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Molecular Typing of *Cryptococcus neoformans*: Taxonomic and Epidemiological Aspects

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Pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) analysis, serotype, and killer toxin sensitivity patterns of a wide range of saprobic, clinical, and veterinary isolates of both varieties of *Cryptococcus neoformans* were examined. *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* differed in chromosomal makeup, RAPD patterns, and killer sensitivity patterns. These results suggest that there are two separate species rather than two varieties. No clear genetic or phenotypic differences were observed among the clinical, saprobic, and veterinary isolates within each taxon. The serotypes differed substantially in their RAPD characteristics. Geographical clustering was observed among the isolates of *C. neoformans* var. *gattii*, but not among the isolates of *C. neoformans* var. *neoformans*. The isolates of each taxon that originated from restricted geographical areas often had identical or similar karyotypes and RAPD patterns, suggesting that clonal reproduction had occurred. The combination of PFGE and RAPD analysis allowed us to distinguish almost all isolates. This combination of techniques is recommended for further research on epidemiological, ecological, and population issues.

Cryptococcus neoformans (Sanfelice) Vuillemin is a zoopathogenic basidiomycetous yeast (teleomorph, *Filobasidiella neoformans* Kwon-Chung) which is usually encountered in the imperfect state. This fungus can cause serious infections, especially in immunocompromised patients. Estimates of the incidence rate in AIDS patients range from 5 to 30%, with the highest numbers occurring in sub-Saharan Africa (21, 30). In human immunodeficiency virus-seropositive individuals, an infection with *C. neoformans* indicates progression to AIDS (40). The problem is aggravated by the emergence of cryptococcal strains that have become resistant to some of the most widely used antifungal agents (11, 19, 33, 34).

According to the current classification, *C. neoformans* consists of the following two varieties: *C. neoformans* var. *neoformans*, with serotypes A, D, and AD (teleomorph, *F. neoformans* var. *neoformans*), and *C. neoformans* var. *gattii* Vanbreuseghem et Takashio, with serotypes B and C (teleomorph, *F. neoformans* var. *bacillispora* Kwon-Chung) (15, 18, 22, 23, 37). The occurrence of recombinants between strains of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* and the demonstration of genetic recombination in the F1 generation have suggested that these taxa are varieties (25, 41), but no genetic analysis of the F2 generation has been performed. In contrast, rather low DNA-DNA reassociation values (range, 55 to 63%) (1) have been observed between isolates of the two varieties, which may reflect genetic divergence between the two taxa. The two varieties differ in karyotype (54), in a number of physiological characteristics (e.g., assimilation of D-proline, D-tryptophan, and L-malic acid), in regulation of creatinine deaminase by ammonia production (2, 13, 31, 38), and in sensitivity to killer toxins of *Cryptococcus laurentii* CBS 139 (3). The two varieties also differ in geographic distribution and habitat. *C. neoformans* var. *neoformans* occurs worldwide, whereas *C. neoformans* var. *gattii* is restricted to the tropics and the southern hemisphere. Both varieties originate from human and animal sources. In the environment, *C. neoformans* var. *neoformans* is frequently isolated from bird droppings and is occasionally isolated from substrates like fermenting fruit juice, wood, and air (28, 30, 46). Saprobic isolates of *C. neoformans* var. *gattii* are usually associated with *Eucalyptus* species (14, 17, 37, 42), but have been isolated from bat guano as well (27). Differentiation between the varieties is usually performed on L-canavanine–glycine–bromthymol blue medium (24, 45) or by testing D-proline assimilation (13).

Nearly all of the AIDS-related *C. neoformans* infections are caused by *C. neoformans* var. *neoformans*, but the two varieties can cause similar neurological syndromes. To diagnose clinical infections and monitor dispersion of strains, technical instruments for cryptococcal genome scanning have been developed (6, 8, 9, 12, 29, 36, 43, 44, 50, 51). Molecular typing techniques have revealed that there is considerable genetic heterogeneity within the species (51, 54). PCR targeted at ribosomal operons has been used to identify the species accurately (53), but has provided insufficient resolution for epidemiological research. A major step forward was the discovery of a plasmid isolated from a *URA5* transformant of *C. neoformans* that could be used to detect genetic polymorphisms (50, 51). Additional probes have subsequently been developed (44), and simultaneous use of these probes gave results consistent with the *URA* plasmid analysis results, thus illustrating the validity of the two typing procedures. Analysis of randomly amplified polymorphic DNA (RAPD) has provided a genetic substructure for each variety (6, 8, 43, 56), but until now no comprehensive study has included both varieties.

In the present study the genetic diversity of the two varieties of *C. neoformans*, including a wide range of environmental and medical isolates, was analyzed by karyotyping and by performing a RAPD analysis with enterobacterial repetitive intergenic consensus (ERIC) primers (48, 52). In addition, serotypes and

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TABLE 1. Origins of the isolates of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* studied

Strain[a]	Origin	Serotype	Mating type	Killer type[b]	RAPD type
C. neoformans var. *neoformans* strains					
CBS 131	Institut Pasteur, Paris, France	AD	?[c]	IV	AA
CBS 132^T	Fermenting fruit juice	D	α	II	AA
CBS 464	Laboratoire de Parasitologie, Paris, France	A	?	IV	AA
CBS 879	Ulcerated cheek	A	α	II	BB
CBS 880	Unknown	A	α	II	BC
CBS 881	36-Year-old woman	A	α	II	BB
CBS 882^T	Nasal tumor of horse, type strain of *Torula nasalis* Harrison	D	α	II	CD
CBS 884	Brain, The Netherlands	A	α	II	BB
CBS 885	Unknown	A	α	II	BC
CBS 886	Unknown	A	α	II	BB
CBS 887	Unknown	A	α	III	BB
CBS 888	Unknown	D	α	II	CD
CBS 889	Unknown	A	?	III	BE
CBS 916	Unknown	A	α	II	BE
CBS 918	Dead white mouse	D	α	III	CD
CBS 939	Unknown	AD	?	III	AF
CBS 950	Tumor	A	?	III	AF
CBS 996^T	Blastomycosis in man, type strain of *Candida psicrophylicus*	A	α	III	BB
CBS 1009	Man	A	α	II	BB
CBS 1143	Cerebrospinal fluid	A	α	II	BF
CBS 1144	Cerebrospinal fluid	A	α	III	BB
CBS 1584	Unknown	A	?	IV	AF
CBS 1931	Soil	A	α	II	BB
CBS 1932	Soil	A	?	II	BB
CBS 1933	Mastitic cow, United States	A	?	II	BC
CBS 1935	Soil	A	α	II	BB
CBS 2771	Cerebrospinal fluid	A	α	II	BB
CBS 4194	Spleen, Germany	D	α	II	CB
CBS 4572	Cerebrospinal fluid	A	α	II	BB
CBS 4868	Sputum, The Netherlands	A	?	II	BB
CBS 5467	Milk from mastitic cow, Switzerland	D	α	III	CD
CBS 5474	Mastitic cow	D	?	III	CD
CBS 5728	Nonmeningitic cellulitis and osteomyelitis, United States	D	α	II	CD
CBS 6885^T	Lesion on bone in man, type strain of *Filobasidiella neoformans*	D	α	II	CD
CBS 6886	Droppings of pigeon	D	a	II	CD
CBS 6900	Genetic offspring of CBS 6885 × CBS 6886	D	α	II	CD
CBS 6901	Genetic offspring of CBS 6885 × CBS 6886	D	a	III	CD
CBS 6961	Man, Oklahoma	A	α	II	DE
CBS 6995	Cerebrospinal fluid, Illinois	A	a	IV	CD
CBS 6999	Pigeon droppings, Thailand	A	α	II	BB
CBS 7000	Pigeon droppings, Denmark	D	a	III	CD
CBS 7779	Urease-negative isolate from AIDS patient, Argentina	A	?	II	BB
CBS 7812	Cerebrospinal fluid	A	?	I	BC
CBS 7814	Air, Belgium	AD	α	II	CD
CBS 7815	Pigeon droppings, former Czechoslovakia	D	α	II	CD
CBS 7816	Cuckoo droppings, Thailand	A	αa	II	CD
RV 26952	Cerebrospinal fluid, Zaire	A	?	II	BB
RV 46115	Plants, India	A	?	II	BC
RV 46119	Pigeon droppings, India	A	?	II	BB
RV 46129	Pigeon droppings, India	A	?	II	BB
RV 52733	Pigeon droppings, Belgium	D	?	II	AC
RV 52755	Cerebrospinal fluid, Belgium	AD	?	II	AC
RV 53794	Canary bird droppings, Belgium	D	?	II	AC
RV 55446	House dust, Zaire	A	?	II	BA
RV 55447	Air inside house, Zaire	A	?	II	BC
RV 55451	Cockroach, Zaire	A	?	III	BC
RV 55980	Canary bird droppings, Belgium	A	?	II	BB
RV 56126	Cerebrospinal fluid from AIDS patient, Belgium (visited Haiti)	A	?	II	GF
RV 56883	Canary bird droppings, Belgium	A	?	II	BB
RV 56894	Canary bird droppings, Belgium	A	?	II	BB
RV 58145	Wood, Zaire	A	?	II	BC
RV 58146	Wood, Zaire	A	?	II	EG
RV 59351	Parrot droppings, Belgium	A	?	II	BA
RV 59369	Parrot droppings, Belgium	A	?	II	BA
RV 59379	Air in zoo, Belgium	A	?	II	BB
RV 60047	Skin cryptococcosis, Belgium	D	?	II	BC
RV 61756	Man, Belgium (visited Zaire)	AD	?	II	AA
RV 61790	Man, Belgium	A	?	II	BA
RV 62210	Cerebrospinal fluid from AIDS patient, Belgium	A	?	II	BA
RV 62692	Skin cryptococcosis, Belgium	D	?	II	CD
RV 63214	Cerebrospinal fluid from AIDS patient, Zaire	A	?	II	BB

Continued on following page

TABLE 1—*Continued*

Strain[a]	Origin	Serotype	Mating type	Killer type[b]	RAPD type
RV 63642	Cerebrospinal fluid from AIDS patient, Brazil	A	?	II	BA
RV 64610	AIDS patient, Rwanda	A	?	II	CE
RV 64612	AIDS patient, Rwanda	A	?	II	BA
RV 65631	Cerebrospinal fluid, Zaire	A	?	II	BB
RV 65662	Man, Portugal (visited Venezuela)	A	?	II	FC
RV 66025	Cryptococcoma, Belgium	A	?	II	BC
RV 66055	AIDS patient, Rwanda	A	?	II	BA
RDA 1335	AIDS patient no. 1, Rotterdam, The Netherlands	A	?	II	BA
RDA 1340	AIDS patient no. 1, Rotterdam, The Netherlands	A	?	II	BA
RDA 1371	AIDS patient no. 2, Rotterdam, The Netherlands	A	?	II	BA
RDA 1369	AIDS patient no. 3, Rotterdam, The Netherlands	A	?	II	BA
RDA 1373	AIDS patient no. 3, Rotterdam, The Netherlands	A	?	II	BA
RDA 1445	AIDS patient no. 4, Rotterdam, The Netherlands	A	?	II	BA
RDA 1419	AIDS patient no. 5, Rotterdam, The Netherlands	D	?	I	FF
RDA 1549	AIDS patient no. 6, Rotterdam, The Netherlands	A	?	II	BA
RDA 1589	AIDS patient no. 7, Rotterdam, The Netherlands	A	?	II	B-
RDA 1006	AIDS patient no. 8, Rotterdam, The Netherlands	A	?	II	BA
RDA 4092	AIDS patient no. 9, Rotterdam, The Netherlands	A	?	II	BA
RDA 4094	AIDS patient no. 10, Rotterdam, The Netherlands	A	?	II	BA
RDA 4054	AIDS patient no. 11, Rotterdam, The Netherlands	A	?	II	BA
RDA 4091	AIDS patient no. 12, Rotterdam, The Netherlands	A	?	II	BA
C. neoformans var. *gattii* strains					
CBS 883	Infected skin, syntype of *Candida hondurianus*	B	?	V	GH
CBS 919^T	Meningoencephalic lesion, type strain of *Torulopsis neoformans* var. *sheppei*	B	?	V	GH
CBS 1622	Tumor	B	?	V	GH
CBS 1930	Sick goat, Aruba	B	?	VII	HI
CBS 1934	Mastitic cow, United States	B	?	V	GJ
CBS 2502	Suspected case of tubercular meningitis	B	?	V	GJ
CBS 5757	Unknown	B	α	V	GH
CBS 5758	Unknown	C	α	VI	DK
CBS 6289	Subculture of type strain RV 20186	B	a	VII	IJ
CBS 6290	Man, Republic of Congo	B	?	V	GJ
CBS 6955^T	Spinal fluid, type strain of *Filobasidiella bacillispora*, California	C	a	VI	DG
CBS 6956	Sputum, Washington	B	α	VIII	IN
CBS 6992	Man	B	α	V	GH
CBS 6993	Man, California	C	α	VI	DK
CBS 6994	Cerebrospinal fluid, New Jersey	C	α	IX	DG
CBS 6996	Man	B	α	VII	EL
CBS 6997	Cerebrospinal fluid, California	B	α	VI	DK
CBS 6998	Cerebrospinal fluid, Thailand	B	a	X	EM
CBS 7229^T	Meningitis, type strain of *C. neoformans* var. *shanghaiensis*, People's Republic of China	B	?	V	GH
CBS 7523	*Eucalyptus camaldulensis*, Australia	B	?	V	GH
CBS 7740	Cerebrospinal fluid, Punjab, India	B	?	VII	GH
CBS 7741	Cerebrospinal fluid, Punjab, India	B	?	VII	GH
CBS 7742	Cerebrospinal fluid, Punjab, India	B	?	VI	GH
CBS 7747	Seedling of olive, Australia	B	?	V	GH
CBS 7748	Air in hollow *Eucalyptus camaldulensis*, Australia	B	?	V	GH
CBS 7749	Bark of *Eucalyptus camaldulensis*, Australia	B	?	V	GH
CBS 7750	Bark debris of *Eucalyptus camaldulensis*, California	B	?	VII	HI
RV 5265	Cerebrospinal fluid, Zaire	B	?	?	GJ
RV 20186^T	Cerebrospinal fluid, Zaire	B	a	VII	GJ
RV 54130	Second isolate of *C. neoformans* var. *shanghaiensis*	B	?	VI	GJ
RV 66095	Cerebrospinal fluid, Brazil	B	?	X	GJ
ATCC 32269	Subculture of type strain of *C. gattii*	B	a	?	GJ
NIH B-3939	Subculture of type strain of *C. gattii*	B	a	?	GJ

[a] CBS, Centraalbureau voor Schimmelcultures Yeast Division; RV, Institute of Tropical Medicine; NIH, National Institutes of Health; ATCC, American Type Culture Collection.

[b] Killer sensitivity types as described by Boekhout and Scorzetti (3).

[c] ?, not known.

killer toxin sensitivity patterns were analyzed, and epidemiological features are discussed below.

MATERIALS AND METHODS

Fungal strains. Medical and saprobic isolates of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* were obtained from the collections of the Yeast Division, Centraalbureau voor Schimmelcultures (Delft, The Netherlands), the Laboratory of Mycology, Institute of Tropical Medicine (Antwerp, Belgium), the Department of Bacteriology, University Hospital Rotterdam (Rotterdam, The Netherlands), and D. Ellis (Adelaide, Australia). Additional isolates were obtained from the National Institutes of Health (Bethesda, Md.) and the American Type Culture Collection (Rockville, Md.). The varieties to which the strains belonged were confirmed by performing color reaction tests on L-canavanine–glycine–bromthymol blue medium, D-proline assimilation tests, and killer toxin sensitivity tests. A total of 92 *C. neoformans* var. *neoformans* strains and 32 *C. neoformans* var. *gattii* strains were studied (Table 1). The strains were maintained on 1% yeast extract–0.5% peptone–4% glucose agar slants at 10°C.

TABLE 2. Distribution of serological types in clinical, saprobic, and veterinary isolates of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*

Type of isolates	*C. neoformans* var. *neoformans*				*C. neoformans* var. *gattii*		
	No. of isolates	% of isolates that are:			No. of isolates	% of isolates that are:	
		Serotype A	Serotype D	Serotype AD		Serotype B	Serotype C
Saprobic	25	72	24	4	5	100	0
Clinical	34	83	13	4	19	85	15
Veterinary	5	20	80	0	2	100	0
Other	1	100	0	0	0	0	0

Serotyping. The antigens used for production of antisera were prepared from the following strains: RV 56164 (serotype A), isolated from canary bird droppings in Belgium; RV 68038 (serotype D), isolated from wood in Burundi; RV 20185 (= ATCC 32267) (serotype B); and RV 45978 (= ATCC 34880) (serotype C). Polyclonal antisera were raised by immunizing female New Zealand White rabbits with heat-killed *C. neoformans* cells. The antisera used for final serotyping in a slide agglutination test were first adsorbed with a mixture of cells of the other serotypes by using the method described by Wilson et al. (55).

Killer toxin sensitivity. Killer toxin sensitivity was analyzed by using protocols described elsewhere (3). The following killer strains were used: *C. laurentii* (Kufferath) Skinner CBS 139, CBS 7235, and CBS 7857; *Cryptococcus podzolicus* (Bab'eva et Reshetova) Golubev CBS 7717; *Cryptococcus humicola* (Daszewska) Golubev CBS 4281; and *Filobasidium capsuligenum* Rodrigues de Miranda CBS 4736. Ten sensitivity types were discerned (3).

PFGE analysis. The cells used for pulsed-field gel electrophoresis (PFGE) were grown in 1% yeast extract–0.5% peptone–4% glucose broth at 25°C with shaking at 180 rpm. Agarose plugs were prepared as described by De Jonge et al. (10) by using Novozym 234 (Novo Industri AS, Bagsvaerd, Denmark). Electrophoresis was performed in a contour-clamped homogeneous electric field (CHEF) DR-II apparatus (Bio-Rad, Veenendaal, The Netherlands) by using the following conditions: 30 h with a ramping pulse time from 100 to 300 s, followed by 40 h with a ramping pulse time from 400 to 600 s. Initially, 110 V was applied, but during later experiments 100 V was applied. Gels were made with chromosome grade agarose (Bio-Rad) in 0.5× TBE and were electrophoresed at 12°C. Commercially available plugs of *Saccharomyces cerevisiae* and *Hansenula wingei* (= *Pichia canadensis*) (both obtained from Bio-Rad) were used as molecular size standards. After electrophoresis was completed, the gels were stained with 0.5 μg of ethidium bromide per ml for 30 min, destained with distilled water for 30 min, and photographed with a UV transilluminator at 300 nm. The sizes of chromosomes were calculated by comparing the running distances with a plot of running distance versus chromosome size for the standards. Karyotypes were also compared by using the program Gelcompar (Applied Maths, Ghent, Belgium).

RAPD analysis. DNA for the RAPD analysis was isolated from colonies grown for 2 or 3 days on solid Sabouraud medium at 30°C. Cells were harvested by suspending them in 1 ml of 20 mM sodium citrate (pH 5.8)–1 M sorbitol containing 10 mg of Novozym 234 per ml. The suspension was incubated at 37°C for 2 h, and spheroplasts were collected by centrifugation. DNA was isolated by lysing the spheroplasts in guanidinium isothiocyanate-containing buffers, and this was followed by affinity purification with Celite (Acros, Geel, Belgium) (4). DNA was dissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA and stored at −20°C.

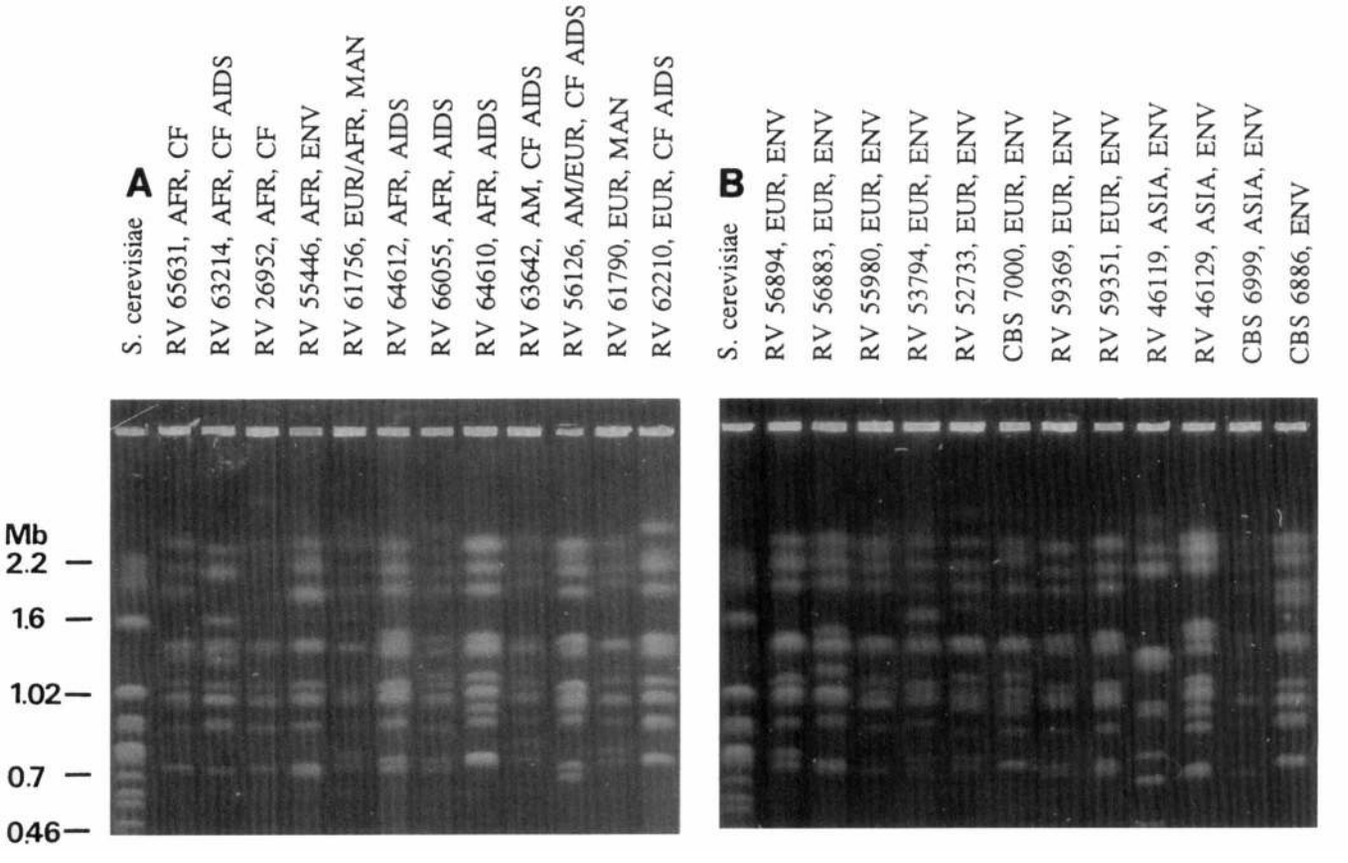

FIG. 1. Karyotypes of strains of *C. neoformans* var. *neoformans*. (A) Clinical isolates. (B) Environmental isolates. Abbreviations: AFR, Africa; EUR, Europe; AM, America; CF, cerebrospinal fluid; ENV, environmental. *S. cerevisiae* was the standard used.

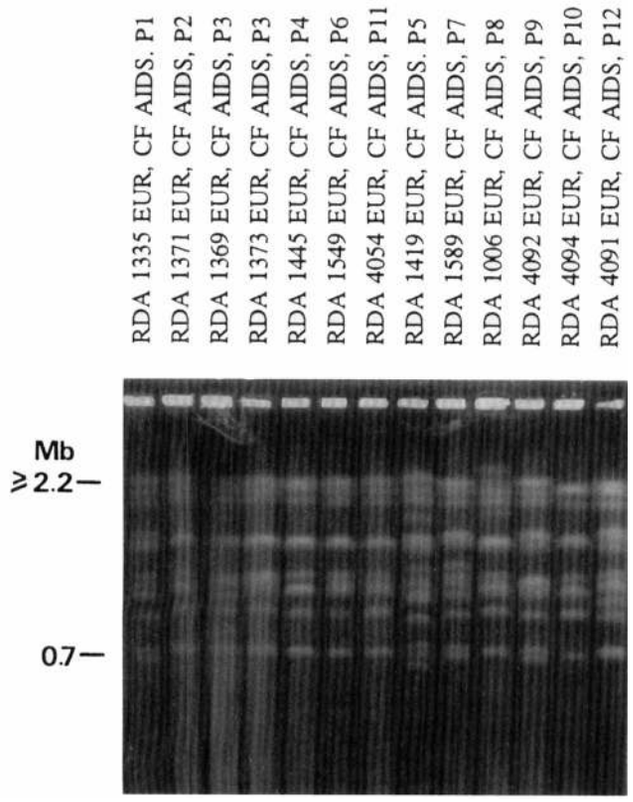

FIG. 2. Karyotypes of isolates of *C. neoformans* var. *neoformans* from Rotterdam AIDS patients. Abbreviations: CF, cerebrospinal fluid; P, patient; EUR, Europe.

Concentrations were determined by comparing the ethidium bromide staining intensities of aliquots with the staining intensities of known amounts of bacteriophage lambda DNA. The RAPD analysis was performed by using the following cycling parameters: predenaturation at 94°C for 4 min, followed by 35 cycles consisting of 1 min 94°C, 1 min 25°C, and 2 min 74°C (20, 48). Approximately 50 ng of DNA was amplified by using primers ERIC1 and ERIC2 (50 pmol in 100 μl). The DNA band patterns obtained with these two primers were inspected visually and were designated by using uppercase letters. If the patterns obtained with the same primer differed in more than two bands, a novel type was defined.

RESULTS

Serotyping. The serotype data are listed in Table 1. A serological comparison of the clinical, veterinary, and saprobic isolates (Table 2) showed that most of the clinical and saprobic isolates of *C. neoformans* var. *neoformans* were serotype A organisms, but four of the five veterinary isolates were serotype D organisms. All of the saprobic and veterinary isolates and most of the clinical isolates of *C. neoformans* var. *gattii* were serotype B organisms.

Killer sensitivity. The killer sensitivity data are shown in Table 1. The two varieties had different killer sensitivity patterns. Isolates of *C. neoformans* var. *neoformans* belonged to killer sensitivity types I to IV. About 80% of these organisms were type II organisms, 13% were type III organisms, 4% were type IV organisms, and 2% were not sensitive at all (type I). No clear relationship among serotype, source of isolation, and geography was observed. About 43% of the *C. neoformans* var. *gattii* isolates were killer sensitivity type V organisms, 23% were type VII organisms, 20% were type VI organisms, 7% were type X organisms, 3% were type VIII organisms, and 3% were type IX organisms. All six of these six killer sensitivity types were represented by the American isolates studied. The African and Asian populations contained fewer killer sensitivity types, and in the Australian population only one type (type V) was identified. The only California isolate studied from *Eucalyptus* sp. was a type VII organism.

Karyotyping by PFGE. The karyotype analysis revealed considerable heterogeneity among the isolates (Fig. 1 through 4). Most isolates of *C. neoformans* var. *neoformans*, whether they were clinical, veterinary, or saprobic, produced a variety of band patterns; these patterns usually consisted of 11 to 13 bands, but the patterns contained between 9 and 21 bands (Fig. 1, 2, and 4 and Table 3). The sizes of the individual chromosomes usually ranged between ca. 0.7 and 2.7 Mb, but in some isolates (e.g., CBS 132^T [T = type strain], CBS 464, CBS 916, CBS 5467, and RV 26952) a larger chromosome (≥3.1 Mb) was present (Fig. 1 and 4). In some isolates a smaller chromosome was present; e.g., RV 46119 had a ca. 500-kb chromosome, CBS 950 had a ca. 600-kb chromosome, CBS 464 had a ca. 370-kb chromosome, CBS 916 had a ca. 300-kb chromosome, and RV 52755 had a ca. 520-kb chromosome. No consistent differences were apparent among the clinical, veterinary, and saprobic isolates or among the serotype A, D, and AD isolates. The overall patterns consisted of four or five to eight or nine bands between ca. 0.7 and 1.4 Mb, frequently with a doublet or triplet at ca. 1 and 1.4 Mb, and three or four bands between 1.8 and 2.7 Mb. A number of isolates contained a more or less continuous series of chromosomes. This was the case in CBS 132, the type strain of *C. neoformans* var. *neoformans*, and in CBS 464 and RV 52755 (Fig. 4). The genome sizes of selected isolates of *C. neoformans* var. *neoformans* that had no or a few doublet chromosomes varied between 15 and 27 Mb (Table 4).

The lengths of the chromosomes of *C. neoformans* var. *gattii* isolates usually varied between ca. 500 kb and 2.7 Mb (Fig. 3 and 4), and the number of chromosomes ranged from 10 to 14 (Table 3). In some isolates (e.g., CBS 919 and CBS 6289) a larger chromosome (≥3.1 Mb) was present (Fig. 3 and 4). The sizes of the smallest chromosomes of most *C. neoformans* var. *gattii* isolates were estimated to vary between ca. 0.5 and 0.6 Mb; the only exception was CBS 6998, whose lowermost band was at ca. 0.75 Mb (data not shown). The overall patterns contained ca. six or seven bands between ca. 0.5 and 1.4 Mb, with a doublet (or triplet) at ca. 1.4 Mb, and two or three bands between ca. 2.0 and 2.4 Mb. All of the serotype C isolates (CBS 5758, CBS 6993, CBS 6994, and CBS 6955) had an additional band at 1.8 Mb, which was present in only one serotype B isolate (CBS 6997). The genome sizes of *C. neoformans* var. *gattii* strains were on average smaller than the genome sizes of *C. neoformans* var. *neoformans* strains (Table 4). Estimates of sizes based solely on isolates that had no or a few doublet chromosomes varied between ca. 12 and 18 Mb.

No differentiation was observed between clinical and saprobic isolates. Three main clusters were identified after a cluster analysis performed with the unweighted pair group method using arithmetic averages (Fig. 5). Cluster 1 contained some of the American isolates, as well as a Chinese isolate; cluster 2 contained Australian, African, and Indian isolates; and cluster 3 contained only American isolates. The saprobic strains from Australia (cluster 2) had similar karyotypes and identical RAPD types. The karyotypes and RAPD patterns of three medical isolates from India (cluster 2) were identical. Also, the karyotypes and RAPD patterns of two meningitis isolates (CBS 7229 and RV 54130) from the People's Republic of China and clinical isolate CBS 919 from the United States were similar.

The karyotypes of type strain RV 20186 of *C. neoformans* var. *gattii* and its subcultures stored in different culture collec-

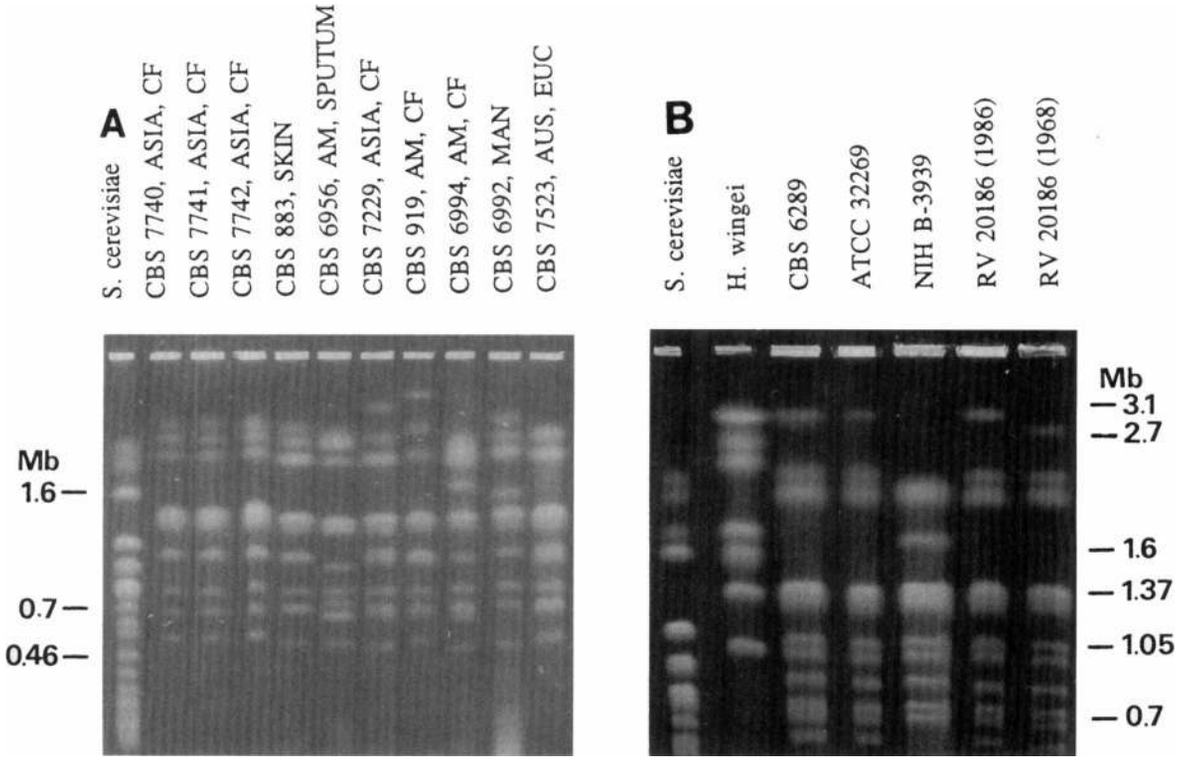

FIG. 3. Karyotypes of selected strains of *C. neoformans* var. *gattii*. (A) Karyotypes of medical and environmental isolates. (B) Karyotypes of subcultures of the type strain of *C. neoformans* var. *gattii* from different culture collections. Abbreviations: ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; NIH, National Institutes of Health; RV, Institute of Tropical Medicine; CF, cerebrospinal fluid; AM, America; AUS, Australia; EUC, *Eucalyptus. S. cerevisiae* and *H. wingei* were the standards used.

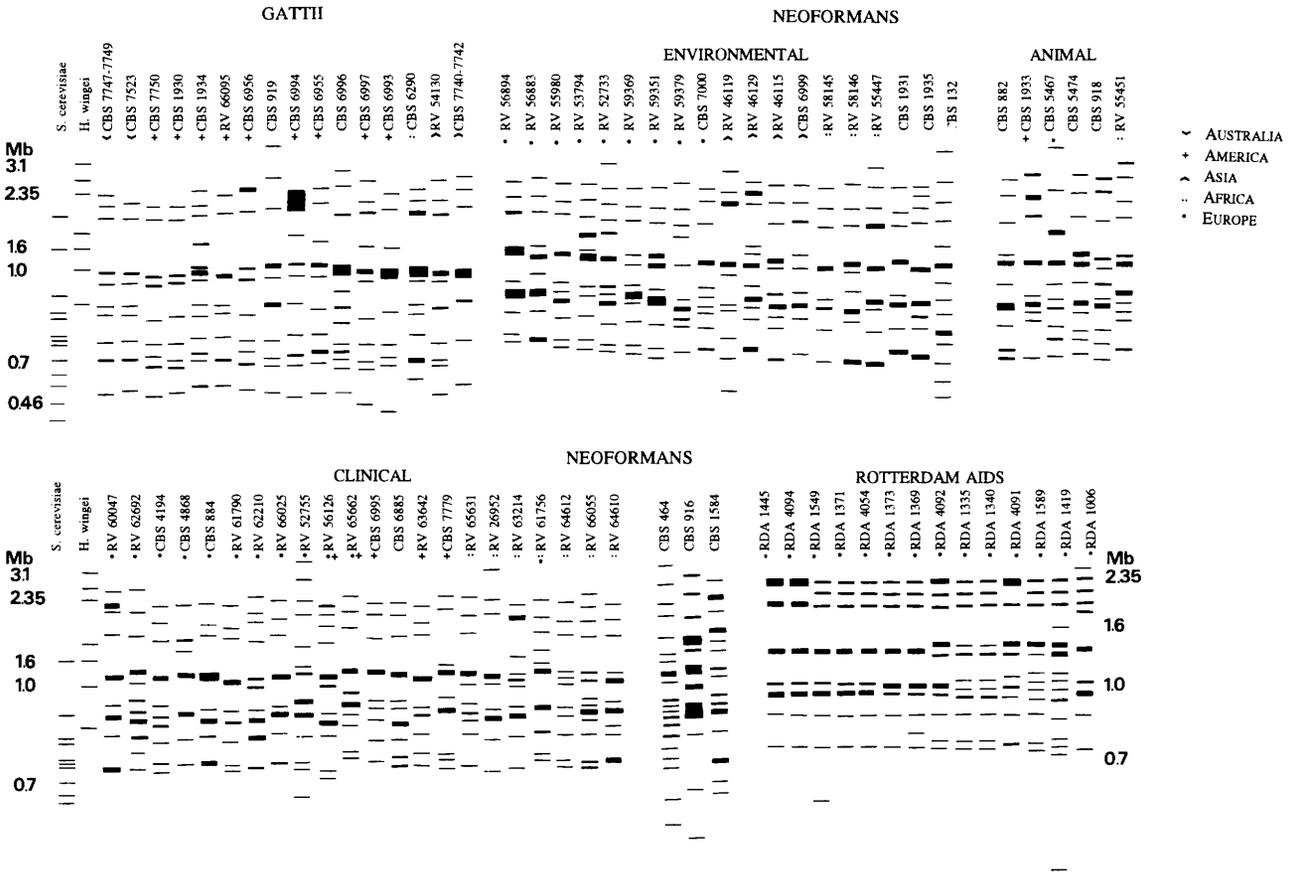

FIG. 4. Schematic representation of karyotypes of *C. neoformans*. (*S. cerevisiae* and *P. canadensis* were the standards used.)

TABLE 3. Distribution of chromosome numbers among isolates of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*

No. of chromosomal DNA bands	% of *C. neoformans* var. *neoformans* isolates			% of *C. neoformans* var. *gattii* isolates (n = 25)
	Clinical (n = 55)	Veterinary (n = 5)	Saprobic (n = 20)	
9	4			
10	7		10	20
11	22		20	8
12	29	20	35	20
13	15	20	20	40
14	9	40	5	12
15	7	20	5	
16	2		5	
17				
18				
19	2			
20	2			
21	2			

TABLE 4. Estimated genome sizes of selected isolates of *C. neoformans*

Taxon	Strain	Estimated genome size (Mb)
C. neoformans var. *neoformans*	CBS 464	27
	CBS 918	19
	CBS 6885	19
	CBS 7779	15
	RV 52755	22
	RV 59379	18.5
	RV 61756	20
C. neoformans var. *gattii*	CBS 883	17
	CBS 919^T	16.5
	CBS 1930	15
	CBS 6289	18
	CBS 6955^T	16
	CBS 6992	16
	CBS 6993	16
	CBS 6996	16
	CBS 6997	18
	CBS 7229^T	17
	CBS 7748	15
	CBS 7750	14
	RV 54130	17
	RV 66095	12

tions (CBS 6289 and ATCC 32269) were identical, whereas subculture NIH B-3939 differed in a number of bands. The latter strain lacked the upper and lower bands and had an additional band at ca. 1.8 Mb (Fig. 3). The largest chromosome of isolate RV 20186^T, which was lyophilized shortly after its discovery in 1968, was found to be somewhat smaller than the largest chromosome of a subculture lyophilized in 1986 after multiple rounds of cultivation on agar slants.

RAPD analysis. Figure 6 shows some of the experimental data obtained when DNA amplification with primers ERIC1 and ERIC2 was performed. The band patterns of the varieties differed. The complete data set obtained with ERIC1 and ERIC2 is presented and interpreted in Table 1. Sixteen different genotypes occurred in *C. neoformans* var. *neoformans*, resulting in an index of variation (i.v.) of 16/91 = 17.5. However, 83% of the *C. neoformans* var. *neoformans* strains belonged to only nine genotypes, which resulted in an i.v. of 11. Eight different genotypes occurred in *C. neoformans* var. *gattii*, resulting in an i.v. of 8/30 = 27. However, 77% of the strains of *C. neoformans* var. *gattii* belonged to only three genotypes (i.v., 13).

Comparisons of genotypes and serotypes, origins, and geographical distribution are presented in Tables 5 through 7. No clear differences among the clinical, saprobic, and veterinary strains were apparent (Table 5). The largest number of RAPD types occurred among strains of clinical origin, but the differences in the i.v.'s appeared to be insignificant. Four of the five

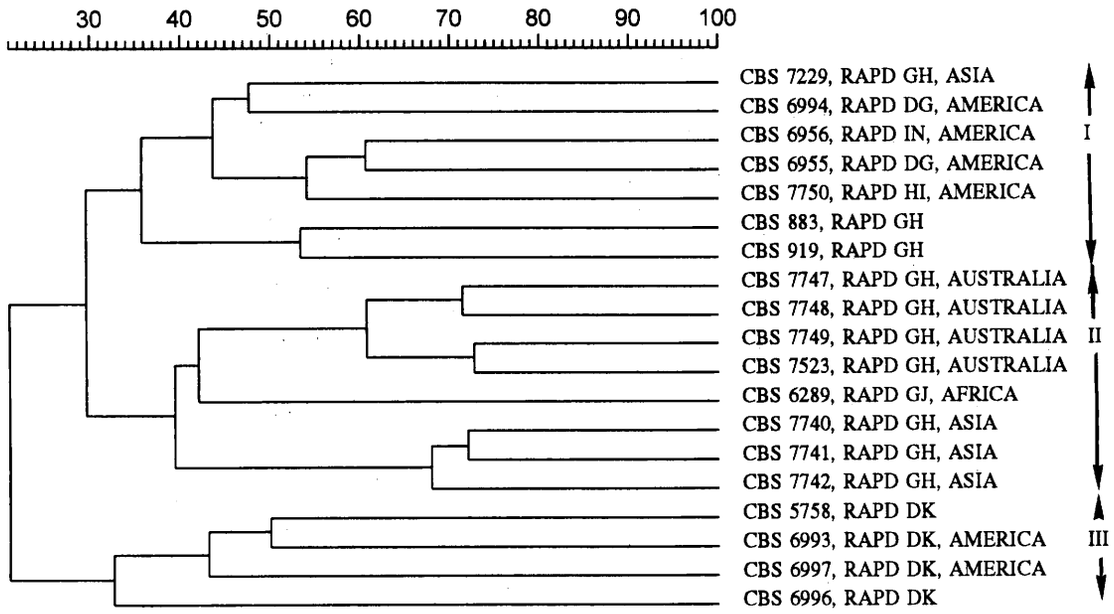

FIG. 5. Unweighted pair group with arithmetic average clustering of karyotypes of *C. neoformans* var. *gattii* and correlation with RAPD types. There are three main clusters (clusters I, II, and III), which correlate well with the RAPD types.

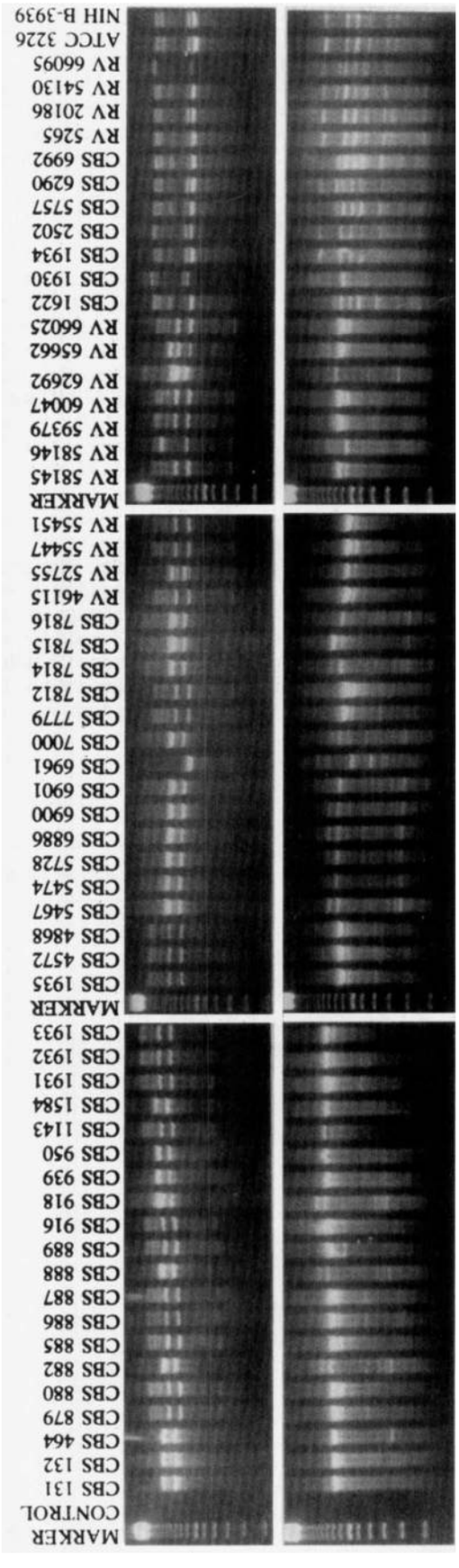

FIG. 6. Variation in RAPD patterns for *C. neoformans* strains as determined with ERIC1 (upper gels) and ERIC2 (lower gels).

TABLE 5. Distribution of RAPD types among clinical, saprobic, and veterinary isolates of *C. neoformans*

Type of isolates	*C. neoformans* var. *neoformans*		*C. neoformans* var. *gattii*	
	RAPD type	No. of strains	RAPD type	No. of strains
Clinical	BA	17	GH	7
	BB	13	GJ	6
	CD	4	DG	2
	BC	3	DK	2
	AA	1	EL	1
	AC	1	EM	1
	AF	1	IN	1
	BF	1		
	CB	1		
	CE	1		
	DE	1		
	FC	1		
	FF	1		
	GF	1		
Saprobic	BB	10	GH	4
	CD	5	HI	1
	BC	4		
	BA	3		
	AC	2		
	AA	1		
	EG	1		
Veterinary	CD	4	HI	1
	BC	1	GJ	1

veterinary isolates of *C. neoformans* var. *neoformans* (80%) were RAPD type CD organisms, and 80% of the isolates from AIDS patients were type BA organisms. Most of the serotype B strains differed from the serotype C strains; the only exception was one RAPD type DK strain (Table 6). About 70% of the serotype D strains (13 of 19 strains) were genotype CD organisms, but only 3% of the serotype A strains (2 of 65 strains) were genotype CD organisms. Strains of *C. neoformans* var. *neoformans* that originated from different continents did not differ widely in genetic structure, whereas the strains of *C. neoformans* var. *gattii* exhibited a geographic substructure (Table 7). The single Australian RAPD type, type GH, also occurred in Asia, but was absent in Africa and the Americas. The Asian, African, and American populations were linked by RAPD type GJ. About 80% of the American isolates (seven of nine isolates) exhibited RAPD types (types DG, DK, HI, and IN) that did not occur on the other continents.

Local isolates from AIDS patients. The Rotterdam isolates from AIDS patients had rather homogeneous karyotypes and RAPD types (Fig. 2 and 4 and Table 1). All of the strains except RDA 1419 were RAPD type BA organisms. Isolate RDA 1419 also differed in its karyotype, its serotype (serotype D), and its killer type (type I) (Table 1). Two isolates obtained from the same patient (RDA 1335 and RDA 1340) had identical karyotypes and RAPD types. However, the karyotypes of two isolates from another patient (RDA 1369 and RDA 1373) differed slightly in the penultimate band (Fig. 2). Two series of isolates (isolates RDA 1371, RDA 1373, RDA 1549, and RDA 4054 and isolates RDA 1445 and RDA 4094) could not be differentiated by the combination of karyotyping and RAPD typing. PFGE revealed more genetic heterogeneity among the Rotterdam isolates than RAPD typing revealed.

TABLE 6. Distribution of RAPD types among serotypes of *C. neoformans*

Serotype	RAPD type	No. of strains
A	BB	23
A	BA	20
A	BC	9
A	AF	2
A	BE	2
A	CD	2
A	AA	1
A	BF	1
A	CE	1
A	DE	1
A	EG	1
A	FC	1
A	GF	1
D	CD	13
D	AC	2
D	AA	1
D	BC	1
D	CB	1
D	FF	1
AD	AA	2
AD	AC	1
AD	AF	1
AD	CD	1
B	GH	12
B	GJ	7
B	HI	2
B	DK	1
B	EL	1
B	EM	1
B	IN	1
C	DK	2
C	DG	2

DISCUSSION

Recently, pulsed-field techniques, DNA fingerprinting, and PCR-mediated procedures have been used to detect genetic variation in *C. neoformans* (5, 6, 8, 9, 12, 26, 36, 43, 51, 54, 56). In these studies usually a limited number of isolates (e.g., isolates originating from a restricted geographic area or isolates belonging to only one of the varieties) have been studied.

The observed differences in genetic makeup, mycotoxin sensitivity patterns, and serology between the two varieties bring into question the conspecific status of these taxa. If the two entities interbreed, we do not understand how the genetic differences are maintained, as homogenization of the population would be expected. However, if reproduction is clonal, as suggested previously (6), the presence of mutually exclusive genetic patterns suggests that different species are involved. Therefore, because of intermediate DNA-DNA hybridization values (1), we have concluded that two separate species may be involved. If this turns out to be true, the binomials *Cryptococcus bacillisporus* Kwon-Chung et Bennett (synonym, *C. neoformans* var. *gattii* Vanbreuseghem et Takashio) and *Filobasidiella bacillispora* Kwon-Chung should be used for the second taxon in its anamorphic and teleomorphic stages, respectively.

Karyotype analysis has revealed strain-specific band patterns (12, 36). Groups of strains with similar or identical karyotypes usually have identical RAPD types. The first analysis of the *C. neoformans* genome revealed 12 or 13 chromosomes in the type strain of *C. neoformans* var. *neoformans*, CBS 132 (10). Somewhat later, three different karyotype patterns were observed (one for serotype A isolates, one for serotype D isolates, and one for serotype B or C isolates) (39). Perfect et al. (35), however, found considerable variation in the band patterns within all four serotypes. Wickes et al. (54) observed differences between the karyotypes of *C. neoformans* var. *gattii* and *C. neoformans* var. *neoformans*, and this was largely confirmed by our results. However, some isolates of *C. neoformans* var. *neoformans* (e.g., CBS 132^T, CBS 464, CBS 916, and CBS 1584) contained smaller chromosomes (up to ca. 0.3 Mb), which fell into the size range of *C. neoformans* var. *gattii* chromosomes. When used with caution, karyotypes may provide estimates of genome size. A serious problem in estimating genome sizes on the basis of karyotypes is that comigrating bands may occur, and different estimates are used for individual chromosomes. Perfect et al. (35) estimated that the genome size of *C. neoformans* is between 15 and 17 Mb, with the number of bands ranging between 10 and 12. Later, Wickes et al. (54) calculated considerably larger genome sizes, ca. 21 to 24.5 Mb, with 13 chromosomes on average in *C. neoformans* var. *gattii* and 12 chromosomes in *C. neoformans* var. *neoformans*. Our results revealed considerable variation in genome size in both varieties, suggesting that there may be differences in ploidy and/or aneuploidy within the species. The genome size of *C. neoformans* var. *neoformans* was found to be somewhat larger than the genome size of *C. neoformans* var. *gattii*.

We observed some differences among the karyotypes of the type strains of *C. neoformans* var. *gattii* maintained in different culture collections. These differences were supported by DNA fingerprinting results (21a), but our RAPD patterns were iden-

TABLE 7. Geographic distribution of RAPD types of *C. neoformans*

Location	*C. neoformans* var. *neoformans*		*C. neoformans* var. *gattii*	
	RAPD type	No. of strains	RAPD type	No. of strains
America	CD	3	GJ	2
	BA	1	DG	2
	BB	1	DK	2
	BC	1	HI	2
	DE	1	IN	1
Europe	BA	16		
	BB	6		
	CD	5		
	AC	3		
	BC	2		
	AA	1		
	CB	1		
	FC	1		
	FF	1		
	GF	1		
Africa	BA	3	GJ	4
	BB	3		
	BC	3		
	CE	1		
	EG	1		
Asia	BB	3	GH	4
	BC	1	EM	1
	CD	1	GJ	1
Australia			GH	4

tical. The following two explanations may account for this phenomenon: (i) instability of karyotypes and DNA fingerprints during prolonged cultivation on agar slants, and (ii) replacement of strains. We favor the first option, since instability of karyotypes has been observed in *C. neoformans* after mutagenesis (2a). Moreover, the rRNA genes occur on the largest chromosomes (54), and differences in the copy number of these genes may result in length polymorphisms as well.

It has been suggested that the karyotypes of saprobic isolates of *C. neoformans* var. *gattii* vary less than the karyotypes of clinical isolates (26). However, our finding of identical karyotypes and RAPD patterns for three clinical strains of *C. neoformans* var. *gattii* from India (32) seems to contradict this observation. Although the Asian clinical isolates differ slightly from the Australian *Eucalyptus* isolates, we do not consider the differences significant.

Different PCR typing strategies may result in detection of different amounts of genetic heterogeneity (6, 8, 42, 43; this study). It is evident that RAPD analysis or PCR fingerprinting resolves clusters that, for instance, are not separated by serotyping. Immediate clinical application of the RAPD approach is still controversial, as conflicting results have been obtained when multiple isolates from a single patient have been studied (7, 16). There are no generally accepted procedures for interpreting RAPD band patterns. Moreover, using different PCR protocols and/or different primers in different laboratories may result in different results (47–49). It has been suggested that there may be a relationship between isolate characteristics and issues like body location or risk factors for the development of disseminated meningoencephalitis (12). However, Varma et al. (51) challenged these ideas after they encountered comparable genetic diversity in isolates from AIDS patients, non-AIDS patients, and the environment. Our karyotype and RAPD type results are consistent with the latter observation. Therefore, it seems likely that infection is usually acquired by inhalation of saprobically living *C. neoformans.*

Our results suggest that RAPD type BA is dominant in AIDS patients, but this hypothesis may be biased by the sample containing a rather large number of isolates obtained from a local population of AIDS patients (strains RDA 1335 through RDA 4091). Discrimination of all Rotterdam isolates by using PFGE and RAPD analysis was not possible. The relatively homogeneous karyotypes with concordant RAPD patterns, serotypes, and killer types of these isolates suggest that clonal expansion of the fungus occurred in this geographically restricted area, as has been revealed by linkage disequilibrium studies in other areas (6). The observed local genetic homogeneity seems to contradict the results of other studies in which only a very small number of strains was found to be identical when PFGE and DNA fingerprinting were used (12, 36). Strains of *C. neoformans* var. *neoformans* from mastitic cows mainly were serotype D and RAPD type CD organisms, suggesting that there is some degree of genetic differentiation. However, more strains of veterinary origin need to be investigated to settle this issue. Our RAPD data suggest that there is geographical differentiation among populations of *C. neoformans* var. *gattii*. When previously published DNA fingerprint patterns (51) were compared with strain origins, a comparable geographic pattern was obtained. There seems to be considerable genetic divergence between the Australian and American populations, with the Asian and African populations somewhat intermediate. It has been suggested that this pattern may be correlated with the existence of some geographic and/or genetic substructure in the *Eucalyptus* hosts (14a).

The following points summarize our results: (i) *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* differ in genetic makeup and may represent separate species; (ii) no genetic differences were observed among clinical, veterinary, and saprobic isolates of either variety; (iii) both varieties have various numbers of chromosomes and various genome sizes, which probably reflect differences in ploidy and/or aneuploidy; (iv) multiple isolates from a patient may exhibit minor karyotype differences; (v) karyotypes may not be stable after prolonged preservation on agar slants; (vi) RAPD typing data suggest that there is a geographic substructure in *C. neoformans* var. *gattii*; (vii) isolates from a local population of AIDS patients have relatively homogeneous PFGE and RAPD patterns; and (viii) the combination of PFGE and RAPD analysis is useful in epidemiological research.

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