Evidence of Recombination in Mixed-Mating-Type and α-Only Populations of Cryptococcus gattii Sourced from Single Eucalyptus Tree Hollows*

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Disease caused by the pathogenic yeast Cryptococcus gattii begins with the inhalation of an infectious propagule. As C. gattii is heavily encapsulated, this propagule is most likely to be a basidiospore. However, most C. gattii strains are infertile in laboratory crosses, and population studies indicate that recombination and dispersal are very restricted. In addition, strains of the α mating type predominate, which would not be expected in a mating population. C. gattii comprises four genetically distinct molecular genotypes, designated VGI to VGIV. C. gattii molecular type VGI has a strong association with Eucalyptus camaldulensis and can be found in high numbers in E. camaldulensis hollows. Previous work on isolates obtained from E. camaldulensis suggested that environmental populations of C. gattii are highly fragmented, have limited ability to disperse, and are confined to individual tree hollows. In the current study, we examined large numbers of isolates from three separate hollows for evidence of recombination. In two hollows, the α and a mating types were present in approximately equal numbers. The third hollow had α cells only and was from a region where α isolates have never been found. Statistical analysis of multilocus genotypes revealed recombining subpopulations in the three Eucalyptus hollows. Recombination was equally present in the α-α and α-only populations. This is consistent with recent studies that have found evidence suggestive of α-α mating in C. gattii and Cryptococcus neoformans and raises the possibility this may be a widespread phenomenon, allowing these fungi to recombine despite a paucity of a mating partners.

Cryptococcus gattii is a basidiomycete yeast that causes potentially fatal disease in a diverse range of hosts, including, birds, reptiles, domestic and wild mammals, and humans (9, 14, 29, 37). In contrast to Cryptococcus neoformans, a major pathogen in AIDS patients, C. gattii causes disease predominantly in immunocompetent hosts and is only rarely associated with immunocompromised patients (43).

C. gattii can be divided into four genetically distinct genotypes, designated VGI to VGIV (17). VGI has a widespread global distribution and is the most prevalent genotype in Australia, accounting for 92% of clinical isolates and almost all environmental isolates (44). VGII has a more restricted distribution, being found predominantly in the Northern Territory and Western Australia (12). While the incidence of disease caused by C. gattii VGI is generally low level and sporadic (11), disease caused by C. gattii VGII can cause outbreaks in restricted areas, such as an ongoing outbreak among humans and animals on Vancouver Island (23) and in goats in Spain (3) and a relatively high prevalence of disease in Arnhem Land in northern Australia and in Papua New Guinea (6).

An association between C. gattii VGI isolates and the detritus within the hollows of certain Australian tree species is well documented, particularly Eucalyptus camaldulensis and Eucalyptus tereticornis (15, 42), with occasional isolation being made from other eucalypts and native trees (30). The respiratory tract is the primary portal of entry in all species and although cryptococcosis is acquired from the environment, the exact nature of the infectious propagule for C. gattii remains unclear. The encapsulated yeast, measuring 10 to 60 μm (9) is unlikely to be able to penetrate to the lung alveoli to initiate disease, and although desiccated yeast cells are small enough to enter the terminal bronchi, they have low viability (54) and are not highly infectious (47). C. gattii has a defined sexual state (teleomorph: Filobasidiella bacillispora) (31) with two mating types, a and α, and a dipolar mating system that produces basidiospores. Spores are infectious in a mouse inhalation model (31); however, although mating has been observed in some laboratory crosses under conditions of nutrient deprivation, many strains, particularly those of genotype VGI, appear to be infertile (7).

In C. neoformans, starvation conditions have been reported to produce asexual basidiospores via a process termed haploid or monokaryotic fruiting (33, 55). Recent evidence suggests that this phenomenon is actually a form of sexual mating involving α mating-type cells only (33). The reported capacity of two α isolates to mate is a potential explanation for an observed predominance of the α mating type both clinically and environmentally. Although α-α mating has not been directly observed in C. gattii, studies examining strains from an ongoing outbreak on Vancouver Island have found diploid isolates of α mating type with heterozygous alleles at their α mating locus (17), suggesting that the hypervirulent VGII strains arose as a result of α-α mating. It is therefore hypothesized that basidiospores created in sexually recombining environmental popula-
tions, whether in traditional α-α or in α-α mating, are an important source of C. gattii infection in humans and animals.

It has so far not proven possible to observe C. gattii sexual structures in environmental samples, and population genetic approaches have therefore been undertaken to detect recombination. Halliday et al. found a unique environmental population consisting of equal proportions of mating types in Renmark, South Australia (20), and analyzed a collection of isolates from several different E. camaldulensis hollows for evidence of recombination (21). Significant linkage disequilibrium was found, indicating the likelihood that the members of this population were not recombining. However, it was apparent that isolates grouped according to their tree of origin, and α and α isolates found together in a single hollow were very closely related, which would not be expected if each mating type reproduced asexually. It is therefore conceivable that E. camaldulensis hollows contain discrete populations within which mating is occurring, with limited exchange of isolates among hollows.

The key to assessing recombination in a natural population is ensuring that members of the population examined have had the opportunity to mate (5). Since genetically distinct subpopulations can arise through physical or genetic barriers that prevent interbreeding, it is also important to select a discrete population and to refine this if necessary so that subpopulations are analyzed individually (8). By selecting well-supported clusters from phylogenograms derived from multilocus data, small recombining populations that were geographically restricted to certain regions of Australia could be identified in C. gattii VGI (6). The same analysis could not detect recombination in VGI isolates from clinical disease, however, which appeared to be highly related and with a strongly clonal genetic structure. As most clinical disease in Australia is caused by VGI isolates, understanding the ecology and life cycle of C. gattii VGI is important for disease management and control strategies. The eucalypt niche is clearly important in the environmental maintenance of C. gattii VGI. The aim of the current study was therefore to examine whether exchange of genetic markers has occurred in C. gattii populations derived from individual eucalypt tree hollows. Hollows found to contain both mating types (22) and a hollow from an area where only α cells have been found were targeted. We report the first evidence of genetic recombination in both single-mating-type and mixed-mating-type populations of C. gattii VGI.

MATERIALS AND METHODS

Cryptococcus gattii isolates. Samples of detritus collected from Gundagai, New South Wales, during field trips in 2004 and 2006 and those collected previously from Renmark, South Australia, by Halliday et al. (20) were used for isolation. Gundagai is located approximately 685 km upstream of Renmark in the Murray-Darling Basin, a large river tributary system draining much of southeast Australia into the southern ocean via the Murray River (Fig. 1). Each population studied was obtained from a single eucalypt hollow. Populations E13 and E15 were obtained from hollows on Renmark trees 13 and 15, respectively, which had been previously found to contain both mating types (21). Population G1 was from a hollow on a single tree in Gundagai. Both Renmark trees were situated on Rail Creek, an anastomosing branch of the Murray River. Renmark tree 13 was situated ~20 m from Rail Creek, and the hollow sampled had a narrow opening at ground level approximately 5 cm wide that ran vertically to a second opening ~3 m above the ground. Tree 15 was on the riverbank, and the hollow was ~4 m above the ground. Several large openings led into a large horizontal region with accumulated detritus, which descended into the trunk of the tree.

The Eucalyptus sampled in Gundagai was approximately 20 m from the Murray-Darling River, one of the major rivers that drain into the Murray River. The hollow was ~6 m from the ground and had a single large opening that ran horizontally through the branch and descended into the tree trunk. Large accumulations of detritus were present for the first visible meter of hollow. Samples of dry detritus were taken from ~60 cm inside the hollow of the Gundagai tree and Renmark tree 15. The samples from Renmark tree 13 were taken from inside the hollow at ground level. All samples were stored in sealed plastic bags in the dark at room temperature. For the isolation of fungal cultures, approximately 15 grams of detritus was suspended in 40 ml of 0.9% (wt/vol) NaCl solution, which was agitated and allowed to settle. One milliliter of the supernatant was plated on bird seed agar and incubated for 7 days at 25°C. Presumptive C. gattii colonies were selected based on their brown color (45) and mucoid appearance, subcultured onto Sabouraud dextrose agar (SDA) for purity, and confirmed as C. gattii by growth and color on L-canavanine–glycine–bromothymol blue agar. Isolates were stored on SDA at 4°C. Thirty-eight isolates were analyzed for molecular and mating types from tree 13, 33 from tree 15, and 32 from the Gundagai tree. An additional 100 isolates sampled from the area surrounding the Gundagai tree were analyzed for mating type. Recombination analysis was conducted on a subset of isolates, with 27 isolates included in population E15, 27 in E13, and 25 in G1.

Mating and molecular typing. DNA extraction was based on the Novozyme 234 dodécyltrimethylammonium bromide and hexadecyltrimethylammonium bromide method described previously by others (32), with the following modifications: approximately 1.5 g (wet weight) of cells was harvested from cultures grown on SDA at 30°C for 48 h, and the protoplasting solution was made with 10 mg/ml Novozyme 234 in SCE buffer (100 mM sodium citrate, 1 M sorbitol, 10 mM EDTA, pH 8).

Mating-type analysis was conducted by PCR coamplification with the two primer sets MFe (upper and lower) and STE20a (upper and lower), which are specific to regions in the α and a mating-type loci, respectively (21, 22). PCR amplifications were carried out in a 50-μl reaction volume containing 1× PCR buffer [100 mM KCl, 60 mM (NH₄)₂SO₄, 200 mM Tris-HCl (pH 8.8), 20 mM MgSO₄, 1% Triton X-100, and 1 mg/ml bovine serum albumin], 5% glycerol, 6.25 μM of each deoxynucleoside triphosphate, 0.2 μM of each primer, 2.5 U of Pfu DNA polymerase, and 1 μl chromosomal DNA and made up to volume with sterile water. Amplification conditions were 5 min at 94°C, 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension time of 72°C for 10.5 min. Ten microliters of each sample was electrophoresed in a 2% agarose–Tris-acetate–EDTA gel, which was stained with ethidium bromide and photographed under UV illumination. Strains of known mating type were used as controls.

Molecular type was determined by restriction fragment length polymorphism analysis using a modification of the method previously described by Meyer et al. (38). The URA5 gene was amplified with primers URA5f and URA5r in a 50-μl reaction mixture containing 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, 0.2 μM of each deoxynucleoside triphosphate, 3 μM MgCl₂, 1.5 U AmpliTaq DNA polymerase, 0.2 μM of each primer, and 1 μl

chromosomal DNA (5 μg/μl). PCR amplification conditions were 94°C for 2 min; 35 cycles of 94°C for 45 s, 61°C for 1 min, and 72°C for 2 min; and a final extension of 72°C for 10 min. PCR products (20 μl) were digested with Sau96I and HhaI for 3 h at 37°C and electrophoresed on a 3% Tris-acetate-EDTA gel at 10 V/cm for 2 h. Standard isolates of known molecular type were used as controls.

**AFLP analysis.** The amplified fragment polymorphism (AFLP) fingerprinting protocol was based on a technique described previously (50), using the AFLP primers EcoRI-TG and Mse-CA, with minor modifications as described previously (21). Selective amplification products were diluted 100-fold, and 1 μl was added to 0.5 μl of Genescan-400HD ROX standard size marker (Applied Biosystems) mixed with 10 μl of Hi-Di formamide. Samples were 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. Electrophoresis was performed at the Sydney University Prince Alfred Macromolecular Analysis Centre. Samples from the different tree hollows were included on a single gel to eliminate any run-to-run variation that might cause the different tree hollow populations to appear different from one another.

**Data analysis.** Amplified fragments of between 50 and 400 bp were converted into a binomial matrix using LecPCR (40a), which scored the presence of a fragment (1) or its absence (0) in each taxon. Polymorphic loci were considered to be bands of the same mobility that were present or absent in 20 to 80% of the population. Loci with identical allele distributions were eliminated. To test whether a recombining or clonal population structure, the index of association (Iₐ) and rBarD, and tree length (Tₑ) were assessed, as described by Campbell et al. (6).

Linkage disequilibrium is a measure of nonrandom association between alleles at different loci. (Iₐ) and rBarD (which is a variation of Iₐ designed for use in recombined populations with fewer than 20 loci) are statistical tests used to detect clonality by assessing whether linkage disequilibrium occurs among genetic loci that are not physically linked. In clonal populations the genome is inherited as an entire, uncombined unit, whereas sexual populations re assort polymorphic alleles so that genetic linkage between loci is reduced or lost. Both tests were implemented using Multilocus (version 1.0b) (1). Tₑ, implemented using PAUP* (version 4.0b), is a permutation test that calculates the lengths of parsimonious trees that are generated from the data set. Clonal populations produce well-resolved trees with short branch lengths and few character state changes. Recombining populations require branch swapping to accommodate exchanged alleles and therefore result in significantly longer trees with poor resolution.

For all tests, the observed value is compared to values generated from 1,000 artificially recombining data sets that have been created from the observed data by shuffling the alleles at each locus among members of the population. The null hypothesis that recombination is occurring is rejected if the observed value is statistically different from the range of values produced from the artificially recombining data set; otherwise it is accepted.

Phylogenetic trees were constructed using PAUP* (version 4.0b). Parsimonious trees were drawn, and those with a score higher than 1,000 were retained during a heuristic search. The best of these trees were then used to create a semistrict consensus tree. Statistical support was obtained by bootstrapping with 1,000 replicates.

Genetic differentiation between populations and subpopulations was assessed as θ, which is an estimate of Wright’s Fₛₑ, the standard measure used to detect gene flow and differentiation among populations (51). θ is calculated as (Q − q)/(1 − q), where Q is the probability that two different genes in a population are the same allele and q is the probability that these are different alleles. If two different populations have the same allele frequencies, then Q = q and θ = 0; if they are fixed for different alleles, then Q = 1, q = 0, and θ = 1. Values were calculated from the binomial data matrix constructed as described above using Multilocus. Statistical support was obtained by comparing the observed θ to values produced from 1,000 randomizations of the data, which simulates what would be expected to occur in a population undergoing panmictic gene flow.

**RESULTS**

**Mating types and molecular types of the tree hollow populations.** Populations E13 and E15 had isolates of the α and α mating types in statistically equal proportions (P = 0.99 and P = 0.95, respectively). In contrast the G1 population consisted of α mating-type isolates only. An additional 100 isolates obtained from the surrounding area were also all α. All isolates were molecular type VGI.

**Recombination occurs in subpopulations within each tree hollow.** The presence or absence of AFLP fragments was scored in a binomial data matrix and used to derive Iₐ and rBarD values and to construct phylogenetic trees. Twenty-seven isolates from each eucalypt hollow population had unique multilocus genotypes and were used in the AFLP analysis. A total of 115 polymorphic loci were scored in the E15 population, 60 were scored in E13, and 75 in population G1. The analysis of all populations included 214 polymorphic loci, many of which were present in only one population.

**Neighborhood-joining phylogenograms and semistrict and majority-rule consensus trees were constructed from the best trees found in a heuristic search of parsimony trees generated by PAUP* (version 4.0b). The three algorithms gave trees with overall similar topologies. Semistrict parsimony consensus trees are shown for each population (Fig. 2a to c), and a majority-rule consensus tree is shown for population G1 (Fig. 2d). Only bootstrap values of ≥70 were retained.**

The consensus tree derived for population E13 (Fig. 2a) had some internal structure, and the population was divided into two clusters supported by a bootstrap value of 95. There was strong support (≥90) for two terminal branches, including one that connected isolates of different mating type (E13w = α and E13z = a). The E15 phylogram had no significant bootstrap support for its topology (Fig. 2b). A key observation was that α and a isolates were interspersed throughout both the E13 and E15 phylograms. The semistrict consensus tree for population G1 had no significant bootstrap support and very little overall structure (Fig. 2c); however, distinct groupings were evident on the majority-rule consensus tree (Fig. 2d).

When analyzed overall, each population derived from a single tree hollow had Tₑ and Iₐ values that lay well outside those derived from artificially recombining populations, allowing the hypothesis of random recombination to be strongly rejected (P < 0.001) (Table 1). As subpopulations may exist in tree hollows on a micro scale, these were targeted by choosing clusters from the semistrict or majority-rule consensus trees that contained related isolates without evidence of internal structure. Any cluster containing five or more isolates was examined, and Iₐ, rBarD, and Tₑ values were derived. Eleven clusters were found to have a structure that was consistent with a recombining population. Two recombining subpopulations, E13sp1 and E13sp2, which each contained five isolates, were present in E13 (Fig. 2a). Three distinct recombining subpopulations were identified in population E15 (E15sp1, -2, and -3) (Fig. 2b), and it was possible to extend E15sp2 to include three of the isolates from E15sp3 before evidence of recombination was lost (E15sp4). Four distinct recombining subpopulations (G1sp1 to -4) were identified in G1, with a fifth consisting of isolates from G1sp3 and G1sp4. Tₑ, Iₐ, and rBarD values are shown for each population and subpopulation in Table 1, along with the range of values generated by artificially recombining their multilocus genotypes. To check that small subpopulations would not appear to be recombining by chance, tests were performed on collections of five to eight isolates taken from
FIG. 2. Phylograms derived from environmental C. gattii populations using PAUP* (v4.0b) and semistrict (a to c) or majority-rule (d) consensus with a parsimony algorithm. (a) Population E15. (b) Population E13. (c and d) Population G1. Recombining subpopulations are indicated on the right of each phylogram. Only bootstrap values of ≥70 are shown.
the two genetically distinct subclusters seen in E13. In all cases, recombination was strongly rejected \((P < 0.001)\), indicating that erroneously finding recombining populations would be unlikely.

**Genetic differentiation among populations and subpopulations.** Complete genetic differentiation, assessed by \(\theta\), was evident between populations E13, E15, and G1, suggesting that gene flow does not occur between different tree hollows (Table 2). \(\theta\) analysis also supported the separation of the two major clusters in E13 identified in Fig. 2a, indicating little or no gene flow between these two groups present in a single tree hollow \((P < 0.001)\). Populations E13sp1, -2, and -3 were likewise genetically differentiated, with no gene flow detectable between these groups of isolates \((P = 0.002)\). The four recombining populations within G1 divided into two clusters: G1sp1 and -2 and G1sp3 and -4. No gene flow was detected between these two clusters \((P = 0.004\) and \(P < 0.001)\) or between the subpopulations G1sp1 and G1sp2 \((P = 0.007)\).

Genetic differentiation was equally high between E13 and E15, located 200 m apart, and between E13 and G1, which are separated by 685 km. This was also evident by MDS, where isolates from E13 were genetically distinct (Fig. 3). MDS indicated some overlap among isolates in populations E15 and G1, however. This subset, designated E15/G1, had borderline evidence of gene flow when analyzed by \(\theta\) (Table 2) \((P = 0.031)\).

MDS found no separation of isolates of the \(a\) and \(\alpha\) mating types in either population E13 or E15. This indicates that genetic relatedness is independent of mating type and is further evidence of recombination within these populations.

**DISCUSSION**

The increasing importance of fungal disease over recent decades has stimulated studies of fungal genetics and epidemiology (6, 14, 34), with a predominant focus on clinical isolates. However, as disseminated fungal disease is initiated by inhalation of an infectious propagule from the environment (9), any comprehensive analysis must focus, at least in part, on the habitat and population genetics of environmental isolates. Given that cryptococcosis is a life-threatening disease that is difficult to treat and fully resolve (9), a focus on prevention of the initial infection from the environment is an essential aspect of minimizing the impact on human and animal populations.

**Recombination occurs in mixed-mating-type and \(a\)-only environmental C. gattii populations.** This is the first report showing strong statistical evidence of recombination in environmental C. gattii populations. Recombination was apparent regardless of the presence of cultured isolates of the \(a\) mating type. The possibility remains that there are some \(a\) isolates in the Gundagai region that were not detected in our samples. However, the 133 isolates examined, which included 33 from the eucalypt hollow and 100 from the surrounding area, were all \(\alpha\). Where they exist, \(a\) cells can be successfully recovered

**TABLE 1.** Recombination analysis of C. gattii populations and subpopulations

<table>
<thead>
<tr>
<th>Population (n)</th>
<th>No. of loci</th>
<th>(T_L (P))</th>
<th>(T_L) range</th>
<th>(I_A/BaD (P))</th>
<th>(I_A) range</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15 (27)</td>
<td>115</td>
<td>555 (0.001)</td>
<td>790–830</td>
<td>10.08 (&lt;0.001)</td>
<td>-0.23–0.32</td>
</tr>
<tr>
<td>E13 (27)</td>
<td>60</td>
<td>212 (0.001)</td>
<td>363–395</td>
<td>7.94 (&lt;0.001)</td>
<td>-0.22–0.46</td>
</tr>
<tr>
<td>G1 (25)</td>
<td>75</td>
<td>323 (0.001)</td>
<td>410–560</td>
<td>5.27 (&lt;0.001)</td>
<td>-0.003–0.07</td>
</tr>
<tr>
<td>E13sp1 (6)</td>
<td>6</td>
<td>9 (0.539)</td>
<td>7–11</td>
<td>0.029 (0.357)</td>
<td>-0.15–0.32</td>
</tr>
<tr>
<td>E13sp2 (6)</td>
<td>17</td>
<td>31 (0.781)</td>
<td>26–33</td>
<td>-0.009 (0.637)</td>
<td>-0.04–0.11</td>
</tr>
<tr>
<td>E15sp1 (6)</td>
<td>10</td>
<td>15 (0.113)</td>
<td>13–19</td>
<td>0.05 (0.148)</td>
<td>-0.09–0.23</td>
</tr>
<tr>
<td>E15sp2 (5)</td>
<td>30</td>
<td>29 (0.994)</td>
<td>24–30</td>
<td>-0.732 (0.999)</td>
<td>-0.77–1.79</td>
</tr>
<tr>
<td>E15sp3 (9)</td>
<td>28</td>
<td>73 (0.192)</td>
<td>67–82</td>
<td>0.249 (0.175)</td>
<td>-0.60–1.10</td>
</tr>
<tr>
<td>E15sp4 (10)</td>
<td>31</td>
<td>84 (0.105)</td>
<td>79–94</td>
<td>0.27 (0.11)</td>
<td>-0.52–0.8</td>
</tr>
<tr>
<td>G1sp1 (5)</td>
<td>12</td>
<td>13 (0.203)</td>
<td>11–15</td>
<td>-0.068 (0.986)</td>
<td>-0.083–0.219</td>
</tr>
<tr>
<td>G1sp2 (6)</td>
<td>17</td>
<td>28 (0.97)</td>
<td>23–29</td>
<td>-0.008 (0.619)</td>
<td>-0.049–0.114</td>
</tr>
<tr>
<td>G1sp3 (5)</td>
<td>16</td>
<td>24 (0.77)</td>
<td>20–26</td>
<td>-0.032 (0.905)</td>
<td>-0.056–0.124</td>
</tr>
<tr>
<td>G1sp4 (7)</td>
<td>30</td>
<td>58 (0.971)</td>
<td>51–60</td>
<td>-0.124 (0.633)</td>
<td>-0.690–1.404</td>
</tr>
<tr>
<td>G1sp5 (5)</td>
<td>9</td>
<td>13 (0.916)</td>
<td>10–14</td>
<td>-0.032 (0.767)</td>
<td>-0.101–0.256</td>
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**TABLE 2.** Genetic differentiation among C. gattii populations and subpopulations

<table>
<thead>
<tr>
<th>Populations (n)</th>
<th>No. of loci</th>
<th>(\theta) Value</th>
<th>(\theta) Range</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E13/G1/E15 (79)</td>
<td>214</td>
<td>0.226</td>
<td>-0.016–0.051</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E15/E13 (54)</td>
<td>196</td>
<td>0.28</td>
<td>-0.0214–0.082</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E13/G1 (52)</td>
<td>154</td>
<td>0.322</td>
<td>-0.019–0.085</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E15/G1 (52)</td>
<td>175</td>
<td>0.101</td>
<td>0.0209–0.075</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Subset E15/G1 (14)</td>
<td>94</td>
<td>0.118</td>
<td>-0.098–0.244</td>
<td>0.031</td>
</tr>
</tbody>
</table>

**FIG. 3.** Nonmetric MDS plot showing the genetic relationships among the E13, E15, and G1 populations. A clear separation is seen between populations E13 and E15, with no separation of mating types within each population. While a separation of isolates from E15 and G1 is apparent, there is an overlapping area containing 14 isolates from both populations, which is designated subset E15/G1.
from the environment (20). To date, however, despite extensive sampling around Australia, isolates of the a mating type have been recovered from only two regions: Renmark in South Australia and Balranald in New South Wales (20; N. Saul, unpublished data). All other regions have yielded exclusively a cells, and it is likely that in Gundagai, the a mating type is absent or at a very low prevalence.

α-α mating in Cryptococcus has been demonstrated in the laboratory (33), and recent studies suggest that this may also occur to some extent in nature (17, 33), but whether it is a common or rare phenomenon is not known. Differentiated mating types can be seen as a form of self-incompatibility that prevents inbreeding. The current study raises the question of whether the Gundagai tree hollow population is unique or whether same-sex incompatibility has failed in C. gattii as a species and recombination can occur in other α-only populations. The evolution to self-compatibility is a common trend among invasive species of plants and fungi (10). This acts to ensure sexual reproduction when it may be difficult or impossible to find a compatible mate, and it is thought that it also may help organisms adapt to novel niches (28). Ensuring sexual reproduction in the absence of a suitable mating partner has precedence in other fungi, as well as in higher eukaryotes, with various levels of regulation controlling the switch from heterothallism to homothallism (28). For example, Saccharomyces cerevisiae can switch mating types to become capable of mating. Like mating-type switching, α-α mating could result in the mating of two genetically identical cells, a process termed “haplo-selling” (28). This enables renewal of the genome (28, 39, 40) and, while reducing allelic diversity, allows for increased selection of favorable alleles and the removal of deleterious ones.

Given the imbalance of mating types in C. gattii, the advantages of bypassing the normal mating-type barrier are particularly evident, as a dispersing yeast cell would be unlikely to encounter a compatible mate in most regions. We therefore propose that α-α mating is likely to be found in other environmental C. gattii populations, where it provides most of the advantages of sex without the need for the a mating type.

Refining population choice enables recombination to be detected. The original study of recombination by Halliday and Carter (21) suggested that the population that had been analyzed consisted of reproductively isolated subpopulations, in which recombination might occur. Further evidence for this was seen in the study by Campbell et al. (6), where recombination was found by narrowing populations to geographically distinct clusters identified on a phylogram. In the current study, even single tree hollows consisted of distinct subpopulations that were identified only by phylogenetic analysis. E. camaldulensis hollows can extend 3 to 4 meters into the trunk of the tree and possess several openings ranging in size from 10 centimeters to half a meter across. Studies on environmental microbial diversity have shown that there can be significantly different microenvironments across small distances, with marked changes in microbial populations (19, 24, 49). It is therefore likely that isolates collected from a single eucalypt hollow could come from multiple different microenvironments and from independent populations. As isolates were recovered from detritus accumulated in horizontal regions of the hollow, these may originate from areas with different abiotic and biotic conditions that might influence the resident C. gattii populations. In addition, the three eucalypts sampled showed evidence of animal habitation, and fungal transfer could be partially mediated by arboreal marsupials. Populations E15 and G1, which were sampled from relatively large hollows might therefore have higher levels of gene flow, increased diversity, and a more complex population structure than E13, which came from a small hollow that would be less conducive to animals.

Could limitations in the populations or data result in an erroneous observation of recombination? Artifacts generated in the creation of polymorphic markers by AFLP are possible; however, this is more likely to lead to a conclusion of clonality (21, 41) and will tend to remove geographic or temporal structure rather than create it (16). Recombination could be incorrectly concluded if excessive homoplasy occurs due to frequent mutational hot spots in the genome (25), but this would also result in few population divisions (16). In this study isolates clearly segregated according to their eucalypt of origin, and populations within single tree hollows were highly subdivided (Fig. 2a to d and 3). We therefore argue that the most feasible explanation for the lack of linkage disequilibrium is recombination.

Genetic differentiation occurs among geographically separate C. gattii populations. The widespread homogeneity of C. gattii across Australia (44) has been recently questioned, and genetic differentiation has been observed across both large geographical areas (6, 17) and highly restricted environments (21). This new finding is partially due to the development of higher-resolution markers and more powerful statistics and highlights the need to revisit old questions as new techniques become available (53). Consistent with the work of Halliday and Carter (21), strong genetic differentiation was observed between the two Eucalyptus hollow populations sampled from Renmark (P < 0.001) (Table 2). However, MDS analysis, while showing strong separation between E13 and E15, indicated an overlap between isolates from G1 and E15 (Fig. 3). Viewed as a whole, there was no gene flow between the two areas (P > 0.001) (Table 2). However, if outlying isolates were excluded, the subset of overlapping isolates had borderline evidence of gene flow (P = 0.031) (Table 2). This raises the question of why two populations derived from eucalypts 200 meters apart had no detectable gene flow, whereas two populations separated by more than 600 kilometers appeared to be connected. Long-range dispersal has precedence in a number of different fungal pathogens (2, 4), and pan-continental dispersal can be facilitated by air currents that can move lightweight spores over large distances (2, 32). Borderline gene flow has also been detected between recombining C. gattii VGII populations derived from Sydney and Arnhem Land, more than 2,000 kilometers apart (8).

The smaller hollow sampled on tree E13 might impede the dispersal of C. gattii by physical processes. E. camaldulensis trees are distributed along the Murray-Murrumbidgee river system (Fig. 1), which flows from the Snowy Mountains in the east through Gundagai toward Renmark in the west. These rivers are subject to periodic flooding events, and although not frequent today (due to drought and depletion of the river systems through irrigation), floodwaters may have reached the heights of the tree hollows in the recent past (36). Studies of
marine organisms have shown that genetic signals of dispersal can be absent in geographically close populations (0.1 to 100 m) but can be more marked in geographically separate populations (1 to 1,000 km) (27). C. gattii has been isolated from aquatic environments (26, 35) and has caused disease in marine mammals (46), suggesting that it can survive in water from aquatic environments (26, 35) and has caused disease in northern Australia: existence of an environmental source other than known host eucalypts. Trans. R. Soc. Trop. Med. Hyg. 91:547–550.


34. Malik, R., M. Krockenberger, G. Cross, R. Doneley, D. Madill, D. Blacks, P.


48. Reference deleted.


