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Approaches on genetic polymorphism of *Cryptococcus* Species Complex

Meng Li [1], Min Chen [1], Weihua Pan [1]

[1] Department of Dermatology, Shanghai Key Laboratory of Molecular Mycology & PLA Key Laboratory of Fungal Diseases, ChangZheng Hospital Second Military Medical University, Shanghai

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1. ABSTRACT

The *Cryptococcus* species complex is the significant pathogenic fungi that result in over 1 million cases of cryptococcosis each year in the world. Proper strategy for genetic polymorphism study of the *Cryptococcus* species complex is crucial to unfold genetic population structure, spread profile and pathogenicity of these pathogenic yeasts worldwide. Currently, an array of approaches, including serotype, PCR fingerprinting, amplified fragment length polymorphisms (AFLP), analysis of DNA sequence, and matrix-assisted laser desorption/ionization time of flight mass spectrometry(MALDI-TOF MS) etc. improved our understanding of genetic diversity or phylogenetic of *Cryptococcus* species complex. This review synthesizes existing techniques of cryptococcal genetic polymorphism and raises issues that remain to be addressed.

2. INTRODUCTION

Cryptococcus species complex are basidiomycetous encapsulated yeast species including *Cryptococcus neoformans* (*C.neoformans*)and *Cryptococcus gattii* (*C.gattii*), which may cause the infections of central nervous system, respiratory system and skin, etc. worldwide (1-5). Although the incidence of cryptococcosis in AIDS patients has decreased because of the introduction of the highly active antiretroviral treatment(HAART) (6,7), cryptococcosis remains a serious disease, with a mortality rate of 10 to 30% in regions where access to treatment is limited (6). Actually, *Cryptococcus* species complex continue to be the most likely cause of fungal meningitis in immunocompromised or immunocompetent patients (1,8,9) Currently, *C. neoformans* is classified into three serotypes: serotype A, serotype D and serotype AD, or two varieties: *C. neoformans var. grubii (*serotype A), *C. neoformans var.*

neoformans (serotype D), and AD hybrids (1,2,10,11). In contrast to *C. neoformans, C.gattii* is divided into two serotypes: serotype B and serotype C, which has been regarded as a tropical and subtropical pathogenic organism until reports that *C. gattii* isolates infected immunocompetent hosts living in Vancouver Island, British Columbia, where temperate climate is (12-15). Additionly, C*ryptococcus* Species complex are heterothallic yeasts with two alternative mating type, α or a, which can multiply by budding or sexual reproduction,respectively (16). The previous studies on mating types of *Cryptococcus* species complex revealed that contrary result of virulence was unfolded between α and a mating type of some serotype strains (17,18).

Recently, the development of molecular techniques has greatly improved our understanding of genetic diversity of *Cryptococcus* species complex (1, 10-14, 19). Several different molecular typing methods have been used in epidemiological and genotypic analysis of the *Cryptococcus* species complex, including electrophoretic karyotyping(20,21), random amplified polymorphic DNA (RAPD) (19,22), PCR fingerprinting (10,14), restriction fragment length polymorphism (RFLP) analysis (14,23,24), AFLP analysis (25), sequencing analysis of single locus (26-28) and multi locus (13,14,29). However, disparate molecular methods produced different results, and different classification data about genetic diversity of *Cryptococcus* species complex is coexisted at present. Hence, it seems useful to review the current molecular biological techniques employed to analyze genetic diversity of *Cryptococcus* species complex in order to demonstrate their usefulness, possible problems and establish linkages among different results of genetic diversity in *Cryptococcus* specie complex.

3. TECHNOLOGICAL ADVANCES

3.1. Immunological approaches

Serotyping is a widely used method to differentiate groups within the *Cryptococcus* species complex (1). The separation into different serotypes is based on antigenic differences resulting from variation in capsular polysaccharides (30). In 1982, Kwon-Chung *et al.* found that *Cryptococcus* species complex isolates could be differentiated by culturing the isolates on L-canavanine-glycine-bromothymol blue medium (CGB test) for clear and accurate distinction among serotype A ,D, B and C strains (31). However, several studies in after years shown that one defect of this CGB medium was the possibility of erroneous results, creating unreliable experimental data in laboratories where no other identification methods are available. Moreover, false positive reactions have been reported, suggesting that CGB medium alone is not sufficient to accurately discriminate between the two species (32). Currently, this CGB medium is still widely used because of its comparatively reliability and convenience (1, 14). Nowadays, t3his agar medium has become a classical method to differentiate *C. neoformans* from *C. gattii* isolates (1, 14). In addition, serotyping is also performed by using agglutination with commercial (Crypto Check Kit; Iatron Labs, Tokyo, Japan) or “homemade” antisera components Actually, Crypto Check Kit was once used as standard method (1,14) to differentiate the serotypes of *Cryptococcus* species complex since 1990s, but Crypto Check Kit was not in producation since 2000 because of lower demand. In 1996, Belay T.*et al.* found that *C. neoformans* was classified into three serotypes based on capsular agglutination reactions: serotype A (*C. neoformans* var. *grubii*), serotype D (*C. neoformans* var. *neoformans*), and serotype AD (hybrids strains) (33). So serotype AD strains began to be accurately separated from clinical or environmental isolates of *Cryptococcus* species complex, and virulence and genetic background of hybrids *Cryptococcus species* complex strains gradually being unfolded (25). In 2000, Nakamura Y firstly proposed that serotype identification of *Cryptococcus species* complex could be based on analysis of the sequence of *CAP59* gene (34). In 2007, A. Enache-Angoulvant *et al.* described a new molecular method for serotype identification of *Cryptococcus* species complex based on the sequence characteristics of a fragment of the *CAP59* gene required for capsule biosynthesis again (35). In our study (data not shown), nearly half of the samples(n=109) could not be amplified by the current primer set of the *CAP59* gene, although the primer set used in this study contains two sites of degenerate bases according to the related sequences from GeneBank. Meanwhile, another primer set (13) used to amplify samples in this study (data not shown) also indicated that the current primer sets of the *CAP59* gene may lack coverage. In our opinion, this method is good to discriminate serotype A and D of *C. neoformans* but it is extremely difficult to do so for B, C and hybrid serotypes, that is why more effective primers need to be designed for the *CAP59* gene. Also in 2007, S. Ito-Kuwa *et al.* proposed another method for serotype identification by using a set of four primers for multiplex PCR amplification (36).Whereas, in our study (data not shown), this technique also is not very effective to identificate five serotypes of *Cryptococcus* species complex like S. Ito-Kuwa *et al.*decribed in the article, and too few *C. gattii* isolates were included (36). Actually, there is no good method available to serotype isolates in the *C. gattii* at present.

In our opinion, it is an important significance to research serotypes for the genetic polymorphism of *Cryptococcus* species complex. However, there is no a unique method to solve this problem, currently. It is necessary to integrate traditional and modern methods combined, and develop new approach in the further.

3.2. Nucleic acid-based methodology

3.2.1. DNA Barcoding techniques

Currently, molecular techniques play an increasingly important role on genetic diversity and epidemiological molecular analyses of *Cryptococcus* species complex. Although EK and RAPD patterns have been successively applied to the study of the initial stage on genetic diversity of *Cryptococcus* species complex (20,21), both these techniques were not reproducible and steady enough even in the same Lab. In 1999, Meyer W *et al.*proposed that PCR fingerprint patterns based on M13 microsatellite DNA or $(GACA)_4$ primer identified eight

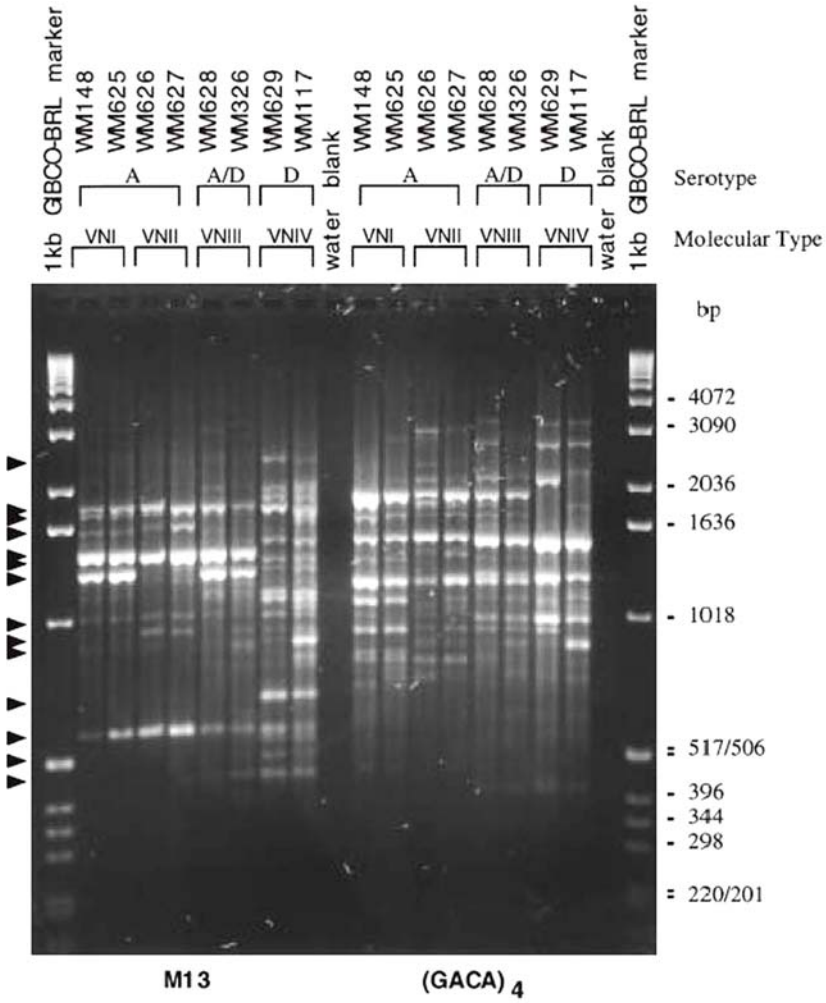

Figure 1. Eight major molecular types of *Cryptococcus* species complex isolates using PCR fingerprint by M13 or $(GACA)_4$ primer. Strains: WM148 and WM625, serotype A, major molecular type VNI, subtype VNIa;WM626 and WM627, serotype A, major molecular type VNII, subtypes VNIIa and VNIIb, respectively;WM628 and WM326, serotype A/D, major molecular type VNIII,subtypes VNIIIa and VNIIIc, respectively;WM629 and WM117,serotype D, major molecular type VNIV, subtypes VNIVa and VNIVb, respectively. Main bands used for the identification of the major molecular types are indicated by arrows(21).

major molecular types , VNI-VNIV and VGI-VGIV (Figure 1), among *Cryptococcus* species complex isolates worldwide except an unique molecular type, VNB, originated from Botswana (18). Actually, PCR fingerprint patterns based on M13 microsatellite DNA or $(GACA)_4$ primer has been used as the major typing technique in the ongoing global molecular epidemiologic survey of *Cryptococcus* species complex (14,21), dividing >600 clinical and environmental isolates into eight major molecular types: VNI (var.*grubii*, serotype A), VNII (var. *grubii*, serotype A), VNIII(serotype AD), VNIV (var. *neoformans*, serotype D), VGI,VGII, VGIII, and VGIV (*C.gattii*, serotypes B and C) (21,22). Several years later, the molecular types were recently confirmed by RFLP analysis of the orotidine monophosphate pyrophosphorylase (URA5) gene and the phospholipase (PLB1) gene (25), and AFLP patterns which also used as other major typing technique identified ten major molecular types among *Cryptococcus* species complex worldwide (14). In 2008, Yao *et al.* established two PCR-RFLP analyses, based on the *CAP1* and *GEF1* genes, could be used for simultaneous identification of the molecular and mating types of the eight major molecular types isolates of the *Cryptococcus* species complex (23). Yao's technique seems more effective and convenient than previous techniques used in genetic diversity analyses of *Cryptococcus* species complex, but this method also needs more empirical testing in the furture.

In our view, AFLP genotyping patterns is also very reliable and widely recognized which compared to M13-PCR fingerprint (14, 25), and these two techniques can be another alternative. Although the PCR fingerprinting method has been widely used in studies of genetic diversity within *Cryptococcus* species complex isolates, AFLP genotyping technique is much more sensitive except expensive. In our opinion, PCR fingerprint patterns based on M13 primer could be viewed as a simplified version of

Table 1. Linkages among different method results of varieties, serotypes and genotypes within *Cryptococcus* species complex

Species	Serotype (31)	AFLP type (19)	Molecular type(21)	IGS type (48)	ITS type(27)	Luminex type (65)
C.neoformans						
var. grubii	A	1	VNI	1a/1b	1	CNN1b
	A	1A	VNB	1a	1	CNN1b
	A	1B	VNII	1c	1	CNN1b
var.neoformans	D	2	VNIV	2a/2b/2c	2	CNN2d
	AD	3	VNIII		1/2	
C. gattii						
C. gattii	B/C	4A	VGI	4c	7	CNG4c
C. gattii	B/C	4B	VGI	4a/4b	3/7	CNG4c
C. gattii	B/C	5A/5C	VGIII	5	5	CNG5b
C. gattii	B	5B	VGIII	5	5	CNG5b
C. gattii	B/C	6	VGII	3	4	CNG3
C. gattii	B/C	7	VGIV	6	6	CNG6

AFLP genotyping patterns because good concordance between these various fingerprinting approaches has been observed in many previous studies (14,29).To summarise the molecular types and serotypes within *C. neoformans*, molecular types VNI (Serotype A,AFLP 1) and VNII isolates (Serotype A,AFLP1B) belong to serotype A (var. *grubii*) and VNIII (Serotype AD,AFLP3) or VNIV isolates(SerotypeD,AFLP2) belong to serotype AD or serotype D (var. *neoformans*).(See Table 1).Remarkablely, no correlation between serotype and molecular type has been found for *C. gattii* (22).

Nowdays, microsatellites are becoming increasingly popular as a molecular typing tool (37). Microsatellites (also referred to as short tandem repeats, STRs) are genomic sequences consisting of tandemly repeated short motifs up to 6 nucleotides (38). Mutations occur at a high frequency at these sites, namely 10^{-2}-10^{-6} per generation compared to only 10^{-9} for point mutations as detected by MLST (39). Mutations in microsatellites usually result in changes in the copy number of the repeats. In other fungi, molecular typing using microsatellites turned out to be more discriminatory than MLST (40). Typing assays for *A. fumigatus* based on microsatellites offer the most advantages in terms of speed, reproducibility, discriminatory power and costs and do not suffer from the drawbacks mentioned for previous typing methods.

In *Cryptococcus neoformans*, This method use a 9-marker microsatellite panel consisting of 3 dinucleotide repeat markers, 3 trinucleotide repeat markers and 3 tetranucleotide repeat markers. In each panel, the distinction between the 3 markers is made using different fluorescent labels. Within each panel, one of the amplification primers carried a fluorescent label consisting of either FAM (6-carboxyfluorescein), HEX (hexachlorofluorescein) or TET (tetrachlorofluorescein). Three subpanels (CNA2, CNA3 and CNA4 respectively) of 3 markers each were amplified using a multicolor multiplx PCR approach. A fourth colour that needs to be detected is for the internal lane standard that needs to be added to each sample in order to determine the relative mobility of the DNA fragments (41).

Amplified products were analyzed on a high resolution capillary electrophoresis platform allowing precise determination of repeat numbers in each marker. Once the samples have been run, most platforms come with software that will interpret the results and may produce a list of peaks that are present in the sample. PCR amplification of sequences containing microsatellites leads to the well-known phenomenon of so-called stutter peaks (42). Fragments with a high number of repetitions produce more stutter peaks than fragments with low or moderate numbers of repetitions. Microsatellite data was analyzed using the multistate categorical similarity coefficient.

Microsatellite complexes (MC's) were defined as groups of 2 or more genotypes differing by a maximum of 2 markers. Within each MC, the amount of variation is attributable to only one or two microsatellite markers as the likely result of instability of these specific markers. Thereby, isolates within an MC, there was very limited to no variation in the less discriminatory markers. The difference between the MC's is attributable to multiple microsatellite markers (≥3markers difference). In *Cryptococcus neoformans*, the nine marker microsatellite panel yielded a discriminatory power of greater than 0.993 (37).

The STRs assay has proven to be a very useful instrument in the identification of epidemiologically related isolates. This is supported by the evidence that the panel of microsatellite markers as molecular typing targets for *C. neoformans var. grubii* and simultaneously allow distinguishing between isolates from clinical and environmental origin (37).

Loop-mediated isothermal DNA amplification(LAMP), firstly described by Notomi *et al* in 2000, involves the utilization of an isothermal step for DNA amplification, by using specially designed primer sets and a DNA polymerase with strand-displacement activity (43). This novel nucleic acid amplification method was firstly evaluated for the detection of hepatitis B virus (44), It allows the rapid, specific amplification of DNA under isothermal conditions by using a distinctive DNA polymerase with strand-displacement activity. The approach has been applied in the filed of bacteria, virus and parasites. In 2004, Endo. S firstly used the method for detection of gp43 of *Paracoccidiodes brasiliensis* (45) Lucas.S *et al* developed a LAMP-based method to identify the serotypes A, D and B/C of the *Cryptococcus* species complex by using CAP59 allele-specific primers and found

it could also allows the identification of the C. *neoformans* variety and /or hybrid origin (46)

Generally, PCR fingerprint patterns based on M13 primer and AFLP patterns are regarded as the standard/classic technique to understand genetic diversity among strains of *Cryptococcus* species complex among these molecular fingerprinting approaches, currently (14,29).

However, PCR fingerprinting patterns is not highly reproducible in different laboratories (21). Furthermore, numbers detected with faintly stained bands and the intensity varies somewhat with the staining process, make allocation to subtypes of the major groups difficult (21). In our study (data not shown), it was difficult to discriminate between fingerprinting patterns of VNI and VGI isolates. As for AFLP genotyping technique, it is not conducive to the widespread use of this technology because it is expensive and requiring high experimental techniques and equipments. Thus, it is necessary to put molecular fingerprinting methods and DNA sequencing technologies combined application of genetic diversity in *Cryptococcus* species complex isolates for more objective and comprehensive study. Nowadays, *ITS* region, *IGS* region or *CAP59* gene sequencing methods were chosen most frequently to assist in the determination of the genetic diversity within *Cryptococcus* species complex.

3.2.2. DNA sequences analysis approaches

It is no doubt that development of the DNA sequencing technique greatly improved our understanding of genetic diversity of *Cryptococcus* species complex. Several sequence analyses such as the internal transcribed spacer (ITS) region including the 5.8S rRNA gene (26), IGS gene region (27), mitochondrial cytochrome b gene and RPR8 gene (28), etc. have been successfully carried out on phylogenetic and genetic diversity analysis of *Cryptococcus* species complex. Furthermore, phylogenetic analysis of ITS or IGS gene sequence is regarded as fast and informative technique to identify *Cryptococcus* species complex isolates (47). Kastu M *et al.*once described *Cryptococcus* species complex into seven genotypes according to specific combinations of eight nucleotide differences located at sequence of ITS gene region among all of five serotypes strains. Diaz MR's study also showed that IGS sequence analyses proved to be a powerful technique for the delineation of *Cryptococcus* species complex at genotypic and subgenotypic levels because six genotypes were divided that genotypes 1 (*C. neoformans* var. *grubii*), genotype 2 (*C. neoformans* var. *neoformans*), and genotypes 3, 4, 5 and 6 represented by *C. ga*ttii (48). Despite these studies of a single locus have been proved to be powerful and repeatable for genotyping the two *C. neoformans* varieties and for subtyping within *C. gattii*. However, results of these techniques based on single locus sequencing is difficult to be compared each other and evaluated the genetic diversity of *Cryptococcus* species complex on a systemic and exhaustive level (49). Hence, sequencing multiple loci of *Cryptococcus* species complex became necessary, for reflecting genetic background of *Cryptococcus* species complex on a higher level and another point of view (Figure 2). There is no doubt that great advantages exists with MLST technique than other single locus sequencing method except increased experiments and fund. Currently, MLST analysis of all haploid molecular genotypes present within *Cryptococcus* species complex was gradually accepted as a standard technique worldwide. However, previous researches that sequenced multiple loci merely used either *C. neoformans* isolates or *C. gattii* isolates (50). Several studies used *C. neoformans* as well as *C. gattii* isolates, but only few *C. gattii* isolates were included (51,52) or the *Cryptococcus* species complex isolates that were studied differed for the various loci (49,52). In 2007, Bovers M *et al.* used multi-locus sequence typing included six losi (*ITS*, *IGS1*,*CNLAC1*,*RBP1*,*RBP2*,and *TEF1* genes) to investigate monophyletic lineages within all six haploid genotypic isolates, and the results supported the current classification of *Cryptococcus* species complex (47).However, multilocus sequence typing (MLST), a method based on the detection of single nucleotide polymorphisms (SNPs), lacks discriminatory power for typing organisms with low levels of genetic diversity (53).

Whole Genome Sequence Typing (WGST) is a methodology that maximizes the data available for inference of genetic diversity, revealing more SNPs than MLST. The method has been applied successfully to distinguish among highly related isolates of the fungus *Coccidiodes immiti* (54). Recently, JD. Gillece *et al* used whole genome sequence typing to perform fine-scale phylogenetic analysis on 20 C. *gattii* isolates, most of which are responsible for the outbreak infection in US Pacific Northwest (55). The research has identified over 100 SNPs among eight VGIIc isolates as well as unique genotypes for each of the VGIIa, VGIIb and VGIIc isolates.It revealed definitively that the VGIIc subtype of *C. gattii,* is genetically distinct from both the VGIIa and VGIIb subtypes (56). As a new generation sequencing methodology, WGST has been shown its power to generate a unique genetic fingerprint for each isolate, effectively subtyping a "clonal" population.

3.3. New technique based on proteomics

The approaches referred above including PCR fingerprinting, AFLP, RAPD, DNA sequencing definitely improved our understanding genetic diversity or phylogenetic of *Cryptococcus* species complex. However, these molecular methods are time-consuming and expensive for the identification of isolates.

Mass spectrometry is the analytic technique used to analyze the mass to charge ratio of various compounds. The most wildly used method to date for the analysis of biomolecules is matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). The technique was firstly proposed to identify bacterial species in 1996 by several teams (57,58). Later, the work was extended to fungal cells in 2000 (59)

Now, MALDI-TOF MS has been proved successfully by several groups to differentiate yeast and fungi. Marklein et.al proved that it could identify correctly

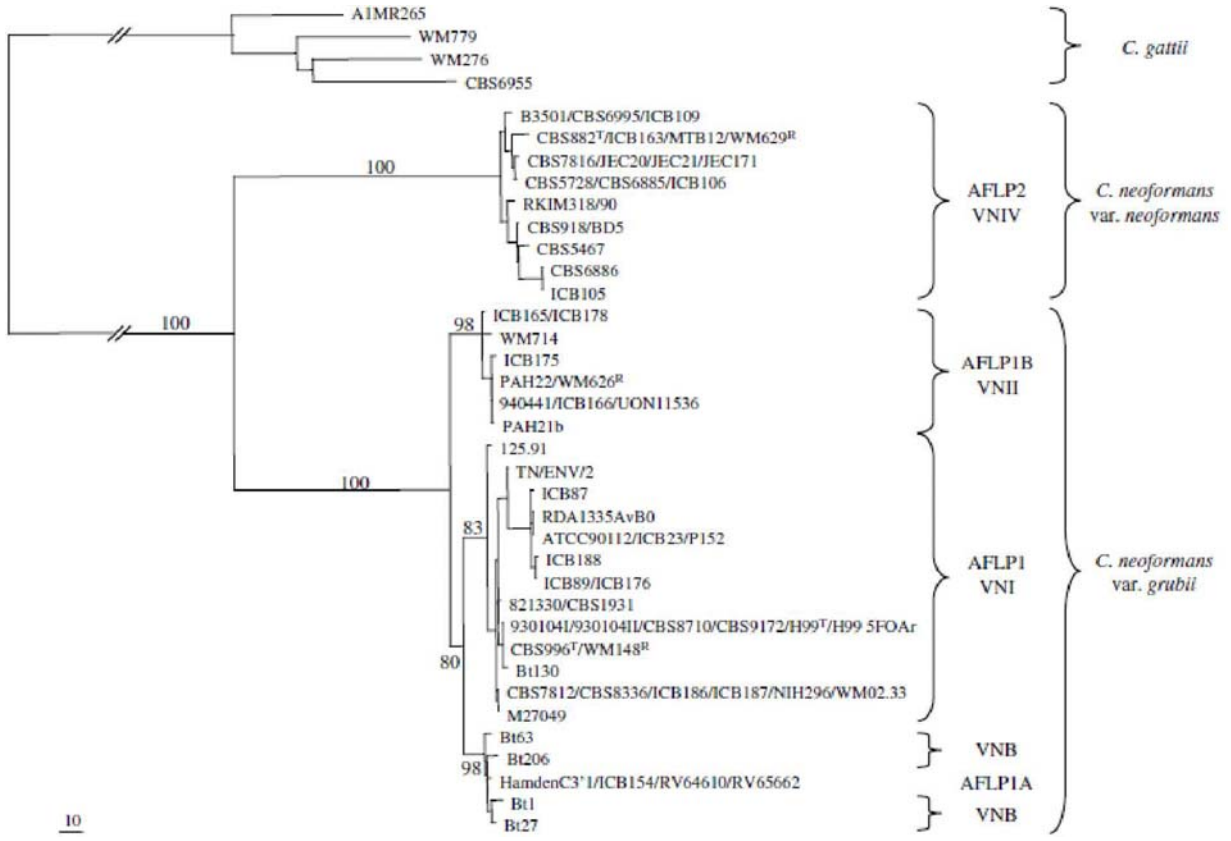

Figure 2. Phylogenetic tree of *Cryptococcus* species complex isolates by analysis of the concatenated data set (RPB1, RPB2, CNLAC1, TEF1, IGS1 and ITS). Presented is one of 60 most parsimonious trees (length 847; consistency index 0.902; retention index 0.961) computed with gaps treated as new state. Data consisted of 3932 characters of which 459 characters were parsimony informative. Bootstrap values (1000 replicates) are indicated for the main branches (49).

247 clinical Candida isolates (in all 267) of 15 different species.In a study performed by van Veen et.al showed comparably high results of identification, 85% of 61 yeasts isolates from 12 different species were correctly identified (60,61). E. De Carolis *et al* reported that the MALDI-TOF MS could successfully identify 103 clinical isolates from 55 species of Aspergillus Fusarium and Mucorales, 91 of 94 isolates were identified to the species level, the rest 3 to the genus level (62).. In another study , MALDI-TOF MS showed its potency to identify dermatophyte and Neoscytaliduim species, both of which cause dermatomycoses (63).. Carolina et.al used this technique to identify 164 *Cryptococcus. neoformans* and *Cryptococcus. gattii* isolates and found the obtain mass spectra correctly identified 100% of all isolates. In addition, all isolates were clearly separated according to their major molecular type (64).

3.4. Others

In 2007, a Luminex suspension array had been developed for identification of *Cryptococcus* species complex isolates. This suspension array correctly identified haploid isolates in all of 58 clinical isolates. Furthermore, hybrid isolates possessing two alleles of the Luminex probe region could be identified as hybrids (65).

4. CONCLUSIONS

Currently, *Cryptococcus* species complex remains the most important cause of fungal meningitis in immunocompromised or immunocompetent patients. It is a increasingly important significance for outline the recent advances on molecular approaches to analyze genetic diversity of *Cryptococcus* species complex isolates on more objective and comprehensive point of view. Generally, the standardized MLST combined the AFLP or M13-PCR fingerprint technique seems a reasonable approach to analyze genetic background and diversity of *Cryptococcus* species complex isolates at present. MALDI-TOF MS, as a new technique applied in fungus, has been proven to perform well in genotype identification of *Cryptococcus* species complex .More approaches should be developed to differentiate genotypes of *Cryptococcus* species complex isolates in the further.

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6. REFERENCE

1. Casadevall A and Perfect JR. *Cryptococcus neoformans*. *ASM Press, Washington, DC*. (1998)

2. Lin XR and Heitman J. The Biology of the *Cryptococcus neoformans* Species Complex. *Microbiology* 60, 69-105 (2006)

3. J.C. Christianson, W. Engber, D. Andes, Primary cutaneous cryptococcosis in immunocompetent and immunocompromised hosts. *Med Mycol* 41(3), 177-188 (2003)

4. J.R. Harris, S.R. Lockhart, E. Debess, N. Marsden-Haug, M. Goldoft, R. Wohrle, S. Lee, C. Smelser, B. Park, T. Chiller, Cryptococcus gattii in the United States: clinical aspects of infection with an emerging pathogen. *Clin Infect Dis* 53(12), 1188-1195 (2011)

5. Danial LF and Nestor LM. Pulmonary Cryptococcosis in Immunocompetent Patients: CT Findings in 12 Patients. *American Roentgen Ray Society* 185(3), 622–626 (2005)

6. Mirza SA, Phelan M, Rimland D, Graviss E, Hamill R, Brandt ME, Gardner T, Sattah M, de Leon GP, Baughman W, Hajjeh RA.. The changing epidemiology of cryptococcosis: an update from populationbased active surveillance in 2 large metropolitan areas,1992-2000. *Clin Infect Dis* 36(6), 789-794 (2003)

7 R. Bruno, P. Sacchi, G. Filice, Overview on the incidence and the characteristics of HIV-related opportunistic infections and neoplasms of the heart: impact of highly active antiretroviral therapy, *AIDS* 17 Suppl, 1S83-87 (2003)

8. Bicanic T and Harrison TS. Cryptococcal meningitis. *Br Med Bull* 72, 99-118 (2004)

9. M. Roy, T. Chiller, Preventing deaths from cryptococcal meningitis: from bench to bedside, *Expert Rev Anti Infect Ther* 9(9), 715-717 (2011)

10 Franzot SP, Salkin IF, Casadevall A,. *Cryptococcus neoformans var. grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. *J Clin Microbiol* 37, 838-840 (1999)

11. Kwon-Chung KJ and Varma A. Do major species concepts support one, two or more species within *Cryptococcus neoformans*? *FEMS Yeast Res* 6(4), 574-587 (2006)

12. Sorrell TC. *Cryptococcus neoformans* variety *gattii*. *Med Mycol* 39(2)155-168. (2001)

13. Fraser JA, Giles SS, Wenink EC, Geunes-Boyer SG, Wright JR, Diezmann S, Allen A, Stajich JE, Dietrich FS, Perfect JR, Heitman J. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature*; 437(7063)1360-1364 (2005)

14. Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, Macdougall L, Boekhout T, Kwon-Chung KJ, Meyer W. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). *Proc Natl Acad Sci USA* 101(49), 17258-63(2004)

15. Bartlett KH, Kidd SE, Kronstad JW, The emergence of *Cryptococcus gattii* in British Columbia and the Pacific Northwest. *Curr Infect Dis Rep* 10(1)58–65 (2008)

16. Kwon-Chung KJ. A new species of Filobasidiella, the sexual state of *Cryptococcus neoformans* B and C serotypes. *Mycologia* 68(4), 943-946 (1976)

17. Brian L, Wickes.The role of mating type and morphology in *Cryptococcus neoformans* pathogenesis. *Int J Med Microbiol* 292(5-6), 313-329 (2002)

18.Nielsen K, Cox GM, Litvintseva AP, Mylonakis E, Malliaris SD, Benjamin DK Jr, Giles SS, Mitchell TG, Casadevall A, Perfect JR, Heitman J.*Cryptococcus neoformans* α Strains preferentially disseminate to the central nervous system during coinfection. *Infection and Immunity* 73(8), 4922–4933 (2005)

19.Boekhout T, van Belkum A, Leenders AC, Verbrugh HA, Mukamurangwa P, Swinne D, Scheffers WA. Molecular typing of Cryptococcus neoformans: taxonomic and epidemiological aspects. *Int J syst Bacteriol* 47(2), 432-442 (1997)

20. Perfect JR, Ketabchi N, Cox GM, Ingram CW, Beiser CL.Karyotyping of *Cryptococcus neoformans* as an epidemiological tool. *J Clin Microbiol* 31(12), 3305-3309 (1993)

21. Meyer W, Marszewska K, Amirmostofian M, Igreja RP, Hardtke C, Methling K, Viviani MA, Chindamporn A, Sukroongreung S, John MA, Ellis DH, Sorrell TC. 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* 20(8), 1790-9 (1999)

22. Chen SC, Brownlee AG, Sorrell TC, Ruma P, Nimmo G.. Identification by random amplification of polymorphic DNA of a common molecular type of Cryptococcus neoformans var. neoformans in patients with AIDS or other immunosuppressive conditions. *J Infect Dis* 173(3), 754-758 (1996)

23. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E; IberoAmerican Cryptococcal Study Group. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* 9(2), 189-195 (2003)

24. Feng XB, Yao ZR, and Liao WQ, Simultaneous identification of molecular and mating types within the *Cryptococcus species* complex by PCR-RFLP analysis. *J Med Microbiol* 57(12), 1481-1490 (2008)

25. Latouche GN, Huynh M, Sorrell TC, Meyer W. PCR-restriction fragment length polymorphism analysis of the phospholipase B (PLB1) gene for subtyping of *Cryptococcus neoformans* isolates. *Appl Environ Microbiol* 69(4)2080-2086 (2003)

26. Litvintseva AP, Lin X, Templeton I, Heitman J, Mitchell TG. Many globally isolated AD Hybrid strains of *Cryptococcus neoformans* originated in Africa. *PLoS Pathogens* 3 (8), 1109-1117 (2007)

27. Diaz MR, Boekhout T, Kiesling T, Fell JW. Comparative analysis of the intergenic spacer regions and population structure of the species complex of the pathogenic yeast Cryptococcus neoformans.*al*. *FEMS Yeast Res* 5(12), 1129-1140 (2005)

28. Butler MI, Poulter RTM. The PRP8 inteins in Cryptococcus are a source of phylogenetic and epidemiological information. *Fungal Genet Biol* 42(5), 452–463 (2005)

29. Ngamskulrungroj P, Gilgado F, Faganello J, Litvintseva AP, Leal AL, Tsui KM, Mitchell TG, Vainstein MH, Meyer W. Genetic diversity of the *Cryptococcus species* Complex suggests that *Cryptococcus gattii* deserves to have varieties. *PLoS ONE* 4 (6), 1-18 (2009)

30. Cherniak R, Sundstrom JB. Polysaccharide antigens of the capsule of *Cryptococcus neoformans*. *Infect Immun* 62(5), 1507–1512 (1994)

31. Kwon-Chung KJ, Polacheck I, Bennett JE. Improved diagnostic medium for separation of *Cryptococcus-neoformans* var *neoformans* (serotype-A and serotype-D) and *Cryptococcus neoformans* var *gattii* (serotype-B and serotype-C). *J Clin Microbiol* 15(3), 535-537(1982)

32. Khan ZU, Al-Anezi AA, Chandy R, Xu J. Disseminated cryptococcosis in an AIDS patient caused by a canavanineresistant strain of *Cryptococcus neoformans* var. *grubii*. *J Med Microbiol* 52(3), 271-275(2003)

33. Belay T, Cherniak R, and Kozel TR. Serotyping of *Cryptococcus neoformans* by dot enzyme assay. *J Clin Microbiol* 34(2), 466-470 (1996)

34. Y. Nakamura, Molecular analyses of the serotype of Cryptococcus neoformans. *Nihon Ishinkin Gakkai Zasshi* 42(2) 69-74 (2001)

35. Enache-Angoulvant A, Chandenier J, Symoens F, Lacube P, Bolognini J, Douchet C, Poirot JL, Hennequin C. Molecular Identification of *Cryptococcus neoformans* Serotypes. *J Clin Microbiol* 45(4), 1261-1265 (2007)

36. Ito-Kuwa S, Nakamura K, Vidotto V. Serotype identification of *Cryptococcus neoformans* by multiplex PCR. *Mycoses* 50, 277-281 (2007)

37. Illnait-Zaragozi MT, Martínez-Machín GF, Fernández-Andreu CM, Boekhout T, Meis JF, Klaassen CH. Microsatellite typing of clinical and environmental Cryptococcus neoformans var. grubii Isolates from Cuba shows multiple genetic lineages. *PLoS ONE* 5(2), 9124 (2010)

38. Bart-Delabesse E, Humbert J F, Delabesse E, Bretagne S. Microsatellite markers for typing Aspergillus fumigatus isolates. *J Clin Microbiol* 36(9), 2413-8(1998)

39. Meyer W, Aanensen DM, Boekhout T, Cogliati M, Diaz MR, Esposto MC, Fisher M, Gilgado F, Hagen F, Kaocharoen S, Litvintseva AP, Mitchell TG, Simwami SP, Trilles L, Viviani MA, Kwon-Chung J. Consensus multi-locus sequence typing scheme for Cryptococcus neoformans and Cryptococcus gattii. *Med Mycol* 47(6)561-70 (2009)

40. laassen CH. MLST versus microsatellites for typing Aspergillus fumigates isolates. *Med Mycol* 47, Suppl 1 S27-33. (2009)

41. De Valk, H. A., Meis, J. F. & Klaassen, C. H. Microsatellite based typing of Aspergillus fumigatus: strengths, pitfalls and solutions. *J Microbiol Methods* 69(2), 268-72 (2007)

42. Pasqualotto, A. C., Denning, D. W. & Anderson, M. J. A cautionary tale: lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. *J Clin Microbiol* 45(2), 522–8 (2007)

43. T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28(12), E63 (2000)

44. K. Nagamine, T. Hase, T. Notomi, Accelerated reaction by loop-mediated isothermal amplification using loop Primers. *Mol Cell Probes* 16(3), 223-229 (2002)

45. S. Endo, T. Komori, G. Ricci, A. Sano, K. Yokoyama, A. Ohori, K. Kamei, M. Franco, M. Miyaji, K. Nishimura, Detection of gp43 of Paracoccidioides brasiliensis by the loop-mediated isothermal amplification (LAMP) method. *FEMS Microbiol. Lett* 234(1), 93-97 (2004)

46. S. Lucas, L.M.M. da, O. Flores, W. Meyer, I. Spencer-Martins, J. Inacio, Differentiation of Cryptococcus neoformans varieties and Cryptococcus gattii using CAP59-based loop-mediated isothermal DNA amplification. *Clin Microbiol Infect* 16(6), 711-714. (2010)

47. Meyer W, Kidd S, Latouche GN, *et al.* Global molecular epidemiology offers hints towards ongoing speciation within *Cryptococcus neoformans*. In: Abstracts

of the 5th International Conference on Cryptococcus and Cryptococcosis, *Adelaide: South Australian Postgraduate Medical Education Association* (2002)

48. Diaz MR, Boekhout T, Theelen B, Fell JW. Molecular Sequence Analyses of the intergenic Spacer(IGS) associated with rDNA of the two varieties of the pathogenic yeast, *Cryptococcus neoformans*. *System Appl Microbiol* 23(4), 535-545 (2000)

49. Bovers M, Hagen F, Kuramae EE, Boekhout T.. Six monophyletic lineages identified within *Cryptococcus neoformans* and *Cryptococcus gattii* by multi-locus sequence typing. *Fungal Genetics and Biology* 45(4), 400-421 (2008)

50. Barreto de Oliveira MT, Boekhout T, Theelen B, Hagen F, Baroni FA, Lazera MS, Lengeler KB, Heitman J, Rivera IN, Paula CR..*Cryptococcus neoformans* shows a remarkable genotypic diversity in Brazil. *J Clin Microbiol* 42(3), 1356-1359 (2004)

51. Xu J, Vilgalys R, Mitchell TG, Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. *Mol Ecol* 9(10), 1471–1481 (2000)

52. Sugita T, Ikeda R, Shinoda T,. Diversity among strains of *Cryptococcus neoformans* var. *gatii* as revealed by a sequence analysis of multiple genes and a chemotype analysis of capsular polysaccharide. *Microbiol Immunol* 45(11), 757-768 (2001)

53. Balajee, S. A., Gribskov, J. L., Hanley, E., Nickle, D. & Marr, K. A. Aspergillus lentulus sp. nov., a new sibling species of A. fumigatus. *Eukaryot Cell* 4(3), 625-32. (2005)

54. D.M. Engelthaler, T. Chiller, J.A. Schupp, J. Colvin, S.M. Beckstrom-Sternberg, E.M. Driebe, T. Moses, W. Tembe, S. Sinari, J.S. Beckstrom-Sternberg, A. Christoforides, J.V. Pearson, J. Carpten, P. Keim, A. Peterson, D. Terashita, S.A. Balajee, Next-generation sequencing of Coccidioides immitis isolated during cluster investigation, *Emerging Infect Dis* 17(2), 227-232 (2011)

55. J.D. Gillece, J.M. Schupp, S.A. Balajee, J. Harris, T. Pearson, Y. Yan, P. Keim, E. DeBess, N. Marsden-Haug, R. Wohrle, D.M. Engelthaler, S.R. Lockhart, Whole genome sequence analysis of Cryptococcus gattii from the Pacific Northwest reveals unexpected diversity, *PLoS One* 6(12), e28550 (2011)

56. S.E. Kidd, H. Guo, K.H. Bartlett, J. Xu, J.W. Kronstad, Comparative gene genealogies indicate that tw clonal lineages of Cryptococcus gattii in British Columbia resemble strains from other geographical areas. *Eukaryotic Cell* 4(10), 1629-1638 (2005)

57. Krishnamurthy T, Ross PL. Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. *Rapid Commun Mass Spectrom*10 (15), 1992-6 (1996)

58. Claydon MA, Davey SN, Edwards-Jones V, Gordon DB. The rapid identification of intact microorganisms using mass spectrometry. *Nat Biotechnol* 14(11), 1584-6 (1996).

59. Li TY, Liu BH, Chen YC. Characterization of Aspergillus spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 14(24), 2393-400 (2000)

60. Marklein G, Josten M, Klanke U, Müller E, Horré R, Maier T, Wenzel T, Kostrzewa M, Bierbaum G, Hoerauf A, Sahl HG. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. *J Clin Microbiol* 47(9), 2912-7 (2009)

61. van VSQ, Claas EC, Kuijper EJ. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. *J Clin Microbiol* 48(3), 900-7 (2010)

62. De Carolis E, Posteraro B, Lass-Flörl C, Vella A, Florio AR, Torelli R, Girmenia C, Colozza C, Tortorano AM, Sanguinetti M, Fadda G. Species identification of Aspergillus, Fusarium and Mucorales with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Microbiol Infect* 18(5), 475-84 (2012)

63. Alshawa K, Beretti JL, Lacroix C, Feuilhade M, Dauphin B, Quesne G, Hassouni N, Nassif X, Bougnoux ME. Successful identification of clinical dermatophyte and Neoscytalidium species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 50(7), 2277-81 (2012)

64. Firacative C, Trilles L, Meyer W. MALDI-TOF MS enables the rapid identification of the major molecular types within the Cryptococcus neoformans/C. gattii species complex. *PLoS One* 7(5), e37566 (2012)

65. Bovers M, Diaz MR, Hagen F, Spanjaard L, Duim B, Visser CE, Hoogveld HL, Scharringa J, Hoepelman IM, Fell JW, Boekhout T.. Identification of genotypically diverse *Cryptococcus neoformans* and *Cryptococcus gattii* isolates by Luminex xMAP technology. *J Clin Microbiol* 45(6), 1874-1883. (2007)

Abbreviations: AFLP: amplified fragment length polymorphisms; MLST: multilocus sequence typing; RAPD: random amplified polymorphic DNA; ITS: the internal transcribed spacer; MALDI-TOF MS: matrix-assisted laser desorption/ionization time of flight mass spectrometry RFLP: restriction fragment length polymorphism; LAMP: loop-mediated isothermal amplification; WGST: whole genome sequence typing

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Send correspondence to: Pan Weihua, Department of Dermatology ,Shanghai Key Laboratory of Molecular Mycology & PLA Key Laboratory of Fungal Diseases, ChangZheng Hospital Second Military Medical University, Shanghai, 200032, China, Tel: 86-021-81885494, Fax: 86-021-81885494, E-mail: weihuapan@126.com