

Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans (= *Filobasidiella neoformans*) is a significant emerging fungal pathogen of humans. To understand the evolution of this pathogen, 34 strains were obtained from various locations around the world and fragments of four genes were sequenced from each. These strains represented all three varieties and five serotypes. The four sequenced genes are: (i) the mitochondrial large ribosomal subunit RNA; (ii) the internal transcribed spacer region of the nuclear rRNA, including ITS1, 5.8S rRNA subunit and ITS2; (iii) orotidine monophosphate pyrophosphorylase; and (iv) diphenol oxidase. Phylogenetic analyses indicated considerable divergence among lineages, which corresponded to the current classification of *C. neoformans* into three varieties. However, there is no apparent phylogeographic pattern. Significant incongruences were observed among gene genealogies. The analyses indicated that the major lineages in *C. neoformans* diverged tens of millions of years ago but have undergone recent dispersion and hybridization.

Keywords: Cryptococcosis, gene genealogy, genetic polymorphism, hybridization

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Introduction

Recognized over a century ago, *Cryptococcus neoformans* is an important pathogenic basidiomycetous yeast of humans and other mammals throughout the world. The incidence of human cryptococcosis has increased dramatically over the past two decades, primarily due to the growing population of immunocompromised hosts (Lederberg *et al.* 1992; Mitchell & Perfect 1995; Casadevall & Perfect 1998). Two varieties of this encapsulated yeast have been traditionally recognized, *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*, and each variety has two predominant capsular serotypes: serotypes A and D in *C. neoformans* var. *neoformans* and serotypes B and C in *C. neoformans* var. *gattii* (Aulakh *et al.* 1981; Kwon-Chung *et al.* 1982a; Kwon-Chung *et al.* 1982b; Kabasawa *et al.* 1991; Mitchell *et al.* 1992; Brandt *et al.* 1995; Mitchell & Perfect 1995; Boekhout *et al.* 1997).

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The nucleotide sequences described in this paper have been deposited in the GenBank database (accession nos. AF140082–AF140217).

In recent years, the serotypes are determined by reactivity with commercially available monoclonal antibodies to the surface capsular polysaccharide (Kabasawa *et al.* 1991). Some strains are untypeable with these reagents. Others serotyped as AD and classified as strains of *C. neoformans* var. *neoformans* (Mitchell & Perfect 1995; Casadevall & Perfect 1998). Recently, a new variety, *C. neoformans* var. *grubii*, was proposed for strains of serotype A (Franzot *et al.* 1999). These varieties and major serotypes differ in their ecology, molecular and morphological characteristics, epidemiology, pathogenicity, physiology and geographical distribution as summarized in Table 1 (Aulakh *et al.* 1981; Kwon-Chung *et al.* 1982a; Kwon-Chung *et al.* 1982b; Kabasawa *et al.* 1991; Casadevall *et al.* 1992; Mitchell *et al.* 1992; Wickes *et al.* 1994; Brandt *et al.* 1995; Mitchell & Perfect 1995; Boekhout *et al.* 1997; Franzot *et al.* 1999). Sexual reproduction with the species has been demonstrated in the laboratory (Kwon-Chung 1975, 1976), but the extent to which sexual reproduction occurs in nature is unknown.

Despite the different patterns of geographical distribution, strains of all three varieties have been isolated on every continent except Antarctica (Casadevall & Perfect

Table 1 Taxonomy, ecology and epidemiology of *Cryptococcus neoformans*

| Serotype | Taxonomy | | | |
|--------------------------------|--|--|--|-------------|
| | <i>C. neoformans</i> var. <i>grubii</i> A | <i>C. neoformans</i> var. <i>neoformans</i> D | <i>C. neoformans</i> var. <i>gattii</i> B | C |
| Main geographical distribution | Worldwide | Northern Europe | Tropical and subtropical | |
| Environmental reservoir | Soil and avian faeces | | Eucalyptus trees | |
| Mating type allele | alpha | a and alpha | a and alpha | a and alpha |
| Epidemiological prevalence: | | | | |
| Non-AIDS patients | | | | |
| Global: temperate | 50–91% | 3–70% | ~5% | ~1% |
| Global: tropical | ~30% | < 1% | ~55% | ~15% |
| USA (excluding Southern CA) | ~90% | ~4% | ~4% | ~2% |
| Southern CA (USA) | ~48% | < 1% | ~37% | ~14% |
| AIDS patients | | | | |
| Global (excluding France) | ~96% | ~2% | < 1% | < 1% |
| France | ~80% | ~20% | 0 | 0 |

Modified from Mitchell & Perfect 1995; Casadevall & Perfect 1998; Franzot *et al.* 1999.

1998). However, it is not known whether each continent had all varieties as the split among continents (the 'historical' hypothesis), or the presence of all varieties in all continents is the result of recent dispersal events (the 'recent dispersal' hypothesis). Assuming that sexual recombination occurs in nature, the 'historical' hypothesis predicts that strains of different varieties and serotypes from the same geographical region should be phylogenetically more similar to each other than to strains from other continents. In contrast, the 'recent dispersal' hypothesis predicts that strains of the same variety and serotype from different geographical regions might be phylogenetically more similar to each other than to strains of other varieties or serotypes from the same geographical regions. The ecological association of *C. neoformans* with pigeons (Casadevall & Perfect 1998) suggests that *C. neoformans* could be transported readily from one location to another. In addition, the dispersal of *C. neoformans* could be abetted by human travel.

The application of molecular markers has revolutionized the analysis of the natural history of many organisms, clarifying modes of reproduction, dispersion, colonization, speciation and hybridization (e.g. Tibayrenc *et al.* 1991; Sibley & Boothroyd 1992; Lenski 1993; Maynard Smith *et al.* 1993; Avise 1994; Goodwin *et al.* 1994; Guttman & Dykhuizen 1994; Koufopanou *et al.* 1997; Xu *et al.* 1997; Anderson & Kohn 1998; Taylor *et al.* 1999). While hybridization between morphologically or phylogenetically distinct taxa is common in plants, there are few confirmed cases of hybridization in fungi. For example, based on cytology, intergenic transcribed spacer (ITS) sequences and amplified fragment length polymorphisms (AFLP), Brasier *et al.* (1999) identified a new *Phytophthora* pathogen that most likely resulted from hybridization between a *Phytophthora*

cambivora-like species and an unknown taxon similar to *P. fragariae*. Using the beta-tubulin and rRNA gene sequences, Tsai *et al.* (1994) isolated multiple, but divergent copies of the beta-tubulin gene in some strains of fungal endophytes of the tall fescue grass (*Festuca arundinacea*). Based on the phylogenetic information of beta-tubulin, they inferred that at least three hybridization events occurred with *Epichloë* species during the diversification of the fungal endophytes of tall fescue grass (Tsai *et al.* 1994). Morphological, structural, and molecular evidence for hybridization has also been reported in a few other fungal groups, including *Ophiostoma* (Brasier *et al.* 1998), *Fusarium* (O'Donnell & Cigelnik 1997), and the poplar rust *Melampsora* (Spiers & Hopcroft 1994). However, there has been no report of hybridization in human pathogenic fungi.

The evolution of organisms is influenced by their mode of reproduction, which may be clonal, sexual, or a combination of both. With human pathogens, these patterns of population genetic variation and speciation may impact public health and the management of infectious diseases (Tibayrenc *et al.* 1990; Lederberg *et al.* 1992; Levy 1992). This study used a multiple gene genealogical approach to examine the patterns of molecular evolution in *C. neoformans*.

During clonal evolution, intact genetic material is transmitted asexually and vertically from one generation to the next, and evolutionary changes result exclusively from mutations. Consequently, the genealogies of unrelated genes from clonally reproducing organisms will be identical (Maynard Smith *et al.* 1993; Anderson & Kohn 1998; Taylor *et al.* 1999). Furthermore, assuming a mutation rate, the degree of molecular difference between lineages can be used to infer the approximate time of their divergence (Stephens & Nei 1985; Li *et al.* 1987; Nei 1987; Guttman & Dykhuizen

1994). Over time, continued divergence coupled with geographical isolation and ecological specialization among lineages may produce new species (Nei 1987; Avise 1994). In contrast, sexual evolution is governed by recombination, which fosters the horizontal transfer of genetic material among individuals. The genealogies of unrelated genes from sexually reproducing organisms may differ. Frequent recombination provides a powerful force to reduce the genetic divergence among lineages within a species. Hence, the analyses of multiple gene genealogies can be used to infer clonality, dispersion, speciation, recombination, and hybridization (Avise 1994; Carbone *et al.* 1999; Taylor *et al.* 1999).

Applying these concepts, we developed a multiple gene genealogy approach to understand the genealogical history of the human pathogen *C. neoformans*. The following questions are addressed. First, what is the phylogeographic pattern of molecular variation in *C. neoformans*? Will the

phylogenetic evidence support the hypothesis of ancient origins and maintenance of all varieties on every continent? Second, will molecular evidence from different genes support the traditional classification of *C. neoformans* into different varieties and serotypes? Third, is there significant incongruence among gene genealogies to provide evidence of hybridization in natural populations?

Materials and methods

Strains

The 34 isolates analysed in this paper were collected from a variety of geographical locations (Table 2, Fig. 1) and deposited at the Medical Mycology Research Laboratory at Duke University Medical Center. All except one (B3501) were obtained directly from clinical or environmental sources. Isolates were identified and confirmed by morphological,

| Strain | Geographic origin | Serotype | Source | Year |
|-----------|-------------------|----------|---------------------------|------|
| M0013 | NC, USA | A | CSF of AIDS patient | 1991 |
| M0061 | NC, USA | A | Skin pustule of patient | 1992 |
| J10 | NYC, USA | A | AIDS patient | 1993 |
| CN-A | California, USA | A | CSF of patient | 1937 |
| Cuba2 | Cuba | A | CSF of AIDS patient | 1995 |
| CN444.91 | Brazil | A | CSF of patient | 1997 |
| CN2109.91 | Brazil | A | CSF of patient | 1997 |
| E1 | Brazil | A | Environment | 1997 |
| ZG280 | China | A | Environment | 1976 |
| CN108.97 | India | A | CSF of patient | 1997 |
| KW4 | Kuwait | A | CSF of patient | 1996 |
| MMRL744 | Italy | A | CSF of patient | 1995 |
| MMRL750 | Italy | A | CSF of patient | 1995 |
| CN124.91 | Tanzania | A | CSF of patient | 1991 |
| B3501 | Laboratory strain | D | K. J. Kwon-Chung, NIH | 1972 |
| B10 | NYC, USA | D | Environment | 1994 |
| J9 | NYC, USA | D | AIDS patient | 1993 |
| MMRL751 | Italy | D | CSF of patient | 1995 |
| MMRL757 | Italy | D | CSF of patient | 1995 |
| CN-D | Denmark | D | Environment | 1981 |
| ZG289 | China | D | Environment | 1991 |
| CN111.97 | India | B | Skin of patient | 1997 |
| ZG284 | China | B | Blood of patient | 1981 |
| CN-B | Zaire | B | CSF of patient | 1970 |
| CN119.95 | NC, USA | C | CSF of patient | 1995 |
| CN-C | California, USA | C | CSF of patient | 1981 |
| CN139.97 | Colombia | C | CSF of patient | 1997 |
| CN140.97 | Colombia | C | CSF of patient | 1997 |
| CN106.97 | India | C | CSF of patient | 1997 |
| CN110.97 | India | AD | CSF of patient | 1997 |
| KW5 | Kuwait | AD | CSF of patient | 1996 |
| CN196.88 | NC, USA | AD | Bronchial tube of patient | 1988 |
| M0024 | NC, USA | UT | CSF of AIDS patient | 1991 |
| M0053 | NC, USA | UT | CSF of AIDS patient | 1992 |

Table 2 Strains of *Cryptococcus neoformans* used in this paper

NC, North Carolina; NYC, New York City; CSF, cerebrospinal fluid.

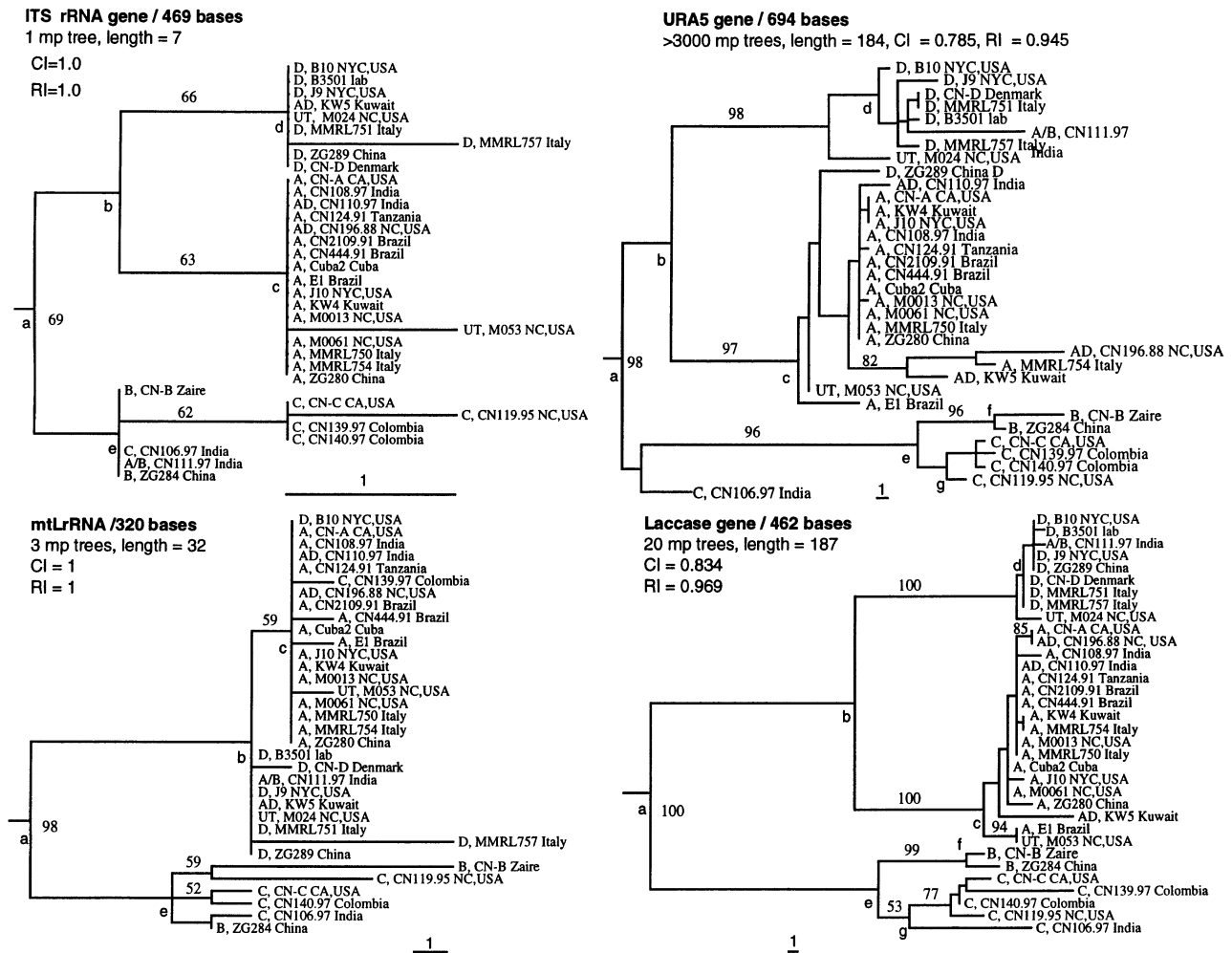


Fig. 1 One most parsimonious tree for 34 isolates of *Cryptococcus neoformans* from each of the four gene regions sequenced. CI, consistency index; RI, retention index. Numbers above each branch are bootstrap values > 50% and based on 500 replicates. For URA5 and LAC trees, branches with > 50% of bootstrap support were also strict consensus branches. Strain designations indicate serotype identification, isolate name, and geographical origin (CA, California; NYC, New York City; NC, North Carolina, all from USA). With the exception of five strains (see text and Fig. 2), all major phylogenetic groups correspond to traditional classifications. Of the two serologically untypeable strains, one (M0024) clustered consistently with the serotype D group and the other (M0053) clustered consistently with the serotype A group. Two of the three strains of serotype AD, CN110.97 and CN196.88, clustered consistently with the serotype A group; while the other (KW5) lacked a consistent affinity with any of the serotypes. Scale bar represents one nucleotide substitution. GenBank accession nos are AF140082–AF140115 (ITS), AF140116–AF140149 (mtLrRNA), AF140150–AF140183 (LAC) and AF140185–AF140217 (URA5).

biochemical and serological tests (Kwon-Chung *et al.* 1982a; Kwon-Chung *et al.* 1982b; Kabasawa *et al.* 1991; Casadevall & Perfect 1998). Among the 34 isolates, 14 are serotype A; seven serotype D; five serotype C; two serotype B; three serotype AD; and two were untypeable. Strain CN111.97 from India reacted very strongly with serotype B antiserum and very weakly with serotype A antiserum.

DNA manipulations

DNA was isolated from each strain as described previously by Xu *et al.* (2000). Portions of four genes were studied, one from the mitochondrial genome and three from the nuclear

genome: (i) the mitochondrial large ribosomal subunit RNA (mtLrRNA); (ii) the internal transcribed spacer region of the nuclear rRNA (ITS), including ITS1, 5.8S rRNA subunit and ITS2; (iii) diphenol oxidase (Laccase or LAC); and (iv) orotidine monophosphate pyrophosphorylase (URA5). Oligonucleotide primers designed to amplify the four gene segments were as follows, 5'–3': (i) mtLrRNA (White *et al.* 1990), forward: gaccctatgcagctctactg, reverse: ttatccctagcgtaactttatc; (ii) ITS (White *et al.* 1990) (on chromosome 2), forward (ITS1): tccgtaggtgaaacctg, reverse (ITS4): tctccgcttattgatatgc; (iii) LAC (Williamson 1994) (on chromosome 5 or 6), forward: ggcgactattatcgt, reverse: ttctggagtggctagagc; and (iv) URA5 (Casadevall *et al.* 1992)

Table 3 Genes, their genomic locations, and the number of nucleotides analysed in this paper

| Gene | Genomic location* | Fragment size (bp) | Ambiguous alignment region† | Unambiguously aligned bases analysed |
|---------|-----------------------------|--------------------|---|--------------------------------------|
| mtLrDNA | Mitochondrion | 320 | 0 | 320 |
| ITS | Nucleus (Chr. 1 or 2) | 522 | 1–20 490–522 | 469 |
| LACCASE | Nucleus (Chr. 4, 5, 6 or 7) | 565 | 1–34 74–119 218 372–376 506–524 | 460 |
| URA5 | Nucleus (Chr. 5, 6) | 744 | 1–48 | 696 |

*Data from refs. White *et al.* 1990, Wickes *et al.* 1994, and Williamson 1994.

†Sequence alignments are available from J. Xu (jpxu@acpub.duke.edu).

(on chromosome 5 or 6), forward: acgctgcctgtactaa, reverse: ggacatgatgaggagt. Chromosomal locations of the three nuclear genes were determined previously by Wickes *et al.* (1994) and Williamson (1994) based on the Southern hybridization profiles of these genes to blots with electrophoretically separated chromosomes of a serotype D strain (Table 3, Wickes *et al.* 1994; Williamson 1994), with the largest band named chromosome 1. However, different varieties, serotypes or strains can present different chromosomal hybridization profiles for the same set of genes (Table 3, Wickes *et al.* 1994).

Each polymerase chain reaction (PCR) contained 10 µL (~1 ng) of diluted genomic DNA template, 0.5 unit of *AmpliTaq* DNA polymerase, 0.2 µM of each primer and 0.2 mM of each deoxyribonucleotide triphosphate in a total volume of 50 µL. The following PCR conditions were used to amplify all genes: 3 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C, and lastly, 7 min of extension at 72 °C. PCR products were cleaned by using Wizard spin columns (Promega) and sequenced using an Applied Biosystems Prism 373 or 377 automated sequencer with dRhodamine-labelled terminators (PE Applied Biosystems), following the manufacturer's instructions. Sequences were generated for both strands. Sequences were aligned and optimized visually. Only unambiguous alignments were used in the phylogenetic and distance analyses (Table 3). Sequence alignments are available from J. Xu at jpxu@acpub.duke.edu.

Data analyses

Phylogenetic analyses of individual genes and all four genes combined, as well as the partition homogeneity tests were all performed with PAUP 4.0 (Swofford 1999). Maximum parsimony trees were identified using heuristic searches based on 500 random sequence additions for each gene region (Maddison 1991; Swofford 1999). Statistical

support for phylogenetic groupings for individual genes and congruence among genes was assessed by bootstrap analysis using 500 replicate data sets (sampled from phylogenetically informative characters) with the random addition of sequences during each heuristic search (Farris *et al.* 1995; Swofford 1999). Midpoint rooting was used to root all four gene trees as appropriate outgroup sequences were not available for every gene. Higher level phylogenetic studies using nuclear-encoded large-subunit and small-subunit RNA gene sequences suggest that *Filobasidiella depauperata* is the closest sibling species to *Cryptococcus neoformans* (Mitchell *et al.* 1992; Casadevall & Perfect 1998). Attempts to amplify URA5 and LAC genes from *F. depauperata* using *C. neoformans* primers were unsuccessful. However, phylogenetic analyses using ITS and mtLrRNA sequences from *F. depauperata* as the outgroup placed the root of the *C. neoformans* trees as indicated in Fig. 1 (results not shown).

To distinguish between the 'historical' hypothesis and the 'recent dispersal' hypothesis as explanations for the widespread geographical distribution of *C. neoformans*, we calculated all pairwise Jukes–Cantor distances (Jukes & Cantor 1969) between strains from the combined data set of four genes. Strains were then grouped according to five geographical regions: North America (including Cuba 2, $n = 12$), South America ($n = 5$), Asia ($n = 9$), Europe ($n = 5$) and Africa ($n = 2$). Student *t*-test was used to compare genetic differences within and between continents. The lack of significant difference between groups would be consistent with the hypothesis of recent dispersal.

To estimate genetic divergence among lineages, we tested whether the four genes evolved according to the molecular clock model. For each gene, Kimura-2 parameter distances were calculated from the maximum-likelihood tree (Felsenstein 1981; Swofford 1999) based on empirical nucleotide frequencies. Maximum-likelihood estimates of the most parsimonious trees with and without a molecular clock demonstrated that while LAC and URA5 genes

evolved in a marginally nonclock-like manner ($0.01 < P < 0.05$), both ITS and mtLrRNA genes did not differ significantly from molecular clock expectations ($P > 0.1$).

Estimates of the time of divergence and hybridization assumed the consensus mutation rate of 2×10^{-9} per nucleotide per year for protein coding genes (Stephens & Nei 1985; Li *et al.* 1987; Nei 1987; Koufopanou *et al.* 1997). However, different genes exhibited different degrees of sequence divergence. Hence, the mutation rates for ITS and mtLrRNA genes were adjusted to those of LAC and URA5, as determined by the relative Kimura-2 parameter distances and assuming the same starting time for divergence in all genes. Using this method, the mutation rate for ITS was estimated as 8.4×10^{-11} , and for mtLrRNA as 6×10^{-10} .

Estimates of the time when hybridization and recombination occurred in each putative hybrid were based on the maximum-likelihood tree for each gene. The Kimura-2 parameter distance was calculated between the most recent node of the hybrid to the tip of the phylogeny (i.e. the hybrid). Among the four genes, the estimate of age show that the earliest hybridization event in each isolate is the maximum age of hybridization.

Results and discussion

Phylogenetic analyses and time since divergence

Of the 1945 unambiguously alignable sites, a total of 318 were variable, including 225 sites that were phylogenetically informative (Table 3, Fig. 1). Parsimony analysis was used to infer the genealogy of each gene (Swofford

1999; Fig. 1). The four genes showed different levels of divergence: LAC is the most divergent, followed by URA5 and mtLrRNA; ITS is the least divergent. The four genealogies showed very similar phylogenetic groupings for most strains (Fig. 1). Analyses by other methods (e.g. Neighbour-Joining, UPGMA, and Maximum-likelihood) revealed the same phylogenetic patterns as the maximum parsimony method (Smouse 1998; Swofford 1999). The phylogenetic relationships largely support the traditional and current classification of *Cryptococcus neoformans* into three varieties and four serotypes: *C. neoformans* var. *neoformans* (serotype D), *C. neoformans* var. *grubii* (serotype A), and *C. neoformans* var. *gattii* (serotypes B and C).

Assuming that the rate of molecular evolution of protein-coding genes is similar to that of other organisms (Li *et al.* 1987; Nei 1987; Koufopanou *et al.* 1997), we estimated the time of divergence into varieties and serotypes. Tests to determine whether the overall evolution of the four genes deviated significantly from molecular clock models (see Materials and methods) were not rejected ($P < 0.01$). Therefore, the distances between individual nodes and terminal taxa can be calculated from maximum-likelihood phylogenies (Swofford 1999). The distances from predominant nodes (labelled in Fig. 1) to their tips are presented in Table 4. The overall molecular divergences between varieties and serotypes are much greater than the divergence within varieties and serotypes (Fig. 1, Table 4). If we assume a neutral mutation rate of 2×10^{-9} per nucleotide per year (Li *et al.* 1987; Nei 1987; Koufopanou *et al.* 1997) for protein coding genes (LAC and URA5), the divergence between *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* occurred about 18.5 million years ago (Ma), and the

Table 4 Estimates of divergence (Kimura-2 parameter distance) among gene lineages in *Cryptococcus neoformans*

| Taxon groups | Node | Genes | | | | Time since divergence in million years* |
|--|------|----------|---------|---------|---------|---|
| | | mtLrRNA | ITS | URA5 | LAC | |
| <i>C. neoformans</i> var. <i>gattii</i> with <i>C. neoformans</i> var. <i>neoformans</i> and <i>C. neoformans</i> var. <i>grubii</i> | a | 0.02037† | 0.00310 | 0.04748 | 0.11296 | 37 |
| Between <i>C. neoformans</i> var. <i>neoformans</i> and <i>C. neoformans</i> var. <i>grubii</i> | b | 0.00225 | 0.00205 | 0.03035 | 0.04685 | 18.5 |
| Within <i>C. neoformans</i> var. <i>grubii</i> | c | 0.00032 | 0.00013 | 0.00580 | 0.01348 | 4.5 |
| Within <i>C. neoformans</i> var. <i>neoformans</i> | d | 0.00225 | 0.00026 | 0.01033 | 0.00121 | 3.3 |
| Between serotypes B and C | e | 0.01178 | 0.00078 | 0.01038 | 0.03158 | 9.5 |
| Within Serotype B | f | 0.01178 | 0.00078 | 0.00425 | 0.00637 | 2.6 |
| Within Serotype C | g | 0.01178 | 0.00028 | 0.00370 | 0.02610 | 6.4 |

*Time since divergence are calculated based on the weighted average of divergence for two protein coding genes (LAC and URA5), assuming a neutral mutation rate of 2×10^{-9} per nucleotide per year for protein coding genes (Stephens & Nei 1985; Nei 1987). The divergence estimated between serotypes A and D is similar to that by Franzot *et al.* (1999). This high level of divergence supports the view that serotypes A and D historically deserve separate variety status (Franzot *et al.* 1999).

†Values in the Table for individual genes represent the Kimura-2 parameter distance between the most recent coalescent node between clades (labelled in Fig. 1) and the tip of the maximum-likelihood phylogenies.

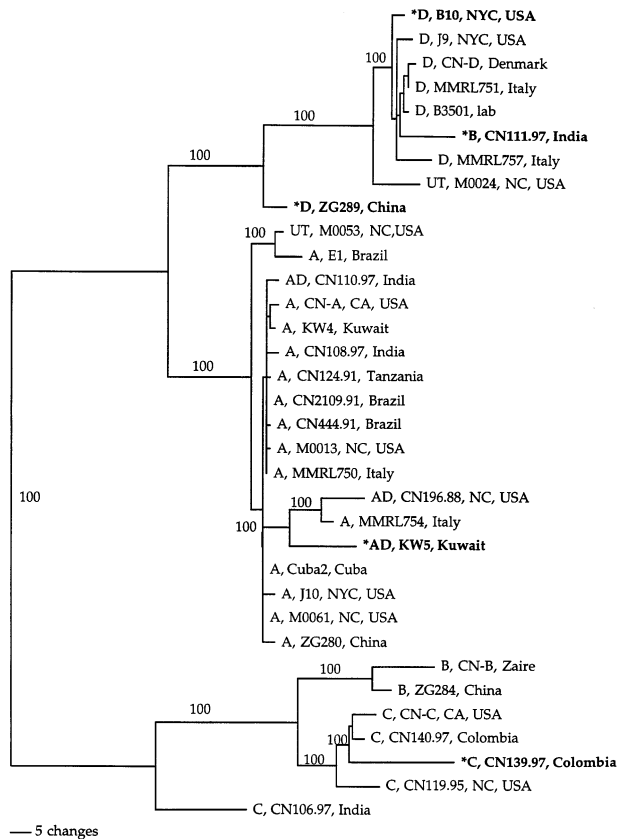


Fig. 2 One of over 6000 equally parsimonious trees inferred from the combined gene genealogies. The tree has a consistency index of 0.7859, retention index of 0.9335, and a length of 453 steps. The scale bar represents five nucleotide changes. The numbers (100) above branches represent that all maximum parsimony trees (> 6000) shared the same groupings, i.e. the strict consensus branches. Strains in boldface had inconsistent genealogical placements among the four genes (Fig. 1, Table 5).

split between serotypes B and C in *C. neoformans* var. *gattii* occurred about 9.5 Ma. The split between *C. neoformans* var. *gattii* and the other two varieties occurred about 37 Ma (Table 4).

This divergence into varieties and serotypes is similar or higher than that of many well-studied sibling species pairs (Stephens & Nei 1985; Li *et al.* 1987; Nei 1987; Avise 1994). Therefore, these results suggest that different varieties of *C. neoformans* are potentially valid phylogenetic species. However, because strains of different varieties and serotypes are able to mate in the laboratory (Kwon-Chung 1975, 1976; 1982b; Kwon-Chung *et al.* 1982a; Casadevall & Perfect 1998) and likely in nature (Fig. 1 and see below), it is not appropriate to recommend separating *C. neoformans* into different species.

Although the results demonstrated significant divergence among lineages corresponding to the recognized varieties and serotypes (Fig. 1), a few strains were notably

discordant. Two (CN110.97, CN196.88) of the three strains of serotype AD grouped consistently with serotype A strains, while the other (KW5) had inconsistent groupings. Therefore, it is possible that AD strains arose from multiple origins. One of the two serologically untypeable strains (M0024) grouped consistently with strains of serotype D, while the other (M0053) grouped consistently with strains of serotype A. The URA5 and LAC gene sequences demonstrated monophyly for strains of serotypes B and C, but the analyses of ITS and mtLrRNA genes were inconclusive (Fig. 1, see also below for strain CN111.97).

The combined gene genealogical analysis demonstrated overall similarity to the phylogenetic relationships derived from individual genealogies (Fig. 1 and Fig. 2). However, the two protein coding genes showed much more divergence and were phylogenetically more informative than the ITS and mtLrRNA genes. Indeed, genealogical information from ITS and mtLrRNA is not apparent in the combined analysis (Fig. 1 and Fig. 2). For example, strains of serotype B in the combined analysis are better separated from strains of serotype C (Fig. 2). In addition, the inconsistent placements of five strains (B10, CN111.97, ZG289, KW5, and CN139.97) in the four gene genealogies are eclipsed in the combined phylogeny (see below).

Recent hybridization among ancient lineages in *C. neoformans*

Strains B-10, ZG289, CN111.97, CN139.97, and KW5 were inconsistently placed in the four genealogies (Fig. 1). Each of these isolates was grouped by different genes with at least one other serotype or variety (Fig. 1). When all the strains are included, phylogenetic incongruence among the four genealogies was statistically significant (Farris *et al.* 1995; Swofford 1999) (ILD test, $P < 0.001$). However, when these five strains were excluded from the analysis, the four genealogies became congruent (ILD test, $P > 0.1$). The genealogical congruence tests were unrelated to the traditional taxonomic classification based on serotypes or varieties, but purely based on the observed sequence data. Furthermore, unlike some other fungal genealogical studies where the number of phylogenetically informative characters were small and competing phylogenies were sometimes difficult to distinguish (e.g. Carbone *et al.* 1999), our results showed significant divergence among lineages and similar unambiguous clustering of individual gene sequences in each lineage. Therefore, the simplest explanation for the different phylogenetic placement of these five strains is the occurrence of hybridization among varieties and serotypes.

There are at least two options for the occurrence of these hybridization events. It is possible that varieties and serotypes hybridized and recombined throughout the history of the species. This hypothesis predicts that divergence would not be evident among varieties and serotypes

Table 5 Estimates for the age of hybridization

| Strain | Geographic origin | Serotype | Gene | | Time range of recombination (in million years)* |
|----------|-------------------|----------|--|---------------------------------------|---|
| | | | Within the identified serotype clade | In other serotype clade(s) (serotype) | |
| B10 | NYC, USA | D | ITS, URA5, LAC | mtLrRNA (A) | 0–0.54 |
| ZG289 | China | D | ITS, mtLrRNA, LAC | URA5(A) | 0–2.36 |
| CN111.97 | India | B/A | ITS | mtLrRNA (D) URA5 (D), LAC (D) | 0–2.62 |
| CN139.97 | Colombia | C | ITS, URA5, LAC | mtLrRNA (A) | 0–0.54 |
| KW5 | Kuwait | AD | ITS (D), mtLrRNA (D), URA5 (A), LAC (A) | 0–3.12 | |

*Age of recombination is based on a mutation rate of 2×10^{-9} for protein coding genes LAC and URA5, with adjustments for the other two genes, ITS and mtLrRNA (see text).
NYC, New York City.

from the same geographical region. However, our results contradict this prediction as most strains of different serotypes are consistently divergent from each other (Table 4, Fig. 1). Most trees support the conventional taxonomic relationships among serotypes and varieties (Table 4, Fig. 1).

An alternative possibility is that divergence among varieties and serotypes was a major evolutionary force in the history of the species, and that hybridization has occurred only recently. Several lines of evidence support the argument of recent hybridization. First, individual gene sequences from recombinant isolates were always placed within a defined cluster (Fig. 1, Table 4). Novel gene lineages, which might represent ancestral hybrid origins, were not detected. A second line of evidence supporting a recent origin of hybrids derives from analysis of coalescent patterns in phylogenetic trees, which can be used to infer the relative timing of evolutionary divergence and hybridization. Under a coalescent model of molecular evolution, the relative age of a gene sequence from its divergence from another sequence can be determined by tracing back to a node of common ancestry in the phylogenetic tree. For all five recombinant strains, the most immediate coalescent nodes of gene sequences were always nearer to the tips of each phylogeny than the most basal coalescent node, which included all isolates of an individual serotype (Fig. 1, Tables 4 and 5). Therefore, the minimum age of recombination events for all five hybrid strains can be set to the present, at the time of sampling (Table 5).

Our estimates of the time for these recombination events ranged from possibly as far back as 3.12 Ma (strain KW5, assuming mutation rate of 2×10^{-9} for LAC and URA) to the present (time 0 for all recombinants) (Table 5). Therefore, the earliest recombination event that produced these five isolates (3.12 Ma) is estimated to be only about

a third to less than one tenth of the time since *C. neoformans* began to diverge into varieties and serotypes (Tables 3 and 4). Varying the mutation rate would alter the ages of divergence and recombination, but not the relative recency of recombination events compared to ancient divergence among lineages. The recent hybridizations were likely related to the recent dispersal of strains of divergent lineages (see below).

Recent dispersal in C. neoformans

None of the four gene genealogies exhibited a pattern of molecular variation that correlated with the geographical origins of the isolates (Fig. 1). Within the whole biological species of *C. neoformans*, there is no evidence for genetic isolation by geographical distance from our analysis of any of the four genes (data not shown). For example, in all four genes, strain CN124.91 from Tanzania was more similar to strain M0013 from North Carolina, USA, than to strain CN-B from Zaire (Fig. 1). Unfortunately, no isolates from Australia were included in this study.

Table 6 presents the mean Jukes–Cantor genetic distance between pairs of strains from the same or different geographical areas. The *t*-tests revealed no significant difference in genetic diversity between samples from any two continents (Table 6). The genetic distances between strains within each continent were all similar to each other. Each continent also had high standard deviations in their pairwise genetic distances between strains. Furthermore, the pairwise distances between strains from different continents were not significantly higher than those between strains from the same continent (Table 6). Therefore, the lack of significant divergence among strains of the same variety and/or serotype from different geographical regions and the significant divergence among strains of different varieties and/or serotypes from the same geographical

Table 6 Mean sequence dissimilarity (Jukes–Cantor distance \pm standard deviation) between pairs of strains of *Cryptococcus neoformans* from the same or different continents

| | North America | South America | Europe | Asia | Africa |
|--|---------------------|---------------------|---------------------|---------------------|---------------------|
| North America (including strain Cuba 2, $n = 12$) | 0.0415 \pm 0.0316 | 0.0434 \pm 0.0335 | 0.0365 \pm 0.0280 | 0.0407 \pm 0.0318 | 0.0535 \pm 0.0397 |
| South America ($n = 5$) | 0.0492 \pm 0.0354 | 0.0494 \pm 0.0295 | 0.0450 \pm 0.0334 | 0.0491 \pm 0.0394 | |
| Europe ($n = 1$) | | | 0.0298 \pm 0.0215 | 0.0399 \pm 0.0282 | 0.0604 \pm 0.0374 |
| Asia ($n = 9$) | | | | 0.0459 \pm 0.0325 | 0.0529 \pm 0.0406 |
| Africa ($n = 2$) | | | | | 0.0913 |

Students *t*-tests demonstrated no significant difference in genetic dissimilarity between pairs of strains from the same or different continents ($P > 0.05$ in all comparisons).

region are consistent with the hypothesis of recent global dispersal in *C. neoformans*.

Apart from their geographical differences, the 34 strains analysed in this study also differed in the time of isolation and type of the specimen (Table 2). Because of the small sample size, it is statistically inappropriate to determine the contribution of isolation time and site to the genetic variation. However, it is unlikely that these factors contributed significantly to the sequence variations. First, compared to the history of the species and individual lineages, the 60 years difference between the earliest and the latest isolations is negligible and significant sequence variation within individual genes is unlikely to have occurred during this time. Second, it has been repeatedly demonstrated that isolates from different body sites of the same host are usually genetically identical (e.g. Brandt *et al.* 1993; 1995; 1996a; 1996b; Casadevall & Perfect 1998). Third, genetically similar or identical strains from patients and their immediate environments have also been demonstrated (summarized in Casadevall & Perfect 1998).

Considering the significant differentiation among lineages, the apparent lack of geographical clustering in *C. neoformans* is somewhat surprising (Figs 1 and 2, Tables 4 and 6). This pattern of population genetic variation is consistent with recent dispersal of *C. neoformans* by humans or other carriers. For example, pigeon droppings are a significant environmental source of *C. neoformans* (Casadevall & Perfect 1998) and association of pigeons and humans may have facilitated the dispersal of *C. neoformans* to many parts of the world. Other demographic and ecological forces may also promote the cosmopolitan dissemination of *C. neoformans* (Mitchell & Perfect 1995; Casadevall & Perfect 1998). For example, it has been suggested that strains of *C. neoformans* var. *gattii* associated with *Eucalyptus* trees in Southern California (Table 1) resulted from the transplantation of *Eucalyptus* trees from Australia (Casadevall & Perfect 1998).

The evidence here does not indicate the geographical origin of each variety and serotype nor the direction of

dissemination. To investigate the origin of each taxon, more strains of each variety and serotype from numerous geographical regions must be analysed. It is hypothesized that the original focus should contain the highest genetic diversity (Avisé 1994; Goodwin *et al.* 1994; Xu *et al.* 1997). For example, since *C. neoformans* var. *gattii* is associated with *Eucalyptus* trees and that *Eucalyptus* is indigenous to Australia, it would be interesting to examine whether populations of *C. neoformans* var. *gattii* from Australia have higher genetic diversity than those from other geographical areas.

Evolutionary and epidemiological implications

The implications of recent dispersion and hybridization among ancient lineages of *C. neoformans* are broad. First, it is likely that the recent dispersion and hybridization in *C. neoformans* are coupled events. The mechanisms of dispersion facilitated by humans and other hosts or vectors could place strains of divergent lineages in close proximity, and subsequent mating among these strains could generate hybrids. Similar suggestions have been applied to other fungal species (Burnett 1983; Tsai *et al.* 1994; Brasier 1995; Brasier *et al.* 1998).

Second, evidence for recent hybridization among divergent lineages in *C. neoformans* suggests that evolutionary divergence and speciation in this human pathogen may be reversing. Recombination and genetic homogenization in *C. neoformans* may indeed be accelerated by the increased global migration of humans and other hosts, as well as by an ever increasing number of susceptible hosts (Lederberg *et al.* 1992). Continued hybridization among divergent lineages could reduce their differences in geographical distribution, ecology, epidemiology, pathobiology and genetic characteristics (Table 1).

Third, our study is relevant to the taxonomy in *C. neoformans*. At present, strains of serotype AD have no clear varietal associations. Furthermore, where should the five strains with inconsistent genealogies be placed? To which

variety do meiotic progenies from crosses between strains of different varieties belong? Under these circumstances, we think it is more appropriate to use the biological species concept and retain one species name (i.e. *C. neoformans*), while recognizing that ancient lineages and significant genetic heterogeneity exist among global populations.

Last, hybridization may also lead to the emergence and evolution of new and possibly more virulent lineages of *C. neoformans* through mechanisms of hybrid evolution (Burnett 1983; Brasier 1995; Ersek *et al.* 1995; Rieseberg 1997). It has been demonstrated that hybridization among fungal plant pathogens in *Phytophthora* and *Ophiostoma* generated new lineages of plant pathogens with novel ecological specialization and virulence (Spiers & Hopcroft 1994; Tsai *et al.* 1994; Brasier *et al.* 1998, 1999). Sexual crosses among ecologically and epidemiologically distinct lineages in *C. neoformans* could produce recombinant genotypes that are better able to exploit natural and clinical environments. Such possibilities indicate that the effect of hybridization on the epidemiology, pathogenesis and evolution of human fungal pathogens warrants additional and vigorous investigation.

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