   | 4   | 0.25 | 0.125|
| CN13    | Nanchang | 359| <4  | <0.5| 2   | 0.25 | 0.125|
| CN16    | Nanchang | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN18    | Nanchang | 5  | <4  | <0.5| 8   | 0.5  | 0.5|
| CN22    | Nanchang | 5  | <4  | <0.5| 2   | 0.125| 0.06|
| CN23    | Nanchang | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN24    | Nanchang | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN34    | Nanchang | 5  | <4  | <0.5| 4   | 0.125| 0.125|
| CN35    | Nanchang | 5  | <4  | <0.5| 2   | 0.25 | 0.25|
| CN39    | Nanchang | 5  | <4  | <0.5| 1   | 0.125| 0.06|
| CN57    | Nanchang | 5  | <4  | <0.5| 2   | 0.125| 0.06|
| CN58    | Nanchang | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN7     | Nanchang | 5  | <4  | <0.5| 2   | 0.125| 0.125|
| CN71    | Nanchang | 5  | <4  | <0.5| 2   | 0.25 | 0.06|
| CN81    | Nanchang | 5  | <4  | <0.5| 1   | 0.25 | 0.125|
| CN85    | Nanchang | 359| <4  | <0.5| 2   | 0.125| 0.125|

Continued
Table 5. Susceptibilities of the 86 C. neoformans isolates from Jiangxi Province against five common antifungal drugs. ST: sequence type as determined based on the combined sequences at the seven loci. FCA: Fluconazole. AMB: Amphotericin B. ITR: Itriconazole. VRC: Voriconazole.

| Isolate | Location | ST | FCA | AMB | ITR | VRC |
|---------|----------|----|-----|-----|-----|-----|
| CN93    | Nanchang | 5  | <4  | <0.5| 2   | 0.125| 0.06 |
| CN46    | Pingxiang| 5  | <4  | <0.5| 2   | 0.125| 0.06 |
| CN15    | Shangrao | 5  | <4  | 1   | 4   | 0.125| 0.06 |
| CN29    | Shangrao | 5  | 4   | 1   | 8   | 0.5  | 0.25 |
| CN30    | Shangrao | 32 | <4  | 0.5 | 4   | 0.25 | 0.125|
| CN32    | Shangrao | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN33    | Shangrao | 5  | <4  | <0.5| 2   | 0.125| 0.06 |
| CN4     | Shangrao | 5  | 4   | <0.5| 1   | 0.125| 0.06 |
| CN40    | Shangrao | 5  | <4  | <0.5| 2   | 0.125| 0.06 |
| CN42    | Shangrao | 5  | 4   | <0.5| 4   | 0.25 | 0.125|
| CN52    | Shangrao | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN54    | Shangrao | 5  | <4  | <0.5| 4   | 0.25 | 0.25 |
| CN67    | Shangrao | 5  | <4  | <0.5| 2   | 0.25 | 0.25 |
| CN70    | Shangrao | 5  | <4  | <0.5| 2   | 0.125| 0.06 |
| CN75    | Shangrao | 5  | <4  | <0.5| 4   | 0.25 | 0.125|
| CN80    | Shangrao | 5  | <4  | <0.5| 1   | 0.125| 0.06 |
| CN84    | Shangrao | 5  | <4  | <0.5| 2   | 0.125| 0.125|
| CN66    | Xinyu    | 5  | <4  | <0.5| 4   | 0.25 | 0.25 |
| CN91    | Xinyu    | 5  | <4  | <0.5| 2   | 0.125| 0.06 |
| CN17    | Yichun   | 5  | <4  | <0.5| 8   | 0.5  | 0.25 |
| CN25    | Yichun   | 226| <4  | <0.5| 2   | 0.125| 0.06 |
| CN38    | Yichun   | 5  | <4  | <0.5| 2   | 0.25 | 0.125|
| CN43    | Yichun   | 5  | <4  | <0.5| 8   | 0.5  | 0.25 |
| CN60    | Yichun   | 5  | <4  | <0.5| 4   | 0.25 | 0.25 |
| CN82    | Yichun   | 186| <4  | <0.5| 2   | 0.125| 0.06 |
| CN20    | Yingtan  | 5  | <4  | 1   | 4   | 0.125| 0.125|
| CN55    | Yingtan  | 5  | <4  | <0.5| 4   | 0.25 | 0.25 |
| CN83    | Yingtan  | 186| <4  | <0.5| 4   | 0.25 | 0.125|
| CN90    | Yingtan  | 186| <4  | <0.5| 1   | 0.125| 0.125|

ST5 was the predominant sequence type. In addition, both unique STs and evidence for long distance dispersals were found among most surveyed regions in China. However, different from previous studies in China, our results identified that most patients in Jiangxi Province with cryptococcosis had underlying risk factors associated with compromised immunity. At present, the mechanism for the predominance of ST5 in East Asian populations is not known. One possibility is that ST5 is more virulent than other sequence types to East Asians. Another possibility is that ST5 was the founder clone in East Asia that has adapted to the local ecological niches. Additional investigations are needed in order to test these possibilities.

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Author Contributions
L.-H.H., J.X., and N.Z.: conceived and designed the experiments; Y.-H.C., F.Y., Z.-Y.B., J.-M.H., N.Z., Q.-S.Z., and Y.-P.H. performed the experiments; Y.-H.C. and J.X. analyzed the data; Y.-H.C., L.-H.H., and J.X. drafted the manuscript. All co-authors reviewed and approved the manuscript.

Additional Information
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