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ARTICLE

Molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii* in China between 2007 and 2013 using multilocus sequence typing and the DiversiLab system

H.-T. Dou · Y.-C. Xu · H.-Z. Wang · T.-S. Li

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Abstract The purpose of this study was to investigate the molecular characteristics of 83 clinical *Cryptococcus neoformans*/*C. gattii* species complex isolated in Beijing, China, between 2007 and 2013. Restriction fragment length polymorphism of the gene *URA5* (*URA5*-RFLP), multilocus sequence typing (MLST), and automated repetitive polymerase chain reaction (rep-PCR; DiversiLab system) were performed to genotype these cryptococcal isolates. There was an excellent correlation amongst the three methods; however, PU157 was assigned as VNII according to *URA5*-RFLP, while it was classified as VNI by the DiversiLab system analysis. PU157 was finally identified as VNB by seven-locus MLST analysis. Moreover, though AD hybrids could not be processed by MLST, ideal results could be obtained by the DiversiLab system. The genotype VNI accounted for 95.2 % (79/83) of isolates. Besides one strain of VNB, VNIII, and VGI each, a strain of VGII was detected in our study, which was isolated from a patient from the temperate region in North China. In addition, the most common MLST sequence type (ST) was ST5, accounting for 91.6 % (76/83), followed by ST31, ST63, ST182, ST295, ST296, and ST332. ST295, ST296, and ST332 were new STs. Except for isolate PU157 (VNB), identical results were obtained quickly and accurately through the DiversiLab system compared to MLST and *URA5*-RFLP. The discovery of VNB and VGII in the temperate climate regions of China suggested that the population structure of *C. neoformans* and *C. gattii* should be explored more extensively. Our results also showed that the DiversiLab system can be used in the genotyping of *C. neoformans* and *C. gattii*.

H.-T. Dou · Y.-C. Xu
Department of Clinical Laboratory, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China

H.-Z. Wang
Department of Clinical Laboratory, Beijing Di-Tan Hospital, Capital Medical University, Beijing, China

T.-S. Li (✉)
Department of Infectious Diseases, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, No. 1, Shuaifuyuan Road, Dongcheng District, Beijing 100730, China
e-mail: litsh5046@sina.com

Introduction

Cryptococcosis is a life-threatening mycosis. It is mainly caused by *Cryptococcus neoformans* and *C. gattii*, which can affect not only immunocompromised patients, but also immunocompetent individuals [1]. *Candida* spp., *Aspergillus* spp., and *Cryptococcus* spp. are the leading causes of fungal disease on the global scale [2, 3]. *C. neoformans* can be found worldwide, mostly in pigeon droppings, while *C. gattii* was formerly discovered in *Eucalyptus camaldulensis* and originally considered to be restricted to tropical or subtropical regions [4]. However, as *C. gattii* was discovered in an outbreak on Vancouver Island in 1999, which eventually spread to British Columbia, Canada, and the US Pacific Northwest, and as the three independent outbreaks of *C. gattii* were found in North America, it has gained the attention of many countries [1, 5–8].

Generally, there are eight molecular types involved in the *C. neoformans*/*C. gattii* species complex [9]. *C. neoformans* can be divided into *C. neoformans* var. *grubii* (serotype A; VNI and VNII), *C. neoformans* var. *neoformans* (serotype D; VNIV), and a hybrid between them (serotype AD; VNIII). *C. gattii* is composed of VGI, VGII, VGIII, and VGIV, which correspond to serotypes B or C [10]. Based on amplified fragment length polymorphism (AFLP) genotyping, *C. neoformans* var. *grubii* can also be divided into VNI, VNII,

and VNB [9, 11, 12]. The hybrids between *C. neoformans* and *C. gattii* (serotype BD) were also reported [13, 14].

Several molecular techniques have been used to study the epidemiology of the *C. neoformans*/*C. gattii* species complex, such as karyotype analysis [15], polymerase chain reaction (PCR) fingerprinting by using M13 [10, 16], restriction fragment length polymorphism of the gene *URA5* (*URA5*-RFLP) [10, 17–19], AFLP [20, 21], random amplification of polymorphic DNA (RAPD) [16], multilocus sequence typing (MLST) [9, 11, 22], and multilocus microsatellite typing (MLMT) [23], etc.

Automated repetitive PCR (DiversiLab system) [24] has been used in the strain identification and typing of some species of yeasts and molds [25–30]. The genotyping of *C. neoformans* and *C. gattii* using the DiversiLab system has not yet been reported.

The aims of this study were: (1) to reveal the molecular population structure of *C. neoformans* and *C. gattii* in China from 2007 to 2013 and (2) to verify the feasibility of the DiversiLab system used in the genotyping of *C. neoformans* and *C. gattii*.

Materials and methods

Isolates

A total of 83 clinical *Cryptococcus* spp. from 76 different patients from Peking Union Medical College Hospital (PUMCH) and Beijing Di-Tan Hospital (BDTH) in Beijing, China between 2007 and 2013 were studied. The clinical information of the 83 isolates can be found in Table 1. The following standard reference isolates of *C. neoformans* and *C. gattii* were included: WM148 (= CBS10085=ATCC MYA-4564, VNI, serotype A), WM626 (= CBS10084= ATCC MYA-4565, VNII, serotype A), WM628 (= CBS10080=ATCC MYA4566, VNIII, serotype AD), WM629 (= CBS10079=ATCC MYA-4567, VNIV, serotype D), WM179 (= CBS10078=ATCC MYA-4560, VGI, serotype B), WM178 (= CBS10082=ATCC MYA-4561, VGII, serotype B), WM161 (= CBS10081=ATCC MYA-4562, VGIII, serotype B), WM779 (= CBS10101=ATCC MYA-4563, VGIV, serotype C) [16], and H99 (= CBS10515= CBS8710, VNI, serotype A) [15]. All the isolates were maintained on Sabouraud dextrose agar medium (Becton Dickinson, Sparks, MD, USA) during our investigation.

Phenotype identification

All the isolates were subcultured onto Sabouraud dextrose agar medium at 37 °C for 48–72 h and identified by VITEK 2 Compact (bioMérieux SA, France). Each isolate was also inoculated on canavanine-glycine-bromthymol blue (CGB) agar [31] at 37 °C for at least 1 month.

DNA extraction

Isolates were grown on Sabouraud dextrose agar medium at 37 °C for 48–72 h. Genomic DNA was then extracted using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions.

Determination of serotype by PCR

The primer pairs JOHE2596/JOHE3241 and JOHE2596/JOHE3240 [32] were selected to separately amplify the genomic DNA of all the isolates to determine their serotype. JOHE2596/JOHE3241 is specific for serotype A, while JOHE2596/JOHE3240 is specific for serotype D.

URA5-RFLP analysis

The *URA5* gene of each isolate was amplified with primers URA5 and SJ01 [10]. PCR reactions and *URA5*-RFLP analysis were performed according to previously described methods [10].

MLST analysis

MLST analysis consists of seven unlinked loci, including six housekeeping genes, namely, *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and the non-coding region IGS1 [9]. Each locus of every specimen was bidirectionally sequenced. These sequences were uploaded to the MLST Database for the *Cryptococcus neoformans*/*C. gattii* species complex (http://mlst.mycologylab.org). An allele number was assigned to each sequence. Seven allele type (AT) numbers and a sequence type (ST) number will be given to each specimen after being compared to the MLST Database website and new AT and ST numbers will be assigned for the new sequences.

The concatenated sequences of the seven loci were aligned with the MEGA v.6.06 software (http://megasoftware.net) [33], along with those of reference cryptococcal isolates reported in previous studies [10, 11, 32, 34, 35]. The phylogenetic tree was constructed using the maximum likelihood method based on the Kimura two-parameter model with 1,000 replications in the bootstrap test.

Each sequence of the seven MLST loci for all tested isolates except isolate PU43 was submitted to GenBank to obtain accession numbers.

Table 1 Clinical and molecular characteristics of the 83 clinical cryptococcal isolates in this study

Isolate	Sex	Age (years)	Specimen	Isolated date	Underlying condition	Outcome	Genotype[c]	ST[k]	Location[l]
PU1[a]	M	51	Abscess	May 2007	Immunocompetent	Alive	VNI	ST5	Tongxian, BJ
PU2	F	33	CSF[d]	Jul 2007	Unknown	Died	VNI	ST5	Tanghe, HN
PU3	M	63	CSF	Sep 2007	Unknown	Alive	VNI	ST5	Linfen, SX
PU4	F	17	CSF	Nov 2007	Unknown	Alive	VNI	ST5	Queshan, HN
PU5	M	63	Blood	Jun 2008	Dermatomyositis	Died	VNI	ST5	Chaoyang, BJ
PU7	M	77	CSF	Jan 2008	DM[e]	Alive	VNI	ST5	Shijingshan, BJ
PU8	M	63	CSF	Apr 2008	Immunocompetent	Alive	VGII	ST182	Hengshui, HB
PU9	M	77	Pulmonary tissue	May 2008	Unknown	Unknown	VNI	ST5	Zhengding, HB
PU10	M	63	CSF	Jun 2008	Dermatomyositis	Died	VNI	ST5	Chaoyang, BJ
PU11	M	45	Blood	Jan 2009	Cushing's syndrom	Died	VNI	ST5	Luohe, HN
PU12	F	25	Blood	May 2009	Unknown	Unknown	VNI	ST5	Taining, FJ
PU13	F	18	Blood	Oct 2009	SLE[f]	Alive	VNI	ST5	Mentougou, BJ
PU15	M	32	CSF	Feb 2009	Unknown	Unknown	VNI	ST5	Unknown, BJ
PU16	F	32	CSF	Feb 2009	Immunocompetent	Alive	VNI	ST5	Anxian, SC
PU17	M	62	Pulmonary tissue	Mar 2009	Unknown	Unknown	VNI	ST5	Nanyang, HN
PU18	M	14	CSF	Dec 2009	Immunocompetent	Alive	VNI	ST5	Xinle, HB
PU19	F	29	CSF	May 2009	SLE	Alive	VNI	ST5	Yongqing, HB
PU20	M	74	Sputum	Dec 2009	Pulmonary carcinoma	Unknown	VNI	ST5	Sanmenxia, HN
PU21	M	45	Sputum	Jan 2009	Cushing's syndrom	Died	VNI	ST5	Luohe, HN
PU22	F	63	Sputum	Feb 2009	Hypercortisolism	Died	VNI	ST5	Huludao, LN
PU23	F	77	Sputum	Aug 2009	Breast carcinoma	Died	VNI	ST5	Xicheng, BJ
PU24	F	41	Pulmonary tissue	Sep 2009	Pulmonary carcinoma	Died	VNI	ST5	Fengtai, BJ
PU25	F	62	Sputum	Dec 2009	Rheumatoid	Alive	VNI	ST5	Chaoyang, BJ
PU26	F	41	Blood	Mar 2010	SLE	Died	VNI	ST5	Nankang, JX
PU27	M	51	CSF	May 2010	HIV[g](+)	Unknown	VNI	ST5	Anyang, HN
PU28	F	60	Pulmonary tissue	Mar 2010	Sarcoidosis	Unknown	VNI	ST5	Jiyuan, HN
PU29	M	42	Pulmonary tissue	Mar 2010	Unknown	Unknown	VNI	ST5	Unknown, LN
PU30	M	45	CSF	Mar 2010	Immunocompetent	Alive	VNI	ST5	Qingdao, SD
PU31	F	13	CSF	Mar 2010	SLE	Alive	VNI	ST5	Taiyuan, SX
PU32	M	52	Blood	Apr 2010	Arteritis	Died	VNI	ST5	Dalian, LN
PU33	M	62	Sputum	Jul 2010	Immunocompetent	Alive	VNI	ST5	Zhangjiakou, HB
PU34	M	72	Sputum	Apr 2010	CRF[h]	Unknown	VNI	ST5	Dongcheng, BJ
PU35	F	37	Blood	May 2011	DPLD[i]	Unknown	VNI	ST5	Liu'an, AH
PU36	F	54	Pulmonary tissue	May 2011	Unknown	Unknown	VNI	ST5	Anyang, HN
PU37	M	47	Pulmonary tissue	Sep 2011	Unknown	Unknown	VNI	ST5	Haidian, BJ
PU38	F	23	CSF	Sep 2011	Immunocompetent	Alive	VNI	ST5	Unknown, Hubei
PU39	F	74	CSF	Sep 2011	Unknown	Alive	VNI	ST5	Fangshan, BJ
PU40	F	58	CSF	May 2011	Myasthenia gravis	Died	VNI	ST5	Jixian, TJ
PU41	F	25	CSF	Nov 2011	SLE	Died	VNI	ST5	Langfang, HB
PU42	F	38	CSF	Apr 2011	SLE	Unknown	VNI	ST5	Fuzhou, FJ
PU43	M	44	CSF	Mar 2011	Immunocompetent	Alive	NA[J]	NA	Haidian, BJ
PU44	M	27	CSF	May 2011	Immunocompetent	Alive	VNI	ST5	Shantou, GD
PU45	F	24	Pulmonary tissue	Jul 2011	Unknown	Alive	VNI	ST5	Dandong, LN
PU46	F	51	Blood	May 2012	Cushing's syndrom	Died	VNI	ST5	Tongzhou, BJ
PU47	F	39	CSF	Jun 2012	Immunocompetent	Alive	VNI	ST5	Xi'an, SX
PU48	M	67	CSF	May 2012	CRF	Died	VNI	ST5	Nanyang, HN
PU49	M	29	CSF	Jul 2012	Immunocompetent	Alive	VNI	ST5	Qinghuangdao, HB
PU50	F	31	CSF	Aug 2012	SLE	Died	VNI	ST5	Jinan, SD
PU51	M	31	Blood	Jun 2012	CRF; lupus nephritis	Unknown	VNI	ST5	Cangzhou, HB

Table 1 (continued)

Isolate	Sex	Age (years)	Specimen	Isolated date	Underlying condition	Outcome	Genotype[c]	ST[k]	Location[l]
PU52	F	76	CSF	Nov 2012	Unknown	Alive	VNI	ST5	Changchun, JL
PU55	F	37	Bronchial aspiration	May 2011	DPLD	Unknown	VNI	ST5	Liu'an, AH
PU56	M	74	CSF	Mar 2013	Unknown	Unknown	VNI	ST5	Chaoyang, BJ
PU57	M	31	CSF	Jun 2012	CRF; lupus nephritis	Unknown	VNI	ST5	Cangzhou, HB
PU64	M	30	Blood	Mar 2013	HIV(+)	Alive	VNI	ST5	Wuhan, Hubei
PU65	M	30	CSF	Mar 2013	HIV(+)	Alive	VNI	ST5	Wuhan, Hubei
PU66	F	32	CSF	Mar 2013	SLE	Alive	VNI	ST5	Fengtai, BJ
PU67	M	55	Pulmonary tissue	Mar 2013	Immunocompetent	Alive	VNI	ST5	Shijiazhuang, HB
PU68	F	49	Abscess	Apr 2013	Pyoderma gangrenosum	Alive	VNI	ST5	Liaocheng, SD
PU99	M	42	CSF	Jan 2011	Unknown	Unknown	VGI	ST332	Guangzhou, GD
PU145	M	39	Subcutaneous tissue	Jun 2013	Immunocompetent	Alive	VNI	ST5	Fengtai, BJ
PU146	F	51	CSF	Jun 2013	DM	Died	VNI	ST31	Chongwen, BJ
PU147[b]	F	28	CSF	Dec 2008	HIV(+)	Alive	VNI	ST5	Nanchang, JX
PU148	M	55	Blood	Mar 2011	HIV(+)	Alive	VNI	ST5	Guiyang, Guizhou
PU149	M	41	CSF	Apr 2009	HIV(+)	Alive	VNI	ST5	Chaoyang, BJ
PU150	F	44	CSF	Apr 2009	HIV(+)	Alive	VNI	ST5	Jiangjin, CQ
PU151	M	60	CSF	Apr 2009	HIV(+)	Died	VNI	ST5	Wenzhou, ZJ
PU152	M	35	BALF	Sep 2009	HIV(+)	Alive	VNI	ST5	Huludao, LN
PU153	M	47	Blood	Jan 2010	HIV(+)	Died	VNI	ST5	Changping, BJ
PU154	F	37	CSF	Feb 2011	HIV(+)	Alive	VNI	ST5	Xicheng, BJ
PU155	F	50	Blood	Mar 2010	HIV(+)	Alive	VNI	ST5	Pingding, SX
PU156	M	50	CSF	May 2010	HIV(+)	Died	VNI	ST5	Changchun, JL
PU157	M	25	Blood	Jan 2012	HIV(+)	Died	VNB	ST295	Baoding, HB
PU158	F	8	Blood	Aug 2010	HIV(+)	Alive	VNI	ST5	Xingtai, HB
PU159	M	28	CSF	Jan 2011	HIV(+)	Alive	VNI	ST5	Taiyuan, SX
PU160	F	22	CSF	Jan 2011	HIV(+)	Died	VNI	ST5	Tongzhou, BJ
PU161	M	35	CSF	Jan 2011	HIV(+)	Alive	VNI	ST296	Qingzhou, SD
PU162	M	38	CSF	Mar 2011	HIV(+)	Alive	VNI	ST63	Tongzhou, BJ
PU163	M	33	CSF	Jul 2013	HIV(+)	Died	VNI	ST5	Xintai, SD
PU164	M	33	Blood	Jul 2013	HIV(+)	Died	VNI	ST5	Xintai, SD
PU165	F	33	CSF	Jul 2013	SLE	Died	VNI	ST5	Zhengzhou, HN
PU166	F	33	Blood	Jul 2013	SLE	Died	VNI	ST5	Zhengzhou, HN
PU167	F	58	CSF	Aug 2013	HIV(+)	Alive	VNI	ST5	Tongzhou, BJ
PU169	M	62	CSF	Oct 2013	DM; tuberculosis	Alive	VNI	ST5	Shunyi, BJ

[a] Isolates in **bold** were collected from Peking Union Medical College Hospital

[b] Isolates in underlined font were collected from Beijing Di-Tan Hospital

[c] Determined by seven MLST loci (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, *URA5*)

[d] *CSF* cerebrospinal fluid

[e] *DM* diabetes mellitus

[f] *SLE* systemic lupus erythematosus

[g] *HIV* human immunodeficiency virus

[h] *CRF* chronic renal failure

[i] *DPLD* diffuse parenchymal lung disease

[j] *NA* not applicable

[k] *ST* sequence type

[l] Abbreviations in this column denote different municipalities or provinces in China: *BJ* Beijing; *HN* Henan; *SX* Shanxi; *HB* Hebei; *FJ* Fujian; *SC* Sichuan; *LN* Liaoning; *JX* Jiangxi; *SD* Shandong; *AH* Anhui; *TJ* Tianjin; *GD* Guangdong; *CQ* Chongqing; *ZJ* Zhejiang; *JL* Jilin

rep-PCR DNA fingerprinting

All the samples and reference isolates were performed rep-PCR using the DiversiLab® Fungal Kit (bioMérieux SA, France). The amplified fragments were separated by electrophoresis in a microfluidics DNA LabChip (bioMérieux SA, France) on an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Further data analysis was performed with the web-based DiversiLab software v.3.4 (http://pumc.diversilab.com). The band-based modified Kullback–Leibler distance method was used for calculating the percent similarities.

Results

Phenotypic identification

All 83 isolates were identified as *C. neoformans* by VITEK 2 Compact. CGB medium inoculated with PU8 turned completely blue after being incubated at 37 °C for 5 days, with the same result for PU99. CGB medium inoculated with the remaining 81 isolates remained yellow a month later. With CGB medium, PU8 and PU99 were identified as *C. gattii*, while the other 81 isolates were *C. neoformans*.

Determination of serotype

PU43 could be amplified not only by JOHE2596/JOHE3241 but also by JOHE2596/JOHE3240, and was confirmed to be serotype AD. However, neither of the two primer pairs could amplify the DNA of PU8 and PU99, which were deduced to be serotypes B or C. The remaining 80 isolates (96.4 %), including PU157, could be amplified by JOHE2596/JOHE3241 but not JOHE2596/JOHE3240, and proved to be serotype A.

URA5-RFLP analysis

The *URA5*-RFLP profiles of nine isolates (PU1, PU28, PU35, PU41, PU48, PU55, PU146, PU161, and PU162) were identical to those of H99 and WM148, which belong to VNI, while PU157, PU43, PU99, and PU8 were assigned as VNII, VNIII, VGI, and VGII, respectively (Fig. 1). The *URA5*-RFLP patterns of the remaining 70 clinical isolates in this study were the same as those of H99 and WM148. Altogether, 79 out of 83 isolates (95.2 %) were identified as VNI.

MLST analysis

A total of seven different MLST STs were recognized, three (ST295, ST296, and ST332) of which were novel and four (ST5, ST31, ST63, and ST182) of which had been reported

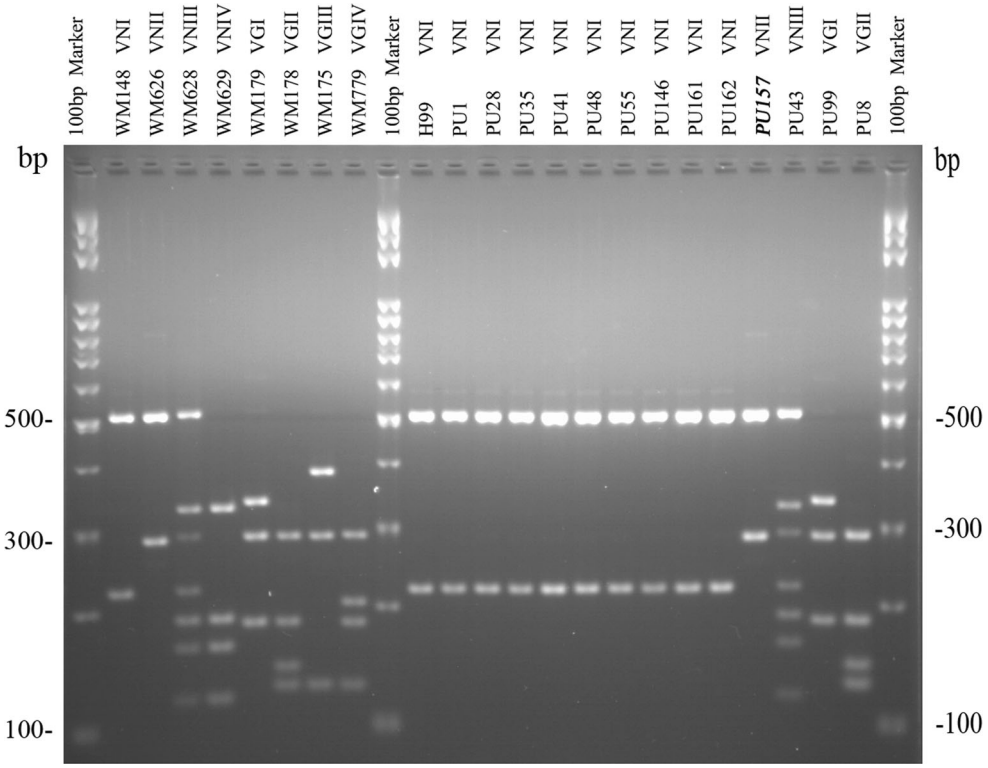


Fig. 1 *URA5*-RFLP profiles generated by double digestion with restriction endonuclease *Sau*96I and *Hha*I. PU157 in ***bold italics*** was recognized as VNII, but later identified as VNB by seven-locus MLST analysis

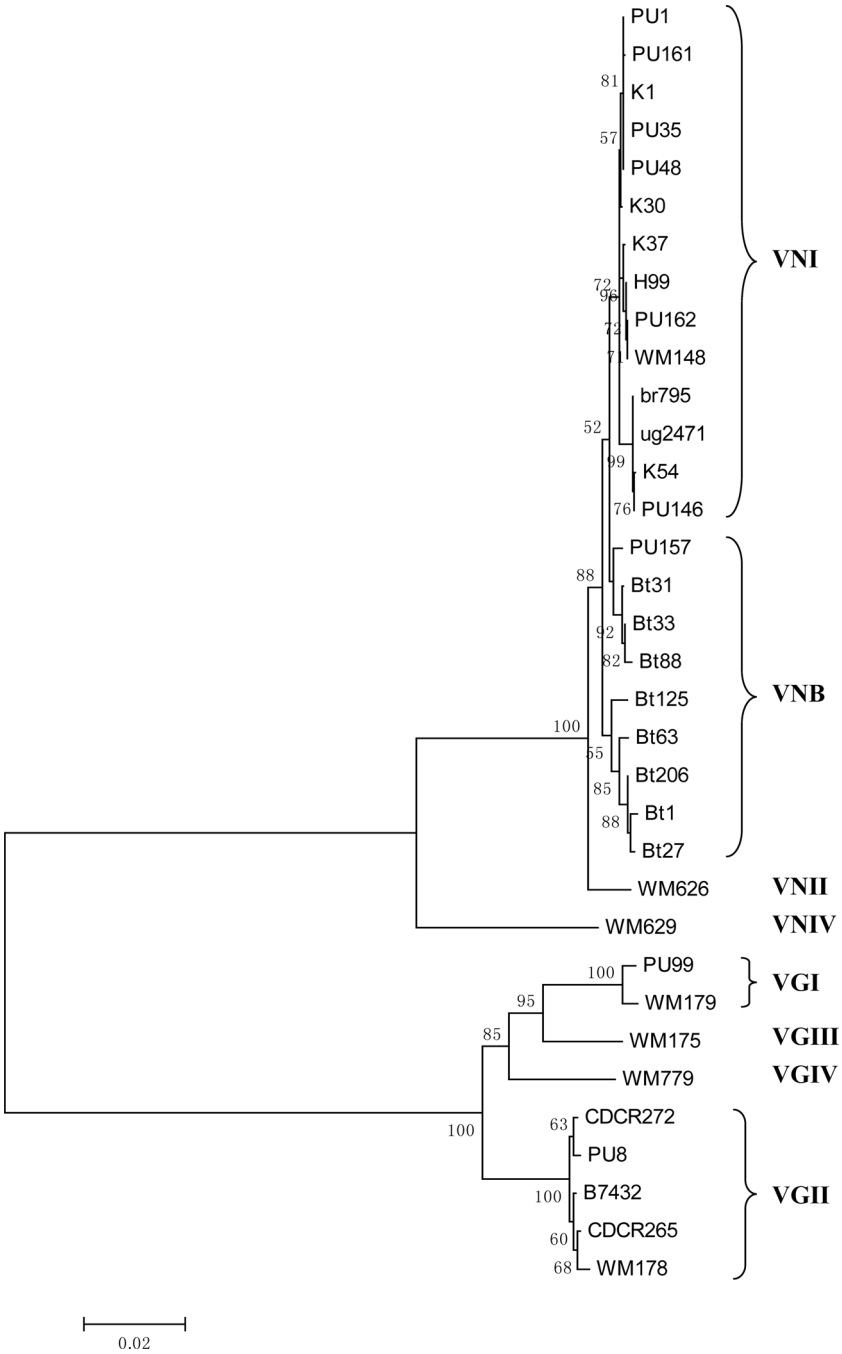
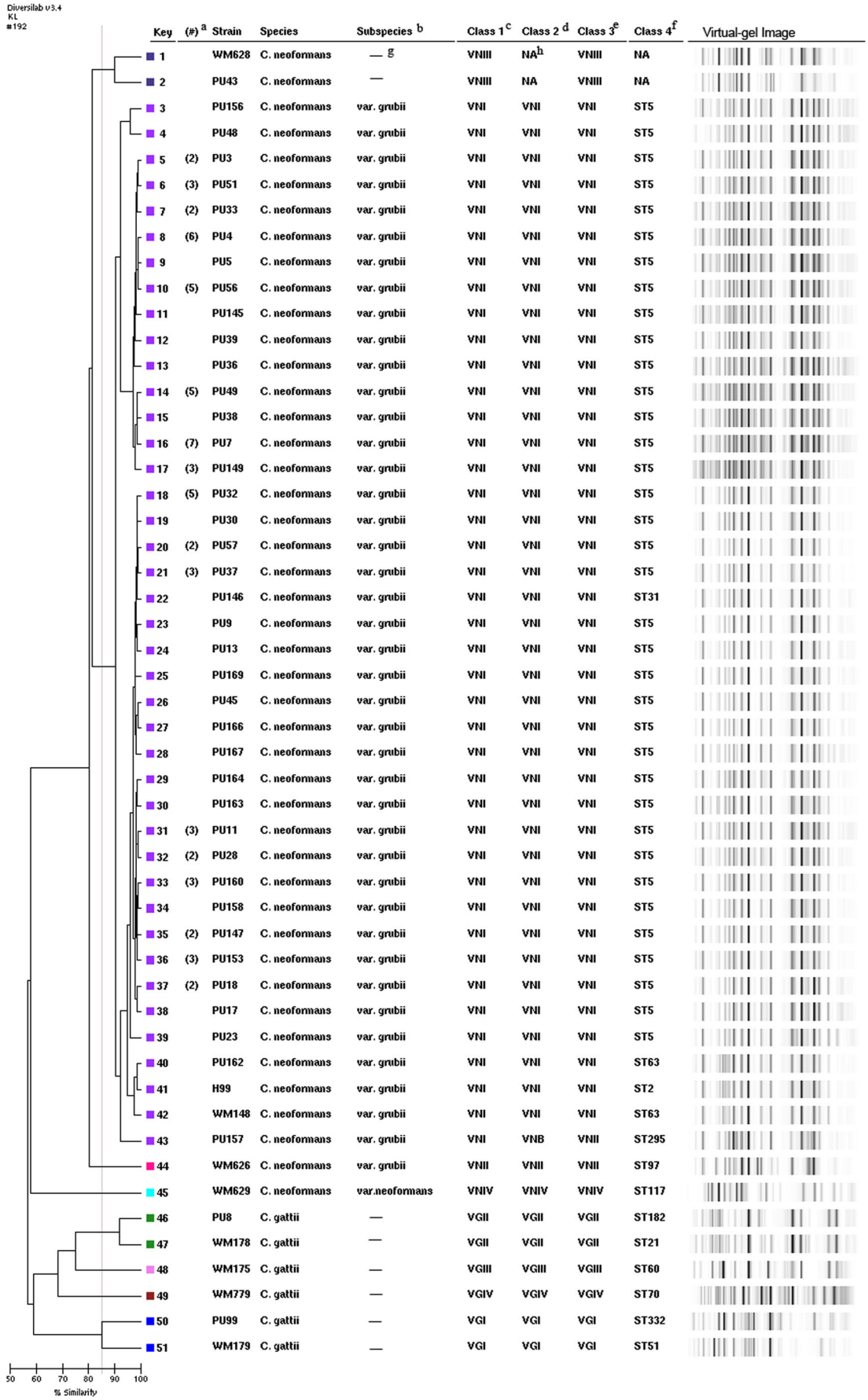


Fig. 2 Phylogenetic tree constructed using the maximum likelihood method with 1,000 replications in the bootstrap test based on the concatenated seven MLST loci (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, and *URA5*) of part of the tested isolates identified as PU and other global cryptococcal isolates from previous studies. Only the bootstrap values >50 % are shown

previously. The most common ST was ST5 (VNI), which accounted for 91.6 % (76/83). ST31, ST63, and ST296 belonged to VNI, while ST295, ST332, and ST182 represented VNB (PU157), VGI (PU99), and VGII (PU8), respectively (Table 1). All the sequences submitted to GenBank acquired their accession numbers between KF864215 and KF890168.

Some loci of isolate PU43 had double peaks in their peak sequence diagram because of the AD hybrid, and PU43 was not analyzed in MLST. In total, the sequence length of the seven concatenated MLST loci was 4,272 base pairs. The phylogenetic tree constructed by the maximum likelihood method showed that most isolates (PU1, PU35, PU48, and PU161) clustered with K1 [34], which correlated with the M5

Fig. 3 rep-PCR-based dendrogram and virtual gel image of the DiversiLab system for the 83 clinical and nine standard reference isolates of *C. neoformans* and *C. gattii*. [a] Number of isolates for which the similarity was no less than 99 % compared with the isolates adjacent to them. [b] Varieties. [c] Genotype concluded by the DiversiLab system. [d] Genotype determined by seven-loci MLST (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, and *URA5*) analysis. [e] Genotype assigned by *URA5*-RFLP. [f] ST generated by the seven MLST loci. [g] –, no varieties. [h] NA, not applicable


Diversilab v3.4
KL
#192
Key (#)[a] Strain Species Subspecies[b] Class 1[c] Class 2[d] Class 3[e] Class 4[f] Virtual-gel Image
1 WM628 C. neoformans —[g] VNIII NA[h] VNIII NA
2 PU43 C. neoformans — VNIII NA VNIII NA
3 PU156 C. neoformans var. grubii VNI VNI VNI ST5
4 PU48 C. neoformans var. grubii VNI VNI VNI ST5
5 (2) PU3 C. neoformans var. grubii VNI VNI VNI ST5
6 (3) PU51 C. neoformans var. grubii VNI VNI VNI ST5
7 (2) PU33 C. neoformans var. grubii VNI VNI VNI ST5
8 (6) PU4 C. neoformans var. grubii VNI VNI VNI ST5
9 PU5 C. neoformans var. grubii VNI VNI VNI ST5
10 (5) PU56 C. neoformans var. grubii VNI VNI VNI ST5
11 PU145 C. neoformans var. grubii VNI VNI VNI ST5
12 PU39 C. neoformans var. grubii VNI VNI VNI ST5
13 PU36 C. neoformans var. grubii VNI VNI VNI ST5
14 (5) PU49 C. neoformans var. grubii VNI VNI VNI ST5
15 PU38 C. neoformans var. grubii VNI VNI VNI ST5
16 (7) PU7 C. neoformans var. grubii VNI VNI VNI ST5
17 (3) PU149 C. neoformans var. grubii VNI VNI VNI ST5
18 (5) PU32 C. neoformans var. grubii VNI VNI VNI ST5
19 PU30 C. neoformans var. grubii VNI VNI VNI ST5
20 (2) PU57 C. neoformans var. grubii VNI VNI VNI ST5
21 (3) PU37 C. neoformans var. grubii VNI VNI VNI ST5
22 PU146 C. neoformans var. grubii VNI VNI VNI ST31
23 PU9 C. neoformans var. grubii VNI VNI VNI ST5
24 PU13 C. neoformans var. grubii VNI VNI VNI ST5
25 PU169 C. neoformans var. grubii VNI VNI VNI ST5
26 PU45 C. neoformans var. grubii VNI VNI VNI ST5
27 PU166 C. neoformans var. grubii VNI VNI VNI ST5
28 PU167 C. neoformans var. grubii VNI VNI VNI ST5
29 PU164 C. neoformans var. grubii VNI VNI VNI ST5
30 PU163 C. neoformans var. grubii VNI VNI VNI ST5
31 (3) PU11 C. neoformans var. grubii VNI VNI VNI ST5
32 (2) PU28 C. neoformans var. grubii VNI VNI VNI ST5
33 (3) PU160 C. neoformans var. grubii VNI VNI VNI ST5
34 PU158 C. neoformans var. grubii VNI VNI VNI ST5
35 (2) PU147 C. neoformans var. grubii VNI VNI VNI ST5
36 (3) PU153 C. neoformans var. grubii VNI VNI VNI ST5
37 (2) PU18 C. neoformans var. grubii VNI VNI VNI ST5
38 PU17 C. neoformans var. grubii VNI VNI VNI ST5
39 PU23 C. neoformans var. grubii VNI VNI VNI ST5
40 PU162 C. neoformans var. grubii VNI VNI VNI ST63
41 H99 C. neoformans var. grubii VNI VNI VNI ST2
42 WM148 C. neoformans var. grubii VNI VNI VNI ST63
43 PU157 C. neoformans var. grubii VNI VNB VNII ST295
44 WM626 C. neoformans var. grubii VNII VNII VNII ST97
45 WM629 C. neoformans var.neoformans VNIV VNIV VNIV ST117
46 PU8 C. gattii — VGII VGII VGII ST182
47 WM178 C. gattii — VGII VGII VGII ST21
48 WM175 C. gattii — VGIII VGIII VGIII ST60
49 WM779 C. gattii — VGIV VGIV VGIV ST70
50 PU99 C. gattii — VGI VGI VGI ST332
51 WM179 C. gattii — VGI VGI VGI ST51
50 60 70 80 90 100
% Similarity

genotype identified by 12 MLST loci typing [11]. Only one isolate (PU162) clustered closely with WM148 and H99, which belonged to the M1 genotype [11]. Only one isolate (PU146) clustered with K54 [34], the M4 genotype [11]. PU157 was even closer to the VNB cluster than the VNI cluster, which was, therefore, identified as VNB. PU8 was classified as VGII and PU99 was determined as VGI (Fig. 2). The remaining 73 tested samples not shown in Fig. 2 had identical sequences of the seven MLST loci with PU1 et al., and were assigned as VNI (Table 1).

DiversiLab typing

An 85 % similarity threshold was chosen to determine the genotype of tested isolates. Isolates among the VNI group showed polymorphism. The nine reference isolates and all 83 cryptococcal isolates in our study were successfully genotyped with the DiversiLab system. The dendrogram showed that most of the isolates (n=79; 95.2 %) clustered together with WM148 (VNI), and were classified as VNI. Samples with strain similarity (≥99 %) were condensed in the dendrogram due to space constraints. PU43 and MW628, the AD hybrids, clustered together in the dendrogram. PU8 clustered with WM178 (VGII), while PU99 clustered with WM179 (VGI). PU43, PU99, and PU8 were determined as VNIII, VGI, and VGII, respectively (Fig. 3). PU157 was identified as VNI by the DiversiLab system analysis.

Discussion

Until now, the reports concerning the molecular epidemiology of the *Cryptococcus* species complex in China were insufficient and less comprehensive. Chen et al. [17] analyzed 129 clinical *Cryptococcus* spp. isolates from China using *URA5*-RFLP and PCR fingerprinting M13 analyses, and showed that 93.0 % (120/129) belonged to *C. neoformans* VNI, while 7.0 % (9/129) were *C. gattii* VGI. With almost the same methods, Feng et al. [18] studied 115 clinical cryptococcal isolates in China and revealed that 89.6 % (103/115) represented VNI, along with two isolates of VNIII, one VNIV, eight VGI, and one VGII.

Our data also showed that VNI was the most common genotype. Meanwhile, we revealed the MLST STs of the 82 isolates of *C. neoformans* and *C. gattii* in our study. A total of 76 VNI (91.6 %) were identified as MLST ST5, followed by one each of ST31, ST63, and ST296 for VNI, one ST295 for VNB, one ST332 for VGI, and one ST182 for VGII. Some studies have shown that ST5 was the most frequent ST in China and other Asian countries [19, 36]. A total of 476 isolates of *C. neoformans* var. *grubii* from eight Asian countries, including 86 Chinese clinical *Cryptococcus* isolates from the Second Military Medical University, were analyzed by MLST analysis. Seventy-four of 86 Chinese cryptococcal isolates were identified as ST5, followed by five ST53, two ST194, and one each of ST31, ST93, ST186, ST191, and ST195 [36]. However, the three dominating MLST STs in Thailand were ST44, ST45, and ST46 [37].

C. gattii has not yet been reported in temperate regions in China. A strain of *C. gattii* VGII (PU8) from the temperate climate region located on longitude 115°~116° and latitude 37°~38° in China was detected in our study. Few VGII isolates had been reported in China previously, all of which were distributed in the subtropical climate zones of South China [18, 38]. Our VGII (ST=182) strain was different from XH91 reported as VGII in China in 2008 [18]. It was also different from the prevalent outbreak strain VGIIb (ST=7) on Vancouver Island from 1999 to 2003 [5], though they clustered closely in Fig 2.

C. neoformans VNB has not yet been reported in Asian countries till now. It had been found in Botswana [11], Italy [12], Portugal, Rwanda, Brazil, and Venezuela [32] etc. A strain of *C. neoformans* VNB (PU157) was discovered in our study. PU157 was finally determined as *C. neoformans* VNB by seven-loci MLST analysis. It belonged to MLST ST295, a novel ST. According to the *URA5*-RFLP results, the VNII-1 isolates of *C. neoformans* were identified as VNII. The phylogenetic tree based on the combined sequences of *GPD1*, IGS1, *PLB1*, and *URA5* revealed that those VNII-1 isolates were VNB [39]. Similar results occurred in our study; PU157 was first assigned as VNII according to its *URA5*-RFLP pattern (Fig. 1), and it seemed to belong to VNI by the DiversiLab system typing (Fig. 3). In the phylogenic tree constructed on the base of the concatenated seven MLST loci, PU157 clustered relatively closer to VNB (Fig. 2).

There was a good correlation between the DiversiLab system and MLST as well as *URA5*-RFLP in genotyping the *Cryptococcus* species complex. Additionally, the total time consumed in the performance of the DiversiLab system for 12 samples lasted less than 4 h. Of particular note is that the AD hybrid can be recognized by the DiversiLab system, which cannot be handled by MLST. Had a reference strain of VNB been included in our study, PU157 may have had an accurate result from the DiversiLab system.

However, the downfall of the DiversiLab system in the genotyping of *Cryptococcus* spp. is that, as there is no database for *Cryptococcus* spp. at present, the reference standard isolates of *Cryptococcus* spp. need to be included simultaneously for the first performance. And the limitation of our study was that there were not enough cryptococcal isolates with diversity in our collection, which suggested us to further investigate the population structure of *C. neoformans* and *C. gattii* in China and validate the ability of the DiversiLab system in genotyping cryptococcal isolates.

In summary, our results showed that the discovery of *C. neoformans* VNB and *C. gattii* VGII in the temperate climate regions in China warrant further investigation concerning the molecular epidemiology of *C. neoformans* and *C. gattii*. Moreover, our results demonstrated that the DiversiLab system could be used in the genotyping of *C. neoformans* and *C. gattii* due to its convenience and effectiveness. However, the resolution and reproducibility of the system needs to be further verified with more cryptococcal isolates of different genotypes.

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Conflict of interest The authors declare that they have no conflict of interest.

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