Genotyping of the Cryptococcus neoformans/ C. gattii *Species Complex*

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Fungal infections are increasing worldwide, yet little is known about their global epidemiology – which makes it difficult to develop an effective clinical response. Of particular concern are the basidiomycetous yeasts of the *Cryptococcus neoformans/C. gattii* species complex, which are the second most common cause of life-threatening fungal diseases in humans and animals worldwide (1). Although the incidence of disease is relatively steady in developed countries, in the developing world, it continues to escalate dramatically, and highly virulent strains of cryptococcosis that have emerged in parts of the developing world are now spreading to developed countries (1). Such fungal migration is best illustrated by recent (and ongoing) disease outbreaks in humans and animals on Vancouver Island, Canada (2), and in animals in Western Australia (D. Ellis, personal communication). This trend will be most likely exacerbated by global warming, as the causative fungal agents of cryptococcosis flourish in subtropical and tropical climates. Australia is one of many countries in which both cryptococcal species are endemic. Cryptococcal infection costs in Australia alone are about $8 million a year (Australian/New Zealand Mycosis Interest Group, unpublished data). As well as endangering people with HIV/AIDS, the disease poses a particularly high risk to the indigenous population. In Arnhem Land especially, where there is a high density of infectious particles, the disease has been found to be present subclinically, lying dormant in humans and animals (R. Malik, personal communication). In view of these facts, it is important to understand the global genotype structure of the cryptococcal population, to apply and develop typing methods, which will give laboratory technicians, researchers and clinicians the tools to assess the clinical risk associated with specific fungal genotypes. This will allow researchers to identify the emergence of highly virulent strains, improve therapy through early detection, and assist with an appropriate public health response, enabling a quick and effective intervention to prevent large-scale disease outbreaks.

Taxonomic Classification and Clinical Association

C. neoformans, the agent of cryptococcosis, had been considered a homogeneous species until 1949 when the existence of four serotypes was revealed based on the antigenic properties of its polysaccharide capsule [3]. Currently, the etiologic agent of cryptococcosis is classified into two species: 1) *C. neoformans* (also known as *Filobasidiella neoformans* when in the teleomorph stage), with two varieties: *C. neoformans* var. *grubii* (serotype A) [4] and *C. neoformans* var. *neoformans* (serotype D), as well as an AD hybrid; and 2) *C. gattii* (serotypes B and C) (also known as *F. bacillospora* when in the teleomorph stage) [5] (**Fig. 1**). *C. neoformans* causes disseminated cryptococcosis in both normal hosts and individuals with impaired immunity. *C. neoformans* var. *grubii* is the major fungal pathogen of patients with AIDS, affecting up to 30% of such patients in developing countries. It is estimated that it causes 2,740 deaths a day in the Sub-Saharan African HIV/AIDS population alone (Centers for Disease Control and Prevention, USA, unpublished data). *C. gattii* affects patients with normal immunity, causing major neurological morbidity. Strains of these species are the leading causes of potentially fatal fungal meningoencephalitis and pneumonia. The infection is assumed to be acquired via inhalation of infectious propagules (desiccated yeast cells [blastoconidia] or basidiospores) from the environment (1).

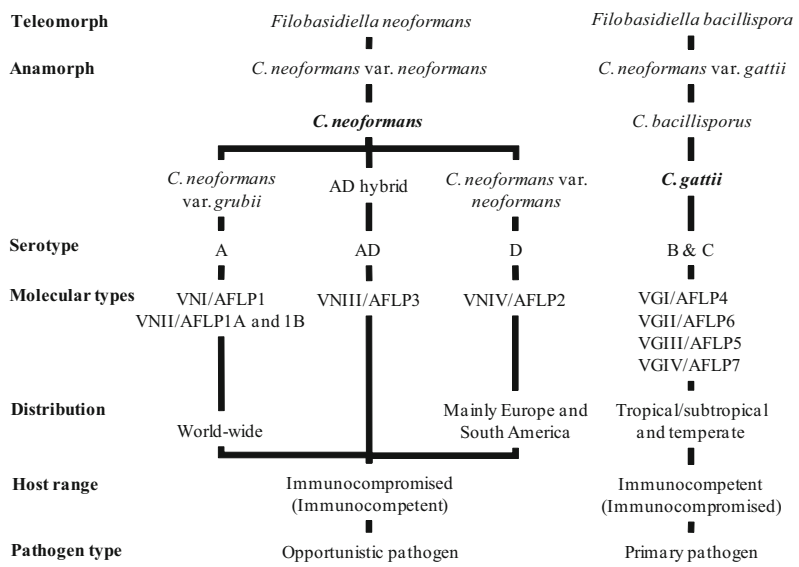

Fig. 1. The taxonomic, molecular, epidemiological and clinical correlation within the *C. neoformans/C. gattii* species complex.

Molecular Grouping

With the development of numerous molecular typing techniques, more intra-species genetic diversity and inter-species hybrid strains of AB and BD serotypes have been revealed. This led to the formulation of the *C. neoformans/C. gattii* species complex, with the number of scientifically valid species within this species complex still a controversial issue.

Due to the high importance of the *C. neoformans/C. gattii* species complex as human fungal pathogens, several research groups are focusing on the molecular determination of the number of genetically diverse sub-groups within each serotype. The molecular methods employed include DNA fingerprinting (6) and PCR fingerprinting based on microsatellite- (M13) or minisatellite-specific primers (e.g., $(GACA)_4$ or $(GTG)_5$) (7), random amplification of polymorphic DNA (RAPD) analysis (8), amplified fragment length polymorphism (AFLP) analysis (9), restriction fragment length polymorphism (RFLP) analysis of the *URA5* (7) and *PLB1* genes (10), the use of intergenic spacer (IGS) sequences (11), multi-locus sequence typing (MLST) (2, 12, 13) and multi-locus microsatellite typing (MLMT) (14). These analyses have revealed associations between the geographic origins of particular genotypes, implying an epidemiologic significance of certain genotypes.

The use of different genotyping methods resulted in various nomenclatures of the identified sub-groups (**Table 1**) (13). The currently recommended nomenclature, by the working group 'Genotyping of *Cryptococcus neoformans* and *C. gattii*' of the International Society of Human and Animal Mycoses (ISHAM), includes eight major molecular types, VNI–VNIV for *C. neoformans* and VGI–VGIV for *C. gattii* (**Fig. 1**) (13). This correlates with the current concept of two species and represents the global population structure based on more than 2,000 *C. neoformans* and *C. gattii* isolates as identified by two major molecular typing methods, PCR fingerprinting using primers specific for microsatellite (M13) or minisatellite $(GACA)_4$ DNA (7) and AFLP analysis (9). The molecular types of *C. neoformans* are thought to be correlated with the serotypes: *C. neoformans* var. *grubii*, serotype A, consists of molecular types VNI and VNII; the hybrid serotype AD comprises VNIII; and *C. neoformans* var. *neoformans*, serotype D, corresponds to VNIV. *C. gattii* consists of VGI, VGII, VGIII, and VGIV, which are all thought to correspond to both serotypes B or C (**Fig. 1**) (7, 13). Standard strains representing each of the eight major molecular types have been published (13) and are deposited at the CBS-Fungal Biodiversity Centre (http://www.cbs.knaw.nl), the American Type Culture Collection (http://www.atcc.org) or the Fungal Genetic Stock Center (http://www.fgsc.net).

Global Distribution of the Major Molecular Types

The worldwide distribution of the eight major molecular types within the *C. neoformans/C. gattii* species complex is based on the integrated analysis of 2,755 cryptococcal isolates obtained via *URA5*-RFLP (9), PCR-fingerprinting (7), AFLP (9) and MLST (13) analysis of 2,075 isolates collected within the international collaborative network coordinated by W. Meyer (7, 9, 15, W. Meyer, unpublished data) and additional published data from 680 isolates (16-18). The majority of the 2,046 clinical, 68 veterinary and 604 environmental isolates were *C. neoformans* isolates, with the molecular type VNI (serotype A) being globally the predominant molecular type (63% of the clinical/veterinary and 41% of environmental isolates were this type, **Fig. 2**).

Infections due to the four *C. gattii* major molecular types are substantially less common, totalling 20% compared

Table 1. Concordance of different molecular typing methods used for the *C. neoformans/C. gattii* species complex.

	C. neoformans* var. *grubii			**AD hybrid**	***C. neoformans* var. *neoformans***	***C. gattii***			
Serotype	A	A	A	AD	D	B/C	B/C	B/C	B/C
PCR-fingerprinting molecular type **Reference 7** **Reference 18**	VNI VN6 (VN5)	VNII	VNII VN7	VNIII VN3/VN4	VNIV VN1 (VN2)	VGI	VGII	VGIII	VGIV
AFLP genotype **Reference 9** **Reference 12**	AFLP1 VNI	AFLP1A/ AFLP1B VNB	AFLP1A/ AFLP1B VNII	AFLP3	AFLP2	AFLP4A/ AFLP4B	AFLP6	AFLP5A/ AFLP5B/ AFLP5C	AFLP7
***URA5* RFLP type** **Reference 7**	VNI	VNII	VNII	VNIII	VNIV	VGI	VGII	VGIII	VGIV
***PLB1* RFLP type** **Reference 10**	A1		A2	A3	A4	A5	A6	A7	A8
ITS genotype **Reference 11**	ITS1	ITS1	ITS1	ITS1/ITS2	ITS2	ITS3/ITS7	ITS4	ITS5	ITS6
IGS genotype **Reference 21**	1A/1B	1A	1C	2C	2A/2B/2C	4	3	5	6

to 80% caused by the four major *C. neoformans* molecular types. Independent analysis of the environmental isolates suggests that the two species are equally present among the globally isolated strains, 48% *C. neoformans* and 52% *C. gattii*.

Comparing clinical/veterinary and environmental *C. gattii* isolates revealed that most of the clinical isolates belong to VGI (9%), with only 7% belonging to VGII, while among the environmental isolates 35% belong to VGII and only 9% belong to VGI. This shift toward VGII isolates may be due to the extensive sampling efforts in connection with the Vancouver Island cryptococcosis outbreak that was caused by this molecular type. A clear difference was observed in the distribution of *C. neoformans* and *C. gattii*, with a global prevalence of *C. neoformans* but a higher prevalence of *C. gattii* in the Americas and the Southern hemisphere (**Fig. 2 and 3**). *C. gattii* was only rarely isolated from patients or the environment in Europe, including Russia, and parts of Asia, especially China, Thailand and Japan (**Fig. 2 and 3**).

C. neoformans, molecular type VNI, serotype A, is causing the majority of infections worldwide in immunocompromised patients (83.9%). All major molecular types of *C. neoformans* caused consistently more infections in immunocompromised patients (78.3%) compared to immunocompetent patients (21.7%), and all major molecular types of *C. gattii* caused consistently more infections in immunocompetent patients (85.3%) compared to immunocompromised patients (14.7%). Globally, the majority of infections in immunocompetent patients were caused by isolates of the *C. gattii* molecular types VGI and VGII. A strikingly different picture was obtained when only taking into account the Chinese isolates, where the majority of infections in immunocompetent patients were caused by *C. neoformans*, molecular type VNI (16). Similar findings have been reported from Korea (J. Kwon-Chung, personal communication). The reasons for this dramatic shift are unknown. This finding is especially intriguing because the opposite is the case in nearby countries such as India, Thailand and Japan, where the majority of infections caused by *C. neoformans* molecular type VNI are found in immunocompromised patients (W. Meyer and Y. Mikami, personal communication).

A shift in the geographical distribution was observed for the molecular types VNIII (AD hybrids) and VNIV (serotype D), which have been previously described mainly from Southern Europe, including Italy and France (19). Our data show that they also occur in relatively high numbers in Latin America, mainly Mexico, Colombia, Brazil and Chile (**Fig. 3**).

When analysing the environmental data alone, a dramatic shift in the distribution of the major molecular types was found. In South America, the most common molecular types isolated in the environment are VNI (46%) and VGII (36%), while in North America, VGII is the most common one (61%), followed by VNI (29%). This is most likely due to the large Vancouver Island cryptococcosis outbreak in Canada, which is caused by VGII isolates. In contrast, in Oceania, the most commonly found molecular type is VGI (70%) and in Europe, VNI (40%). Due to the small number of isolates, five and 13 respectively, no comments can be made for Africa and Asia. The high number of VGII isolates found in the Americas (**Fig. 1**), in connection with the phylogenetic placement of VGII as an ancestral population within the *C. gattii* clade (20), is in agreement with the original reporting of *C. gattii* being found in high numbers in South America (19). This reinforces the possibility that the origin of this species was South America (20), from where it dispersed globally. In addition it also became clear that *C. gattii* has extended its ecological niche from being restricted to tropical and subtropical areas as previously reported (20), to temperate regions, e.g., Argentina, Canada and Greece (**Fig. 3**).

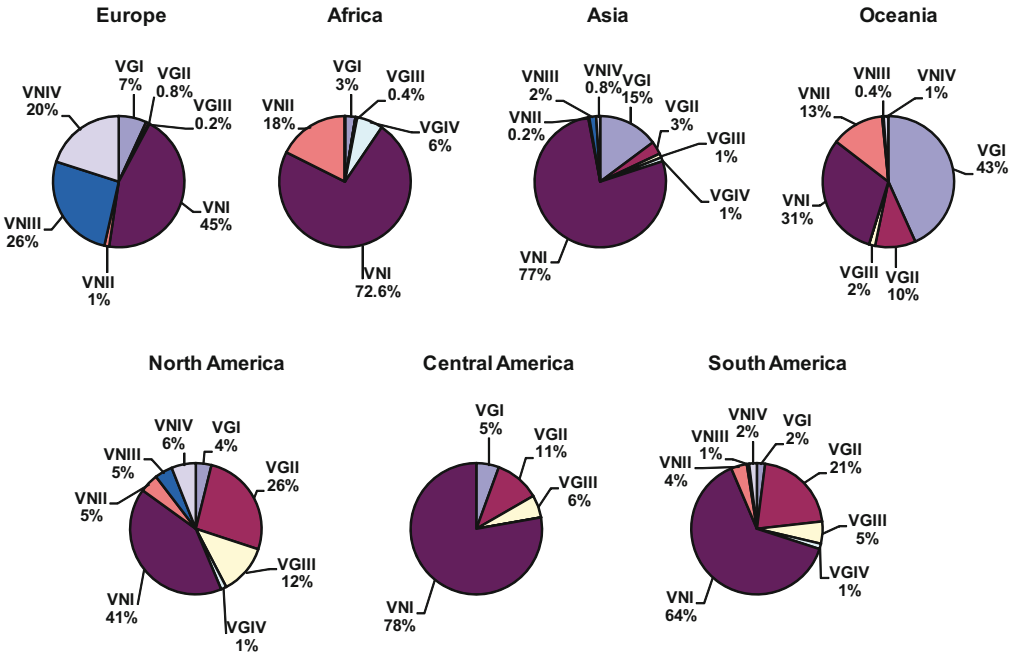

Fig. 2. Global distribution of the eight major molecular types of the *C. neoformans*/*C. gattii* species complex identified among 2,755 globally collected isolates (7, 9, 15-18, W. Meyer, unpublished data) using either URA5-RFLP, PCR-fingerprinting, AFLP and MLST analysis according to geographical regions/continents of origin.

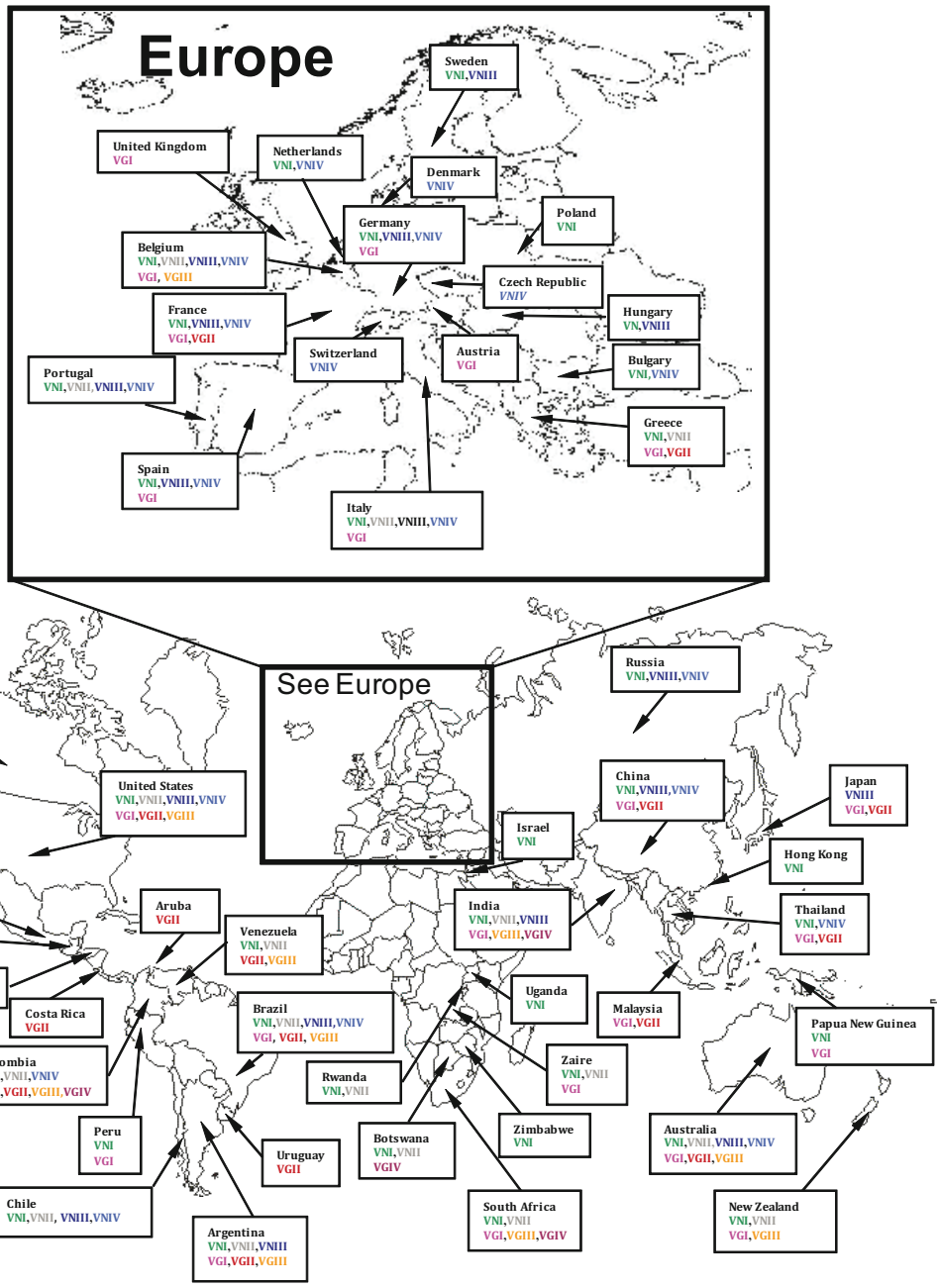

Fig. 3. Presence of the eight major molecular types of the *C. neoformans/C. gattii* species complex identified among 2,755 globally collected isolates (7, 9, 15-18, W. Meyer, unpublished data) using either URA5-RFLP, PCR-fingerprinting, AFLP and MLST analysis per studied country.

Concluding Remarks

Over the last two decades, an increasing number of molecular typing studies have been carried out to understand the genetic variation of cryptococcal strains, with the two major typing methods being PCR fingerprinting (7) and AFLP analysis (9). These studies have led to the establishment of the *C. neoformans/C. gattii* species complex comprising at least seven monophyletic lineages/major molecular types (VNI, VNII, VNIV, VGI, VGII, VGII, VGIV), excluding the serotype AD hybrid strains (VNIII) (**Fig. 1**). It was found that *C. gattii* has a much larger global distribution than previously considered (19) and that this species is not restricted to tropical and subtropical regions (**Fig. 3**). It was also found that the molecular types VNIII (the AD hybrids) and VNIV (serotype D) are not only mainly found in Southern Europe as previously reported (20), but are also present in the Americas (**Fig. 3**). Having established a baseline of the global molecular type distribution, we are now able to apply highly standardised methods such as MLST typing to investigate the global population diversity of the *C. neoformans/C. gattii* species complex, to study the gene flow between cryptococcal populations, to investigate recombination and to identify the spread of individual genotypes or, more specifically, emerging highly virulent strains around the globe.

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