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Clonality and Recombination in Genetically Differentiated Subgroups of *Cryptococcus gattii*†

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***Cryptococcus gattii* is a pathogenic yeast that together with *Cryptococcus neoformans* causes cryptococcosis in humans and animals. High numbers of viable *C. gattii* propagules can be obtained from certain species of Australian *Eucalyptus camaldulensis* trees, and an epidemiological link between *Eucalyptus* colonization and human exposure has been proposed. However, the highest prevalence of *C. gattii* cryptococcosis occurs in Papua New Guinea and in regions of Australia where the eucalypt species implicated to date are not endemic. This study investigated the population structure of three geographically distinct clinical and veterinary populations of *C. gattii* from Australia and Papua New Guinea. All populations that consisted of a genotype found frequently in Australia (VGI) were strongly clonal and were highly differentiated from one another. Two populations of the less common VGII genotype from Sydney and the Northern Territory had population structures inferring recombination. In addition, there was some evidence of reduced genetic differentiation between these geographically remote regions. In a companion study presented in this issue, VGII isolates were overwhelmingly more fertile than those of the VGI genotype, giving biological support to the indirect assessment of sexual exchange. It appears that the VGI genotype propagates clonally on eucalypts in Australia and on an unknown substrate in Papua New Guinea, with infection initiated by an unidentified infectious propagule. VGII isolates are completing their life cycles and may be dispersed via sexually produced basidiospores, which are also likely to initiate respiratory infection.**

Fungal organisms employ a diverse array of mechanisms in sexual reproduction. In about 20% of known fungal species, however, a sexual stage has never been documented (18). Phylogenetic analysis has found these apparently asexual fungi to frequently have their closest relatives among the sexual fungi, which implies a recent loss of sexual functions (27). In addition, molecular studies reveal some purportedly asexual species to possess a recombining population structure, indicating that cryptic sex occurs via an unknown mechanism (5, 13, 39, 47). Some fungal species do appear to be genuinely asexual, but as most fungi can release asexual propagules, often in large numbers, under favorable conditions, the possibility remains that clonality is restricted to the population under study, and a sexual population of the same species occurs elsewhere (11, 48).

Sex is considered important for maintaining eukaryotic species in the long term. Experimental studies of yeasts have found sexual strains to have a significantly increased rate of adaptation to harsh environmental conditions compared to asexual strains (14). Completion of the fungal life cycle is therefore likely to be a fundamental part of growth in a stable ecological system. Finding clonal behavior in sexually competent species often points to recent disturbances such as habitat fragmentation or importation of foreign species into favorable, naïve areas. Conversely, finding recombination may help define the natural ecological niche of the organism. In pathogenic eukaryotes, an understanding of whether or not sexual recombination occurs is important for the development of successful diagnostic and treatment procedures (41, 42). In recombining organisms, genetic material governing virulence or antimicrobial resistance traits can be passed between strains and reassorted to produce novel combinations. A necessary assumption in a recombining population is that any strain could acquire virulence factors and become pathogenic.

Cryptococcus gattii and the closely related *Cryptococcus neoformans* are emerging yeast pathogens causing cryptococcosis, a disease which varies in severity from essentially asymptomatic to severe, life-threatening meningoencephalitis. While *C. neoformans* is a major AIDS pathogen, *C. gattii* is uncommon in AIDS patients, and most cases occur in otherwise apparently healthy people. *C. gattii* is more geographically restricted than *C. neoformans* and is largely confined to tropical and subtropical regions. An association between this yeast and certain

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species of *Eucalyptus* was reported by Ellis and Pfeiffer (9, 31) and may in part explain the relatively high incidence of *C. gattii* cryptococcosis in Australia and in subtropical regions to which eucalyptus trees have been exported.

Within *C. gattii*, a number of genetically distinct subgroups occur, designated VGI to VGIV (8), or amplified fragment length polymorphism (AFLP) groups 4 to 7 (3) (T. Boekhout, personal communication). VGI (AFLP 5) is the most widespread molecular type and accounts for most clinical and environmental isolates in Australia. VGII (AFLP 6) is mostly restricted to the Northern Territory (NT) and Western Australia but is more common in South America, the northwestern United States, and Canada, in particular, Vancouver Island (28, 45). VGIII (AFLP 4) appears to be common in Colombia and has been found in India and the United States, while VGIV has been reported in Africa and Central America (8, 28). Diversity between each molecular type is high, and it is likely that gene flow between these types is infrequent or absent.

An analysis of mating type and recombination in a *C. gattii* VGI population collected from *Eucalyptus camaldulensis* trees within a limited geographic region in Australia found that although both mating types could be recovered in an approximately 1:1 ratio (17), strong linkage disequilibria occurred between genetic loci, and it was concluded that this population was clonal (17). In addition, an analysis of the relationship between isolate genotype and host tree indicated that dispersal and genetic exchange between host trees was negligible. This, together with the finding of high levels of *C. gattii* cryptococcosis in Papua New Guinea (PNG) (24), where the normal host eucalypts are not found, has challenged the assumption that *Eucalyptus* is the only environmental niche for *C. gattii*.

The current study set out to examine further populations of *C. gattii* for evidence of genetic exchange. Clinical populations were targeted, with the assumption that infections are likely to have been initiated by sexually produced basidiospores (37). The two human clinical populations were obtained from patients living in Papua New Guinea and the Northern Territory of Australia. Both regions do not harbor the usual *Eucalyptus* host trees, therefore eliminating the possibility of clonal propagation on these tree species. The third population included veterinary isolates from the Sydney region. As animals are far more limited in their geographic range than humans, this population was considered less likely to contain isolates from genetically isolated regions, which might skew the analysis. Molecular type, mating type, and recombination were assayed in all populations. We report evidence of both clonality and sexual recombination in separate *C. gattii* populations.

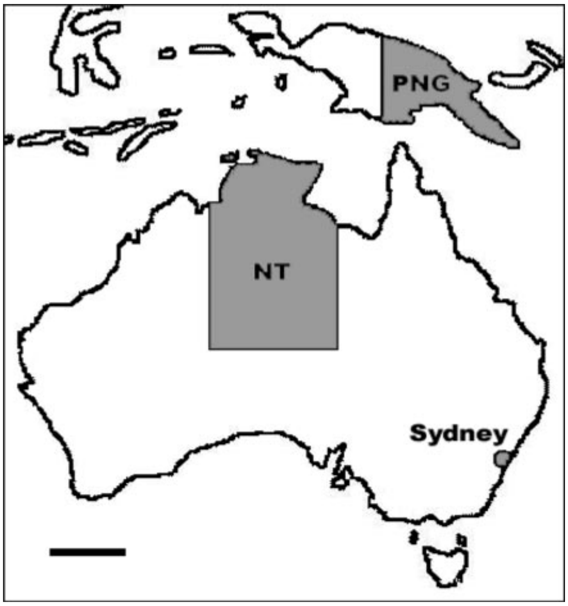

FIG. 1. Regional map showing areas from which isolates were obtained. Scale bar, 1,000 km.

MATERIALS AND METHODS

Strains and media. All strains used in this study are listed in the supplemental material (see Table S1 in the supplemental material). Isolates from PNG and the NT were from human clinical infections (Fig. 1). The majority of the NT isolates were from indigenous patients. The Sydney isolates were from a variety of veterinary sources including domestic cats and dogs and some native wildlife (30). Mating type reference strains CBS5757 (serotype B, MATα) and CBS6998 (serotype B, MAT**a**) were obtained from the Centraalbureau voor Schimmelcultures culture collection, and molecular type standard reference strains WM179 (VGI/AFLP 4), Ram2 (VGII/AFLP 6), WM161 (VGIII/AFLP 5), and WM779 (VGIV/AFLP 7) were from the Molecular Mycology Research Laboratory culture collection at Westmead Hospital. Isolates were obtained from source laboratories as either freeze-dried cells or on plates and were cultured on Sabouraud dextrose agar.

DNA extraction. Chromosomal DNA extraction was based on the Novozyme 234 dodecyltrimethylammonium bromide and hexadecyltrimethylammonium bromide method described previously by Wen et al. (44), with the following modifications: approximately 0.75 g of cells (wet weight) grown on Sabouraud dextrose agar was collected, the protoplasting solution was made with 10 mg/ml of Novozyme 234 in SCE buffer (100 mM sodium citrate, 1 M sorbitol, 10 mM EDTA), and all centrifugation steps were performed at 13,000 × *g*. The DNA pellet was resuspended in 100 μl of Tris-EDTA buffer (100 mM Tris-HCl, 0.1 mM EDTA [pH 8]). DNA was diluted 1:10 for PCR amplification.

Mating type analysis. Mating type analysis was carried out by PCR amplification using MFα primers (upper and lower sets) and the $STE20_{SF}$**a** primer sets (15, 17) which are specific to the mating type regions of α and **a** mating type cells, respectively. PCR amplifications were performed in 50-μl reaction mixtures containing 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 5% glycerol, 6.25 μM concentrations of each deoxynucleoside triphosphate, 0.2 μM concentrations of each primer, 2.5 U of *Taq* DNA polymerase, and 1 μl of diluted template DNA. Amplification conditions for PCR were 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min and a final extension step at 72°C for 7 min. All amplifications were carried out in a Perkin-Elmer 2400 thermal cycler. Ten microliters of each amplification product was electrophoresed at 10 V/cm in 2% agarose gels containing 0.5 ng/ml ethidium bromide. The gels were visualized by UV transillumination and photographed. Culture collection strains of known mating type were used as controls (see Table S1 in the supplemental material).

Molecular type analysis. Molecular type was determined by DNA fingerprinting and restriction fragment length polymorphism (RFLP) analysis. Fingerprinting was performed using the simple repetitive sequence primer $(GACA)_4$ (2, 29). Amplification conditions were 94°C for 5 min followed by 35 cycles of 94°C for 20 seconds, 50°C for 30 seconds, and 72°C for 20 seconds and a final extension step at 72°C for 7 min. Ten microliters of each amplification product was electrophoresed on 2% agarose Tris-acetate-EDTA gels and visualized by UV transillumination. The protocol used to determine molecular type via RFLP was modified from a method described previously by Meyer et al. (28). The *URA5* gene was amplified in 50 μl containing 50 ng DNA, 1× PCR buffer (10 mM Tris-HCl, [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM of deoxynucleoside triphosphate, 3 mM MgAc, 1.5 units AmpliTaq DNA polymerase, and 50 ng of each of the primers URA5 and SJO1. PCR amplification was 1 cycle of 94°C for 3 min and 35 cycles of 94°C for 45 s, 61°C for 1 min, and 72°C for 2 min followed by 1 cycle of 72°C for 10 min. PCR products were digested with Sau96I and HhaI for 3 h and separated on 3% agarose gels by electrophoresis at 10 V/cm for 2 h. Standard strains of known molecular type were used as controls (see Table S1 in the supplemental material).

AFLP. The AFLP fingerprinting protocol was based on the technique described previously by Vos et al. (43) AFLP primers EcoRI-TG–MseI-CA and

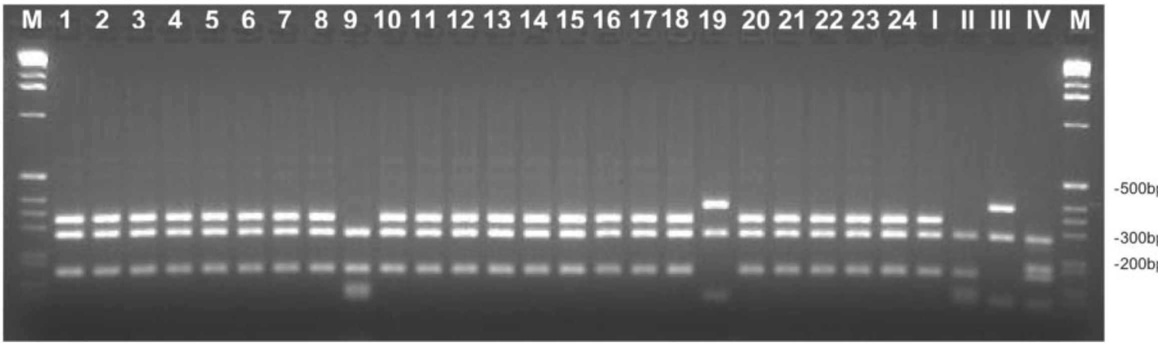

FIG. 2. Molecular typing profiles generated via RFLP analysis of *URA5*. Lane M, 1Kb Plus DNA ladder (Invitrogen); lanes 1 to 24, sample strains from PNG; lanes I to IV, molecular type standard strains WM179 (VGI), Ram2 (VGII), WM161 (VGIII), and WM779 (VGIV). All samples shown are molecular type VGI except lane 9, which is VGII, and lane 19, which is VGIII.

EcoRI-CA–MseI-GT were used with minor modifications to the procedure described previously by Halliday and Carter (16). All amplifications were carried out in a Perkin-Elmer 2400 thermal cycler.

AFLP fragment detection and analysis. A total of 0.5 μl of Genescan-400HD ROX standard size marker (Applied Biosystems) was mixed with 10 μl of Hi-Di Formamide (Applied Biosystems) and aliquoted into 0.5-ml Eppendorf tubes. Selective amplification products were diluted 100-fold in distilled H_2O, and 1 μl of this was added to the ROX standard. Samples were then denatured at 94°C for 5 min and placed on ice for 5 min before being loaded onto an ABI 3700 analyzer. Samples were autoanalyzed using the GS400HD analysis module. Data collection, fragment sizing, and pattern analyses were done with GeneScan, version 3.1, analysis software (Applied Biosystems). Polymorphic loci were defined as bands of the same mobility present in some isolates and absent in others. For each polymorphic locus, there were two possible alleles, which were scored as 1 when the amplified fragment was present and 0 when the fragment was absent. Data matrices were produced using LecPCR (40). A polymorphic locus was included in the analysis only if it was present or absent in at least five of the isolates, was strongly amplified, and was greater than 40 bp and less than 500 bp in size.

Data analysis. The index of association (I_A) (32), RbarD (1), and tree length (T_L) tests (5) were used to distinguish between recombining and clonal modes of reproduction. I_A and RbarD are statistical tests that measure the degree of nonrandom association between alleles at different loci (linkage disequilibrium), with the RbarD algorithm adjusting for limited numbers of loci, and were calculated by using the Multilocus, version 1.0b, software (1). The T_L test uses the permutation test in PAUP* (version 4.0b4a) to calculate the length of phylogenetic trees by treating the isolates as taxa and the alleles at each locus as phylogenetic characters with two character states. Both analyses involved comparing the values for the observed data set with the values for 1,000 artificially recombining data sets. Artificially recombining data sets were constructed by randomly shuffling the alleles for each locus between members of the population while keeping the proportions of alleles at each locus constant. The inability to distinguish between the observed data set and the artificially recombined data sets supports the null hypothesis of sexual recombination, whereas a significant difference between the data sets supports clonality (5, 6, 16). Genetic differentiation between populations, assessed as theta (θ), which is an estimate of Wright's F_{st}, the standard measure of population subdivision, was also calculated using the Multilocus, version 1.0b, software (1). This compares loci present in different populations to give a measure of the amount of gene flow between populations. If different populations have the same allele frequencies at all loci, then θ will be zero, indicating no genetic differentiation. Conversely, if the populations are fixed for different alleles at all loci, then θ will be 1, indicating total genetic differentiation. The significance of θ is again assessed by comparing the observed data set with 1,000 artificially produced data sets in which the alleles are randomized among populations.

TABLE 1. Molecular and mating types of *C. gattii* isolates from Australia and PNG

Population	No. (%) of isolates				
	Molecular type			Mating type[a]	
	VGI	VGII	VGIII	α	**a**
PNG (n = 37)	31 (83.8)	2 (5.4)	4 (10.8)	35 (94.6)	2 (5.4)
NT (n = 21)	9 (42.9)	12 (57.1)	0	19 (90.5)	2 (9.5)
Sydney (n = 23)	17 (73.9)	6 (26.1)	0	23 (100)	0
Total (n = 81)	57 (70.4)	20 (24.7)	4 (4.9)	77 (95.1)	4 (4.9)

[a] All **a** mating type strains were the VGI molecular type.

RESULTS

Molecular type analysis and distribution. DNA fingerprinting using $(GACA)_4$ primers and RFLP analysis of the *URA5* gene were used to generate digestion profiles for the 81 isolates which were compared with those produced for each molecular type standard (Fig. 2). Of the 81 isolates, 70% were molecular type VGI, 25% were VGII, and 5% were VGIII. Within the PNG population, the vast majority of isolates (84%) were molecular type VGI, 5% were VGII, and 11% were VGIII. In the NT population, VGI isolates were outnumbered by VGII by 43% to 57%. The Sydney population contained 74% VGI and 26% VGII isolates (Table 1). No VGIII isolates were found in the Australian populations.

Mating type analysis. MFα primers amplified a 109-bp fragment in the culture collection strain of a known α mating type (CBS5757) but failed to amplify any fragment in the control **a** mating type strain (CBS6998). The $STE20a_{SF}$ primers were similarly used to amplify a 219-bp fragment from the **a** mating type only. Mating type analysis of the 81 isolates indicated a significant bias towards the α mating type, with a 77:4 MATα:MAT**a** ratio. Within population groups, the ratio of MATα:MAT**a** varied. In the PNG population, this was 35:2; in the NT population, the ratio was 19:2; and the Sydney veterinary population did not include any MAT**a** isolates among the 23 isolates tested (Table 1). All MAT**a** strains were molecular type VGI.

AFLP data analysis. A total of 55 polymorphic loci were identified across the isolates from the three populations with allele frequencies varying in each population (see Table S2 in the supplemental material). Seventy-six percent of loci were polymorphic in all populations. Five loci were polymorphic in the PNG and NT populations but were fixed in the Sydney population, four were restricted to the NT and Sydney populations, and two were restricted to the PNG and Sydney populations. The PNG and NT populations each had a single polymorphic locus that was fixed in the other two populations. A comparison of molecular types revealed nine loci that were polymorphic in only the VGI isolates and four that were unique to VGII.

Population genetic and phylogenetic analyses of multilocus genotype data. (i) Phylogenetic analysis. The phylogenetic analysis program PAUP* (version 4.0b4a) (38) was used to produce a parsimony phylogram from PAUP*-compatible files generated by Multilocus (version 1.0b) software (1) (Fig. 3). Only bootstrap values greater than 60 were included on the phylogram. Most isolates clustered first according to molecular type and then according to geographic location. Three PNG VGI isolates and one VGII isolate from the NT branched with the two VGII and four VGIII isolates from PNG (Fig. 3). All these isolates had relatively distinct genetic profiles, and long-branch attraction may be responsible for this apparent clustering.

(ii) Index of association data for the three geographically isolated populations. I_A, RbarD, and T_L values were generated for each molecular type containing five or more isolates within each of the three populations. The analysis was confined to molecular type groups, as the significant level of genetic variation between molecular types would skew the results in favor of clonality (13). I_A, RbarD, and T_L values for all VGI isolates and for each geographically separate VGI population were significantly different from their associated recombining data sets, indicating clonality (Table 2). Tests of subgroups, evident on the phylogram (indicated by branches labeled C1 to C4 in Fig. 3), also gave strongly clonal results. In contrast, the Sydney VGII veterinary population returned values for each test that lay well within the range of values obtained for the recombined data sets, and the null hypothesis of recombination could not be rejected (P = 0.428 to 0.488). When isolate V21, which grouped more closely with two NT isolates (NT14 and H16), was removed from the analysis, the P value increased significantly (P = 0.974 to 0.999). Analysis of the complete NT VGII population gave a significant difference between the observed and the randomly generated data sets indicating clonality. Analysis was then conducted on subgroups evident on the phylogram (NT1 and NT2) within this population. The eight NT VGII isolates grouping on branch NT1 (Fig. 3) still indicated clonality (P = 0.001); however, five isolates within this group, on branch NT2 (Fig. 3), gave values that strongly suggested recombination (P = 0.849 to 0.989) (Table 2).

(iii) Genetic differentiation between populations. The θ values obtained for VGI groups in Sydney, the NT, and PNG were all highly significant, indicating strong genetic differentiation between each of these regions (Table 3). When Sydney veterinary VGII isolates were compared with all VGII isolates from the NT, significant differentiation was also seen. However, when the comparison was confined to NT isolates that branched with the veterinary isolates on the phylogram (Fig. 3), significance dropped to a P value of 0.039, indicating the possibility of allele sharing between these populations.

DISCUSSION

Recombination occurs in *C. gattii* populations belonging to molecular type VGII. We report here evidence of recombination occurring in two VGII populations of *C. gattii* from distinct geographic regions of Australia. In Australia, VGII is a less common cause of *C. gattii* cryptococcosis than VGI, and there have been few isolations of VGII from the environment (3, 7, 22, 33, 34). However, a high proportion of VGII isolates from clinical samples in the Northern Territory has been reported, which was also found in the isolates analyzed in the current study. Most interestingly, VGII isolates have recently been found to be the cause of the first documented "outbreak" of cryptococcosis on Vancouver Island, British Colombia (12, 23). To date, this outbreak has resulted in over 60 human infections including 4 deaths and in excess of 250 animal infections (19, 25, 36). However, while most of the Vancouver Island VGII isolates were fertile in mating studies, where typeable, all were of the α mating type and genetic diversity was extremely restricted, suggesting a clonal epidemic structure (12, 23).

VGI isolates from Australia and PNG have a clonal population structure. In contrast to VGII, all VGI populations had highly clonal structures. Clonality was particularly strong in the largest VGI group from Papua New Guinea. PNG has a relatively high prevalence of cryptococcal disease. This, coupled with the absence of the usual *Eucalyptus* host (24), made it a promising candidate as a center of origin of *C. gattii* in which the fungus might complete its life cycle in connection with its true ecological niche. Instead, the appearance of the PNG VGI cluster on the phylogram (Fig. 3) is more suggestive of a clonal bloom with all isolates very closely related and appearing to stem from a recent common ancestor. *C. gattii* has never been isolated from the environment in PNG despite concerted attempts and targeting of likely environmental niches (24). Our data suggest, as with *Eucalyptus* species in Australia, that a favorable substrate on which extensive clonal propagation occurs might exist. Interestingly, two **a** mating type strains occurred within the PNG cluster, and it is possible that some inbreeding occurs but with such closely related strains that clonality is not disrupted.

Indirect assessment of recombination is linked to biological mating in *C. gattii*. Analyzed as a whole, the NT VGII population returned a clonal population structure, and it was not until subgroups on the phylogram were identified that evidence of recombination was found. This shows the value of an initial phylogenetic analysis in which substructure within populations can be identified. Assessing recombination in a population that is genetically subdivided will skew results in favor of clonality, as any alleles that are fixed across the division will appear linked (13). Clonality can also be erroneously concluded when loci are physically linked or when clone mates are oversampled from a population (32). It is more difficult to incorrectly conclude that recombination has occurred, although this can happen if high levels of homoplasy, due, for example, to hypermutation, occur (20).

Our major concern in the current study was that the recombining populations found were small, which in turn reduced the number of polymorphic loci. I_A and RbarD values were generated for several small (n = 5) randomly chosen groups of VGI isolates selected from clonal populations; these consistently remained clonal, indicating that population size was not affecting our results (data not shown). Homoplasy was also considered unlikely, as the level of genetic diversity observed in the recombining populations was not particularly high, with conserved loci outnumbering polymorphic loci by 28 to 6 in the NT population and 40 to 10 in the Sydney population.

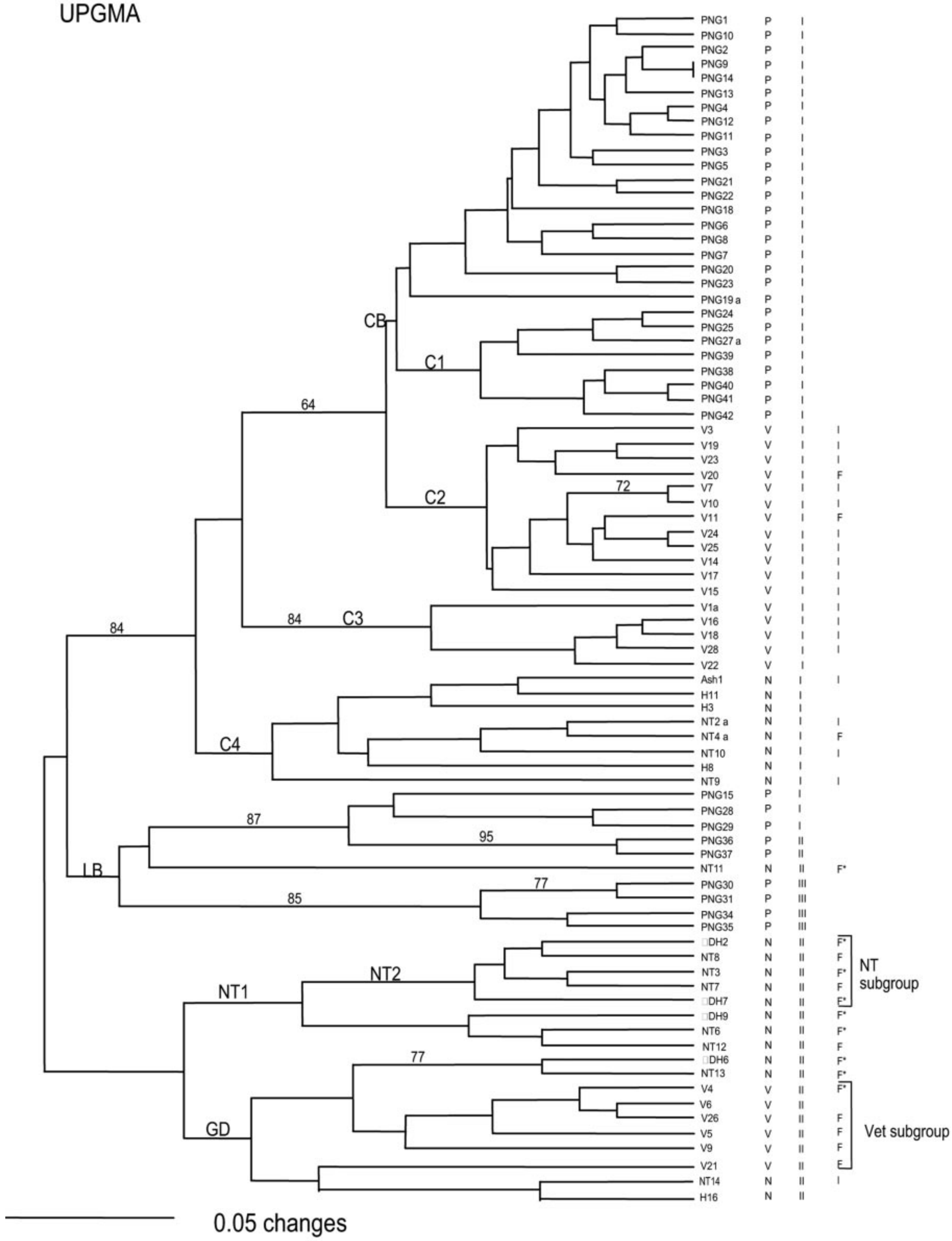

FIG. 3. Unweighted-pair group method using average linkages (UPGMA) phylogram of clinical populations of *C. gattii* from Australia and Papua New Guinea. The phylogenetic tree was generated with PAUP*, version 4.0b4a, software (D. L. Swofford, Sinauer Associates, Inc., Sunderland, Mass.). Isolate names are at branch tips, and the first column (from left) indicates geographic sources P (PNG isolates), N (Northern Territory isolates), and V (Sydney [veterinary] isolates). The second column denotes molecular or VG type I, II, or III. The third column denotes isolates tested for fertility indicated by I (infertile), F (fertile), or F∗ (robustly fertile). NT subgroup and veterinary (Vet) subgroup brackets indicate recombining populations. **a** indicates MAT**a** isolate. Labels above branches indicate clonal subgroups (C1 to C4), PNG clonal bloom (CB), long-branch attraction cluster (LB), NT VGII subgroups (NT1 and NT2), and genetically undifferentiated subgroup (GD). Numbers above lines are bootstrap values (only values of >60 are shown). Most isolates segregated according to molecular type and geographic location.

TABLE 2. Recombination test results for *C. gattii* populations and subpopulations

Population	VGI value (*P* value)			VGII value (*P* value)		
	I_A	RbarD	T_L	I_A	RbarD	T_L
PNG	0.720 (0.001)	0.040 (0.001)	72 (0.001)			
NT	1.110 (0.001)	0.040 (0.001)	22 (0.005)	2.430 (0.001)	0.070 (0.001)	83 (0.001)
NT1 subgroup				1.978 (0.001)	0.152 (0.001)	22 (0.002)
NT2 subgroup				−0.334 (0.849)	−0.067 (0.849)	8 (0.989)
Sydney	1.880 (0.001)	0.070 (<0.001)	75 (0.001)	0.065 (0.428)	0.007 (0.428)	17 (0.488)
Sydney (V21 excluded)				0.646 (0.974)	0.043 (0.974)	22 (0.999)

The most significant evidence supporting recombination in these populations comes from the concurrent study by us and colleagues investigating mating in Australian *C. gattii* strains (5a). That study identified 27 fertile Australian isolates capable of mating, and of these isolates, 18 have been included in the current analysis. The overwhelming majority of strains showing any sign of fertility and all "robust maters" were of molecular type VGII. When superimposed on the results of the indirect analysis used in this study, the recombining populations and the isolates shown to be capable of mating in the laboratory were strongly correlated (Fig. 3). However, several NT isolates outside the recombining group were also fertile. It is possible that these were sampled from separate, genetically unconnected populations in which recombination occurs. The Northern Territory is a very large region (almost twice the size of the state of Texas), and individual infections might be acquired from very geographically separate areas or even outside this region, given the propensity of humans to travel.

Genetic differentiation between populations. Previous studies have indicated widespread homogeneity of *C. gattii* throughout Australia (34, 35). The development of more highly discriminating molecular techniques, combined with powerful statistical analyses, revealed significant genetic differentiation between VGI isolates from Sydney, NT, and PNG. This result was not surprising considering the geographic distance between these populations. Genetic differentiation between the VGII populations was considerably lower, and only borderline differentiation was seen between the Sydney population and NT isolates that branched with it on the phylogram (Fig. 3) ($P = 0.039$). Again, the number of isolates in this group is small, and further analysis with additional isolates will help clarify whether any genetic exchange is occurring. Aerial dispersal over hundreds of kilometers is presumed to have occurred with some plant pathogens (4), and it is possible that sexual spores could allow long-distance dispersal of *C. gattii*.

Mating type, recombination, and clonality: implications for cryptococcosis. *C. gattii* populations in Australia appear to reproduce clonally or sexually, and in the current study, together with data in the accompanying paper on mating, this segregates strongly with molecular type. These two different reproductive modes influence the ecology of *C. gattii* in the environment, which may in turn have implications for the acquisition of cryptococcal disease. First, our recombination analysis together with studies of *C. neoformans* suggest that although the pathogenic cryptococci are sexual species, extensive clonal propagation occurs on certain favorable substrates: pigeon guano for *C. neoformans*, decaying eucalyptus detritus in tree hollows for *C. gattii* VGI in Australia, and unknown substrates for *C. gattii* VGI in PNG and VGII on Vancouver Island. The outbreak on Vancouver Island and the high incidence of *C. gattii* cryptococcosis in PNG indicate that asexually reproducing *C. gattii* isolates can be an important source of cryptococcal infection. A pertinent question remains regarding the nature of the infective propagule, which is considered unlikely to be a vegetatively produced encapsulated yeast cell (45). Basidiospores are produced via monokaryotic fruiting from some *C. neoformans* var. *neoformans* strains, and interestingly, this process has recently been shown to involve diploidization of cells of the same mating type (α) and the development of recombinant progeny (26); however, basidiospore development via monokaryotic fruiting has never been seen in *C. gattii* (46), but it is possible that particular environmental conditions are required to initiate this process.

Second, our study has identified two recombining populations, and given the data on fertility, it is likely that all of the Australian VGII isolates in this study have a sexual origin. The two geographic VGII populations appear quite different in their association with cryptococcosis. In Sydney, VGII isolates have rarely been reported from human infections (34), yet this molecular type was relatively common among the veterinary isolates. This might reflect a difference in host habits, as animals generally spend more time outdoors and in closer proximity to vegetation than humans. In contrast, the NT has one of the highest incidences of *C. gattii* cryptococcosis in the world (10, 21). It is tempting to speculate that this might be related to the presence of fertile and sexually active VGII isolates and the associated production of infectious basidiospores. If *C. gattii* VGII appears to be adapting and migrating into more temperate areas, such as Vancouver Island, this could have implications for the further emergence of *C. gattii* cryptococcosis.

TABLE 3. Genetic differentiation between populations

Population	θ value (*P* value)			
	VGI		VGII	
	NT	Sydney	Sydney	GD[a] (Sydney)
PNG	0.394 (0.001)	0.289 (0.001)		
NT		0.399 (0.001)	0.257 (0.002)	
GD[a] (NT)				0.270 (0.039)

[a] GD, genetically undifferentiated cluster (Fig. 3).

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