

Genotypes of Clinical and Environmental Isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* in Korea

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Abstract Multilocus sequence typing analysis was applied to determine the genotypes of 147 (137 clinical and 10 environmental) *Cryptococcus neoformans* and three clinical *Cryptococcus gattii* isolates from 1993 to 2014 in Korea. Among the 137 clinical isolates of *C. neoformans*, the most prevalent genotype was ST5 (n = 131), followed by ST31 (n = 5) and ST127 (n = 1). Three *C. gattii* strains were identified as ST57, ST7, and ST113. All environmental isolates were identified as *C. neoformans* with two genotypes, ST5 (n = 7) and ST31 (n = 3). Our results show that *C. neoformans* isolates in Korea are genetically homogeneous, and represent a close genetic relationship between clinical and environmental isolates.

Keywords *Cryptococcus gattii*, *Cryptococcus neoformans*, Genotype, Multilocus sequence typing

The *Cryptococcus neoformans/Cryptococcus gattii* species complex is the causative agent of cryptococcosis, especially in immunodeficient hosts. Both species contain a number of genetically diverse subgroups and differ in their ecology and epidemiology [1, 2]. *C. neoformans* comprises two varieties and two serotypes (A and D), whereas *C. gattii* comprises two serotypes (B and C) [1]. *C. neoformans* has been associated worldwide with pigeon excreta and is responsible for more than 90% of the cases of cryptococcal infections [1, 3]. Until recently, *C. gattii* was considered restricted to the tropical and subtropical climate zones and thought to be related mainly to *Eucalyptus* trees [4]. However, a recent outbreak of molecular type VGII of *C. gattii* in the Pacific Northwest of North America, a temperate

area, indicated an environmental shift of this species [5, 6].

Due to the number of genetically diverse subgroups within each serotype, several molecular typing methods, including PCR fingerprinting, amplified fragment length polymorphism, PCR-restriction fragment length polymorphism (RFLP) analysis of the orotidine monophosphate pyrophosphorylase (*URA5*) gene, random amplification of polymorphic DNA analysis, multilocus microsatellite typing, and multilocus sequence typing (MLST) have been developed to study the molecular epidemiology of *C. neoformans* and *C. gattii* [7-10]. Based on genetic differences, eight distinct molecular types have been recognized in two species: VNI and VNII (*C. neoformans* var. *grubii*, serotype A); VNIII (serotype AD, hybrid); VNIV (*C. neoformans* var. *neoformans*, serotype D); and VGI, VGII, VGIII, and VGIV (*C. gattii*, serotypes B and C). Within *C. neoformans* var. *grubii*, VNI is the most prevalent molecular type worldwide, especially in Asian countries, whereas VGI has been the most prevalent type of infection due to *C. gattii* [10, 11]. Further genetic analysis has divided the VGII genotype into three subtypes: VGIIa, VGIIb, and VGIIc [12]. Among the molecular typing methods, MLST has shown a high degree of intraspecies discriminatory power as well as reproducibility between different laboratories. This method can be used to trace the putative origin of pathogenic populations [8, 13]. Recently, a standardized MLST scheme for genotyping of the *C. neoformans/C. gattii* species complex has been established by the International Society of Human and Animal Mycoses (ISHAM) working group to enable the standardization and

Mycobiology 2015 September, 43(3): 360-365
<http://dx.doi.org/10.5941/MYCO.2015.43.3.360>
pISSN 1229-8093 • eISSN 2092-9323
© The Korean Society of Mycology

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Received July 29, 2015

Revised August 25, 2015

Accepted August 31, 2015

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acquisition of global genotypic data [10]. Our previous data showed that the predominant genotype of 46 clinical *C. neoformans* isolates from 2008 to 2012 by MLST analysis was VNI-ST5 (95.7%), and the remaining genotype was VNI-ST31 (4.3%) [14]. Most *C. neoformans* isolates in the Far East Asian countries Korea, Japan, and China belong to VNI-ST5 with low genetic diversity. However, minor MLST genotypes identified within populations represent a greater genetic diversity among these countries [15-17]. Although considerable data on the global epidemiology of the *C. neoformans/C. gattii* species complex are available, information on the molecular epidemiology of Korean strains remains limited. In this study, we investigated the genotypes of the previously studied and untyped *C. neoformans* and *C. gattii* isolates from clinical and environmental sources using MLST, and compared them with the genotypic diversity among the Korean strains of the *C. neoformans/C. gattii* species complex.

A total of 140 clinical isolates of *Cryptococcus* species were obtained from medical centers located in the Seoul (n = 117) and Busan (n = 23) areas between 1993 and 2014, and 10 environmental *Cryptococcus* species were isolated from pigeon droppings in Seoul (n = 3) and Busan (n = 7) during the same period, as previously described [18]. The identification of species strain was determined using the canavanine-glycine-bromothymol blue agar test [19] and serotyping was performed using a slide agglutination kit (Crypto Check; Iatron Laboratories, Tokyo, Japan), following

the manufacturer's instructions. The reference strains used in this study were as follows: WM148 (serotype A, VNI); WM626 (serotype A, VNII); WM628 (serotype AD, VNIII); WM629 (serotype D, VNIV); WM179 (serotype B, VGI); WM178 (serotype B, VGII); WM175 (serotype B, VGIII); and WM779 (serotype C, VGIV). Genomic DNA was extracted from each isolate using a Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Mating typing and molecular typing by *URA5*-RFLP were performed as previously described [20, 21]. MLST analysis was performed according to an ISHAM consensus scheme of seven unlinked genetic loci (*CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and *IGS1*). DNA from each isolate was amplified by PCR in a 20 µL reaction volume for each of the seven MLST loci by using the primers (Table 1) and protocols as described previously [8, 10]. Each locus was subsequently sequenced using the Applied Biosystems 3730 sequencer with the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA). Alleles at each locus were assigned numbers (allele types) upon comparison with those identified in the global collection, resulting in a 7-digit profile for each isolate. Each unique allelic profile was concatenated and assigned a sequence type (ST) according to the MLST scheme (<http://mlst.mycologylab.org>). The phylogenetic tree from the combined DNA sequences of the 7 genes was inferred using neighbor joining by MEGA 5.2 software [22].

Table 1. Primers used for MLST analysis of *Cryptococcus neoformans* (CN) and *C. gattii* (CG) isolates

Locus	Primer name	Primer sequence	Amplification conditions	Ref.
<i>CAP59</i>	CAP59F	5'-CTCTACGTCGAGCAAGTCAAG-3'	94°C 3 min, 35 cycles (94°C 30 sec, 60°C 30 sec, 72°C 1 min), 72°C 5 min	[10]
	CAP59R	5'-TCCGCTGCACAAGTGATACCC-3'		
<i>GPD1</i>	GPD1F	5'-GGTTGTCAAGGTTGGAATCAACGG-3'	96°C 5 min, 35 cycles (96°C 30 sec, 62°C 30 sec, 72°C 1 min), 72°C 5 min	[10]
	GPD1R	5'-GGAGCGGAAATGACGACCTTCTT-3'		
<i>IGS1</i>	IGS1F	5'-ATCCTTTGCAGACGACTTGA-3'	94°C 3 min, 35 cycles (94°C 30 sec, 58°C 30 sec, 72°C 1 min), 72°C 5 min	[10]
	IGS1R	5'-GTGATCAGTGCATTGCATGA-3'		
<i>LAC1</i>	LAC1F	5'-GGCGATACTATTATCGTA-3'	95°C 4 min, 35 cycles (95°C 30 sec, 50°C 30 sec, 72°C 1 min), 72°C 5 min	[8]
	LAC1R	5'-TTCTGGAGTGGCTAGAGC-3'		
<i>PLB1</i>	PLB1F	5'-CTTCAGGCGGAGAGAGGTTT-3'	94°C 3 min, 35 cycles (94°C 30 sec, 58°C 30 sec, 72°C 1 min), 72°C 5 min	[10]
	PLB1R	5'-GATTTGGCGTTGGTTTCAGT-3'		
<i>SOD1</i> for CN	SODCN1F	5'-TCTAATCGAAATGGTCAAGG-3'	94°C 3 min, 35 cycles (94°C 45 sec, 56°C 30 sec, 72°C 1 min), 72°C 5 min	[10]
	SODCN1R	5'-CGCAGCTGTTTCGTCTGGATA-3'		
<i>SOD1</i> for CG	SODCG1F	5'-GATCCTCACGCCATTACG-3'	94°C 3 min, 35 cycles (94°C 45 sec, 56°C 30 sec, 72°C 1 min), 72°C 5 min	[10]
	SODCG1R	5'-GAATGATGCGCTTAGTTGGA-3'		
<i>URA5</i>	URA5F	5'-ATGTCCTCCCAAGCCCTCGAC-3'	94°C 3 min, 35 cycles (94°C 45 sec, 63°C 1 min, 72°C 2 min), 72°C 5 min	[10]
	URA5R	5'-TTAAGACCTCTGAACACCGTACTC-3'		

MLST, multilocus sequence typing; Ref., reference.

Table 2. Distribution of the molecular types among *Cryptococcus neoformans* and *C. gattii* isolates in Korea

Source	Molecular type					Total
	VNI	VNIV	VGI	VGII	VGIII	
Clinical	136	1	1	1	1	140
Environment	10	0	0	0	0	10
Total, n (%)	146 (97.3)	1 (0.7)	1 (0.7)	1 (0.7)	1 (0.7)	150

Among the 140 clinical strains of the *C. neoformans/C. gattii* species complex, 136 isolates were *C. neoformans* var. *grubii* serotype A, and one was *C. neoformans* var. *neoformans* serotype D. The remaining three were *C. gattii* serotype B.

All environmental isolates were *C. neoformans* var. *grubii* serotype A. The most common molecular type by *URA5*-RFLP analysis was VNI among clinical isolates (97%) and environmental isolates (100%), and less frequent molecular

Table 3. The allelic profiles and sequence types of selected *Cryptococcus neoformans* and *C. gattii* isolates

Strain	Year	Region	Source	Molecular type	Mating type	Allelic profile							ST
						<i>CAP59</i>	<i>GPD1</i>	<i>IGS1</i>	<i>LAC1</i>	<i>PLB1</i>	<i>SOD1</i>	<i>URA5</i>	
Cn1	1993	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn2	1993	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
SC3	1993	Busan	Clinical	VGI	α	16	5	3	5	5	65	12	57
Cn4	1993	Busan	Clinical	VNIV	α	17	21	28	13	14	1	16	127
ECn6	1993	Busan	Environment	VNI	α	1	3	1	5	2	1	1	5
ECn7	1993	Busan	Environment	VNI	α	1	3	1	5	2	1	1	5
Cn44	1995	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn45	1995	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn8	1996	Busan	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn9	1996	Busan	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn14	1997	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn15	1997	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn51	1999	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
SC52	1999	Seoul	Clinical	VGIII	α	18	3	1	32	20	38	19	113
Cn19	2000	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn20	2000	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn22	2001	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
ECn23	2002	Busan	Environment	VNI	α	1	3	1	5	2	1	1	5
ECn24	2002	Busan	Environment	VNI	α	1	1	10	3	2	1	1	31
ECn25	2002	Busan	Environment	VNI	α	1	1	10	3	2	1	1	31
ECn26	2002	Busan	Environment	VNI	α	1	3	1	5	2	1	1	5
ECn27	2002	Busan	Environment	VNI	α	1	3	1	5	2	1	1	5
Cn29	2003	Seoul	Clinical	VNI	α	1	1	10	3	2	1	1	31
Cn58	2004	Busan	Clinical	VNI	α	1	1	10	3	2	1	1	31
Cn64	2005	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn41	2005	Busan	Clinical	VNI	α	1	1	10	3	2	1	1	31
Cn83	2005	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
SC89	2006	Seoul	Clinical	VGII	α	2	6	10	4	2	15	2	7
Cn94	2006	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn95	2007	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn100	2008	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn102	2008	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn104	2009	Busan	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn105	2009	Busan	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn137	2010	Seoul	Clinical	VNI	α	1	1	10	3	2	1	1	31
Cn163	2010	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn164	2011	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn165	2012	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn146	2012	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
ECn01	2012	Seoul	Environment	VNI	α	1	3	1	5	2	1	1	5
ECn02	2012	Seoul	Environment	VNI	α	1	1	10	3	2	1	1	31
ECn03	2012	Seoul	Environment	VNI	α	1	3	1	5	2	1	1	5
Cn170	2013	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn171	2013	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn172	2013	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn173	2013	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn180	2014	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn181	2014	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn182	2014	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5
Cn183	2014	Seoul	Clinical	VNI	α	1	3	1	5	2	1	1	5

ST, sequence type.

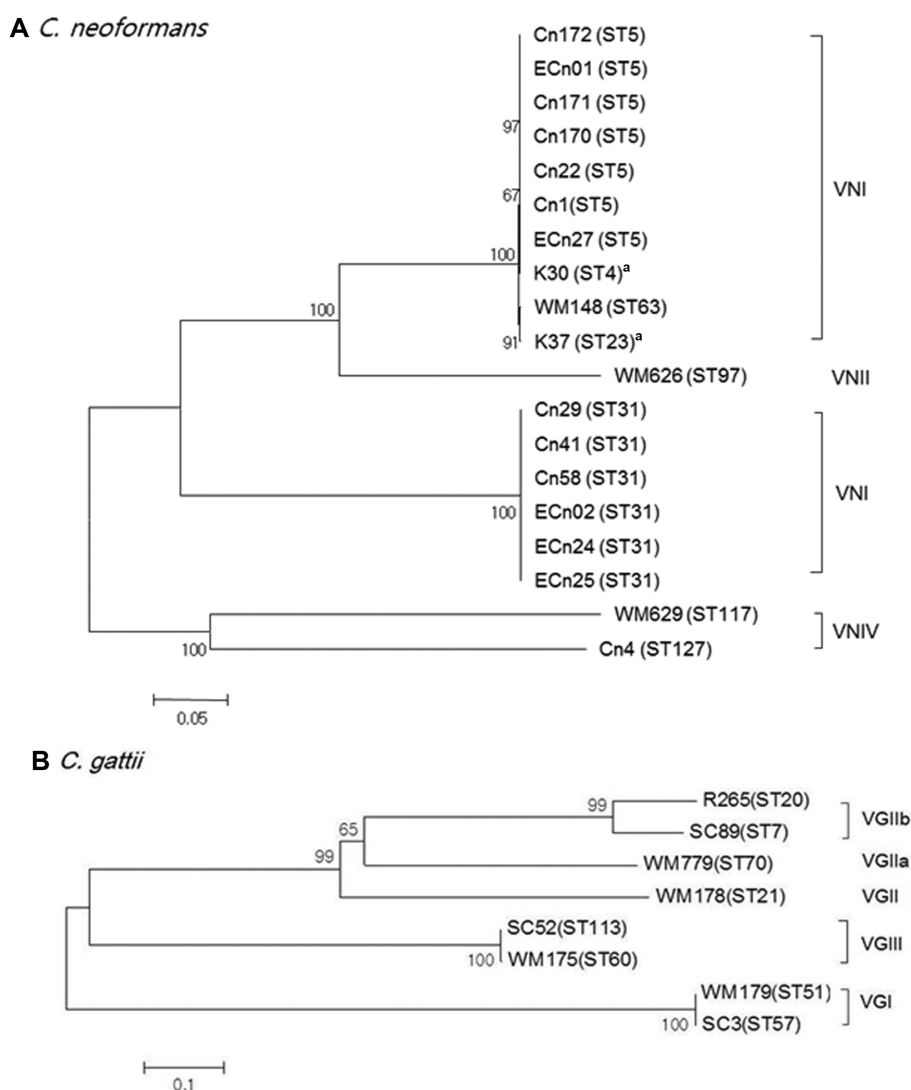


Fig. 1. Phylogenetic analysis of the Korean isolates and reference strains of *Cryptococcus neoformans* (A) and *C. gattii* based on neighbor-joining analysis of the concatenated seven International Society of Human and Animal Mycoses consensus multilocus sequence typing loci using the program MEGA 5.2. Numbers on each branch indicate the bootstrap values > 50%, based on 500 replications. ^aReference strains (Choi *et al.* [15]).

types VNIV, VGI, VGII, and VGIII were also identified (Table 2). All isolates were mating type α . The MLST results and other information for the selected isolates are summarized in Table 3. MLST analysis divided the 137 *C. neoformans* isolates into three STs, including ST5 (96%, $n = 131$), ST31 ($n = 5$), and ST127 ($n = 1$). Ten environmental isolates were also identified as ST5 ($n = 7$) and ST31 ($n = 3$). Our data demonstrate that the clinical and environmental strains in Korea are genetically homogeneous, with most of the genotypes classified as ST5 that was also found to be the major type among the Chinese and Japanese populations [16, 17]. However, the occurrence of the ST31 genotype in clinical and environmental isolates showed the genetic relatedness among the Asian countries including India and Thailand [11, 23]. MLST data for three *C. gattii* isolates showed the presence of ST57, ST7, and ST113. A

phylogenetic tree was constructed by the neighbor-joining method based on a concatenated data set from 7 loci for MLST (Fig. 1). The selected strains of *C. neoformans* showed three STs belonging to molecular types VNI and VNIV. Three *C. gattii* were STs belonging to molecular types VGI, VGII, and VGIII. On combining current and previous data, the rare isolates ST127, ST57, and ST113 were found to be unique to Korea. The ST7 type of *C. gattii* strain was identical to the less virulent Vancouver Island outbreak strain, VGIIb [12]. Previous studies suggested that the high prevalence of VNIIc-M5 (VNI-ST5) among non-HIV infected persons in Korea may be due to the cryptococcal ecology in Korea, and the possibility of the occurrence of VNIIc-M5 in the environment [15]. In this study, we confirmed a strong linkage between clinical and environmental isolates of VNI-ST5 and VNI-ST31 genotypes, which might

be the major causative genotypes of cryptococcosis in Korea. *C. gattii* infections are quite rare in temperate regions, especially Korea and Japan. Therefore, limited information on the molecular characteristics of the Korean *C. gattii* strain is available. Chen *et al.* [24] reported that nine *C. gattii* strains isolated from 1980 to 2006 were found to be the VGI molecular type, which is commonly isolated in South East Asia. Wu *et al.* [17] reported that two *C. gattii* were identified as ST44 (VGII) and ST159 (VGI) by MLST. In Asia, most genetic studies of the *C. neoformans/C. gattii* species complex have been performed in Thailand and India. The majority of the genotypes among the Thai cryptococcal isolates were ST4 and ST6 clones of *C. neoformans* molecular type VNI with 13 different STs, and ST7 of *C. gattii* molecular type VGII, the data of which indicated a significant genetic variation among different Asian regions [23]. Recently, increases in immunocompromised patients with malignant diseases and the elderly population in Korea have led to a higher incidence of invasive fungal infections such as cryptococcosis. Therefore, the clinical features and molecular epidemiology of the pathogenic *C. neoformans/C. gattii* species complex in Korea need to be continuously studied.

In conclusion, *C. neoformans* isolates in Korea are genetically homogeneous, with the majority of isolates belonging to ST5, and a strong linkage exists between clinical and environmental isolates. In addition, the occurrence of rare genotypes may serve as an important indicator of global epidemiologic analysis.

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