Molecular epidemiology, environmental dispersion and antifungal susceptibility of Cryptococcus grubii and C. gattii prevalent in India

Anuradha Chowdhary
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Cover: Front Indian ink slide of Cryptococcus neoformans
Back Syzygium cumini, Delhi, India

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Molecular epidemiology, environmental dispersion and antifungal susceptibility of Cryptococcus grubii and C. gattii prevalent in India

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Chapter 1

General Introduction
Introduction

Cryptococcus neoformans (Sanfelice) Vuillemin, the classical etiologic agent of cryptococcosis, is currently recognized as a species complex, comprising C. neoformans var. grubii, serotype A, C. neoformans var. neoformans, serotype D, and C. gattii, serotypes B and C (Kwon-Chung et al., 2011, Simwami et al., 2011). Cryptococcosis is a life-threatening, opportunistic fungal infection of worldwide distribution, including India, especially occurring in the human immunodeficiency virus (HIV) positive patients (Casadevall & Perfect, 1998; Chakrabarti et al., 2000; Khanna et al., 2000; Lakshmi et al., 2007; Thakur et al., 2008). C. neoformans and C. gattii differ significantly with regard to their geographical distribution and ecological niches (Casadevall & Perfect, 1998; Kwon-Chung et al., 2002). The vast majority of cryptococcal infections, particularly in immunocompromised patients, are caused by C. neoformans var. grubii whereas C. gattii accounts for a smaller proportion of cases though it frequently infects immunocompetent patients in tropical and subtropical regions. In the past decade, a more virulent genotype of C. gattii (AFLP6A/VGIIa; AFLP6C/VGIIc) has emerged as a primary pathogen on Vancouver Island and its adjoining areas in Canada and the USA, revealing extension of this pathogen’s geographical domain to the temperate climate (Kidd et al., 2004; Datta et al., 2009; Byrnes et al., 2010).

Historical

Cryptococcus neoformans was isolated for the first time in 1894 by Sanfelice from peach juice in Italy (Sanfelice, 1894). He described it as an encapsulated yeast, demonstrated its pathogenicity for laboratory animals and named it Saccharomyces neoformans. In the same year, Busse (1894) and Buschke (1895) in Germany reported the first human case which they described as Saccharomyces haminis. For the next 57 years, this pathogenic yeast was known only from clinical cases, until the renowned medical mycologist, Chester Emmons (1951) reported 4 incidental isolations of C. neoformans in the USA during an investigation of 716 soil samples for Histoplasma capsulatum, employing the mouse-inoculation technique. Subsequently, Emmons (1955) reported frequent isolations of virulent C. neoformans strains from pigeon nests bearing old excreta, indicating that it was an environmental reservoir for the pathogen. His pioneering work stimulated worldwide studies which demonstrated that desiccated pigeon and other avian fecal matter constituted the most important natural habitat of C. neoformans. Fritz Staib and coworkers in Germany refocused attention on the pathogen’s original isolation from peach juice by Sanfelice when they reported its isolation from a ripe peach fruit (Staib et al., 1973; 1974) and demonstrated in vitro colonization by C. neoformans of dried leaves, stems and other parts of plants under defined laboratory conditions (Staib et al., 1972a; 1972b). In 1986, C. neoformans was isolated from wood samples collected from a hollow tree trunk inside an aviary in the Zoological Garden, Antwerp, Belgium (Bauwens et al., 1986). In this paper, reference was made to unpublished observations of Danielle Swinne on the isolation of C. neoformans var. neoformans from saw dust of tropical trees, Entandrophragma species, in a sawmill in Kinshasa, Congo. It was stated that although the isolation of C. neoformans from bark and wood from avaiaries could be due to contamination with bird droppings, it was nevertheless possible that some trees could provide a natural habitat for C. neoformans. Further investigations on the role of wood in the natural history of C. neoformans was therefore suggested. The environmental niche of C. gattii remained unknown for nearly two decades after this taxon was described by Vanbreuseghem and Takashio (1970). Attention to its natural habitat in trees was first drawn by Ellis and Pfeiffer (1990) who isolated serotype B strains from the debris of leaves and flowers of Eucalyptus camaldulensis in Australia.

Classification of the Cryptococcus species complex

Cryptococcal organisms belong to the Filobasidiella clade of the Tremellales (Order Tremellomycetes), and the genus Cryptococcus includes over 100 species, the overwhelming number of which are not pathogenic to mammals ( Fonseca et al., 2011). The most important pathogenic species are C. neoformans and C. gattii species complex, which includes C. neoformans var. neoformans and C. neoformans var. grubii (Table 1). Interspecies hybrids of C. neoformans and C. gattii may occur both in vitro and in vivo, and the distinction of these organisms as two separate species has been recently confirmed by molecular techniques and genomic analysis (Bovers et al., 2006; 2008). Their classification was originally based on serotype as determined by their capsular antigens, and they were divided as C. neoformans var. neoformans serotype D, C. neoformans var. grubii serotype A, with an additional hybrid serotype AD (diploid hybrid), and C. gattii serotype B or C (Kwon-Chung et al., 2011; Simwami et al., 2011). Genotyping has now replaced serological typing for the classification of these organisms, as the molecular genotype is more precise and aids in understanding relationships both within and between geographical areas. Genotyping of Cryptococcus spp. relies on various molecular methods, including PCR fingerprinting, amplified fragment length polymorphisms, restriction fragment length polymorphism, random amplification of polymorphic DNA, and multilocus sequence typing. (Boekhout & van Belkum, 1997; Meyer et al., 1999; 2003; Boekhout et al., 2001; Kidd et al., 2004; Meyer et al., 2009; Hagen et al., 2010). C. neoformans var. grubii (serotype A) isolates are molecular types VNI and VNJ, whereas C. neoformans var. neoformans is molecular type VNIV and the hybrid serotype AD is molecular type VNIII. C. gattii isolates are classified as VG1, VGII, VGIII, and VGIV. The B and C serotypes of C. gattii are not specific to any molecular types. Genetic subtypes exist within each molecular type and reflect different strains.
Phenotypical identification
Cryptococcus gattii differs from C. neoformans in various aspects, including a contrasting human host profile and a reduced susceptibility to certain antifungal drugs (Lin & Heitman, 2006; Khan et al., 2007; Hagen et al., 2010; Chowdhary et al., 2011b). (Table?) Since C. gattii is an emerging pathogen, it is important for the clinical microbiology laboratory to differentiate it from its closely relative C. neoformans. A proficiency testing survey administered by the New York State Department of Health indicated that only 5% of the clinical laboratories surveyed identified C. gattii, whereas the rest misidentified it as C. neoformans (New York State Department of Health, 2005). In comparison, the situation in developing countries is expected to be even more unsatisfactory. Various phenotypic techniques have been used for differentiating C. gattii from C. neoformans, including use of canavanine-glycine-bromothymol blue agar (CGB), glycine-cycloheximide-phenol red agar, creatine-dextrose bromothymol blue thymine agar and creatine-dextrose bromothymol blue agar (Kwon-Chung et al., 1978, 1982a; Salkin et al., 1982; Min & Kwon-Chung, 1986; Iroanenu et al., 1994). The CGB agar was reported to give fewer false-positive and false-negative results than the others. Currently, available commercial methods for yeast identification, such as API 20 AUX (bioMerieux, Paris, France), Vitek (bioMerieux), and MicroScan (Siemens, Erlangen, Germany) do not differentiate between C. neoformans and C. gattii. However, recently, matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has been successfully used to differentiate C. neoformans from C. gattii (McTaggart et al., 2011a). Serotyping is useful for differentiation of C. gattii from C. neoformans but the only commercial kit previously available for serotyping (Crypto-Check kit; Iatron Inc., Tokyo, Japan) is no longer manufactured. However, multiplex PCR and liquid array-based methods have been recently reported for differentiation of C. neoformans and C. gattii (Bovers et al., 2007; Feng et al., 2008), but this technology is not yet routinely utilized by most clinical laboratories. McTaggart et al. (2011b) evaluated an algorithm, incorporating commercial rapid biochemical tests, differential media and DNA sequence analysis for a rapid and accurate differentiation of C. gattii and C. neoformans. CGB agar or IGS sequencing differentiated these isolates within 48 h. On CGB, 25 of 27 (93%) C. gattii strains induced a blue color change in contrast to 0 of 86 C. neoformans isolates. Neighbour-joining cluster analysis of IGS sequences differentiated C. neoformans var. grubii, C. neoformans var. neoformans and C. gattii.

Molecular Identification
Over the past two decades, a variety of molecular techniques have been introduced for identification and typing of pathogenic fungi. Many of these techniques, as opposed to classical phenotypic characterization, have the potential to provide rapid, sensitive, and specific identification of the C. neoformans species complex. Molecular fingerprinting techniques, e.g. random amplified polymorphic DNA (Boekhout & van Belkum, 1997), restriction fragment length polymorphism (RFLP) (Meyer et al., 2003; Kidd et al., 2004), pulsed-field gel electrophoresis (Boekhout et al., 1997), luminex technology (Bovers et al., 2007; Diaz & Fell, 2005) amplified fragment length polymorphism (AFLP) (Boekhout et al., 2001; Hagen et al., 2010), PCR fingerprinting with minisatellite (M13) or microsatellite primers (GACA4 or CTG5) (Meyer et al., 1999; Meyer et al., 2003; Kidd et al., 2004), karyotypes (Boekhout et al., 1997), sequencing (Diaz et al., 2000; Katsu et al., 2004; Bovers et al., 2008a; 2008b; McTaggart et al., 2011b), multilocus microsatellite typing (Illnait-Zaragozi et al., 2010a,b), mating type locus (Cogliati et al., 2006) and multilocus sequence typing (MLST) (Litvintseva et al., 2006; Hiremath et al., 2008; Meyer et al., 2009; Chowdhary et al., 2011a) are techniques that have been applied to characterize the genetic heterogeneity of the C. neoformans species complex.

Restriction enzyme analysis of PCR products (REA-PCR), also called RFLP could not distinguish C. neoformans from C. gattii after enzymatic digestion of the PCR products with four restriction enzymes (Vilgalys & Hester, 1990). This was followed by PCR based molecular techniques. Meyer et al. (1995) tested several hybridization probes as single oligonucleotide primer PCR assays. Hybridization probes used for this approach were based on hypervariable and repetitive DNA sequences, such as the M13 phage core sequence, (GTG)ₙ, and (GACA)ₙ. Random amplification of polymorphic DNA is a promising method to distinguish not only C. neoformans from C. gattii but also the different serotype A and D versus the less polymorphic DNA fingerprints of serotype B and C. Based on PCR fingerprinting, AFLP analysis, analysis of the orotidine monophosphate pyrophosphorylase (URAS) and phospholipase (PLB1) genes by RFLP and MLST, C. neoformans and C. gattii have been further classified into several distinct genotypes: AFLP1/VNI and AFLP1A/AFLP1B/ VNII (C. neoformans var. grubii, serotype A), AFLP2/VNI (C. neoformans var. neoformans, serotype D), AFLP3/VNII (hybrid serotype AD), AFLP4/VGI, AFLP6A/VGila, AFLP6B/VGlib, AFLP6C/ VGiic, AFLPS/VGII, AFLP7/VGIV and AFLP10/VGIV (C. gattii, serotype B/C). In addition, hybrids of C. neoformans var. neoformans and C. gattii of C. neoformans var. grubii and C. gattii belong to genotypes AFLP8 and AFLP9, respectively (Bovers et al., 2006, 2008a).

Although more labour intensive and costly, DNA sequencing is rapidly becoming a common procedure in most clinical laboratories because of its greater discriminatory power than that provided by differential culture media and biochemical tests. In laboratories where DNA sequencing is routinely available, IGS, internal transcribed spacer (ITS) and D2 region of the fungal 28S large ribosomal subunit distinguish C. neoformans from C. gattii and these are optimal methods for identification of Cryptococcus species. However, sequencing of the D2 region of the 28S large ribosomal subunit may not be able to reliably distinguish C. neoformans var. grubii and C. neoformans var. neoformans (Klein et al., 2009). Similarly, sequencing of the ITS region differentiates C. gattii but it has poor discrimination (≥99.5% similarity) between C. neoformans var. neoformans and C. neoformans var. grubii (McTaggart et al., 2011b). Sugita et al. (2001), applied multi locus sequence typing (MLST), using the sequences of the IGS, ITS, topoisomerase (TOPI) and a locus encoding for a capsule associated protein (CAP59). Various MLST studies were performed to investigate whether or not the C. neoformans/C. gattii species complex consists of multiple species (Bovers et al., 2008b, 2009; Ngamskulrungroj et al., 2009; Xu et al., 2009). To analyse the population structure of C. neoformans and C. gattii, MLST studies from different geographic regions, especially endemic regions in Africa, India, Australia and Vancouver Island have emerged during the last few years (Byrnes et al., 2009a, 2009b, 2010; Carricone et al., 2011; Chen et al., 2008; Chowdhary et al., 2011a; Fraser et al., 2005; Hiremath et al., 2008;
Kidd et al., 2005; Litvintseva et al., 2003, 2007, 2009, 2011; Simwami et al., 2011; Xu et al., 2009). Hiremath et al. (2008) have analysed the structure of environmental populations of *C. neoformans* var. grubii, comprising 78 isolates originating from decayed wood in trunk hollows of 9 tree species in 5 geographical locations spread over the Union Territory of Delhi, Bulandshahr and Hathras (Uttar Pradesh), Amritsar (Punjab) and Amroli (Haryana) in north-western India. The isolates were subjected to MLST, using five gene fragments. All of the isolates were found to be molecular type VNI and mating type (α) strains, unambiguous evidence for recombination was observed which supported the hypothesis that strains of *C. neoformans* may undergo sexual reproduction on decaying wood of various host tree species. Interestingly, despite the lack of mating type (α) strains, unambiguous evidence for recombination was observed which supported the hypothesis that strains of *C. neoformans* may undergo sexual reproduction on decaying wood of various host tree species. The population structure of 109 isolates of *C. gattii*, serotype B, originating from the wood detritus of trees and the surrounding soil from nine different tree species at seven north-western locations, i.e., Amritsar, Union Territories of Chandigarh and Delhi, Amroli, Bulandshahr, Hathras and Meerut and one in Tamil Nadu, namely, Tiruvannamalai in south India was analysed by MLST, using nine gene fragments (Chowdhary et al., 2011a). Population genetic analyses revealed limited evidence of recombination but unambiguous evidence for clonal reproduction and expansion. The consensus MLST scheme was proposed using seven unlinked nuclear loci CAP59, GPD1, IGS1, LAC1, PLB1, SOD1 and URA5 (Meyer et al., 2009).

**Environmental prevalence**

The success in demonstration of *C. neoformans* in soil and avian excreta in early studies by the pioneers of Medical Mycology, Chester Emmons (1951), Libero Ajello (1958) and Maxwell Littman (1959) was achieved by employing the mouse inoculation technique. Investigations on the natural habitat of *C. neoformans* were further stimulated when Fritz Staib (1962) developed and introduced niger seed based agar, a selective medium for its rapid isolation and presumptive identification. The first environmental isolation of *C. neoformans* in India was made from old pigeon excreta in Delhi (Sethi et al., 1966), employing the mouse-inoculation technique. These observations were confirmed by Padhye and Thirumalachar (1967) from Pune and Gugnani and Sethi (1968) from Delhi who reported the isolation of *C. neoformans* from soil collected from a pigeon house situated within the National Zoological Park, New Delhi, and also from an old building inside the B.R. College Agricultural Farm, Agra. Subsequently, Khan et al. (1978) reported a more frequent association (55.4%) of *C. neoformans* with old pigeon excreta in a Centre for Bird Hospital, Delhi. This was followed by a larger study, covering 489 diverse natural substrates and employing Staib’s niger seed agar as a selective isolation medium. *C. neoformans* was reported from 38 of 253 (15%) old avian excreta investigated (Pal et al., 1979). Outside of India, *C. neoformans* has been reported from 5/97 (5.2%) excreta samples of swallow (*Hirundo rustica*) in Iran (Hedayati et al., 2011), 24% of chicken faeces in Thailand (Kuroki et al., 2004) and from 25.5% of excreta of caged passerine and psittacine birds in Brazil (Lugarini et al., 2008). It seems pertinent to point out here that avian excreta is primarily a natural habitat of *C. neoformans*, although *C. gattii*, serotype B has been sporadically isolated from this substrate (Abegg et al., 2006). Nielsen et al. (2007) have reported that pigeon guano supported in vitro growth of both species, and it allowed a prolific mating of *C. neoformans* but not of *C. gattii*. Consequently pigeon guano represents a fundamental but not a realized environmental niche for *C. gattii*. Worldwide literature reports have firmly established that desiccated excreta of pigeons and other avian species are an excellent natural substrate for the growth and multiplication of *C. neoformans* in the environment. In saprobic settings, *C. neoformans* is inhibited by UV light and temperature exceeding 44°C. It can catabolize high concentration of urea, catecholamines and other nitrogenous compounds in pigeon excreta (Fiskin et al., 1990). Also, it can produce laccase and become melanised (Nosanchuk et al., 1999) which provides some protection against UV radiation, temperature extremes and oxidative compounds. The melanin chelates silver and perhaps other toxic heavy metals that protects the fungus against degrading enzymes (Rosas & Casadevall, 2001; Garcia-Rivera & Casadevall, 2001). The ability to produce urease, enables it to thrive on urea and other nitrogenous compounds in the excreta. *C. neoformans* may produce potentially infectious basidiospores in the pigeon excreta. In sites with pigeon manure harbouring *C. neoformans*, air sampling has demonstrated aerosols of yeast cells and basidiospores (Litvintseva et al., 2011). Therefore, desiccated pigeon excreta constitute a plausible source of human cryptococcosis.

**Soil**

*Cryptococcus neoformans* and *C. gattii* have been frequently reported from soil and dust. However, these isolations are from samples that contained excreta of pigeons, other avian species or bats (Emmons, 1955; Ajello, 1958; Casadeval & Perfect, 1998). In a survey of soil-inhabiting human pathogenic fungi in India, Gugnani and Shrivastav (1972) reported the isolation of *C. neoformans* from four out of 308 soil samples, employing the mouse-inoculation technique. Three of the 4 positive soil samples were rich in bat guano and collected from a historical monument, whereas the remaining positive sample contained traces of decomposed plant material and originated from a riverine site. Recently, Randhawa et al. (2008) have reported isolation of both *C. neoformans* and *C. gattii* from soil surrounding the base of a number of host trees harboring these pathogens in decayed wood of their trunk hollows. Depending upon the site of investigation, the prevalence in soil ranged from 11 to 50% for *C. neoformans*, 14–57% for *C. gattii* and 7–11% for concomitant occurrence of both the species in the same soil sample. These findings are in concordance with the results of an extensive study on characterization of environmental sources of *C. gattii* including soil samples collected from within one meter of the base of many host trees investigated in British Columbia, Canada, and the Pacific Northwest of the US (Kidd et al., 2007b).

**Trees, decayed wood, other plant debris**

The environmental habitat of *C. gattii* was unknown until investigators from Australia in the early 1990’s reported its association with debris of Eucalyptus trees (Ellis & Pfeiffer, 1990; Pfeiffer & Ellis, 1992). This work led to a number of worldwide studies, focusing on plant debris, especially decayed wood inside trunk hollows, to investigate it as a natural habitat of *C. gattii* and *C. neoformans* (Lazera et al., 1993; 1996; 2000; Callejas et al., 1998; Randhawa et al., 2000; Restrepo et al., 2000; Krockenberger et al., 2002; Granados et al., 2005; Ribeiro
et al., 2006). In a retrospective analysis of their data (1992-2004), Granados and Castaneda, employing a logistic regression model and log-transformed Pearson correlations, explored the relationship between the occurrence of serotypes of the *C. neoformans* species complex in tree samples and the climatic conditions in Colombia (Granados & Castaneda, 2006). Their results suggested that climatic conditions, mainly humidity, temperature, evaporation and solar radiation affected the environmental occurrence of the various serotypes. Contrary to the Australian experience, investigations done in India indicated that *C. gattii* was rarely associated with *Eucalyptus* trees, and that the most important host tree for *C. gattii*, as well as *C. neoformans* in northwestern India was *Syzygium cumini*. *Cryptococcus gattii* was reported for the first time from India in association with trees by Chakrabarti et al. (1997) who isolated it from 5 of 354 (1.4%) plant debris samples of *Eucalyptus camaldulensis* in Punjab. A low prevalence (0.4%) for *C. gattii* in flowers of *E. tereticornis* and in bark of *E. camaldulensis* for *C. neoformans var. grubii* has also been reported by Gugnani et al. (2005). On the other hand, *C. gattii* was not found in any of the 86 *E. camaldulensis* trees sampled by Abraham et al. (1997) in Vellore, South India. This was in agreement with the negative results for *C. gattii* reported by Swinne et al. (1994) who investigated 657 *Eucalyptus* samples collected in Rwanda, Africa, Hamasha et al. (2004) from Jordan and Ergin et al. (2004) from Turkey who investigated 500 and 1175 plant debris samples related to *Eucalyptus* trees, respectively. In yet another study, covering 732 environmental samples in Vancouver Island, Canada, *C. gattii* was not found in any of the *Eucalyptus* debris samples although it was isolated from several native tree species such as alder (*Alnus* spp.), cedar (*Cedrus* spp.), Douglas fir (*Pseudotsuga menziesii*), Garry oak (*Quercus garryana*) and grand fir (*Abies grandis*) (Kidd et al., 2004; 2007b). Besides, the results were negative for *C. gattii* in plant 498 bark samples (*E. tereticornis* - 104, *E. camaldulensis* - 98, unidentified *Eucalyptus* species - 188) collected from Delhi, Dehradun (Uttar Pradesh) and Amritsar (Punjab) in north-western India. However, one isolate of *C. neoformans var. grubii* was isolated, which provided the first evidence in India of decayed wood in trunk hollows of living trees as a potential ecologic niche other than avian excreta for *C. neoformans var. grubii* (Randhawa et al., 2001). The afore-mentioned study led to more intensive investigations that aimed to characterize the environmental reservoirs of *C. gattii* and *C. neoformans*. It was found that *Ficus religiosa* trees harbored only *C. neoformans var. grubii* in their trunk hollows or fissures, whereas this variety and *C. gattii*, serotype B, were both equally distributed (prevalence 10.6%) in decayed wood inside trunk hollows of the 66 *S. cumini* trees investigated. Furthermore, *C. gattii* was repeatedly isolated on 36/44 (82%) occasions from 7 *S. cumini* known positive trees sampled longitudinally over a period of 689 days. These data strongly supported a long-term colonization of decayed wood inside trunk hollows of *S. cumini* by both the pathogens.

**Reproduction and population structure**

*Cryptococcus neoformans* and *C. gattii* are haploid yeasts that predominantly reproduce asexually, i.e. by budding (Figure 1). However, they also possess a bipolar mating system, with mating types *MATa* and *MATa* (*Kwon-Chung, 1975; 1976a*). Mating may occur if cells of opposite mating types meet (*Kwon-Chung, 1975; 1976a; 1976b*). Mating involves fusion between cells of opposite mating type (α and α), resulting in conversion from a haploid budding yeast form to a dikaryotic mycelial form that ultimately produces basidia and basidiospores, which may serve as infectious propagules. The vast majority of clinical and environmental isolates are predominantly mating types α which have been linked to virulence. The discovery that monokaryotic fruiting under laboratory conditions represents a novel type of sexual reproduction involving only one of the two mating types, most commonly *MATa* revealed that same sex or unisexual reproduction could profoundly influence the population structure. In fact, recent population genetic studies provide robust evidence that both α-α opposite sex mating and α-α unisexual mating occur in nature in both *C. neoformans* and *C. gattii*, with the potential to influence the evolutionary trajectory and the production of infectious spores. Initial reports showed that during mating mitochondria are inherited from the parent opposite sex mating and α-α unisexual mating occur in nature in both *C. neoformans* and *C. gattii*, with the potential to influence the evolutionary trajectory and the production of infectious spores. *Cryptococcus neoformans* cells may also reproduce by haploid fruiting, a process that involves diploidization and meiosis (Lin et al., 2005) and occurs in response to nitrogen starvation and/or desiccation (Wickes et al., 1996). Haploid fruiting might occur through self-diploidization or through cell-cell fusion (Lin et al., 2005). Haploid fruiting resembles mating, but there are some differences. Mating occurs between cells of opposite mating types (*Kwon-Chung, 1976*; *Kwon-Chung & Popkin, 1976*), whereas haploid fruiting involves cells of the same mating type (*Wickes et al., 1996*). During haploid fruiting the nuclei fuse in the hyphae (Lin et al., 2005), whereas during mating the fusion of nuclei occurs in the basidium (*Kwon-Chung, 1976*). Furthermore, clamp connections of hyphae produced during mating are fused, whereas clamp connections produced during haploid fruiting are unfused (*Wickes et al., 1996*).
During haploid fruiting, basidia with viable basidiospores are formed, albeit at a lower frequency than in a standard MATa-MATa mating (Wickes et al., 1996). Although haploid fruiting has first been described in MATa isolates of all serotypes (Wickes et al., 1996), it has also been observed in a few MATa isolates (Tscharke et al., 2003). Interestingly, one of the environmental C. gattii isolates from Vancouver Island is a diploid homoygous MATa isolate (Fraser et al., 2005), which may have been generated by aberrant haploid fruiting. Haploid fruiting, or more specifically for the resulting filamentation, may fulfil several purposes. First, it may increase the chance of finding a mating partner (Wang et al., 2000; Hull & Heitman, 2002). An indication of this is the stimulation of haploid fruiting of MATa cells in response to MFA pheromone (Shen et al., 2002). Secondly, it may increase the foraging capacity under low nutrient conditions, as is indicated by the observation that haploid fruiting of MATa cells is enhanced by overexpression of the MFA1 pheromone gene, which is induced under starvation conditions (Davidson et al., 2000; Shen et al., 2002). Recently, a phenomenon called same-sex mating, i.e. mating between two non-isogenic MATa cells, has been described (Lin et al., 2005; Yan et al., 2007). The isolation of serotype A MATa-serotype D MATa environmental isolates (Litvintseva et al., 2005a) suggests that same-sex mating may occur in the environment. Although mating of C. neoformans or C. gattii can be induced under laboratory conditions (Kwon-Chung, 1975; Kwon-Chung & Popkin, 1976), it has never been found in the environment. In addition, past studies have found evidence for a clonal population structure (Brandt et al., 1993; 1996; Chen et al., 1995; Franzot et al., 1997). However, when C. neoformans var. grubii and var. neoformans were studied separately, the null hypothesis of recombination, which indicates sexual reproduction, could no longer be rejected (Taylor et al., 1999). In addition, analysis of the CNLAC1 and URA5 genes for AD hybrid isolates showed that recombination occurred within each variety (Xu & Mitchell, 2003). Furthermore, evidence for recombination was found in subpopulations of var. grubii (Litvintseva et al., 2003; 2005a), var. neoformans (Litvintseva et al., 2005a) and in subpopulations of C. gattii AFLP6 (Campbell et al., 2005a). In summary, C. neoformans and C. gattii predominantly reproduce clonally, but in some subpopulations sexual reproduction may occur.

**Mating-type locus**

Fungal sexual reproduction is genetically regulated by the mating-type (MAT) locus, a specialized region of the genome that is idiomorphic or allelic between different sexes, regulates the sexual cycle which differs between cells of opposite mating type. Cryptococcus neoformans and C. gattii possess a single MAT locus, which is unusually large, i.e. more than 100 kb (Lengeler et al., 2002; Fraser et al., 2004; Fraser et al., 2005; Ren et al., 2005). It encodes more than twenty genes, including homeodomain genes which establish cell type identity, genes involved in pheromone production and sensing, components of a MAP kinase cascade, essential genes, and genes which do not seem to have a function in mating (Lengeler et al., 2002; Fraser et al., 2004). Evidence suggests that the ancestor of C. neoformans had two unlinked sex determining regions, which expanded by acquisition of genes of related function. A chromosomal translocation fused the two regions, which resulted in a tripolar intermediate mating system that collapsed into a bipolar system. In this bipolar system, recombination suppressing inversions occurred, which resulted in the currently known MAT loci (Fraser et al., 2004). The Cryptococcus species complex yeasts have a bipolar mating system involving a and α cells. One unique feature of the C. neoformans serotype A and C. gattii populations is that the vast majority (> 99.9% in serotype A) of clinical and environmental isolates are mating type α; mating type a strains are extremely rare, except in a population isolated from the sub-Saharan region in Africa, in which MATa makes up 10% of the population (Litvintseva et al., 2003). Interestingly, the majority of environmental and clinical isolates belongs to MATa. (Kwon-Chung & Bennett, 1978; Jong et al., 1982; Hironaga et al., 1983; Schmeding et al., 1984; Madrenys et al., 1993; Takeo et al., 1993; Chen et al., 1995; Halliday et al., 1999; Ordóñez & Castañeda, 2001; Okhusu et al., 2002; Yan et al., 2002; Casali et al., 2003; Fraser et al., 2003; Litvintseva et al., 2003; Huerfano et al., 2003; Trilles et al., 2003; Campbell et al., 2005a; Campbell et al., 2005b; Litvintseva et al., 2005a; 2005b; Escandón et al., 2006; Saracci et al., 2006; Abegg et al., 2006; Okabayashi et al., 2006; Viviani et al., 2006). However, in some cases C. gattii MATa and MATa isolates have been found in an 1:1 ratio and MATa isolates sometimes even outnumbered the MATa isolates (Halliday et al., 1999; Escandón et al., 2006), which indicates that in some niches MATa isolates are just as common as MATa isolates. Unequal inheritance of the MAT-locus could provide an explanation for the excess of MATa isolates (same?). However, in laboratory crossings MATa and MATa isolates were either obtained in an 1:1 ratio (Kwon-Chung et al., 1976a; Kwon-Chung, 1980; Kwon-Chung et al., 1982a; 1982b; Keller et al., 2003; Wickes et al., 1996; Litvintseva et al., 2003; Tscharke et al., 2003; Nielsen et al., 2003) or an excess of MATa progeny was obtained (Kwon-Chung et al., 1982a; Tscharke et al., 2003). It has also been suggested that MATa isolates might be more virulent than MATa isolates. Virulence studies have been conducted comparing congenica and α strains. In both the serotype D JEC20/JEC21 and NIH433a/NIH433α backgrounds, α cells are more virulent than a cells (Kwon-Chung et al., 1992; Heitman et al., 1999). However, in the serotype D background, the congenic strains (B-3501a/B-3501α) are equally virulent (Kwon-Chung et al., 2003; 2005a; 2005b; Escandón et al., 2006; Saracci et al., 2006; Abegg et al., 2006; Okabayashi et al., 2006; Viviani et al., 2006). In addition, Barchiesi et al. (2005) found that the presence of a serotype A-MATa allele is associated with virulence. Finally, virulence studies carried out with serotype D congenic pairs of different genetic background showed that the genetic background plays a significant role in determining the effect of mating type on virulence (Nielsen et al., 2005b). These studies indicate that MATa can be associated with virulence, although the genetic background of the isolate determines whether an effect is present.

**Epidemiology**

Cryptococcus neoformans and C. gattii are life-threatening etiologic agents of fungal meningitis, with an increasing number of global cases occurring in HIV/AIDS patients but more so in developing countries. An estimated one million cases of cryptococcal meningitis occur globally per year in AIDS patients, resulting in approximately 625,000 deaths (Park et al., 2009). India has the second largest burden of cryptococcosis due to an estimated population of 3.1 million to 9.4 million persons living with HIV/AIDS (UNAIDS, 2006). The
country has a documented high prevalence (1.7–4.7%) of cryptococcosis in persons with HIV/AIDS (Kumarasamy et al., 2003; Vajpayee et al., 2003). The large global burden of cryptococcosis presents a number of challenges to public health, particularly in resource-deficient regions of high HIV prevalence in sub-Saharan Africa and South/Southeast Asia. Cryptococcal meningitis (CM) is an AIDS-defining illness in 25–30% and 64–91% of cases in South-East Asia and sub-Saharan Africa, respectively. (Heyderman et al., 1999; Bogaerts et al., 1999; Gordon et al., 2000; Schutte et al., 2000; French et al., 2003; Kim et al., 2003; Kumarasamy et al., 2003; Bekondi et al., 2006; McCarthy et al., 2006; Kong et al., 2007; Park et al., 2009). A recent study by Park et al. (2009) estimates 958,000 cases of CM occur each year (range: 371,700–1.54 million). Table 2 summarizes the estimated HIV-related CM cases in different areas of the world. The region with the greatest number of cases was Sub-Saharan Africa, with 720,000 cases per year followed by South and Southeast Asia, with 120,000 cases per year. Western and Central Europe (500 cases) and Oceania (100 cases) had the fewest estimated cases. On the basis of these data and the estimates of case fatality, approximately 624,725 deaths (range: 124,956–1,124,494) were associated with CM. When comparing deaths in Sub-Saharan Africa, it is clear that it is one of the leading causes of infection-related mortality, possibly even causing more deaths than more common infections such as tuberculosis.

Table 2. Estimated global distribution of cryptococcal meningitis cases in HIV patients. (After Park et al., 2009).

<table>
<thead>
<tr>
<th>Geographic region</th>
<th>Number of cases (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-Saharan Africa</td>
<td>720,000 (144,000–1,296,000)</td>
</tr>
<tr>
<td>South and South-East Asia</td>
<td>120,000 (24,000–216,000)</td>
</tr>
<tr>
<td>Eastern Europe and Central Asia</td>
<td>27,200 (5400–49,000)</td>
</tr>
<tr>
<td>Western and Central Europe</td>
<td>500 (100–1000)</td>
</tr>
<tr>
<td>North Africa and Middle East</td>
<td>6500 (1300–11,600)</td>
</tr>
<tr>
<td>North America</td>
<td>7800 (1600–14,000)</td>
</tr>
<tr>
<td>Caribbean</td>
<td>7800 (1600–14,100)</td>
</tr>
<tr>
<td>Latin America</td>
<td>54,400 (10,900–97,900)</td>
</tr>
</tbody>
</table>

Scope of this Thesis

Cryptococcus neoformans and C. gattii are free living saprobes in nature, infecting human and animal hosts when air-borne infectious propagules (yeast cells or basidiospores) are inhaled. As the infection is acquired from exogenous sources and is ordinarily not transmissible from one infected individual to another, it is vitally important to have first-hand knowledge of environmental prevalence of C. neoformans and C. gattii with a view to designing any possible control measures against cryptococcosis. The outbreak of human and animal cryptococcosis on Vancouver Island due to C. gattii indicated that exposure to environmental sources such as colonized trees and soil led to pulmonary and disseminated cryptococcosis. Besides, it is necessary to make environmental isolations of C. neoformans and C. gattii in order to probe their genetic structure. Furthermore, the advances made in genotyping techniques and their application to environmental isolates will address some important questions, such as, is there any evidence of ecological specialization among C. neoformans isolates from pigeon guano and those from decayed wood or other plant debris? The studies conducted in the present thesis contributes to our knowledge of the environmental distribution, molecular ecology and antifungal susceptibility profiles of C. gattii and C. neoformans prevalent in north-western India.

Part I deals with the application of molecular biological techniques to investigate the population structure of Indian C. neoformans and C. gattii inhabiting north-western India. Chapter 2 describes the structure of environmental populations of C. neoformans var. grubii, isolates originating from decayed wood in trunk hollows of 9 tree species in 5 geographical locations, i.e. Union Territory of Delhi, Bulandshahr and Hathras (Uttar Pradesh), Amritsar (Punjab) and Amrouli (Haryana) in north-western India. The isolates were subjected to MLST, using five gene fragments. In addition their mating types were determined and population analysis was done. Chapter 3 discusses population structure, recombination, clonal expansion and multilocus sequence typing in environmental populations of C. gattii serotype B, originating from wood detritus of trees and the surrounding soil from nine different tree species at seven north-western locations, i.e. Amritsar, Union Territories of Chandigarh and Delhi, Amrouli, Bulandshahr, Hathras and Meerut and one in Tamil Nadu, namely, Tiruvannamalai in south India. Multi locus sequence typing was conducted for all isolates using nine gene fragments. In Part II the first chapters 4–6 are devoted to antifungal susceptibility profiles, serotypes, genotypes and mating types of environmental and clinical C. neoformans and C. gattii isolates from north-western India. Chapter 4 is the first antifungal susceptibility testing report on environmental isolates from India. It was based on 117 isolates of C. neoformans, serotype A, and 65 of C. gattii, serotype B, originating from decayed wood in trunk hollows of F. religiosa and S. cumini trees. The study compares the MICs of amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole for C. gattii and C. neoformans highlighting the absence of primary resistance in Indian environmental isolates. The results presented in chapter 5 show that C. neoformans var. grubii and C. gattii serotype B isolates differ in their susceptibilities to the azoles fluconazole and voriconazole. The methodologies used i.e., RPMI agar by disc diffusion test and Etest, yielded comparable results notably C. gattii isolates showed...
significantly reduced susceptibility vis-à-vis of C. neoformans isolates. **Chapter 6** describes serotypes, genotypes and mating types of 308 isolates of C. neoformans var. gruibi and C. gattii serotype B originating from clinical and environmental sources from north-western India. Their genotypes were determined based on two methods: (i) PCR fingerprinting using (GACA)4 and M13 phase core sequences as single primers and (ii) DNA sequences at the URA5 locus. **Chapter 7** represents a multicenter Asian comparative study of antifungal susceptibility and genetic diversity of the C. neoformans var. gruibi by microsatellite typing of approximately 500 isolates from seven Asian countries, including 60 isolates from North India. **Chapter 8** is based on an international study of wild-type susceptibility endpoint distributions and epidemiologic cutoff values for amphotericin B and fluocytosine for C. neoformans var. gruibi and C. gattii. Wild type (WT) MIC distributions were constructed to estimate epidemiologic cutoff values (ECVs) for C. neoformans and C. gattii versus amphotericin B and fluocytosine. A total of 3,590 amphotericin B and 3,045 fluocytosine CLSI MICs for C. gattii and 985 and 853 MICs for C. gattii, respectively were gathered in 9-16 (amphotericin B) and 8-13 (fluocytosine) laboratories (Europe, United States, Australia, Brazil, Canada, India, and South Africa) and aggregated for the analyses. **Part III** is an account of work aimed to describe the environmental prevalence and distribution of both C. gattii and C. neoformans in north-western India. **Chapter 9** provides an update of the environmental prevalence of C. neoformans and C. gattii in India. The primary ecological niche of both pathogens is decayed wood in trunk hollows of a wide spectrum of host trees, representing 18 species, is discussed. Distribution of both species in soil and air in close vicinity of their tree hosts is discussed. The study also describes an overwhelming number of C. neoformans strains belonged to genotype AFLP1/VNI, var. gruibi (serotype A), whereas C. gattii strains were genotype AFLP4/VGI, serotype B. All of the environmental strains of C. neoformans and C. gattii were mating type α (MATα). **Chapter 10**, highlights the widespread prevalence of C. neoformans and C. gattii in decayed wood inside trunk hollows of a wide spectrum of host trees and from soil near the base of various host trees from Delhi and several places in the Indian states of Uttar Pradesh, Haryana, Tamil Nadu and Chandigarh Union Territory. In addition the data on high prevalence, fungal population density, perennial colonization and aerial isolations from decayed wood in trunk hollows of S. cumini trees, the well documented primary environmental niche of C. gattii and C. neoformans in north-western India is discussed. **Chapter 12** describes the seasonal variations in the prevalence of C. neoformans var. gruibi and C. gattii in decayed wood inside trunk hollows of diverse tree species. In **Chapter 13** the previously described techniques to isolate Cryptococcus from environmental sources were successfully applied in a sampling study in the temperate climate of the Netherlands. The tropical fungus C. gattii was for the first time isolated outside its normal niche in (sub) tropical areas in Northern Europe. In addition C. neoformans was for the first time isolated from a living tree in the Netherlands. The general discussion and final conclusion are found in **Chapter 14**.

**References**


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Sanjay S. Hiremath Anuradha Chowdhary Tusharantak Kowshik Harbans S. Randhawa Sheng Sun Jianping Xu
Chapter 2

Summary

The basidiomycete yeast *Cryptococcus neoformans* is a cause of significant morbidity and mortality in immunocompromised hosts throughout the world. The sporadic nature of the infection and the limited empirical evidence for direct human-to-human transmission have led to the belief that infections in humans are predominantly caused by the inhalation of basidiospores from environmental sources. Therefore, analysing the structure of environmental populations of *C. neoformans* can significantly increase our understanding of its ecology, evolution and epidemiology. Decaying wood is a rich source of organic and inorganic compounds and is known to be a suitable ecological niche for many micro-organisms, including *C. neoformans*. However, relatively little is known about the population structure of *C. neoformans* sampled from decaying wood. In this study, we analysed samples of *C. neoformans* var. *grubii* colonizing decaying wood in tree hollows of nine tree species in five geographical locations (Delhi, Bulandshahar, Hathras, Amritsar and Amroli) in north-western India. Multilocus sequence typing was conducted using five gene fragments for each of 78 isolates. All isolates belonged to mating type *α*. Population-genetic analyses identified no evidence for significant differentiation among populations belonging to either different geographical areas or different host tree species. Interestingly, despite the lack of mating type *α* strains in our survey, we found unambiguous evidence for recombination in our population analyses. Our results are consistent with the hypothesis of long-distance dispersal and recombination in environmental populations of this species in India.

Introduction

*Cryptococcus neoformans* var. *grubii* is the most common causative agent of cryptococcosis and can lead to significant morbidity and mortality in hosts with a defective T-cell-mediated immunity (Casadevall & Perfect, 1998). It has a global distribution and has been isolated from both human and environmental sources such as avian guano, soil, fruits, vegetables and decaying hollows of more than ten species of trees in different parts of the world (Randhawa et al., 2003; Reimão et al., 2007). Current surveys suggest that the prevalence of cryptococcosis caused by strains of *C. neoformans* var. *grubii* varies among geographical regions, probably a reflection of their variable frequencies in different environments (Bennett et al., 1977; Casadevall & Perfect, 1998; Tintelnot et al., 2004). *C. neoformans* var. *grubii* represents strains of serotype A, one of the five serotypes (A, B, C, D and AD) defined based on their cell-surface antigenic properties within the pathogenic cryptococcal species complex.

Like those of other micro-organisms in nature, environmental conditions for *C. neoformans* may change and fluctuate significantly, both spatially and temporally, in nutrient levels, temperature, water availability, etc. Under conditions of low nutrient availability, low moisture and a temperature in the range of 15–33 °C, mating and sexual reproduction between strains of opposite mating types may occur, resulting in the production of basidiospores. These sexual spores are smaller than vegetative cells and can disperse easily by wind or other means to other environmental niches, including human hosts. The mating system of this heterothallic basidiomycete is controlled by one locus with two alternative alleles: MATa and MATα (Kwon-Chung, 1975, 1976). Previous studies have shown that the majority of clinical and environmental isolates belong to the MATa mating type (Kwon-Chung & Bennett, 1978; Yan et al., 2002). Interestingly, a laboratory MATα strain that belonged to serotype D (one of three serotypes, A, D and AD, in *C. neoformans*) has been shown to be more virulent than its congenic MATa strain in a murine model of cryptococcosis (Kwon-Chung et al., 1992). Although the details and the relative frequencies of mating in natural environments are little known, in the laboratory, both opposite-sex mating (MATa–MATα) and same-sex mating (MATα–MATα) have been demonstrated (Kwon-Chung, 1976; Keller et al., 2003; Nielsen et al., 2003; Lin et al., 2005). A recent study indicated that the MATa locus was among several genomic regions that contribute quantitatively to same-sex mating and haploid fruiting in *C. neoformans* (Lin et al., 2006). Evidence for same-sex mating (MATα–MATα) in nature was recently reported between strains of serotypes A and D (Lin et al., 2007). Same-sex mating has also been hypothesized to be responsible for generating the major genotype of a recent outbreak of cryptococcosis on Vancouver Island, British Columbia, Canada (Fraser et al., 2005).

Sexual reproduction, between strains either of opposite sexes or the same sex, can potentially increase genetic variation in natural populations of organisms. The diversity of genotypes resulting from sexual reproduction can increase the efficiency of natural selection and may enhance the chances of successfully colonizing novel environments (Weismann, 1904; Goddard et al., 2005). However, unlike those in the majority of plants and animals, where sexual reproduction can be easily detected, the detection of mating and sexual reproduction in micro-organisms requires the analysis of genotypic data from multiple strains.
Population structure in Cryptococcus neoformans

Chapter 2

Significant associations (linkage disequilibrium) among alleles at the population level are consistent with sexual reproduction while significant associations (linkage disequilibrium) are indicative of clonal reproduction. Other signatures of clonal reproduction include over- or under-representations of certain genotypes and genealogical congruence among genes from unlinked loci (e.g. Xu, 2005b). Previous studies have employed a variety of molecular markers to demonstrate the modes of microbial reproduction in nature and to determine how evolutionary as well as ecological factors can influence the modes of reproduction and the structures of microbial populations (Maynard-Smith et al., 1993, Avise, 1994; Xu, 2005b), including those of C. neoformans. For example, an MLST study of the population structure of C. neoformans var. grubii involving 102 representative strains from different parts of the world showed that the global population contained three genetically distinct subgroups (Litvintseva et al., 2006). Ten of the 102 strains were MATa and they were all from Botswana. The remaining 92 were MATa and were from different parts of the world, including Botswana. As expected, the Botswana population that contained strains of both mating types showed evidence of recombination and sexual reproduction (Litvintseva et al., 2003, 2006). In contrast, the remaining geographical populations that contained only MATa strains were largely clonal, with no clear evidence of recombination. Similar analyses of natural populations of a closely related species, Cryptococcus gattii, that contained exclusively or predominantly MATa strains identified at least three distinct lineages in this species but no conclusive evidence for recombination (Kidd et al., 2005). However, in another study of C. gattii populations, evidence for both clonality and recombination was obtained, with clonality found for samples belonging to one molecular type, VGI, and evidence of recombination found for samples of a different molecular type, VGII (Campbell et al., 2005).

In this study, we analysed 78 strains of C. neoformans var. grubii isolated from nine tree species distributed in five geographical areas in north-western India. The mating types of these strains were determined based on direct PCR using mating-type-specific primers (Yan et al., 2002) and all strains were found to have the MATa mating type. A multigene genealogical approach was used to analyse the population samples. DNA sequences were obtained for each strain from each of the five genetic loci distributed in different parts of the genome. Our population-genetic analyses identified no evidence of population subdivision based on either the host tree species or their geographical origins. Interestingly, while clonality and clonal dispersal among geographical regions were clearly evident in our analysed populations, there was also unambiguous evidence for recombination in this MATa population of C. neoformans var. grubii.

Methods

Samples of C. neoformans

The decaying wood inside the trunk hollows of nine species of trees was sampled for Cryptococcus neoformans (serotypes A and D) between 2003 and 2006. Strains were collected by taking swabs from the decayed wood and inoculating the swabs on modified niger seed agar (Randhawa et al., 2005). The colonies were screened for morphological (microscopy) and physiological characteristics consistent with C. neoformans (VITEK 2 system, bioMérieux). Isolates were then serotyped using the Crypto-check slide agglutination test (latron). Details of the 78 strains obtained for this study are presented in Table 1. The strains analysed here are available upon request from Drs H. Randhawa and J. Xu.

DNA extraction and PCR amplification

A previously described technique (Xu et al., 2000) was used to extract high-molecular-mass genomic DNA from all 78 strains. Fragments of four nuclear DNA genes and one mitochondrial gene were chosen for PCR amplification and analyses. To avoid the potential bias in linkage disequilibrium due to physical linkage between markers, the five marker loci used in this study were located on separate chromosomes, as determined based on the completed JEC21 genome (Loftus et al., 2005). The nuclear DNA markers used for amplification were: (1) CAP1 (on chromosome 4) located within the mating locus and encoding a capsule-synthesis-associate protein; (2) FTR1 (on chromosome 3), which encodes a high-affinity iron permease; (3) LAC (on chromosome 7), which encodes the laccase/diphenol oxidase; (4) the internal transcribed spacer (ITS) region, which comprises ITS1, 5.8S rRNA and ITS2 (on chromosome 2); and (5) the mitochondrial large-subunit RNA (mttLrRNA) (Xu et al., 2000). The JEC21 genome assembly from the Stanford Genome Technology Center (http://www.tigr.org/tdb/e2k1/cna1/) was used to identify the loci and design primers when required. Fragments of 731bp (CAP1), 788 bp (FTR1), 486 bp (LAC), 468 bp (ITS) and 439 bp (mttLrRNA) were obtained by using the following primer pairs (5′→3′): CAP1, GTTCTCGTACCACATCTTC (forward) and CTTACCTTCCAGTCGCC (reverse); FTR1, GTTCTCGTACCACATCTTC (forward) and TCTCCAGTCTCGCCTCTTC (reverse); LAC, GCGAGTATCCCTAAGGTA (forward) and TCTGGACTGAGCGGTCAGAC (reverse); ITS, TCGTAGTTGGAACCTCCG (forward) and TCTCCGCTTTACTGATCG (reverse); mttLrRNA, CGCTAAATCAGATCCTCG (forward) and TTATCCCTAATCCTTATAT (reverse). Each PCR reaction comprised 2.1 μl (approx 1 ng) template DNA, 8 μl GoTaq (Taq DNA polymerase+MgCl2, at a final concentration of 1.5 mM, supplied by Promega,), and 0.2 μM of each primer to make up a total volume of 16 μl. The thermocycling profile included an initial denaturing temperature of 95 °C for 4 min followed by 40 cycles of 95 °C for 1 min, annealing temperature for 1 min, and 72 °C for 1 min. The final extension step was 72 °C for 7 min. Annealing temperatures for amplifying the five loci were: 59.2 °C for CAP1, 56.8 °C for FTR1, 50 °C for LAC, 50 °C for ITS, and 46 °C for mttLrRNA. The resulting PCR products were purified using the MicroCLEAN kit (Microzone) and sequenced at the MoBix Laboratory, McMaster University, using an ABI3100 automated DNA sequencer.
Mating type determination using molecular methods

The mating types of isolates were determined by PCR amplification using primer pairs designed from the sequences of the mating-type-specific STE12 and STE20 genes. These primers were specific to serotype- and/or mating-type-specific. The specific primer sequences, amplification conditions, gel electrophoresis and data screening followed those described by Yan et al. (2002).

Phylogenetic analysis

Sequences from the 78 strains for each gene locus were aligned using CLUSTAL_X version 2 (Thompson et al., 1997). The alignments were then imported into SeaView (Galtier et al., 1996) and edited manually. Orthologues for the five genes in two model strains were retrieved for comparison from the completed genomes of serotype A (H99 strain, http://www.broad.mit.edu/annotation/genome/Cryptococcus_neoforms/Home.html) and serotype D (JEC21 strain, http://www.tigr.org/). Phylogenetic analysis was performed using PAUP*4.0b10 (Swofford, 1996). Maximum-parsimony trees were constructed for all the individual fragments by conducting heuristic searches based on 500 random sequence additions. Bootstrap analyses to assess the support for the clades were also conducted using 1000 replicate samples of phylogenetically informative characters. The serotype D strain JEC21 was used as an out-group for all the analysis. For the LAC gene fragment, where extensive sequence information is available and is used to delineate the molecular subtypes (VNI, VNII and VNB) within C. neoformans var. grubii, strains representative of each of the three types from Litvintseva et al. (2003) were analysed together with our data.

Clonality and recombination

Three complementary tests were conducted to examine evidence for clonality and recombination in the Indian population of C. neoformans var. grubii. In the first test, we examined evidence for genealogical incongruence among the five genes. This test was conducted using the partition homogeneity test (PHT) or incongruence length difference (ILD) test (Farne et al., 1994) implemented in PAUP*4.0b10 (Swofford, 1996). The assumption made in this test is that the whole genome evolves as one single unit in strictly clonally reproducing organisms and as a result, genealogies of different gene fragments should be congruent in such organisms. In contrast, in the case of a recombining population structure, there would be incongruence among the different gene genealogies because different genes could have different histories. For this test, we used 100 replicates, each with 10 random sequence addition replicates using the NNI branch-swapping algorithm and saving a maximum of 1000 trees per replicate. This test was conducted for both the entire dataset and also the two largest individual populations, from Delhi and Amritsar, respectively. It has been shown that relying on this test alone for inferences of recombination could produce false-positive results when the consistency indices for individual gene trees are not high (Barker & Lutzoni, 2002). To avoid this potential problem, we also conducted the following two population genetic tests for evidence of recombination.

In the second test, we examined allelic associations among alleles from different loci using two common statistics in population genetics: the index of association ($I_A$) (Maynard-mith et al., 1993) and RbarD (Agapow & Burt, 2001) tests. $I_A$ and RbarD were estimated using Multilocus, version 1.0b (Agapow & Burt, 2001). The $I_A$ analyses the variance of the distances between all possible pairs of multilocus genotypes. The null hypothesis is that there is random association of alleles. Statistical significance was derived by comparing the observed dataset to 10000 artificially recombined datasets. During the process of generating the artificially recombined datasets, we permitted random shuffling of alleles at each locus within a population but kept the proportions of alleles at each locus constant. If there were linkage disequilibrium due to a lack of recombination, the observed $I_A$ should be significantly higher than the randomized recombining dataset. RbarD is a modified measure of $I_A$ but adjusted for the number of loci. The adjustment helps facilitate better comparison between populations and studies.

In the third test, we calculated the phylogenetic incompatibility by looking for the proportion of loci with all possible recombinant genotypes. This test looks for the evidence of recombination in a population by essentially looking for incongruence in different regions of the genome. The statistical significance of this test was inferred by comparing the number of incompatible pairs of loci in the observed dataset to those from a randomly shuffled dataset, using the program Multilocus, version 1.0b (Agapow & Burt, 2001).

Population structure

We assessed the genetic structure of the Indian samples of C. neoformans using three different analyses. In the first, we conducted the T-PTP (topology-dependent permutation tail probability) test (Faith, 1991) to determine whether host tree species or the geographical locations of strains had a significant influence on the topologies of phylogeny. For this test, reshuffled datasets were generated using Multilocus version 1.0b (Agapow & Burt, 2001) and the tree lengths were estimated using PAUP* version 4.0b10 (Swofford, 1996). If there was no significant structure identified with respect to either host tree species or geographical origins, the optimal tree score for the observed data without any constraint would be significantly shorter than for trees with constrained topologies. In the second test, population differentiation analysis was done by calculating $I_A$ (Weir, 1996). In the third test, we calculated the phylogenetic incompatibility by looking for the proportion of loci with all possible recombinant genotypes. This test looks for the evidence of recombination in a population by essentially looking for incongruence in different regions of the genome. The statistical significance of this test was inferred by comparing the number of incompatible pairs of loci in the observed dataset to those from a randomly shuffled dataset, using the program Multilocus, version 1.0b (Agapow & Burt, 2001).
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Mating assays

The abilities to mate and reproduce sexually were examined for representative strains in this study. Briefly, 16 strains representing all 15 multilocus genotypes (MGs) identified in this study were chosen (see below; these strains are marked in Table 1). Each of the 16 strains was mated with three standard mating testers for *C. neoformans* independently. The three testers were JEC20 (MATα), JEC21 (MATα) and H99 (MATα). JEC20 and JEC21 belong to *C. neoformans* var. *neofor mans* (serotype D) while H99 belongs to *C. neoformans* var. *grubii* (serotype A). To prepare for mating, strains were first grown on YEPD (yeast extract-peptone-dextrose) medium at room temperature for 3 days. Approximately equal numbers of cells from mating partners were then mixed on V8-Juice agar medium (5% [v/v] V8 juice, 3 mM KH₂PO₄ and 4% [w/v] agar at pH 7). The plates were then incubated in the absence of light at room temperature (–22 °C) for 21 days. For negative controls, we separately inoculated the three tester strains as well as the 16 representative strains from India onto V8-Juice agar medium and incubated under the same condition as described above for mating mixtures. For positive controls, we mated JEC20 and H99. In addition, we constructed several pairs of same-sex matings between strains from India (all MATα). Filamentation and basidiospore formation were recorded by light microscopy for each of the matings and controls.

Results

Molecular variation

We were able to amplify all five DNA fragments for all the 78 isolates collected for this study. The results of PCR using the STE12 and STE20 gene primers showed that all isolates belonged to the α mating type. Our direct sequencing of PCR products identified no ambiguous nucleotide sites at any of the five genes for any of the 78 strains, consistent with haploidy. In total, we obtained 2912 nucleotides for each strain. The entire alignment had 11 parsimony-informative sites and 5 parsimony-uninformative sites. No polymorphic site was observed within the sequenced regions for the 16 multilocus genotypes among the 78 strains, consistent with haploidy.

To compare the multilocus genotypes identified here with those reported previously, we compared our sequences with those in the GenBank using BLASTN. Our analyses identified that all our sequences had the closest matches to sequences of strains of *C. neoformans* var. *grubii*, consistent with our expectation. Of the three gene fragments analysed here, LAC has been the most widely used in previous studies. As a result, a significant number of allelic sequences exist for the LAC gene in GenBank and some of these sequences have been used to define several molecular types within *C. neoformans* (Litvintseva et al., 2003, 2006). We therefore analysed our sequences with eight representative sequences from the three different molecular types to determine the likely molecular type(s) of our strains. The eight representative sequences of the LAC gene included five of VNI, two of VNB and one of VNII. Our analyses identified that all 78 strains showed the highest similarity to strains of the VNI molecular type (Figure S1a).

Lack of genetic differentiation between populations

The θ value, a measure of population subdivision, showed no significant differentiation among geographical or host-tree-based populations and the observed value lies well within the distribution of the randomized datasets (only results based on geographical origins are shown in Figure 2). The amount of genetic differentiation observed between populations based on either geographical location (Table 2) or host tree species (Table 3) was also measured by Nei’s D and we observed no significant difference between these populations. The lack of significant genetic differentiation is also supported by a lack of monophyletic pattern in the T-PTP tests based on either geographical origins or host tree species, with T-PTP values of 1.00 and 0.60 respectively. While we observed a positive correlation between geographical distance and pairwise population genetic distance, the correlation was statistically not significant (data not shown).

Evidence for both clonality and recombination

As described above, the evidence for clonality and recombination was examined using three tests. The first was the partition homogeneity test or incongruence length difference test. This test showed no statistically significant heterogeneity among the five markers used in this study. This test was conducted for the total sample of 78 strains as well as separately for each of the two largest geographical populations (Delhi and Amritsar). None of the samples shown any significant incongruence between genealogies from different markers (entire dataset \( n=78 \), \( P=0.27 \); Delhi \( n=47 \), \( P=0.23 \); Amritsar \( n=21 \), \( P=0.75 \)). This result suggests that the overall population structure is consistent with significant clonality and clonal ex-

vious evidence of strain clustering based on either geographical origin or host tree species (Figure S1). Indeed, all major multilocus genotypes are shared among strains from multiple geographical regions or host tree species. Among the 78 strains, the number of haplotypes for the individual gene fragments was as follows: four for LAC, three for CAP1, eight for FTR1 (Figure S1), and one each for ITS and mtLRNA (Table 1). In total, the entire dataset yielded 16 multilocus genotypes among the 78 strains (Figure 1). Almost all the genotypes identified here were represented in the largest subpopulation, from Delhi (Figure 1). The second-largest subpopulation (Amritsar) had two haplotypes for each of CAP1, FTR1 and LAC.

To compare the multilocus genotypes identified here with those reported previously, we compared our sequences with those in the GenBank using BLASTN. Our analyses identified that all our sequences had the closest matches to sequences of strains of *C. neoformans* var. *grubii*, consistent with our expectation. Of the three gene fragments analysed here, LAC has been the most widely used in previous studies. As a result, a significant number of allelic sequences exist for the LAC gene in GenBank and some of these sequences have been used to define several molecular types within *C. neoformans* (Litvintseva et al., 2003, 2006). We therefore analysed our sequences with eight representative sequences from the three different molecular types to determine the likely molecular type(s) of our strains. The eight representative sequences of the LAC gene included five of VNI, two of VNB and one of VNII. Our analyses identified that all 78 strains showed the highest similarity to strains of the VNI molecular type (Figure S1a).
### Table 1. Population structure in Cryptococcus neoformans

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Source tree/geographical origin</th>
<th>Year isolated</th>
<th>Multilocus genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>INN95_7363_3</td>
<td>Delhi/MH, India</td>
<td>2006</td>
<td>1 1 1 1 1</td>
</tr>
<tr>
<td>INN95_7362_3</td>
<td>Delhi/MH, India</td>
<td>2006</td>
<td>1 1 1 1 1</td>
</tr>
<tr>
<td>INN95_7361_3</td>
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<td>2006</td>
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<tr>
<td>INN95_7360_3</td>
<td>Delhi/MH, India</td>
<td>2006</td>
<td>1 1 1 1 1</td>
</tr>
</tbody>
</table>

*Allele for markers (Bollet left to right): CAPI, FTR1, LAC1, ITS, MGIVX.

### Table 2. Population structure in Cryptococcus neoformans

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Source tree/geographical origin</th>
<th>Year isolated</th>
<th>Multilocus genotype</th>
</tr>
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<tbody>
<tr>
<td>INN20_326B-DDD_1</td>
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<tr>
<td>INN20_326B-DDD_1</td>
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<td>2004</td>
<td>1 1 1 1 1</td>
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<td>2004</td>
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<tr>
<td>INN20_326B-DDD_1</td>
<td>Delhi/Amritsar</td>
<td>2004</td>
<td>1 1 1 1 1</td>
</tr>
</tbody>
</table>

*Allele for markers (Bollet left to right): CAPI, FTR1, LAC1, ITS, MGIVX.

**Fertility of C. neoformans var. grubii strains from India**

Among the 48 crosses that we set up between 16 representative strains and the three testers, only seven successfully produced hyphal filaments and basidiospores. All seven were with strain JEC20 (the MATa mating tester) and none with JEC21 or H99. The results are consistent with our molecular determination of mating types and suggest that at least some of the strains are sexually fertile. While the positive control cross JEC20×H99 mated successfully, none of the negative controls worked. In addition, after more than 3 weeks of incubation, none of the crosses between strains of the same sex, MATa, produced any hyphal filaments.
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Discussion

C. neoformans var. grubii is among the most important fungal pathogens in humans. Understanding its population structure and ecology in natural environments could have significant medical implications. In this study, our population-genetic analyses showed evidence for extensive gene flow among the environmental populations of this organism in India.

Furthermore, we identified evidence for both clonality and recombination in this sample, which contained exclusively the MATa mating type. Our results are consistent with the hypothesis that C. neoformans might be able to complete its sexual life cycle on decaying wood of several tree species in India. A recent study by Xue et al. (2007) demonstrated that mating and sexual reproduction between strains of opposite mating types can occur for both C. neoformans and C. gattii on the model plant Arabidopsis thaliana and a natural tree host of C. gattii, the eucalyptus.

All of the host tree species investigated in this study are native to India. These tree species included Syzygium cumini (Java plum/jamun tree), Polyalthia longifolia (Indian mast tree), Mimusops elengi (bullet wood), Azadirachta indica (neem tree), Manilkara hexandra (mangos), Acacia nilotica (thorn mimosa), Cassia fistula (golden shower tree) and Mangifera indica (mango). In particular, one tree species, P. longifolia, is found around many homes throughout India. All the trees from which C. neoformans var. grubii was sampled were very close to human habitation. In addition to these tree species, C. neoformans var. grubii has also been recovered from the hollows of other tree species such as Cassia grandis (pink shower tree), Senna multijuga (November shower tree) and Ficus microcarpa (fig tree) (Lazera et al., 1996). Our results demonstrated that the populations of C. neoformans var. grubii in different tree species are not genetically different and that there is frequent gene flow among different tree species. The spread of this micro-organism between different hosts could have been mediated by wind-aided dispersal of basidiospores or desiccated vegetative cells. The five cities from where the strains were isolated in this study are among the most densely populated regions in India. This is especially true of Delhi, where human population density and migration are both very high. The high rate of human migration could also contribute to the dispersal of C. neoformans var. grubii among different locations. In addition, tree-dwelling animals such as birds and squirrels can potentially aid in the spread and dispersal of this organism. At the global level, there is evidence for limited genetic differentiation and frequent gene flow among geographical populations of C. neoformans var. grubii (Xu et al., 2000; Litvintseva et al., 2003, 2006). Short- and long-distance dispersals have been re-
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Ported previously for many fungal species, including saprobes, and plant and animal pathogens (Xu, 2005b). The limited genetic variation found here among strains from the different hosts and geographical regions suggests that the Indian population of C. neoformans var. grubii might be of recent origin and/or that the dispersals were very frequent and recent. While the widespread over-representation of certain genotypes is consistent with clonality, the identification of phylogenetic incompatibility and low $I_A$ values also suggested the presence of recombination in the Indian environmental population of C. neoformans var. grubii. These results are consistent with the structures of many microbial populations, characterized by unambiguous recombination, clonal dispersal and expansion of a few genotypes (e.g. Maynard-Smith et al., 1993; Xu, 2005b).

The evidence for recombination in a MATα population of C. neoformans var. grubii is surprising. Previous analyses of samples of C. neoformans var. grubii have indicated that clonality was the dominant feature of reproduction in natural populations. This was somewhat expected because in most previous studies, the strains analysed from either human or environmental sources belonged to mating type $\alpha$. There were two exceptions where evidence for recombination was detected in C. neoformans var. grubii. The first was an analysis of 14 hybrid strains of serotypes AD where both mating types $a$ and $\alpha$ strains from serotypes A and D were reconstructed using gene genealogies. The reconstructed serotype A and D populations both showed evidence of recombination (Xu & Mitchell, 2003). The second example was from humans in Botswana, where both mating types were found and, not surprisingly, evidence for recombination as well (Litvintseva et al., 2003).

Our current study is unique in that there was clear evidence for recombination in a sample where all the isolates belonged to one mating type, $\alpha$. MATα presents the detailed mechanism for our observed recombination is not known. However, there are several possibilities. The first is that, unlike in most previous studies (e.g. Litvintseva et al., 2003; Xu et al., 2000), the samples analysed here were all from a geographically more restricted region, in north-Geographical region Delhi Bulandshahar Hathras Amritsar Amrouli

Geographical distance $D$ between geographical populations

<table>
<thead>
<tr>
<th>Geographical region</th>
<th>Delhi</th>
<th>Bulandshahar</th>
<th>Hathras</th>
<th>Amritsar</th>
<th>Amrouli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delhi</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bulandshahar</td>
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<td>Hathras</td>
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<tr>
<td>Amrouli</td>
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<td>0.053</td>
<td>0.059</td>
<td>0.118</td>
<td>0.120</td>
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</tbody>
</table>

Table 2. Genetic differences among populations of C. neoformans var. grubii in India: Nei's genetic distance $D$ between geographical populations

<table>
<thead>
<tr>
<th>Host tree species</th>
<th>Syzygium cumini</th>
<th>Polyalthia longfolia</th>
<th>Mimusops elengi</th>
<th>Azadirachta indica</th>
<th>Manilkara hexandra</th>
<th>Cassia fistula</th>
<th>Mangifera indica</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=26)</td>
<td>0</td>
<td>0.022</td>
<td>0.050</td>
<td>0.024</td>
<td>0.024</td>
<td>0.007</td>
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<tr>
<td>(n=14)</td>
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<td>0.082</td>
<td>0.068</td>
<td>0.082</td>
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<tr>
<td>(n=4)</td>
<td>0.050</td>
<td>0.054</td>
<td>0.059</td>
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<tr>
<td>(n=7)</td>
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<tr>
<td>(n=3)</td>
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<td>0.084</td>
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<td>0.118</td>
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<tr>
<td>(n=14)</td>
<td>0.007</td>
<td>0.015</td>
<td>0.059</td>
<td>0.084</td>
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<td>(n=5)</td>
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<td>0.118</td>
<td>0.064</td>
<td>0.053</td>
<td>0.059</td>
<td>0.118</td>
</tr>
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</table>

Table 3. Genetic differences among populations of C. neoformans var. grubii in India: Nei's genetic distance $D$ between populations from different host tree species

<table>
<thead>
<tr>
<th>Population</th>
<th>$I_A$ (Pvalue)</th>
<th>RbarD (Pvalue)</th>
<th>Phylogenetic incompatibility (Pvalue)</th>
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<tr>
<td>Total (n=78)</td>
<td>0.1433 (0.1388)</td>
<td>0.0360 (0.1388)</td>
<td>0.5 (&lt;0.0001)</td>
</tr>
<tr>
<td>Delhi (n=47)</td>
<td>0.1519 (0.102)</td>
<td>0.0385 (0.102)</td>
<td>0.7 (&lt;0.0001)</td>
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<tr>
<td>Amritsar (n=21)</td>
<td>0.0454 (0.363)</td>
<td>0.0114 (0.363)</td>
<td>0.8 (0.108)</td>
</tr>
</tbody>
</table>

Table 4. Multilocus linkage disequilibrium analyses for samples of C. neoformans var. grubii from India (subpopulations with sample sizes greater than 5 are also shown)

*IA, index of association.
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hollows, might be highly conducive to mating. A recent study indicated that both C. neoformans and C. gattii could complete their sexual reproductive life cycle in plants (Xue et al., 2007). Most previous studies of environmental samples of C. neoformans var. grubii have focused on pigeon guano. Whether populations of C. neoformans var. grubii from different ecological niches (e.g. pigeon guano and soil) in India show population structures similar to that from tree hollows remains to be examined.

The sexual recombination detected here could be achieved through one of two pathways. The first possibility is that recombination was due to same-sex mating between MATa strains in natural populations of C. neoformans var. grubii. Same-sex mating has been observed in the laboratory (Lin et al., 2005; Yan et al., 2007) and in nature between strains of serotypes A and D (Lin et al., 2007). The second hypothesis is that the recombination observed here was between strains of opposite mating types. Even though we were unable to isolate any MATa strains from India, it is possible that MATa strains are there but are very rare in nature in India and difficult to isolate. Our result that some of these isolates can mate with MATa but not MATα strains in the laboratory suggests that these MATa strains might be present in the environment in India to help maintain the sexual fertility of MATα strains. However, more extensive sampling would be required to distinguish between these two hypotheses. We would like to emphasize that the evidence for recombination was found not only for the whole sample but also for the subpopulations in Delhi and Amritsar. This finding suggests that many geographical regions in India (and potentially other places in other parts of the world) could be hotspots for sexual mating and the dispersal of basidiospores of this organism.

A combined sexual and asexual mode of reproduction is common for many microbial species, including many human pathogens (Xu, 2005b). It was recently shown that sexual reproduction could incur a cost in fitness (Xu, 2005a). It is also well known that sexual reproduction can produce a large number of recombinant genotypes that could speed up adaptation to novel environments (Weismann, 1904; Goddard et al., 2005). In C. neoformans, while mating could occur between strains of the same or different sexes, it remains to be seen if the two alternative forms of mating have different adaptive significance. In addition, we know very little of the specific conditions in nature under which this organism undergoes same-sex mating as opposed to mating between opposite mating types. In the laboratory setting, low nitrogen, low moisture and ambient temperature are conducive to mating. The nutritional and other physicochemical features of tree hollows from where C. neoformans were isolated are unknown at present. Ecological and physiological studies of natural environments conducive for C. neoformans mating will provide further insights into the mechanisms of evolution and population structuring of this important fungal pathogen.

Acknowledgement

This work was supported by grants from the Natural Science and Engineering Research Council of Canada (J.X.), Genome Canada (J.X.), and the Premier’s Research Excellence Award (J.X.). We thank the Indian National Science Academy for the award of a Young Scientist position to H.S.R. and to the Department of Science & Technology, New Delhi, for the award of a Young Scientist research grant to A.C.

Table 5. Allelic combinations showing evidence of recombination in the environmental population of C. neoformans var. grubii from India (a) All four possible allelic combinations are found between alleles 1 and 3 of locus FTR1 and alleles 1 and 2 of locus LAC. (b) All six possible allelic combinations are found between alleles 1, 3, and 5 of locus FTR1 and alleles 1 and 2 of locus CAP1. The number of strains for each genotype is shown in the table.

<table>
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<tr>
<th>(a)</th>
<th>LAC (allele 1)</th>
<th>LAC (allele 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTR1 (allele 1)</td>
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<td>42</td>
</tr>
<tr>
<td>FTR1 (allele 3)</td>
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<td>14</td>
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</table>

<table>
<thead>
<tr>
<th>(b)</th>
<th>CAP1 (allele 1)</th>
<th>CAP1 (allele 2)</th>
</tr>
</thead>
<tbody>
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<td>4</td>
</tr>
<tr>
<td>FTR1 (allele 3)</td>
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<td>5</td>
</tr>
<tr>
<td>FTR1 (allele 5)</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 3. Distribution of IA values. The observed index of association (IA) value was within the distribution of IA obtained from 10000 artificially recombed datasets (P=0.1816).
Chapter 3

Genetic differentiation, recombination and clonal expansion in environmental populations of Cryptococcus gattii in India

Anuradha Chowdhary
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Tusharantak Kowshik
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Jianping Xu
Summary

Cryptococcus gattii is a ubiquitous eukaryotic pathogen capable of causing life-threatening infections in a wide variety of hosts, including both immuno-compromised and immuno-competent humans. Studying environmental populations of this pathogen is especially critical in countries like India with an ongoing AIDS epidemic and an increasing number of immuno-compromised patients. In this study, we analyzed 109 isolates of C. gattii obtained from hollows of nine tree species from eight geographic locations in India. Multilocus sequence typing was conducted for all isolates using nine gene fragments. All 109 isolates belonged to the VGI group and were mating type α. Population genetic analyses revealed limited evidence of recombination but unambiguous evidence for clonal reproduction and expansion. However, the observed clonal expansion has not obscured the significant genetic differentiation among populations from either different geographic areas or different host tree species. A positive correlation was observed between genetic distance and geographic differentiation among populations from either different geographic areas or different host tree species. The results obtained here for environmental populations of this pathogen is especially critical in countries like India with an ongoing AIDS epidemic and an increasing number of immuno-compromised patients.

Introduction

Cryptococcus gattii is a member of the Cryptococcus neoformans species complex (CNSC) which is primarily a haploid saprobyte but capable of infecting both immuno-competent and immuno-compromised humans and a variety of animal hosts, including (Chen et al. 2000; Casadevall & Perfect 1998; Kerkering et al. 1981). Based on antigenic properties of the polysaccharide galactoxylomannan (GXM) capsule, CNSC is traditionally classified into four haploid serotypes, namely: serotype A (C. neoformans var. grubii), serotypes B and C (C. gattii), and serotype D (C. neoformans var. neoformans), and diploid/aneuploid hybrids of strains of serotypes A and D (serotype AD) (Casadevall & Perfect 1998; Xu et al. 2002; Xu & Mitchell 2003). Because cryptococcal infections are almost exclusively acquired from environmental sources (Casadevall & Perfect, 1998), it is important to study the ecology and population biology of this organism in its natural environments.

The first report on environmental isolation of C. gattii was by Ellis and Pfeiffer in Australia who investigated plant debris under the canopy of Eucalyptus trees as a reservoir of this pathogen (Ellis & Pfeiffer 1990a). This was followed by numerous studies which recovered C. gattii from various tree species in United States, Mexico, Canada, Colombia, Argentina, Brazil, Paraguay, India, Italy, Jordan, Egypt, Africa, and Papua New Guinea (Pfeiffer & Ellis 1991; Launerson et al. 1997; Chakrabarti et al. 1997; Mahmoud, 1999; Montenegro & Paula 2000; Sorell, 2001; Davel et al. 2003; Randhawa et al. 2003, 2006,2008; Granados & Castañoeda 2005, 2006; Kidd et al. 2005, 2007; Xu 2010). Besides, C. gattii has been reported from a variety of other environmental sources such as soil, fruits, vegetables, freshwater, seawater, insect frass and desiccated excreta of a few species of caged birds (Casadevall & Perfect 1998, Abegg et al. 2006). However, decayed wood inside trunk hollows of trees, bark or other plant debris remains its main ecologic niche.

Based on molecular fingerprinting and gene genealogical analyses, C. gattii has been divided into four distinct lineages VGI, VGII, VGIII and VGIV (Kidd et al. 2005; Bovers et al. 2008; Ngamskulrungroj et al. 2009). VGI is the most commonly isolated genotype group occurring worldwide including several Asian countries and Australia where it is predominantly associated with Eucalyptus trees (Chen et al. 2000; Meyer et al. 2003; Randhawa et al. 2008, 2010). VGI has been recovered from Colombia, the Pacific Northwest region of North America and also from certain parts of Australia (Trilles et al. 2003; Kidd et al. 2005; Escandón et al. 2006). During the last decade, the VGII genotype was responsible for the outbreak of cryptococcosis on Vancouver Island in British Columbia, Canada, and in the states of Washington and Oregon in the US (Stephen et al. 2002; Kidd et al. 2005; Fraser et al. 2003, 2005; Byrnes et al. 2010). This outbreak was significant because it showed that C. gattii was capable of causing life-threatening infections in apparently healthy individuals in a temperate climate. VGIII and VGIV are relatively rare genotypes but have been recovered from both environmental and clinical samples (Kidd et al. 2005; Bovers et al. 2008). VGIV has been implicated as etiologic agent of cryptococcosis in AIDS patients in sub-Saharan Africa (Litvinsteva et al. 2005). More extensive studies are required to confirm if the seemingly non-random distribution of the four distinct lineages among geographic regions is due to inadequate studies or represents true geographical or biological differences among the lineages.
An important factor influencing the patterns of genetic variation in natural populations of *C. gattii* is the mode of reproduction. *C. gattii* is heterothallic with a bipolar mating system controlled by a single locus with two alternative alleles: MATα and MATα (Kwon-Chung 1975, 1976). As in the majority of microorganisms (Xu 2010), asexual reproduction is expected to be common in natural populations of *C. gattii*. Asexual reproduction leads to linkage disequilibrium and the propagation of clonal lineages within a population, and through dispersal, to clonal expansion across ecological niches and geographic regions. On the other hand, sexual reproduction recombines genetic materials from different strains, results in linkage equilibrium and the formation of basidiospores which may likely be the dominant infectious particles of cryptococcal infections (Sukroongreung et al. 1998; Giles et al. 2009; Velagapudi et al. 2009). Because of their small size and low water content, basidiospores are better adapted for dispersal than vegetative cells. Successful mating between MATα-MATα and MATα-MATα parents has been carried out in the laboratory. However, among the four lineages of *C. gattii*, evidence for recombination has been found only in natural populations of the VGI and VGII lineage in Australia and in the VGII lineage in North America (Campbell et al. 2005a, b; Fraser et al. 2005; Saul et al. 2008; Xu et al. 2009).

Likewise, there is very limited information on the geographic structure of environmental populations for any of the four lineages in *C. gattii*. In this study, we analyzed 109 strains of *C. gattii* associated with nine different tree species in eight geographic locations in India. For each strain, nine nuclear loci previously identified to be polymorphic in *C. gattii* were sequenced. All the isolates in our study were found belonging to the VGI lineage and had the MATα mating type. These data were analyzed to address the following specific questions. First, how extensive is clonality in natural populations of *C. gattii* VGI in India? Is there any evidence of recombination? Second, what are the relationships among ecological and geographic populations of this lineage? In a recent survey of environmental populations of *C. neoformans* var. *grubii* in India (Hiremath et al. 2008), we found unambiguous evidence for recombination and extensive gene flow among ecological and geographic populations. Our results here showed both similarities and differences in population structure between *C. gattii* VGI and *C. neoformans* var. *grubii* from similar habitats and geographic origins in India.

**Materials and Methods**

**Environmental Isolates of *C. gattii***

The *C. gattii* isolates investigated originated from decaying wood inside tree trunk hollows, from the bark of trees and from soil that lay within 1m around the base of the sampled trees (Randhawa et al. 2008). The sampled sites contained no avian guano or bird nests. The sampled host trees represented 9 species, 8 of which were native to India, i.e., *Syzgium cumini, Mimusops elengi, Azadirachta indica, Cassia fistula, Polyalthia longifolia, Manilkara hexandra, Acacia nilotica*, and *Tamarindus indica*; the remaining test trees belonged to the genus *Eucalyptus*, an import from Australia. The environmental surveys were conducted from 2001-2008 in seven locations in Northern India: Delhi, Meerut, Bulandshahar, Hathras, Amritsar, Amrouli, Chandigarh and one location in Southern India: Tiruvannamalai. Samples were collected and processed, using a swabbing technique as described in Randhawa et al. (2005). Brown yeast colonies were purified by streaking and were further screened for morphological (microscopy) and physiological features (VITEK 2 system, bioMérieux, l’Étoile, France) characteristic of *C. gattii*. The isolates were then serotyped using the Crypto-check slide agglutination test, which was based on monoclonal antibodies specific for the variable capsular polysaccharide (latron, Tokyo, Japan). For samples with positive *C. gattii* cultures, only one isolate from each individual tree at each geographic location was used for subsequent population genetic analyses. In total, 109 independent *C. gatti* strains (Table 1) were obtained and maintained in a -80°C freezer. Genomic DNA was isolated from each strain using a protocol described previously (Xu et al. 2000a) and these samples were used for further genotyping analyses. The strains analyzed here are available upon request from Dr. Harbans Randhawa and Dr. J-P Xu.

**Mating type determination**

We used the mating type- (MATα and MATα) and serotype- (A, B, and D) specific primers of the mating related genes STE12 and STE20 to screen and identify both the mating type and serotype for each of the strains. The primer sequences and procedures for PCR amplification and gel electrophoresis followed those described previously (Yan et al. 2002).

**Multilocus Sequence Typing**

Fragment of nine nuclear loci were chosen for strain genotyping and for population genetic analyses. These markers have been used in previous studies and include the consensus markers by the Cryptococcus MLST consortium (Meyer et al. 2009). However, despite repeated tries, <10% of the strains were successfully amplified using the recommended primers to amplify the SOD1 gene fragment. As a result, the SOD1 locus was not analyzed further here. The gene name, its chromosomal location, analyzed fragment length, and the specific PCR primers for each of the nine gene fragments studied here are as follows: (1) *CAP1*, chromosome IV, part of the MATα locus, encodes a capsule-synthesis associated protein; 700bp, 5'-CGTTCGGAGTACAGAGGAACATC-3' (forward) and 5'-CTCTACTGGCATCAGGTAATCT-3' (reverse); (2) *URA5*, chromosome VIII, encodes an orotate phosphoribosyl pyrophosphate transferase, 563bp, 5'-ATGTCCTCCCAAGCCCTCGAC-3' (forward) and 5'-AGGCAAAGAGCAGAGGTAATCT-3' (reverse); (3) *FTR1*, chromosome III, encodes a high affinity iron permease, 704bp, 5'-GGCGATACTATTATCGTA-3' (forward) and 5'-TCTCAGGCTCGCCATCTTC-3' (reverse); (4) *CAP5*, chromosome I, a capsule-synthesis associated protein, 502bp, 5'-CTCCTAGCTGCAAGATTCAAGAACCACATCCTCAAG-3' (forward) and 5'-TCCGTGCAACAGTGAATCC3-3' (reverse); (5) *PLB1*, chromosome XII, a phospholipase B probably involved in cell invasion; 853bp, 5'-CTTACGAGCCGAGAGGATTT-3' (forward) and 5'-GATTGCGGCTTTGGTTTCT-3' (reverse); (6) *GPD1*, chromosome VII, encodes an octate phosphoribosyl pyrophosphate transerase, 563bp, 5'-ATGCTCCCTCCAAGCCCTCGAC-3' (forward) and 5'-TAAAGACTCTGCAACACCTGACTC3-3' (reverse); (7) *GPD1*, chromosome VII, encodes glyceraldehyde-3-phosphate dehydrogenase, 856bp, 5'-CCACCGAAACCCTCTCTGATA-3'.
Each PCR reaction mixture comprised 2.0μL (~1ng) template DNA, 8μL GoTaq (Taq DNA polymerase + MgCl₂, at a final concentration of 1.5mM, supplied by Promega, USA), 0.2μM of each primer, and 5.8μl of sterile distilled water to make up a total volume of 16μL. The thermo cycling profile included an initial denaturing step at 95°C for 4min. This was followed by 40 cycles of: denaturation at 95°C for 1min., primer-specific annealing temperature for 1min., and finally primer extension at 72°C for 7min. Primer-specific annealing temperatures for amplifying the nine gene fragments were: 59.2°C for CAP1, 50°C for LAC, 56°C for FTR1, 55°C for CAP59, 56°C for PLB1, 50°C for URA5, 56°C for GPD1, 54°C for IDE and 55°C for IGS. The resulting PCR products were purified using the MicroCLEAN kit (Microzone, Ottawa, Canada) and sequenced using the same primers at the MoBio Laboratory, McMaster University, using the ABI310 automated DNA sequencer.

Phylogenetic analyses
To compare the sequences in our strains to those of model sequenced strains in CNSC, orthologs for all the nine gene loci were retrieved from the following reference genomes: C. gattii strain R265 (Serotype B, VGII, http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans_b/Home.html), C. gattii strain WM276 (Serotype B, VGI, http://www.bcgsc.ca/project/cryptococcus/), C. neoformans var. grubii strain H99 (Serotype A, http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans/Home.html) and C. neoformans var. neoformans strain JEC21 (Serotype D, http://www.tigr.org/tdb/e2k1/cna1). Sequence alignment was done using Clustal X version 2 (Thompson et al. 1997) separately for each gene fragment. The alignments were manually inspected and adjusted if necessary. Maximum-parsimony trees were constructed for each of the individual gene fragments by conducting heuristic searches based on 500 random sequence additions using PAUP* 4.0b10 (Swofford, 2002). Bootstrap analyses to assess the support for the clades were also conducted using 1000 replicate samples of phylogenetically informative characters. Serotypes A and D were used as outgroup strains for all the analyses.

Mode of reproduction
The number of alleles at each locus and the number of multilocus genotypes were obtained based on the gene sequences from individual phylogenetic trees. The multilocus genotype matrix was exported into the program Multilocus, version 1.0b (Agapow & Burt 2001) to test the extent of departure from linkage equilibrium using three statistical measures: the Index of Association (I(J)) (Brown et al. 1980), rBarD and phylogenetic incompatibility. I(J) is the ratio of the observed variance in the association of alleles among loci to the corresponding expected variance based on random associations. Significant associations among alleles at different loci are inconsistent with random recombination but consistent with clonality. The expected value of allelic associations increases with the number of loci analyzed, therefore, the second index rBarD, which is independent of the number of loci, was also used. The third test calculates the proportion of loci that when compared in pairwise combinations, show phylogenetic incompatibility. In the simplest case of phylogenetic incompatibility, for two loci with two alleles each, if all four possible genotypes are found in the population, these two loci are called phylogenetically incompatible. Phylogenetic incompatibility suggests recombination in the population. The underlying principles, methods of calculations and interpretations of the results for all three tests are described in the Multilocus program manual (Agapow & Burt 2001).

Population Structure
The relationships between and among populations were analyzed based on geographic locations and/or associated host tree species. Two programs were used to determine the extent of genetic differentiations among populations. The first statistic q (Weir, 1996) was calculated using Multilocus version 1.0b (Agapow & Burt 2001). The value of q ranges from 0 (i.e. no genetic differentiation and frequent gene flow) to 1 (i.e. complete genetic isolation). In the second, we used AMOVA to estimate the variance in allele frequencies among the subpopulations. We compared the distribution of the observed variance in allele frequency among subpopulations to the expected distribution based on resampled data (10000 times in our study) while assuming no differentiation. During resampling, the subpopulation sizes are held constant but individuals are reassigned to different subpopulations to simulate gene flow to generate genetically undifferentiated subpopulations. A significant difference between the observed variance in allele frequency and the expected variance assuming no differentiation would indicate that the subpopulations were genetically differentiated. Furthermore, the relationships between geographic distance and genetic distance among geographic populations were examined using Mantel’s test. All the above tests were conducted using GenAIex 6.3 (Peakall & Smouse 2006).

Results
Mating type and genotype of strains of C. gattii from India
Our analyses identified that only primers specific to the C. gattii serotype B, MATα mating type successfully amplified DNA fragments at the STE12 and STE20 genes for all 109 strains. Our results thus indicate that all 109 isolates analyzed here belong to the MATα mating type of C. gattii. At the nine nuclear loci used for DNA sequence-based strain typing, while the majority of the strains were successfully amplified and resulted in clear sequences, we were unable to obtain DNA sequences for a variable number of strains at each of the nine loci, despite repeated trials using different amplifying conditions including different annealing temperatures such as temperature gradients. In this study, the total number of strains and the total number of aligned base pairs for each locus are as follows: (a) CAP1 (99 strains; 700bp aligned) (b) LAC (98 strains; 448bp aligned) (c) FTR1 (104 strains; 704bp aligned) (d) CAP59 (94 strains; 502bp aligned) (e) PLB1 (93 strains; 853bp aligned) (f) URA5 (80 strains; 563bp aligned) (g) GPD1 (85 strains; 856bp aligned) (h) IDE (98 strains; 758bp aligned) (i) IGS (96 strains; 740bp aligned). None of the sequences obtained here showed any evidence
Cryptococcus gattii from India

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Our analyses identified that, compared to the other model strains used, sequences from strain WM276 (which belongs to the VGI group) clustered most closely with all our isolates (Figures 1 and 2). The conclusion that all our strains are in the VGI group was supported by a more extensive data that included multiple strains within each of the four lineages VGI to VGIV (Bovers et al. 2008; Kidd et al. 2005; Ngamskulrungroj et al. 2009). A representative figure showing the strain-clustering pattern using the IDE gene sequences is presented in Figure 2. Given that the out-group-rooted trees and the mid-point rooted trees were identical in topology, all the model strains (WM276, R265, JEC21 & H99) have been trimmed off the trees in order to enhance the visual differences among strains on the phylogenetic trees (Supplementary Figures S1a-S1j).

Table 1. Summary information for the nine loci amplified from Cryptococcus gattii isolates from India.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr.</th>
<th>Primer sequence (5’→3’)</th>
<th>Temperature (°C)</th>
<th>Fragment size (bp)</th>
<th>Variable sites</th>
<th>Number of strains</th>
<th>Number of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP1</td>
<td>IV</td>
<td>F:GGTTCCGCTACGAGAGAGGGA R:CCCTAAGCCTCAAGCTGCC</td>
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<td>700</td>
<td>8 (8,0)</td>
<td>99</td>
<td>6</td>
</tr>
<tr>
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<td>I</td>
<td>F:CCCTAGGTCGAGCAAGTAAGGC R:TGTCCTGAGCAAGCTGACCC</td>
<td>55</td>
<td>502</td>
<td>8 (6,2)</td>
<td>94</td>
<td>7</td>
</tr>
<tr>
<td>FTR1</td>
<td>III</td>
<td>F:GTTCTGCAATACACTTCTC R:TCTCAACTTCGCACATCT</td>
<td>58.6</td>
<td>704</td>
<td>15 (13,2)</td>
<td>104</td>
<td>8</td>
</tr>
<tr>
<td>GPD1</td>
<td>VII</td>
<td>F:CCACGGAAACTCCCTAGGATA R:CTCTATTAGCCACCTCTCT</td>
<td>56</td>
<td>856</td>
<td>7 (7,0)</td>
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<tr>
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<td>758</td>
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<td>98</td>
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<tr>
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<tr>
<td>URA5</td>
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<td>50</td>
<td>563</td>
<td>6 (6,0)</td>
<td>80</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 1. Summary information for the nine loci amplified from Cryptococcus gattii isolates from India.

a. Chromosome location of the sequenced gene fragment.
b. Total number of variable nucleotide sites among the sequenced isolates for each gene fragment. The first number in the parentheses refers to the number of parsimony informative variable sites and the second refers to the number of parsimony uninformative sites.
c. Number of strains where this locus was successfully amplified.

Among the 109 strains, the numbers of variable sites for each of the loci were as follows: (1) CAP1 (8 variable nucleotide sites, all parsimony-informative); (2) LAC (8 variable nucleotide sites, all parsimony-informative); (3) FTR1 (15 variable nucleotide sites, 13 of them parsimony-informative); (4) CAP59 (8 variable nucleotide sites, 6 of them parsimony-informative); (5) PLB1 (8 variable nucleotide sites, 5 of them parsimony-informative); (6) URA5 (6 variable nucleotide sites, all parsimony-informative); (7) GPD1 (7 variable nucleotide sites, all parsimony-informative); (8) IDE (8 variable nucleotide sites, 7 of them parsimony-informative); and (9) IGS (18 variable nucleotide sites, 17 of them parsimony-informative). The GenBank accession numbers for the unique allelic sequences at all nine loci are FJ604481-FJ604535.

The combined total number of aligned nucleotides among the strains was 5916bp. The entire alignment had 77 parsimony informative characters and 9 parsimony uninformative characters. These polymorphic sites resulted in 20 unique multilocus genotypes for the 109 sequenced strains (Table 1). The MLST genotypes are represented as MGI-MGXIX (Figure 1). Our genealogical analyses showed three consistent groupings in each individual locus as well as in the combined dataset (Figure 1). The first group includes one multilocus genotype MGXI. The five strains from this lineage were recovered only from Syzygium cumini trees from the city of Bulandshahar. The second group includes two multilocus genotypes MGXII and MGXIII. All strains in this group were recovered from Azadirachta indica trees. MGXII, which consists of five strains, was found only in Bulandshahr and the lone MGXIII strain was recovered from Delhi. The remaining 17 multilocus genotypes were in the third group. All three groups received >95% bootstrap support for the MP analyses in each of the individual gene genealogies as well as in the combined gene genealogy.
Evidence for limited recombination but predominant clonal reproduction of *C. gattii* in India

The \( I_p \) values were calculated for each geographic sub-population with more than five isolates and for the entire sample. Our analyses identified that all \( I_p \) values for each subpopulation and for the total sample were significantly higher than the simulated datasets obtained assuming random mating and recombination (Figure 3). We repeated the same test for subpopulations based on host tree species and obtained similar results, also rejecting the null hypothesis of random mating and recombination for each tree species-based sub-population (data not shown). Similarly, the \( rBarD \) values confirmed that there was no evidence of random mating and recombination in either the host tree species-based subpopulations or geography-based subpopulations (Table 2). In addition, the \( I_p \) values and the \( rBarD \) values calculated using clone-corrected samples also rejected the hypothesis of random mating and recombination for all analyzed samples. Our results thus indicate that the environmental samples of *C. gattii* from India are predominantly clonal. However, despite the overwhelming evidence for clonal population structure for *C. gattii* populations in India, the phylogenetic incompatibility test identified clear cases of phylogenetic incompatibility between three pairs of loci (Table 3). Specifically, loci pairs CAP59 (Chromosome I) and FTR1 (Chromosome III); CAP59 and IDE (Chromosome XII); and FTR1 and URA5 (Chromosome VIII) each had two alleles randomly associated with each other in the Delhi population of *C. gattii* (and hence the total sample as well, Table 3). These four loci are on different chromosomes. Our results are thus consistent with limited but unambiguous evidence of recombination in the Delhi population of *C. gattii*.

To examine further whether samples from different host tree species differ in their patterns of linkage disequilibrium, we examined allelic associations among the nine loci for each sample with multiple isolates. Specifically, the following four host tree species had multiple samples: *Mimusops elengi*, *Azadirachta indica*, *Syzygium cumini*, and *Tamarindus indica*. Similar to the geography-based analyses, all three measures \( I_p \), \( rBarD \), and phylogenetic compatibility, rejected the hypothesis of random mating and suggested clonality as the dominant mode of reproduction for populations of this species from different trees in India (data not shown). However, the population from *Syzygium cumini* showed three cases of phylogenetic incompatibility (out of 36 pairwise loci comparison), consistent with limited recombination. In contrast, none of the populations from other three tree species showed any evidence of phylogenetic incompatibility.

Genotype distribution

We assessed the diversity of multilocus genotypes across geographic locations and host tree species by calculating the genotype diversity \( h \) (where \( p_i \) is the frequency of the \( i \)-th genotype), using GenAlEx 6.3 (Peakall & Smouse 2006). Bulandshahar was found to have the highest genotype diversity \( n=13; h=0.648±0.008 \), followed sequentially by Delhi \( n=64; h=0.332±0.603 \), Meerut \( n=5; h=0.113±0.057 \) and Tiruvannamalai \( n=7; h=0 \). The rest of the geographical locations had less than 5 strains and were not included in this analysis. The highest genotypic diversity associated with host tree species was found in *Azadirachta indica* \( n=12; h=0.588±0.040 \), followed by *Syzygium cumini* \( n=49; h=0.475±0.062 \), *Mimusops elengi* \( n=49; h=0.199±0.021 \) and *Tamarindus indica* \( n=7; h=0 \). The remaining tree species had less than five isolates recovered from them and were not included in the analyses. The difference in genotype diversity was due to the degrees of over-representation of certain genotypes for different geographic locations and/or different host tree species (Figure 1). For example, MGIII was the most common genotype shared by 36 isolates from two locations, Delhi and Amrouli and from five different host tree species. In contrast, MGIII represented by 7 strains was exclusively from Tiruvannamalai. None of the geographic populations or any of the host trees investigated here contained all the 20 multilocus genotypes.

Relationships among geographic and host tree populations of *C. gattii* from India

The statistic \( q \) showed that both host tree-based and geography-based populations of *C. gattii* from natural environments in India were overall significantly differentiated. The AMOVA results indicated that geographic separation contributed 39% of the overall genetic variation, the host tree species contributed 21%, with the remaining 40% came from within individual populations. All three levels of contributions were significantly greater than 0 (\( p<0.001 \)). Indeed, the pairwise \( \phi_{PT} \) values between geographic populations and host tree-based populations were all statistically significant (Tables 4 and 5). The Mantel test showed that genetic distances and geographic distances among the geographic populations were positively correlated (\( p<0.010 \), with a correlation coefficient of 0.321 (Figure 5). Overall, our results suggest that both geographic separation and host tree contributed significantly to the overall genetic variation among environmental populations of *C. gattii* in India.

Discussion

Mating type distribution

In this study, we surveyed the genotypes of strains of *C. gattii* from the detritus of trees and the surrounding soil from several locations in India. We found that all 109 isolates analyzed here belonged to one mating type, *MATa*. This result is consistent with the global pattern of mating type distribution in CNSC in favor of the *MATa* mating type (e.g., Kwon-Chung and Bennett, 1978; Yan et al. 2002). For example, in our recent population analyses of *C. neoformans* var. *grubii* from similar environmental sources in India, we found that all 78 analyzed strains belonged to the *MATa* mating type (Hiremath et al. 2008). Though *MATa* strains of both *C. neoformans* and *C. gattii* have been isolated from patients in India (Jain et al. 2008), as far as we know, there is yet no conclusive evidence for the existence of *MATa* strains in environmental populations of *CNSC* in India. Several hypotheses have been proposed to explain the predominance of *MATa* strains both in clinical specimens and in the natural environments (Yan et al. 2002). For example, many *MATa* strains are capable of haploid fruiting to generate abundant basidiospores that may be easily dispersed across ecological niches and geographic regions (Wickes et al. 1996). In contrast, though there are exceptions, *MATa* strains are typically incapable of haploid fruiting (Wickes et al. 1996; Tschcharke et al. 2003). In addition, genetic factors within the MAT locus or closely linked to...
The mating type locus might play a role in the biased distribution in favor of MATα.

It should be noted that while the majority of surveys identified MATα as the dominant mating type allele for C. gattii, a few geographic populations of VGI in southern Australia possessed the MATa allele at a frequency similar to that of the MATα allele (Halliday et al. 1999). Indeed, it’s possible that the absence of MATα mating type in environmental samples of CNSC in India might be due to our inadequate sampling and/or insufficient analyses. For example, four of the 57 strains of CNSC from 39 patients in India analyzed in the Jain et al. (2005) study were MATa. The four MATa strains included three of C. neoformans var. grubii and one of C. gattii. If these patients acquired their infections in India, the results would suggest that environmental populations of CNSC must contain MATa strains. In addition, serotype AD strains have also been reported from India (Banerjee et al. 2001). Previous analyses have identified that serotype AD strains are recent hybrids between strains of serotypes A and D and mostly between those of opposite mating types (Xu et al. 2002, the multilocus genotypes correspond to those in Figure 1).

Table 2. Summary information of strains of C. gattii from India analyzed in this study

<table>
<thead>
<tr>
<th>Geographic location (latitude and longitude)</th>
<th>Host plant</th>
<th>Sample size</th>
<th>Multilocus genotype(s) (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amritsar (31.37N, 74.55E)</td>
<td>Syzygium cumini</td>
<td>1</td>
<td>XIV (1)</td>
</tr>
<tr>
<td>Amroli (21.3N, 72.85E)</td>
<td>Syzygium cumini</td>
<td>3</td>
<td>I (1); V (1); IX (1)</td>
</tr>
<tr>
<td>Bulandshahr (28.24N, 77.75E)</td>
<td>Azadirachta indica</td>
<td>5</td>
<td>XII (5)</td>
</tr>
<tr>
<td>Chandigarh (30.44N, 76.47E)</td>
<td>Syzygium cumini</td>
<td>8</td>
<td>VI (1); IX (1); XVII (2)</td>
</tr>
<tr>
<td>Delhi (28.54N, 77.12E)</td>
<td>Azadirachta indica</td>
<td>7</td>
<td>I (1); VI (1); X (1); XIII (1)</td>
</tr>
<tr>
<td></td>
<td>Cassia fistula</td>
<td>2</td>
<td>XX (1)</td>
</tr>
<tr>
<td></td>
<td>Eucalyptus spp.</td>
<td>1</td>
<td>I (1)</td>
</tr>
<tr>
<td></td>
<td>Manilkara hexandra</td>
<td>2</td>
<td>IV (1); XIII (1)</td>
</tr>
<tr>
<td></td>
<td>Mimusopsis elengi</td>
<td>21</td>
<td>I (1); II (1); VIII (1)</td>
</tr>
<tr>
<td></td>
<td>Polyalthia longifolia</td>
<td>4</td>
<td>VI (1); IX (1); XV (1)</td>
</tr>
<tr>
<td></td>
<td>Syzygium cumini</td>
<td>36</td>
<td>I (13); II (2); IV (1); V (1); VI (1); VII (3); XV (2); XVI (2); XVII (1); XX (7); XX (2)</td>
</tr>
<tr>
<td>Hathras (27.22N, 78.02E)</td>
<td>Azadirachta indica</td>
<td>2</td>
<td>V (1)</td>
</tr>
<tr>
<td>Meerut (29.01N, 77.45E)</td>
<td>Syzygium cumini</td>
<td>5</td>
<td>VII (1); IX (4)</td>
</tr>
<tr>
<td>Tiruvannamalai (12.15N, 80.78E)</td>
<td>Tamarindus indica</td>
<td>7</td>
<td>III (7)</td>
</tr>
</tbody>
</table>

1. The multilocus genotypes correspond to those in Figure 1.
C. neoformans var. grubii from similar geographic areas and ecological niches in India, as observed in an earlier study (Hiremath et al. 2008). As far as we know, no similar comparisons have been made for environmental populations of C. neoformans and C. gattii from India. Chapter 3

Table 3. Multilocus linkage disequilibrium analyses for samples of Cryptococcus gattii from India (sub-populations with sample sizes greater than 10 are also shown)

<table>
<thead>
<tr>
<th>Population</th>
<th>Sample type</th>
<th>I_A (p value)</th>
<th>RbarD (p value)</th>
<th>Phylogenetic compatibility (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Indian</td>
<td>All strains (n=109)</td>
<td>2.567 (p&lt;0.001)</td>
<td>0.325 (p&lt;0.001)</td>
<td>0.917 (p&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>Unique genotypes (n=20)</td>
<td>1.679 (p&lt;0.001)</td>
<td>0.211 (p&lt;0.001)</td>
<td>0.917 (p&lt;0.001)</td>
</tr>
<tr>
<td>Delhi sample</td>
<td>All strains (n=64)</td>
<td>1.824 (p&lt;0.001)</td>
<td>0.243 (p&lt;0.001)</td>
<td>0.917 (p&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>Unique genotypes (n=15)</td>
<td>0.987 (p&lt;0.001)</td>
<td>0.124 (p&lt;0.001)</td>
<td>0.917 (p&lt;0.001)</td>
</tr>
<tr>
<td>Buaanidhaur</td>
<td>All strains (n=13)</td>
<td>5.411 (p&lt;0.001)</td>
<td>0.678 (p&lt;0.001)</td>
<td>1 (p=0.001)</td>
</tr>
<tr>
<td></td>
<td>Unique genotypes (n=4)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\( a \) index of association
\( b \) Sample sizes are too small for these tests to be meaningful so results are not presented.

2003; Yan & Xu 2003; Lin & Heitman 2006; Lin et al. 2007). Specifically, two types of crosses have been found responsible for the majority of serotype AD strains: serotype A MATa X serotype D MATa and serotype D MATa X serotype A MATa. If serotype AD strains found in India were derived from either of the above two types of crosses, the result would suggest two possibilities. One, the hybrids were formed in India and that the natural population of C. neoformans in India contained the MATa mating type. Two, the hybrids were formed elsewhere and then dispersed into India. More extensive environmental sampling and more detailed analyses might reveal whether strains of MATa of the C. neoformans species complex exist in the natural environment in India.

Multilocus sequence typing in CNSC

Although largely successful, a number of isolates could not be amplified with the recommended primers and conditions at each of the nine loci, including the six consensus loci recommended by the Cryptococcus MLST Consortium (Meyer et al. 2009). Furthermore, only about 10% of the isolates were successfully amplified at the SOD1 locus, another gene fragment recommended for MLST of CNSC strains by the Consortium. All strains and DNA samples were successfully amplified using the universal fungal ITS1 and ITS4 primers (data not shown) as well as the C. gattii-specific MATa primers at the STE12 and STE20 loci. In addition, each strain had at least four of the nine loci successfully amplified and sequenced. Therefore, we believe that the failed amplifications were not due to inadequate quality or quantity of DNA. Furthermore, fresh DNA samples were re-extracted and purified and different amplifying conditions were used for strains that failed the initial PCR amplifications. It is likely that mutations at the primer sites were the causes for the failed amplifications. We believe a critical examination using more diverse strains should be conducted to derive a set of primers and PCR conditions to make the multilocus sequence typing system for C. gattii and CNSC in general more robust.

Clonality and recombination

Given the unisexual nature of the samples of C. gattii in our study, the results of linkage disequilibrium and clonality in our samples were expected. Our evidence for clonality came not only from significant allelic associations between and among loci (i.e. linkage disequilibrium) but also from over-representations of several multilocus genotypes. Interestingly, our analyses also identified evidence of recombination. Previous studies of Australian samples of C. gattii showed a similar pattern: pervasive evidence of clonality and limited but unambiguous evidence for recombination (Halliday et al. 1999, 2003; Campbell et al. 2005 a; Saul et al. 2008). Indeed, evidence for recombination was found in samples of relatively broad distributions as well as that within a single tree hollow containing strains of only a single MATa mating type (Saul et al. 2008). Phylogenetic incompatibilities found in C. gattii in the Saul et al. (2008) study as well as in our current study supports the concept that same-sex mating may play a role in environmental populations of C. gattii (Fraser et al. 2005). Such a possibility is also supported by mitochondrial population genetic studies where evidence for recombination was observed within the VGI lineage (Xu et al. 2009). Furthermore, there were clear signals of horizontal gene transfer in the mitochondrial genomes between lineages VGI, VGII and VGV (Bovers et al. 2009; Xu et al. 2009).

Population structure

Molecular phylogenetic analyses identified that all 109 strains analysed here belonged to the VGI lineage. As described in Introduction, this lineage seemed to a broader geographic distribution than lineages VGII and VGIV (Litvintseva et al., 2010; Xu et al. 2010). However, its broad distribution in India and other parts of the world has not obscured the genetic differentiation observed among geographic and ecological populations of the VGI lineage in India. Such differentiations contrast with the limited genetic differences among samples of C. neoformans var. grubii from similar geographic areas and ecological niches in India, as observed in an earlier study (Hiremath et al. 2008). As far as we know, no similar comparisons have been made for environmental populations of C. neoformans and C. gattii.
in other parts of the world. At present, the reasons for the significant differentiations and for the differences between these two closely related species in CNSC are unknown. Two possibilities might contribute to the observed differences between the two species. In the first, the genetic differences observed are due to the lack of differentiation observed earlier for C. neoformans var. grubii, which might be due to incomplete or biased sampling in one or both studies. Indeed, despite over 8 years of sampling (from 2001 to 2008), our sample sizes among geographic regions and from different host trees were far from balanced and represented only a limited number of locations in India. If this possibility were true, the greater samples size analysed here than in previous studies (109 versus 78 strains, Hiremath et al. 2008) would suggest that the conclusion obtained here should be more robust. The second possibility is that the genetic differentiations among populations of C. gattii in India represent their true ecological and geographic differences. The significant geographic contribution to the overall genetic differences in C. gattii was also supported by the positive correlation between genetic distance and geographic differences among strains and populations. Increasingly, evidence indicates that tree debris is likely the primary environmental niche for C. gattii, which might be due to incomplete or biased sampling in one or both studies. Indeed, despite over 8 years of sampling (from 2001 to 2008), our sample sizes among geographic regions and from different host trees were far from balanced and represented only a limited number of locations in India. If this possibility were true, the greater samples size analysed here than in previous studies (109 versus 78 strains, Hiremath et al. 2008) would suggest that the conclusion obtained here should be more robust.

Table 4. Genetic differentiation between pairs of geographical populations of C. gattii from India.

<table>
<thead>
<tr>
<th>Geographical Region</th>
<th>Bulandshahr (n=13)</th>
<th>Delhi (n=64)</th>
<th>Meerut (n=5)</th>
<th>Trivannamalai (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delhi</td>
<td>0.243**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meerut</td>
<td>0.264</td>
<td>0.108*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trivannamalai</td>
<td>0.442*</td>
<td>0.413**</td>
<td>0.649</td>
<td></td>
</tr>
</tbody>
</table>

* 0.01 < P < 0.05; ** P < 0.01. Values represent *p* values between the respective population pairs.

Implications for microbial biogeography

The contrasting results in population structure between C. neoformans var. grubii and C. gattii VGI in India could be of broad interests to microbial population geneticists and microbial biogeographers. Based on microbial dispersal abilities and their susceptibilities to environmental conditions, the patterns of microbial distribution may be classified into one of four broad types (Xu, 2005; 2010; Martiny et al. 2006). The first is that the microorganisms are randomly distributed across all geographic regions and ecological niches with no dispersal barriers and no impact from ecological conditions. The second is that no dispersal barriers exist for microorganisms but contemporary environmental variation determines their distribution. This is the commonly called Baas-Becking hypothesis that states ‘everything is everywhere’ and that ‘the environment selects’ (Baas-Becking, 1934). The third is that dispersal is a barrier thus historical events determine microbial distribution pattern while contemporary environmental conditions play relatively little roles. The fourth possibility is that the distributions were impacted by both historical events (i.e. limited dispersal) and contemporary environmental conditions. The patterns of genetic variation we identified for geographic and ecological populations of C. neoformans var. grubii from India (Hiremath et al. 2008) would be consistent with the first distribution pattern while that of C. gattii VGI in this study would be consistent with the other three possibilities. A more extensive sampling from more locations and more trees might allow us to distinguish the three possibilities for C. gattii genotype distributions, and enabling a comprehensive and critical evaluation of individual geographic and ecological factors to the overall genetic variation between

Figure 5. A Mantel test between genetic distance (allelic differences at nine loci) and geographical distances (in km, calculated based on longitudinal and latitudinal coordinates) between all pairs of strains of C. gattii from India (P < 0.001).
the two species in India. Current evidence suggests that the four lineages (i.e. VGI, VGII, VGIII and VGIV) within C. gattii are not randomly distributed among geographic regions. Specifically, VGI is the most commonly isolated genotype group in several Asian countries (e.g. Litvintseva et al. 2010; Xu et al. 2010). VGII is the most frequently recovered from Australia, Colombia and the Pacific Northwest region of North America (Trilles et al. 2003; Kidd et al. 2005; Escandón et al. 2006). In contrast to lineages VGI and VGII, lineages VGIII and VGIV are relatively rare (Kidd et al. 2005; Bovers et al. 2008). There are two possibilities for the non-random distribution of the four distinct lineages among geographic regions. The first is that the observed pattern is due to incomplete sampling. The second is that our current knowledge represents their true geographical and/or biological differences among the four lineages. If the spatial pattern is confirmed with more extensive sampling across many more geographic regions, a key area of research would be to identify the contributions of historical events, dispersal abilities and contemporary environmental conditions that could have impacted the current distribution patterns. At present, relatively little is known about the contributions of these factors to spatial distributions of most microorganisms. Expanded sampling, coupled with genotypic and functional analyses of various groups of strains in the C. neoformans species complex, could help make this group of organisms a model system for studying microbial biogeography.

In conclusion, our multiclonal gene genealogical analyses of environmental samples of C. gattii from India identified that all of the isolates had the MATa mating type and belonged to the geographically broadly distributed VGI group. The populations of C. gattii showed both similarities to and differences from those of C. neoformans var. grubii prevalent in similar ecological niches and geographic locations in India. Specifically, both lineages showed strong evidence of clonality and limited but unambiguous evidence of recombination. However, different from C. neoformans var. grubii, we found very limited gene flow among the samples of C. gattii from either different geographic areas or different host tree species. The potential ecological and genetic factors for such divergent patterns as well as the implications of these patterns on the epidemiology of cryptococcal infections in humans remains to be explored.

Acknowledgements

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References


Chapter 4

Antifungal susceptibility of *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from decayed wood of trunk hollows of *Ficus religiosa* and *Syzygium cumini* trees in north-western India

Zia U. Khan
Harbans S. Randhawa
Tusharantak Kowshik
Anuradha Chowdhary
Rachel Chandy
Summary

We present antifungal susceptibility data on environmental isolates of *Cryptococcus neoformans* (serotype A, n = 117) and *Cryptococcus gattii* (serotype B, n = 65) cultured from decayed wood of trunk hollows of *Ficus religiosa* and *Syzygium cumini* trees. Susceptibilities to amphotericin B, fluconazole, ketoconazole, itraconazole and voriconazole were determined by using Etest. The MICs were read after 48 h as per the guidelines provided by the manufacturer. The MIC90s and susceptibility ranges for *C. neoformans* isolates were as follows: 0.094 (0.004–0.25) mg/L of amphotericin B, 4 (0.032–12) mg/L of fluconazole, 0.094 (0.004–0.75) mg/L of itraconazole, 0.064 (0.002–0.19) mg/L of ketoconazole, and 0.047 (0.006–0.125) mg/L of voriconazole, whereas for *C. gattii* isolates these were 0.125 (0.023–0.5) mg/L of amphotericin B, 8 (0.032–16) mg/L of fluconazole, 0.75 (0.006–2) mg/L of itraconazole, 0.125 (0.003–0.19) mg/L of ketoconazole, and 0.094 (0.004–0.125) mg/L of voriconazole. A comparison of the geometric means of MICs (mg/L) revealed that *C. gattii* was less susceptible than *C. neoformans* to amphotericin B (0.075 versus 0.051, P = 0.0003), fluconazole (2.912 versus 2.316, P = 0.003), itraconazole (0.198 versus 0.0344, P < 0.0001), ketoconazole (0.072 versus 0.037, P < 0.0001), and voriconazole (0.045 versus 0.023, P < 0.0001). The antifungal susceptibility data obtained in this study indicate that the occurrence of primary resistance among environmental isolates of *C. neoformans* serotype A and *C. gattii* serotype B is rare, and serotype B isolates are less susceptible than serotype A isolates.

Introduction

Cryptococcosis is one of the leading pulmonary and meningeal mycoses of worldwide occurrence. The disease predominantly occurs in immunocompromised patients with underlying predisposing factors, such as organ transplantation, haematological malignancies, and advanced human immunodeficiency virus disease. The causative species include *Cryptococcus neoformans* (serotypes A, D, AD) and *Cryptococcus gattii* (serotypes B and C). Epidemiologically, these species differ from each other mainly with respect to geographic distribution, preference for natural habitat, pathobiology and host infectivity (immunocompetent versus immunocompromised) and genetics (Casadevall & Perfect, 1998; Kwon-Chung et al., 2006). Therapeutic management of cryptococcal infections usually consists of amphotericin B therapy with or without 5-flucytosine, whereas fluconazole is the drug of choice for prophylaxis and maintenance therapy. Owing to frequent instances of disease relapse, there is growing concern among clinicians about emergence of antifungal resistance in *C. neoformans* and *C. gattii* during therapy or prophylaxis (Berg et al., 1998; Brandt et al., 2001). Most reports of resistance have emerged in the setting of cryptococcal meningoitis in AIDS patients after prolonged prophylaxis with fluconazole (Armengou et al., 1996; Paugam et al., 1994). This problem could have more serious dimensions in developing countries of Southeast Asia and Africa where large numbers of HIV-infected patients exist, and resources to treat the disease are inadequate or patients might receive suboptimal doses of fluconazole. In this context, recent reports of increased resistance to fluconazole in clinical isolates of *C. neoformans* originating from Cambodian (Chandenier et al., 2004; Sar et al., 2004) and Indian (Datta et al., 2003) patients are noteworthy. Studies on antifungal susceptibilities of environmental isolates of *C. neoformans* or *C. gattii* are scanty and are based on a small number of isolates (Currie et al., 1995; Franzot et al., 1996; Yildiran et al., 2000; Alves et al., 2001; Moraes et al., 2003; Souza et al., 2005; Tay et al., 2005; Pedroso et al., 2006). No such information is available from Asia or Africa where cryptococcosis is a significant problem in HIV/AIDS patients. In this communication, we present data for a large number of environmental isolates of *C. neoformans* (n = 117) and *C. gattii* (n = 65) cultured from decayed wood inside trunk hollows of living trees of *Ficus religiosa* and *Syzygium cumini* in north-western India.

Materials and Methods

Collection and identification of isolates

One hundred and eighty-two environmental isolates of *C. neoformans* serotype A (n = 117) and *C. gattii* serotype B (n = 65) from decayed wood inside trunk hollows of *S. cumini* living trees in Delhi/New Delhi metropolitan area, Amritsar city (Punjab), Meerut Cantt (Uttar Pradesh) and from *F. religiosa* trees in New Delhi cultured over a 6 year period (Randhawa et al., 2003; 2005; 2006) were included for antifungal susceptibility testing. Identification of the *C. neoformans* and *C. gattii* isolates was initially done by the characteristic brown pigment developing in their yeast-like colonies on simplified niger seed medium (Paliwal & Randhawa, 1978) with seed concentration increased to 70 g/L, and by verification of physiological characteristics employing the Vitek 2 Yeast ID system (bioMerieux, Marcy-
l’Étoile, France). Confirmation of C. gattii isolates was done by their ability to grow on canavanine-glycine-bromothymol medium which was marked by change in colour of the medium from greenish yellow to blue. In case of any doubt, identity was also confirmed by D-proline assimilation (Dugalt et al., 1987). Serotyping of the isolates was done by Crypto Check kit (Iatron Laboratories Inc., Tokyo, Japan).

**Etest**

The *in vitro* activity of the antifungal agents was determined by the Etest (AB Biodisk, Solna, Sweden) in accordance with the manufacturer’s instructions. The Etest was performed by inoculating 150 mm Petri dishes containing 60 mL of RPMI-1640 agar supplemented with 2% glucose and buffered to pH 7.0 with MOPS as recommended by the CLSI (formerly NCCLS) (CLSI, 2002). The inoculum was applied with cotton swabs using growth suspension prepared in 0.85% NaCl with a turbidity equivalent to that of a 0.5 McFarland standard. Plates were incubated at 35°C and read after 48–72 h. Reference strains *Candida albicans* ATCC 90 028 and *Candida parapsilosis* ATCC 22 019 were used for quality control. In the absence of CLSI susceptibility breakpoints for Cryptococcus species, only data on MICs for the isolates are presented. The isolates were tested in a blinded manner without knowing their species or the serotypes.

**Statistical analysis**

Scattergrams of the MICs for serotype A and serotype B isolates were compared by the Mann–Whitney test. Statistical analyses were performed with GraphPad Prism version 3.00 (San Diego, CA, USA). Statistical significance was defined as a *P* value < 0.05.

**Results**

**In vitro susceptibility**

The comparative data on MIC against amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole as determined by Etest for *C. gattii* and *C. neoformans* isolates are presented in Figure 1. The MICs and susceptibility ranges for *C. neoformans* isolates (*n* = 117) were as follows: 0.094 (0.004–0.25) mg/L of amphotericin B, 4 (0.032–12) mg/L of fluconazole, 0.094 (0.004–0.75) mg/L of itraconazole, 0.064 (0.002–0.19) mg/L of ketoconazole, and 0.047 (0.006–0.125) mg/L of voriconazole, whereas for *C. gattii* isolates (*n* = 65) these were 0.125 (0.023–0.5) mg/L of amphotericin B, 8 (0.032–16) mg/L of fluconazole, 0.75 (0.006–2) mg/L of itraconazole, 0.125 (0.003–0.19) mg/L of ketoconazole, and 0.094 (0.004–0.125) mg/L of voriconazole.

**Relationship with serotypes**

A comparison of the geometric means (GMs) of MICs (mg/L) revealed that *C. gattii* serotype B showed significantly reduced susceptibility compared with *C. neoformans* serotype A to amphotericin B (0.075 versus 0.051, *P* = 0.0003), fluconazole (2.912 versus 2.316, *P* = 0.003), itraconazole (0.198 versus 0.0344, *P* < 0.0001), ketoconazole (0.072 versus 0.037, *P* < 0.0001), and voriconazole (0.045 versus 0.023, *P* < 0.0001) (Figure 1).

**Discussion**

There is paucity of reports on *in vitro* antifungal susceptibilities of environmental isolates of *C. neoformans* and *C. gattii* and these are based on small sample size (Currie et al., 1995; Franzot et al., 1996; Yildiran et al., 2000; Alves et al., 2001; Moraes et al., 2003; Souza et al., 2005; Tay et al., 2005; Pedroso et al., 2006). While most of the studies included isolates obtained from pigeon or bird droppings, (Currie et al., 1995; Franzot et al., 1996; Yildiran et al., 2000; Alves et al., 2001; Moraes et al., 2003; Souza et al., 2005) the others have not indicated the source of origin (Moraes et al., 2003; Pedroso et al., 2006). Our study is noteworthy in that it presents antifungal susceptibility data on the largest number of environmental isolates of *C. neoformans* and *C. gattii* reported so far, and all of them came from tree trunk hollows. In all probability, these isolates had no previous exposure to any of the antifungal agents tested in this study and hence the MIC values we have presented should be intrinsic. A comparison of the susceptibility profiles of environmental isolates published during 1996–2005 revealed that MICs values of antifungal agents have not changed noticeably (Currie et al., 1995; Franzot et al., 1996; Yildiran et al., 2000; Alves et al., 2001; Moraes et al., 2003; Souza et al., 2005; Tay et al., 2005). These observations are reinforced by our results. No noticeable differences were observed in the MICs for our environmental isolates obtained between 2000–02 and 2003–05, which are also in conformity with the global trends in antifungal susceptibility of clinical isolates of *C. neoformans* (Archibald et al., 2004; Calvo et al., 2001; Pfaller et al., 2005). As reported in previous studies on environmental (Yildiran et al., 2000; Souza et al., 2005) and clinical isolates, (Maxwell et al., 2003; van Duin et al., 2004) voriconazole exhibited highest activity against our environmental isolates of *C. gattii* as well as *C. neoformans*.

No CLSI susceptibility breakpoints for Cryptococcus species are currently available for any of the antifungal agents. Moreover, relationship of *in vitro* susceptibility results with clinical outcome is not yet clearly understood. Several technical problems pertaining to *in vitro* antifungal susceptibility of Cryptococcus species on RPMI medium used in CLSI methodology warrant attention (Ghanoum et al., 1992; Espinel-Ingroff et al., 1996). For instance, RPMI medium poorly identifies strains putatively resistant to amphotericin B and, therefore, to overcome this problem the use of yeast nitrogen base (YNB) medium or antibiotic 3 medium has been proposed (Ghanoum et al., 1992; Espinel-Ingroff et al., 1996; Jessup et al., 1998; Rex et al., 1995; Warnock et al., 1998). However, the MICs obtained on YNB medium in a recent comparative study did not predict the early clinical outcome better than the MICs obtained with RPMI medium (Dannaoui et al., 2006). Inconsistencies have also been observed between the MICs obtained on RPMI medium by Etest and CLSI micro-dilution method (Espinel-Ingroff et al., 1996; Warnock et al., 1998; Dannaoui et al., 2006; Aller et al., 2000). Etest on RPMI medium has been shown to yield higher MICs of fluconazole and lower MICs of amphotericin B than the CLSI method of broth dilution (Dannaoui et al., 2006).

It is well known that *C. neoformans* (serotypes A, D and AD) and *C. gattii* (serotypes B and C) differ from each other in several characteristics, including epidemiology, pathogenicity, and clinical manifestations. Besides, minor to significant differences in the susceptibilities of the two species have been reported. Chen et al. (2000) reported that *C. gattii* isolates were less susceptible than *C. neoformans* to amphotericin B (*P* < 0.001). Likewise, a difference in
Figure 1. Comparison of fluconazole, itraconazole voriconazole, ketoconazole and amphotericin B MICs for *C. neoformans* serotype A and *C. gattii* serotype B isolates.

Susceptibilities to azoles (one dilution difference) has been reported (Calvo et al., 2001). Using CLSI microdilution method, Trilles et al. (2004) found that while *C. gattii* was less susceptible to azoles than *C. neoformans*, this difference was not so conspicuous with respect to amphotericin B. This is contrary to the study of Chen et al., (2000) who demonstrated that *C. gattii* is significantly less susceptible than *C. neoformans* to amphotericin B. A comparison of GMs of MICs in our study, however, revealed that *C. gattii* was less susceptible than *C. neoformans* to amphotericin B. A comparison of *C. gattii* with *C. neoformans* revealed that the occurrence of primary resistance against amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole among environmental isolates of *C. neoformans* serotype A and *C. gattii* serotype B is rare, and serotype B isolates are less susceptible than serotype A isolates.

Transparency declarations
None to declare.

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Chapter 4

Susceptibility of environmental Cryptococcus isolates

References


Cryptococcus neoformans, serotype A and Cryptococcus gattii, serotype B isolates differ in their susceptibilities to fluconazole and voriconazole

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Summary

This study presents antifungal susceptibility data on environmental isolates of Cryptococcus neoformans, serotype A (n = 32) and C. gattii, serotype B (n = 18) to fluconazole and voriconazole employing disk diffusion and Etest methods. Disk diffusion test was performed on Mueller-Hinton agar as recommended in the Clinical and Laboratory Standards Institute (CLSI) document, M44-A. For comparison, disk diffusion test and Etest were also performed on RPMI 1640 agar supplemented with 2% glucose. The plates were incubated at 35°C and read after 48 h. Comparison of geometric mean (GM) of inhibition zone diameters revealed that C. gattii isolates were significantly less susceptible than C. neoformans isolates to fluconazole (p = 0.001) and voriconazole (p < 0.0001). Similar results were obtained on RPMI agar by disk diffusion test and Etest showing significantly reduced susceptibility for C. gattii isolates. Notwithstanding the differences in the susceptibilities of the two species to fluconazole and voriconazole, they appeared susceptible as per the CLSI breakpoints recommended for some Candida species. To what extent these differences in the susceptibilities of C. neoformans and C. gattii impact therapeutic management of cryptococcosis is unclear, although some studies reported less favorable response in cases caused by the latter species.

Introduction

The genus Cryptococcus contains two pathogenic species, namely Cryptococcus neoformans and Cryptococcus gattii. Currently, the two species comprise three varieties, five serotypes and eight genotypes. C. neoformans has been classified into serotype A, D, and AD, whereas C. gattii into serotype B and C (Chayakulkeeree & Perfect, 2008). The strains belonging to serotype A represent C. neoformans var. grubii, whereas those of serotype D represent C. neoformans var. neoformans. Based on molecular characterization, genotypes VN I to VN IV have been assigned to C. neoformans and genotypes VG I to VG IV to C. gattii (Chayakulkeeree & Perfect, 2008). The majority of infections, particularly in immunocompromised patients, are caused by C. neoformans, whereas C. gattii accounts for smaller proportion of cases often occurring in immunocompetent patients in tropical and subtropical regions. Exposure to colonized soil or trees enhances the possibility of inhalation of airborne propagules (yeast forms and basidiospores) and may cause pulmonary and central nervous system disease. Recent reports of human cryptococcosis in Vancouver Island due to C. gattii (genotype VGIIa, VGIIb) with evidence of strong link to common environmental exposure is noteworthy as the species extends its geographic distribution to temperate climate (Kidd et al., 2004).

Most of the studies on in vitro antifungal susceptibility have been carried out on clinical or environmental isolates of C. neoformans using broth dilution and/or Etest methods (Franzot & Hamdan, 1996; Warnock et al., 1998; Aller et ., 2000; Maxwell et al., 2003; Pfaller et al., 2004). There is paucity of data comparing C. neoformans and C. gattii susceptibilities to different antifungal agents by disk diffusion test. However, employing broth dilution methods, a few studies have reported that clinical isolates of C. gattii are relatively less susceptible than C. neoformans to amphotericin B (Chen et al., 2000) 5-flucytosine (Chen et al., 2000) and azoles (Calvo et al., 2000; Fernandez et al., 2003; Gomez-Lopez et al., 2008; Torres-Rodriguez et al., 2008). Recently, Dannaoui et al (2006) reported that even within C. neoformans species, serotype D isolates showed significantly lower minimum inhibitory concentrations (MICs) than serotype A isolates to amphotericin B and fluconazole. In an earlier publication (Khan et al., 2007), we have reported that environmental C. gattii, serotype B isolates were significantly higher MICs than C. neoformans, serotype A isolates to amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole. In this communication, we present comparative results of antifungal susceptibility testing to fluconazole and voriconazole by disk diffusion test and Etest. The test isolates had originated from a common source of decaying wood of tree hollows and were not exposed to antifungal agents tested in the study.

Materials and Methods

Source of isolation and identification of the isolates

All the environmental isolates of C. neoformans and C. gattii were cultured from decayed wood obtained from trunk hollows of Syzygium cumini and Ficus religiosa trees (Randhawa et al., 2003; 2006). The isolates were identified by their typical microscopic morphology.
showing encapsulated yeast cells, ability to develop characteristic brown pigmentation on simplified niger seed agar medium (Paliwal & Randhawa, 1978) and by verification of biochemical characteristics employing Vitek2 yeast identification system (bioMérieux, Marcy-l’Etoile, France). Confirmation of C. gattii isolates was done by their ability to grow on canavanine-glycine-bromothymol medium (changing the color of the medium from greenish yellow to blue) and by D-proline assimilation (Dufait et al., 1987). Serotyping was done by Crypto Check kit (Iatron Laboratories Inc., Tokyo, Japan).

**Disk diffusion and Etest**

Disk diffusion testing was performed as described in the CLSI document No. M44-A using Mueller-Hinton agar (MHA) supplemented with 2% glucose and 0.5µg/ml methylene blue dye (CLSI, 2004). The Etest (AB Biodisk, Solna, Sweden) was done in accordance with the manufacturer’s instructions on RPMI-1640 medium supplemented with 2% glucose and buffered to pH 7.0 with 0.165 M 3-N-morpholino propane-sulphonic acid as described previously (CLSI, 2002) With a view to obtaining comparative results, we also performed disk diffusion test with RPMI-1640 medium. Fluconazole (25-µg) and voriconazole (1-µg) susceptibility disks were obtained from Becton-Dickinson (Sparks, Md, USA). The inoculated plates were incubated at 35°C and read after 48 h. The inhibition zone diameter and minimum inhibitory concentration (MIC) end points were read visually. Reference strains Candida albicans ATCC 90028 and Candida parapsilosis ATCC 22019 were used for quality control. In absence of susceptibility breakpoint for Cryptococcus species, we used CLSI interpretive criteria recommended for Candida species (CLSI, 2004; 2006). Accordingly, corresponding susceptibility breakpoints for fluconazole disk diffusion test and Etest were as follows: susceptible (S) ≥19 mm and <8 µg/ml, susceptible dose-dependent (S-DD) 15-18 mm and 16-32 µg/ml, and resistant (R) ≤14 mm and ≥64 µg/ml, respectively. The susceptibility breakpoints for voriconazole disk diffusion test were S, ≥17 mm; SDD, 14 to 16 mm; and R, <13 mm diameter of inhibition zones (Pfaller et al., 2006) The corresponding Etest breakpoints were S, <1 µg/ml; SDD, 2 µg/ml; and R, >4 µg/ml (CLSI, 2006).

**Statistical analysis**

Differences in the geometric mean of zone diameters and Etest MICs for serotype A and serotype B isolates were compared by the Mann-Whitney test. The diameters of inhibition zone (mm) surrounding the fluconazole and voriconazole disks at 48 h of incubation were plotted against their respective E-test MICs read at 48 h. The least squares method and weighted least squares methods were used to calculate regression lines using SPSS version 14.0. Statistical analyses were performed with GraphPad Prism version 3.00 (San Diego, CA, USA). Statistical significance was defined as a p-value < 0.05.

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**Results**

A comparison of antifungal susceptibility data obtained by disk diffusion and E test for C. neoformans and C. gattii is presented in Tables 1-2 and Figures 1-3. Using 25-µg fluconazole disk, the geometric mean (GM) values of inhibition zone diameters on MHA and RPMI medium of C. neoformans, serotype A isolates were 25 (range 16-34) mm and 29 (range 22-35) mm, and of C. gattii serotype B isolates, these were 21 (range 18-26) mm and 24 (range 15-32) mm, respectively (Tables 1-2). Likewise, with voriconazole 1-µg disks, GM of inhibition zone on MHA and RPMI medium were 38 (range 31-50) mm and 41 (range 32-46) mm for serotype A isolates, and 32 (range 27-39) mm and 34 (range 25-43) mm for serotype B isolates, respectively (Tables 1-2). The Etest MIC<sub>50</sub> and MIC<sub>90</sub> values of for C. gattii and C. neoformans were 6 and 12 µg/ml, and 2 and 6 µg/ml for fluconazole, whereas for voriconazole these values were 0.032 and 0.064 µg/ml, and 0.023 and 0.064 µg/ml, respectively (Table 2). The comparison of GM of inhibition zone diameters of disk diffusion tests performed on MHA and RPMI medium by Mann-Whitney test revealed that C. gattii, serotype B isolates were significantly less susceptible than C. neoformans, serotype A isolates to both fluconazole and voriconazole (p < 0.001 and p < 0.0001) and voriconazole (p < 0.0001) and voriconazole (p < 0.0001), respectively. Similarly, a comparison of geometric mean of MICs of Etest revealed that serotype B isolates were significantly less susceptible than serotype A isolates for fluconazole (4.3 µg/ml vs. 2.2 µg/ml, p < 0.008) as well as voriconazole (0.041 vs. 0.026 µg/ml, p < 0.003) (Table 1, Figs. 1-2). There was a significant agreement between zone diameter and Etest MICs for fluconazole (y = 29.04 – 0.42 x; R=0.367, p = 0.009) and voriconazole (y = 42.24 – 100.35 x; R = 0.347, p = 0.008) calculated by least squares method (Figs. 3A, 3B). Thus, there was a significant agreement between zone diameter and Etest MICs for fluconazole (y = 29.04 – 0.42 x; R=0.367, p = 0.009) and voriconazole (y = 42.24 – 100.35 x; R = 0.347, p = 0.008) calculated by least squares method (Figs. 3A, 3B). Thus, the isolates exhibiting narrower inhibition zone by disk diffusion test mostly showed higher MICs by Etest.

Taking into consideration CLSI approved susceptibility breakpoints for Candida species, both the drugs showed very good in vitro activity with few exceptions. For fluconazole, 2 of the C. gattii isolates that fell in S-DD category (cut-off value ≤14mm) with zone diameters of 15 and 18 mm yielded corresponding Etest MICs of 8 and
Table 2. Comparison of susceptibility of C. gattii, serotype A and C. neoformans, serotype B, isolates to fluconazole and voriconazole by disk diffusion test and E-test using RPMI medium

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>Antifungal agents</th>
<th>Disk diffusion inhibition zone (mm)</th>
<th>E-test MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>GM* Range</td>
<td>MIC50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GM</td>
<td>MIC 90</td>
</tr>
<tr>
<td>C. neoformans, serotype A (32)</td>
<td>Fluconazole</td>
<td>22-35</td>
<td>0.006-0.125</td>
</tr>
<tr>
<td>C. gattii, serotype B (18)</td>
<td>Fluconazole</td>
<td>15-32</td>
<td>0.38-16</td>
</tr>
<tr>
<td>C. neoformans, serotype A (32)</td>
<td>Voriconazole</td>
<td>22-35</td>
<td>0.016-0.094</td>
</tr>
<tr>
<td>C. gattii, serotype B (18)</td>
<td>Voriconazole</td>
<td>25-34</td>
<td>0.016-0.094</td>
</tr>
</tbody>
</table>

*A Geometric mean

Discussion

This study is noteworthy that it presents antifungal susceptibility data on environmental isolates of C. gattii and C. neoformans, which apparently have not been previously exposed to fluconazole and voriconazole. While the observations on significantly higher MICs of C. gattii isolates to azoles are in conformity with earlier reports (Calvo et al., 2000; Fernandes et al., 2003; Trilles et al., 2004; Khan et al., 2007; Gomez-Lopez et al., 2008; Torres-Rodriguez et al., 2008), our study provides additional concordant data derived from disk diffusion test. The geometric mean of inhibition zone diameters of fluconazole and voriconazole was significantly lower for C. gattii isolates than C. neoformans isolates and this was also reflected in Etest MICS (Table 2). Taking into consideration CLSI susceptibility breakpoints recommended for Candida species (CLSI, 2004), all our C. gattii and C. neoformans isolates were susceptible/S-DD to fluconazole and susceptible to voriconazole, (Table 2). Similar results were obtained by Etest and demonstrated a good correlation with inhibition zone diameter values (Figs. 3A-3B). Despite significant intrinsic differences in MICs and inhibition zone diameters between the two species (Table 2), both the drugs showed good in vitro activity, mostly yielding breakpoint values far less than those recommended for susceptible Candida isolates (CLSI, 2004; 2006; Pfaffer et al., 2006).

The underlying mechanisms for differential antifungal susceptibilities of the two Cryptococcus species (Calvo et al., 2000; Chen et al., 2000; Fernandes et al., 2003; Gomez-Lopez et al., 2008; Torres-Rodriguez et al., 2008) or between the serotypes of the same species (Dannaoui et al., 2006) are not fully understood. An important factor implicated in susceptibility to antifungal agents is the melanin content of the cell wall, which may prevent the drug from reaching its target site (Gomez & Nosanchuk, 2003; Ikeda et al., 2003; Nosanchuk et al., 2006). It is yet to be determined if C. gattii and C. neoformans isolates differ in their ability to synthesize melanin in vitro and/or in vivo. Recently, Alvarado–Ramirez et al. (2008) reported a much higher laccase activity among C. gattii isolates than the C. neoformans isolates that were recovered from goats that died of cryptococcosis. In this context, it may be pertinent to mention that some literature reports indicate a less favorable response to antifungal therapy and relatively worse prognosis of infections caused by C. gattii than those caused by C. neoformans (Sorrell, 2001; Speed & Dunt, 1995).

Both C. neoformans and C. gattii can synthesize melanin or melanin-like pigments from a variety of natural substrates containing diphenolic/indolic compounds (Nosanchuk & Casadevall, 2006; Eisenman et al., 2007). Attention may be drawn here that the primary

Figure 1. Comparison of fluconazole and voriconazole zones of inhibition (mm) of C. neoformans, serotype A (n = 32) and C. gatti, serotype B (n = 18) isolates by disk diffusion test read at 48 hr. Bars represent the geometric mean of zone of inhibition with 95% confidence intervals.

Figure 2. Comparison of fluconazole and voriconazole, MICs (µg/ml) of C. neoformans, serotype A (n = 32) and C. gatti, serotype B (n = 18) isolates by E-test. Bars represent the geometric mean of MICs with 95% confidence intervals. MIC scale is in log2 base.
The ecological niche of \textit{C. gattii} is decaying wood (Randhawa et al., 2006; Chayakulkeeree & Perfect, 2008), where lignin is degraded by phenoloxidase/laccase enzymes that could possibly be associated with melanin synthesis (Kojima et al., 1990). By contrast, \textit{C. neoformans} is mostly found in association with pigeon excreta, although more recently it has also been reported from tree hollows (Randhawa et al., 2003; 2006; Chayakulkeeree & Perfect, 2008). Whether the preferential occurrence of the two species in a particular natural habitat contributes to their differential susceptibilities to antifungal agents is unclear. A recent report by Chaskes et al., (2008) suggests that pigment production by \textit{C. gattii} and \textit{C. neoformans} may be dependent on the type of the substrate available in the medium. For example, \textit{C. gattii} produced large amount of brown intracellular pigment(s) on fructose D-tryptophan glycine medium, whereas \textit{C. neoformans} produced only smaller amount of the brown pigments. Furthermore, pigments produced by \textit{C. gattii} from D-tryptophan were distinct and were not related to melanin formation from 3, 4-dihydroxyphenylalanine.

While studying the melanization properties of our environmental isolates on simplified Niger seed agar (Paliwal & Randhawa, 1978) and tobacco agar (Khan et al., 2004), we observed more intense pigmentation of the colonies of \textit{C. gattii} isolates than the \textit{C. neoformans} isolates (Khan et al., 2007). Although seems speculative, it is possible that \textit{C. gattii} isolates following their primary isolation from tree hollows may undergo some degree of attenuation in their capacity to produce melanin on laboratory media. This may possibly explain the higher (Calvo et al., 2001; Fernandes et al., 2003; Khan et al., 2007; Gomez-Lopez et al., 2008; Torres-Rodriguez et al., 2008) and the lower or similar (Morgan et al., 2006; Thompson et al., 2008) MICs of azoles reported in different studies for \textit{C. gattii} in comparison with \textit{C. neoformans}.

In conclusion, our disk diffusion susceptibility data reinforce the observations that \textit{C. gattii} isolates are significantly less susceptible to fluconazole and voriconazole than \textit{C. neoformans} isolates. To what extent these differences in the antifungal susceptibilities of the two species have implications for therapeutic management of cryptococcosis, is an area for further research.

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\textbf{Competing interest:} None to declare.

\textbf{Ethical approval:} Not required.
Chapter 5

C. neoformans and C. gattii susceptibility

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In vitro antifungal susceptibility profiles and genotypes of 308 clinical and environmental isolates of *Cryptococcus neoformans* var *grubii* and *Cryptococcus gattii* serotype B from north-western India

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Antifungal susceptibilities of Cryptococcus spp. Chapter 6

Summary

Cryptococcus neoformans and C. gattii are etiologic agents of cryptococcosis, a major opportunistic systemic mycosis of increasing global importance. We report the antifungal susceptibility profiles of clinical and environmental isolates of Cryptococcus neoformans var. grubii, genotype VNI/AFLP1, (n= 246) and Cryptococcus gattii serotype B, genotype VGI/AFLP4 (n= 62), originating from patients and environmental sources in north-western India. All of the C. neoformans var grubii and C. gattii isolates were mating type α. Employing the broth microdilution method, both of the species were susceptible to the antifungals tested except two clinical C. neoformans var grubii isolates which were resistant to 5-flucytosine (MIC >64 µg ml⁻¹). The data on geometric mean of MICs revealed that antifungals tested except two clinical C. neoformans var grubii isolates which were resistant to 5-flucytosine (MIC >64 µg ml⁻¹). The data on geometric mean of MICs revealed that antifungals tested except two clinical C. neoformans var grubii isolates which were resistant to 5-flucytosine (MIC >64 µg ml⁻¹). The data on geometric mean of MICs revealed that antifungals tested except two clinical C. neoformans var grubii isolates which were resistant to 5-flucytosine (MIC >64 µg ml⁻¹). The data on geometric mean of MICs revealed that antifungals tested except two clinical C. neoformans var grubii isolates which were resistant to 5-flucytosine (MIC >64 µg ml⁻¹). The data on geometric mean of MICs revealed that antifungals tested except two clinical C. neoformans var grubii isolates which were resistant to 5-flucytosine (MIC >64 µg ml⁻¹). The data on geometric mean of MICs revealed that antifungals tested except two clinical C. neoformans var grubii isolates which were resistant to 5-flucytosine (MIC >64 µg ml⁻¹).

Cryptococcus neoformans var grubii isolates were significantly less susceptible to fluconazole, itraconazole and voriconazole (P <0.0001). Also, MIC₅₀ of C. gattii was two-fold higher than that of C. neoformans var grubii for fluconazole, itraconazole and voriconazole. However, no statistically significant difference in susceptibility of the two Cryptococcus species was observed against amphotericin B and 5-flucytosine. Furthermore, the environmental C. neoformans var grubii isolates were significantly less susceptible to fluconazole, itraconazole and 5-flucytosine (P <0.0001) than the clinical isolates. A continued surveillance of antifungal susceptibility of clinical and environmental strains of C. neoformans and C. gattii would be desirable for monitoring the emergence of any resistant strains in order to ensure more successful therapy of cryptococcosis.

Introduction

Cryptococcosis is a life-threatening, opportunistic fungal infection of world-wide distribution, including India, especially in the HIV positive population (Casadevall & Perfect 1998; Chakrabarti et al., 2000; Khanna et al., 2000; Lakshmi et al., 2007; Thakur et al., 2008). It has two major etiologic agents, namely, Cryptococcus neoformans and Cryptococcus gattii. Cryptococcus neoformans has serotypes A, D and AD, whereas C. gattii has serotypes B and C. The strains belonging to serotype A represent C. neoformans var grubii, whereas those of serotype D represent C. neoformans var neoformans. Cryptococcus neoformans var and C. gattii differ significantly with regard to their geographical distribution and ecological niches (Casadevall & Perfect 1998; Kwon-Chung et al., 2002). Based on molecular studies, employing PCR fingerprinting, Amplified Fragment Length Polymorphisms (AFLP) analysis, analysis of the orotidine monophosphate pyrophosphorylase (URAS) and phospholipase (PLB1) genes by Restriction Fragment Length Polymorphism (RFLP) and Multilocus Sequence Typing (MLST), C. neoformans and C. gattii have been further classified into several distinct genotypes. They are VNI/AFLP1 and VNI/AFLP1A/AFLP18 (C. neoformans var grubii, serotype A), VNI/AFLP2 (C. neoformans var neoformans, serotype D), VNI/AFLP3 (hybrid serotype AD), VGI/AFLP4, VGI/AFLP5, VGI/AFLP7 and AFLP7 and AFLP10 (C. gattii, serotype B/C). In addition, hybrids of C. neoformans var neoformans and C. gattii of C. neoformans var grubii and C. gattii belong to genotype AFLP8 and AFLP9, respectively. A vast majority of cryptococcal infections, particularly in immunocompromised patients, are caused by C. neoformans, whereas C. gattii accounts for a smaller proportion of cases, often occurring in immunocompetent patients in tropical and subtropical regions. However, in the past decade, a more virulent genotype of C. gattii, VGIa/VGIlc, has emerged as a primary pathogen on Vancouver Island and its adjoining areas in Canada and the United States, indicating extension of its geographical domain to the temperate climate (Kidd et al., 2004; Byrnes et al., 2010). The outbreak of human and animal cryptococcosis on Vancouver Island due to C. gattii indicated that exposure to environmental sources such as colonised trees and soil led to pulmonary and disseminated cryptococcosis. In India, we have reported a widespread colonization of decayed wood inside trunk hollows of diverse tree species by C. neoformans var grubii and C. gattii serotype B (Randhawa et al., 2006, 2008; Hiremath et al., 2008). The objective of this study was to compare antifungal susceptibility profiles of clinical isolates with those of environmental isolates of C. neoformans var grubii and C. gattii serotype B originating from decayed wood of diverse tree species and from their surrounding soil in north-western India.
Methods

Fungal isolates
Three hundred and eight isolates, comprising 246 fungal isolates

Methods

the URA5 locus (Meyer et al., 1999); and (ii) DNA sequences at

Gene expression

and 5-flucytosine, 0.03-16 µg ml⁻¹ for amphotericin B, and 0.015-8 µg ml⁻¹ for itraconazole and voriconazole. The yeasts inoculum was adjusted to a concentration of 0.5-2.5 x10⁴ cells ml⁻¹ in RPMI medium as measured by spectrophotometer, and an aliquot of 0.1 ml was added to each well containing various concentrations of antifungal drugs. Drug-free and yeast-free controls were included and microplates were incubated at 35 °C for 72 h.

Following the CLSI recommendations, two quality control strains, Candida krusei (ATCC 6258) and Candida parapsilosis (ATCC 22019) were used with each test. The reproducibility of the in vitro results was assessed by determining minimum inhibitory concentrations (MICs) for all strains twice on two different days. The MIC end points were read visually after 72 h and defined for fluconazole, voriconazole, itraconazole and 5-flucytosine as the lowest drug concentration that caused a prominent decrease in growth (50%) vis-a-vis the controls. For amphotericin B, the MIC was defined as the lowest concentration at which there was 100% inhibition of growth compared with the drug-free control wells.

Statistical analysis

Statistical differences between MIC values of various groups of strains were assessed, using the Mann-Whitney test. Statistical significance was defined as P-value <0.05. Statistical analyses were performed with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

Results

The results of antifungal susceptibility testing of C. neoformans var grubii, genotype VNI/AFLP1 and C. gattii type B, genotype VGI/AFLP4 are summarized in Table 1. All of the isolates of C.neoformans var grubii and C. gattii showed low MICs to all the antifungals tested except two clinical of C. neoformans var grubii which had high MICs against 5-flucytosine (MIC >64 µg ml⁻¹). However, there were some notable differences in antifungal susceptibility of the two species and within each species depending upon origin of strains from environmental or clinical sources. Specifically, the geometric means (GM) of MICs of the C. gattii type B sample were significantly higher than those of C. neoformans var. grubii for fluconazole (GM 6.996 versus 2.614 µg ml⁻¹, P<0.0001), itraconazole (GM 0.244 versus 0.112 µg ml⁻¹, P<0.0001) and voriconazole (GM 0.138 versus 0.056 µg ml⁻¹, P<0.0001). Similarly, MIC₅₀ of C. gattii was two-fold higher than that of C. neoformans var grubii for fluconazole (8 versus 4 µg ml⁻¹), itraconazole (0.5 versus 0.25 µg ml⁻¹) and voriconazole (0.25 versus 0.125 µg ml⁻¹). However, no statistically significant difference in susceptibility of the two Cryptococcus species was observed against amphotericin B and 5-flucytosine. Interestingly, in comparison with clinical isolates, the environmental C. neoformans var grubii isolates exhibited significantly reduced susceptibility to fluconazole (GM 3.639 versus 2.19 µg ml⁻¹, voriconazole, and amphotericin B). Further dilutions of each antifungal agent were prepared with RPMI 1640 medium with glucose without bicarbonate (Sigma), buffered to pH 7 with 0.165M 3-N-morpholinepropanesulfonicacid (Sigma). The drug dilutions were dispensed in 96-well microdilution plates, sealed and frozen at -70 °C until needed. The final concentrations of the drugs ranged from 0.125-64 µg ml⁻¹ for fluconazole and 5-flucytosine, 0.03-16 µg ml⁻¹ for amphotericin B, and 0.015-8 µg ml⁻¹ for itraconazole and voriconazole.

Antifungal susceptibility testing

In vitro antifungal susceptibility testing was determined, using the Clinical and Laboratory Standards Institute broth microdilution method (CLSI, 2008). The tested antifungal drugs included amphotericin B (Sigma, St. Louis, Mo), fluconazole, voriconazole (Pfizer, Groton, CT), itraconazole (LeePharma, Hyderabad, India), and 5-flucytosine (Sigma). Stock solutions were prepared in water (fluconazole and 5-flucytosine) or dimethyl sulfoxide (itraconazole, voriconazole, and amphotericin B). Further dilutions of each antifungal agent were prepared with RPMI 1640 medium with glucose without bicarbonate (Sigma), buffered to pH 7 with 0.165M 3-N-morpholinepropanesulfonicacid (Sigma). The drug dilutions were dispensed in 96-well microdilution plates, sealed and frozen at -70 °C until needed. The final concentrations of the drugs ranged from 0.125-64 µg ml⁻¹ for fluconazole and 5-flucytosine, 0.03-16 µg ml⁻¹ for amphotericin B, and 0.015-8 µg ml⁻¹ for itraconazole and voriconazole. The yeasts inoculum was adjusted to a concentration of 0.5-2.5 x10⁴ cells ml⁻¹ in RPMI medium as measured by spectrophotometer, and an aliquot of 0.1 ml was added to each well containing various concentrations of antifungal drugs. Drug-free and yeast-free controls were included and microplates were incubated at 35 °C for 72 h.

Following the CLSI recommendations, two quality control strains, Candida krusei (ATCC 6258) and Candida parapsilosis (ATCC 22019) were used with each test. The reproducibility of the in vitro results was assessed by determining minimum inhibitory concentrations (MICs) for all strains twice on two different days. The MIC end points were read visually after 72 h and defined for fluconazole, voriconazole, itraconazole and 5-flucytosine as the lowest drug concentration that caused a prominent decrease in growth (50%) vis-a-vis the controls. For amphotericin B, the MIC was defined as the lowest concentration at which there was 100% inhibition of growth compared with the drug-free control wells.

Statistical analysis

Statistical differences between MIC values of various groups of strains were assessed, using the Mann-Whitney test. Statistical significance was defined as P-value <0.05. Statistical analyses were performed with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

Results

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### Table 1.

<table>
<thead>
<tr>
<th>Species and Source</th>
<th>MIC (µg ml⁻¹)</th>
<th>MIC (µg ml⁻¹)</th>
<th>MIC (µg ml⁻¹)</th>
<th>MIC (µg ml⁻¹)</th>
<th>MIC (µg ml⁻¹)</th>
<th>MIC (µg ml⁻¹)</th>
<th>MIC (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC50</td>
<td>MIC90</td>
<td>MIC50</td>
<td>MIC90</td>
<td>MIC50</td>
<td>MIC90</td>
<td>MIC50</td>
</tr>
<tr>
<td>C. neoformans var. grubii</td>
<td>0.062</td>
<td>0.250</td>
<td>0.062</td>
<td>0.250</td>
<td>0.062</td>
<td>0.250</td>
<td>0.062</td>
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<tr>
<td>Clinical (n=180)</td>
<td>0.062</td>
<td>0.250</td>
<td>0.062</td>
<td>0.250</td>
<td>0.062</td>
<td>0.250</td>
<td>0.062</td>
</tr>
<tr>
<td>Environmental (n=60)</td>
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<td>0.250</td>
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<td>0.250</td>
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<td>Total (n=240)</td>
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<td>0.250</td>
<td>0.062</td>
<td>0.250</td>
<td>0.062</td>
</tr>
</tbody>
</table>

**Discussion**

This study is noteworthy for documenting the in vitro antifungal susceptibilities of clinical and environmental isolates of *C. neoformans* var. *grubii* (n=246) and *C. gattii* (n=62) to amphotericin B, 5-flucytosine and other antifungals. The results demonstrate that C. gattii isolates were significantly less susceptible than C. neoformans var. grubii which is in conformity with several earlier reports (Jain et al., 2003; Trilles et al., 2006). Interestingly, 3 serial isolates from 3 patients showed a four-fold increase in fluconazole MICs over a period of 4 serial isolates over 1-3 months from a patient showed a four-fold increase in fluconazole MICs.

![Figure 1. Comparison of fluconazole, itraconazole, voriconazole, and 5-flucytosine MICs for *C. gattii* and *C. neoformans* var. *grubii*.](image)

*Note: MIC values were determined using the microbroth dilution method.*
their virulence. Thus the inherently higher level of heteroresistance to fluconazole of \textit{C. gattii} strains may be another factor that influences the MICs of the strains resulting in the variability of results of \textit{in vitro} antifungal susceptibility reported in different studies.

As reported previously (Yildiran et al., 2000; van Duin et al. 2004; Souza et al., 2005; Khan et al., 2007), voriconazole exhibited the highest inhibitory activity against the isolates of \textit{C. neoformans} var \textit{grubii} (GM 0.056 µg ml\(^{-1}\)) and \textit{C. gattii} (GM 0.138 µg ml\(^{-1}\)). Itraconazole MICs were in the range of 0.25-0.5 µg ml\(^{-1}\) in 84 % of \textit{C. gattii} serotype B and in 14% of \textit{C. neoformans} var \textit{grubii} isolates. This is in consonance with the results of Iqbal et al. (2010) who tested 43 clinical isolates of \textit{C. gattii} from patients in Oregon, U.S.A. Twenty-three percent of their isolates had itraconazole MICs >1 µg ml\(^{-1}\) whereas 55.8% revealed MICs in the range of 0.25-0.5 µg ml\(^{-1}\). Interestingly, their VGI and VGIII isolates had comparatively low fluconazole MICs while a majority with MICs of 16 to 32 µg ml\(^{-1}\) were of the subtype VGIIc. Similarly, all of our AFLP 4/VGI \textit{C. gattii} isolates revealed MICs of ≤ 16 µg ml\(^{-1}\) which is consistent with the report of Hagen et al. (2010) on \textit{C. gattii} that showed lower MICs for AFLP 4/VGI isolates (1.401 and 2.467 µg ml\(^{-1}\)) versus the higher MICs for AFLP 6/ VGII (4.961 and 5.638 µg ml\(^{-1}\)) isolates against 5-flucytosine and fluconazole, respectively. Concerning susceptibility to 5-flucytosine, less than 2% of \textit{C. neoformans} isolates have been reported as resistant to it prior to treatment (Scholer & Polak, 1984) which is comparable to our results of 1.2 % (2/162) for clinical \textit{C. neoformans} var \textit{grubii} isolates. However, concern for any emergence of resistance during treatment with this drug alone has led to its use in combination with amphotericin B in patients with cryptococcosis (Perfect et al., 2010). All of the patients whose serial isolates showed increase in azole and amphotericin B MICs were HIV-positive. It may be added in this context that relapses in patients with AIDS associated cryptococcosis are often due to deterioration of the host immune function rather than to an increase in MICs (Witt et al., 1996). However, a rising MIC of fluconazole has been implicated in clinical relapse in patients with AIDS-associated cryptococcal meningitis (Paugam et al., 1994; Birley et al., 1995; Currie et al., 1995; Armengou et al., 1996; Aller et al., 2000; Berg et al., 1998; Davey et al., 1998).

Our significantly lower susceptibility of environmental \textit{C. neoformans} var \textit{grubii} isolates to fluconazole, itraconazole and 5-flucytosine than that of clinical isolates is contrary to the findings of some investigators who found that antifungal susceptibility was not related to the clinical or environmental origin of strains (Franzot & Hamdan 1996; Moraes et al., 2003; Trilles et al., 2004). Of relevance here is the report of Soares et al. (2005) stating that a solitary isolate of \textit{C. neoformans} var \textit{grubii} from pigeon excreta was resistant to fluconazole (MIC 64 µg ml\(^{-1}\)). Likewise, in another report from Brazil, one of their environmental isolates of \textit{C. neoformans} var \textit{neoformans} was found to be resistant to itraconazole and 3 additional isolates exhibited high MICs of 16-32 µg ml\(^{-1}\) against fluconazole (Costa et al., 2010). Furthermore, a fluconazole resistant strain isolated from an immunocompetent patient without exposure to this azole has also been reported, indicating the existence of primary resistance in environmental strains to fluconazole (Orni-Wasserlauf et al., 1999).

Keeping in view these emerging reports of resistance in the environmental strains, a continued surveillance for emergence of antifungal resistance in clinical and environmental strains of \textit{C. neoformans} and \textit{C. gattii} would be desirable for more successful therapy of cryptococcosis.

\textbf{Acknowledgements}

Acknowledgement is made to the Indian National Science Academy, New Delhi, for the award of an Hon. Scientist position to H. S. R. and to Dr. M Rahman for assistance with statistical analysis of the data. This work was financially supported by the Department of Science and Technology, Government of India (F.No.SR/50/HS-62/2008) and the Indian Council of Medical Research, New Delhi (HIV/50/107/2008).
References


Introduction

*Cryptococcus neoformans* and *C. gattii* are encapsulated basidiomycetous yeasts that can cause life-threatening infections in humans. According to the current classification, *C. neoformans* consists of two varieties, namely variety *grubii* (serotype A) and variety *neoformans* (serotype D). *C. neoformans* and *C. gattii* differ in ecology, biochemistry, molecular characteristics, and their ability to cause disease (Bover et al., 2008; Lin & Heitman, 2006). *C. neoformans* is known as an opportunistic pathogen because it mainly infects immunocompromised patients, whereas *C. gattii* is considered a primary pathogen that infects otherwise healthy individuals (Springer & Chaturvedi, 2010). Clinical characteristics of *C. neoformans* and *C. gattii* differ as well. The latter species causes more frequently cryptococcomas and has a lower susceptibility to antifungal agents, resulting in prolonged treatment and a higher mortality rate when compared to *C. neoformans* (Perfect et al., 2010).

Cryptococcus neoformans var. grubii continues to be the most important cause of fungal meningitis in immunocompromised patients. The global burden of cryptococcal infections among HIV-infected individuals is estimated at nearly one million new cases per year (Park et al., 2009). In South Asia and Southeast Asia, the incidence of HIV infection is the second-highest with over 4.5 million HIV-infected patients. The prevalence of cryptococcal meningitis was estimated to be 13.6 and 120 per thousand HIV-infected individuals per year among HIV-infected patients in these two regions, respectively (Park et al., 2009). In contrast, most cases of cryptococcosis in East Asia, especially in China and Japan, where HIV prevalence is low, have been reported from apparently immunocompetent patients and were mostly caused by *C. neoformans* var. *grubii* (Shen et al., 2007; Chen et al., 2008; Choi et al., 2010; Xu et al., 2011). Similarly, Vietnamese patients with cryptococcal meningitis were usually infected by this variety, but here it manifests in both immunocompromized and immunocompetent patients (Day et al., 2011).

Most cryptococcal meningitis cases in Asia are caused by *C. neoformans* (Xu et al., 2011). Notwithstanding the clinical importance of the fungus in this part of the world, a systematic survey on the genetic diversity of the pathogen has not been performed. Genotyping of isolates may reveal differences in host range and clinical symptoms. Recently, a genotyping study of Vietnamese clinical isolates using amplified fragment length polymorphism (AFLP) revealed two genotypes, VN1y and VN1s with the former as the major genotype for isolates originating from non-HIV-infected patients (Day et al., 2011). For epidemiological studies of the *C. neoformans/C. gattii* complex, several molecular methods have been used, such as AFLP, M13-based PCR fingerprinting, Multi-locus Sequence Typing (MLST) and analysis of the intergenic spacer (IGS) ribosomal DNA sequences (Boekhout et al., 2001; Kidd et al., 2004; Diaz et al., 2005; Meyer et al., 2009).

Microsatellite analysis is a genotyping technique that is becoming increasingly popular for molecular typing of medically important fungi (de Valk et al., 2007; Illnait-Zaragozi et al., 2010a; Rudramurthy et al., 2011). Microsatellites, also referred to as short tandem repeats (STRs), are genomic sequences that consist of tandem repeated short motifs (Klaassen, 2009). Mutations in microsatellites may lead to a change in the number of repeats creating genotypic diversity. In some fungi, e.g. *Aspergillus fumigatus* and *A. flavus*, molecular typing using microsatellites proved to be more discriminating than MLST (Klaassen, 2009;
Rudramurthy et al., 2011). In our study, microsatellite typing was applied to estimate the extent of genetic diversity and the epidemiological relationships of a collection of clinical cryptococcal isolates that originated from East, South and Southeast Asia, and the Middle East. Moreover, a set of environmental isolates from Japan and Thailand was included. In vitro antifungal susceptibility was determined for amphotericin B, fluconazole, fluconazole, itraconazole, voriconazole, posaconazole and isavuconazole. The aims of this study were: (i) to analyze the genotypic diversity as well as the distribution of C. neoformans var. grubii from different geographical regions in Asia, (ii) to relate the genetic background of the cryptococcal isolates to disease status and origin from human body, (iii) to test the in vitro antifungal susceptibility of the isolates against seven antifungal drugs, and (iv) to determine if differences in susceptibility correlate with the observed genotypic diversity.

Materials and Methods

Isolates and media
A total of 427 clinical isolates of Cryptococcus neoformans var. grubii were obtained from the collections of the Chinese Cryptococcus Reference Centre at the Second Military Medical University, Shanghai, China (n = 115); Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (n = 79); Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India (n = 61); Sappasitthaprasong Hospital, Ubon Ratchathani, Thailand (n = 60); Department of Parasitology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia (n = 40); Department of Microbiology, Meiji Pharmaceutical University, Tokyo, Japan (n = 28); Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand (n = 20); Department of Microbiology, Faculty of Medicine, Health Sciences Centre, Kuwait University, Jahriya, Kuwait (n = 10); Mycology Unit, Mycology Division, Department of Laboratory Medicine and Pathology, Hamad Medical Corporation, Doha, Qatar (n = 5) and Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Thailand (n = 9) (Supplement Table 1). Furthermore, 66 environmental isolates were obtained from the Department of Microbiology, Faculty of Medicine, Chiang Mai University, Thailand (n = 57) and the Department of Microbiology, Meiji Pharmaceutical University, Tokyo, Japan (n = 9) (Supplement Table 2). Two-hundred and thirty-seven isolates originated from HIV-infected patients and 156 isolates were obtained from HIV-negative patients. Thirty-four out of 427 clinical isolates were from patients with unknown HIV status. Species identification was initially performed by standard mycological methods (Hazen et al., 2003). l-Canavanine-glycine-bromothymol blue (CGB) medium was used to distinguish between C. neoformans and C. gattii isolates (Kwon-Chung et al., 1982). Cryptococcus isolates were stored in sterile 2ml screw-capped tubes containing porous beads (Microbank, ProLab Diagnostics, Richmond Hill, ON, Canada) at -80°C until further use.

Mating- and serotype analysis by PCR, and microsatellite typing
Genomic DNA extraction was performed as previously described (Hagen et al., 2010). All PCR amplifications for mating- and serotyping were carried out in a total volume of 20 µl containing 0.1 mM dNTPs, 0.5 U of Taq DNA polymerase (Gentaur, Brussels, Belgium), 1 µl of template genomic DNA (100 ng/µl) and 0.5 µl of the forward and reverse primers, as described by Bovers et al., (2006) in 1x PCR reaction buffer containing 50 mM MgCl₂ (Barreto de Oliveira et al., 2004). Microsatellite analysis was performed using nine microsatellite markers (Illnait-Zaragozi et al., 2010a). However, instead of three multiplex PCRs, each locus was amplified in a separate PCR reaction and reaction products containing different fluorescent labels were pooled prior to analysis. One microliter of combined reaction product was mixed with 8.75 µl water and 0.25 µl ET-550R ROX Size Standard (GE Healthcare, Diegem, Belgium). Samples were analyzed on a MegaBACE 500 automated DNA analysis platform (GE Healthcare) equipped with a 48 capillary array according to the instructions of the manufacturer. Repeat numbers were assigned using Fragment Profiler v1.2 (GE Healthcare), imported into BioNumerics v6.0 software (Applied Maths, Sint Martens-Latem, Belgium) and analyzed using the multistate categorical similarity coefficient. According to previously described criteria, microsatellite complexes (MCs) were defined as groups of two or more microsatellite genotypes that differ by a maximum of two loci (Illnait-Zaragozi et al., 2010a).

Antifungal susceptibility testing
In vitro antifungal susceptibility testing of amphotericin B (AMB; Bristol Myers Squibb, Woerden, The Netherlands), fluconazole (SFC; Valeant Pharmaceuticals, Zoetermeer, The Netherlands), fluconazole (FLU; Pfizer Central Research, Sandwich, Kent, United Kingdom), itraconazole (ITR; Janssen Cilag, Tilburg, The Netherlands), posaconazole (POS; Schering-Plough Corp., Kenilworth, NJ, USA), voriconazole (VOR; Pfizer Central Research) and isavuconazole (ISA; Basilea Pharmaceutica, Basel, Switzerland) was performed using the standard broth microdilution method as described in CLSI document M27-A3 (CLSI, 2008). The minimal inhibitory concentrations (MIC) were read optically after 72 h of incubation at 35°C. For AMB, the MIC was defined as the lowest concentration of drug showing no yeast growth. For the other antifungal compounds, the MIC was defined as the lowest concentration that caused a prominent reduction of yeast growth (≥50%). Candida krusei ATCC6258 and Candida parapsilosis ATCC22019 were used as quality controls. The resistance breakpoints of FLU and 5FC were taken from CLSI document M27-A3 (CLSI, 2008) as follows: ≥16 µg/ml for FLU; ≥32 µg/ml for 5FC. According to Nguyen and Yu (1998) the resistance breakpoint of AMB is ≥2 µg/ml. Interpretive criteria of ITR and the new azoles have not been proposed yet for C. neoformans. However, a MIC ≤1 µg/ml was suggested as the susceptibility breakpoint for ITR, VOR, POS and ISA (Pfaller et al., 2004; 2005a; 2005b; Souza et al., 2010).

Data analysis
The Simpson’s index of diversity was calculated to assess the genotypic diversity of microsatellite genotypes among the different populations (Simpson, 1949). The value obtained is scaled from zero to one, where a value of one indicates that all isolates are
different, and a value of zero means that all belong to the same genotype. A Chi-square and Fisher exact tests were applied to examine the association between MCs and isolate categories, including HIV status, sample sources (environmental or clinical), and geographical origin. In vitro antifungal susceptibility testing results were statistically analyzed using the t-test method. *P* values less than 0.05 were considered significant for all statistical methods. The statistics were analyzed using StatsDirect v2.7.8 (StatsDirect, Cheshire, United Kingdom).

**Results**

**Mating-type and serotype analysis**

Of the 427 clinical *C. neoformans* var. *grubii* isolates obtained from HIV-positive patients (*n* = 237), HIV-negative patients (*n* = 156), and those with an unknown HIV status (*n* = 34), 426 isolates (99.8%) belonged to mating-type *α* and serotype *A* (*αA*), and one isolate (0.2%) from a HIV-negative patient in China belonged to mating-type *α* and serotype *AD* (*αAAd*). All 66 environmental isolates of *C. neoformans* var. *grubii* obtained from avian droppings from Chiang Mai, Thailand (*n* = 57) and Tokyo, Japan (*n* = 9) were mating type *α* and serotype *A* (Supplementary Tables 1 and 2).

**Microsatellite genotyping**

The genetic diversity of 492 isolates of *C. neoformans* var. *grubii* (*αA*) and one *αAAd* hybrid isolate was analyzed by microsatellite typing. Within this collection of 493 isolates, 265 different genotypes were found. These 265 genotypes were distributed over eight microsatellite complexes (MCs), which were defined as described previously (Illnait-Zaragozi et al., 2010a). Of the eight observed MCs, five were previously found in a collection of cryptococcal isolates from Cuba (i.e., MC1, MC2, MC3, MC8 and MC12), while three novel MCs were observed in the Asian collection presented here. These were assigned MC15, MC16, and MC17 (Figure 1A, and Supplementary Tables 1 and 2). Twenty-four of the 493 isolates obtained from HIV-positive patients (*n* = 237), HIV-negative patients (*n* = 136), and those with an unknown HIV status (*n* = 34) were mating type *α* and serotype *A* (*αA*), and one isolate (0.2%) from a HIV-negative patient in China belonged to mating-type *α* and serotype *AD* (*αAAd*). All 426 isolates (99.8%) belonged to mating-type *α* and serotype *A* (*αA*), and one isolate (0.2%) from a HIV-negative patient in China belonged to mating-type *α* and serotype *AD* (*αAAd*). All 66 environmental isolates of *C. neoformans* var. *grubii* obtained from avian droppings from Chiang Mai, Thailand (*n* = 57) and Tokyo, Japan (*n* = 9) were mating type *α* and serotype *A* (Supplementary Tables 1 and 2).

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The distribution of environmental and clinical isolates among MCs was studied using two different, and a value of zero means that all belong to the same genotype. A Chi-square and Fisher exact tests were applied to examine the association between MCs and isolate categories, including HIV status, sample sources (environmental or clinical), and geographical origin. In vitro antifungal susceptibility testing results were statistically analyzed using the t-test method. *P* values less than 0.05 were considered significant for all statistical methods. The statistics were analyzed using StatsDirect v2.7.8 (StatsDirect, Cheshire, United Kingdom).

**Table 1. Distribution of microsatellite complexes (MCs) between different countries. The predominant MCs in each country are indicated in bold.**

<table>
<thead>
<tr>
<th>MCs</th>
<th>China (n)</th>
<th>Japan (n)</th>
<th>India (n)</th>
<th>Indonesia (n)</th>
<th>Thailand (n)</th>
<th>Kuwait (n)</th>
<th>Qatar (n)</th>
<th>Total number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>MC2</td>
<td>102 (88.7)</td>
<td>14 (37.8)</td>
<td>4 (9.8)</td>
<td>182 (80.89)</td>
<td>2 (20)</td>
<td>2 (20)</td>
<td>3 (30)</td>
<td>24 (4.9)</td>
</tr>
<tr>
<td>MC3</td>
<td>1 (0.9)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (0.2%)</td>
</tr>
<tr>
<td>MC8</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>MC12</td>
<td>8 (6.9)</td>
<td>0 (0%)</td>
<td>2 (3.3)</td>
<td>0 (0%)</td>
<td>3 (1.33)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>13 (2.6)</td>
</tr>
<tr>
<td>MC15</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>6 (9.8)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>9 (1.8)</td>
</tr>
<tr>
<td>MC16</td>
<td>0 (0%)</td>
<td>20 (54.1)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>21 (4.3)</td>
</tr>
<tr>
<td>MC17</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>15 (37.5)</td>
<td>3 (1.33)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>18 (3.7)</td>
</tr>
<tr>
<td>None MCs</td>
<td>4 (3.5)</td>
<td>3 (8.1)</td>
<td>5 (8.2)</td>
<td>2 (5)</td>
<td>5 (2.22)</td>
<td>3 (30)</td>
<td>2 (40)</td>
<td>24 (4.9)</td>
</tr>
<tr>
<td>Total</td>
<td>115</td>
<td>37</td>
<td>61</td>
<td>102</td>
<td>425</td>
<td>10</td>
<td>12</td>
<td>493</td>
</tr>
</tbody>
</table>

The genotypic diversity of *C. neoformans* var. *grubii* was found to be the most diverse (*D* = 1.000) in the Kuwait and Qatar populations, followed by the Japanese (*D* = 0.998), Indonesian (*D* = 0.994), Indian (*D* = 0.983), Chinese (*D* = 0.975), and Thai populations (*D* = 0.968) (Table 2). The genetic diversity within MCs showed values of the diversity index close to 1, thus indicating that each MC contained a high level of genetic diversity. A significant correlation between MCs and HIV-status was observed by the Chi-square (*p* < 0.0001) and Fisher exact tests (*p* < 0.0001) (Figures 1A and C, and Table 3). The majority of isolates from HIV-negative patients belonged to MC2 that accounted for 104 out of 156 isolates from HIV-negative patients (66.67%), followed by MC16 (*n* = 13, 8.33%). In contrast, MC8 was the predominant MC containing isolates from HIV-positive patients (138 of 237, 58.2%), followed by MC2 (*n* = 30, 12.7%) and MC17 (*n* = 16, 6.8%). The distribution of environmental and clinical isolates among MCs was studied using two populations from Tokyo, Japan and Chiang Mai, Thailand. In both cases, the clinical and environmental isolates belonged to the same MCs (Figure 1D and Table 4). MC2 and MC8 were the common MCs in the Thai population. MC8 contained 27 out of 41 clinical isolates and 44 out of 57 environmental isolates, whereas MC2 contained 12 out of 41 clinical isolates and 8 out of 57 environmental isolates. Among the Japanese isolates, MC16 contained 16 out of 28 clinical isolates and 4 out of 9 environmental isolates, and MC2 contained 11 out of 28 clinical isolates and 3 out of 9 environmental isolates. The combined results of the Thai and Japanese populations showed a correlation between MCs and the clinical or environmental origin (*p* = 0.0006, Chi-square; *p* = 0.0002, Fisher exact test). However, no such correlation was observed within the Thai population from Chiang Mai (*p* = 0.1106, Chi-square; *p* = 0.0957, Fisher exact test) nor within the Japanese population from Tokyo (*p* = 0.9192, Chi-square; *p* = 0.9999, Fisher exact test).
Above and next page, Figure 1. Genotypic variation of *C. neoformans* isolates from different Asian countries by microsatellite typing: (a) Minimum spanning tree based on a multistate categorical analysis representing 429 *C. neoformans* var. *grubii* isolates from different countries. Each circle represents a unique genotype. The size of the circle corresponds to the number of isolates within that genotype. Numbers and connecting lines correspond to the number of different markers between genotypes. Genotypes with identical colors are part of a microsatellite complex (MC). Circles without color are unique genotypes that are not part of a MC.; (b) Same as A, but now showing the genotypes from different geographic locations. Different colors correspond to different countries.; (c) Same as A and B, but now showing the genotypes from clinical and environmental sources.; (d) Same as A, B and C, but now showing the genotypes of Thai and Japanese population from clinical and environmental sources.

In vitro antifungal susceptibility testing

The MIC values of all *C. neoformans* var. *grubii* isolates for the seven antifungal compounds tested are listed in Table 5. Almost all cryptococcal isolates were susceptible to AMB, ITR, FLU, VOR, POS and ISA. Notably, 18 clinical isolates (3.7%) from Indonesia (*n* = 13), Thailand (*n* = 4) and China (*n* = 1) were resistant to 5FC with MIC ≥32 µg/ml (Supplementary Table 3). Most of 5FC resistant isolates occurred in MC8 (*n* = 8) and MC17 (*n* = 7), and two belonged to MC3 (Supplementary Table 4). Approximately 39% of all MC17 isolates were resistant to 5FC. When compared to 3.4% and 5.3% of MC8 and MC3, respectively, this found to be highly significant (*p*<0.0001, Chi-square; *p*<0.0001, Fisher exact test). In addition, ten FLU-resistant isolates (2%) occurred in different countries, including China (*n* = 2), India (*n* = 1), Indonesia (*n* = 5), and Thailand (*n* = 2) and belonged to MC2 (*n* = 3), MC3 (*n* = 2), MC8 (*n* = 2), MC17 (*n* = 2) and one isolate from India (number 25_17) that could not be assigned.
Cryptococcus neoformans Isolates from Asia

Chapter 7

Africa and North America (Simwami study showed that the Asian population was genetically less diverse than those occurring in 2005). Recently, a population biology study using MLST analysis of complexes (MCs), including four new ones. Simpson’s index of diversity, however, showed that genetic diversity within each individual population and each MCs was very high. The genotypic structure of the C. neoformans var. grubii isolates differed widely among the populations from the different countries. This finding supports the hypothesis that local geographic differences occur in Asia between populations from the different countries. This finding supports the hypothesis that local environmental and climate (Franzot et al., 2011). Furthermore, fifteen of the 115 Chinese isolates (Supplementary Table 1) were investigated previously by M13 PCR fingerprinting and were identified as genotype VNIC (Chen et al., 2008). All these isolates were clustered in MC2 and supported the relatively limited genetic diversity among Chinese strains. Thus, it seems that the genetic diversity of cryptococcal isolates from Asian countries is lower than that of populations occurring in other parts of the world. The minimum spanning tree based on microsatellite analysis showed that MC8 contained mostly Thai isolates (Figure 1B), thus supporting the presence of limited genetic diversity among this population as described before (Mayer et al., 1999; Sukroongreung et al., 2001; Sriburee et al., 2004; Simwami et al., 2011). However, other Thai isolates from HIV-infected patients and bird excreta from the North and Northeast occurred in MCs that comprised also isolates from China and Japan (i.e., MC2 and MC16). Can these findings be explained by assuming a relation with bird migration, especially the East Asia-Australian flyway, through which pathogenic microorganisms such as C. neoformans var. grubii may disperse between China and Thailand (Olsen et al., 2006)? The scattered distribution of isolates from Kuwait and Qatar may be due to migration of foreign workers from Southeast Asia who may have carried isolates that were obtained from their country of origin, similar as has been demonstrated for African immigrants in France (Garcia-Hermoso et al., 1999). Further support for this hypothesis is that young children in USA acquired the organism from their surrounding environments (Goldman et al., 2001). Isolates belonging to MC8 contained mainly cryptococcal isolates from HIV-infected patients, whereas MC2 and MC16 comprised mostly isolates from non-HIV-infected patients (Figure 1C). A recent report from Vietnam revealed two genotypic clusters based on AFLP analysis, viz., VNIG and VNId, that contained isolates from HIV-negative and positive patients, respectively (Day et al., 2011). Thus genotypic differences were seen between both patient categories based on AFLP analysis and this observation is reminiscent the differences we observed in the current microsatellite data. However, no comparison could be made between the AFLP- and microsatellite typing because, unfortunately, none of the Vietnamese isolates was available for our studies.

In Japan and Thailand, environmental isolates co-occurred with clinical isolates in MC2, MC8, and MC16 (Figure 1A, C and D, and Table 3), and these results suggest a genetic relatedness between environmental and clinical cryptococcal isolates in these countries. Such a relationship has been suggested before (Yamamoto et al., 1995; Franzot et al., 1997; Casali et al., 2003; Currie et al., 2004; Delgado et al., 2005), but is at stake with a recent analysis from Cuba where the clinical isolates largely belonged to a different MC than those obtained from the environment (Illnait-Zaragozi et al., 2010a). At present, Table 3. Distribution of microsatellite complexes (MCs) from patients according to HIV status. The predominant MCs in each HIV status are indicated in bold.

<table>
<thead>
<tr>
<th>MCs</th>
<th>HIV positive n (%)</th>
<th>HIV negative n (%)</th>
<th>Unknown status n (%)</th>
<th>Total number of isolates n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1</td>
<td>6 (2.5)</td>
<td>6 (3.85)</td>
<td>2 (5.88)</td>
<td>14 (3.3)</td>
</tr>
<tr>
<td>MC2</td>
<td>30 (12.7)</td>
<td>104 (66.67)</td>
<td>6 (17.65)</td>
<td>140 (32.8)</td>
</tr>
<tr>
<td>MC3</td>
<td>30 (12.7)</td>
<td>7 (4.99)</td>
<td>1 (2.94)</td>
<td>38 (8.9)</td>
</tr>
<tr>
<td>MC8</td>
<td><strong>138 (58.2)</strong></td>
<td><strong>4 (2.56)</strong></td>
<td><strong>19 (55.88)</strong></td>
<td><strong>161 (37.7)</strong></td>
</tr>
<tr>
<td>MC12</td>
<td>2 (0.8)</td>
<td>8 (5.13)</td>
<td>0</td>
<td>10 (2.3)</td>
</tr>
<tr>
<td>MC15</td>
<td>3 (1.3)</td>
<td>5 (3.2)</td>
<td>0</td>
<td>8 (1.9)</td>
</tr>
<tr>
<td>MC16</td>
<td>1 (0.4)</td>
<td>13 (8.33)</td>
<td>3 (8.83)</td>
<td>17 (4)</td>
</tr>
<tr>
<td>MC17</td>
<td>16 (6.8)</td>
<td>1 (0.64)</td>
<td>1 (2.94)</td>
<td>18 (4.2)</td>
</tr>
<tr>
<td>None MCs</td>
<td>11 (4.6)</td>
<td>8 (5.13)</td>
<td>2 (5.88)</td>
<td>21 (4.9)</td>
</tr>
<tr>
<td>Total</td>
<td><strong>237</strong></td>
<td><strong>156</strong></td>
<td><strong>34</strong></td>
<td><strong>427</strong></td>
</tr>
</tbody>
</table>
the observed differences between the genetic relationship of environmental and clinical isolates in different parts of the globe are not easily explained, but it may be that different environmental niches are occupied in different locales. Susceptibility analysis of 493 C. neoformans isolates to seven antifungals yielded no change in MIC ranges and MIC50, and MIC90 values of AMB and FLU when compared to previous studies (Brandt et al., 2001; Illnait-Zaragozi et al., 2008; Thompson et al., 2009). The MIC ranges and values for ITR and the three novel antifungal agents POS, ISA, and VOR were slightly changed to those reported previously (Illnait-Zaragozi et al., 2008; Thompson et al., 2009; Hagen et al., 2010). The MIC50 and MIC90 values for ISA were lower than those observed for the other antifungal agents, thus corroborating previous observations ((Illnait-Zaragozi et al., 2008; Thompson et al., 2009). Importantly, resistance in approximately 4% of the isolates to SFC was observed. Most SFC-resistant isolates came from Indonesia and Thailand where this drug is not in use (Chotianapund et al., 2007; Ganiem et al., 2009). In our study, we found that approximately 40% of all MC17 isolates were resistant to SFC. These findings suggest that intrinsic resistance to SFC occurs among Southeast Asian isolates of C. neoformans, especially in MC17 and in Indonesia. Intrinsic resistance to SFC in C. neoformans is uncommon and occurs with a low incidence of approximately 1-2% as reported from the USA in the 1990s (Vermes et al., 2000; Perea & Patterson, 2002). The reason for this high number of SFC-resistant isolates in the Southeast Asian region is not clear, and needs further study. Furthermore, resistance to FLU was observed. The FLU-resistant isolates occurred in different countries, including China, India, Indonesia, and Thailand. C. neoformans may develop resistance to FLU after treatment (Perfect & Cox, 1999; Perea & Patterson, 2002). Five out of 10 FLU resistant isolates (1 from MC3, 1 from MC8, and 3 from MC17) from Indonesia were also not susceptible to SFC. Our findings suggested that isolates in certain MCs, especially MC17, may be prone to a decreased susceptibility to antifungals, especially FLU and SFC. The observed double resistance to SFC and FLU has not been reported before, and may pose a risk for the patients infected with such isolates.

Table 4. Distribution of C. neoformans isolates from clinical and environmental samples from Thailand and Japan in microsatellite complexes (MCs). The predominant MCs in each sample type in these countries are indicated in bold.

<table>
<thead>
<tr>
<th>Total number of isolates</th>
<th>MC1</th>
<th>MC2</th>
<th>MC3</th>
<th>MC4</th>
<th>MC5</th>
<th>MC6</th>
<th>MC7</th>
<th>MC8</th>
<th>MC9</th>
<th>MC10</th>
<th>MC11</th>
<th>MC12</th>
<th>MC13</th>
<th>MC14</th>
<th>MC15</th>
<th>MC16</th>
<th>MC17</th>
<th>MC18</th>
<th>MC19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand isolates</td>
<td>22</td>
<td>18</td>
<td>15</td>
<td>13</td>
<td>10</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Japan isolates</td>
<td>8</td>
<td>13</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5. The MIC range, MIC50, MIC90, and geometric mean MIC (µg/mL) for all 493 C. neoformans isolates from clinical and environmental origin of isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Antifungal agent</th>
<th>MIC Range</th>
<th>MIC50</th>
<th>MIC90</th>
<th>Geometric Mean MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>All C. neoformans isolates (n = 493)</td>
<td>Amphotericine B</td>
<td>0.063 - 1</td>
<td>0.25</td>
<td>0.252</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5-Flucytosine</td>
<td>&lt;0.063 - &gt;64</td>
<td>4</td>
<td>3.465</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Fluconazole</td>
<td>0.125 - 32</td>
<td>2</td>
<td>2.299</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.063</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.049</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>&lt;0.016 - 0.25</td>
<td>0.063</td>
<td>0.061</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Isavuconazole</td>
<td>&lt;0.016 - 0.125</td>
<td>0.031</td>
<td>0.027</td>
<td>0.063</td>
</tr>
<tr>
<td>Isolates from HIV-positive patients (n = 237)</td>
<td>Amphotericine B</td>
<td>0.063 - 1</td>
<td>0.25</td>
<td>0.236</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5-Flucytosine</td>
<td>&lt;0.063 - &gt;64</td>
<td>4</td>
<td>3.794</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Fluconazole</td>
<td>0.125 - 32</td>
<td>2</td>
<td>2.545</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.062</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.056</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.062</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Isavuconazole</td>
<td>&lt;0.016 - 0.125</td>
<td>0.031</td>
<td>0.024</td>
<td>0.063</td>
</tr>
<tr>
<td>Isolates from HIV-negative patients (n = 156)</td>
<td>Amphotericine B</td>
<td>0.063 - 1</td>
<td>0.25</td>
<td>0.254</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5-Flucytosine</td>
<td>&lt;0.063 - &gt;64</td>
<td>4</td>
<td>3.091</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Fluconazole</td>
<td>0.125 - 32</td>
<td>2</td>
<td>2.054</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.056</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.062</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.062</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Isavuconazole</td>
<td>&lt;0.016 - 0.125</td>
<td>0.031</td>
<td>0.021</td>
<td>0.063</td>
</tr>
<tr>
<td>Clinical isolates from Thailand and Japan (n = 69)</td>
<td>Amphotericine B</td>
<td>0.063 - 0.5</td>
<td>0.25</td>
<td>0.294</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5-Flucytosine</td>
<td>&lt;0.063 - &gt;64</td>
<td>4</td>
<td>3.429</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Fluconazole</td>
<td>0.25 - 32</td>
<td>2</td>
<td>2.356</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.056</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.094</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.090</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Isavuconazole</td>
<td>&lt;0.016 - 0.063</td>
<td>0.063</td>
<td>0.037</td>
<td>0.063</td>
</tr>
<tr>
<td>Environmental isolates from Thailand and Japan (n = 66)</td>
<td>Amphotericine B</td>
<td>0.25 - 0.5</td>
<td>0.25</td>
<td>0.294</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5-Flucytosine</td>
<td>&lt;0.063 - &gt;64</td>
<td>4</td>
<td>2.930</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Fluconazole</td>
<td>0.125 - 32</td>
<td>2</td>
<td>2.103</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.125</td>
<td>0.094</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.063</td>
<td>0.056</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>&lt;0.016 - 0.5</td>
<td>0.125</td>
<td>0.090</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Isavuconazole</td>
<td>&lt;0.016 - 0.063</td>
<td>0.063</td>
<td>0.037</td>
<td>0.063</td>
</tr>
</tbody>
</table>
Isolates from HIV-infected and non-HIV-infected patients did not differ in MIC values of 5FC, FLU, and VOR, but the MIC ranges of isolates from HIV-infected patients were broader than those of isolates from non-HIV-infected patients. The highest MIC values of isolates from HIV-infected patients were 16 times higher for 5FC and FLU and two to four times as high as those from non-HIV-infected patients, respectively.

This is the first extensive report on in vitro antifungal susceptibility and genotyping of clinical and environmental isolates of C. neoformans from several Asian countries. Based on the in vitro susceptibility test results, the patients in the region seem to receive appropriate antifungal therapy with AMB plus SFC for induction therapy followed by consolidation/maintenance therapy of FLU. AMB is still the most effective agent to treat an infection with C. neoformans (Perfect et al., 2010). A Chinese study showed that the risk of death in patients with cryptococcosis who did not receive AMB-based initial therapy was about 7-9 times higher than those given AMB (Perfect & Cox, 1999). Another study from Thailand used different combinations of antifungal therapies, i.e., AMB alone, AMB plus SFC, AMB plus FLU, or triple antifungals (AMB plus SFC and FLU) to treat cryptococcal meningitis showed that treatment of AMB combined with SFC remains a powerful treatment strategy that results in rapid clearance of the pathogen (Brouwer et al., 2004). Our study did not reveal a significant change in the MICs of AMB for C. neoformans, and therefore, initial treatment with AMB remains the recommended choice. However, given the well-tolerated nature and an excellent activity against Cryptococcus strains, the new generation of triazoles may become an important addition to the currently used antifungals.

In summary, genotypic differences in microsatellite patterns occur between C. neoformans populations from the Asian countries studied. However, the overall genetic diversity is low and most of the countries had a unique distribution of MCs. Good in vitro antifungal activity was observed, but SFC and FLU resistant, as well as SFC/FLU double resistant isolates occurred.

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Disclaimers

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Chinese Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

Transparency declarations

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References


posaconazole, and fluconazole against 4,169 clinical isolates of Candida spp. and Cryptococcus neoformans collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. Diagnostic Microbiology and Infectious Disease 48: 201-205.


Clinical breakpoints (CBPs) have not been established for the Cryptococcus neoformans-Cryptococcus gattii species complex. Wild type (WT) MIC distributions were constructed in order to establish epidemiologic cutoff values (ECVs) for C. neoformans and C. gattii versus amphotericin B and flucytosine. A total of 3,590 amphotericin B and 3,045 flucytosine CLSI broth microdilution MICs for C. neoformans and C. gattii, respectively (including those for 42 to 259 AFLP1-AFLP3/VNII-VNIV isolates) and 985 and 853 MICs for C. neoformans and C. gattii, respectively (including those for 1,002 AFLP1/VNI and 8 to 39 AFLP1a-AFLP2-AFLP3/VNII-VNIV isolates) were gathered in 9 to 16 (amphotericin B) and 8 to 13 (flucytosine) independent laboratories (Europe, the United States, Australia, Brazil, Canada, India, and South Africa) and aggregated for the analyses. Additionally, 442 amphotericin B and 313 flucytosine MICs measured using the CLSI alternative Yeast Nitrogen Base broth (CLSI-YNB) instead of the RPMI-1640 (CLSI-RPMI) medium as well as 237 Etest amphotericin B MICs for C. neoformans were evaluated. CLSI-RPMI ECVs for distributions originating in at least three laboratories were (percentages of laboratories contributing data was too low to certain that the differences were due to factors other than assay variation. ECVs may aid in the detection of isolates with acquired resistance mechanisms.

Introduction

Among the non-Candida yeasts, the Cryptococcus neoformans-Cryptococcus gattii species complex has been the most common species recovered from clinical isolates (32.9% of 8,717 isolates) as well as the second most common severe fungal infection after those caused by Candida spp. in certain regions (Pfaller et al., 2009b, Pfaller et al., 2010b). Infections caused by C. neoformans var. grubii (serotype A) are common worldwide among immunocompromised hosts followed by C. neoformans var. neoformans (serotype D) (Bovers et al., 2008). On the other hand, C. gattii (serotypes B and C) is isolated mostly in areas where this species is endemic among both immunocompromised as well as non-immunocompromised patients and these infections are more difficult to treat (Harris et al., 2011, Lin & Heitman, 2006).Irrespective of the cryptococcal species, it has been estimated that cryptococcal infections are associated with high mortality rates (≥12.7%). Using molecular methodology at least eight molecular types (also referred as molecular or AFLP genotypes, AFLP1-3/VNI-VNIV [serotypes A, D, and AD] and AFLP4- AFLP7/VGI-VGIV [serotypes B and C]) have been identified among the four serotypes (Bovers et al., 2008, Byrnes et al., 2011, Hagen et al., 2010, Trilles et al., 2008). Although several newer antifungal agents are available, the polyene amphotericin B and especially its lipid formulations remain important therapeutic choices for the systemic treatment and prevention of cryptococcal infections caused by C. neoformans and C. gattii (Ostrosky-Zeichner et al., 2003, Perfect & Cox, 1999). Among the three lipid amphotericin B formulations, liposomal amphotericin B has been licensed for the treatment of cryptococcal meningitis among AIDS patients (Ostrosky-Zeichner et al., 2003, Perfect et al., 2010). However, relapses are frequent. The polyenes bind to ergosterol in the cell membrane, which leads to pore formation and eventually cell death (Vanden Bossche et al., 1994). In contrast, flucytosine inhibits DNA replication and protein synthesis. In polyene-resistant Candida and Cryptococcus isolates, ergosterol content has been lower than in susceptible isolates (Kelly et al., 1994). Several enzymes (purine-ribosyltransferase) are involved in the mode of action and resistance to flucytosine (Espinel-Ingroff, 2008, Whelan, 1987, White & Hoot, 2011). Flucytosine in combination with amphotericin B is one the recommendations for the treatment of cryptococcal infections (Kanani & Perfect, 2008).

The availability of reference methodologies has enabled the recognition of resistance and the proposal of clinical breakpoints (CBPs) as well as epidemiologic cutoff values (ECVs) for Candida spp. and Aspergillus spp. and most available antifungal agents by both Clinical and Laboratory Standards Institute (CLSI) and the European Committee of Antibiotic Susceptibility Testing (AFST-EUCAST) methodologies (Espinel-Ingroff et al., 2010, Espinel-Ingroff et al., 2011a, Espinel-Ingroff et al., 2011b, Pfaller et al., 2009a, Pfaller et al., 2010a, Pfaller et al., 2010c, Pfaller et al., 2011, Rodriguez-Tudela et al., 2008). CLSI amphotericin B MICs for most C. neoformans and C. gattii isolates are ≤0.5 μg/ml and flucytosine MICs ≤8 μg/ml (Brandt et al., 2001, De Bedout et al., 1999, Hospenthal & Bennett, 1998, Illnait-Zaragozi et al., 2008, Perkins et al., 2005, Pfaller et al., 2009a, White & Hoot, 2011), but acquired resistance is frequent during flucytosine monotherapy (Block et al., 1973, Hospenthal & Bennett, 1998). In the last few years, antifungal susceptibility differences have been
reported between these two species as well as among the molecular types and serotypes (Calvo et al., 2001, Chong et al., 2010, Chowdhary et al., 2011, Hagen et al., 2010, Iqbal et al., 2010, Thompson et al., 2009, Trilles et al., 2012). However, neither CBPs nor ECVs are available for either C. neoformans or C. gattii versus amphotericin B or flucytosine. In the absence of CBPs, ECVs could help to characterize the susceptibility of these two species to amphotericin B, its lipid formulations and flucytosine and to monitor the emergence of strains with mutations that could lead to reduced antifungal susceptibility to these agents.

We are proposing ECVs (see “Definitions” below) for amphotericin B and flucytosine against C. neoformans (AFLP1/VNI and non-molecular typed isolates) and C. gattii (AFLP4/VGI and AFLP6/VGII and non-molecular typed isolates). The purpose of the study was (i) to define wild-type (WT) susceptibility endpoint distributions (see “Definitions” below) of each species/molecular/AFLP genotype and agent combination by using aggregated CLSI-RPMI broth MICs of amphotericin B and flucytosine gathered in 8 to 16 laboratories (3,590 to 3,054 species/molecular/AFLP genotype and agent/combination dependent) in Europe, the United States, Australia, Brazil, Canada, India, and South Africa and (ii) to propose ECVs with data originating in at least three of these laboratories. The 442 amphotericin B and 313 flucytosine MICs that were obtained using the alternative CLSI-Yeast Nitrogen Base (CLSI-YNB) broth (CLSI, 2008a, CLSI, 2008b) and the 237 amphotericin B Etest MICs for C. neoformans were analyzed separately.

Materials and Methods

Isolates Each isolate from unique clinical specimens was tested at each medical center: the University of Texas Health Science Center, San Antonio, TX; the University of Iowa College of Medicine, Iowa, IA; the University of Texas Health Science Center, Houston, TX; Centers for Disease Control and Prevention, Atlanta, GA; Duke University Medical Center, Durham, NC; VCU Medical Center, Richmond, VA; the Canisius Wilhelmina Hospital, Department of Medical Microbiology and Infectious Diseases, Nijmegen, the Netherlands; the HPA Centre for Infections, Kingsdown, Bristol, United Kingdom; the Innsbruck Medical University, Innsbruck, Austria; the Hospital General Universitario Gregorio Marañón, Madrid, Spain; Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra Majadahonda-Pozuelo, Spain; the Universitat Rovira i Virgili Sant Llorenç, Reus, Spain; the University of Alberta, Edmonton, Alberta, Canada; the Adolfo Lutz Institut, Sao Paulo, Brazil; Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; the Instituto de Pesquisa Clinica Evandro Chagas-FIOCRUZ, Rio de Janeiro, Brazil and Women’s and Children’s Hospital, North Adelaide, Australia. Species and molecular/AFLP genotype identification were performed at each medical center using standard methodologies (Chowdhary et al., 2011, Hagen et al., 2010, Hagen et al., 2012, Iqbal et al., 2010, Pan et al., 2012, Trilles et al., 2012). We have aggregated the maximum available CLSI data from each laboratory and agent (a total of 3,590 to 3,054 MICs of amphotericin B and flucytosine MICs for C. neoformans [including those for 1,002 AFLP1/ VNI isolates] and 985 and 853 MICs for C. gattii [including those including those for 42 to 259 AFLP4/-/AFLP7/VGI-VGIV isolates]) (Chowdhary et al., 2011, Hagen et al., 2010, Hagen et al., 2012, Iqbal et al., 2010, Pan et al., 2012, Trilles et al., 2012). A set of 313 flucytosine and 442 amphotericin B MICs obtained using CLSI-YNB broth instead of the CLSI-RPMI medium (CLSI, 2008a) as well as 237 Etest amphotericin B MICs for C. neoformans were also available; both of these sets were analyzed separately. One or both quality control (QC) isolates (Candida parapsilosis ATCC 22019 and C. krusei ATCC 6258) were used by the participant laboratories (CLSI, 2008a, CLSI, 2008b); Table 1 depicts the QC data for these isolates.

### Table 1. QC MICs for quality control (QC) strains used in the 13 (flucytosine) and 16 (amphotericin B) laboratories according to the CLSI broth microdilution method a

<table>
<thead>
<tr>
<th>QC isolate</th>
<th>Antifungal</th>
<th>QC MIC Range</th>
<th>QC MIC Range</th>
<th>QC MIC Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parapsilosis ATCC 22019</td>
<td>AMB 1-4(2) 100</td>
<td>FCT 8-32 (16) 99.6</td>
<td>AMB 1-4(2) 100</td>
<td>FCT 8-32 (16) 99.6</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>AMB 1-4(2) 100</td>
<td>FCT 0.12-0.5(0.25) 97.9</td>
<td>AMB 1-4(2) 100</td>
<td>FCT 0.12-0.5(0.25) 97.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>QC isolate</th>
<th>Antifungal</th>
<th>QC MIC Range</th>
<th>QC MIC Range</th>
<th>QC MIC Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parapsilosis ATCC 22019</td>
<td>AMB 1-4(2) 100</td>
<td>FCT 0.12-0.5(0.25) 97.9</td>
<td>AMB 1-4(2) 100</td>
<td>FCT 0.12-0.5(0.25) 97.9</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>AMB 1-4(2) 100</td>
<td>FCT 0.12-0.5(0.25) 97.9</td>
<td>AMB 1-4(2) 100</td>
<td>FCT 0.12-0.5(0.25) 97.9</td>
</tr>
</tbody>
</table>

**Antifungal susceptibility testing**

In order to include MIC results in the total set of available CLSI aggregated data from the participant laboratories (Table 2), amphotericin B and flucytosine MICs were obtained at each center by following the CLSI M27-A3 broth microdilution method (standard RPMI-1640 broth [0.2% dextrose], final inoculum concentrations that ranged from 0.4 x 10^3 to 5 x 10^4 CFU/ml and 72 h of incubation); MICs were the lowest drug concentrations that produced prominent growth inhibition (≥50%) compared to growth control (CLSI, 2008a). Testing conditions and interpretation of MICs were otherwise identical for those isolates grown in YNB broth. The set of amphotericin B Etest MICs were obtained following the manufacturer’s instructions and endpoints were rounded up to the nearest two-fold dilution. MIC data for the two QC reference strains utilized during the years of testing in each center were obtained in the same manner (CLSI, 2008a, CLSI, 2008b).
Table 2. Wild-type (WT) MIC distributions of amphotericin B and flucytosine for Cryptococcus neoformans-Cryptococcus gattii species complex

<table>
<thead>
<tr>
<th>Species</th>
<th>Antifungal Agent</th>
<th>Medium</th>
<th>No. of Labs</th>
<th>No. of isolates</th>
<th>aMIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. neoformans</td>
<td>AMB, FCT</td>
<td>RPMI</td>
<td>N</td>
<td>13</td>
<td>121</td>
</tr>
<tr>
<td>VNI (APR1)</td>
<td>Y</td>
<td>4</td>
<td>2002</td>
<td>2</td>
<td>122</td>
</tr>
<tr>
<td>VNI (APR1a)</td>
<td>Y</td>
<td>2</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>VNI (APR1c)</td>
<td>Y</td>
<td>2</td>
<td>15</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>VNI (APR1d)</td>
<td>Y</td>
<td>2</td>
<td>39</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>All strains</td>
<td>Both</td>
<td>1000</td>
<td>6</td>
<td>83</td>
<td>415</td>
</tr>
<tr>
<td>C. neoformans</td>
<td>YNB</td>
<td>N</td>
<td>1</td>
<td>462</td>
<td>5</td>
</tr>
<tr>
<td>VNI (APR1)</td>
<td>Y</td>
<td>4</td>
<td>2002</td>
<td>2</td>
<td>122</td>
</tr>
<tr>
<td>VNI (APR1a)</td>
<td>Y</td>
<td>2</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>VNI (APR1c)</td>
<td>Y</td>
<td>2</td>
<td>15</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>VNI (APR1d)</td>
<td>Y</td>
<td>2</td>
<td>39</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>All strains</td>
<td>Both</td>
<td>1000</td>
<td>6</td>
<td>83</td>
<td>415</td>
</tr>
<tr>
<td>C. gattii</td>
<td>AMB, FCT</td>
<td>RPMI</td>
<td>N</td>
<td>4</td>
<td>120</td>
</tr>
<tr>
<td>VGI (APR4)</td>
<td>Y</td>
<td>5</td>
<td>259</td>
<td>1</td>
<td>49</td>
</tr>
<tr>
<td>VGI (APR6)</td>
<td>Y</td>
<td>4</td>
<td>122</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>VGI (APR6a)</td>
<td>Y</td>
<td>3</td>
<td>200</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>VGI (APR6b)</td>
<td>Y</td>
<td>2</td>
<td>106</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>VGI (APR6c)</td>
<td>Y</td>
<td>2</td>
<td>42</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>VGI (APR6d)</td>
<td>Y</td>
<td>2</td>
<td>42</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>VGI (APR6f)</td>
<td>Y</td>
<td>2</td>
<td>42</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>VGI (APR7)</td>
<td>Y</td>
<td>2</td>
<td>84</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>All strains</td>
<td>Both</td>
<td>195</td>
<td>2</td>
<td>9</td>
<td>165</td>
</tr>
</tbody>
</table>

Definitions
- aMIC=amphotericin B; FCT= flucytosine.; b RPMI=RPMI-1640 as described by the CLSI M27-A3; YNB= yeast nitrogen base as described by the CLSI M27-A3 (8); c Number of laboratories contributing data to each MIC (minimal inhibitory concentration) distribution. amphotericin B and flucytosine MICs as determined by CLSI broth microdilution method (8), Amphotericin B Etest MICs as determined according to manufacturer's instructions. d Modal MIC for each distribution is underlined.

Results and Discussion
Several amphotericin B and flucytosine MIC distributions were obtained for C. neoformans (Table 2): (i) CLSI-RPMI aggregated data from 13 and 10 laboratories, respectively, for non-molecular/AFLP typed isolates and (ii) from two to four laboratories for AFLP typed isolates (Table 2). The aggregated MIC distributions obtained at least three laboratories for non-molecular/AFLP typed isolates and (ii) from two to four laboratories for AFLP typed isolates (Table 2).

The aggregated data from laboratories for non-molecular/AFLP typed isolates and (ii) from two to four laboratories for AFLP typed isolates (Table 2). The aggregated data from laboratories for non-molecular/AFLP typed isolates and (ii) from two to four laboratories for AFLP typed isolates (Table 2). The aggregated data from laboratories for non-molecular/AFLP typed isolates and (ii) from two to four laboratories for AFLP typed isolates (Table 2). The aggregated data from laboratories for non-molecular/AFLP typed isolates and (ii) from two to four laboratories for AFLP typed isolates (Table 2).
gattii may aid in the evaluation of clinical isolates by identifying those strains that may have acquired resistance mechanisms and serve as an early warning of emerging changes in the susceptibility patterns of these organisms. Even though cryptococcal meningitis and other infections decreased with the use of antiretroviral therapies, these infections are still a major problem among immunosuppressed patients and in certain geographical areas. Amphotericin B and flucytosine continue to be important therapeutic agents for cryptoccocal infections (Ostrosky-Zeichner et al., 2003, Perfect et al., 2010).

Variability is expected when MICs from different laboratories are compared despite standardization efforts (CLSI, 2008a, CLSI, 2008b). In prior reports, amphotericin B MICs measured in RPMI were for two of the three VGII/AFLP6 subtypes (AFLP6a/VGIIa and AFLP6c/VGIIc). Our modes may indicate individual interpretations of MIC endpoints or it may be due to the use of different lots of antifungal powders or both. The aggregated amphotericin B and flucytosine MIC distributions for C. neoformans and C. gattii (for non-molecular and molecular genotyped isolates) are depicted in Table 2; this table also lists the single-laboratory distributions for C. neoformans (YNB and Etest data). The highest amphotericin B modal MICs (0.5 μg/ml) measured in RPMI were for C. gattii non-molecular typed and AFLP6/VGI isolates; the modal MIC for all other distributions was 0.25 μg/ml. Amphotericin B modes observed in the data from most individual contributing laboratories were similar (0.25 or 0.5 μg/ml) for both species. The exceptions were the mode (0.12 μg/ml) from one laboratory for C. neoformans (non-molecular typed isolates) and for AFLP2/VNI and AFLP3/VNI, but the number of isolates was low for the latter two sets. Overall, our values reflect amphotericin B MICs or geometric means (0.25 and 0.5 μg/ml) obtained in previous studies for similar sets of non-molecular or molecular typed isolates (Chong et al., 2010, Dannaoui et al., 2006, Illnait-Zaragozi et al., 2008, Perkins et al., 2005, Thompson et al., 2009); results have been higher (>0.5 μg/ml) in other studies (Brandt et al., 2001, De Bedout et al., 1999). For C. neoformans, amphotericin B MICs measured in YNB appeared to be higher (mode, 1 μg/ml) than those measured in RPMI. By contrast, amphotericin B MICs observed using the Etest were lower (mode, 0.12 μg/ml) (Table 2). Whether the YNB and Etest data would be different from those results obtained using the RPMI method is unclear, because each set was from a single laboratory. However, both modes were different than those observed in 12 of the 13 laboratories using RPMI for the similar set of non-molecular typed isolates. In prior reports, amphotericin B MICs have been either 0.12 μg/ml (Dannaoui et al., 2006) or 1 μg/ml (Brandt et al., 2001, 2005, De Bedout et al., 1999). These results underline the variability of susceptibility tests results using different methodologies as well as the need to evaluate MIC distributions before ECV definition.

Flucytosine modes were more species, molecular type and medium-dependent (Tables 2 and 3). The mode for C. neoformans non-molecular typed and AFLP1/VNI isolates was the same (4 μg/ml), but more variability was observed for C. gattii, with the lowest mode for the small set of non-molecular typed isolates (1 μg/ml) and the highest value (8 μg/ml) for two of the three AFLP6/VGI subtypes (AFLP6a/VGIa and AFLP6c/VGIC). Our modes reflect the differences found between the susceptibility of C. neoformans (MICs < 4 μg/ml) and C. gattii to flucytosine (MICs ≥ 2 μg/ml) as well as among the latter species genotypes (MICs < 1 to 4 μg/ml) (Chong et al., 2010, Thompson et al., 2009), where the highest value was for AFLP6/VGII. YNB yielded a higher mode (8 μg/ml) than that for the similar set of

Table 3. Epidemiologic cutoff values (ECVs) for amphotericin B and flucytosine species complex as obtained in 3 to 16 laboratories by the CLSI M27-A3 broth microdilution method

<table>
<thead>
<tr>
<th>Species</th>
<th>Antifungal</th>
<th>Range</th>
<th>Mode</th>
<th>Calculated Statistical ECV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25%</td>
</tr>
<tr>
<td>C. neoformans</td>
<td>AMB</td>
<td>0.03-4</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>VNI/AFLP1</td>
<td>0.03-2</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>C. neoformans</td>
<td>FCT</td>
<td>0.125-64</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>C. gattii</td>
<td>AMB</td>
<td>0.06-1</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>VGI/AFLP4</td>
<td>0.03-1</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>VGI/AFLP1</td>
<td>0.125-2</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>VGI/AFLP6a</td>
<td>0.06-1</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>C. gattii</td>
<td>FCT</td>
<td>0.25-8</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>VGI/AFLP4</td>
<td>0.125-64</td>
<td>4</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>VGI/AFLP6</td>
<td>0.25-64</td>
<td>2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>% Over statistical ECV</td>
<td>29%</td>
<td>97.5%</td>
<td>99%</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Percentages of isolates of Cryptococcus neoformans-Cryptococcus gattii species complex above the amphotericin B and flucytosine wild-type (WT) distribution as obtained in 3 to 16 laboratories by the CLSI M27-A3 broth microdilution method

<table>
<thead>
<tr>
<th>Species or genotype</th>
<th>Anti-fungal</th>
<th>Statistical ECV ≥95%</th>
<th>Statistical ECV ≥97.5%</th>
<th>Statistical ECV ≥99%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. neoformans</td>
<td>AMB</td>
<td>1 (1.3%)</td>
<td>1 (1.3%)</td>
<td>2 (0.1%)</td>
</tr>
<tr>
<td>VNI/AFLP1</td>
<td>0.5 (2.8%)</td>
<td>1 (0.2%)</td>
<td>1 (0.2%)</td>
<td></td>
</tr>
<tr>
<td>C. neoformans</td>
<td>FCT</td>
<td>16 (1.4%)</td>
<td>16 (1.2%)</td>
<td>32 (1.1%)</td>
</tr>
<tr>
<td>VNI/AFLP1</td>
<td>8 (3.4%)</td>
<td>8 (3.4%)</td>
<td>16 (2.4%)</td>
<td></td>
</tr>
<tr>
<td>C. gattii</td>
<td>AMB</td>
<td>1 (0%)</td>
<td>1 (0%)</td>
<td>1 (0%)</td>
</tr>
<tr>
<td>VGI/AFLP4</td>
<td>0.5 (0.8%)</td>
<td>0.5 (0.8%)</td>
<td>0.5 (0.8%)</td>
<td></td>
</tr>
<tr>
<td>VGI/AFLP6</td>
<td>1 (0.8%)</td>
<td>1 (0.8%)</td>
<td>2 (0.8%)</td>
<td></td>
</tr>
<tr>
<td>VGI/AFLP6a</td>
<td>0.5 (2.5%)</td>
<td>0 (0%)</td>
<td>1 (0%)</td>
<td></td>
</tr>
<tr>
<td>C. gattii</td>
<td>FCT</td>
<td>4 (3.6%)</td>
<td>8 (0%)</td>
<td>8 (0%)</td>
</tr>
<tr>
<td>VGI/AFLP4</td>
<td>4 (3.4%)</td>
<td>4 (3.4%)</td>
<td>4 (3.4%)</td>
<td></td>
</tr>
<tr>
<td>VGI/AFLP6</td>
<td>16 (2.9%)</td>
<td>16 (2.9%)</td>
<td>16 (2.9%)</td>
<td></td>
</tr>
</tbody>
</table>

* Data from laboratories using the CLSI RPMI broth
* Calculated epidemiological cutoff values (ECVs) comprising ≥95 or ≥97.5% or ≥99% of the statistically modeled population.

ECV, μg/ml (% of observations above each statistical ECV)

<table>
<thead>
<tr>
<th>Species or genotype</th>
<th>Anti-fungal</th>
<th>Statistical ECV ≥95%</th>
<th>Statistical ECV ≥97.5%</th>
<th>Statistical ECV ≥99%</th>
</tr>
</thead>
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<tr>
<td>C. neoformans</td>
<td>AMB</td>
<td>1 (1.3%)</td>
<td>1 (1.3%)</td>
<td>2 (0.1%)</td>
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<tr>
<td>VNI/AFLP1</td>
<td>0.5 (2.8%)</td>
<td>1 (0.2%)</td>
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</tr>
<tr>
<td>C. neoformans</td>
<td>FCT</td>
<td>16 (1.4%)</td>
<td>16 (1.2%)</td>
<td>32 (1.1%)</td>
</tr>
<tr>
<td>VNI/AFLP1</td>
<td>8 (3.4%)</td>
<td>8 (3.4%)</td>
<td>16 (2.4%)</td>
<td></td>
</tr>
<tr>
<td>C. gattii</td>
<td>AMB</td>
<td>1 (0%)</td>
<td>1 (0%)</td>
<td>1 (0%)</td>
</tr>
<tr>
<td>VGI/AFLP4</td>
<td>0.5 (0.8%)</td>
<td>0.5 (0.8%)</td>
<td>0.5 (0.8%)</td>
<td></td>
</tr>
<tr>
<td>VGI/AFLP6</td>
<td>1 (0.8%)</td>
<td>1 (0.8%)</td>
<td>2 (0.8%)</td>
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</tr>
<tr>
<td>VGI/AFLP6a</td>
<td>0.5 (2.5%)</td>
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<tr>
<td>C. gattii</td>
<td>FCT</td>
<td>4 (3.6%)</td>
<td>8 (0%)</td>
<td>8 (0%)</td>
</tr>
<tr>
<td>VGI/AFLP4</td>
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<td>4 (3.4%)</td>
<td>4 (3.4%)</td>
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</tr>
<tr>
<td>VGI/AFLP6</td>
<td>16 (2.9%)</td>
<td>16 (2.9%)</td>
<td>16 (2.9%)</td>
<td></td>
</tr>
</tbody>
</table>
Amphotericin and flucytocine wild-type susceptibility

Chapter 8

Amphotericin and flucytocine wild-type susceptibility

Although the mechanisms of amphotericin B resistance in C. neoformans are poorly understood, despite amphotericin B use since the 1960s, they are better elucidated for non-molecular typed C. neoformans isolates measured in RPMI (Table 2). Since this value was based on results from a single-laboratory, it is not clear if this discrepancy is medium or reader related. However, modes in each participant laboratory for C. neoformans (non-molecular typed and molecular/AFLP typed isolates) were either 2 or 4 μg/ml (data not shown in Tables 2 and 3). Based on these data and the wide geographical range over which MICs have been collected in the present study, we are confident of our data validity.

Table 3 depicts the proposed amphotericin B and flucytosine ECVs for the aggregated distributions of C. neoformans and C. gattii (molecular or non-molecular typed isolates) where the data originated in three or more laboratories (using the methodology that comprised >95%, >97.5% and >99% of the modeled population). The CLSI amphotericin B ECV was 1 μg/ml for C. neoformans and C. gattii non-molecular typed distributions and AFLP6/VGII (encompassing 98.5, 100 and 99.2% of the isolates, respectively) and 0.5 μg/ml for AFLP1/VNI, AFLP4/VGI and AFLP6a/VGIIa (encompassing 97.2, 99.2 and 97.5% of the isolates, respectively). It is interesting that an amphotericin B MIC of 2 μg/ml is anecdotally believed to be the breakpoint for resistance and yet here can be perceived as a WT value. The flucytosine ECV for C. neoformans non-molecular typed and AFLP1/VNI distributions were 16 and 8 μg/ml (encompassing 98.6 and 96.6% of the isolates, respectively). For C. gattii, flucytosine ECVs were either 4 μg/ml (non-molecular typed and AFLP4/VGI) or 16 μg/ml (AFLP6/VGII), encompassing 96.4, 95.7 and 97.1% of the isolates. Because only two laboratories contributed data for the other molecular typed isolates and both amphotericin B and flucytosine distributions in YNB were obtained in a single-laboratory, ECVs were not proposed for these distributions (Table 1). Tentative values of 1 μg/ml (amphotericin B, encompassing 98.4% of the isolates) and 16 μg/ml (flucytosine, encompassing 97.1% of the isolates) can be suggested for YNB MICs (data not shown in Table 3). Visual ECVs were similar to those using the >95% of the modeled population and some higher ECVs were observed using >95% of the modeled populations (Turnidge et al., 2006). The frequency of amphotericin B and flucytosine MICs above the ECV (non-WT) varied according to the distribution analyzed (Table 4). The rate of non-WT MICs was lower for amphotericin B distributions of both species (0 to 2.8%) than those for flucytosine (1.4 to 4.8%). The rate of flucytosine non-WT MICs was lower among all C. neoformans (4.3%) than among all C. gattii (10.8%), but almost the same for amphotericin B (4.3 and 4.1% respectively). Although cryptococcal infections by both species are clinically similar, there is a delayed treatment response and other complications in infections caused by C. gattii than those due to C. neoformans (Perfect et al., 2010). In vitro resistance to amphotericin B (0.6 to 5.3%) and flucytosine (2.2 to 46%) has been reported among smaller number of C. neoformans isolates; although the susceptible cutoff for amphotericin B has been consistent (≥2 μg/ml), it has been variable for flucytosine (≥8 and ≥32 μg/ml) in the different studies (Brandt et al., 2001, De Bedout et al., 1999, Perkins et al., 2005, Thompson et al., 2009). All these results underscore the utility of antifungal susceptibility testing and WT cutoffs as a practical tool to detect amphotericin B and flucytosine resistance among Cryptococcus isolates; species identification is clinically useful. Again, these results also indicate that ECVs should be species- and for this fungal group molecular/AFLP type-specific.

Although the mechanisms of amphotericin B resistance in C. neoformans are poorly understood, despite amphotericin B use since the 1960s, they are better elucidated for flucytosine (Espinel-Ingroff et al., 2008, Kanafani & Perfect, 2008, Perfect & Cox, 1999, Schwarz et al., 2007, Whelan, 1987, White & Hoot, 2011). In addition, the link between genetic mutations and high MICs of these two agents for Cryptococcus spp. have not been determined. Susceptibility testing by M27-A3 methodologies demonstrated that high MICs for C. neoformans were predictive of clinical failure for the azoles and to a lesser degree for both polyenes and flucytosine (Perfect & Cox, 1999). Amphotericin B MICs of >2 μg/ml have been associated with clinical failure, a defect in the target enzyme sterol delta 7-isomerase, other mutations in the sterol biosynthesis pathway (e.g., 5,6 desaturase), or an increase in the efflux of the drug from the fungal cell (Hospenthal and Bennett, 1998, Joseph-Horne et al., 1996, Kelly et al., 1994). C. neoformans primary as well as acquired resistance to flucytosine was frequently reported when its use began in the 1970s and high flucytosine MICs correlated with failure (as high as 57%) in patients receiving this agent alone (Block et al., 1973, Hospenthal & Bennett, 1998, Whelan, 1987). Resistance to this agent occurs by mutations within the pyrimidine pathway (either cytosine deaminase or uracil phosphoribosyltransferase) specifically linked in haploid C. neoformans to a single mutation at either the FCY1 or the FCY2 gene (Whelan, 1987). Poor correlation between amphotericin B and flucytosine CLSI MICs with clinical outcome has been reported (Dannaoui et al., 2006) but the number of patients was low (4 to 19 to in each set of therapy success or failure groups), geometric mean MICs were similar (amphotericin B, 0.22 to 0.39 μg/ml; flucytosine 2.24 and 2.87 μg/ml) and other factors could have influenced the outcome (some patients were HIV-positive). It is noteworthy that the combination of these two agents is synergistic in vitro despite resistance to flucytosine (Schwarz et al., 2007). Therefore, much needs to be examined and determined regarding the relationship between non-WT strains and resistance mechanisms.

In conclusion, the ECVs of amphotericin B (0.5 to 1 μg/ml) and of flucytosine (4 to16 μg/ml) proposed in this study for the C. neoformans-C. gattii species complex were species- and molecular/AFLP type-specific. Further investigation should determine the relationship between molecular mechanisms of amphotericin B and flucytosine resistance and our proposed non-WT values. Some of the distributions were small (especially for various C. neoformans molecular genotypes) and continuing surveillance should either corroborate or extend the information provided in the present study. In the absence of CBPs, these ECVs may aid in detecting isolates with reduced susceptibility to amphotericin B and flucytosine (non-WT isolates) harboring resistance mechanisms and they should be included in the revised version of the CLSI M27-A3 document.
Chapter 9

Environmental prevalence of Cryptococcus neoformans and C. gattii in India: An update

Anuradha Chowdhary
Harbans Singh Randhawa
Anupam Prakash
Jacques F. Meis
Environmental prevalence of C. neoformans and C. gattii in India

Chapter 9

Summary

An overview of work done to-date in India on environmental prevalence, population structure, seasonal variations and antifungal susceptibility of Cryptococcus neoformans and Cryptococcus gattii is presented. The primary ecologic niche of both pathogens is decayed wood in trunk hollows of a wide spectrum of host trees, representing 18 species. Overall, C. neoformans showed a higher environmental prevalence than that of C. gattii which was not found in the avian habitats. Apart from their arboreal habitat, both species were demonstrated in soil and air in close vicinity of their tree hosts. In addition, C. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta. An overwhelming number of strains belonged to genotype AFLP1/VNI, var. neoformans showed a strong association with desiccated avian excreta.

Introduction

Cryptococcus neoformans (Sanfelice) Vuillemin, the classical etiologic agent of cryptococcosis, is currently recognized as a species complex, comprising C. neoformans var. grubii, serotype A, C. neoformans var. neoformans, serotype D, and C. gattii, serotypes B and C (Kwon-Chung et al., 2011, Simwami et al., 2011). Cryptococcus neoformans and C. gattii differ significantly in their geographic distribution and ecologic niches. A vast majority of cryptococcal infections, particularly in immunocompromised patients, are caused by C. neoformans var. grubii whereas C. gattii accounts for a smaller proportion of cases but it frequently infects immunocompetent patients in tropical and subtropical regions. Cryptococcus neoformans is a life-threatening etiologic agent of fungal meningitis, with an increasing number of global cases occurring in HIV/AIDS patients but more so in developing countries. An estimated one million cases of cryptococcal meningitis occur globally per year in AIDS patients, resulting in approximately 625 000 deaths (Park et al., 2009). India has the second largest burden of cryptococcosis due to an estimated population of 3.1 million to 9.4 million persons living with HIV/AIDS (UNAIDS 2006). The country has a documented high prevalence (1.7 to 4.7%) of cryptococcosis in persons with HIV/AIDS (Kumarasamy et al., 2003; Vajpayee et al., 2003).

The large global burden of cryptococcosis presents a number of challenges to public health, particularly in resource-deficient regions of high HIV prevalence in sub-Saharan Africa and South/Southeast Asia. Multiple strategies, including environmental surveillance, increase in the number of diagnostic laboratories and strengthening of their infrastructure are required to combat the increasing threat to public health.

In the past decade, a more virulent genotype of C. gattii, AFLP6A/VGIIa; AFLP6C/VGIIc, has emerged as a primary pathogen on Vancouver Island and its adjoining areas in Canada and the USA, revealing extension of this pathogen’s geographical domain to the temperate climate (Kidd et al., 2004; Datta et al., 2009; Byrnes et al., 2010). The new genotype has been responsible for over 350 cases of human cryptococcosis, 19 of which were fatal despite aggressive antifungal therapy, and the outbreak is still ongoing (Bartlett et al., 2008; Galanis et al., 2010; Mak et al., 2010). It is noteworthy that the source of this cryptococcal outbreak was traced to extensive colonization of several native trees by C. gattii and its occurrence in soil in the affected temperate region (Kidd et al., 2004, 2007b; Byrnes et al., 2010).

Cryptococcus neoformans and C. gattii are free-living saprobes in nature and they infect their human and animal hosts when air-borne infectious propagules (yeast cells or basidiospores) are inhaled. As the infection is acquired from exogenous sources and it is ordinarily not transmitted from one infected individual to another, it is vitally important to have first-hand knowledge of environmental prevalence of C. neoformans and C. gattii with a view to designing any possible control measures against cryptococcosis. Besides, it is necessary to make environmental isolations of C. neoformans and C. gattii in order to probe their genetic structure. Furthermore, the advances made in genotyping techniques and their application to environmental isolates will address some important questions, such as, is there any evidence of ecological specialization among C. neoformans isolates from pigeon guano and those from decayed wood or other plant debris? This paper aims to present an overview, in global context, of the work done to-date on the environmental prevalence of C. gattii and C. neoformans in India and indicate the gaps in our existing knowledge.
Historical
Cryptococcus neoformans was isolated for the first time in 1894 by Sanfelice from peach juice in Italy (Sanfelice 1894). He described it as encapsulated yeast, demonstrated its pathogenicity for laboratory animals and named it Saccharomyces neoformans. In the same year, (Busse, 1894) and Buschke (1895) in Germany reported the first clinical human case which they described as Saccharomycosis hominis. For the following 57 years, this pathogenic yeast was known only from clinical cases, until the globally renowned medical mycologist, Chester Emmons (1951) reported 4 incidental isolations of C. neoformans in the USA during an investigation of 716 soil samples for Histoplasma capsulatum, employing the mouse-inoculation technique. Subsequently, Emmons (1955) reported frequent isolations of virulent C. neoformans strains from pigeon nests bearing old excreta, indicating that it was an environmental reservoir for the pathogen. His pioneering work stimulated worldwide studies which demonstrated that desiccated pigeon and other avian fecal matter constituted the most important natural habitat of C. neoformans. Fritz Staib and coworkers in Germany refocused attention on the pathogen’s original isolation from peach juice by Sanfelice when they reported its isolation from a ripe peach fruit (Staib et al., 1973; Staib et al., 1974) and demonstrated in vitro colonization by C. neoformans of dried leaves, stems and other parts of various plants under defined laboratory conditions (Staib et al., 1972a; Staib et al., 1972b). In 1986, C. neoformans was isolated from wood samples collected from a hollow tree trunk inside an aviary in the Zoological Garden, Antwerp, Belgium (Bauwens et al., 1986). In this paper, reference was made to unpublished observations of Daniëlle Swinne on the isolation of C. neoformans var. neoformans from saw dust of tropical trees, Entandophragma species, in a sawmill in Kinshasa, Congo. It was stated that although the isolation of C. neoformans from bark and wood from aviaries could be due to contamination with bird droppings, it was nevertheless possible that some trees could provide a natural habitat for C. neoformans. Further investigations on the role of wood in the natural history of C. neoformans was, therefore, suggested.

The environmental niche of C. gattii remained unknown for nearly two decades after this taxon was described by Vanbreuseghem and Takashio (1970). Attention to its natural habitat in trees was first drawn by Ellis and Pfeiffer (1990a) who isolated serotype B strains from debris of leaves and flowers of Eucalyptus camaldulensis in Australia.

Isolation Techniques
Sample collection: Apart from air sampling, the wide variety of environmental samples that have been investigated include soil, avian excreta, decayed wood and bark of trees or other plant debris (Lazéra et al., 1993; Randhawa et al., 2001; Kidd et al., 2007a; Kidd et al., 2007b; Illnait-Zaragozi et al., 2010a). Samples of soil, plant debris or bark from tree trunks are collected in clean, self-sealing polyethylene bags or screw-capped, 2-oz, glass bottles, using metal spatula and forceps after cleaning them with 70% ethanol. Decayed wood from inside tree trunk hollows is scraped with a sterile surgical scalpel and shavings or small pieces of decayed wood are collected (Randhawa et al., 2005). Aerial sampling may be done by exposure of petri-dishes containing simplified Staib’s niger seed medium, or using air samplers (Staib 1962; Paliwal and Randhawa 1978; Randhawa et al., 2006; Kidd et al., 2007b). Currently, a more efficacious sampling technique of swabbing is employed which is briefly described below.

Swabbing: Cotton-tipped swabs can be prepared in-house from bamboo broom sticks, measuring 40-cm-long and 2mm in thickness. The swab sticks are wrapped in craft paper and sterilized by autoclaving. Sampling of decayed wood, bark and plant debris is done by rubbing the swabs moistened with sterile physiological saline, containing gentamycin (25 mg/L). The swab handles are then cut short and transferred to sterile, screw-capped glass bottles (75 x 25 mm) and brought to the laboratory for processing. The swabs are inoculated directly or after suitable dilution on simplified Staib’s niger seed plates. The efficacy of swabbing versus a conventional technique for sampling of decayed wood in tree trunk hollows for isolation of C. neoformans and C. gattii was evaluated by Randhawa et al., (2005). Of 42 known positive wood samples, swabbing was successful for isolation of C. neoformans in 40 (95%) as against 32 (76%) by the conventional technique, and this difference was statistically significant (P<0.01). Moreover, the conventional technique yielded 24% false-negative results in striking contrast to only 5% by swabbing. Besides, swabbing yielded a significantly higher C. neoformans mean colony count per positive sample than did the conventional technique (P<0.005), thus demonstrating the superiority of the former technique. Notably, application of the swabbing technique revealed that over 80% of the S. cuminii trees in one locality of Delhi harboured the C. neoformans species complex in decayed wood inside their trunk hollows. In view of its greater efficacy, the swabbing technique has been widely adopted in environmental studies.

Sample Processing
Floatation-sedimentation technique: Sample processing is done by a floatation-sedimentation technique, previously used for isolation from soil of Coccidioides immitis, the etiologic agent of coccidioidomycosis (Stewart and Meyer 1932). Briefly, a measured quantity of the test soil samples, avian excreta, plant debris, etc. is suspended in sterile physiological saline and mixed thoroughly by vortexing. The suspension is allowed to stand during their isolations in primary cultures, followed by separation of the supernatant.

Direct inoculation: Measured aliquots of the supernatant are directly inoculated on plates of simplified Staib’s niger seed medium or alternative selective media such as sunflower seed agar, caffeic acid agar and L-DOPA medium (Staib 1962; Shaw and Kapica 1972; Paliwal and Randhawa 1978; Hopfer and Blank 1975). Colonies of C. gattii and C. neoformans can be presumptively identified on these media by their characteristic, variably chocolate brown pigment. However, other yeasts, such as melanin positive (Mel+) variants of Cryptococcus laurentii and Cryptococcus cassia may also develop the same pigment (Figs. 1 A,B) and thus likely to be confused with C. gattii and C. neoformans during their isolations in primary cultures.

Mouse inoculation technique: The supernatant is injected intraperitoneally into laboratory mice which are sacrificed after 4-6 weeks. Their visceral organs such as liver and spleen are macerated and inoculated on appropriate mycological media for isolation of C. neoformans and C. gattii or other target pathogenic fungi. This method has become obsolete for the isolation of C. neoformans /C. gattii from environmental sources since the introduction of
Phenotypic and molecular identification: Cryptococcus gattii differs from C. neoformans in various aspects, including a contrasting human host profile and a reduced susceptibility to certain antifungal drugs (Lin and Heitman 2006; Khan et al., 2007; Hagen et al., 2010; Chowdhary et al., 2011b). Since C. gattii is as an emerging pathogen, it is important for the clinical microbiology laboratory to differentiate it from its closely related C. neoformans. A proficiency testing survey administered by the New York State Department of Health indicated that only 5% of clinical laboratories participating in the event were able to correctly identify C. gattii, while the remaining 95% of laboratories surveyed misidentified the isolate as C. neoformans (New York State Department of Health, 2005). In comparison, the situation in developing countries is expected to be far more unsatisfactory.

Various phenotypic techniques have been used for differentiating C. gattii from C. neoformans, including use of canavanine-glycine-bromothymol blue agar (CGB), glycine-cycloheximide-phenol red agar, creatinine-dextrose bromothymol blue thymine agar and creatinine-dextrose bromothymol blue agar (Kwon-Chung et al., 1978, 1982; Salkin et al., 1982; Min and Kwon-Chung 1986; Irokanulo et al., 1994). The CGB agar was reported to give fewer false-positive and false-negative results than the others. Currently available commercial methods for yeast identification, such as API 20 AUX (bioMerieux, Paris, France), Vitek (bioMerieux), and MicroScan (Siemens, Erlangen, Germany) do not differentiate between C. neoformans and C. gattii. However, recently, matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has been successfully used to differentiate C. neoformans from C. gattii (McTaggart et al., 2011a). Serotyping is useful for differentiation of C. gattii from C. neoformans but the only commercial kit previously available for serotyping (Crypto-Check kit; Iatron Inc., Tokyo, Japan) is no longer manufactured.

However, multiplex PCR and liquid array-based methods have been recently reported for differentiation of C. neoformans and C. gattii (Bowers et al., 2007; Feng et al., 2008), but this technology is not yet routinely utilized by most clinical laboratories. McTaggart et al., (2011b) evaluated an algorithm, incorporating commercial rapid biochemical tests, differential media and DNA sequence analysis for a rapid and accurate differentiation of C. gattii and C. neoformans. CGB agar or IGS sequencing differentiated these isolates within 48 hours. On CGB, 25 of 27 (93%) C. gattii strains induced a blue color change in contrast to 0 of 86 C. neoformans isolates. Neighbour-joining cluster analysis of IGS sequences differentiated C. neoformans var. grubii, C. neoformans var. neoformans and C. gattii. Over the past two decades, a variety of molecular techniques have been introduced for identification of pathogenic fungi. Many of these techniques, as opposed to classical phenotypic characterization, have the potential to provide rapid, sensitive, and specific identification of the C. neoformans species complex. Molecular fingerprinting techniques, e.g. random amplified polymorphic DNA (Boekhout and van Belkum 1997), restriction fragment length polymorphism (RFLP) (Meyer et al., 2003; Kidd et al., 2004), pulsed-field gel electrophoresis (Boekhout et al., 1997), sequencing (Diaz et al., 2000; Katsu et al., 2004; Bovers et al., 2008; McTaggart et al., 2011b), multilocus microsatellite typing (Illumit-Zaragozi et al., 2010a,b), mating type locus (Cognati et al., 2006) and multilocus sequence typing (MLST) (Litvintseva et al., 2006; Hiremath et al., 2008; Meyer et al., 2009; Chowdhary et al., 2011a) are techniques that have been applied to characterize the genetic heterogeneity of the C. neoformans species complex. Based on molecular studies, using PCR fingerprinting, AFLP analysis, analysis of the orotidine monophosphate pyrophosphorylase (URAS) and phospholipase (PLB1) genes by RFLP and MLST, C. neoformans and C. gattii have been further classified into several distinct genotypes: AFLP1/VNI and AFLP1A/VLPB/VNI (C. neoformans var. grubii, serotype A), AFLP2/VNIV (C. neoformans var. neoformans, serotype D), AFLP3/VNII (hybrid serotype AD), AFLP4/VGII, AFLP6A/VGIIA, AFLP6B/VGIIIB, AFLP6C/VGIII, AFLP7/VGIV and AFLP10/VGIV (C. gattii, serotype B/C). In addition, hybrids of C. neoformans var. neoformans and C. gattii and of C. neoformans var. grubii and C. gattii belong to genotypes AFLP8 and AFLP9, respectively (Bovers et al., 2006, 2008). Although more labour intensive and costly, DNA sequencing is rapidly becoming a common procedure in most clinical laboratories because of its greater discriminatory power than is generally provided by using differential media and biochemical tests. In laboratories where DNA sequencing is routinely available, IGS, Internal Transcribed Spacer (ITS) and D2 region of the fungal 28S large ribosomal subunit distinguish C. neoformans from C. gattii and are optimal methods for identification of Cryptococcus species. However, sequencing of the D2 region of the 28S large ribosomal subunit may not be able to reliably distinguish C. neoformans var. grubii and C. neoformans var. neoformans (Klein et al., 2009). Similarly, sequencing of the ITS region differentiates C. gattii but has poor discrimination (≥99.5% similarity) between C. neoformans var. neoformans and C. neoformans var. grubii (McTaggart et al., 2011b).

Environmental Prevalence

The success in demonstration of C. neoformans in soil and avian excreta in early studies by the stalwarts of Medical Mycology, Chester Emmons (1951), Libero Ajello (1958) and Maxwell Littman (1959) was achieved by employing the mouse inoculation technique. Investigations on the natural habitat of C. neoformans were further stimulated when Fritz Staib (1962) developed and introduced the niger seed agar, a selective medium for its isolation, which has been further used in many studies. The positive samples came from a heap of dry, mortar-like excreta admixed with dust and feathers found accumulated inside an abandoned uppermost floor of the Arts Faculty Building, University of Delhi, North Campus, where feral pigeons were roosting. The isolated C. neoformans strains were found to be pathogenic to mice experimentally infected intra-cerebrally. These observations were confirmed by Padhye and Thirumalachar (1967) from Pune and Gugnani et al., (1967, 1972) from Delhi who reported the isolation of C. neoformans from old excreta collected from a pigeon house inside the National Zoological Gardens.
Park, New Delhi, and also from an old building inside the B.R. College Agricultural Farm, Agra. Subsequently, Khan et al., (1978) reported a more frequent association (55.4%) of *C. neoformans* with old pigeon excreta in a Charity Bird Hospital, Delhi. This was followed by a larger VPCI study, covering 489 diverse natural substrates and employing Stahb’s niger seed agar as a selective isolation medium. *C. neoformans* was reported from 38 of 253 (15%) old avian excreta investigated (Pal et al., 1979). This report was noteworthy for isolating *C. neoformans* for the first time from the excreta of 5 avian species, namely *Ara ararauna* (Blue-and-yellow Macaw), *Ara chloroptera* (Red-and-green Macaw), *Melopsittacus undulatus* (Common Pet Parakeet), *Centropus sinensis* (Crow Pheasant) and *Estrilda amandava* (Red Munia). No serotyping or varietal identification of the *C. neoformans* isolates was done in any of the afore-mentioned studies. Outside of India, *C. neoformans* has been reported from 5/97 (5.2%) excreta samples of swallow (*hirundo rustica*) in Iran (Hedayati et al., 2011), 24% of chicken faeces in Thailand (Kuroki et al., 2004) and from 25.5% of excreta of caged passerine and psittacine birds in Brazil (Lugarini et al., 2008). It seems pertinent to point out here that avian excreta is primarily a natural habitat of *C. neoformans*, although *C. gattii*, serotype B has been sporadically isolated from this substrate (Abegg et al., 2006).

Nielsen et al., (2007) have reported that pigeon guano supported in vitro growth of both species, and it allowed a prolific mating of *C. neoformans* but not of *C. gattii*. Consequently pigeon guano represents a fundamental but not a realized environmental niche for *C. gattii*. Mussa (1997) reported isolation of *C. neoformans* in 4 of 181 (2.2%) bat guano samples, collected mostly from Delhi. In another study by the VPCI Mycology Group, *C. neoformans* was incidentally isolated from the intestinal contents of one of 155 insectivorous bats belonging to *Rhinopoma hardwickei*, captured from an abandoned, dark and dingy floor of an old school building in Delhi (Khan et al., 1982). Outside of India, a solitary isolation of *C. gattii* from bat guano has been reported from Brazil by Lazéra et al., (1993).

Worldwide literature reports have firmly established that desiccated excreta of pigeons and other avian species are an excellent natural substrate for the growth and multiplication of *C. neoformans* in the environment. In saprobiq settings, *C. neoformans* is inhibited by UV light and temperature exceeding 44°C. It can catabolize high concentration of urea, catecholamines and other nitrogenous compounds in pigeon excreta (Fiskin et al., 1990). Also, it can produce laccase and become melanized (Nosanchuk et al., 1999) which provides some protection against UV radiations, temperature extremes and oxidative compounds. The melanin chelates silver and perhaps other toxic heavy metals that protects the fungus against degrading enzymes (Rosas and Casadevall 2001; García-Rivera and Casadevall 2001). The ability to produce urease, enables it to thrive on urea and other nitrogenous compounds in the excreta. *C. neoformans* may produce potentially infectious basidiospores in the pigeon excreta. In sites with pigeon manure harbouring *C. neoformans*, air sampling has demonstrated aerosols of yeast cells and or basidiospores (Litvintseva et al., 2011). Besides, many investigators have reported the proximity of patients with cryptococcosis to pigeon or other avian habitats (Currie et al., 1994; García-Hermoso et al., 1997; Nosanchuk et al., 2000). In addition, isolates of *C. neoformans* recovered from patients and pigeon guano (Franzot et al., 1997; Currie et al., 1994; Litvintseva et al., 2005) and excreta of a pet magpie, *Pica pica* (Lagrou et al., 2005) were shown to have the same genotype, suggesting zoonotic transmission of cryptococcosis. Therefore, desiccated pigeon excreta constitute a plausible source of human cryptococcosis.

**Role of pigeon as carrier/host?**: Pigeons and most other birds are not hosts to *C. neoformans* and they do not acquire cryptococcosis because their body temperature (average 42.5°C) is too high to allow the growth of this fungal pathogen (Emmons 1955; Littman and Borok 1968). Nevertheless, a number of cases of avian cryptococcosis caused by *C. neoformans* var. *grubii* and *C. gattii* have been reported, but the tolerance of the etiologic isolates to high temperatures was not tested. Also, the infection in these avian cases was restricted to cutaneous sites or the upper respiratory tract (Malki et al., 2003).

The isolation of *C. neoformans* from the feet, beak and gastrointestinal tract of the feral pigeon (*Columba livia*) has been well documented (Swinne-Desgain 1975). In India, Sethi and Randhawa (1968) carried out a study involving experimental feeding of feral pigeons with a virulent strain of *C. neoformans*. None of the infected birds showed any signs of cryptococcal infection although *C. neoformans* could be isolated from the intestinal contents or fresh excreta in 17 of 18 infected pigeons sacrificed over a period of 3 weeks. In addition, the pathogen was recovered from the fresh excreta and intestinal contents of one of the pigeons necropsied on the 36th post-infection day. From the same laboratory, Khan et al., (1978) reported the isolation of *C. neoformans* from the crops of 4 (1.3%) of 319 feral pigeons investigated in Delhi, supporting the view that the pigeon itself is not a reservoir of the pathogen but may serve as its mechanical carrier and disseminator in the environment. In this context reference may be made to Cafarchia et al., (2006) who have suggested a role for birds of prey such as *Falco tinnunculus* (Kestrel) and *Buteo buteo* (Buzzard) as carriers and spreaders of *C. neoformans*.

**Soil**: *Cryptococcus neoformans* and *C. gattii* has been frequently reported from soil and dust. However, these isolations are from samples that contained excreta of pigeons, other avian species or bats (Emmons 1955; Ajello 1958; Casadeval and Perfect 1998). In a survey of soil-inhabiting human pathogenic fungi in India, Gugnani and Shrivastav (1972) reported the isolation of *C. neoformans* from four out of 308 soil samples, employing the mouse inoculation technique. Three of the 4 positive soil samples were rich in bat guano and collected from a historical monument, whereas the remaining positive sample contained traces of decomposed plant material and originated from a riverine site. Recently, Randhawa et al., (2008) have reported isolation of both *C. neoformans* and *C. gattii* from soil surrounding the base of a number of host trees harboring these pathogens in decayed wood of their trunk hollows. These host trees belonged to *Syzygium cumini*, *Mimusops elengi*, *Polyalthia longifolia* and *Azadirachta indica*. Of the 95 soil samples investigated, 25 were positive for *C. neoformans*, 23 for *C. gattii* and 5 for both of the species, yielding a prevalence of 26%, 24% and 5%, respectively. Depending upon the site of investigation, the prevalence in soil ranged from 11-50% for *C. neoformans*, 14-57% for *C. gattii* and 7-11% for concomitant occurrence of both the species in the same soil sample. Concerning the prevalence in soil with regard to individual host tree species, the highest for *C. gattii* at 29% was in the vicinity of *S. cumini* trees as against 25% and 20% in the vicinity of *M. elengi* and *A. indica* trees, respectively. For *C. neoformans*, the highest prevalence in soil was 31% in the vicinity of *S. cumini*, followed by 12.5% each for *M. elengi* and *P. longifolia* trees. None of the 10 control soil samples from an open playground away from the *S. cumini*
trees harbouring *C. neoformans* yielded any isolation of *C. gattii* or *C. neoformans*. These findings are in concordance with the results of an extensive study on characterization of environmental sources of *C. gattii* including soil samples collected from within one meter of the base of many host trees investigated in British Columbia, Canada, and the Pacific Northwest of the US. (Kidd et al., 2007b).

**Trees, decayed wood, other plant debris:** Cryptococcus gattii was reported for the first time from environmental sources in India by Chakrabarti et al., (1997) who isolated it from five of 354 (1.4%) plant debris samples of Eucalyptus trees investigated in Punjab. The positive samples belonged to three Eucalyptus camaldulensis trees, two of which were in the Chak Sarkar Forest and one in the village Periana near Ferozepur, Punjab. No quantitative results were reported such as the pathogen’s population density in any positive sample and no attempts were reported to re-isolate the fungus from the positive *E. camaldulensis* trees. Consequently, epidemiologic significance of the findings remained uncertain. Gugnani et al., (2005) have reported a low prevalence of 0.4% for *C. gattii* in flowers of *E. tereticornis* trees in Delhi and of *C. neoformans* var. *grubii* in the bark of *E. camaldulensis* trees in Chandigarh, Punjab. In common with the preceding report of Chakrabarti et al., (1997), no information was provided on the population density of *C. gattii* and *C. neoformans* in the positive samples. In an environmental study carried out in Vellore, South India, *C. gattii* was not found in any of the 86 *E. camaldulensis* trees sampled (Abraham et al., 1997). This was in agreement with the negative results for *C. gattii* reported by Swinne et al., (1994) who investigated 657 Eucalyptus samples collected in Rwanda, Africa, Hamasha et al., (2004) from Jordan and Ergin et al., (2004) from Turkey who investigated 500 and 1175 plant debris samples related to *Eucalyptus* trees, respectively. Likewise, in an investigation of 732 environmental samples in Vancouver Island, Canada, *C. gattii* has not been found in any of the Eucalyptus debris samples although it was isolated from several native tree species such as alder (*Alnus* spp.), cedar (*Cedrus* spp.), Douglas fir (*Pseudotsuga menziesii*), Garry oak (*Quercus garryana*) and grand fir (*Abies grandis*) (Kidd et al., 2004; Kidd et al., 2007b). Negative results for *C. gattii* were also reported by Randhawa et al., (2001) in an environmental study covering 702 samples of diverse plant material which included 498 bark samples (*E. tereticornis* - 104, *E. camaldulensis* - 98, unidentified Eucalyptus species - 188) collected from Delhi, Dehradun (Uttar Pradesh) and Amritsar (Punjab) in north-western India. However, four isolates of *C. neoformans* var. *grubii* and a number of other yeast-like fungi were isolated. Two of the *C. neoformans* isolates came from wood debris in tree trunk hollows of *Butea monosperma*, ‘the forest flame’ and one from a trunk hollow of a *Tamarindus indica* tree in New Delhi sampled during May/June 1996. The fourth *C. neoformans* var. *grubii* isolate came from the bark of an Eucalyptus tree in Amritsar. These isolations provided the first evidence in India of decayed wood in trunk hollows of living trees as a potential ecologic niche other than avian excreta for *C. neoformans* var. *grubii*. Among the additional Cryptococcus species isolated in this study were *C. laurentii* - 3 isolates and *C. albidus* – 2 isolates. Of these, 2 isolates of *C. laurentii* and one of *C. albidus* originated from the bark of a Eucalyptus tree. Interestingly, the third *C. laurentii* isolate was a rarely reported, melanin forming (Mel') variant that was initially mistaken for *C. neoformans*. It was repeatedly isolated from wood debris inside hollows of an abandoned, old timber beam of sal wood, *Shorea robusta*, sampled serially during Sept. 3, 1993 to March 12, 1996. As the variant isolate possessed phenol-oxidase (melanin forming) activity and it caused lesions in liver and spleen in experimentally infected laboratory mice, the findings are of potential clinical and epidemiologic significance (Mussa et al., 2000).

The afore-mentioned study led to more intensive investigations that aimed to characterize the environmental reservoirs of *C. gattii* and *C. neoformans*. It was found that *F. religiosa* trees harbored only *C. neoformans* var. *grubii* in their trunk hollows, whereas this variety and *C. gattii*, serotype B, were both equally distributed (prevalence 10.6%) in decayed wood inside trunk hollows of the 66 *S. cuminii* trees investigated. Furthermore, *C. gattii* was repeatedly isolated on 36/44 (82%) occasions from 7 *S. cuminii* known positive trees sampled longitudinally over a period of 689 days. Likewise, the over-all isolation frequency of *C. neoformans* var. *grubii* from the two host tree species came to 22/27 (81%) occasions during the same follow-up span. These data strongly supported a long-term colonization of decayed wood inside trunk hollows of *S. cuminii* by both the pathogens. The conclusion was reinforced by high population densities found in wood samples (maximally 6 × 10⁷ cfu/g for *C. gattii* and 8 × 10⁶ cfu/g for *C. neoformans*). No Eucalyptus trees were seen in the localities where *S. cuminii* and *F. religiosa* host trees were sampled. During the next phase of the study (Randhawa et al., 2006), a large number of *S. cuminii* trees were investigated from Delhi and other parts of north-western India, i.e. Amritsar (Punjab), Meerut and Bulandshahr (Uttar Pradesh) and the Chandigarh Union Territory. The results corroborated the strong ecological association of *C. gattii* and *C. neoformans* with *S. cuminii* trees throughout north-western India.

**Host tree species spectrum:** An overview of the environmental studies done in India (Table 1) indicates that 17 species of trees representing 12 families have been documented as hosts of the *C. neoformans* species complex. A decade-long study (2001-2011) of Randhawa and coworkers, revealed that the prevalence of *C. gattii* and *C. neoformans* differed considerably not only from one host tree to another but also among trees of the same host species occurring in a given locality or in different geographic regions. The isolation of *C. neoformans* var. *grubii* from 17 host tree species and of *C. gattii* serotype B from 12 tree species showed that the former has a more widespread arboreal distribution. Of the *C. gattii* host trees, *S. cuminii* in Delhi yielded the highest prevalence (89%), followed by *A. indica* (35 - 40%), *Manilkara hexandra* (33%), *M. elengi* (31%), *Cassia marginata* (20%), *P. longifolia* (7%), *Cassia fistula* and *Acacia nilotica* (5.2% each). Notably, the isolation frequency of *C. gattii* and *C. neoformans* from their host tree species was not related to the phenological state of the trees, which was in agreement with the observations of Lázera et al., (1998), Granados and Castañeda (2005), Kidd et al., (2007b) and Byrnes et al., (2011). Furthermore, *Eucalyptus* species proved to be among the least important host for *C. gattii*. Both of these observations were in striking contrast to the results reported from Australia (Ellis and Pfeiffer 1990a) where *C. gattii* isolations were linked to flowering of the trees, and *Eucalyptus* species were reported as predominant, if not virtually exclusive hosts. However, it is not clear as to what extent this difference can be attributed to the fact that tree species other than those of *Eucalyptus* have received scant attention of the Australian investigators interested in the ecology of *C. gattii* and *C. neoformans*. 

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Environmental prevalence of *C. neoformans* and *C. gattii* in India
For \textit{C. neoformans}, the most important host tree was \textit{A. indica} (prevalence 60%), followed by \textit{S. cumini} (54%), \textit{M. hexandra} (50%), \textit{C. fistula} (47%), \textit{M. elengi}, (15%), \textit{P. longifolia}, (13%) and lower prevalence in the range of 5 - 8% for \textit{Alstonia scholaris}, \textit{F. religiosa} and \textit{Dalbergia sissoo}. Most of the afore-mentioned host tree species had large canopies and conspicuous trunk hollows. As with \textit{C. gattii}, \textit{Eucalyptus} trees were among the least important host trees for \textit{C. neoformans}. It seems pertinent to mention here that a much larger number of host tree species other than those recorded in India have been reported from other countries. This includes more than 10 tree species each from Colombia and Vancouver Island, Canada (Granados and Castañeda 2005; Kidd et al., 2007b). Interestingly, \textit{C. gattii} has also been frequently isolated from a succulent cactus species \textit{Cephalocereus royerii} in the Guanica Dry Forest, Puerto Rico (Loperena-Alvarez et al., 2010). It is anticipated that the current global list of host tree species for the two pathogens (already exceeding 50) will expand considerably with further ecologic studies in as yet unexplored and climatically divergent geographic regions in India or elsewhere.

\textit{Cryptococcus gattii}, serotype \textit{C}, has not yet been reported from environmental or clinical sources in India. Much of the available information about this pathogen has been contributed by Elizabeth Castañeda and co-workers from Colombia where it occurs in association with tropical almond trees (\textit{Terminalia catappa}). In an experimental study aimed at exploring the interaction between serotype \textit{C} and \textit{Terminalia catappa}, they inoculated stems of 30 seedlings with an almond isolate. The isolate caused no visible lesions or microscopic phytopathology, but was recovered in culture up to one year and also demonstrated microscopically in sections of infected stem. It was further observed that the fungus spread from infected stem to soil and back to the seedlings, which was indicative of the affinity of serotype \textit{C} for almond plants and of the potential for long-term establishment of a stable interrelationship (Callejas et al., 1998; Escandón et al. 2002). These observations suggest the probability of an endophytic relationship between \textit{C. gattii} and its host plant \textit{T. catappa}.

### Longitudinal surveillance and population density

Of all the host trees harboring \textit{C. gattii} and \textit{C. neoformans} identified in India, it is \textit{S. cumini} that has been most extensively investigated and documented regarding its epidemiologic importance. In order to determine whether this tree species was transient, intermittently or perennially colonized, 7 of the known positive \textit{S. cumini} trees in Delhi were subjected to a long-term mycological surveillance. Significantly, \textit{C. gattii} and \textit{C. neoformans} were repeatedly re-isolated over a follow up period of 4.2 to 5.2 years. Furthermore, the positive samples carried a population density, ranging from 3 \times 10^{1} - 6 \times 10^{5} CFU/g for \textit{C. gattii} and 2 \times 10^{3} - 8 \times 10^{4} CFU/g for \textit{C. neoformans} (Randhawa et al., 2006). The highest population density of \textit{C. gattii} previously reported in any wood debris sample was 2.6 \times 10^{6} CFU/g in a \textit{Cassia grandis} tree in Brazil (Lazéra et al., 2000) which is 1/230^\text{th} part of the peak density of 6 \times 10^{5} CFU/g in one of the \textit{S. cumini} wood samples. It would have been interesting to compare the population density data of Randhawa et al. (2006) with those of the \textit{Eucalyptus} debris samples reported positive for \textit{C. gattii} from Australia by Ellis and Pfeiffer (1990a), but, unfortunately, no such data are available. From a review of the environmental studies done so far in India and elsewhere, it is clear that the natural habitat of \textit{C. gattii} is not restricted to plant debris of \textit{Eucalyptus} species or any other specific tree species. Instead, it has a generalized ecologic association with decayed wood or other plant debris of a wide spectrum of diverse tree species. The major factor underlying wood colonization by \textit{C. gattii} and \textit{C. neoformans} is probably the ability of these pathogens to produce the enzyme laccase which has been implicated in degradation of wood lignin by Basidiomycetes (Kirk and Farrel 1987; Thurson 1994; Williamson 1994; Eggert et al., 1996).

### Australian hypothesis of global spread of \textit{C. gattii}

In their historically important papers on the ecology of \textit{C. gattii}, Ellis and Pfeiffer (1990 a; b) and Pfeiffer and Ellis (1992) reported that flowers and other plant debris of \textit{E. camaldulensis} and \textit{E. teretocornis} trees constituted the main natural habitat of \textit{C. gattii}. They also hypothesized that \textit{C. gattii} had spread globally to other countries through the Australian export of infected \textit{E. camaldulensis} seeds containing dormant dikaryotic mycelium of \textit{C. gattii}. They believed that \textit{C. gattii} was a smut-like fungus which, however, belongs to an entirely unrelated group of plant pathogens classified under the class Ustilaginomycetes, subphylum Ustilaginomycotina, whereas \textit{C. gattii} belongs to the class Tremellomycetes, subphylum Agaricomycotina. In this probably misplaced analogy with a plant pathogen, it was further speculated that when the infected seeds of \textit{E. camaldulensis} would germinate and grow, the mycelia of \textit{C. gattii} would also grow in the tissues of the seedling and eventually sporulate to produce basidiospores at the time of the host tree’s flowering (Ellis and Pfeiffer 1990b). The authors stressed this hypothesis in various publications despite lack of any supportive scientific evidence and it continues to be cited (Lazéra et al. 2011). The weakness of this hypothesis is apparent from the following observations: One, \textit{C. gattii} has never been isolated from seeds, ovaries or anthers of \textit{E. camaldulensis} or any other \textit{Eucalyptus} species, nor has any histological evidence been presented to demonstrate the presence of dikaryotic mycelium in any of these or other parts of the host tree. Incidentally, attempts, in our laboratory to demonstrate the fungus histologically in situ in decayed wood pieces taken from inside trunk hollows of \textit{S. cumini} positive trees in Delhi were unsuccessful (Randhawa and Kowshik, unpublished data). Two, the smut fungi are plant pathogens, whereas \textit{C. gattii} is essentially an environmental, free-living saprobe which may sporadically infect humans and animals. Three, \textit{C. gattii} has been reported from native tree species in many countries, including those which have no \textit{Eucalyptus} flora. To cite two illustrative examples, \textit{C. gattii} has been isolated from a native jungle tree, \textit{Guettarda acreana}, in a wild tropical forest without anthropic action in Brazil (Fortes et al., 2001) and also from a Douglas fir tree (\textit{Pseudotsuga menziesii}) in Berg en Dal, Nijmegen, the Netherlands, a region where no tropical \textit{Eucalyptus} trees are prevalent (Chowdhary et al., 2012). Last but not the least, the hypothesis is conceptually wrong because it was based on the presumption that \textit{C. gattii} was a constituent of mycobiota, exclusively native to Australia and that the fungus had no autochthonous occurrence in other parts of the world.

### Fruits and Vegetables

Fruits and vegetables do not constitute a natural habitat or ecological niche for \textit{C. neoformans} and \textit{C. gattii} although these pathogens have been sporadically isolated from these substrates. The first report on the occurrence of \textit{C. neoformans} in fruits was by Staib et al., (1972; 1973) from Germany who isolated it from sliced, ripe peach fruits incubated in the laboratory. In another report (López-Martínez and Castañón-Olivares, 1995), \textit{C. neoformans} var. \textit{neoformans} was isolated from 9.4% of fruits and 4.2% of vegetables in
Mexico City. In India, *C. neoformans* has been reported from solitary samples of tomato (*Lycopersicon esculentum*), ‘vegetable sponge’ (*Luffa cylindrica*) and brinjal (*Solanum melongena*) in a survey of 437 samples of a wide variety of vegetables collected from a number of markets in Delhi. Serotyping revealed that 2 of the 3 isolates were serotype A whereas one was untypeable (Misra 1978; Misra and Randhawa 2000). In addition, another study from Delhi has reported isolation of *C. neoformans* from solitary samples of a few additional vegetables and fruits in an investigation covering 254 vegetables and 186 fruits samples (Pal and Mehrotra 1985). Most likely, the positive vegetables and fruits in the afore-mentioned reports carried the pathogen as an environmental contaminant.

**Water, other substrates**

The occurrence of *C. gattii* and *C. neoformans* in water remains as yet unexplored in India. Interestingly, a highly virulent genotype of *C. gattii*, VGII, has been frequently isolated from fresh water as well as marine water in British Colombia, Canada, which is an endemic area for this pathogen (Kidd et al., 2007b). The prevalence rate and population density of the new genotype varied from 16.6% (1.1 ± 2 CFU/100 ml) to 20.7% (5.1 ± 11 CFU/100 ml) in fresh water of lakes and rivers/ creeks whereas it was 21% (2.2 ± 2.0 CFU/100 ml) in seawater. Kidd et al., (2007b) further demonstrated that this *C. gattii* genotype survived best in filtered or unfiltered ocean water at room temperature and distilled water at room temperature.

**Aerial Survey**

Information on the aerial prevalence of *C. neoformans* and *C. gatti* in India is scarce. The first report is by Khan et al., (1978) who isolated *C. neoformans* by aerial exposure of Staub’s niger seed plates in a Charity Bird Hospital, Delhi, which had over 600 pigeons. It was observed that the isolation frequency of *C. neoformans* was much higher on petri-dishes exposed inside the pigeon cages (55%) than in those exposed outside the cages in the hospital room (30%). Notably, the fungus could no longer be isolated from the indoor air after a thorough cleaning and painting of the entire bird hospital. Randhawa and Paliwal (1979) reported negative results for *C. neoformans* in a 2-year, aeromycological study (1975-77) conducted outdoor in the lawns of a postgraduate hostel in the University of Delhi, North Campus. However, other species of *Cryptococcus*, such as, *C. ater*, *C. flavus*, *C. laurentii*, *C. magnus*, *C. terreus*, *C. uniguttulatus* and *C. albidos* were occasionally isolated. Recently, isolations of the *C. neoformans* species complex were frequently made by sampling of air inside tree trunk hollows of *S. cumini* trees in Delhi which were known to harbor *C. neoformans* and *C. gatti* (Randhawa et al., 2006). Attention may be called here to the aerial prevalence of *C. gatti* in Vancouver Island, Canada. The reported *C. gatti* concentrations (CFU/m³) in air samples were found to be significantly higher during the warm, dry summer months, although potentially infectious propagules (less than 3.3 µm in diameter) were present throughout the year (Kidd et al., 2007b).

**Seasonal Variations**

It is understood that abiotic factors such as pH, humidity, temperature, sunlight and wind play an important role in the environmental prevalence and dissemination of *C. neoformans* and *C. gatti*. Most of the work done so far is on the occurrence of *C. neoformans* var. *grubii* in avian excreta which support its growth and multiplication under dry conditions (Ruiz et al., 1981; Caicedo et al., 1999; Montenegro and Paula 2000; Kuroki et al., 2004; Grandos and Castañeda 2005). In vitro studies by Martinez et al., (2001) have shown differences in thermo-tolerance between *C. neoformans* var. *grubii* (serotypes A) and *C. neoformans* var. *neoformans* (serotype D). However, the problem of seasonal variations in environmental prevalence of *C. neoformans* and *C. gatti* remains virtually unexplored. Recently, Randhawa et al., (2011) have reported a retrospective study of seasonal variations in the prevalence of these pathogens in decayed wood inside trunk hollows of a wide spectrum of tree species investigated from five geographical locations in north-western India over a period of 7 years (2000-2007). Climatically, north-western India has five distinct seasons, namely winter, spring, summer, rainy season and autumn. The data analyzed included results of isolation of *C. neoformans* and *C. gatti* from 1,439 decayed wood samples collected from trunk hollows of 518 trees, representing 20 species. Of the 406 isolates of *C. neoformans* species complex, 247 were *C. neoformans* var. *grubii* (serotype A) and 171 were *C. gatti*, serotype B. Both pathogens were isolated during all the seasons, and the overall prevalence of *C. neoformans* var. *grubii* was significantly higher (17.2%) than that of *C. gatti* serotype B (11.9%, P < 0.0001). It indicated that decayed wood was as good, if not a better natural habitat for *C. neoformans* var. *grubii* as for *C. gatti*. Both of the pathogens revealed some seasonal variations in their prevalence, the highest being during the autumn, followed by that in the summer. For *C. gatti*, the prevalence during the winter was significantly less than that during the summer (P <0.02) and the autumn (P <0.02). In contrast, the lowest prevalence of *C. neoformans* var. *grubii* (10.7%) was in the rainy season which was significantly less than that in the autumn (P <0.0001), followed by that in the summer (P <0.0001) and winter (P <0.001). Interestingly, a similar pattern of low prevalence of *C. neoformans* var *grubii* in chicken faeces during rainy season and high prevalence during the dry season has been reported from Thailand (Kuroki et al., 2004). On the other hand, the low prevalence of *C. gatti* in decayed wood during winter was similar to that reported from Bogotá, Colombia, where *C. gatti* had a low population density in bark samples but it was not found in decayed wood of trunk hollows investigated during January and February (Granados and Castañeda 2005). Comprehensive prospective studies are warranted in order to gain an insightful knowledge of any seasonal pattern of prevalence of *C. neoformans* and *C. gatti* not only in decayed wood but also in other natural substrates such as avian excreta and soil.

**Population structure**

Population structure denotes genetic diversity among individuals constituting a population, their operative modes of reproduction, genetic exchange and formation of subgroups which may be determined by geographical, temporal and other ecological factors. Much of the available information on the environmental population structure of *C. neoformans* and *C. gatti* in India has resulted from a collaborative study between the Mycology group of VPCI and Dr. Jianping Xu, Department of Biology, McMaster University, Hamilton, Canada. The main findings of this study are summarized below:

*C. neoformans*: The first paper reported on the structure of environmental populations of *C. neoformans* var. *grubii*, comprising 78 isolates originating from decayed wood in trunk hollows of 9 tree species in 5 geographical locations, i.e. Union Territory of Delhi, Bulandshahr and Hathras (Uttar Pradesh), Amritsar (Punjab) and Amroli (Haryana) in
north-western India (Hiremath et al., 2008). The isolates were subjected to MLST, using five gene fragments. All of the isolates were found to be molecular type VNI and mating type α (MATα). Population-genetic analyses provided no evidence for significant differentiation among populations belonging to either different geographical areas or different host tree species. Interestingly, despite the lack of mating type a (MATα) strains, unambiguous evidence for recombination was observed which supported the hypothesis that strains of *C. neoformans* may undergo sexual reproduction on decaying wood of various host tree species.

**C. gattii**: The second paper in the series dealt with genotyping of 109 isolates of *C. gattii*, serotype B, originating from the wood detritus of trees and the surrounding soil from nine different tree species at seven north-western locations, i.e., Amritsar, Union Territories of Chandigarh and Delhi, Amroli, Bulandshahr, Hathras and Meerut and one in Tamil Nadu, namely, Tiruvannamalai in south India (Chowdhary et al., 2011a). MLST, using nine gene fragments, revealed that all of the *C. gattii* isolates belonged to the mating type, MATα which conforms to the global mating type pattern of *C. neoformans* and *C. gattii*. Molecular phylogenetic analyses identified that all of the 109 strains analyzed belonged to the AFLP4/ VGI lineage which has a wider geographic distribution than lineages AFLP6/VGI, AFLP5/ VGIII and AFLP7/VGIV (Xu 2010; Litvintseva et al., 2011). However, the wide distribution of *C. gattii* in India has not obscured the genetic differentiation among populations from either different geographic areas or different host tree species in India. Population genetic analyses revealed limited evidence of recombination but unambiguous evidence for clonal reproduction and expansion.

Over-all, the serotype distribution of clinical isolates so far reported from India corresponds to the environmental distribution pattern with predominance of serotype A strains, and a low prevalence of serotypes B, D and AD (Banerjee et al., 2004; Padhye et al., 1993). Recently, we reported the serotypes, genotypes and mating types of 308 isolates of *C. neoformans* species complex, originating from clinical and environmental sources. Their genotypes were determined based on two methods: (i) PCR fingerprinting using ([GACA]4 and M13 phage core sequences as single primers and (ii) DNA sequences at the *URA5* locus. All of the 109 strains analyzed belonged to the AFLP4/VGI type, genotype, and mating type α. Of the 246 clinical isolates examined, 233 were MATα and 13 were MATa. The 246 isolates belonged to the mating type, genotype, and serotype *C. gattii* var. grubii isolates, 160 were clinical, originating from 130 patients, and the remaining 86 were from decayed wood of trees and soil. Among the 62 *C. gattii* isolates, 60 were from environmental and two were from clinical sources. The clinical isolates had been collected during 2002 to 2009 from various hospitals in the Union Territories of Delhi and Chandigarh, and the states of Uttar Pradesh and Himachal Pradesh (Chowdhary et al., 2011b). In an earlier study, Jain et al., (2005) determined the genotypes in 57 clinical isolates from India. Of these, 51 (89.4%) belonged to group VNI (*C. neoformans* var. *grubii*, serotype A), one belonged to group VNI (C. neoformans var. *neoformans*, serotype D) and 5 isolates belonged to group VGI (*C. gattii*). Consistent with the global pattern, 90% of the Indian serotype A and B isolates exhibited a MATα mating type. Forty-eight of the 51 *C. neoformans* var. *grubii*, were MATα and 3 were MATa. The solitary isolate of *C. neoformans* var. *neoformans* was MATα, whereas 4 of the 5 *C. gattii* isolates were MATα and one MAT α.

### Table 1. Prevalence of *Cryptococcus neoformans* and *C. gattii* in north-western India

<table>
<thead>
<tr>
<th>Host Tree (Family)</th>
<th>No. Samples (n=197)</th>
<th>Prevalence (%)</th>
<th>Serotyping, mating type, genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucalyptus camaldulensis (Myrtaceae)</td>
<td>354</td>
<td>1.4</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>E. tereticornis (Myrtaceae)</td>
<td>390</td>
<td>0.2</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Butea monosperma (Caesalpinioideae)</td>
<td>40</td>
<td>0.4</td>
<td>B, MATα, VNI</td>
</tr>
<tr>
<td>Tamarindus indica (Papilionaceae)</td>
<td>233</td>
<td>0.4</td>
<td>B, MATα, VNI</td>
</tr>
<tr>
<td>Syzygium cumini (Myrtaceae)</td>
<td>66</td>
<td>10.6</td>
<td>B, MATα, VNI</td>
</tr>
<tr>
<td>E. globulus (Myrtaceae)</td>
<td>19</td>
<td>5.2</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Polyalthia longifolia (Annonaceae)</td>
<td>5</td>
<td>0.1</td>
<td>B, MATα, VNI</td>
</tr>
<tr>
<td>E. marginata (Mimosoideae)</td>
<td>5</td>
<td>10.6</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Aegle marmelos (Rutaceae)</td>
<td>1</td>
<td>0</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Dalbergia sissoo (Faboideae)</td>
<td>13</td>
<td>10.6</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Manilkara hexandra (Sapotaceae)</td>
<td>13</td>
<td>30.7</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Aegle marmelos (Rutaceae)</td>
<td>5</td>
<td>10.6</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Ficus religiosa (Caesalpinioideae)</td>
<td>5</td>
<td>0</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Mimusops elengi (Sapotaceae)</td>
<td>66</td>
<td>30.7</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Aegle marmelos (Rutaceae)</td>
<td>3</td>
<td>0</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Alstonia scholaris (Apocynaceae)</td>
<td>13</td>
<td>30.7</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Manilkara hexandra (Sapotaceae)</td>
<td>13</td>
<td>30.7</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Aegle marmelos (Rutaceae)</td>
<td>5</td>
<td>0</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Dalbergia sissoo (Faboideae)</td>
<td>13</td>
<td>10.6</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Manilkara hexandra (Sapotaceae)</td>
<td>13</td>
<td>30.7</td>
<td>A, MATα, VNI</td>
</tr>
<tr>
<td>Aegle marmelos (Rutaceae)</td>
<td>5</td>
<td>0</td>
<td>A, MATα, VNI</td>
</tr>
</tbody>
</table>

*Attempts at re-isolation were negative, 33% of those were from samples, 16% of those were from samples, and 16% of those were from samples.*
Antifungal Susceptibility Profiles

Resistance to antifungal agents in environmental and clinical strains of *C. neoformans* and *C. gattii* has been a rare global occurrence. Soares et al., (2005) reported a solitary isolate of *C. neoformans* var. *grubii* from pigeon excreta that was resistant to fluconazole, (MIC 64 mg/L). Similar findings were reported from Cuba and those authors concluded that environmental isolates seemed to be less susceptible to fluconazole than clinical ones (Illnait-Zaragozi et al., 2008). Likewise, in another report from Brazil, one of the environmental isolates of *C. neoformans* var. *neoformans* was found to be resistant to itraconazole whereas three additional isolates exhibited high MICs of 16–32 mg/L against fluconazole (Costa et al., 2010). The first antifungal susceptibility testing report on environmental isolates from India was based on 117 isolates of *C. neoformans*, serotype A, and 65 of *C. gattii*, serotype B, originating from decayed wood in trunk hollows of *F. religiosa* and *S. cuminii* trees, employing the Etest method (Khan et al., 2007). A comparison of the geometric mean MICs revealed that *C. gattii* was less susceptible than *C. neoformans* to amphotericin B (0.075 versus 0.051, P=0.0003), fluconazole (2.912 versus 2.316, P=0.0003) itraconazole (0.198 versus 0.034, P<0.0001), ketoconazole (0.072 versus 0.037, P<0.0001), and voriconazole (0.045 versus 0.023, P<0.0001). No primary resistance was observed against amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole which is in consonance with worldwide literature reports that resistance in *Cryptococcus* species complex is rarely observed (Pfaller et al., 2005). In an extension of this work, the same investigators reported the antifungal susceptibility profiles in clinical and environmental isolates of *C. neoformans* var. *grubii*, genotype AFLP1/VNI MATα (n=246), and *C. gattii*, serotype B, genotype AFLP4/VGI, MATα (n=62), using the broth microdilution method (Chowdhary et al., 2011b). Both the species had low MICs to the antifungals tested except for two clinical *C. neoformans* var. *grubii* isolates that were resistant to 5-flucytosine (MIC 64 mg/L). Data on the geometric mean of MICs revealed that *C. gattii* was significantly less susceptible than *C. neoformans* var. *grubii* to fluconazole, itraconazole and voriconazole (P<0.0001). In addition, the MICs of *C. gattii* were twofold higher than that of *C. neoformans* var. *grubii* for fluconazole, itraconazole and voriconazole. However, no statistically significant difference was observed in susceptibility of the two *Cryptococcus* species to amphotericin B and 5-flucytosine. Furthermore, the environmental *C. neoformans* var. *grubii* isolates were significantly less susceptible to fluconazole, itraconazole and 5-flucytosine (P<0.0001) than the clinical isolates. Similar results have been reported previously from Cuba (Illnait-Zaragozi et al., 2008). Hagen et al., (2010) reported that *C. gattii*, showed lower MICs for AFLP4/VGI isolates (1.401 and 2.467 mg/L) versus the higher MICs for AFLP6/VGII isolates (4.961 and 5.638 mg/L) against 5-flucytosine and fluconazole, respectively. Iqbal et al., (2010) tested 43 clinical isolates of *C. gattii* from patients in Oregon, USA. Interestingly, their AFLP4/VGI and AFLP5/VGIII isolates had comparatively low fluconazole MICs, whilst the majority with high MICs of 16–32 mg/L were of subtype AFLP6/VGIIc. In contrast Thompson et al., (2009) reported no differences in the antifungal susceptibilities of the two species.

An earlier study from India had reported only the fluconazole susceptibility profiles and genotypes of 57 clinical isolates, comprising 51 *C. neoformans* var. *grubii*, genotype AFLP1/VNI, one *C. neoformans* var. *neoformans*, genotype AFLP2/VNIV, and five *C. gattii* strains, genotype AFLP6/VGII (Jain et al., 2005). The reported MICs ranged from 8 to 16 mg/L for *C. neoformans* var. *grubii* and 2 to 64 mg/L for *C. gattii*.

Future Perspective

The geoclimatically divergent regions of India with their rich variety of flora and fauna offer a wide scope for further investigations on the environmental prevalence of *C. gattii* and *C. neoformans* and their population structure. Currently, the occurrence of *C. gattii* serotype C in the environment or in clinical material in India is entirely unknown. Likewise, there are scarcely any data concerning the environmental distribution of *C. neoformans* var. *neoformans* (serotype D) which is largely known from Europe. Further studies are required to compare the genetic structure of clinical and environmental strains of both pathogens with a view to probing the extent of their inter-relationship. Such a study may shed a new light on the origin of subgroups of various genotypes. There is little information at present on the prevalence of cryptococcosis in animals or humans in the areas of north-western India where host trees are perennially colonized by *C. gattii* and *C. neoformans*. Comprehensive clinico-mycological investigations are warranted to probe the magnitude of health hazard posed by the environmental prevalence of *C. gattii* and *C. neoformans*.

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Declaration of interest

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Environmental prevalence of *C. neoformans* and *C. gattii* in India

Chapter 9

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**Hamasha AM, Yildiran ST, Gonum A, Saraci MA, Doganci L. (2004).** *Cryptococcus neoformans* varieties from material under the canopies of *Eucalyptus* trees and pigeon dropping samples from four major cities in Jordan. Mycopathologia, 158, 195-9.


Distribution of Cryptococcus gattii and Cryptococcus neoformans in decayed trunk wood of Syzygium cumini trees in north-western India

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Zhun Yan
Jianping Xu
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Chapter 10

**Summary**

The aim of this study is to report the regional distribution of *Cryptococcus gattii* and *Cryptococcus neoformans* in decayed wood inside trunk hollows of *Syzygium cumini* trees (Java plum, Indian black berry) investigated in Amritsar (Punjab), Meerut Cantonment and Bulandshahr (Uttar Pradesh) and Delhi, in north-western India. Two hundred and seventeen wood samples collected from 74 *S. cumini* trees were investigated. This includes 7 known positive *S. cumini* trees in Delhi subjected to a mycological surveillance for perennial colonization by *C. gattii* and *C. neoformans*. *Cryptococcus gattii* showed the highest prevalence (89%) in *S. cumini* trees in Delhi, followed by 27%, 12.5% and 9% prevalence in Bulandshahr, Amritsar City and Meerut Cantonment respectively. In contrast, *C. neoformans* had the highest prevalence (54%) in Amritsar, followed by 44% in Delhi, 9% in Bulandshahr and 0% in Meerut Cantt. Furthermore, 44% of the *S. cumini* trees in Delhi, 9% in Bulandshahr and 8% in Amritsar were concomitantly colonized by both *C. gattii* and *C. neoformans*. A mycological surveillance over 4.8–5.2 years of 7 selected *S. cumini* trees in Delhi revealed perennial colonization by both the *Cryptococcus* species. In addition, air samples taken close to the decayed trunk hollows of 4 of the perennially colonized *S. cumini* trees contained strains of the *C. neoformans* species complex. Of a random sample of 48 isolates serotyped, 26 (54%) were *C. neoformans*, serotype A, and 22 (46%) *C. gattii*, serotype B. Determination of mating type alleles was done in 44 of the isolates, comprising 31 of *C. neoformans*, serotype A and 13 of *C. gattii*, serotype B. All of them proved to be mating type α (MATα). The data on high prevalence, fungal population density, perennial colonization and aerial isolations indicate that decayed wood in trunk hollows of *S. cumini* trees is to-date the main well documented primary environmental niche of *C. gattii* and *C. neoformans* in north-western India. Attention is drawn to the likely health hazard posed by the environmental reservoirs of *C. gattii* and *C. neoformans* occurring in tree trunk hollows in proximity to human and animal habitations.

**Introduction**

Cryptococcosis is a major systemic mycosis of worldwide distribution with predilection for the central nervous system. It is potentially fatal unless diagnosed and treated at an early stage with appropriate antifungal therapy (Sarosi & Davies, 2000; Casadevall & Perfect, 1998). According to current taxonomic nomenclature of the etiologic agent (Kwon-Chung et al., 2002), cryptococcosis is caused by two distinctive species: the predominantly opportunistic pathogen, *Cryptococcus neoformans* (serotypes A, D and AD) and the primary pathogen, *Cryptococcus gattii* (serotypes B and C). Furthermore, based on analysis of URA 5 sequences and DNA fingerprinting patterns two varieties have been proposed for *C. neoformans*, i.e. *C. neoformans* var. *grubii* comprising serotype A isolates and *C. neoformans* var. *neoformans* that is restricted to serotype D isolates (Franzot et al., 1999). The two species differ in their geographic distribution: *C. neoformans* occurs globally whereas *C. gattii* is restricted to countries with tropical and subtropical climate barring its rare genotype VGI/AFLP6 which seems to be emerging in temperate climates as apparent from a recent outbreak of cryptococcosis in Vancouver Island in Canada (Kidd et al., 2004).

Both of the species occur saprobically in nature and the infection is acquired by inhalation of their air-borne yeast cells or basidiospores. Old excreta of pigeons or other avian species is known to be the commonest environmental reservoir of *C. neoformans*, although the species has been reported from many environmental sources such as soil, bat guano, raw vegetables, decayed wood in tree trunk hollows, etc. (Emmons, 1951; 1955; Ajello, 1958; Randhawa et al., 1965; Sethi et al., 1966; Khan et al., 1978; Misra et al., 2000; Lazera et al., 2000). The natural habitat of *C. gattii* was unknown until Ellis and Pfeiffer from Australia in the early 1990s reported its association with debris of *Eucalyptus* trees (Ellis & Pfeiffer, 1990; Pfeiffer & Ellis, 1992 ). Subsequently it was shown that decayed wood in trunk hollows of several tree species in Brazil served as a natural habitat of *Eucalyptus* (Ellis & Pfeiffer, 1990; Pfeiffer & Ellis, 1992 ). Our laboratory reported recently that the most important host tree for *C. gattii* in the Delhi/New Delhi area was Syzygium cumini (Indian black berry, Java plum, black plum), harbouring the fungus in decayed wood in its trunk hollows (Randhawa et al., 2000; 2003; 2005) and not *Eucalyptus camaldulensis* or *Eucalyptus tereticornis* as in Australia. In this communication, we report the results of a wider environmental survey of *C. gattii* and *C. neoformans* in *S. cumini* trees extending to Amritsar City (Punjab), Meerut Cantt. and Bulandshahr (Uttar Pradesh), situated about 450 km in north-west, 70 km and 60 km east of Delhi, respectively. The study also includes the results of attempted aerial isolations of the two pathogens from inside trunk hollows of several trees of *S. cumini* in Delhi known to harbour both or either of the species and demonstrates their perennial colonization in this ecologic niche during the 4.8 to 5.2 years of sampling.
Material and Methods

Collection sites and wood sampling

One hundred and eighty-five decayed wood samples collected from trunk hollows in 74 Syzygium cumini living trees were investigated. The distribution of investigated trees was as follows: 24 in Amritsar City, Punjab, 23 in Meerut Cantt., 11 in Bulandshahr, Uttar Pradesh, and 16 in Delhi. The sampling included reinvestigation of 7 S. cumini trees from Delhi, designated as TT-28, TT-41, TT-44, TT-47, TT-110, TT-115 and TT-151 that had been shown to harbour C. neoformans or C. gattii in trunk hollows during our previous study in 2000–2002 (Randhawa et al., 2003). These trees have been under surveillance for perennial colonization by C. neoformans and/or C. gattii. The S. cumini trees selected for sampling had generally large canopies and appeared to be very old as apparent from the large girth of their extensively decayed trunks.

Sampling of decayed wood was done with an in-house swabbing technique using simplified niger seed agar (NSA) as earlier described by Randhawa et al. (2005). The plates of NSA inoculated with the swabs were incubated at 28°C and observed up to 7 days for isolation of C. gattii and C. neoformans. To determine a rough estimate of viable population density of the C. neoformans species complex in a given test sample, one swab from each trunk hollow was immersed in 10 ml of sterile physiological saline and vortexed for 2 min on a cyclomixer (Remi, Mumbai, India). The swab was taken out and discarded. The sample suspension was allowed to sediment for 30 min, followed by inoculation of duplicate plates of NSA with 100µl aliquots of the supernatant. After 3–4 days of incubation at 28°C, the number of chocolate brown yeast-like colonies compatible with the C. neoformans species complex appearing on NSA plates was counted visually. The number computed was multiplied by 10^6 in order to determine the colony count per swab.

Aerial sampling

For aerial isolations of the two Cryptococcus spp. from trunk hollows, 4 S. cumini trees (TT-28, TT-110, TT-115 and TT-248) were selected. These trees had been shown to be repeatedly positive for C. gattii/C. neoformans in their decayed wood. Ten petri plates containing NSA were exposed one by one for 30 seconds in each tree trunk hollow. After removing its lid, each medium plate was held facing the decayed trunk while aerosols were created by repeated manual pressing of an egg-shaped rubber bulb, measuring 90 mm long and midpoint circumference of 150 mm, that was attached to a 160 mm long rubber tubing (inside diameter 5 mm) ending in a plastic nozzle having an opening with 2 mm diameter. At its proximal end, the rubber bulb was fitted with a metallic valve that controlled one-way flow of air through the nozzle when it was pressed. The exposed media plates were inoculated and observed as in the isolation of fungi by swabbing from decayed wood.

Identification and serotyping

Initial screening of C. gattii and C. neoformans on the inoculated plates was done by microscopic examination of variably brown yeast-like colonies developing on NSA. The suspected colonies were purified by dilution plating and identified by their morphological study and verification of salient physiological characteristics, employing the VITEK 2 System (bio-Mérieux, Marcy-l’Etoile, France) (Randhawa et al., 2000; 2003; Kurtzman & Fell, 1998). Serotyping of the isolates was done by the slide agglutination test based on monoclonal antibodies specific for the variable capsular polysaccharide, employing the commercially available kit, Crypto-Check (Iatron Laboratories, Tokyo, Japan). The number of isolates serotyped was 48.

Mating type determination

The mating types of strains were determined as described previously (Yan et al., 2002). Briefly, genomic DNA was isolated from each strain by the method described by Xu et al. (2000). A total of five pairs of primers were used in PCR reactions to screen the mating types. One was the MATα specific STE12α primer pair. PCR using this primer pair was found to be able to amplify a product from all MATα strains, but none from MATα strains, regardless of their serotype (A, B, C, D, or AD) (Yan et al., 2002). The other four pairs of primers targeted the STE20 gene and they were serotype (A, D) and mating type (α and α) specific. While these four primers have been shown to be effective for identifying the mating types of serotypes A and D strains, they were found to be less effective in serotypes B and C strains. Details of PCR reaction, gel electrophoresis and scoring followed those of Yan et al. (2002). The combined results from these PCR reactions were used to interpret the mating type of individual strains. The number of isolates investigated for mating type was 44; 26 of these originated from S. cumini and 18 from Ficus religiosa decayed wood in Delhi. Thirteen of the S. cumini isolates were C. gattii, serotype B whereas all of the remaining 31 were C. neoformans, serotype A.

Results

Strain isolation

The trunk hollows of S. cumini trees that harboured C. gattii and C. neoformans in their decayed wood varied widely in their dimensions depending upon the extent of decay. Some of the trees were extensively damaged with their hollows vertically extending several meters up their trunks. In primary cultures of wood samples on NSA, smooth and variably mucoid, yeast-like colonies of C. neoformans species complex were distinctly seen after 2–4 days of incubation, exhibiting variable shades of a chocolate brown pigment that darkened with age (Figure 1). At this stage, we occasionally observed chocolate brown, glistening colonies of black yeast associated with decayed wood that resembled the macroscopic features of C. gattii/C. neoformans (Figure 2) with which it could be confused but it was readily differentiated by its microscopic morphology.

Serotyping and mating type determination

Of the 48 isolates of the C. neoformans species complex randomly serotyped, 26 (54%) were found to be C. neoformans serotype A, and 22 (46%) C. gattii serotype B. PCR using the mating type α-specific STE12α primers amplified an expected fragment for all of the 44 strains investigated for their mating type. For serotype A strains, PCR using the STE20α primers produced expected products. None of the other three STE20α primer pairs produced
any PCR product for any of the strains. In addition, negative controls of mating type strains in our collection produced no amplification product (Data not shown). Therefore, our results indicate that all analyzed strains were mating type α (MATα).

Regional distribution

The data on prevalence of \textit{C. neoformans} and \textit{C. gattii} in decayed wood inside trunk hollows of \textit{S. cumini} trees in Amritsar, Meerut Cantt., Bulandshahr and Delhi are presented in Table 1 and depicted in Figure 3. The highest prevalence of \textit{C. gattii}, serotype B (89%), was found in Delhi where 8 out of the 9 \textit{S. cumini} trees investigated were positive. Concomitantly, 44% of these trees yielded \textit{C. neoformans}, serotype A. In strong contrast, \textit{C. gattii} had a prevalence of only 12.5% in Amritsar, 9% in Meerut Cantt. and 27% in Bulandshahr. The predominant species found in \textit{S. cumini} trees in Amritsar was \textit{C. neoformans}, serotype A, showing a prevalence of 54% as against 44% in Delhi where it occurred concomitantly with \textit{C. gattii}, serotype B. Samples from Meerut Cantt. did not yield any isolation of \textit{C. neoformans}. Overall, concomitant occurrence of \textit{C. gattii} and \textit{C. neoformans} in the same trunk hollows was seen in 7 of the 67 (10%) \textit{S. cumini} trees investigated. The viable population density of the \textit{C. neoformans} species complex varied widely in decayed wood of \textit{S. cumini} trees not only from place to place or tree to tree in the same locality but also from site to site in the same trunk hollow. For example, the count was found to be as low as 1 CFU/swab inoculated with material from trunk hollows in \textit{S. cumini} trees, TT-110, whereas it ranged from 3.25×10^3 CFU / swab in Delhi to 2.1×10^7 CFU/swab in Bulandshahr. In a long-term surveillance study on some selected \textit{S. cumini} trees from Delhi (Table 2), decayed wood was found to be colonized by both \textit{C. gattii} and \textit{C. neoformans} during our 4.8–5.2 years of investigation.

Aerial isolations

Air-sampling of trunk hollows of \textit{S. cumini} trees, TT-28, TT-110, TT-115 and TT-248 investigated from Delhi yielded isolates of the \textit{C. neoformans} species complex on both of the dates when tested, i.e. 31 October and 11 November, 2005. Of the 40 NSA plates exposed altogether, 21 (52.5%) were found to contain isolates of the \textit{C. neoformans} species complex. The frequency of aerial isolation and the number of colonies recovered per plate varied widely from tree to tree. Thus, TT-28 yielded a 90% isolation frequency, followed by

<table>
<thead>
<tr>
<th>Place / locality</th>
<th>Collection period</th>
<th>No. of trees investigated</th>
<th>No. trees positive for \textit{C. gattii}</th>
<th>No. trees positive for \textit{C. neoformans}</th>
<th>No. trees positive for both species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amritsar, Panjab.</td>
<td>Nov., 2004 to Jan., 2005</td>
<td>40</td>
<td>4/9 (44.4%)</td>
<td>0/9</td>
<td>4/9 (44.4%)</td>
</tr>
<tr>
<td>Meerut Cantt., U.P.</td>
<td>May-July, 2004</td>
<td>40</td>
<td>2/23 (9%)</td>
<td>0/23</td>
<td>0/23</td>
</tr>
<tr>
<td>Bulandshahr, U.P.</td>
<td>Mar., 2006</td>
<td>22</td>
<td>5/22 (23%)</td>
<td>1/22 (4.5%)</td>
<td>6/22 (27.3%)</td>
</tr>
<tr>
<td>Delhi</td>
<td>May-July, 2004</td>
<td>74</td>
<td>13/16 (81.2%)</td>
<td>2/16 (12.5%)</td>
<td>15/16 (93.8%)</td>
</tr>
<tr>
<td>Total:</td>
<td></td>
<td></td>
<td>41/142 (29%)</td>
<td>11/142 (7.7%)</td>
<td>52/142 (36.5%)</td>
</tr>
</tbody>
</table>

70% in TT–110, 30% in TT–248 and 20% in TT–115. Likewise, the highest mean colony count ranged from 1.5–4.2 in TT–28, followed by 1.2–3.1 in TT–110 and 0.1–0.5 in TT–115 and TT–248. It appeared from these results that the isolation frequency of \textit{C. neoformans} species complex and the number of colonies recovered per plate was higher from trees with deep-seated hollows than from trees with open-faced decay of their trunk. The highest colony count recorded was 10 on a NSA plate exposed to the trunk hollow of \textit{S. cumini} tree, TT-110.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{cryptococcus_gattii.png}
\caption{Innumerable tiny, chocolate brown, yeast-like colonies of \textit{C. gattii} on a simplified Staub’s niger seed agar (NSA) plate, isolated from decayed wood inside trunk hollow of a \textit{S. cumini} tree in Amritsar, seen after 6 days of incubation at 28°C. Interspersed among them are also many cottony white, fluffy mold colonies.}
\end{figure}
Discussion

The results presented above reinforce and extend our earlier observations (Randhawa et al., 2003) that decayed wood in trunk hollows of S. cumini living trees is an important environmental niche of C. gattii, serotype B, and C. neoformans, serotype A, in Delhi and possibly elsewhere in India wherever this tree species may be distributed. It is apparent from the data that the association of C. gattii and C. neoformans with S. cumini as a host tree is not restricted to Delhi but has a wider distribution in northwestern India. It is interesting to note that the prevalence of C. gattii vis-à-vis that of C. neoformans in tree trunk hollows of S. cumini varies widely from place to place. The divergent prevalence of C. gattii or C. neoformans in various places as noted above underscores the complexity of a number of abiotic and biotic factors governing the distribution of the two species in a given environmental niche. Outside of India, C. neoformans had been previously reported from rotten wood and other plant debris inside the tree trunk hollow of a S. cumini (S. jambolana) tree in Rio de Janeiro, Brazil (Lazera et al., 1993). However, it was interpreted as a transient contamination because the investigators failed to repeat the observation with wood material from the same tree or other S. cumini trees in the area.

It seems pertinent to point out that contrary to the literature reports from Australia (Ellis & Pfeiffer, 1990; Pfeiffer & Ellis, 1992) C. gattii has been only sporadically reported from plant debris of Eucalyptus species in India. The first report was by Chakrabarti et al. (1997) who reported C. gattii, serotype B, from flowers of 3 Eucalyptus camaldulensis trees in Ferozpur area, Punjab, whereas the results were negative with 623 additional samples of plant debris of E. camaldulensis, E. tereticornis and E. citriodora. In a subsequent study covering 390 bark samples collected in north-western India from miscellaneous Eucalyptus species, including E. camaldulensis and E. tereticornis, Randhawa et al. (2000) reported a solitary isolation of C. neoformans from an unidentified Eucalyptus tree in Amritsar. As no information was provided in either of the afore-mentioned studies on the number of C. gattii or C. neoformans colonies isolated from the positive samples nor was any attempt made to repeat the isolations from positive sites, the epidemiologic significance of these observations remained unclear. More recently, Gugnani et al. (2005) have reported two isolates each of C. gattii, serotype B and C. neoformans var. grubii from flowers and bark collected under canopies of E. tereticornis and E. camaldulensis trees in Delhi and Chandigarh, respectively. The significance of Eucalyptus trees in the epidemiology of cryptococcosis in India will, however, continue to be elusive until data of a long-term surveillance study of the positive trees including information on population density of the two fungi in the test samples is available. Going by our data on the high prevalence, population density, aerial isolation and perennial colonization (already demonstrated for over 5 years) by C. gattii and C. neoformans in decayed wood of tree trunk hollows of S. cumini, we believe that this tree species is to date the main primary ecological niche for C. gattii and C. neoformans in north-western India. Further investigations are under way to probe the possible role of a number of other tree species as additional hosts to C. gattii and C. neoformans.

It is notable that isolates of the C. neoformans species complex were found in air samples taken close to the decayed trunks of all of the four perennially colonized S. cumini trees investigated. This observation indicated that dispersal of C. gattii and C. neoformans from one colonized tree could possibly occur to other trees by their aerosols which may be generated artificially or by natural disturbances of decayed wood surfaces due to a variety of agents such as wind, insects, birds, etc. Previously, C. gattii had been demonstrated by air sampling conducted under the canopy of Eucalyptus camaldulensis host trees in Australia, and in air samples collected near the hollows of two pink shower (Cassia grandis) trees in Brazil (Lazera et al., 2000; Ellis & Pfeiffer, 1990). On the other hand, attempts at aerial isolation of the fungus from the vicinity of host trees were unsuccessful in two instances, namely, one from India and one from Australia (Chakrabarti et al., 1997; Halliday & Carter, 2003). It is generally believed that cryptococcosis in humans and animals is acquired by inhalation of such aerosols from the environment. It is pertinent to mention in this context that a large scale outbreak of cryptococcosis due to C. gattii, serotype B, involving at least 59 laboratory-confirmed cases in humans and 45 in terrestrial or marine mammals occurred during 2000–2003 on southern Vancouver Island, Canada. The outbreak was related to colonization on Vancouver Island by C. gattii of a large number of local trees such as Douglas fir (the majority), cedar, arbutus, alder, maple, spruce, Garry oak, etc, thus highlighting the risk of cryptococcosis developing in humans and animals following their exposure to such environmental reservoirs of the pathogen (Stephen et al., 2002; Laster et al., 2004).

Until recently, information was lacking on the etiologic role of C. gattii in cryptococcosis in India. Attention to the occurrence of C. gattii in this country was first drawn in 1993 by Padhye et al. (1993) who identified 3 clinical isolates of the species among a collection of 18 strains maintained as C. neoformans in the Mycology Section of Postgraduate Institute of Medical Education and Research, Chandigarh. These 3 C. gattii isolates were subsequently confirmed as the etiologic agents of chronic meningitis in Indian patients. Since then, the fungus has been reported from 6 other cases of meningitis which included one each from Vellore (Tamil Nadu), Bijapur (Karnataka) and Pondicherry in South India, and 3 from New Delhi (Abraham et al., 1997; Peerapur et al., 2000; Khyriem et al., 2004; Banerjee et al., 2004). Keeping in view our observations on the frequent and widespread association of C. gattii with decayed wood of S. cumini trees that are cultivated throughout India, it seems enigmatic that C. gattii has been rarely reported so far in the etiology of cryptococcosis in this country. This may be largely attributable to inadequate awareness in general about the importance of accurate speciation of the isolates of Cryptococcus encountered in diagnostic microbiology laboratories.
Chapter 10

Cryptococcus gattii and C. neoformans in decayed S. cumini trees

References


Chapter 11

The expanding host tree species spectrum of *Cryptococcus gattii* and *Cryptococcus neoformans* and their isolations from surrounding soil in India

H.S. Randhawa
T. Kowshik
Anuradha Chowdhary
K. Preeti Sinha
Z. U. Khan
Sheng Sun
Jianping Xu
Summary

This study reports a widespread prevalence of *C. neoformans* and *C. gattii* from decayed wood inside trunk hollows of 14 tree species representing 12 families and from soil near the base of various host trees from Delhi and several places in the States of U.P., Haryana, Tamil Nadu and Chandigarh Union Territory, all in India. Of the 311 trees belonging to 20 species of 14 families investigated, 64 (20.5 %) were found to contain strains of the *C. neoformans* species complex. The number of trees positive for *C. neoformans var grubii* (serotype A) was 51 (16.3 %), for *C. gattii* (serotype B) 24 (7.7%) and for both *C. neoformans* and *C. gattii* 11 (3.5 %). The over-all prevalence of the *C. neoformans* species complex in decayed wood samples was 19.9% (111 / 556). There was no obvious correlation between the prevalence of these two yeast species and the host tree species. The data on prevalence of *C. gattii* (24%) and *C. neoformans* (26%) in soil around the base of some host trees indicated that soil is another important ecological niche for the two *Cryptococcus* species in India. Among our sampled tree species, eight and six were recorded for the first time as host trees for *C. neoformans var. grubii* and *C. gattii*, respectively. A longitudinal surveillance of 8 host tree species over 0.7 to 2.5 years indicated long term colonization of *Polyalthia longifolia*, *Mimusops elengi* and *Manilkara hexandra* trees by *C. gattii* and *C. neoformans*. The mating type was determined for 153 of the isolates that included 98 strains of serotype A and 55 of serotype B. All of them proved to be mating type α (MAT α). Our observations documented the rapidly expanding spectrum of host tree species for *C. gattii* and *C. neoformans* and indicate that decayed woods of many tree species are potentially suitable ecological niches for both of the pathogens.

Introduction

According to the current taxonomy, cryptococcosis is caused by two distinctive species i.e. *Cryptococcus neoformans* (serotypes A, D, and AD) and *C. gattii* (serotypes B and C) (Kwon-Chung et al., 2002). The former comprises *C. neoformans var. grubii* (serotype A), *C. neoformans var neoformans* (serotype D) and the recent hybrids between strains of serotypes A and D (serotype AD). The environmental habitat of *C. gattii* was unknown until investigators from Australia in the early 1990’s reported its association with debris of *Eucalyptus* trees (Ellis & Pfeiffer, 1990; Pfeiffer & Ellis, 1992). That work led to a number of worldwide investigations focusing on plant debris, especially decayed wood inside trunk hollows, to examine the natural habitat of *C. gattii* as well as *C. neoformans* (Lazera et al., 1993; 1996 ; 2000 ; Callejas et al., 1998; Randhawa et al., 2000; Restrepo et al., 2000; Krockenberger et al., 2002; Granados & Castaneda 2005; Ribeiro et al., 2006). In a retrospective analysis of their data (1992-2004), Granados and Castaneda have explored the relationship between occurrence of the serotypes of the *C. neoformans* species complex in tree samples and the climatic conditions in Colombia, employing a logistic regression model and logged Pearson correlations (Granados & Castaneda, 2006). Their results suggested that climatic conditions, mainly humidity, temperature, evaporation and solar radiation affected the environmental occurrence of the various serotypes. Contrary to the Australian experience, investigations done in India indicated that *C. gattii* was rarely associated with eucalyptus, and the most important host tree for *C. gattii* as well as *C. neoformans* in north-western India was *Syzygium cumini* (Chakrabarti et al., 1997; Abraham et al., 1997; Randhawa et al., 2000; 2003; 2005; 2006). In this communication, we present the results of an extended environmental survey. Our results support the conclusion that the primary ecological niche of *C. gattii* and *C. neoformans* is not any specific tree species but rather the decayed wood of a wide spectrum of tree species belonging to numerous families and genera.

Material and Methods

Collection sites and sampling

Five hundred and fifty-six decayed wood samples collected from inside trunk hollows of 311 trees belonging to 14 families were investigated during 2002-2007. The main criterion in selecting any tree for investigation was the presence of decayed wood in the form of trunk hollows. The selected trees were distributed in urban areas, occurring mostly in public parks, avenues and pavements on roadside. Except the various *Eucalyptus* species which are known to have been imported from Australia, all other species belonged to the indigenous or naturalized Indian flora, Figure 1 depicts a part of an outline map of India showing the locations of various places where samples were collected. The investigated trees belonged to *Polyalthia longifolia* (n = 55), *Mangifera indica* (n = 38) *Azadirachta indica* (n = 36), *Tamarindus indica* (n = 20), *Cassia fistula*, *Alstonia scholaris*, *Acacia nilotica* and *Eucalyptus* spp. (n=19 each ), *Dalbergia sissoo* and *Ficus religiosa* (n = 15 each), *Mimusops elengi* (n=13), *Callistemon equisetifolia*, *Syzygium cumini* and *Zizyphus mauritiana* (n = 7...
Host trees and occurrence in soil of C. gattii and C. neoformans

Chapter 11

Tokyo, Japan).

capsular polysaccharide, employing the commercial kit, Crypto-Check (Iatron Laboratories,
done by the slide agglutination test using monoclonal antibodies specific for the variable
their morphological study and verification of salient physiological characteristics, employing
colonies appearing on NSA plates was done by microscopic examination of melanin positive
(see Figure 2). The results show that C. gattii and C. neoformans are widely distributed in
decayed wood inside trunk hollows of a broad spectrum of tree species (Table 1). Of the 311
trees investigated, 64 (20.5%) yielded isolations of the C. neoformans species complex. In
decayed wood, the C. neoformans species complex was isolated from 111 of the 556 samples
investigated, giving a prevalence of 19.9%. The population density of the C. neoformans
species complex in decayed wood samples ranged from about 100 cfu/ swab in a
(serotype B) was 13 (4.1%) while concomitant occurrence of both of
, 40 of the 64 (62.5%) were positive for
C. gattii (serotype A).

Strain isolation and identification

The procedure for processing of wood samples for isolation and identification of C. gattii
and C. neoformans and for determining their mating types was the same as described in an
earlier paper (Randhawa et al., 2006). To isolate these two yeast species from soil, about 1
gram of each soil sample was suspended in 10 ml of sterile physiological saline fortified with
gentamicin (25 mg / l), vortexed for 2 min and allowed to stand for 30 min. Aliquots of 100
µl of the supernatant were inoculated on plates of simplified Staib’s niger seed agar (NSA),
icubated at 28°C and observed up to 7 days. To estimate the viable population density of
the C. neoformans species complex in a given test sample, one cotton-tipped swab from
each trunk hollow was immersed in 10 ml of sterile physiological saline and vortexed for 2
min on a cyclomixer (Remi, Mumbai, India). The swab was taken out and discarded and the
samples up to a depth of 15 cm have been reported to have a high concentration of C. gattii
(Kidd et al., 2007). soil collection was done from upper layers of the ground, approximate
depth not exceeding 10 cm, using a sterile steel spatula and polythene bags. Our negative
control samples were collected from an open site situated about 15 meters away from the
positive S. cumini host trees in New Police Lines near the University of Delhi, North Campus.

Data on the prevalence of C. neoformans species complex, C. gattii and C. neoformans
inside trunk hollows of 311 trees belonging to about 20 species of 14 families and from soil
collected close to the base of some of the host tree species are presented in Tables 1-3 and
depicted in Fig 2. Our results show that C. gattii and C. neoformans are widely distributed in
deayed wood inside trunk hollows of a broad spectrum of tree species (Table 1). Of the 311
trees investigated, 64 (20.5 %) yielded isolations of the C. neoformans species complex. In
decayed wood, the C. neoformans species complex was isolated from 111 of the 556 samples
investigated, giving a prevalence of 19.9%. The population density of the C. neoformans
species complex in decayed wood samples ranged from about 100 cfu/ swab in a T. indica
tree in Tamil Nadu to as high as 57,000 cfu / swab in an A. indica tree in Amrouli, Haryana.
The number of host trees exclusively harbouring C. neoformans (serotype A) was 40 (12.8
%), exclusively C. gattii (serotype B) was 13 (4.1%) while concomitant occurrence of both
of the species was observed in 11 (3.5%). In other words among the host trees positive for C.
neoformans and / C. gattii, 40 of the 64 (62.5%) were positive for C. neoformans (serotype A).

### Table 1. Prevalence of C. gattii and C. neoformans in decayed wood inside trunk hollows of miscellaneous host tree species in India, 2002-2007

<table>
<thead>
<tr>
<th>Tree species (Common English/Hindi Name and family)</th>
<th>Place/Locality</th>
<th>Collection period</th>
<th>Total trees examined</th>
<th>Cfu/ swab (C. gattii)</th>
<th>Cfu/ swab (C. neoformans)</th>
<th>Total trees positive/Total No. examined (C. n. sp complex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyalthia longifolia (Bast tree, Annonaceae)</td>
<td>Delhi, U.T. Rashanara Garden</td>
<td>May-Jul, 2004</td>
<td>3/2/18</td>
<td>5/18</td>
<td>1/18</td>
<td>5/18 (1/2/18)* 1 × 10^4-1.4 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Delhi Univ</td>
<td>Jun-05</td>
<td>0/23</td>
<td>1/23</td>
<td>0/23</td>
<td>1/23 (1/54) 2 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Other sites</td>
<td>Feb-04</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14 (0.9/16) 2 × 10^4</td>
</tr>
<tr>
<td>Mangifera indica (Mango tree, Magnoliaceae)</td>
<td>Amarnath, Haridwar</td>
<td>Mar-Oct, 2006</td>
<td>0/7</td>
<td>1/7</td>
<td>0/7</td>
<td>1/7 (3/30) 1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Other sites</td>
<td>2004-07</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11 (0.9/11) 1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>District Park, Pitampura &amp; Delhi Univ.</td>
<td>Jun-05</td>
<td>0/23</td>
<td>1/23</td>
<td>0/23</td>
<td>1/23 (1/49) 2 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Delhi Univ</td>
<td>Jun-07</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5 (4.1/10) 1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Other sites</td>
<td>Feb-06</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14 (0.9/14) 2 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Other sites</td>
<td>2004-07</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11 (0.9/11) 1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Delhi, U.T. Delhi Univ, &amp; neighbourhood</td>
<td>Aug-Oct, 2005</td>
<td>2/20</td>
<td>7/20</td>
<td>5/20</td>
<td>8/20 (2/6/5) 1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>District Park, Pitampura &amp; Delhi Univ.</td>
<td>Jun-05</td>
<td>0/23</td>
<td>1/23</td>
<td>0/23</td>
<td>1/23 (1/49) 2 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Delhi Univ</td>
<td>Jun-07</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5 (4.1/10) 1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Other sites</td>
<td>Feb-06</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14 (0.9/14) 2 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Other sites</td>
<td>2004-07</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11 (0.9/11) 1 × 10^4</td>
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<td></td>
<td>Delhi, U.T. Delhi Univ, &amp; neighbourhood</td>
<td>Aug-Oct, 2005</td>
<td>2/20</td>
<td>7/20</td>
<td>5/20</td>
<td>8/20 (2/6/5) 1 × 10^4</td>
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<td>District Park, Pitampura &amp; Delhi Univ.</td>
<td>Jun-05</td>
<td>0/23</td>
<td>1/23</td>
<td>0/23</td>
<td>1/23 (1/49) 2 × 10^4</td>
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<tr>
<td></td>
<td>Delhi Univ</td>
<td>Jun-07</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5 (4.1/10) 1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Other sites</td>
<td>Feb-06</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14</td>
<td>0/14 (0.9/14) 2 × 10^4</td>
</tr>
<tr>
<td></td>
<td>Other sites</td>
<td>2004-07</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11 (0.9/11) 1 × 10^4</td>
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<td>Delhi, U.T. Delhi Univ, &amp; neighbourhood</td>
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<td>Other sites</td>
<td>Feb-06</td>
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<td>0/14</td>
<td>0/14</td>
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<tr>
<td></td>
<td>Other sites</td>
<td>2004-07</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11</td>
<td>0/11 (0.9/11) 1 × 10^4</td>
</tr>
</tbody>
</table>

*Figures in parenthesis denote number of positive wood samples / Number examined. Cfu/ swab = Colony forming units per swab.
of the same species occurring in the same locality or in different geographic regions. Overall, *C. neoformans* had a wider spectrum of host trees than *C. gattii*. Specifically, *C. neoformans* was found associated with as many as 12 tree species whereas *C. gattii* was found in 8. Of the host trees for *C. gattii* in Delhi, *A. indica* showed the highest prevalence of 35%, followed by *M. hexandra* (33%), *M. elengi* (31%), *P. longifolia* (7%), *C. fistula* and *Eucalyptus* species (5% each). For *C. neoformans*, *A. indica* yielded the highest prevalence of 60%, followed by *M. hexandra* (50%), *C. fistula* (47%), *M. elengi* (15%), *A. nilotica* (14%), *P. longifolia* (13%) and relatively lower prevalences in the range of 5-8% for *A. scholaris*, *F. religiosa* and *D. sissoo*. Notably, 8 of the 12 tree species found positive for *C. neoformans* and 6 of the 8 positive for *C. gattii* are recorded for the first time to contain these two species. Table 2 provides the results of a longitudinal surveillance of 23 host trees representing 8 species for colonization by *C. gattii* and *C. neoformans* which were serially isolated from the trunk holes during a period of 0.7 to 2.5 years. The frequency of culture positive wood samples ranged from 50 to 100% in the host trees under surveillance.

The possibility of soil surrounding the host trees as an environmental niche of *C. gattii* and *C. neoformans* was investigated for some trees belonging to *S. cumini*, *M. elengi*, *P. longifolia* and *A. indica* occurring in Delhi, Bulandshahr and Chandigarh (Table 3). Of 95 soil samples examined, 43 (45%) yielded positive cultures of the *C. neoformans* species complex. Further characterization of the isolates revealed that *C. neoformans* occurred in 25, *C. gattii* in 23 and both *C. neoformans* and *C. gattii* in 5 of the soil samples investigated, yielding a prevalence of 26%, 24% and 5%, respectively. The prevalence of the two species in soil varied among sites ranging from 14 - 57% for *C. gattii*, 11-50% for *C. neoformans* and 7 - 11% for concomitant occurrence of both in the same soil sample. There was also variation among soil samples from around different host tree species. For example soil in vicinity of *S. cumini* trees had the highest prevalence for *C. gattii* at 29% whereas, that surrounding the *M. elengi* and *A. indica* trees had a prevalence of 25% and 20%, respectively. For *C. neoformans*, the highest prevalence in soil was 31% in the vicinity of *S. cumini*, followed by 12.5% each for *M. elengi* and *P. longifolia* trees. None of the 10 negative control soil samples from an open play ground away from the *S. cumini* positive trees in New Police Lines, Delhi, yielded any isolation of *C. gattii* or *C. neoformans*.

**Discussion**

The study extends our earlier investigations that demonstrated *S. cumini* (Java plum, Indian black berry, black plum) as a more important host tree species than *Eucalyptus* spp, *Butea monosperma* and *Tamarindus indica* for both *C. gattii* (serotype B) and *C. neoformans* (serotype A) in north-western India (Randhawa et al., 2003; 2005; 2006).The results from our expanded investigation revealed that 8 new host tree species harboured *C. neoformans* and 6 had *C. gattii*. The epidemiological importance of the sampled trees would depend not only on their extent of colonization but also on their geographic distribution. For example, epidemiologic importance of host trees like *M. hexandra* might not be as much as that of host trees with a lower prevalence of *C. gattii* and / or *C. neoformans* such as *C. fistula*, *A. nilotica*, *M. elengi*, etc. This is because *M. hexandra* has a narrower geographic distribution.
Chapter 11

Table 3. Prevalence of C. gattii and C. neoformans in soil around the base of miscellaneous host tree species.*

<table>
<thead>
<tr>
<th>Locality</th>
<th>Host tree</th>
<th>Collection Period</th>
<th>C. gattii (No. examined)</th>
<th>C. neoformans (No. examined)</th>
<th>Total (No. examined)</th>
<th>C. gattii (c.f.u/ g)</th>
<th>C. neoformans (c.f.u/ g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delhi, U.T.</td>
<td>S. cumini</td>
<td>Jan. 2007</td>
<td>7/14</td>
<td>0/14</td>
<td>1/14</td>
<td>1x10^2 - 3x10^3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Feb. 2007</td>
<td>2/18</td>
<td>7/18</td>
<td>9/18</td>
<td>1x10^2 - 3x10^3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Apr. 2007</td>
<td>1/12</td>
<td>8/12</td>
<td>9/12</td>
<td>1x10^2 - 3x10^3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>May 2007</td>
<td>2/2</td>
<td>7/2</td>
<td>9/2</td>
<td>1x10^2 - 3x10^3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>June 2007</td>
<td>5/6</td>
<td>1/6</td>
<td>6/6</td>
<td>1x10^2 - 3x10^3</td>
<td>8x10^2</td>
</tr>
<tr>
<td>Bilaspur, U.P.</td>
<td>M. elengi</td>
<td>Jan. 2007</td>
<td>2/8</td>
<td>1/8</td>
<td>3/8</td>
<td>1x10^2 - 1x10^3</td>
<td></td>
</tr>
<tr>
<td>Chandigarh, U.T.</td>
<td>A. indica</td>
<td>May 2007</td>
<td>1/5</td>
<td>0/5</td>
<td>1/5</td>
<td>1x10^3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>June 2007</td>
<td>0/5</td>
<td>1/5</td>
<td>1/5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Ten control soil samples collected from an open playground in Delhi University, North Campus, yielded negative results.
† C. gattii only
‡ C. neoformans only

C. gattii and C. neoformans proved to be mating type a (MAT a), indicating that this mating type is highly prevalent in the geographic regions sampled. This finding is in conformity with our previous report and with those of Yan et al from U.S.A. and Kidd et al on the isolates from British Columbia (Randhawa et al., 2006; Yan et al., 2002; Kidd et al., 2005).

In this study, 10 of the 14 host tree species were located in the Union Territory of Delhi itself (Table 1). This was because the bulk of tree sampling (75%) was done in this region. A more extensive sampling of trees in other regions will likely reveal a similar preponderance of host tree species for the C. neoformans species complex. Our results support the conclusion that decayed wood of a wide spectrum of tree species is the primary ecological niche or natural substrate for colonization and propagation of these two pathogens. The principal factor implicated in this colonization might be the ability of these pathogens to produce the enzyme laccase. Laccase is known to be involved in the degradation of wood lignin by other Basidiomycetes (Krik & Farrel, 1987; Thurson, 1994; Williamson, 1994; Eggert et al., 1996). This phenomenon is analogous to the presence of creatinine in avian excreta which preferentially supports the saprobic colonization by C. neoformans var. grubii and until recently had been known to be its principal environmental reservoir.

A literature review in 2003 identified 22 tree species belonging to 17 genera and 9 families as hosts of the C. neoformans species complex (Randhawa et al., 2003). A perusal of papers published up to 2007 (Granados & Castaneda, 2005; Kidd et al., 2004; 2007; Reimão et al., 2007) plus data from the present study shows that the number of host tree species has risen to 50. The number will likely increase as more trees are examined. We also note that our isolation of C. gattii from decayed wood of T. indica trees in Tiruvannamalai marks the first environmental demonstration of this pathogen in the south Indian state of Tamil Nadu. Based on the frequency of isolation of the C. neoformans species complex in a longitudinal surveillance, the host tree species are broadly divisible into two categories: One comprised the tree species such as D. sissoo, F. religiosa, A. scholaris, T. indica and Eucalyptus species which were found positive on only one occasion and attempts at re-isolation were negative. The second category comprised eight tree species containing positive cultures on several occasions over a period ranging from 0.7 to 2.5 years. These tree species were either intermittently or perennially colonized but clarification of this point would warrant their further longitudinal surveillance. In contrast, the tree species in the first category appeared to be transient hosts unless their long-term longitudinal surveillance proves it otherwise. Another noteworthy observation was the frequent isolation of C. gattii (14-57%) and C. neoformans (12-31%) from soil near the base of several host tree species with their population density ranging from 1x10^2 to 1x10^4 cfu/g (Table 3). Our data suggest that soil is possibly another important ecological niche of these two pathogens. The overall prevalence in soil for C. gattii (24%) and for C. neoformans (26%) in our study is somewhat lower than the 32% positive results reported from the Pacific Northwest of U.S.A (Kid et al., 2007) but higher than that from the Gulf Islands (13%) off Vancouver Island, Canada (MacDougall et al., 2007). The range of population density for the C. neoformans species complex
reported by MacDougall et al. (2007) was 10 - 36,350 cfu/g of soil whereas it is 100-10,000 cfu/g in the present study. Attention may be further called to a meticulously conducted comprehensive study on the dispersal mechanisms of C. gattii in British Columbia, Canada, by Kidd et al. (2007) who reported on longitudinal surveillance of tree and soil colonization by C. gattii. They demonstrated the association of C. gattii with high traffic locations by isolating it from the wheel-wells of vehicles on Vancouver Island and the mainland, as also from footwear, indicating its anthropogenic dispersal. In addition, dispersal of C. gattii through passive transport by wild and domestic animals was suggested, and C. gattii was isolated from both fresh water and sea water in several locations. Furthermore, increased levels of airborne C. gattii were detected during forestry activities such as wood chipping, the byproducts of which are frequently used in park landscaping. Similar mechanisms of dispersal can be envisaged in north-western India where we have demonstrated that C. gattii and C. neoformans are frequently associated with decayed wood in trunk hollows of a number of host tree species and also occurs in surrounding soil. In their pioneering work, Ellis and Pfeiffer reported that flowers and other plant debris of Eucalyptus camaldulensis and E. tereticornis in Australia constituted the main natural habitat of C. gattii. They believed that C. gattii had spread to other countries through Australian export of infected seeds or seedlings of Eucalyptus spp. (Ellis & Pfeiffer, 1990a; 1990b; Pfeiffer & Ellis, 1992). It was speculated that dormant dikaryotic mycelium of this fungus could over-winter in the ovaries and anthers of E. camaldulensis buds which released its basidiospores at the time of flowering. This postulate, however, has remained untenable due to the lack of isolation of C. gattii from these organs and the lack of histological demonstration of the fungus in ovaries / anthers or seeds of Eucalyptus trees. Consistent with several other reports (Granados & Castaneda, 2005; Swinne et al., 1994) we have found no correlation of the occurrence of either C. gattii or C. neoformans in various host trees with their flowering season. Also, the special association of C. gattii with various Eucalyptus species reported from Australia has not been observed in many other countries (Lazera et al., 2000; Randhawa et al., 2000; Granados & Castaneda, 2005; Swinne et al., 1994). Consequently, Eucalyptus trees cannot be universally considered a primary or principal ecologic niche for this pathogen in regions outside of Australia. In India, C. gattii has been only sporadically isolated from Eucalyptus plant debris (Randhawa et al., 2000; Chakrabarti et al., 1997). In the present study, the solo Eucalyptus tree yielding C. gattii on a solitary occasion was found in close proximity to a number of S. cumini trees in the University of Delhi, North Campus, which we have previously reported as perennially colonized by this pathogen. The ecologic significance of the association of C. gattii with Eucalyptus trees in north-western India is, therefore, uncertain and warrants further investigation. It should be pointed out in this context that in the isolations reported by Gugnani et al. (2005) from Delhi, no information was given on the number of sites or Eucalyptus trees sampled. We would also like to correct their wrong statement that C. gattii was isolated from a banyan tree, Ficus bengalensis, by Randhawa et al. (2003).

Considering the wide environmental distribution of C. gattii in northwestern India and its prevalence with high population density in certain localities, it seems enigmatic that only 6 cases of human cryptococcosis due to this species have so far been recorded (Padhye et al., 1993; Banerjee et al., 2004), i.e., 3 each from Chandigarh and New Delhi. Besides, of the 92 clinical isolates of C. neoformans species complex referred to our laboratory by diagnostic centers in this region, only 2 proved to be C. gattii (Unpublished data). At present, there is little information concerning the prevalence of cryptococcosis due to C. gattii in domestic or wild animals in India. However, a case of pulmonary cryptococcosis in a stray dog and the isolation of C. neoformans species complex from a case of caprine mastitis and from the intestinal contents of an insectivorous bat in Delhi have been reported (Sethi et al., 1967; Pal & Randhawa, 1976; Khan et al., 1982). Besides, a questionable case of cryptococcosis in a bandicoot rat due to C. neoformans var grubii infection has also been recorded (Randhawa et al., 2007). Attention may be called here to an outbreak of cryptococcosis due to C. gattii in Vancouver Island, Canada, in which animal cases exceeded human cases by almost 75%, highlighting their value as a sentinel indicator of this disease (Duncan, 2005). In another noteworthy study, 27% of 177 culture confirmed cases of cryptococcosis in cats and dogs in Australia were caused by C. gattii (O’ Brien et al., 2004). Therefore, it would be highly desirable to investigate the prevalence of cryptococcosis in indigenous animal populations, especially in localities with known environmental reservoirs of C. gattii. Such a study promises to provide a useful clue to the likely public health hazard posed by environmental reservoirs of C. gattii and C. neoformans. Finally, a wider environmental survey covering climatically divergent regions of India would help further elucidate the ecology of C. gattii and C. neoformans.
Acknowledgements

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Conflict of Interest: None

References

Seasonal variations in prevalence of Cryptococcus neoformans var. grubii and Cryptococcus gattii in decayed wood inside trunk hollows of diverse tree species in north-western India: A retrospective study

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T. Kowshik
Anuradha Chowdhary
Anupam Prakash
Z.U. Khan
Jianping Xu
Summary

This study presents a 7-year retrospective analysis of seasonal variations in the prevalence of Cryptococcus neoformans var. grubii and Cryptococcus gattii in decayed wood inside trunk hollows of 518 trees belonging to 20 species in north-western India during 2000-2007. Of the 1439 wood samples investigated, 406 (28.2%) were found to be positive for the Cryptococcus neoformans species complex which included 247 samples from which C. neoformans var. grubii was recovered and 171 which yielded C. gattii. While, both of the pathogens were isolated through all the seasons, the overall prevalence of C. neoformans var. grubii was significantly higher (17.2%) than that of C. gattii var. grubii. The highest prevalence for both of the pathogens was in the autumn, followed by that in the summer. For C. gattii, the lowest prevalence occurred during the winter and for C. neoformans var. grubii during the rainy season. The low prevalence of C. gattii during winter is similar to that reported from Bogota, Colombia, where C. gattii had a low population density in bark samples but it was not found in decayed wood of trunk hollows investigated during the winter months of January and February. The prevalence of C. neoformans var. grubii was significantly lower in the rainy season than in the other portion of the year. This finding is similar to the reported low frequency of isolation (4%) of C. neoformans var. grubii from chicken feces in the rainy season investigated in northern Thailand. Further investigations are warranted to determine the clinical significance of seasonal variations in the prevalence of C. neoformans var. grubii and C. gattii in decayed trunk wood of various trees in climatically divergent regions of India.

Introduction

In earlier publications (Randhawa et al., 2003; 2006; 2008), we reported that decayed wood inside trunk hollows of a wide spectrum of tree species and the soil in vicinity of the colonized trees in north-western India could serve as an important natural reservoir for Cryptococcus neoformans var grubi and Cryptococcus gattii, the principle etiologic agents of cryptococcosis. In addition, our preliminary investigations of a collection of C. neoformans var grubii isolates from decayed trunk wood of trees in this region revealed evidence for long-distance dispersal and recombination (Hiremath et al., 2008). The climate in north-western India varies widely from season to season. The variable climatic factors include fluctuating temperature, relative humidity and rainfall. However, the effects of these climatic factors across the seasons on the prevalence of C. neoformans and C. gattii in decayed wood inside trunk hollows of various trees remain largely unknown. Here we present a summary retrospective analysis of our data on seasonal variations in prevalence of both the pathogens in decayed wood of tree trunk hollows sampled during a 7-year period, from 2000 to 2007.

Materials and Methods

The data analyzed comprised the results of isolation of C. neoformans and C. gattii from a total of 1439 decayed wood samples collected from trunk hollows of 518 trees, representing 20 species. The investigated trees were distributed widely in the north-western states of Punjab, Haryana, U.P, and the Union Territories of Delhi and Chandigarh. Detailed information pertaining to the sites of collection, and methods of sampling, strain isolation and identification of both C. neoformans and C. gattii, can be found in our earlier publications (Randhawa et al., 2003; 2006; 2008; Hiremath et al., 2008). Based on the parameters of temperature, relative humidity, rainfall and various phases in the life cycle of plants, the following 5 seasons have been recognized for the areas covered in our investigation:

**Summer:** (April 21 to June 21): Noted for very high temperature, low relative humidity and low rainfall combined with desiccating high winds. Maximum temperature is usually between 38°C and 42.5°C, which may rise to 45°C and beyond on certain days. Ground vegetation is sparse.

**Rainy:** (June 22 to September 22): Characterized by high temperatures and high relative humidity but with low diurnal range in them. About 80% of the total rainfall (~666.4 mm) occurs during this period. Ground vegetation is lush with the highest ground coverage at this time of the year.

**Autumn:** (September 23 to November 21): Temperature is typically moderate with a high diurnal range. Relative humidity is also moderate but with very low rainfall.
Winter: (November 22 to February 19): Temperature and rainfall both are low. Relative humidity is moderate with a high diurnal range. Typical minimum temperature ranges from 5°C to 10°C but can touch freezing point on certain days.

Spring: (February 20 to April 20): This period is characterized by moderate temperature and moderate relative humidity. However, rainfall is usually low.

The differences in prevalence between C. neoformans var grubii and C. gattii across the seasons were analyzed, using the Chi-square contingency table test, with P values<0.05 considered as statistically significant.

Results and Discussion

The summary results of the seasonal dynamics of prevalence of the two Cryptococcus sp. are presented in Table 1 and depicted in Figure 1. The range of variations in temperature, relative humidity and rainfall across the seasons in north-western India are shown in Figure 2. Of the 1439 wood samples investigated, 406 (28.2%) were found to be positive for the Cryptococcus neoformans species complex which comprised 235 positive for C. neoformans var grubii and 159 for C. gattii. Notably, both of the pathogens were isolated through all the seasons. However, C. neoformans var grubii had a significantly higher overall prevalence (16.3%) than that of C. gattii serotype B (11.1%; P< 0.0001), indicating that decayed wood is as good as, if not a better, natural habitat for C. neoformans than for C. gattii. Based on our molecular typing results, all the C. neoformans var grubii isolates from the trees were classified as molecular type VNI (Hiremath et al., 2008) whereas C. gattii isolates belonged to the molecular type VGI [manuscript under preparation]. Both the pathogens showed seasonal variations in their prevalence. Specifically, the highest prevalence for both the pathogens was found during the autumn, followed by that in the summer. For Cryptococcus gattii, the lowest prevalence occurred during the winter, which was significantly less than that during the summer (P < 0.021) and the autumn (P <0.020). In contrast, the rainy season yielded the lowest prevalence of Cryptococcus neoformans var. grubii (10.6%). This was significantly less than that in the autumn (P <0.0001), followed by that in the summer (P <0.0001) and the winter (P < 0.001). Interestingly, similar results of a low frequency of isolation (4%) during the rainy season as against a high prevalence (24%) of C. neoformans var grubii during the dry season have been reported in an investigation of chicken feces in northern Thailand (Kuroki et al., 2005). Although the prevalence of C. neoformans var grubii in the present study was higher in the spring (14.2%) than that during the rainy season (10.6%), the difference was not statistically significant (P=0.055). Our results of low prevalence of C. gattii in decayed wood during winter are similar to those of Granados and Castañeda (2005) who did not find any C. gattii in decayed wood of tree trunk hollows but only a low population density (cfu/g) of the pathogen in the bark of trees investigated from Bogotá, Colombia, during the winter months of January and February. However, its prevalence was high during April and May. Subsequently, Granados and Castañeda reported a retrospective study (2006), analysing the relationship between the occurrence of various serotypes of the C. neoformans species complex in tree samples and the climatic conditions recorded during samplings in four cities of Colombia, between 1992 and 2004. Their analyses using logistic regression and lagged Pearson correlations indicated that environmental conditions, mainly humidity, temperature and solar radiation, variably affected the occurrence of serotypes, A, B and C in a different manner. It was also noted that serotype A (C. neoformans var grubii) was more thermotolerant and hygrophobic than serotype B. Our data on the prevalence of serotypes A and B during the summer and rainy

![Figure 1. Seasonal variations in isolation frequencies of Cryptococcus neoformans var grubii and Cryptococcus gattii in decayed tree trunk wood, north-western India.](image)

![Figure 2. Variations in temperature, relative humidity and rainfall during various seasons, north-western India.](image)

Table 1. Seasonal variations in prevalence of Cryptococcus neoformans var grubii and Cryptococcus gattii in decayed wood inside trunk hollows of miscellaneous trees in north-western India.

<table>
<thead>
<tr>
<th>Seasons</th>
<th>No. wood samples tested</th>
<th>No. positive for Cryptococcus neoformans sp. complex (%)</th>
<th>No. positive for Cryptococcus neoformans var grubii (%)</th>
<th>No. positive for Cryptococcus gattii (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter</td>
<td>481</td>
<td>128 (26.6)</td>
<td>84 (17.5)</td>
<td>44 (9.2)</td>
</tr>
<tr>
<td>Spring</td>
<td>275</td>
<td>65 (23.6)</td>
<td>39 (14.2)</td>
<td>26 (9.5)</td>
</tr>
<tr>
<td>Summer</td>
<td>176</td>
<td>64 (36.3)</td>
<td>42 (23.9)</td>
<td>28 (15.9)</td>
</tr>
<tr>
<td>Rainy</td>
<td>346</td>
<td>78 (22.5)</td>
<td>37 (10.6)</td>
<td>47 (13.6)</td>
</tr>
<tr>
<td>Autumn</td>
<td>161</td>
<td>71 (44.1)</td>
<td>45 (28.0)</td>
<td>26 (16.2)</td>
</tr>
<tr>
<td>Total</td>
<td>1439</td>
<td>406 (28.2)</td>
<td>247 (17.1)</td>
<td>171 (11.8)</td>
</tr>
</tbody>
</table>
seasons provides further support for their hypothesis (Table 1). While the observed seasonal pattern for C. gattii in our survey is similar to that from Colombia, it is different from that of Kidd et al. (2007) who did not find any seasonal pattern in the prevalence of C. gattii in their survey of the pathogen in decayed wood, soil, water and air carried out in British Columbia, Canada, and the Pacific Northwest of the United States. These divergent results may be attributed to the striking geoclimatic differences between the geographic regions investigated. Another possible reason for the differences is that the C. gattii population investigated by Kidd et al. (2007) belonged to a newly emerged molecular type, VGIIa, and not to VGI which encompasses our C. gattii isolates.

As far as we are aware, this is the first environmental survey on seasonal variations in the prevalence of C. neoformans var grubii and C. gattii in decayed wood of living trees from India. It would be extremely interesting to correlate these results with the prevalence of cryptococcosis in patients in the same region in order to determine their inter-relationship. Unfortunately, no such clinical data are available in India at present. However, such comparisons have been done in Thailand and the USA (Chariyalertsak et al., 1996; Sorvillo et al., 1997). In the Thailand study, 793 cases of cryptococcosis in AIDS patients admitted to Chiang Mai University Hospital were analyzed regarding their prevalence in the dry and rainy seasons but there was no significant difference. In contrast, the U.S. study found a significantly higher prevalence of cryptococcosis in the fall and winter months than in the spring and summer. A comprehensive prospective study including both environmental and clinical data would be needed to determine the potential health impact of seasonal variations in the prevalence of C. neoformans var grubii and C. gattii in decayed wood of various trees in climatically divergent regions of India.

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Declaration of interest

The authors report no conflicts of interest, and they alone are responsible for the content and writing of the paper.

References


Discovery of a temperate climate niche for *Cryptococcus gattii* in Northern Europe, the Netherlands

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Ferry Hagen
Corné H. Klaassen
Jacques F. Meis
Cryptococcus gattii was considered to be geographically restricted to countries with tropical and subtropical climates until 1999 when an outbreak of cryptococcosis in humans and animals occurred in the temperate climate of Vancouver Island in British Columbia, Canada (Kidd et al., 2004). The first report on environmental occurrence of C. gattii in Europe came from the Mediterranean region of Italy by Montagna et al., (1997) who isolated it from 11 of 255 (4.3%) samples of plant detritus of Eucalyptus camaldulensis trees collected from the residential locality of an autochthonous case of cryptococcal meningitis due to C. gattii in Apulia (Montagna et al., 1997). These observations were recently substantiated by the isolation of C. gattii from plant debris of trees belonging to Ceratonia siliqua (carob), Pinus halepensis (stone pine) and E. camaldulensis in Spain (Colom et al., 2011). Herein, we report environmental isolation of the primary pathogenic fungus C. gattii from a forest in Berg en Dal, the Netherlands, extending its geographic distribution to the temperate climate of Northern Europe.

One hundred and twelve decayed wood samples collected from inside trunk hollows of 52 living trees belonging to five families were investigated during April-May, 2011, in Nijmegen, the Netherlands. The trees sampled were chestnut (Castanea sativa, n=24), Douglas fir (Pseudotsuga menziesii, n=17), oak (Quercus macranthera, n=6), walnut (Juglans regia, n=3), and mulberry (Morus alba, n=2). The main criterion in selecting any tree for sampling was its advanced age and presence of large trunk hollows, variably sheltered from sunlight. The sampled sites had no bird nests and were apparently free from avian excreta. The decayed wood samples were collected with an in-house swabbing technique, employing simplified Staib’s niger seed agar (NSA) as described previously (Randhawa et al., 2005). The inoculated plates were incubated at 30°C and periodically observed up to seven days for isolation of C. gattii and C. neoformans. Suspected colonies of Cryptococcus were purified by dilution plating and identified by their morphological and biochemical profiles, including development of blue color on L-canavanine-glyicine bромothymol blue (CGB) medium. Identity of the isolates was confirmed by sequencing the ITS and D1/D2 regions, and they were genotyped using Amplified Fragment Length Polymorphism (AFLP) fingerprinting and Multi-Locus Sequence Typing (MLST). The MLST loci CAP10, CAP59, GPD1, IGS, LAC1, MPD1, PLB1, SOD1, TEF1α and URA5 of the environmental C. gattii isolates were amplified, sequenced and data were compared to MLST data from a large population study (Fraser et al., 2005) with a recently published set of clinical, veterinary and environmental C. gattii isolates from Mediterranean Europe and the Netherlands (Figure) (Colom et al., 2011; Janssens et al., 1957; Hagen et al., 2009). In addition, the mating-type was determined by PCR, using mating-type specific primers for the STE12a and α alleles (Hagen et al., 2010).

Four strains of Cryptococcus neoformans species complex were isolated from 112 decayed wood samples examined from 52 trees. One of these strains, originating from an oak tree (Quercus macranthera), was identified as C. neoformans var. grubii. The remaining three strains, all originating from different hollows in a Douglas fir tree, were identified as C. gattii genotype AFLP4/VGI and mating-type α. The strains were deposited at the CBS-KNAW Fungal Biodiversity Centre (accession numbers CBS12340, CBS12355 and 12356) and the sequences in Genbank (accession number JN982044–JN982073). MLST analysis revealed that our C. gattii isolates are more closely related to the Dutch clinical isolate (Janssens et al., 1957) and to the clinical and environmental C. gattii isolates (AFLP4/VGI) reported previously from the Netherlands and other countries in Europe than to isolates from outside of Europe (Colom et al., 2011; Hagen et al., 2009; 2010). The Dutch autochthonous C. gattii AFLP4/VGI, CBS2502 (earlier identified as C. neoformans) isolate was recovered post-mortem in 1957 from the lungs of a pregnant female patient with cryptococcosis (Janssens et al., 1957). This patient came from a low social economic strata, unlikely to have travelled outside of the Netherlands, who probably acquired the infection indigenous from an environmental source (Janssens et al., 1957). Furthermore, as of now it is apparent that genotype AFLP4/VGI is the genotype of C. gattii prevalent in Europe (Colom et al., 2011; Hagen et al., 2009; 2010). It is pertinent to mention here that, outside of Europe, C. gattii has been earlier reported from Douglas fir trees in Vancouver Island, Canada, although those isolates represented another molecular type, i.e., AFLP6/VGII (Kidd et al., 2007). Genotype AFLP 4/VGI C. gattii isolates have been implicated in human infections in that region but no environmental isolates have been found until now. Our detection of C. gattii in the environment and its previous isolation from a clinical case in the Netherlands is suggestive of the endemic occurrence of this pathogen in the temperate climate of Northern Europe. This is in agreement with the concept emerging from a decade of investigations in Canada and the Pacific Northwest that the geographic distribution of C. gattii extends to the temperate region albeit with another AFLP genotype (Kidd et al., 2004; 2007; Springer & Chaturvedi, 2010). Further environmental studies are likely to reveal a wider spectrum of host trees and higher environmental prevalence of C. gattii in this continent than what is apparent from the available literature.
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References

Cryptococcus neoformans and Cryptococcus gattii are etiologic agents of cryptococcosis, a potentially fatal fungal infection with predilection for the central nervous system. The disease has a worldwide distribution. Patients with AIDS and other immunodeficiencies are especially prone to cryptococcosis. Cryptococcosis is the most common fungal disease in HIV-infected persons, and is the AIDS-defining illness for two-thirds of the HIV-infected population. Recently published estimates suggest that annually approximately one million HIV-positive patients develop cryptococcal meningitis, resulting in 625,000 fatalities. India has the second largest burden of cryptococcosis due to an estimated 5.7 million adults and children living with HIV/AIDS (UNAIDS, 2006). The country has a documented high prevalence (1.7–4.7%) of cryptococcosis in persons with HIV/AIDS (Kumarasamy et al., 2003; Vajpayee et al., 2003). Notwithstanding the over-all paucity of data, an increase in the incidence ranging from 6.5-fold in South India (Khanna et al., 2000) to 15-fold in northwestern India (Chakrabarti et al., 2000) has been reported. Remarkably it is now recognised that not only immunocompromised individuals are affected, as shown by a major outbreak of the primary pathogen C. gattii in North-West USA and British Columbia, Canada with the source the establishment of the so called tropical fungus in the temperate climate of Vancouver island. Despite this striking increase in incidence of cryptococcosis, information about the molecular ecology of C. gattii and C. neoformans in India is largely lacking (Jain et al., 2005). Besides, there is paucity of information on the distribution of Cryptococcus species in the environmental and clinical material. The aim of this thesis was to investigate the environmental prevalence, molecular epidemiology and antifungal susceptibility profiles of the two fungal pathogens C. neoformans and C. gattii prevalent in India. This thesis consists of three main Sections: Part I contains the chapters 2 and 3 that focus on the (molecular) epidemiology of C. neoformans var. grubii and C. gattii in India. Part II includes chapters 4-6, that focus on the antifungal susceptibility pattern, genotypes, serotypes and mating types of both clinical and environmental C. neoformans var. grubii and C. gattii in India. Chapter 7 represents a multicenter Asian comparative study of antifungal susceptibility and genetic diversity of C. neoformans var. grubii by microsatellite typing of approximately 500 isolates from seven Asian countries, including 60 isolates from North-India. In addition in chapter 8, an international multicentric study is included aimed at determining epidemiological cut-off MICs to define wild-type susceptibility endpoint distributions and epidemiological cutoff values for amphotericin B and fluocytosine versus C. neoformans and C. gattii. Finally Part III comprises the chapters 9-13 that are all focussed on the environmental prevalence of C. gattii and C. neoformans in India. Experience with environmental sampling was successfully performed in the Netherlands where both C. neoformans and C. gattii were successfully isolated from the environment constituting first environmental isolation of this yeast from the Netherlands.
disequilibrium (Xu et al., 1999; Xu, 2005b). In these tests, random associations (i.e. linkage equilibrium) among alleles at the population level are consistent with sexual reproduction while significant associations (linkage disequilibrium) are indicative of clonal reproduction. Other signatures of clonal reproduction include over- or under-representations of certain genotypes and genealogical congruence among genes from unlinked loci (e.g. Xu, 2005b). Previous studies have employed a variety of molecular markers to demonstrate the modes of microbial reproduction in nature and to determine how evolutionary as well as ecological factors can influence the modes of reproduction and the structures of microbial populations (Maynard-Smith et al., 1993, Avise, 1994; Xu, 2005b), including those of *C. neoformans*. For example, an MLST study of the population structure of *C. neoformans* var. *grubii* involving 102 representative strains from different parts of the world showed that the global population contained three genetically distinct subgroups (Litvintseva et al., 2006). Ten of the 102 strains were *MATa* and they were all from Botswana. The remaining 92 were *MATa* and were from different parts of the world, including Botswana. As expected, the Botswana population that contained strains of both mating types showed evidence of recombination and sexual reproduction (Litvintseva et al., 2003, 2006). In contrast, the remaining geographical populations that contained only *MATa* strains were largely clonal, with no clear evidence of recombination. Similar analyses of natural populations of a closely related species, *Cryptococcus gattii*, that contained exclusively or predominantly *MATa* strains identified at least three distinct lineages in this species but no conclusive evidence for recombination (Kidd et al., 2005). However, in another study of *C. gattii* populations, evidence for both clonality and recombination was obtained, with clonality found for samples belonging to one molecular type, VGI, and evidence of recombination found for samples of a different molecular type, VGII (Campbell et al., 2005). In our study, we analysed 78 strains of *C. neoformans* var. *grubii* isolated from nine tree species distributed in five geographical areas in north-western India. The mating types of these strains were determined based on direct PCR using mating-type-specific primers (Yan et al., 2002) and all strains were found to have the *MATa* mating type. A multiple-gene genealogical approach was used to analyse the population samples. DNA sequences were obtained for each strain from each of the five genetic loci distributed in different parts of the genome. Our population-genetic analyses identified no evidence of population subdivision based on either the host tree species or their geographical origins. Interestingly, while clonality and clonal dispersal among geographical regions were clearly evident in our analysed populations, there was also unambiguous evidence for recombination in this *MATa* population of *C. neoformans* var. *grubii*.

**Cryptococcus gattii**

*Cryptococcus gattii* is a ubiquitous eukaryotic pathogen capable of causing life-threatening infections in a wide variety of hosts, including both immunocompromised and immunocompetent humans. Since infections by *C. gattii* are predominantly obtained from environmental exposures, understanding environmental populations of this pathogen is critical, especially in countries like India with a large population and with environmental conditions conducive for the growth of *C. gattii*. The first report on environmental isolation of *C. gattii* was by Ellis and Pfeiffer in Australia who identified plant debris under the canopy of *Eucalyptus* trees as a reservoir of this pathogen (Ellis & Pfeiffer, 1990). This was followed by numerous studies which recovered *C. gattii* from various tree species in the USA, Mexico, Canada, Colombia, Argentina, Brazil, Paraguay, India, Italy, Jordan, Egypt, Africa and Papua New Guinea (Pfeiffer & Ellis, 1992; Chakrabarti et al., 1997; Laurentson et al., 1997; Mahmoud, 1999; Montenegro & Paula, 2000; Sorrell, 2001; Davel et al., 2003; Randhawa et al., 2003, 2006, 2008; Granados & Castañeda, 2005; 2006; Kidd et al., 2005, 2007; Xu et al., 2010). In addition, *C. gattii* has been reported from a variety of other environmental sources such as soil, fruits, vegetables, freshwater, seawater, insect frass and desiccated excreta of a few species of caged birds (Casadevall & Perfect, 1998; Abeeg et al., 2006). Of the diversity of environmental niches, decayed wood inside trunk hollows of trees, bark and other plant debris are among the most commonly investigated and where *C. gattii* is frequently found (Xu et al., 2010).

Based on molecular fingerprinting and gene genealogical analyses, *C. gattii* has been divided into four distinct lineages: VGI, VGII, VGIII and VGIV (Kidd et al., 2005; Boevers et al., 2008; Ngaamskulrungruj et al., 2009). VGI is the most commonly isolated genotype group occurring worldwide including several Asian countries and Australia where it is predominantly associated with *Eucalyptus* trees (e.g. Chen et al., 2000; Meyer et al., 2003; Randhawa et al., 2008). VGII has been recovered from Colombia, the Pacific Northwest region of North America and also from certain parts of Australia although not associated with *Eucalyptus* (Trilles et al., 2003; Kidd et al., 2005; Escandón et al., 2006). The last decade has seen the VGII genotype group causing an outbreak of cryptococcosis on Vancouver Island in British Columbia, Canada, and in the states of Washington and Oregon in the USA (Fraser et al., 2003, 2005; Kidd et al., 2005; Byrnes et al., 2010). Unfortunately, this outbreak is still ongoing and will likely continue to the foreseeable future. Ecologically and epidemiologically, this outbreak is significant because it shows that *C. gattii* is capable of causing life-threatening infections in apparently healthy individuals in a temperate climate. In contrast to the broad distributions and medical significance of VGI and VGII genotype groups, VGIII and VGIV are relatively rare genotype groups in both environmental and clinical samples (e.g. Kidd et al., 2005; Boevers et al., 2008; Litvintseva et al., 2010). However, strains of VGIV have been shown contributing to cryptococcosis in AIDS patients in sub-Saharan Africa (Litvintseva et al., 2005).

The mode of reproduction is an important contributor to the patterns of genetic variation in natural populations of all organisms, including *C. gattii*. As in the majority of microorganisms (Xu, 2010), asexual reproduction is expected to be common in natural populations of *C. gattii*. Prolonged asexual reproduction leads to linkage disequilibrium and the propagation of clonal lineages within a population, and through dispersal, to clonal expansion across ecological niches and geographic regions. On the other hand, sexual reproduction recombines genetic materials from different strains, results in linkage equilibrium and, in *C. gattii*, the formation of basidiospores. Because of their small size and low water content, basidiospores are better adapted for dispersal than vegetative cells and are likely the dominant infectious particles of cryptococcal infections (Giles et al., 2009; Velagapudi et al., 2009). However, among the four lineages of *C. gattii*, evidence for recombination has been found only in natural populations of the VGI and VGII lineages in Australia and in the VGII
lineage in North America (Campbell et al., 2005a,b; Fraser et al., 2005; Saul et al., 2008; Xu et al., 2009; Litvintseva et al., 2010). Furthermore, there is very limited information on the geographic structure of environmental populations for any of the four lineages in C. gattii. In this study, we analysed 109 strains of C. gattii associated with nine different tree species originating from the wood detritus of trees and the surrounding soil from nine different tree species at seven north-western locations, i.e. Amritsar, Union Territories of Chandigarh and Delhi, Amrouli, Bulandshahr, Hathras and Meerut and one in Tamil Nadu, namely, Tiruvannamalai in south India. For each strain, nine nuclear loci previously identified to be polymorphic in C. gattii were sequenced. All the isolates in our study were found to belong to the VGI lineage and had the MATα mating type. These data were analysed to address the following specific questions. First, how extensive is clonality in natural populations of C. gattii VGI in India? Is there any evidence of recombination? Second, what are the relationships among ecological and geographic populations of this lineage?

Our results here showed both similarities and differences in population structure between C. gattii VGI and C. neoforms var. grubii from similar habitats and geographic origins in India. Population genetic analyses revealed limited evidence of recombination but unambiguous evidence for clonal reproduction and expansion. However, the observed clonal expansion has not obscured the significant genetic differentiation among populations from either different geographic areas or different host tree species. A positive correlation was observed between genetic distance and geographic distance. The results obtained here for environmental populations of C. gattii showed both similarities and differences with those of the closely related C. neoforms var. grubii from similar locations and host tree species in India.

## Part II. Antifungal susceptibility profiles, genotypes, serotypes and mating types of both clinical and environmental isolates of Indian C. neoforms var. grubii and C. gattii and genotypic differences and multi-resistance in Asian populations of Cryptococcus neoforms var. grubii

### Indian scenario

Resistance to antifungal agents in environmental and clinical strains of C. neoforms and C. gattii has been a rare global occurrence. Soares et al. (2005) reported a solitary isolate of C. neoforms var. grubii from pigeon excreta that was resistant to fluconazole, (MIC 64 mg/L). Similar findings were reported from Cuba and those authors concluded that environmental isolates seemed to be less susceptible to fluconazole than clinical ones (Illnait-Zaragozi et al., 2008). Likewise, in another report from Brazil, one of the environmental isolates of C. neoforms var. neoforms was found to be resistant to iraconazole whereas three additional isolates exhibited high MICs of 16–32 mg/L against fluconazole (Costa et al., 2010). Studies on antifungal susceptibilities of environmental isolates of C. neoforms or C. gattii are scanty and are based on a small number of isolates (Currie et al., 1995b; Franzot & Hamdan, 1996; Yildiran et al., 2000). No such information is available from Asia or Africa where cryptococcosis is a significant problem in HIV/AIDS patients. Chapter 4 was the first antifungal susceptibility testing report on environmental isolates from India based on 117 isolates of C. neoforms, serotype A, and 65 of C. gattii, serotype B, originating from decayed wood in trunk hollows of F. religiosa and S. cumini trees, employing the Etest method. A comparison of the geometric mean MICs revealed that C. gattii was less susceptible than C. neoforms to amphotericin B (0.075 versus 0.051, p = 0.0003), fluconazole (2.912 versus 2.316, p = 0.0003), itraconazole (0.198 versus 0.034, p < 0.0001), ketoconazole (0.072 versus 0.037, p < 0.0001), and voriconazole (0.045 versus 0.023, p < 0.0001)

No primary resistance was observed against amphotericin B, fluconazole, itraconazole, ketoconazole and voriconazole which is in consonance with worldwide literature reports that resistance in Cryptococcus species complex is rarely observed (Pfaller et al., 2005).

Therapeautic management of cryptococcal infections usually consists of amphotericin B therapy with or without 5-flucytosine, whereas fluconazole is the drug of choice for prophylaxis and maintenance therapy. Owing to frequent instances of disease relapse, there is growing concern among clinicians about emergence of antifungal resistance in C. neoforms and C. gattii during therapy or prophylaxis. Most reports of resistance have emerged in the setting of cryptococcal meningitis in AIDS patients after prolonged prophylaxis with fluconazole (Paugam et al., 1994; Birley et al., 1995; Currie et al., 1995; Armeanou et al., 1996; Berg et al., 1998; Davey et al., 1998; Aller et al., 2000). This problem could have more serious dimensions in developing countries of Southeast Asia and Africa where large numbers of HIV-infected patients exist, and resources to treat the disease are inadequate or patients might receive suboptimal doses of fluconazole.

In a larger study (chapter 6) the antifungal susceptibility profiles in clinical and environmental isolates of C. neoforms var. grubii, genotype AFLP1/VNI MATα (n = 246), and C. gattii, serotype B, genotype AFLP4/VGI, MATα (n = 62), using the broth microdilution method were investigated. Both the species had low MICs to the antifungals tested except for two clinical C. neoforms var. grubii isolates that were resistant to 5-flucytosine (MIC 64 mg/L). Data on the geometric mean of MICs revealed that C. gattii was significantly less susceptible than C. neoforms var. grubii to fluconazole, itraconazole and voriconazole (p < 0.0001). In addition, the MIC<sub>50</sub> of C. gattii was 2-fold higher than that of C. neoforms var. grubii for fluconazole, itraconazole and voriconazole. However, no statistically significant difference was observed in susceptibility of the two Cryptococcus species to amphotericin B and 5-flucytosine. Furthermore, the environmental C. neoforms var. grubii isolates were significantly less susceptible to fluconazole, itraconazole and 5-flucytosine (p < 0.0001) than the clinical isolates. Of the 53 serial isolates of C. neoforms var. grubii in this study, collected at least 1 month apart from 23 patients, four serial isolates obtained from four patients receiving antifungal therapy of amphotericin B for 3 weeks and followed by fluconazole prophylaxis (400 mg daily) showed a fourfold increase in fluconazole MICs over a period of 1.5–2.5 months. However, the MIC values did not exceed 4 mg/L. Likewise, one serial C. neoforms var. grubii isolate from a patient showed a fourfold increase in itraconazole MIC but not in fluconazole MIC. A similar increase in amphotericin B MICs was found, which ranged from fourfold to fivefold in four serial isolates over 1–3 months for strains originating from six patients who had received this antifungal drug for 3 weeks. Here again, the increase in the amphotericin B MICs did not exceed 1 mg/L. Interestingly, three serial isolates from three patients showed a fourfold increase in 5-flucytosine MICs, although none of the patients had received 5-flucytosine previously.
Similar results have been reported previously from Cuba (Illnait-Zaragozi et al., 2008). Hagen et al. (2010) reported that C. gattii, showed lower MICs for AFLP4/VGII isolates (1.401 and 2.467 mg/L) versus the higher MICs for AFLP6/VGII isolates (4.961 and 5.638 mg/L) against 5-flucytosine and fluconazole, respectively. Iqbal et al. (2010) tested 42 clinical isolates of C. gattii from patients in Oregon, USA. Interestingly, their AFLP4/VG and AFLP5/VGIII isolates had comparatively low fluconazole MICs, whilst the majority with high MICs of 16–32 mg/L were of subtype AFLP6/VGIII. In contrast Thompson et al. (2009) reported no differences in the antifungal susceptibilities of the two species.

Asian scenario

The prevalence of cryptococcosis in Asia has been rising after the onset of the AIDS epidemic and estimates indicate more than 120 cases per 1,000 HIV-infected individuals per year. Almost all cryptococcal disease cases in both immunocompromised and immunocompetent patients in Asia are caused by C. neoformans var. grubii. Epidemiological studies on C. neoformans in pan-Asia have not been reported. Chapter 7 focuses on the genetic diversity of the fungus by microsatellite typing and susceptibility analysis of approximately 500 isolates from seven Asian countries, including sixty isolates of C. neoformans var. grubii from India. Genetic diversity of Asian isolates of C. neoformans was determined using microsatellite analysis with nine microsatellite markers. The analysis revealed eight microsatellite complexes (MCs) which showed different distributions among geographically defined populations. A correlation between MCs and HIV-status was observed. Microsatellite complex 2 was mainly associated with isolates from HIV-negative patients, whereas MC8 was associated with those from HIV-positive patients. Most isolates were susceptible to amphotericin B, itraconazole, voriconazole, posaconazole, and isavuconazole, but 17.3% and 10% (2%) were found to be resistant to 5-flucytosine and fluconazole, respectively. Importantly, five Indonesian isolates (approximately 12.5% from all Indonesian isolates investigated and 1% from the total studied isolates) were resistant to both antifungals. The majority of 5-flucytosine resistant isolates belonged to MC17. The findings showed a different distribution of genotypes of C. neoformans var. grubii isolates from various countries in Asia, as well as an association of the microsatellite genotypes with the original source of the strains and resistance to 5-flucytosine. Ten fluconazole resistant isolates (2%) occurred in different countries, including China (n = 2), India (n = 1), Indonesia (n = 5), and Thailand (n = 2) and belonged to MC2 (n = 3), MC3 (n = 2), MC8 (n = 2), MC17 (n = 2) and one isolate from India that could not be assigned to any MC (n = 1). The ranges and highest MIC values were those of 5-flucytosine (<0.063 to >64 mg/L), followed by fluconazole (0.125 to 32 mg/L). The lowest MIC range was observed for isavuconazole (<0.016 to 0.125 mg/L), followed by posaconazole (<0.016 to 0.25 mg/L), and voriconazole and itraconazole (both <0.016 to 0.5 mg/L). Among the azoles, fluconazole showed the lowest activity (MIC_{50} = 4 mg/L) and isavuconazole the highest (MIC_{90} = 0.063 mg/L). In summary, genotypic differences in microsatellite patterns occur between C. neoformans populations from the Asian countries studied. However, the overall genetic diversity in Asian countries are low and most of the countries had a unique distribution of MCs.

Despite the MIC data available for Cryptococcus species, clinical breakpoints (CBPs) are not available for the Cryptococcus neoformans-Cryptococcus gattii species-complex. The availability of reference methodologies has enabled the recognition of resistance isolates as well as the proposal of clinical breakpoints (CBPs) and epidemiologic cutoff values (ECVs) for Candida spp. and Aspergillus spp. to most available antifungal agents by both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee of Antibiotic Susceptibility Testing (AFST-EUCAST) (Espinel-Ingroff et al., 2003, 2010; Rodriguez-Tudela et al., 2008; Pfaller et al., 2009a, 2011; Lass-Flör et al., 2009; Messer et al., 2003; CLSI, 2008; Diekema et al., 2009). CLSI MICs are <1 mg/L (amphotericin B) and <8 mg/L (5-flucytosine) for most C. neoformans and C. gattii isolates (Pfaller et al., 2010; White et al., 2011), but acquired resistance is frequent during fluocytosine monotherapy (Block et al., 1973; Hospenthal & Bennett, 1998). In the last few years, antifungal susceptibility differences have been reported between these two species as well as among the molecular types and serotypes (Trilles et al., 2004, 2011; Iqbal et al., 2010; Khan et al., 2007, 2009; Chowdhary et al., 2011; Chen et al., 2000; Calvo et al., 2001; Morgan et al., 2006; Tay et al., 2006; Thompson et al., 2009). However, neither CBPs nor ECVs are available for either C. neoformans or C. gattii versus amphotericin B or fluconazole. In the absence of CBPs, ECVs could help to characterize the susceptibility of these species to amphotericin B, its lipid formulations and fluconazole and to monitor the emergence of strains with mutations that could lead to reduced antifungal susceptibility to these agents. We conducted an international study (chapter 8) and wild type (WT) MIC distributions were constructed to establish ECVs for C. neoformans and C. gattii versus amphotericin B and fluconazole. A total of 3,590 amphotericin B and 3,045 flucytosine CLSI MICs for C. neoformans (including 1,002 VNI and 8-39 for VNII-VNIV isolates) and 985 and 853 MICs for C. gattii, respectively (including 42-259 VGI-VGIV isolates), were gathered in 9-16 (amphotericin B) and 8-13 (fluconazole) laboratories (Europe, United States, Australia, Brazil, Canada, India, and South Africa) and aggregated for the analyses. The purpose of the study was to define WT susceptibility endpoint distributions of each species/molecular type and agent combination by using aggregated CLSI-RPMI broth MICs of amphotericin B and fluconazole gathered in 8 to 16 laboratories (3,590 to 3,045 MICs for C. neoformans and 985 to 853 MICs for C. gattii, species/molecular type and agent/combo combination dependent) in Europe, the United States, Australia, Brazil, Canada, India, and South Africa. ECVs for distributions originating in >3 laboratories were (percentages of isolates for which MICs were <ECV): amphotericin B, 0.5 mg/L for C. neoformans VNI (97.2%) and C. gattii VGI and VGII (99.2% and 97.5%, respectively) and 1 mg/L for C. neoformans (98.5%) and C. gattii non-typed isolates (100%) and VGII (99.2%); fluconazole, 4 mg/L for C. gattii non-typed (96.4%) and VGII (95.7%); 8 mg/L for VNI (96.6%); and 16 mg/L for C. neoformans non-typed (98.6%) and C. gattii VGII (97.1%). ECVs may aid in the detection of isolates with acquired resistance mechanisms. For susceptibility testing to be clinically relevant, the test result should predict with some reliability the clinical outcome when an infected patient is treated with the specific agent evaluated; this test result is the CBP. The amphotericin B and fluconazole ECVs established in the present study for C. neoformans and C. gattii may aid in the evaluation of clinical isolates by identifying those strains that may have acquired resistance mechanisms, and serve as an early warning of emerging changes in the susceptibility patterns of these organisms.
Trees, decayed wood, other plant debris
The success in demonstration of *C. neoformans* in soil and avian excreta in early studies by the pioneers of Medical Mycology, Chester Emmons (1951), Libero Ajello (1958) and Maxwell Littman (1959) was achieved by employing the mouse-inoculation technique. Investigations on the natural habitat of *C. neoformans* were further stimulated when Fritz Staib (1962) developed and introduced niger seed agar, a selective medium for its rapid isolation and presumptive identification. In India, our studies aimed to characterize the environmental reservoirs of *C. gattii* and *C. neoformans*. It was found that *F. religiosa* trees harbored only *C. neoformans* var. *grubii* in their trunk hollows, whereas this variety and *C. gattii*, serotype B, were both equally distributed (prevalence 10.6%) in decayed wood inside trunk hollows of the 66 *S. cumini* trees investigated. Furthermore, *C. gattii* was repeatedly isolated on 36/44 (82%) occasions from 7 *S. cumini* known positive trees sampled longitudinally over a period of 689 days. Likewise, the overall isolation frequency of *C. neoformans* var. *grubii* from the two host tree species came to 22/27 (81%) occasions during the same follow-up span. These data strongly supported a long-term colonization of decayed wood inside trunk hollows of *S. cumini* by both the pathogens. The conclusion was reinforced by high population densities found in wood samples (maximally $6 \times 10^5$ CFU/g for *C. gattii* and $8 \times 10^4$ CFU/g for *C. neoformans*). No *Eucalyptus* trees were seen in the localities where *S. cumini* and *F. religiosa* host trees were sampled. During the next phase of the study, a large number of *S. cumini* trees were investigated from Delhi and other parts of north-western India, i.e. Amritsar (Punjab), Meerut and Bulandshahr (Uttar Pradesh) and the Chandigarh Union Territory. The results corroborated the strong ecological association of *C. gattii* and *C. neoformans* with *S. cumini* trees throughout north-western India.

The prevalence of *C. gattii* and *C. neoformans* differed considerably not only from one host tree to another but also among trees of the same host species occurring in a given locality or in different geographic regions. Table 1 shows the host trees harbouring *C. neoformans* and *C. gattii* investigated in the present thesis work. The isolation of *C. neoformans* var. *grubii* from 12 host tree species and of *C. gattii* serotype B from 8 tree species showed that the former has a more widespread arboreal distribution. Of the *C. gattii* host trees, *S. cumini* in Delhi yielded the highest prevalence (89%), followed by *A. indica* (35–40%), *M. elengi* (31%), *M. indica* (20%), *P. longifolia* (7%), *Cassia fistula* and *Aegle marmelos* (5.2% each). Notably, the isolation frequency of *C. gattii* and *C. neoformans* from their host tree species was not related to the phenological state of the trees, which was in agreement with the observations of Lazera *et al.* (1998), Granados and Castañeda (2005), Kidd *et al.* (2007b) and Byrnes *et al.* (2011). Furthermore, *Eucalyptus* species proved to be among the least important host for *C. gattii*. Both of these observations were in striking contrast to the results reported from Australia (Ellis & Pfeiffer, 1990) where *C. gattii* isolations were linked to flowering of the trees, and *Eucalyptus* species were reported as predominant, if not virtually exclusive hosts. However, it is not clear as to what extent this difference can be attributed to the fact that tree species other than those

### Table 1

<table>
<thead>
<tr>
<th>Host tree</th>
<th>No. samples examined</th>
<th><em>C. gattii</em> Prevalence (%)</th>
<th><em>C. neoformans</em> Prevalence (%)</th>
<th>Serotype, mating type, genotype</th>
</tr>
</thead>
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<tr>
<td>Eucalyptus sp.</td>
<td>20</td>
<td>10.6</td>
<td>0</td>
<td>MAT a, VGI, VN</td>
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<tr>
<td>Syzygium cumini</td>
<td>66</td>
<td>10.6</td>
<td>0</td>
<td>MAT a, VGI, VN</td>
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<tr>
<td>Poinciana longifolia</td>
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<td>10.6</td>
<td>0</td>
<td>MAT a, VGI, VN</td>
</tr>
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<td>Mangifera indica</td>
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<td>10.6</td>
<td>0</td>
<td>MAT a, VGI, VN</td>
</tr>
<tr>
<td>Castanopsis indica</td>
<td>35</td>
<td>10.6</td>
<td>0</td>
<td>MAT a, VGI, VN</td>
</tr>
<tr>
<td>Azadirachta indica</td>
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<td>10.6</td>
<td>0</td>
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</tr>
<tr>
<td>Acacia nilotica</td>
<td>19</td>
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<td>0</td>
<td>MAT a, VGI, VN</td>
</tr>
<tr>
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<td>0</td>
<td>-14.2</td>
<td>MAT a, VNI</td>
</tr>
<tr>
<td>Ficus religiosa</td>
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<td>17.6</td>
<td>0</td>
<td>MAT a, VNI</td>
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<td>17.6</td>
<td>0</td>
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</tr>
<tr>
<td>Mangifera indica</td>
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<td>17.6</td>
<td>0</td>
<td>MAT a, VGI, VN</td>
</tr>
<tr>
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<td>17.6</td>
<td>0</td>
<td>MAT a, VGI, VN</td>
</tr>
<tr>
<td>Aegle marmelos</td>
<td>1</td>
<td>0</td>
<td>17.6</td>
<td>MAT a, VNI</td>
</tr>
<tr>
<td>Alstonia scholaris</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>MAT a, VNI</td>
</tr>
<tr>
<td>Ficus religiosa</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>MAT a, VNI</td>
</tr>
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<tr>
<td>Aegle marmelos</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>MAT a, VNI</td>
</tr>
</tbody>
</table>

*Attempts at re-isolation were negative; **: All of these were bark samples; #: Percentage not given because only a solitary sample was tested
of Eucalyptus have received scant attention of the Australian investigators interested in the ecology of C. gattii and C. neoformans. For C. neoformans, the most important host tree was A. indica (prevalence 60%), followed by S. cumini (54%), M. hexandra (50%), C. fistula (47%), M. elengi, (15%), P. longifolia, (13%) and lower prevalence in the range of 5 - 8% for Alstonia scholaris, F. religiosa and Dalbergia sissoo. Most of the afore-mentioned host tree species had large canopies and conspicuous trunk hollows. As with C. gattii, Eucalyptus trees were among the least important host trees for C. neoformans. It seems pertinent to mention here that a much larger number of host tree species other than those recorded in India have been reported from other countries. This includes more than 10 tree species each from Colombia and Vancouver Island, Canada (Granados & Castaño, 2005; Kidd et al., 2007b). Interestingly, C. gattii has also been frequently isolated from a succulent cactus species Cephalocereus royenii in the Guanica Dry Forest, Puerto Rico (Loperena-Alvarez et al., 2010). It is anticipated that the current global list of host tree species for the two pathogens (already exceeding 50) will expand considerably with further ecologic studies in as yet unexplored and climatically divergent geographic regions in India or elsewhere.

Longitudinal surveillance and population density
Of all the host trees harboring C. gattii and C. neoformans identified in India, it is S. cumini that has been most extensively investigated and documented regarding its epidemiologic importance. In order to determine whether this tree species was transiently, intermittently or perennially colonized, 7 of the known positive S. cumini trees in Delhi were subjected to a long-term mycological surveillance (chapter 10). Significantly, C. gattii and C. neoformans were repeatedly re-isolated over a follow-up period of 4.2–5.2 years. Furthermore, the positive samples carried a population density, ranging from 3 × 10^5 to 6 × 10^6 CFU/g for C. gattii and 2 × 10^8 to 8 × 10^9 CFU/g for C. neoformans. The highest population density of C. gattii previously reported in any wood debris sample was 2.6 × 10^10 CFU/g in a C. neoformans species or any other specific tree species. Instead, it has a generalized ecologic association with decayed wood or other plant debris of a wide spectrum of diverse tree species. The major factor underlying wood colonization by C. gattii and C. neoformans is probably the ability of these pathogens to produce the enzyme laccase which has been implicated in degradation of wood lignin by Basidiomycetes (Kirk & Farrel, 1987; Thurson, 1994; Williamson, 1994; Eggert et al., 1996).

Soil
Cryptococcus neoformans and C. gattii has been frequently reported from soil and dust. However, these isolations are from samples that contained excreta of pigeons, other avian species or bats (Emmons, 1955; Ajello, 1958; Casadeval & Perfect, 1998). We isolated both C. neoformans and C. gattii from soil surrounding the base of a number of host trees harboring these pathogens in decayed wood of their trunk hollows (chapter 11). These host trees belonged to Syzygium cumini, Mimusops elengi, Polyalthia longifolia and Azadirachta indica. Of the 95 soil samples investigated, 25 were positive for C. neoformans, 23 for C. gattii and 5 for both of the species, yielding a prevalence of 26%, 24% and 5%, respectively. Depending upon the site of investigation, the prevalence in soil ranged from 11 to 50% for C. neoformans, 14–57% for C. gattii and 7–11% for concomitant occurrence of both the species in the same soil sample. Concerning the prevalence in soil with regard to individual host tree species, the highest for C. gattii at 29% was in the vicinity of S. cumini trees as against 25% and 20% in the vicinity of M. elengi and A. indica trees, respectively. For C. neoformans, the highest prevalence in soil was 31% in the vicinity of S. cumini, followed by 12.5% each for M. elengi and P. longifolia trees. None of the 10 control soil samples from an open playground away from the S. cumini trees harbouring C. neoformans yielded any isolation of C. gattii or C. neoformans. These findings are in concordance with the results of an extensive study on characterization of environmental sources of C. gattii including soil samples collected from within one meter of the base of many host trees investigated in British Columbia, Canada, and the Pacific Northwest of the US (Kidd et al., 2007b).

Aerial
Information on the aerial prevalence of C. neoformans and C. gattii in India is scarce. We isolated C. neoformans species complex in air samples taken close to the decayed trunks of all of the four perennially colonized S. cumini trees investigated. This observation indicated that dispersal of C. gattii and C. neoformans from one colonized tree could possibly occur to other trees by their aerosols which may be generated artificially or by natural disturbances of decayed wood surfaces due to a variety of agents such as wind, insects, birds, etc. Previously, C. gattii had been demonstrated by air sampling conducted under the canopy of Eucalyptus camaldulensis host trees in Australia, and in air samples collected near the hollows of two pink shower (Cassia grandis ) trees in Brazil (Lazéra et al., 2000; Ellis & Pfeiffer, 1990). On the other hand, attempts at aerial isolation of the fungus from the vicinity of host trees were unsuccessful in two instances, namely, one from India and one from Australia (Chakrabarti et al., 1997; Halliday et al., 2003). It is generally believed that cryptococcosis in humans and animals is acquired by inhalation of such aerosols from the environment. It is pertinent to mention in this context that a large scale outbreak of cryptococcosis due to C. gattii, serotype B, involving humans and terrestrial or marine mammals occurred during 2000-2003 on southern Vancouver Island, Canada and is ongoing. The outbreak was related to colonization on Vancouver Island by C. gattii of a large number of local trees such as Douglas fir (the majority), cedar, arbutus, alder, maple, spruce, Garry oak, etc, thus highlighting the risk of cryptococcosis developing in humans and animals following their exposure to such environmental reservoirs of the pathogen. The reported C. gattii concentrations (CFU/ml) in air samples were found to be significantly higher during the warm, dry summer months, although potentially infectious propagules (less than 3.3 μm in diameter) were present throughout the year (Kidd et al., 2007b).

The vast majority of cryptococcal infections, particularly in immunocompromised patients, are caused by C. neoformans, whereas C. gattii accounts for a smaller proportion of cases, often occurring in immunocompetent patients in tropical and subtropical regions. However, in the past decade, a more virulent genotype of C. gattii, VGIIIa/VGIIc, has emerged as a primary pathogen on Vancouver Island and its adjoining areas in Canada and the USA, indicating extension of its geographical domain to the temperate climate (Kidd et al., 2004;
Byrnes et al., 2010). The outbreak of human and animal cryptococcosis on Vancouver Island due to C. gattii indicated that exposure to environmental sources such as colonized trees and soil led to pulmonary and disseminated cryptococcosis. In India, a widespread colonization of decayed wood inside trunk hollows of diverse tree species by C. neoformans var. grubii and C. gattii serotype B has been documented (Randhawa et al., 2006; 2008; Hiremath et al., 2008). The objective of the studies were to compare antifungal susceptibility profiles of clinical isolates with those of environmental isolates of C. neoformans var. grubii and C. gattii serotype B originating from decayed wood of diverse tree species and from their surrounding soil in northwestern India.

Seasonal variations
It is understood that abiotic factors such as pH, humidity, temperature, sunlight and wind play an important role in the environmental prevalence and dissemination of C. neoformans and C. gattii. Most of the work done so far is on the occurrence of C. neoformans var. grubii in avian excreta which support its growth and multiplication under dry conditions (Ruiz et al., 1981; Caicedo et al., 1999; Montenegro & Paula, 2000; Kuroki et al., 2004; Grandos & Castaneda, 2005). In vitro studies by Martinez et al. (2001) have shown differences in thermo-tolerance between C. neoformans var. grubii (serotypes A) and C. neoformans var. neoformans (serotype D). However, the problem of seasonal variations in environmental prevalence of C. neoformans and C. gattii remains virtually unexplored. We conducted a retrospective study of seasonal variations in the prevalence of these pathogens in decayed wood inside trunk hollows of a wide spectrum of tree species investigated from five geographical locations in north-western India over a period of 7 years (2000–2007) (chapter 12). Climatically, north-western India has five distinct seasons, namely winter, spring, summer, rainy season and autumn. The data analyzed included results of isolation of C. neoformans and C. gattii from 1439 decayed wood samples collected from trunk hollows of 518 trees, representing 20 species. Of the 406 isolates of C. neoformans species complex, 247 were C. neoformans var. grubii (serotype A) and 171 were C. gattii, serotype B. Both pathogens were isolated during all the seasons, and the overall prevalence of C. neoformans var. grubii was significantly higher (17.2%) than that of C. gattii serotype B (11.9%, p < 0.0001). It indicated that decayed wood was as good, if not a better natural habitat for C. neoformans var. grubii as for C. gattii. Both of the pathogens revealed some seasonal variations in their prevalence, the highest being during the autumn, followed by that in the summer. For C. gattii, the prevalence during the winter was significantly less than that during the summer (p < 0.02) and the autumn (p < 0.02). In contrast, the lowest prevalence of C. neoformans var. grubii (10.7%) was in the rainy season which was significantly less than that in the autumn (p < 0.0001), followed by that in the summer (p < 0.0001) and winter (p < 0.001). Interestingly, a similar pattern of low prevalence of C. neoformans var. grubii in chicken faeces during rainy season and high prevalence during the dry season has been reported from Thailand (Kuroki et al., 2004). On the other hand, the low prevalence of C. gattii in decayed wood during winter was similar to that reported from Bogota, Colombia, where C. gattii had a low population density in bark samples but it was not found in decayed wood of trunk hollows investigated during January and February (Grandos & Castaneda, 2005).

Isolation of C. gattii from the temperate climate of the Netherlands
Cryptococcus gattii was considered to be geographically restricted to countries with tropical and subtropical climates until 1999, when an outbreak of cryptococcosis in humans and animals occurred in the temperate climate of Vancouver Island, British Columbia, Canada. Montagna et al. (1997) reported the first environmental C. gattii in Europe from the Mediterranean region of Italy. These observations were recently substantiated by the isolation of C. gattii from plant debris of trees belonging to Ceratonia siliqua (carob), Pinus halepensis (stone pine), and E. camaldulensis in Spain (Colom et al., 2012). An attempt was made to isolate C. gattii from the temperate climate of the Netherlands using the previously described techniques (chapter 13). We isolated the primary pathogenic fungus C. gattii from a forest in Berg en Dal, the Netherlands, which extends its geographical distribution to the temperate climate of northern Europe. One hundred and twelve decayed wood samples were collected from inside trunk hollows of 52 living trees belonging to 5 families during April–May 2011 in the Nijmegen area, the Netherlands. The trees sampled were chestnut (Castanea sativa, n = 24), Douglas fir (Pseudotsuga menziesii, n = 17), oak (Quercus macranthera, n = 6), walnut (Juglans regia, n = 3), and mulberry (Morus alba, n = 2). The decayed wood samples were collected with an in-house swabbing technique by using simplified Staub niger seed agar as described (Randhawa et al., 2005). Identity of the isolates was confirmed by sequencing the internal transcribed spacer and D1/ D2 regions, and they were genotyped by using amplified fragment-length polymorphism (AFLP) fingerprinting and multilocus sequence typing (MLST). The MLST loci CAP10, CAP59, GDP1, IGS, LAC1, MPD1, PLBI, SOD1, TEF1a, and data were compared with MLST data from a large C. gattii population study (Fraser et al., 2005) and with a recently published set of clinical, animal, and environmental C. gattii isolates from Mediterranean Europe and the Netherlands (Figure) (Janssens et al., 1957; Hagen et al., 2009; Colom et al., 2012). In addition, the mating type was determined with PCR by using mating type–specific primers for the STE12a and a alleles (Hagen et al., 2010). MLST analysis showed that the C. gattii isolates in our study are more closely related to the clinical isolate from the Netherlands (Janssens et al., 1957) and to the clinical and environmental C. gattii isolates (AFLP4/VGI) reported from the Netherlands and other countries in Europe than to isolates from outside Europe.

Conclusion and future studies
The present thesis generated data on environmental prevalence, population structure, seasonal variations and antifungal susceptibility of Cryptococcus neoformans and Cryptococcus gattii isolates prevalent in India. An overview of work done emphasizes that the primary ecologic niche of both Cryptococcus neoformans and Cryptococcus gattii pathogens is decayed wood in trunk hollows of a wide spectrum of host trees. Overall, C. neoformans showed a higher environmental prevalence than that of C. gattii which was not found in the avian habitats. Apart from their arboreal habitat, both species were demonstrated in soil and air in close vicinity of their tree hosts. An overwhelming number of C. neoformans strains belonged to genotype AFLP1/VNI, var. grubii (serotype A), whereas C. gattii strains were genotype AFLP4/VGI, serotype B. All of the environmental strains
of *C. neoformans* and *C. gattii* were mating type α (MATα). Interestingly, despite lack of mating type a (MATa) strains, unambiguous evidence for recombination was observed which supported the hypothesis that strains of *C. neoformans* may undergo sexual reproduction on decaying wood of various host tree species. Population genetic analyses of *C. gattii* revealed limited evidence of recombination but unambiguous evidence for clonal reproduction and expansion. However, the observed clonal expansion has not obscured the significant genetic differentiation among populations from either different geographic areas or different host tree species. The antifungal susceptibility data obtained in this study indicate that the occurrence of primary resistance among environmental and clinical isolates of *C. neoformans* serotype A and *C. gattii* serotype B is rare, and serotype B isolates are less susceptible than serotype A isolates.

It is notable that the environmental isolation of the primary pathogenic fungus *C. gattii* from a forest in Berg en Dal, the Netherlands, extended its geographic distribution to the temperate climate of northern Europe. Our detection of *C. gattii* in the environment in the Netherlands suggests that this pathogen is endemic to the temperate climate of northern Europe. This suggestion agrees with the concept emerging from a decade of investigations in Canada and the Pacific Northwest that the geographic distribution of *C. gattii* extends to the temperate region, albeit with another AFLP genotype. Comprehensive prospecitive studies are warranted in order to gain an insightful knowledge of environmental prevalence of *C. neoformans* and *C. gattii* not only in decayed wood but also in other natural substrates such as avian excreta and soil. The geoclimatically divergent regions of India with their rich variety of flora and fauna offer a wide scope for further investigations on the environmental prevalence of *C. gattii* and *C. neoformans* and their population structure. Currently, the occurrence of *C. gattii* serotype C in the environment in India is entirely unknown. Likewise, there are scarcely any data concerning the environmental distribution of *C. neoformans* var. *neoformans* (serotype D) which is largely known from Europe. Further studies are required to compare the genetic structure of clinical and environmental strains of both pathogens with a view to probing the extent of their inter-relationship. Such a study may shed a new light on the origin of subgroups of various genotypes. There is little information at present on the prevalence of cryptococcosis in animals or humans in the areas of north-western India where host trees are perennially colonized by *C. gattii* and *C. neoformans*. Comprehensive clinico-mycological investigations are warranted to probe the magnitude of health hazard posed by the environmental prevalence of *C. gattii* and *C. neoformans*.

### References


Chapter 1
General Discussion and Summary


Samenvatting

Dit proefschrift beschrijft de moleculaire epidemiologie, seizoensgebonden schommelingen in voorkomen in de omgeving en in vitro gevoeligheid van Cryptococcus neoformans en Cryptococcus gattii gisten uit India. Onderzoek laat zien dat de primair ecologische niche van de pathogenen Cryptococcus neoformans en Cryptococcus gattii vermolmd hout is in holtes van stammen van een groot aantal verschillende gastheerbomen. In het algemeen werd Cryptococcus neoformans vaker aangetroffen in de natuur dan Cryptococcus gattii, welke niet werd gevonden in gebieden met vogel uitwerpselen. Beide soorten werden evenwel aangetoond op diverse bomen als ook in de bodem en lucht in de nabijheid van bomen. Het overgrote deel van de soort Cryptococcus neoformans behoorde tot genotype AFLP1/VNI, var. grubii (serotype A), terwijl Cryptococcus gattii vooral bleek te behoren tot genotype AFLP4/VGI, serotype B. Alle onderzochte stammen van Cryptococcus neoformans en Cryptococcus gattii geïsoleerd uit omgevingskweken bleken te behoren tot mating type α (MATα). Interessant is dat, ondanks dat mating type a (MATa) stammen niet werden gevonden, er bewijs voor recombinatie is waargenomen. Dit ondersteunt de hypothese dat Cryptococcus neoformans stammen seksuele reproductie op rottend hout van diverse boomsoorten kunnen ondergaan. Bij populatie genetische analyses van Cryptococcus gattii bleek slechts een beperkt bewijs van deze recombinatie, maar wel een duidelijk bewijs voor clonale voortplanting en verspreiding aanwezig te zijn. Toch heeft de waargenomen clonale expansie de grote genetische differentiatie tussen populaties uit verschillende geografische gebieden of uit verschillende boomsoorten niet vertroebeld. De in vitro gevoeligheidsdata voor anti-schimmel middelen die zijn verkregen in deze studie lieten zien dat het optreden van primaire resistentie bij omgevings- en klinische isolaten van Cryptococcus neoformans serotype A en Cryptococcus gattii serotype B zeldzaam is, maar wel dat serotype B-isolaten minder gevoelig bleken te zijn dan serotype A isolaten.

Een opvallende waarneming is dat de primair pathogene schimmel Cryptococcus gattii ook gevonden is in een bos in Berg en Dal, Nederland, waardoor aangetoond is dat de verspreiding van deze zogenaamde “tropische schimmel” tot in het gematigde klimaat van Noord-Europa doorgedrongen is. Het aantonen van Cryptococcus gattii in de natuur in Nederland doet sterk vermoeden dat deze ziekteverwekker endemisch is in het gematigde klimaat van Noord-Europa. Hoewel zeker nog meer onderzoek nodig is, is deze vondst in overeenstemming met de bevinding, voortgekomen uit een decenniumlange onderzoek in Canada en de Pacific Northwest van de Verenigde Staten, dat de geografische spreiding van Cryptococcus gattii zich uitstrekt tot in gematigde klimaatstreken. Uitgebreide prospectieve studies zijn nodig om inzicht te krijgen in het voorkomen in de natuur van Cryptococcus neoformans en Cryptococcus gattii. Niet alleen vermolmd hout, maar ook andere natuurlijke substraten zoals vogeluitwerpselen en grond zijn potentiële bronnen van deze schimmel.

De grote verscheidenheid in klimaat in de diverse streken van India met hun grote variëteit aan flora en fauna bieden unieke mogelijkheden voor verder onderzoek naar het voorkomen in de omgeving van Cryptococcus gattii en Cryptococcus neoformans en de daarbij behorende populatie structuur. Momenteel is de aanwezigheid in de natuur van Cryptococcus gattii serotype C in India nog volledig onbekend. Ook zijn er nauwelijks gegevens over de verspreiding in het milieu van Cryptococcus neoformans var. neoformans (serotype D), een subtype dat vooral bekend is uit Europa. Verder onderzoek is nodig om de genetische structuur van klinische en omgevingsstammen van deze ziekteverwekkers te vergelijken, met het doel om meer kennis te verwerven over de omvang van hun onderling verband. Een dergelijk onderzoek kan nieuw licht werpen op de oorsprong van de subgroepen uit de verschillende genotypen van Cryptococcus. Op dit moment is er weinig informatie bekend over de prevalentie van cryptococcose bij mens of dier in het noord-westen van India, waar zeer veel bomen zijn gekoloniseerd met Cryptococcus gattii en Cryptococcus neoformans. Uitgebreid klinisch-mycologisch onderzoek is gerechtvaardigd om de omvang van het potentiële gevaar voor de volksgezondheid als gevolg van het voorkomen van Cryptococcus gattii en Cryptococcus neoformans in de omgeving in kaart te brengen.
Epilogue, Curriculum vitae and List of publications
Epilogue

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Curriculum vitae

Anuradha Chowdhary was born in Delhi, India. She obtained her MB,BS (1991) and MD, Microbiology (1996) from the University of Delhi, Lady Hardinge Medical College, Delhi. She received post-doctoral training in Medical Mycology at the Centers for Disease Control and Prevention, Atlanta, USA (2002-2003) and worked as a visiting scientist at the Department of Biology, McMaster University, Hamilton, Ontario, Canada (2009). In 2009 she attended the course “Advanced Laboratory Training in Molecular Medical Mycology” conducted by The University of Melbourne, Australia and Mahidol University, Thailand in collaboration with Howard Hughes Medical Institute, USA. Since 2005 Anuradha Chowdhary is an associate professor in Medical Mycology, Vallabhbhai Patel Chest Institute, University of Delhi. Her longtime research interest, together with Prof. Randhawa at the same institute, has been cryptococcosis. She came in contact with Prof. Mouton and Dr. Meis in 2010 and plans were made to focus research efforts towards a PhD training project at the Radboud University Medical Center and Canisius Wilhelmina Hospital, Nijmegen. To finish and extend her ongoing experiments she worked as a fellow in Nijmegen for 3 months in 2011. She is married with Sunil Kumar and has a 6-year-old daugther Destiny.

List of publications


