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Concordance of Clinical and Environmental Isolates of *Cryptococcus neoformans* var. *gattii* by Random Amplification of Polymorphic DNA Analysis and PCR Fingerprinting

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Sixty-one clinical and forty-nine environmental isolates of *Cryptococcus neoformans* var. *gattii* from Australia and the United States were analyzed by random amplification of polymorphic DNA (RAPD), using 12- to 22-mer primers in pairs, and/or PCR fingerprinting with a single primer derived from the microsatellite core sequence of the wild-type phage M13 (5′ GAGGGTGGCGGTTCT 3′). Three major genetic profiles were identified by both typing techniques. A single RAPD profile (VGI) predominated among clinical isolates (44 of 48, 92%) and isolates from host eucalypts (45 of 45, 100%) from Australia. Of the 94 Australian isolates, 4 (3 clinical and 1 environmental) were assigned to profile VGII; 2 of these were recovered from patients and one was recovered from plant debris from Western Australia. Only one Australian clinical isolate was assigned to profile VGIII. A different distribution of RAPD profiles (four VGIII, two VGII, and one VGI) was found among four clinical and three environmental isolates from the United States. RAPD profiles of 8 of the 101 isolates studied revealed minor genetic variants, 4 of profile VGI and 4 of profile VGII. Genetic concordance between the majority of clinical and environmental isolates in Australia is consistent with the hypothesis that human disease is acquired from exposure to host eucalypts. Profiles of clinical isolates were independent of body site of infection, and profiles of all isolates were stable over time. Analysis by PCR fingerprinting confirmed the RAPD results. A second RAPD profile (VGII) was associated with infection in southwest Western Australia, where the two host eucalypts do not occur naturally. This raises the possibility of an alternative and as yet unidentified natural habitat of *C. neoformans* var. *gattii*. Our results indicate that RAPD analysis is a sensitive and useful method for investigating environmental sources of human infection with this biotype.

A simple biotyping technique, based on biochemical differences, was developed in the early 1980s to distinguish between the two varieties of *Cryptococcus neoformans*, *C. neoformans* var. *neoformans* (serotypes A, D, and AD) and *C. neoformans* var. *gattii* (serotypes B and C) (13). This provided a basis for comparing the clinical course and epidemiology of human cryptococcosis caused by the two biotypes and their ecology. *C. neoformans* var. *neoformans* is the commonest cause of fungal meningitis worldwide, and it has a global distribution and a predilection for immunocompromised individuals, especially those with AIDS (6, 16). In contrast, the distribution of human cryptococcosis due to *C. neoformans* var. *gattii* is geographically restricted, the fungus causes disease predominantly in immunocompetent hosts, and characteristic clinical manifestations include large pulmonary or cerebral cryptococcomas (11, 19, 23).

C. neoformans var. *neoformans* is commonly found in weathered pigeon droppings, although it can also be isolated from the excreta of other birds, soil contaminated with avian guano, and rotting vegetables (12). It has been assumed that inhalation of infectious particles from avian excreta is the major route of human infection (9, 10). Support for an epidemiological linkage between clinical and environmental isolates of *C. neoformans* var. *neoformans* has been obtained recently by using restriction fragment length polymorphism analysis with two DNA probes (7, 26). A number of common profiles were identified among clinical isolates and isolates from excreta of pigeons or caged pet birds in the vicinity of patients (7, 26).

In contrast, the only known environmental niches of *C. neoformans* var. *gattii* are the eucalypts, *Eucalyptus camaldulensis* (river red gum) and *E. tereticornis* (forest red gum) (8, 21). The distribution of human cryptococcosis due to *C. neoformans* var. *gattii* is similar to the distribution of these trees (Hawaii, southern California, Mexico, Brazil, parts of Africa, Southeast Asia, Australia, and Papua New Guinea [8, 12, 15]), although *C. neoformans* var. *gattii* has not been isolated from eucalypts outside Australia and California (20). Comparison of a single Californian environmental isolate with three environmental isolates from Australia by karyotyping revealed that the four isolates were genetically different but related (14). Subsequent analysis by DNA probing confirmed differences between the fingerprints of the same three environmental isolates from Australia and three from California, although the isolates from each country were more similar to each other than to those from overseas (26). Genetic typing of human and environmental isolates from geographically defined locations has not been

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reported; evidence for an association between known environmental sources and human infection remains circumstantial.

We have previously developed a random amplification of polymorphic DNA (RAPD) technique using arbitrary primers of 12- to 22-mers in pairs (4, 5) which clearly distinguishes between the two varieties of *C. neoformans* and between serotypes of *C. neoformans* var. *neoformans*. Five major profiles of *C. neoformans* var. *neoformans* have been previously defined (5). In this study, we compare the RAPD profiles of clinical and environmental isolates of *C. neoformans* var. *gattii* from Australia and the United States to investigate the possible link between human infection and exposure to host eucalypts. PCR fingerprinting, a technique in which single primers specific to microsatellite or minisatellite DNA are used to amplify hypervariable genomic DNA sequences of *C. neoformans*, has previously been shown to distinguish between three of the four serotypes (A, D, and B-C) of *C. neoformans* and to identify individual cryptococcal strains from the United States (17, 18). This method was used to confirm the sensitivity of the RAPD technique used in this study.

MATERIALS AND METHODS

Cryptococcal isolates. Clinical isolates were obtained from patients in New South Wales (NSW), Queensland (QLD), South Australia (SA), Victoria (VIC), Western Australia (WA), Northern Territory (NT), and the Australian Capital Territory (ACT) and from the United States as detailed in Table 1. Of the Australian isolates, 45 were recovered from immunocompetent hosts and 3 were recovered from immunosuppressed patients. Thirty-one environmental isolates were obtained over periods of up to 3 years from *E. camaldulensis* trees in two Australia states (NSW and SA), 14 were obtained from *E. tereticornis* trees in two states (NSW and QLD), and 1 was obtained from plant debris in WA. Details of these isolates are summarized in Table 2. Isolates were recovered from environmental samples as described previously by Ellis and Pfeiffer (8). Briefly, air samples (180 liters/min for 5 min) were collected directly onto *Guizotia abyssinica* agar plates (24) with a model 5203 surface air sampler (Pool Bioanalysis Italiana). *E. camaldulensis* and *E. tereticornis* trees were identified with expert botanical assistance and sampled repeatedly over a period of time in most instances (Table 2). Repeated samples were undertaken, as the presence of *C. neoformans* var. *gattii* may be seasonal and not all trees are consistently positive. Soil and vegetation samples were suspended in 20 ml of sterile distilled water and allowed to stand for 10 min; 0.5-ml aliquots were then streaked onto *G. abyssinica* agar plates and maintained at room temperature for 7 days. The plates were examined carefully, daily, by holding them up to direct light. Small, pinhead colonies exhibiting the brown color suggestive of *C. neoformans* on *G. abyssinica* agar plates were streaked for single colonies. The identities of all such yeast colonies were determined by the API 20C AUX system (BioMerieux, Marcy l'Etoile, France) and biotyped by standard techniques (12). Three isolates from eucalypts in California (two from San Diego and one from near Fort Point, San Francisco) were included for comparison (Table 2). Serotyping was performed by using the Crypto Check agglutination test (Iatron Laboratories Inc., Tokyo, Japan). Isolates were subcultured before use onto Sabouraud dextrose agar plates (Difco Laboratories, Detroit, Mich.) to ensure purity of growth. Cryptococcal colonies were harvested after 72 h of incubation at 30°C and used for DNA preparation.

RAPD analysis. (i) DNA isolation. Genomic DNA was extracted and purified by a modification of the method of Boom and coworkers (2). Approximately 0.5 to 1 g of cells (wet weight) was suspended in an excess of lysis buffer (0.2 M NaOH, 1% sodium dodecyl sulfate [SDS], 10 mM EDTA). The suspension was boiled for 30 min and centrifuged at 12,000 × *g* for 5 min. The supernatant was neutralized with 3 M potassium acetate–2 M acetic acid and centrifuged as before. The resulting supernatant was incubated with 10% Celite 545 resin (Fluka Chemical Corp, Ronkonkoma, N.Y.) prewarmed to 60°C in guanidine HCl (pH 5.5) on a rotary mixer for 4 to 15 h. The DNA-resin mix was poured into a column and washed three times with a solution containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.6), and 2.5 mM EDTA in 50% ethanol. The wash solution was allowed to drain under gravity, and the column was centrifuged briefly to remove excess wash solution. DNA was eluted with Tris-EDTA buffer (10 mM Tris [pH 7.6], 1 mM EDTA) at 55°C. The elution procedure was repeated twice. DNA was quantified spectrophotometrically.

(ii) PCR amplification. Three 20- to 22-mer primers were chosen arbitrarily from laboratory stocks and used in pairs. They were CN1 (5′ TACCCCCGCC CATATTCCAT 3′), MYC1 (5′ GAGGAAGGTGGGGATGACGT 3′), and 5SOR (5′ ATGGGAATACGACGTGCTGTAG 3′). Six 12-mer primers, including FPK1-01 (5′ ACACGGACGTCA 3′), FPK1-05 (5′ ACTTGGCGGCCT 3′), FPK1-07 (5′ ACCCTGCTCATC 3′), FPK1-08 (5′ CAGGCGAAGGTT 3′), FPK1-13 (5′ ACGGCTGGTTCC 3′), and FPK1-20 (5′ CAACAGCCCCCA 3′), were obtained commercially (FPK1-FAPD kit; Bresatec, Thebarton, SA, Aus-

TABLE 1. Clinical isolates of *C. neoformans* var. *gattii*[a]

Isolate no.	Geographical origin	Yr isolated	Clinical source	Serotype
WA-3	WA	1994	Skin	B
WA-7	WA	1991	Lung	B
WA-8	WA	1985	CSF	B
WA-9	WA	1965	Lung	B
WA-10	WA	1987		B
WA-11	WA			B
WA-12	WA	1990	CSF	B
WA-13	WA	1965	CSF	B
WA-14	WA	1994	CSF	B
W-1	NSW	1990	CSF	B
W-7	NSW	1991	CSF	B
W-8	NSW	1991	Lung	B
W-9	NSW	1990	Lung	B
W-27	NSW	1994	Lung	B
RNS-1[b]	NSW	1994	Lung	B
WGH-1	NSW	1994	Lung	B
RPAH-1	NSW	1994	Lung	B
ACT-1	ACT	1991	Lung	B
RM-1	VIC	1989	Lung	B
RM-2	VIC	1989	CSF	B
RM-3	VIC	1989	Lung	B
AU-1	VIC	1988	CSF	B
AU-2	VIC	1990	CSF	B
F-7	VIC	1991	CSF	B
F-8[c]	VIC	1991	CSF	B
F-9	VIC	1992	CSF	B
F-10	VIC	1982	Bone	B
F-11	VIC	1987	Lung	B
F-12	VIC	1988	Brain	B
F-13	VIC	1991	CSF	B
F-14[c]	VIC		CSF	B
LK-16	QLD	1992	Lung	B
PAH-13	QLD	1994	Lung	B
PAH-23	QLD	1994		B
QML-1	QLD	1994	Lung	B
IMVS-1	SA	1990	CSF	B
IMVS-2	SA	1990	CSF	B
IMVS-3	SA	1992	Lung	B
IMVS-4	SA	1989	Lung	B
QEH-1	SA	1985	Lung	B
QEH-2	SA	1985	Lung	B
ASH-1	NT	1994	CSF	B
RDH-2	NT	1994	Lung	B
NT-2[d]	NT			B
NT-3[d]	NT			B
NT-4[d]	NT			B
NT-5[d]	NT			B
NT-6[d]	NT			B
n32	DUMC			B
n33	DUMC			C
371-B	UCLA		CSF	B
373-B	UCLA			B
381-B	UCLA			B
385-B	UCLA			B
396-B	UCLA			B
380-C	UCLA			C
381-C	UCLA			C
384-C	UCLA			C
ATCC 32609	ATCC	1981	Lung	B
ATCC 34878	ATCC	1981		B
ATCC 32608	ATCC	1981	CSF	C
ATCC 34880	ATCC	1981	CSF	C

[a] Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); DUMC, Duke University Medical Center (Durham, N.C.); UCLA, University of California at Los Angeles (Los Angeles); CSF, cerebrospinal fluid.
[b] Isolated from a patient with sarcoidosis.
[c] Recovered from HIV-infected individuals.
[d] Isolates from patients residing in Arnhem Land, NT.

TABLE 2. Distribution and sources of 49 environmental isolates of *C. neoformans* var. *gattii* analyzed by RAPD

Geographical location	Tree type	No. of sites sampled	Period of sampling[a]	Isolate source	Isolate no.
Balranald (western NSW)	*E. camaldulensis*	3	12-89–12-90 (2 collections, 12 mo apart)	Bark, wood Fruit, soil Woody debris	E401, E410 E402 E403, E404, E405, E406, E407, E409
Tocumwal (River Murray, NSW)	*E. camaldulensis*	1	1-91	Woody debris	E414
Mt. Annan (Greater Sydney, NSW)	*E. tereticornis*	1	11-91–12-94 (2 collections, 3 yr apart)	Woody debris	E486, E487, E493
Currumbin (Gold Coast)	*E. tereticornis*	1	1-91–12-93 (3 collections)	Woody debris	E412, E413, E415, E416, E417, E418, E419, E420, E421
Barossa Valley (SA)	*E. camaldulensis*	7	12-89–11-92 (5 collections over 3 yr)	Debris in tree hollow Leaf debris Bark Air sample Plant debris Fruit	E40, E55, E59, E71, E81, E95 E5, E4, E101 E35, E36, E37, E17, E108 E14, E29, E38 E9, E30, E49, E50, E58 E107
Busselton (WA)	Plant debris near fence of paddock	1	1993	Plant debris	E141
San Francisco, Calif.	*E. camaldulensis* (or hybrid)	1		Woody debris	E451
San Diego, Calif.	*Eucalyptus* spp. *E. citriodora*	1 1		Woody debris Woody debris	E698 E697

[a] Dates, month-year.

tralia). CN1, MYC1, and 5SOR were used in pairs. The 12-mer primers were used in the following combinations: FPK1-01–FPK1-05, FPK1-05–FPK1-07, FPK1-05–FPK1-13, and FPK1-08–FPK1-20. PCR was performed in 25-μl volumes, each containing 10 ng of DNA, 3 mM magnesium chloride (12-mer primers) or 6 mM magnesium choride (20- to 22-mer primers), 200 μM (each) deoxynucleoside triphosphate (Promega Corporation, Sydney, NSW, Australia), 10 pmol of each primer in combination, and 0.8 U of *Taq* DNA polymerase (Advanced Biotechnologies, Sydney, NSW, Australia) under recommended buffer conditions. Amplification was carried out in an MJ Research PTC-100-60 thermal cycler (Bresatec) as follows: denaturation at 93°C for 3 min, annealing at low stringency (10 cycles of 35°C, 72°C, and 93°C, each for 1 min) followed by high-stringency annealing (20 cycles at 55°C, 72°C, and 93°C for 1 min each), with a final extension step at 72°C for 5 min. The parameters were changed for the primer pair 5SOR-CN1 to include an annealing temperature of 40°C in the first 10 cycles and 30 rather than 20 cycles at the high-stringency step.

Products of amplification were separated by electrophoresis on 7 to 10% polyacrylamide gels and visualized by silver staining by using a modification of the method of Bassam and Caetano-Anolles (1), in which 0.6 μg, and not 2.0 μg, of sodium thiosulfate was used per ml in the developing step. RAPD assays of each DNA preparation were repeated at least once. In addition, DNA preparations from two separate cultures of each isolate were run in parallel. Analysis of further DNA preparations from selected isolates on different occasions over a 2-year period under identical test conditions was performed to confirm the reproducibility of the method. Bands were included in the analysis if they were visible, regardless of the intensity of the strain. RAPD profiles were classified into major patterns if all seven primer pairs produced different and reproducible patterns. Minor variants within the major profile were designated with a lowercase letter (a, b, etc.) if a consistent difference was noted with one primer pair and with an uppercase letter (A, B, etc.) if a difference was noted with more than one, but not all, of the seven primer pairs. Analysis showed subsequently that band differences occurred with one, three, or all seven primer pairs. The reproducibility of the method has previously been confirmed (5).

PCR fingerprinting. (i) DNA isolation. DNA was extracted as described previously (17). Briefly, cryptococcal cells contained in a microcentrifuge tube were ground in liquid nitrogen with a miniature pestle (Kontes, Vineland, N.J.) and resuspended in 700 μl of extraction solution preheated to 55°C. The stock solution contained 100 mg of triisopropylnaphthalene sulfonic acid (Serva, Heidelberg, Germany), 600 mg of *para*-aminosalicylic acid (Serva), 10 ml of deionized water, 2.5 ml of buffer (1 M Tris-HCl, 1.25 M NaCl, 0.25 M EDTA [pH 8.0]), and 7.5 ml of Tris-EDTA buffer-saturated phenol. After incubation for 2 min at 55°C, 500 μl of chloroform was added, incubation was continued for a further 2 min at 55°C, the mixture was centrifuged at 2,500 × *g* for 10 min, and the aqueous phase was transferred to a new microcentrifuge tube. The extraction was repeated, first with an equal volume of phenol-chloroform, 1:1, and then with an equal volume of chloroform alone. Genomic DNA was precipitated from the final aqueous phase by the addition of 0.03 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold 96% ethanol, incubation for at least 1 h or overnight at −20°C, and centrifugation at 4°C for 30 min at 14,000 × *g*. The DNA pellet was then washed with 70% ethanol, dried in air, resuspended in deionized water, and stored at 4 or −20°C.

(ii) PCR amplification. PCR fingerprinting was performed as outlined previously (17, 18). The minisatellite-specific core sequence of the wild-type phage M13 (5′ GAGGGTGGCGGTTCT 3′) was used as a single primer in the PCR (27). Amplifications were performed in 50-μl volumes, each containing 25 ng of DNA; 10 mM Tris-HCl (pH 8.0); 50 mM KCl; 1.5 mM $MgCl_2$; 3 mM magnesium acetate; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP (Boehringer GmbH, Mannheim, Germany); 30 ng of primer; and 2.5 U of Amplitaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.). PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler (model 480) as follows: denaturation at 93°C for 20 s, annealing at 50°C for 30 s, and extension at 72°C for 20 s, followed by a final extension cycle at 72°C for 6 min. Prior to analysis, samples were concentrated to approximately 20 μl (Speed Vac; Savant, Hicksville, N.Y.) and analyzed by electrophoresis in 1.4% agarose gels in 1× Tris-borate-EDTA buffer for 13 h at 3 V/cm. Amplification products were detected by staining gels with ethidium bromide and photographing under UV light. PCR fingerprinting patterns were assigned according to the major bands that were typical for that pattern; bands of approximately 380, 600, 700 to 850, and 3,000 bp in size were taken into consideration in grouping the strains. Bands were included in the analysis if they were visible, independent of their intensity (18).

RESULTS

Collection sites. Environmental sites from which *C. neoformans* var. *gattii* was cultured and their relationship to the natural distribution of *E. camaldulensis* and *E. tereticornis* in Australia are shown in Fig. 1. Multiple collections were made from seven sites (Barossa Valley) within a 15-km radius of the domicile of a patient with pulmonary cryptococcosis due to *C. neoformans* var. *gattii*, over a 3-year period, as detailed in Table

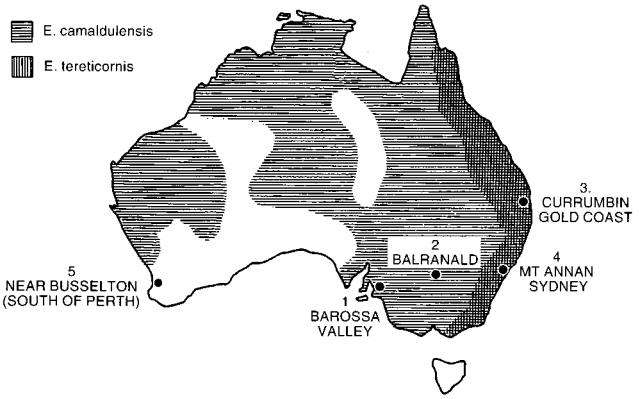

FIG. 1. Collection sites in Australia. 1, Barossa Valley, 50 km north of Adelaide. Collections made from single *E. camaldulensis* trees growing at each of seven sites. 2, Balranald, 500 km east of Adelaide. Collections made from a single *E. camaldulensis* tree. 3, Currumbin, Gold Coast, 80 km southeast of Brisbane. Collections made from a single *E. tereticornis* tree. 4, Mt. Annan Botanical Gardens, 70 km west of Sydney. Collections made from a stand of five *E. tereticornis* trees. 5, Busselton, 200 km southeast of Perth. Collections made from unidentified plant debris accumulated along a farm fence line.

2. Sampling was also performed in the environs of two patients living in Greater Sydney but without success. Environmental isolates from California were obtained from an *E. camaldulensis* (or hybrid) tree in San Francisco and from two eucalypts in the San Diego Zoo area, one of which was identified as *E. citriodora* and the other of which could not be identified to species level accurately but may have been a hybrid *E. camaldulensis* (7a). The distribution of the 48 Australian clinical isolates based on domiciles of the patients is shown in Fig. 2. The geographical origins and clinical sources of all the isolates studied (where known) are summarized in Table 1.

Reproducibility of RAPD profiles. Analysis of separate DNA preparations of the same strain over a 2-year period produced identical RAPD patterns in all instances, as did DNA preparations from two separate cultures of the same isolate analyzed

FIG. 2. Distribution of 48 cases of *C. neoformans* var. *gattii* infection in Australia. The number of cases in each location is as follows: Greater Perth, 8; Fremantle, 1; Alice Springs, 1; Arnhem Land, 6; Greater Brisbane, 3; Canberra, 1; Greater Adelaide, 5; Barossa Valley, 1; Greater Sydney, 5 (the remaining four dots on the map in NSW represent one case each); Greater Melbourne, 9 (the remaining four locations in Victoria represent one case of infection each).

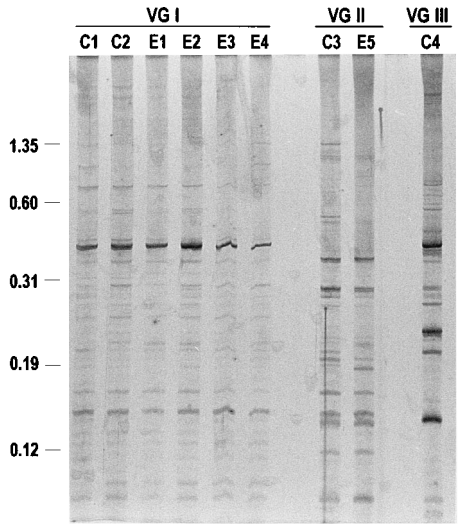

FIG. 3. RAPD profiles of *C. neoformans* var. *gattii* amplified with the primer pair FPK1-05–FPK1-07 illustrating the three major RAPD profiles as indicated in the top row. Molecular size reference markers (ϕX174 DNA digested with *Hae*III) in kilobases are indicated on the left. The other lanes contain amplified DNA from clinical (C) and environmental (E) isolates designated as follows: lane C1, W-1; lane C2, IMVS-4; lane E1, E50; lane E2, E403; lane E3, E420; lane E4, E486; lane C3, WA-12; lane E5, E1411; lane C4, QEH-2.

at the same time run in parallel. Variations in the intensities of amplified fragments from separate DNA preparations of the same isolate or from separate PCR amplifications performed on the same DNA preparation were observed. RAPD profiles were therefore assigned according to the number and position of bands present and not according to their intensity (5).

Characterization of RAPD profiles. Three major fingerprint patterns, designated VGI (var. *gattii* I) to VGIII were identified among the 52 clinical and 49 environmental isolates of *C. neoformans* var. *gattii* analyzed by RAPD. Examples of the three major RAPD profiles after amplification with the primer pair FPK1-05–FPK1-07 are shown in Fig. 3. The slight variations observed in the banding patterns around the 0.19-kb reference marker in lanes C3 and E5 were not consistently observed; the representative isolates were therefore assigned to the same profile. Within these major profiles, variants (see Materials and Methods) were distinguished. Amplification with the primer combinations 5SOR-CN1, MYC1-5SOR, and FPK1-05–FPK1-07 revealed minor differences in banding patterns which distinguished the subtypes VGIIA and VGIIB within profile VGII (Fig. 4). The use of the primer pairs FPK1-08–FPK1-20 and FPK1-05–FPK1-13 distinguished the subtypes VGIa and VGIb, respectively, within profile VGI (data not shown). All the primer combinations distinguished between the three major RAPD patterns. The relationship between RAPD profile, source of isolate (environmental or clinical), and country of origin is shown in Table 3. Of the 48 Australian clinical isolates, 44 (92%) were assigned to profile VGI, 3 (6.3%) were assigned to profile VGII (1 to VGII and 2 to VGIIB), and 1 (2%) was assigned to profile VGIII. All were serotype B. Three of the four American clinical isolates were assigned to profile VGIII (ATCC 34878, ATCC 32608, and ATCC 43880); one of these was serotype B, and the other two were serotype C. The remaining American isolate (ATCC 32609, serotype B) belonged to RAPD profile VGIIA (Fig. 4). Two of the three Australian clinical isolates (WA-8 and WA-12) and the only environmental isolate (E141) assigned to

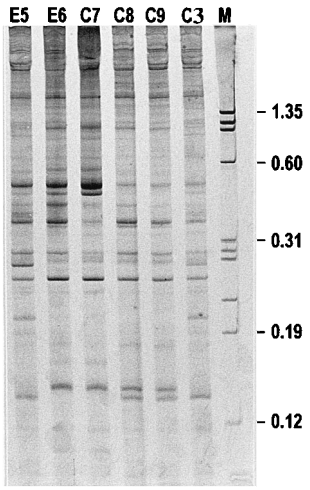

FIG. 4. Representative gel showing variation within RAPD profile VGII (amplification with 5SOR-CN1). M, molecular size reference markers as described in the legend to Fig. 3. Other lanes contain DNA from cryptococcal isolates as follows: lane E5, E1411 (plant debris, WA); lane E6, E451 (*E. camaldulensis*, San Francisco); lane C7, ATCC 32609; lane C8, WA-8 (clinical isolate, WA); lane C9, W-7 (clinical isolate, NSW); lane C3, WA-12 (clinical isolate, WA). Isolates E451 and ATCC 32609 were assigned to profile VGIIA, whereas isolates WA-8 and W-7 were assigned to profile VGIIB.

profile VGII were obtained from WA. The third clinical isolate assigned to profile VGII (W-7) was obtained from a patient living on the central coast of NSW. This patient was a construction engineer who had travelled widely throughout Australia but who had not visited WA. The isolate assigned to profile VGIII was obtained from a patient living in SA whose travel history was unobtainable. RAPD profiles were independent of the clinical source of the isolates. The number of patients with underlying immunosuppression ($n = 3$) was too small to test for a correlation with RAPD profile.

Among the Australian eucalypt isolates, all 45 were serotype B and were assigned to profile VGI; these included 4 minor variants, 2 of profile VGIa and 2 of profile VGIb. The RAPD profile of the single environmental isolate from WA was obtained from the fence line of a field containing sheep with cryptococcosis and was identified as VGII (serotype B). RAPD profiles of isolates obtained from material collected from different components of the same tree at the same time and from the same tree over a period of time were, with the exception of those noted above, identical with all the primer pairs. RAPD profiles of the three American environmental isolates were VGI, VGIIA, and VGIII as shown in Table 3.

PCR fingerprinting. To confirm the sensitivity of the RAPD method, 8 clinical and 20 environmental cryptococcal isolates were also studied by PCR fingerprinting. In addition, 10 clinical isolates from the United States previously analyzed by this technique were included for comparison (Table 1) (18). The minisatellite-specific M13 core sequence primer successfully amplified hypervariable DNA fragments from all isolates. Three major PCR fingerprints (patterns 1 to 3), which corresponded to the three profiles established by RAPD analysis (VGI to VGIII), were observed. Examples of each of these three general PCR fingerprint patterns are illustrated in Fig. 5 (pattern 1, lane 16; pattern 2, lane 19; pattern 3, lane 17). All isolates tested were grouped accordingly. Australian environmental isolates from the Barossa Valley, SA; Mt. Annan, NSW; Balranald, NSW (isolate E402); and the Gold Coast, QLD (isolate E415) were assigned to pattern 1. The profiles of these isolates were identical, with the exception of isolates E402 and E406, whose fingerprints revealed only minor variations (Fig. 5); the RAPD profile of isolate E406 was a variant of profile VGI (VGIb). The remaining environmental isolate (E416) from the Gold Coast was assigned to pattern 3; this isolate belonged to RAPD profile VGI. Three clinical isolates from Australia (W-8, WA-3, and IMVS-4) were assigned to pattern 1, one isolate (W-7) was assigned to pattern 2, and the remaining isolate (QEH-2) was assigned to pattern 3. The fingerprints of the American clinical and environmental isolates revealed the same three major patterns (Fig. 5). Six of the 11 American serotype B isolates and all of the serotype C isolates studied were assigned to pattern 3, pattern 2 was represented by strains E451 and ATCC 32609, while strains E697 and UCLA 371-B were assigned to pattern 1. However, each isolate was distinguished by a unique PCR fingerprint, indicating strain variation between them.

DISCUSSION

Evidence for an epidemiological link between the natural habitat of *C. neoformans* var. *gattii* and human infection remains circumstantial. The global distribution of the putative host trees, *E. camaldulensis* and *E. tereticornis*, approximates that of human cases (8). Despite the conduct of environmental searches in Australia and elsewhere, no other natural source of *C. neoformans* var. *gattii* has been identified. Examination of a large number of *C. neoformans* var. *gattii* isolates by RAPD analysis in the present study has shown genetic concordance between the majority of clinical (92%) and eucalypt (100%) isolates in Australia, which is supportive of the epidemiological association between this natural cryptococcal reservoir and human clinical disease. In addition, we have recently demonstrated that the koala, a species of animal indigenous to Australia whose diet includes the leaves of the two host eucalypts, is colonized with and can develop disease due to *C. neoformans* var. *gattii*, with a RAPD profile (VGI) identical to that of eucalypt-derived isolates (22).

TABLE 3. RAPD profiles of clinical and environmental isolates of *C. neoformans* var. *gattii*

Isolate type, country of origin, and tree type	RAPD profile (no. of isolates)		
	VGI	VGII	VGIII
Environmental			
Australia			
E. camaldulensis	31[a]		
E. tereticornis	14		
Plant debris		1	
United States			
E. camaldulensis or hybrid		1[b]	
Eucalyptus spp.[c]			1
E. citriodora	1		
Clinical			
Australia	44	3[d]	1
United States		1[b]	3

[a] Twenty-seven isolates, VGI; two isolates, VGIa (from two air samples obtained 2 months apart from hollow of same tree in the Barossa Valley); two isolates, VGIb (from debris obtained from the same tree on the same day [Balranald]).
[b] VGIIA.
[c] Species uncertain (perhaps hybrid *E. camaldulensis*).
[d] One isolate, VGII; two isolates, VGIIB.

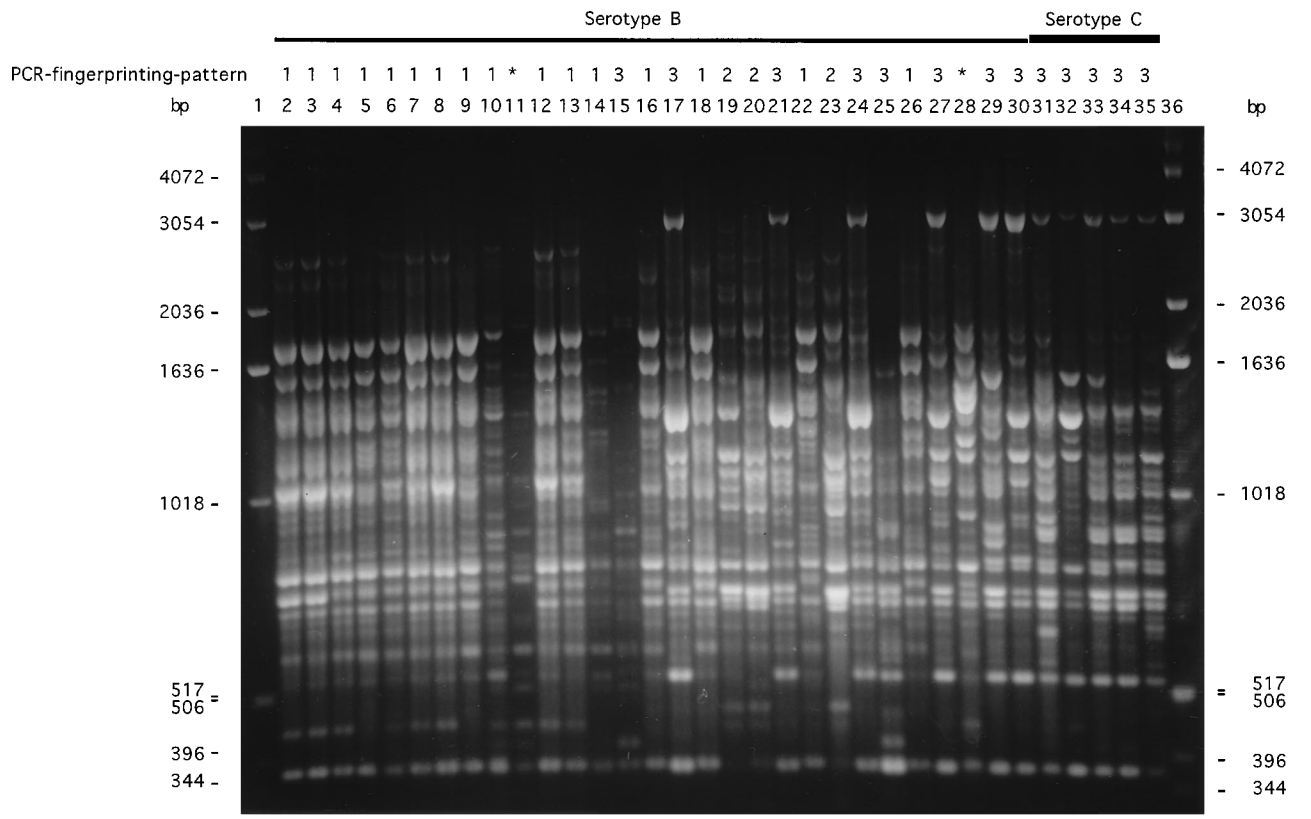

FIG. 5. Electrophoretic separation of PCR fingerprints of clinical and environmental isolates of *C. neoformans* var. *gattii* by amplification of genomic DNA with the M13 core sequence. Lanes 1 and 36, 1-kb ladder (GIBCO-BRL); lanes 2 through 8, isolates E4, E81, E14, E29, E35, E38, and E95, respectively (Barossa Valley); lane 9, isolate IMVS-4; lanes 10 and 11, isolates E402 and E406, respectively (Balranald); lanes 12 and 13, isolates E485 and E487, respectively (Mt. Annan); lanes 14 and 15, isolates E415 and E416, respectively (Gold Coast); lanes 16 through 19, isolates WA-3, QEH-2, W-8, and W-7, respectively; lanes 20 to 22, isolates E451 (San Francisco) and E689 and E697 (San Diego), respectively; lanes 23 through 35, isolates ATCC 32609, ATCC 34878, DUMC n32, UCLA 371-B, UCLA 373-B, UCLA 381-B, UCLA 385-B, UCLA 396-B, ATCC 34880, DUMC n33, UCLA 380-C, UCLA 381-C, and UCLA 394-C, respectively. *, strains that were not able to be grouped in either of the three PCR fingerprint patterns.

Although our data suggest that human disease is acquired from exposure to eucalypt-associated *C. neoformans* var. *gattii*, important questions remain. We noted a close correlation between human and environmental isolates of profile VGI in those areas which are covered by tracts of the host eucalypts. However, seven of nine clinical isolates from WA assigned to profile VGI were obtained from patients living in the Perth-Fremantle area. Neither *E. camaldulensis* nor *E. tereticornis* occurs naturally in the southwestern region of WA. The occurrence of the infections may be explained by human exposure during travel to an area of endemicity; by the occurrence, as yet unreported, of one or both eucalypt species in this area and/or the artificial introduction of some trees, as is known to have occurred in the Perth area (7a); or by the existence of an additional environmental source of *C. neoformans* var. *gattii*. The present study does not allow us to distinguish between these possibilities; however, we have previously observed that *C. neoformans* var. *gattii* is more readily recovered from natural stands of host eucalypts in undisturbed locations than from lone trees in urban environments (7a). It is of particular interest that of the three Australian clinical isolates assigned to RAPD profile VGII in our study, two were obtained from patients who lived in the southwest of WA; the single environmental isolate with profile VGII was likewise obtained from WA. Infection with this genetic type in WA supports the possibility of an environmental source of *C. neoformans* var. *gattii* other than the two known host eucalypt species, as all eucalypt isolates to date have been of RAPD profile VGI.

Comparison of the 94 Australian cryptococcal isolates with 7 from the United States confirmed that isolates from each continent were largely distinct, as has been reported previously with smaller numbers of isolates analyzed by DNA hybridization with the probe UT-4p (26). The concordance of molecular type between three clinical isolates and one environmental isolate and between a fourth clinical isolate and a second environmental isolate from the United States is consistent with the eucalypt species as a natural habitat for *C. neoformans* var. *gattii* in the United States.

It is of interest to note the contrast between the homogeneity of fingerprints in our clinical cryptococcal isolates and the heterogeneity reported by analysis by alternative typing methods of smaller numbers of isolates. For example, 11 genetic profiles were identified among 24 isolates by DNA probing in a study which included isolates obtained from six countries (26). Seven profiles were identified among 12 Australian isolates in this study; however, no information on the source patients was available (26). Karyotyping of isolates of *C. neoformans* var. *gattii* from the United States has also revealed heterogeneity (14), as has a PCR-based technique, also used in this study, directed at minisatellite repeat sequences in *C. neoformans* (18). The latter technique demonstrated that 12 strains of *C. neoformans* var. *gattii* (6 serotype B and 6 serotype

C) had one major PCR fingerprinting pattern. Variations in this major banding pattern, especially within the higher-molecular-weight bands, permitted the identification of individual strains (18). These findings raise the possibility that the homogeneity observed by us arises from the relative insensitivity of the RAPD technique rather than a true homogeneity of the isolates. Brandt and coworkers reported identity of U.S.-derived serotype B (three isolates) and serotype C (three isolates), with resemblance of a single serotype B isolate to the serotype C strains, by RAPD (3). RAPD analysis of 21 clinical and 8 environmental isolates of *C. neoformans* var. *neoformans* in Nagasaki, Japan, revealed that 18 of the 29 isolates belonged to a single pattern and that 6 of the remaining 11 belonged to one other profile (28). In that study, the relative homogeneity of isolates was confirmed by DNA hybridization with the UT-4p probe (28).

PCR fingerprinting was used in this study to validate the sensitivity of the RAPD technique. In previous experiments studying over 100 American clinical and environmental isolates of *C. neoformans*, 12 of which were *C. neoformans* var. *gattii* (17, 18), it was shown that single primers, specific to simple repetitive (microsatellite DNA) sequences [(CA)8, (CT)8, (GTG)5, and (GACA)4] as well to minisatellite DNA sequences (core sequences of the wild-type phage M13), were able to generate individual strain-specific DNA polymorphisms. All strains had a unique banding pattern (18). In contrast, the PCR fingerprints of Australian environmental and clinical isolates of *C. neoformans* var. *gattii* exhibited a much higher (approximately 99%) genetic homology than did isolates from the United States (72% [17, 18]). Most of the Australian isolates tested had identical PCR fingerprinting profiles. The results indicate that there is greater genetic variability among isolates of *C. neoformans* var. *gattii* from the United States than among those from Australia and confirm the observations made by RAPD analysis. RAPD analysis also confirmed the close similarity (two identical and one very similar) observed by DNA probing (26) and the identity by karyotyping (14) of the same three Australian environmental isolates studied independently by ourselves and in the United States. Our method is a simple and reproducible one for the investigation of potential environmental sources of infection with *C. neoformans* var. *gattii* and for the comparison of isolates from different countries.

In conclusion, we have demonstrated that there is substantial genetic homogeneity among Australian isolates of *C. neoformans* var. *gattii* by using RAPD analysis. Pattern similarities extend to separate isolates from the same tree, from neighboring trees, and from trees of different geographic areas. There is a regional correlation between clinical and environmental isolates. Fingerprints of environmental and clinical isolates from the United States differ from those obtained in Australia. The existence of infection caused by the uncommon genetic type (VGII) of *C. neoformans* var. *gattii* in southwest WA, and perhaps in NSW, underlines the importance of continuing to define the range of ecological niches of *C. neoformans* var. *gattii*. Southwest WA is a focus of animal infection with *C. neoformans* var. *gattii*; RAPD analysis and PCR fingerprinting of these isolates would be valuable in the search for a new cryptococcal habitat, as animals are much less mobile than humans. Further epidemiological studies investigating genetic diversity in this biotype of *C. neoformans* from a range of geographical locations are indicated.

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