The Predominance of a Specific Genotype of Cryptococcus neoformans var. Grubii in China and Japan

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1. Introduction

Members of the Cryptococcus neoformans/C. gattii species complex are basidiomycetous yeasts, which are the etiological agents of cryptococcosis [1]. Among such opportunistic pathogens, many microbial phenotypes have been clearly correlated with virulence, including polysaccharide capsule production, formation of melanin, and secretion of various proteins [2]. The infection proceeds by inhalation and spreads to the central nervous system causing meningitis. Cryptococcosis caused by C. neoformans is predominantly

AIDS, while infections caused by C. gattii often occur in immunocompetent patients [3]. The species complex is classified into two species: C. neoformans (serotypes A, D, and AD) and C. gattii (serotypes B and C) [4]. C. neoformans is divided into two varieties and one hybrid, namely, C. neoformans var. grubii (serotype A), C. neoformans var. neoformans (serotype D), and a hybrid of both varieties (serotype AD). More recently, it was suggested to separate the Cryptococcus neoformans/C. gattii species complex into seven species [1], but for this study, the traditional classification was followed. C. neoformans var. grubii has been isolated
worldwide [4–8] and causes most of the cryptococcal infections in HIV-infected patients [9]. Cryptococcus species typically reside in a variety of environmental niches, mainly old avian droppings (especially aged pigeon droppings) [4, 5, 10, 11], decaying wood [12–14], and soil contaminated with these droppings and/or decaying wood alone [10, 13].

Several studies have focused on the molecular determinants of the number of genetically diverse subgroups within each serotype [15–18]. Molecular methods employed to define these subgroups revealed associations between geographic origin and particular genotypes, implying an epidemiologic significance of specific genotypes. Although, in the past decade, molecular genotyping methods have been applied extensively to characterize the population genetic structure of C. neoformans, only a few reports have been published on genotype analyses of C. neoformans var. grubii from China and Japan [19–23]. Therefore, we were prompted to conduct a genetic characterization of C. neoformans var. grubii in China and Japan using previously established microsatellite markers [17]. Microsatellites, also known as simple sequence repeats (SSR) or short tandem repeats (STRs), are highly polymorphic and spread throughout all genomes, including humans, lower eukaryotes, and fungi [24, 25]. We have previously reported the usefulness of multilocus microsatellite typing (MLMT) using three specific microsatellite-amplifying PCR primer sets (CNG1, CNG2, and CNG3) for the characterization of the genotype structure of C. neoformans var. grubii [17, 26]. To date, 38 MLMT types have been recognized globally among C. neoformans var. grubii isolates [17].

In the current study, we applied MLMT to characterize the genotypes of 52 strains isolated in China and Japan. To place the Chinese and Japanese cryptococcal isolates in a global context and to compare the herein used MLMT typing technique with the typing techniques used elsewhere, 22 of 52 Chinese and Japanese strains were randomly selected and subjected to the restriction fragment length polymorphism analysis of the orotidine monophosphate pyrophosphorylase gene (URA5-RFLP), M13 PCR-finger-printing, and multilocus sequence typing (MLST) analysis.

2. Methods

2.1. Ethics Statement. This study was approved by the institutional ethics committee (Guiyang Hospital of Guizhou Aviation Industry Group Research Ethics Committee, permission number: 2015-037).

2.2. PCR Amplification, DNA Sequencing, and Multilocus Microsatellite Typing (MLMT)

2.2.1. Strains Used. Thirty-nine isolates of C. neoformans var. grubii from China (including 30 clinical isolates, eight environmental isolates, and one isolate from an unknown source) and 13 isolates from Japan (6 clinical isolates and seven isolates from unknown sources) were studied (Table 1). Of the strains isolated in China, seven were from Beijing, nine (including five environmental strains) from Nanjing, five from Shanghai, and 15 from Guangzhou, including three environmental strains. All of the environmental strains were isolated from pigeon droppings in China. Of the 13 isolates collected in Japan, seven were from Chiba Prefecture (central Japan), and six were from Nagasaki Prefecture (western Japan) (Table 1). Clinical samples included CSF (cerebrospinal fluid, 25 strains), BAL (bronchial lavage, three strains), TBLB (transbronchial lung biopsy, one strain), and sputum (one strain). All strains were initially identified as C. neoformans by standard microbiological methods and growth characteristics on Canavanine-Glycine-Bromothymol blue (CGB) agar [27]. The strains were grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) slants and incubated at 30°C for 48–72h before DNA extraction.

2.2.2. DNA Extraction. Genomic DNA was extracted as previously described [26]. Briefly, 3–4 loops of yeast cells from PDA slants were suspended in TE buffer (10 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ EDTA, and pH 8.0). Tris-HCl (pH 8.0) was added to the washed yeast cells and boiled. After treatment with chloroform-isooamyl alcohol, the aqueous phase was separated. The aqueous layer was mixed with isopropanol and 3 mL ammonium acetate. The samples were centrifuged, and the resulting nucleic acid pellets were washed, dried, and finally resuspended in TE buffer.

2.2.3. PCR for Mating-Type Determination. The mating type was determined according to Halliday and Carter [28] using specific primers for the amplification of both a and a loci.

2.2.4. MT Analysis. MT types were determined as previously reported [17, 26]. Namely, PCR primers, which amplify three microsatellite loci, designated CNG1, CNG2, and CNG3, were used. The microsatellite locus CNG1 has repeats of the “TA” motif, with repeat numbers ranging from 9 to 13, resulting in five microsatellite allele types. The locus CNG2 has reproductions of the “GA” motif, with repeat numbers ranging from 7 to 12, resulting in 6 microsatellite allele types. Finally, the microsatellite locus CNG3 has repeats of the “CAT” motif, with repeat numbers ranging from 5 to 12, resulting in 8 microsatellite allele types. These microsatellites were amplified using Ready-To-Go PCR beads (Amersham Pharmacia Co., Piscataway, NJ, USA), a set of primers (CNG1, CNG2, and CNG3) at a final concentration of 1 μM, and 25 pg of genomic DNA, in a volume of 25 μL using a GeneAmp 9600 thermocycler (Perkin-Elmer Inc., USA). The PCR conditions were as previously described [17, 26]. The amplified PCR products were purified using a PCR product pre-sequencing kit (ExoSAP-IT; USA Corp., Cleveland, OH, USA), and the DNA sequences were determined by an automatic sequencer (ABI PRISM® 3100; PE Applied Biosystems, Tokyo, Japan). MLMT allele types were determined by calculating the number of repeat numbers in each locus, and the MLMT type was determined by combining the repeat allele numbers of the three different motifs [26].

2.3. RA5-RFLP, M13 PCR-Fingerprinting, and MLST Analysis Using the ISHAM Consensus MLST Scheme for the C. neoformans/C. gattii Species Complex

2.3.1. Used. To place the Chinese and Japanese strains in a global context, 22 out of the 52 strains used in MLMT analysis were randomly selected from the cultures isolated in
Table 1: List of *Cryptococcus neoformans* var. *grubii* strains studied, their sources, country of origin, CNG-Allele type, and multilocus microsatellite type (MLMT).

| IFM strain number | Source of isolation | Country of origin | CNG1 allele type (TA repeat number) | CNG2 allele type (GA repeat number) | CNG3 allele type (CAT repeat number) | MLMT type |
|-------------------|---------------------|-------------------|-------------------------------------|-------------------------------------|-------------------------------------|-----------|
| 45835 (WM09.168)  | Unknown             | CH, Japan         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45836             | Unknown             | CH, Japan         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45839             | Unknown             | CH, Japan         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45840             | Unknown             | CH, Japan         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45841             | Unknown             | CH, Japan         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45842 (WM09.169)  | Patient             | CH, Japan         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45843             | Unknown             | CH, Japan         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 40045 (WM09.170)  | Unknown             | N, Japan          | 1 (9)                               | 3 (10)                              | 2 (7)                               | 2         |
| 46652 (WM09.171)  | Sputum              | N, Japan          | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 46654             | TBLB                | N, Japan          | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 46655 (WM09.172)  | CSF                 | N, Japan          | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 46658             | BAL                 | N, Japan          | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 59                | BAL                 | N, Japan          | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 55983 (WM09.173)  | CSF                 | BJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 55984             | CSF                 | BJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 55985             | CSF                 | BJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 55986 (WM09.174)  | CSF                 | BJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 55987             | CSF                 | BJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 56847 (WM09.176)  | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 56848             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 56849             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 56850 (WM09.177)  | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 56851             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45712             | CSF                 | NJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45717 (WM09.181)  | CSF                 | NJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 47272 (WM090.175) | CSF                 | BJ, China         | 2 (11)                              | 5 (12)                              | 6 (12)                              | 14        |
| 47273             | CSF                 | BJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45721 (WM09.178)  | Patient             | SH, China         | 5 (14)                              | 5 (12)                              | 2 (7)                               | 39        |
| 45722             | Patient             | SH, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45723 (WM09.179)  | Patient             | SH, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45725             | Patient             | SH, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45726 (WM09.180)  | Patient             | SH, China         | 1 (9)                               | 3 (10)                              | 1 (5)                               | 34        |
| 45760             | Pigeon dropping     | NJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45764 (WM09.185)  | Pigeon dropping     | NJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45766 (WM09.186)  | Pigeon dropping     | NJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45768             | Pigeon dropping     | NJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 45772 (WM09.187)  | Pigeon dropping     | NJ, China         | 4 (13)                              | 5 (12)                              | 2 (7)                               | 29        |
| 52696 (WM09.188)  | BAL                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 52364 (WM09.189)  | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 52366             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 52368             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 52370 (WM09.190)  | CSF                 | GZ, China         | 3 (12)                              | 4 (11)                              | 2 (7)                               | 16        |
| 52372             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 52376             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 52378             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 52379             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 52380             | CSF                 | GZ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
China and Japan. In addition, the following standard strains of the eight major molecular types of the C. neoformans/C. gattii species complex have been included for significant molecular type identification: WM148 (VNI, serotype A, CSF, HIV-, Australia), WM626 (VNII, serotype A, CSF, HIV-, Australia), WM628 (VNIII, serotype AD, CSF, HIV+, Australia), WM629 (VNIV, serotype D, Blood, HIV+, Australia), WM179 (VGII, serotype B, CSF, HIV-, Australia), WM178 (VGI, serotype B, CSF, HIV+, Australia), WM175 (VGIII, serotype B, Eucalypt, USA), and WM779 (VGIV, serotype C, Cheetah, South Africa).

### 2.3.2. DNA Extraction.
For DNA extraction, the strains were grown on Sabouraud dextrose agar slants at 37°C for two days. Then, the cells were collected by centrifugation, and the cell pellets were frozen in liquid nitrogen. The frozen cells were ground with a miniature pestle (1.5 cm diameter). The tubes were kept in the freezer at −20°C overnight, and ground cells were mixed with lysis buffer and 2-mercaptoethanol. The samples were mixed vigorously and then incubated at 65°C for 1 h (vortexed at least once during incubation). DNA was extracted as described previously [29].

### 2.3.3. URA5-RFLP Analysis.
PCR amplification of the URA5 gene was conducted using the forward primer URA5 (5′-ATGTCCCTCCCAAGCCCTCAGACTCCG-3′), and the reverse primer SJ01 (5′-TTAAGACCTCTGA ACACCGTACTC-3′) as previously described [30]. A portion of the PCR products was then double digested with the restriction enzymes Sau961 and HhaI. The digestion products were separated on a 3% agarose gel. The major molecular types were determined by manual comparison with the RFLP patterns of standard strains of the eight major molecular types.

### 2.3.4. M13 PCR-Fingerprinting.
Strain typing was performed using PCR-fingerprinting with the core sequence of the wild-type phage M13-specific primer (5′-GAGGGTGCGGGTTCT T-3′) [7] using 10 ng μL⁻¹ genomic DNA and Ready-To-Go PCR beads (Amersham Pharmacia Co., Piscataway, N.J., USA) in a volume of 25 μL as previously illustrated [30, 31]. The main molecular types were determined by manual comparison with the PCR-fingerprinting patterns of standard strains of the eight major molecular types. Strain genotypes were identified based on their individual banding patterns.

### 2.3.5. MLST Analysis.
Strains were randomly selected for MLST analysis based on seven unlinked loci, i.e., CAP59, which encodes a capsular-associated protein; GPDI, which encodes glyceraldehyde-3-phosphate dehydrogenase; IGS1, which encodes a ribosomal RNA intergenic spacer; LASC1, which encodes laccase; PLBI, which encodes phospholipase; SOD1, which encodes Cu, Zn superoxide dismutase; and URA5, which encodes orotidine monophosphate pyrophosphorylase. Each genetic locus was amplified using the primers and amplification parameters described by the International Society for Human and Animal Mycology (ISHAM) Cryptococcal Working Group for genotyping C. neoformans and C. gattii and analyzed as previously reported [32, 33]. Allele and sequence types were determined against the MLST webpage of the University of Sydney (https://mlst.mycologylab.org).

### 3. Results

#### 3.1. Distribution of the MLMT Types of C. neoformans var. Grubii Strains in Chinese and Japanese Cultures.
Based on the repeat numbers of each microsatellite, the combined MLMT types were determined and are shown in Table 1. The 52 C. neoformans var. grubii strains were classified into 7 MLMT types (MLMT-2, MLMT-14, MLMT-16, MLMT-17, MLMT-29, MLMT-34, and MLMT-39), with the major one being MLMT-17 (88.5%, 46 out of the 52 strains). MLMT-39 was identified as a novel MLMT type with a unique SSR allele at the microsatellite region in CNG1 (DDBJ accession number AB488809). The most common repeat number of the “TA” motif of the microsatellite locus CNG1 was 12, and 46 strains belong to this repeat number group in MLMT-17. However, the number of repeats of the “GA” and “CAT” motifs of the microsatellite loci CNG2 and CNG3 in MLMT-17 was 12 and 7, respectively, and 49 and 50 out of the 52 strains, respectively, presented these repeat numbers. The distribution of the MLMT types in Chinese and Japanese cultures is shown in Figure 1(a), in comparison with the MLMT types obtained globally (except for cultures from China, Brazil, and Japan; Figure 1(b)), and the MLMT types obtained from Brazilian cultures are shown in Figure 1(c). Among the 52 strains, 39 Chinese isolates were classified into 6 MLMT types: MLMT-14 (1 strain), MLMT-16 (1 strain), MLMT-17 (34 strains), MLMT-29 (1 strain), MLMT-34 (1 strain), and MLMT-39 (1 strain). The regional MLMT-type distribution

#### Table 1: Continued.

| IFM strain number | Source of isolation | Country of origin | CNG1 allele type (TA repeat number) | CNG2 allele type (GA repeat number) | CNG3 allele type (CAT repeat number) | MLMT type |
|-------------------|---------------------|-------------------|-------------------------------------|-------------------------------------|-------------------------------------|-----------|
| 45718 (WM09.182)  | CSF                 | NJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 48163             | Unknown             | NJ, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 57499 (WM09.191)  | Pigeon dropping     | GY, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 57500             | Pigeon dropping     | GY, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |
| 57502             | Pigeon dropping     | GY, China         | 3 (12)                              | 5 (12)                              | 2 (7)                               | 17        |

BAL: bronchial lavage; TBLB: transbronchial lung biopsy; CSF: cerebrospinal fluid; BJ: Beijing; CH: Chiba; GY: Guiyang; GZ: Guangzhou; N: Nagasaki; NJ: Nanjing; SH: Shanghai.
patterns of the Chinese isolates are shown in Figure 2. Thirteen Japanese isolates were classified into 2 MLMT types, namely, MLMT-2 (1 strain) and MLMT-17 (12 strains), with the latter MLMT type being predominant amongst the Japanese isolates studied. The MLMT-17 type was highly prevalent in both countries, with prevalence ratios being in China at 87.2% and Japan at 92.3%.

3.2. High Prevalence of the MLMT-17 Type in China and Japan. Information on the 36 strains from patients, including 25 strains from CSF, three strains from BAL, one strain from TBLB, and one strain from sputum, is shown in Table 1. For the remaining nine strains, no patient information was available. A correlation analysis between clinical status and MLMT type revealed that the MLMT-17 type predominates the clinical samples regardless of their isolation site. Out of the eight environmental isolates, seven strains were of the MLMT-17 type (87.5%), being the major type in Chinese and Japanese pigeon droppings. The remaining strain was of the MLMT-29 type.

3.3. VNI/ST5(M5) Is One Specific Genotype among Chinese and Japanese C. neoformans var. grubii Isolates. To compare the herein applied typing method to other typing methods and to place them into the global molecular epidemiology of the C. neoformans/C. gattii species complex, 22 randomly selected strains from the 52 C. neoformans var. grubii isolates from China and Japan were studied for MLMT typing and subjected to URA5-RFLP analysis, M13 PCR-fingerprinting, and MLST typing (Table 2). URA5-RFLP analysis showed that all 22 isolates were of molecular type VNI (Table 2). PCR-fingerprinting using the minisatellite specific primer M13 showed a significant pattern that is shared among most of the studied isolates. The pattern can be seen in all 22 VNI isolates. MLST of the 22 strains for seven loci revealed the presence of a predominant MLST type, VNI/ST5 (M5), to which 19 of the isolates (86%) belonged. The results of MLST typing compared to those of the MLMT typing are shown in Table 2, confirming the predominance of one specific genotype among Chinese and Japanese C. neoformans var. grubii isolates.
Strain IFM 47272 belongs to the MLST type ST32 (M4) (MLMT-14), and strain IFM 45722 belongs to the MLST type ST31 (M4b) (MLMT-29). One of the isolates, IFM 40045, represents a novel, previously unidentified MLST sequence type ST66 (MLMT-2). The strains IFM 45721, IFM 45726, and IFM 52370 belong to the same MLST type as ST5 (M5), but they were classified into different MLMT types, namely, MLMT-39, MLMT-34, and MLMT-16, respectively (Table 2).

### 4. Discussion

Studies have previously reported the usefulness of MLMT using the sequences of three different microsatellite regions for the genotyping of *C. neoformans* isolates [17, 26]. To date, 176 strains of *C. neoformans* var. *grubii* have been classified into 38 MLMT types [17, 26]. In the present study, newly isolated 52 strains of the *C. neoformans* var. *grubii* from China and Japan were classified into 7 MLMT types using the same MLMT method. Most of these (46 strains) were classified into one major genotype, MLMT-17 (88.6%). In both countries, the MLMT-17 type was highly prevalent. The prevalence ratios of MLMT-17 in China were 87.2% and Japan at 92.3%, suggesting that clonal reproduction among the populations of *C. neoformans* var. *grubii* in the two countries may be the main reproductive style. This was also previously observed in Taiwan, where two isolates of *C. neoformans* var. *grubii* were classified as the MLMT-17 type [26]. There was only one allele type difference between the major type (MLMT-17) and the three other

| No. of *C. neoformans* var. *grubii* isolates in China | Beijing | Guangzhou | Nanjing | Shanghai | Guiyang |
|--------------------------------------------------------|---------|-----------|---------|----------|---------|
| 12 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 |

**Figure 2**: MLMT-type distribution of 39 *Cryptococcus neoformans* var. *grubii* strains isolated from Beijing, Guangzhou, Nanjing, Shanghai, and Guiyang in China. MLMT: multilocus microsatellite typing.
types (MLMT-16, MLMT-29, and MLMT-39), indicating that these are genetically very closely related. Therefore, microevolution in \textit{C. neoformans} var. \textit{grubii} strains in China and Japan might be a reasonable explanation for the presence of MLMT-16, MLMT-29, and MLMT-39 [34]. These results suggest a predominance of one specific MLMT type (MLMT-17) among the Chinese and Japanese isolates.

Furthermore, strain IFM 45772 (MLMT-29) was classified as VNI according to major molecular type and pattern. It differed from MLST type ST5 (M5) and was classified as MLST type ST31 (M4b) due to sequence differences in the \textit{GPD1}, \textit{IGS1}, and \textit{LAC1} loci. Further studies on the strains of the MLMT-29 type are essential, considering that the strain IFM 45772 was isolated from pigeon droppings. In contrast, the isolation sites of the three previously studied strains are unknown [26]. The remaining type MLMT-2 is different from MLMT-17 in terms of sequences of two loci, CNG1 and CNG2. We previously isolated MLMT-2 from Costa Rica [26]. In addition, we also reported about 12 strains belonging to the MLMT-2 type from pigeon droppings and clinical samples from Brazil [26]. As such, strains of the MLMT-2 genotype are also considered to be widely distributed in South and Central America as well as East Asia. Finally, the strain IFM 47272 with the genotype MLMT-14 was isolated in China. This genotype was previously isolated from Brazil (2 strains), again suggesting a wide distribution.

In addition, we found that MLMT typing and M13 PCR-fingerprinting are highly concordant. Our results agree well with a report in which most of the Chinese strains of the

\begin{table}
\begin{center}
\begin{tabular}{lccccccccccc}
Strain no. & IFM no. & \text{CAP59} & \text{GPD1} & \text{IGS1} & \text{LAC1} & \text{PLB1} & \text{SOD1} & \text{URA5} & \text{MLST} & \text{ST} & \text{MLMT type} & \text{Major molecular type*} \\
\hline
WM09.168 & 45835 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.169 & 45842 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.170 & 40045 & 7 & 1 & 1 & 12 & 1 & 1 & 2 & 66 & 2 & VNI \\
WM09.171 & 46652 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.172 & 46655 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.173 & 55983 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.174 & 55986 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.175 & 47272 & 1 & 1 & 10 & 3 & 4 & 1 & 1 & 32 & M4 & 14 & VNI \\
WM09.176 & 56847 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.177 & 56850 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.178 & 45721 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 39 & VNI \\
WM09.179 & 45723 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.180 & 45726 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 34 & VNI \\
WM09.181 & 45717 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.182 & 45718 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.185 & 45764 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.186 & 45766 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.187 & 45772 & 1 & 1 & 10 & 3 & 2 & 1 & 1 & 31 & M4b & 29 & VNI \\
WM09.188 & 52696 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.189 & 52364 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.190 & 52370 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
WM09.191 & 57499 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 16 & VNI \\
c48 & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
jp1088 & & 1 & 3 & 1 & 5 & 2 & 1 & 1 & 5 & M5 & 17 & VNI \\
A3 11 & & 7 & 1 & 1 & 2 & 1 & 1 & 2 & 23 & M3 & VNI \\
br795 & & 1 & 1 & 10 & 3 & 4 & 1 & 1 & 32 & M4 & VNI \\
ug2463 & & 1 & 1 & 1 & 3 & 2 & 1 & 5 & 6 & M10a & VNI \\
ug2471 & & 1 & 1 & 10 & 3 & 2 & 1 & 1 & 31 & M4b & VNI \\
H99 & & 7 & 1 & 1 & 1 & 1 & 1 & 2 & 2 & M1b & VNI \\
WM148 & & 7 & 1 & 1 & 18 & 1 & 1 & 1 & 63 & M1 & VNI \\
WM626 & & 2 & 14 & 14 & 8 & 11 & 11 & 27 & 97 & M7 & VNI \\
WM629 & & 16 & 21 & 30 & 19 & 13 & 1 & 19 & 117 & VNIV & \\
\hline
\end{tabular}
\end{center}
\caption{ISHAM consensus scheme MLST types of selected Chinese and Japanese strains in comparison with the MLST types obtained previously, the MLMT types, and the URA5-RFLP patterns.}
\end{table}

Note: *Meyer et al. [33]; Litvintseva et al. 2006. 1Determined by URA5-RFLP analysis or M13 PCR-fingerprinting. ISHAM: international society for human and animal mycology; MLST: multilocus sequence typing; MLMT: multilocus microsatellite typing; ST: sequence type.
C. neoformans var. grubii belong to one particular group, which was based on M13 PCR-fingerpointing [21]. In addition to the application of MLMT to typing strains of the haploid major molecular types of C. neoformans, we also found that AD hybrid strains show different profiles of the CNG1, CNG2, and CNG3 loci, which are easily differentiated from those of the haploid C. neoformans var. grubii strains (data not shown). Therefore, it is possible to determine the AD hybrid strains from the haploid C. neoformans var. grubii strains by MLMT typing. The present results also persistently encourage the hypothesis that human cryptococcosis can be acquired from an immediate environmental reservoir, based on the isolation of the MLMT-17 type from patients and the environment, such as pigeon droppings [5, 11, 30].

Also, M13 PCR-fingerpointing illustrated that most of the studied strains (86.4%) exhibited an identical band pattern, the VNI clade, which includes the clinic and environmental isolates. MLST analysis also revealed a common sequence type (86.4%), ST5 (M5). Three out of the four environmental isolates that were recovered from pigeon excreta also belonged to the sequence type ST5 (M5). This suggests that cryptococcal infections in Chinese patients could be due to the inhalation of cryptococcal cell-contaminated droplets from pigeon excreta. However, more environmental samples should be studied to verify the source of the infection undoubtedly. MLST analysis of strain IFM 47272 revealed that this isolate belongs to the sequence type ST32 (M4). The sequence alignment of the 7 MLST loci showed an identical pattern to the international reference strain br795 isolated in Brazil, which belongs to the MLMT-14 type [17]. Therefore, further detailed environmental studies of the isolation site are warranted, as, currently, only two strains are available. MLST analysis of the isolate IFM 45722 (MLMT-29) showed that it is identical with sequence type M4b and is grouped with the international reference strain ug2471, which we assessed in our previous study [26], where we isolated three strains of this genotype in Japan. The isolate IFM 40045 (MLMT-2) did not group with any of the reference strains for the MLST locus LAC1 and was subsequently identified as a new sequence type. However, MLMT typing confirmed the presence of this genotype in Brazil and Costa Rica [17].

Recent multi-institutional studies by Chen et al. [21], Khayhan et al. [31], and Kaorharrin et al. [6] revealed that the Asian C. neoformans var. grubii populations of Thailand, China, and Japan show limited genetic diversity and demonstrate a largely clonal mode of reproduction compared to the global MLST dataset. These reports suggest that ST5 (M5) is the major MLST genotype among C. neoformans var. grubii isolates in China and Japan, and the sequence types ST4 (M4) and ST6 (M6) are more predominant in Thailand [6, 30]. High distribution ratios of C. neoformans var. grubii strains with the MLST type ST5 (M5) in Japan were also recently reported by Umeyama et al. [35] and MIhara et al. [36]. The prevalence of the ST5 (M5) genotype of C. neoformans var. grubii in Korea was also reported by Park et al. [37]. Furthermore, the Asian population of C. neoformans var. grubii has been shown to be genetically less diverse than those occurring in Africa [38, 39] and Europe [40], and one genotype is predominant in China [21, 22]. In this study, the presented analysis using MLMT confirmed the predominant distribution of a specific genotype MLMT-17/VNI/ST5 (M5) in China and Japan, reinforcing the findings of recent reports [6, 21, 22, 35, 36].

Some studies [9, 26, 41] have indicated that due to the burden of HIV infection, its associated cryptococcosis is more prevalent in other countries, especially in South Africa. And with increasing immigration, more subtypes of C. neoformans var. grubii would emerge. Although the situation in China is considered to be more complex, further analysis by MLMT typing using more extensive samples from clinical and environmental sources in China and Japan is needed to improve our understanding of the infection route of cryptococcosis in these countries.

### 5. Conclusion

In conclusion, We identified for the first time that a predominant distribution of a specific genotype of C. neoformans var. grubii, MLMT-17/VNI/ST5 (M5) in China and Japan. This genotype is also considered as the major genotype among Asian countries, but more general conclusions require more extensive investigations. Continuous monitoring of the genotype distribution of cryptococcosis is important for the investigation of HIV-associated cryptococcosis in Asian countries.

### Data Availability

The materials described in this work can be made available to interested researchers upon completion of the necessary agreements between institutions. Data generated for this study are included in the figures and additional materials.

### Conflicts of Interest

The authors declare that they have no conflict of interest.

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### References

[1] F. Hagen, K. Khayhan, B. Theelen et al., “Recognition of seven species in the Cryptococcus gattii / Cryptococcus neoformans species complex,” *Fungal Genetics and Biology*, vol. 78, pp. 16–48, 2015.

[2] J. A. Alsop, “Virulence mechanisms and Cryptococcus neoformans pathogenesis,” *Fungal Genetics and Biology*, vol. 78, pp. 55–58, 2015.
[3] K. J. Kwon-Chung, J. A. Fraser, T. L. Doering et al., “Cryptococcus neoformans and Cryptococcus gattii, the etiologic agents of cryptococcosis,” Cold Spring Harbor Perspectives in Medicine, vol. 4, no. 7, article a019760, 2014.

[4] K. J. Kwon-Chung, T. Boekhout, J. W. Fell, and M. Diaz, “(1557) proposal to conserve the name Cryptococcus gattii against C. honduarius and C. bailliiispuros (Basidiomycota, Hymenomyctes, Tremellomyctidae),” Taxon, vol. 51, no. 4, pp. 804–806, 2002.

[5] E. I. Nweze, F. A. Cheka, U. E. Dibua, C. Eze, and U. S. Onoja, “Isolation of Cryptococcus neoformans from environmental samples collected in southeastern Nigeria,” Revista do Instituto de Medicina Tropical de São Paulo, vol. 57, no. 4, pp. 295–298, 2015.

[6] S. Kaocharoen, P. Ngamskulrungroj, C. Firacative et al., “Molecular epidemiology reveals genetic diversity amongst isolates of the Cryptococcus neoformans/C. gattii species complex in Thailand,” PLoS Neglected Tropical Diseases, vol. 7, no. 7, article e2297, 2013.

[7] J. Rhodes, C. A. Desjardins, S. M. Sykes et al., “Tracing genetic exchange and biogeography of Cryptococcus neoformans var. grubii at the global population level,” Genetics, vol. 207, no. 1, pp. 327–346, 2017.

[8] M. Chan, D. Lye, M. K. Win, A. Chow, and T. Barkham, “Clinical and microbiological characteristics of cryptococcosis in Singapore: predominance of Cryptococcus neoformans compared with Cryptococcus gattii,” International Journal of Infectious Diseases, vol. 26, pp. 110–115, 2014.

[9] R. Rajasingham, R. M. Smith, B. J. Park et al., “Isolation and characterisation of Cryptococcus neoformans and Cryptococcus gattii from the environment in Beijing, China,” Mycopathologia, vol. 199, 2015.

[10] M. Kangogo, H. Boga, W. Wanyoike, and C. Bii, “Isolation and characterisation of Cryptococcus neoformans and Cryptococcus gattii from environmental sources in Nairobi, Kenya,” East African Medical Journal, vol. 91, no. 8, pp. 281–285, 2014.

[11] G. S. Alves, A. K. Freire, A. D. Bentes et al., “Molecular typing of environmental Cryptococcus neoformans/C. gattii species complex isolates from Manaus, Amazonas, Brazil,” Mycoses, vol. 59, no. 8, pp. 509–515, 2016.

[12] P. Escandón and E. Castañeda, “Supervivencia a largo plazo de las especies Cryptococcus neoformans y Cryptococcus gattii en muestras ambientales almacenadas procedentes de Colombia,” Revista Iberoamericana de Micología, vol. 32, no. 3, pp. 197–199, 2015.

[13] C. A. Lara, R. O. Santos, R. M. Cadete et al., “Identification and characterisation of xylanolytic yeasts isolated from decaying wood and sugarcane bagasse in Brazil,” Antonie Van Leeuwenhoek, vol. 105, no. 6, pp. 1107–1119, 2014.

[14] C. P. Girish Kumar, D. Prabu, H. Mitan, Y. Mikami, and T. Menon, “Environmental isolation of Cryptococcus neoformans and Cryptococcus gattii from living trees in Guindy National Park, Chennai, South India,” Mycoses, vol. 53, no. 3, pp. 262–264, 2010.

[15] M. Muñoz, M. Camargo, and J. D. Ramirez, “Estimating the intra-taxa diversity, population genetic structure, and evolutionary pathways of Cryptococcus neoformans and Cryptococcus gattii,” Frontiers in Genetics, vol. 9, p. 148, 2018.

[16] Y. H. Choi, P. Ngamskulrungroj, A. Varma et al., “Prevalence of the VN1c genotype of Cryptococcus neoformans in non-HIV-associated cryptococcosis in the Republic of Korea,” FEMS Yeast Research, vol. 10, no. 6, pp. 769–778, 2010.

[17] J. Zhu, Y. Kang, J. Uno et al., “Comparison of genotypes between environmental and clinical isolates of Cryptococcus neoformans var. grubii based on microsatellite patterns,” Mycopathologia, vol. 169, no. 1, pp. 47–55, 2010.

[18] P. F. Herkert, J. F. Meis, G. L. de Oliveira Salvador et al., “Molecular characterization and antifungal susceptibility testing of Cryptococcus neoformans sensu stricto from southern Brazil,” Journal of Medical Microbiology, vol. 67, no. 4, pp. 560–569, 2018.

[19] A. P. Litvintseva and T. G. Mitchell, “Population genetic analyses reveal the African origin and strain variation of Cryptococcus neoformans var. grubii,” PLoS Pathogens, vol. 8, no. 2, article e1002495, 2012.

[20] H. Dou, H. Wang, S. Xie, X. Chen, Z. Xu, and Y. Xu, “Molecular characterization of Cryptococcus neoformans isolated from the environment in Beijing, China,” Medical Mycology, vol. 55, no. 7, pp. 737–747, 2017.

[21] J. Chen, A. Varma, M. R. Diaz, A. P. Litvintseva, K. K. Wollenberg, and K. J. Kwon-Chung, “Cryptococcus neoformans strains and infection in apparently immunocompetent patients, China,” Emerging Infectious Diseases, vol. 14, no. 5, pp. 755–762, 2008.

[22] Y. H. Chen, F. Yu, Z. Y. Bian et al., “Multilocus sequence typing reveals both shared and unique genotypes of Cryptococcus neoformans in Jiangxi Province, China,” Scientific Reports, vol. 8, no. 1, p. 1495, 2018.

[23] W. Pan, K. Khayhan, F. Hagen, H. Fagen et al., “Resistance of Asian Cryptococcus neoformans serotype A is confined to few microsatellite genotypes,” PLoS One, vol. 7, no. 3, article e32868, 2012.

[24] H. Ellegren, “Microsatellites: simple sequences with complex evolution,” Nature Reviews. Genetics, vol. 5, no. 6, pp. 435–445, 2004.

[25] M. L. Vieira, L. Santini, and A. L. Diniz, “Microsatellite markers: what they mean and why they are so useful,” Genetics and Molecular Biology, vol. 39, no. 3, pp. 312–328, 2016.

[26] A. Hanafy, S. Kaocharoen, A. Jover-Botella et al., “Multilocus microsatellite typing for Cryptococcus neoformans var.grubii,” Medical Mycology, vol. 46, no. 7, pp. 685–696, 2008.

[27] J. K. Woon-Chung, I. Polacheck, and J. E. Bennett, “Improved diagnostic medium for separation of Cryptococcus neoformans var. neoformans (serotypes A and D) and Cryptococcus neoformans var. gattii (serotypes B and C),” Journal of Clinical Microbiology, vol. 15, no. 3, pp. 533–537, 1982.

[28] C. L. Halliday and D. A. Carter, “Clonal reproduction and limited dispersal in an environmental population of Cryptococcus neoformans var gattii isolates from Australia,” Journal of Clinical Microbiology, vol. 41, no. 2, pp. 703–711, 2003.

[29] W. Meyer, K. Marszewska, M. Amirmostofan et al., “Molecular typing of global isolates of Cryptococcus neoformans var. neoformans by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA-a pilot study to standardize techniques on which to base a detailed epidemiological survey,” Electrophoresis, vol. 20, no. 8, pp. 1790–1799, 1999.

[30] N. Vélez and P. Escandón, “Distribution and association between environmental and clinical isolates of Cryptococcus neoformans in Bogotá-Colombia, 2012-2015,” Memórias do Instituto Oswaldo Cruz, vol. 111, no. 10, pp. 642–648, 2016.

[31] K. Khayhan, F. Hagen, W. Pan et al., “Geographically structured populations of Cryptococcus neoformans variety grubii in Asia correlate with HIV status and show a clonal population structure,” PLoS One, vol. 8, no. 9, article e72222, 2013.
[32] W. Meyer, A. Castañeda, S. Jackson, M. Huynh, E. Castañeda, and the IberoAmerican Cryptococcal Study Group, “Molecular typing of IberoAmerican Cryptococcus neoformans isolates,” *Emerging Infectious Diseases*, vol. 9, no. 2, pp. 189–195, 2003.

[33] W. Meyer, D. M. Aanensen, T. Boekhout et al., “Consensus multi-locus sequence typing scheme for Cryptococcus neoformans and Cryptococcus gattii,” *Medical Mycology*, vol. 47, no. 6, pp. 561–570, 2009.

[34] Y. Chen, R. A. Farrer, C. Giambardino et al., “Microevolution of serial clinical isolates of Cryptococcus neoformans var. grubii and C. gattii,” *mBio*, vol. 8, no. 2, 2017.

[35] T. Umeyama, H. Ohno, F. Minamoto et al., “Determination of epidemiology of clinically isolated Cryptococcus neoformans strains in Japan by multilocus sequence typing,” *Japanese Journal of Infectious Diseases*, vol. 66, no. 1, pp. 51–55, 2013.

[36] T. Mihara, K. Izumikawa, H. Kakeya et al., “Multilocus sequence typing of Cryptococcus neoformans in non-HIV associated cryptococcosis in Nagasaki, Japan,” *Medical Mycology*, vol. 51, no. 3, pp. 252–260, 2013.

[37] S. H. Park, M. Kim, S. I. Joo, and S. M. Hwang, “Molecular epidemiology of clinical Cryptococcus neoformans isolates in Seoul, Korea,” *Mycobiology*, vol. 42, no. 1, pp. 73–78, 2014.

[38] M. Van Wyk, N. P. Govender, T. G. Mitchell, and A. P. Litvintseva, “Multilocus sequence typing of serially collected isolates of Cryptococcus from HIV-infected patients in South Africa,” *Journal of Clinical Microbiology*, vol. 52, no. 6, pp. 1921–1931, 2014.

[39] Y. Chen, A. P. Litvintseva, A. E. Frazzitta et al., “Comparative analyses of clinical and environmental populations of Cryptococcus neoformans in Botswana,” *Molecular Ecology*, vol. 24, no. 14, pp. 3559–3571, 2015.

[40] M. Cogliati, R. R. Zamfirova, A. M. Tortorano, M. A. Viviani, and The Fimua Cryptococcosis Network, “Molecular epidemiology of Italian clinical Cryptococcus neoformans var. grubii isolates,” *Medical Mycology*, vol. 51, no. 5, pp. 499–506, 2013.

[41] M. W. Tenforde, A. E. Shapiro, B. Rouse et al., “Treatment for HIV-associated cryptococcal meningitis,” *Cochrane Database of Systematic Reviews*, vol. 7, no. 7, article Cd005647, 2018.