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Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*

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Amplified fragment length polymorphism (AFLP) genotyping of isolates of the pathogenic fungus *Cryptococcus neoformans* suggested a considerable genetic divergence between the varieties *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* on the one hand versus *C. neoformans* var. *gattii* on the other. This divergence is supported by additional phenotypic, biochemical, clinical and molecular differences. Therefore, the authors propose the existence of two species, *C. neoformans* (Sanfelice) Vuillemin and *C. bacillisporus* Kwon-Chung, which differ in geographical distribution, serotypes and ecological origin. Within each species three AFLP genotypes occur, which differ in geographical distribution and serotypes. Differences in ecological origin (AIDS patients, non-AIDS patients, animals or the environment) were found to be statistically not significant. In *C. neoformans* as well as in *C. bacillisporus* one of the genotypes represented a hybrid. The occurrence of hybridization has consequences for the reproductive biology of the species, as new genotypes with altered virulence or susceptibility to antifungal drugs may arise through the exchange of genetic material.

Keywords: *Cryptococcus neoformans*, systematics, hybrids, geography, serotypes, AFLP

INTRODUCTION

Cryptococcus neoformans is a clinically important basidiomycetous yeast (Howard & Kwon-Chung, 1995). The fungus belongs to the order Tremellales (jelly fungi) of the Hymenomycetes (Fell *et al*., 2000), a group of fungi commonly occurring on woody substrates. The species is known in both the asexual (anamorph) and sexual (teleomorph) state, for which the respective names *Cryptococcus neoformans* (Sanfelice) Vuillemin and *Filobasidiella neoformans* Kwon-Chung are used. In nature the fungus occurs, to our present knowledge, only in the asexual state. *C. neoformans* can cause life-threatening infections in humans, 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 (Mitchell & Perfect, 1998), while other estimates set the upper limit at 6–12% (Casadevall & Perfect, 1995). The main sites of infection are the lungs and the central nervous system, including cerebrospinal fluid (CSF), while most skin infections are probably due to disseminated systemic infections (Casadevall & Perfect, 1998; Schupbach *et al*., 1976).

According to the current classification, the species consists of three varieties: *C. neoformans* var. *neoformans* (serotype D), *C. neoformans* var. *grubii* Franzot *et al*. (serotype A) (Franzot *et al*., 1999), both comprising the teleomorph *F. neoformans* var. *neoformans* Kwon-Chung, and *C. neoformans* variety *gattii* Vanbreuseghem & Takashio (serotypes B and C) with the teleomorph *F. neoformans* var. *bacillispora* Kwon-Chung (Franzot *et al*., 1999; Kwon-Chung, 1975, 1976; Kwon-Chung & Bennett, 1984; Kwon-Chung *et al*., 1978, 1982a, b; Pfeiffer & Ellis, 1993). Variety *grubii*

Abbreviations: AFLP, amplified fragment length polymorphism; CCV, cophenetic correlation value; CSF, cerebrospinal fluid; IGS, intergenic spacer; RAPD, random amplified polymorphic DNA; UPGMA, unweighted pair group method using arithmetic means.

Table 1. Origins of isolates of *C. neoformans* and *C. bacillisporus* studied

Strain	AFLP genotype	Serotype*	Origin†	Location
C. neoformans				
CBS 132	3	D	Fermenting fruit juice, T *Cryptococcus neoformans*	Italy
CBS 464	3	A*	Unknown	France
CBS 879	1	A	Ulcerated cheek	Unknown
CBS 882	2	D	Nasal tumour of horse, T *Torula nasalis*	USA
CBS 886	1	A	Unknown	Unknown
CBS 887	1	A	Unknown	Unknown
CBS 888	2	D	Unknown	Unknown
CBS 889	1	A*	Unknown	Unknown
CBS 916	1	A	Unknown	Unknown
CBS 918	2	D	Dead white mouse	Netherlands
CBS 939	3	D*	Unknown	Unknown
CBS 950	3	AD*	Tumour	Unknown
CBS 996	1	A	Blastomycosis from man, T *Candida psicrophylicus*	Argentina
CBS 1144	1	A	CSF	Unknown
CBS 1931	1	A	Soil	Unknown
CBS 1932	1A	A	Soil	Unknown
CBS 1935	1	A	Soil	Unknown
CBS 2771	1	A	CSF	Unknown
CBS 4194	2	D	Spleen	Germany
CBS 5467	2	D	Milk from mastitic cow	Switzerland
CBS 5474	2	D	Mastitic cow	Unknown
CBS 5728	2	D	Non-meningitic cellulitis	USA
CBS 5756	1	A	Unknown	Unknown
CBS 6885 (NIH 12)	2	D	Lesion on bone in man, T *Filobasidiella neoformans*	USA
CBS 6886 (NIH 430)	2	D	Pigeon droppings	Denmark
CBS 6900 (NIH B-3501)	2	D	Genetic offspring of CBS 6885 × 6886	
CBS 6901 (NIH B-3502)	2	D	Genetic offspring of CBS 6885 × 6886	
CBS 6961	1	A*	Man	USA
CBS 6995	2	D*	CSF non-AIDS patient	USA
CBS 6999	1	A	Pigeon droppings	Thailand
CBS 7000	2	D	Pigeon droppings	Denmark
CBS 7779	1	A	AIDS patient, urease-negative	Argentina
CBS 7812	1	A	CSF non-AIDS patient	Unknown
CBS 7814	2	D*	Air	Belgium
CBS 7815	2	D	Pigeon droppings	Former Czechoslovakia
CBS 7816	2	D*	Cuckoo droppings	Thailand
CBS 7821	3	AD	Single basidiospore 6886 × 7814 (ATCC 42161)	
CBS 7822	2	D*	Single basidiospore 6885 × 6886	
CBS 7824	?	D*	Single basidiospore 7816	
CBS 7825	?	AD	Single basidiospore 7816	
CBS 8336	1	A	Decaying wood of *Cassia* tree	Brazil
CBS 8337	1	A	Decaying wood of *Cassia* tree	Brazil
CBS 8710	1	A	Hodgkin's disease, T *Cryptococcus neoformans* var. *grubii*	USA
Hamdan 214-L	1A	A	AIDS patient	Brazil
Hamdan 299	1A	A	AIDS patient	Brazil
Hamdan 822b	1	A	AIDS patient	Brazil
Hamdan C3-1	1A	A	Pigeon droppings	Brazil
Hamdan C31	1A	A	AIDS patient	Brazil
Hamdan F2′1	1	A	Pigeon droppings	Brazil
Hamdan I3′1	1	A	Pigeon droppings	Brazil

Table 1 (*cont.*)

Strain	AFLP genotype	Serotype*	Origin†	Location
Hamdan MCP-2	1A	A	Pigeon droppings	Brazil
Hamdan WP	1	A	AIDS patient	Brazil
J10	1	A	AIDS patient	USA
J11	1A	A	AIDS patient	USA
J15	1	A	AIDS patient	USA
J22a	3	D	AIDS patient	USA
J40	3	D	AIDS patient	USA
J51	1	A	AIDS patient	USA
J9	2	D*	AIDS patient	USA
NIH 192	1	A	Desert soil	USA
NIH 193	1	A	Soil	USA
NIH 296	1	A	Non-AIDS patient	USA
NIH 311	1	A	Non-AIDS patient	USA
NIH 443	1	A	Soil	USA
NIH 449	1	A	Non-AIDS patient	USA
P050	1	A	AIDS patient	Zimbabwe
P056	1	A	AIDS patient	Zimbabwe
P090	1	A	AIDS patient	Zimbabwe
P139	1	A	AIDS patient	Zimbabwe
P140	1	A	AIDS patient	Zimbabwe
P141	1	A	AIDS patient	Zimbabwe
P152	1	A	AIDS patient	Zimbabwe
P172	1	A	AIDS patient	Zimbabwe
RV26952	1	A	CSF, non-AIDS patient	Zaire
RV46115	1	A	Plants	India
RV46119	1	A	Pigeon droppings	India
RV46129	1	A	Pigeon droppings	India
RV52733	3	D	Pigeon droppings	Belgium
RV52755	3	D*	CSF, non-AIDS patient	Belgium
RV53794	3	D	Canary droppings	Belgium
RV55446	1	A	House dust	Zaire
RV55451	1	A	Cockroach	Zaire
RV55980	1	A	Canary droppings	Belgium
RV56126	1A	A	CSF, AIDS patient	Belgium (visited Haiti)
RV56883	1	A	Canary droppings	Belgium
RV56894	1	A	Canary droppings	Belgium
RV58145	1	A	Wood	Zaire
RV58146	1A	A	Wood	Zaire
RV59351	1	A	Parrot droppings	Belgium
RV59369	1	A	Parrot droppings	Belgium
RV59379	1	A	Air in zoo	Belgium
RV60074	1	A*	Skin cryptococcosis, non-AIDS patient	Belgium
RV61756	1A	AD	Man	Belgium (visited Zaire)
RV61790	1	A	Man	Belgium
RV62210	1	A	CSF, AIDS patient	Belgium
RV62692	2	D	Skin cryptococcosis	Belgium
RV63214	1	A	CSF, AIDS patient	Zaire
RV63642	1	A	CSF, AIDS patient	Brazil
RV64610	1A	A	AIDS patient	Rwanda
RV64612	1	A	AIDS patient	Rwanda
RV65361	1	A	CSF, probably AIDS patient	Zaire
RV65662	1A	A	AIDS patient	Portugal
RV66025	1	A	Cryptococcoma, non-AIDS patient	Belgium
RV66055	1	A	AIDS patient	Rwanda
WM164	1A	A	Pigeon droppings	Australia

Table 1 (*cont.*)

Strain	AFLP genotype	Serotype*	Origin†	Location
WM361	1	A	CSF AIDS patient	Thailand
WM364	1	A	CSF AIDS patient	Thailand
WM374	1	A	CSF AIDS patient	Thailand
WM375	1	A	CSF AIDS patient	Thailand
WM553	1A	A	House dust	Brazil
WM554	1	A	Dust from pigeon	Brazil
WM555	1	A	Dust from pigeon	Brazil
WM712	1	A	Cat paranasal	Australia
WM713	1	A	Cat paranasal	Australia
WM714	1	A	Cat paranasal	Australia
WM715	1	A	Pine needles	Australia
WM716	1	A	Woody debris of *Eucalyptus camaldulensis*	Australia
WM719	1	A	AIDS patient	South Africa
WM720	1	A	Pigeon droppings	India
WM721	1	A	Pigeon droppings	India
WM722	1	A	Pigeon droppings	India
WM723	1	A	Environmental isolate	USA
WM724	1	A	Debris of *Eucalyptus*	USA
WM725	1	A	Debris, zoo hippo cage	USA
110B	1	A	Environmental isolate	France
122A	1	A	Environmental isolate	France
12A	1	A	Environmental isolate	France
13A	1	A	Environmental isolate	France
20B	2	D	Environmental isolate	France
22A	2	D	Environmental isolate	France
385D	2	D	Unknown	USA?
57B	1	A	Environmental isolate	France
RDA 1335 AvB0	1	A	AIDS patient no. 1	Netherlands
RDA 1340 AvB1	1	A	AIDS patient no. 1	Netherlands
RDA 4092 AvB10	1	A	AIDS patient no. 9	Netherlands
RDA 4094 AvB11	1	A	AIDS patient no. 10	Netherlands
RDA 4054 AvB12	1	A	AIDS patient no. 11	Netherlands
RDA 4091 AvB13	1	A	AIDS patient no. 12	Netherlands
RDA 1371 AvB2	1	A	AIDS patient no. 2	Netherlands
RDA 1369 AvB3	1	A	AIDS patient no. 3	Netherlands
RDA 1373 AvB4	1	A	AIDS patient no. 3	Netherlands
RDA 1549 AvB7	1	A	AIDS patient no. 6	Netherlands
B10	1	A	Environmental isolate	USA
B3	1	A	Environmental isolate	USA
B5	1	A	Environmental isolate	USA
BA1	3	D*	AIDS patient	France
BA3	3	AD*	AIDS patient	France
BA4	3	AD*	AIDS patient	France
BA5	3	AD*	AIDS patient	France
BD1	2	D	AIDS patient	France
BD2	1	A	AIDS patient	France
BD3	2	D	AIDS patient	France
BD5	2	D	AIDS patient	France
C. bacillisporus				
CBS 883	4B	B	Infected skin, syntype *C. hondurianus*	Honduras
CBS 919	4A	B	Meningoencephalic lesion, T *Torulopsis neoformans* var. *sheppei*	USA
CBS 1622	4A	B	Tumour	Unknown
CBS 1930	6	B	Sick goat	Aruba

Table 1 (*cont.*)

Strain	AFLP genotype	Serotype*	Origin†	Location
CBS 1934	4A	B	Mastitic cow	USA
CBS 5757	4A	B	Unknown	Unknown
CBS 5758	5C	C	Unknown	Unknown
CBS 6289	4A	B	Subculture of type strain of *C. neoformans* var. *gattii* (RV20186)	Zaire
CBS 6290	4A	B	Man	Zaire
CBS 6955 (NIH 191)	5C	C	CSF, T *Filobasidiella bacillispora*	USA
CBS 6956 (NIH 444)	6	B	Sputum	USA
CBS 6992 (NIH 17)	4A	B	Man	USA
CBS 6993 (NIH 18)	5C	C	Man	USA
CBS 6994 (NIH 34)	5C	C	CSF	USA
CBS 6997 (NIH 298)	5C	B	CSF	USA
CBS 7229	4B	B	Meningitis, T *C. neoformans* var. *shanghaiensis*	China
CBS 7523	4B	B	*Eucalyptus camaldulensis*	Australia
CBS 7740	4B	B	CSF	India
CBS 7742	4B	B	CSF	India
CBS 7747	4B	B	Olive seedling	Australia
CBS 7748	4B	B	Air in hollow *Eucalyptus camaldulensis*	Australia
CBS 7749	4B	B	Bark debris of *Eucalyptus camaldulensis*	Australia
CBS 7750	6	B	Bark debris of *Eucalyptus camaldulensis*	USA
CBS 8684	6	B	Nest of wasp	Uruguay
CBS 8755 (HOO58-I-682)	5A	C	Detritus of almond tree	Colombia
CBS 8756 (HOO58-I-818)	5A	C	Detritus of almond tree	Colombia
CDC B-5751	4B	B	CSF, non-AIDS patient	India
CDC 5765	4B	B	Flowers of *Eucalyptus camaldulensis*	India
NIH 139	5C	C	Non-AIDS patient	USA
NIH 178	5C	C	Non-AIDS patient	USA
NIH 189	5B	B	Non-AIDS patient	USA
NIH 190	5B	B	Non-AIDS patient	USA
RV20186	4A	B	CSF	Zaire
RV5265	4A	B	CSF	Zaire
RV54130	4B	B	Second isolate of *C. neoformans* var. *shanghaiensis*	China
RV66095	4A	B	CSF non-AIDS patient	Brazil
WM161	5B	B	Debris of *Eucalyptus* spp.	USA
WM176	4B	B	*Eucalyptus citriodora*	USA
WM717	4B	B	Woody debris of *Eucalyptus tericornis*	USA
WM718	4B	B	Woody debris of *Eucalyptus tericornis*	USA
WM726	5B	B	*Eucalyptus citriodora*	USA
WM727	4A	B	Debris of *Eucalyptus* from car park of zoo	USA
WM728	5B	B	Debris of *Eucalyptus* from car park of zoo	USA
380C	5C	C	Unknown	Unknown
381C	5C	C	Unknown	USA
384C	5C	C	Patient	USA
385C (= NIH 18)	5B	C	Unknown	USA
48A	4A	B	Lung of a goat	Spain
52A	4A	B	Brain of a goat	Spain
55A	4A	B	Lung of a goat	Spain
56A	4A	B	Gut of a goat	Spain
59A	4A	B	Lung of a goat	Spain
60A	4A	B	Lung of a goat	Spain

*In the case of anomalous results, serotypes were redetermined using immunofluorescence with monoclonal antibody (Dromer *et al.*, 1993) and agglutination with the IatronO kit (Ikeda *et al.*, 1982). These are indicated with an asterisk.

† T, type strain.

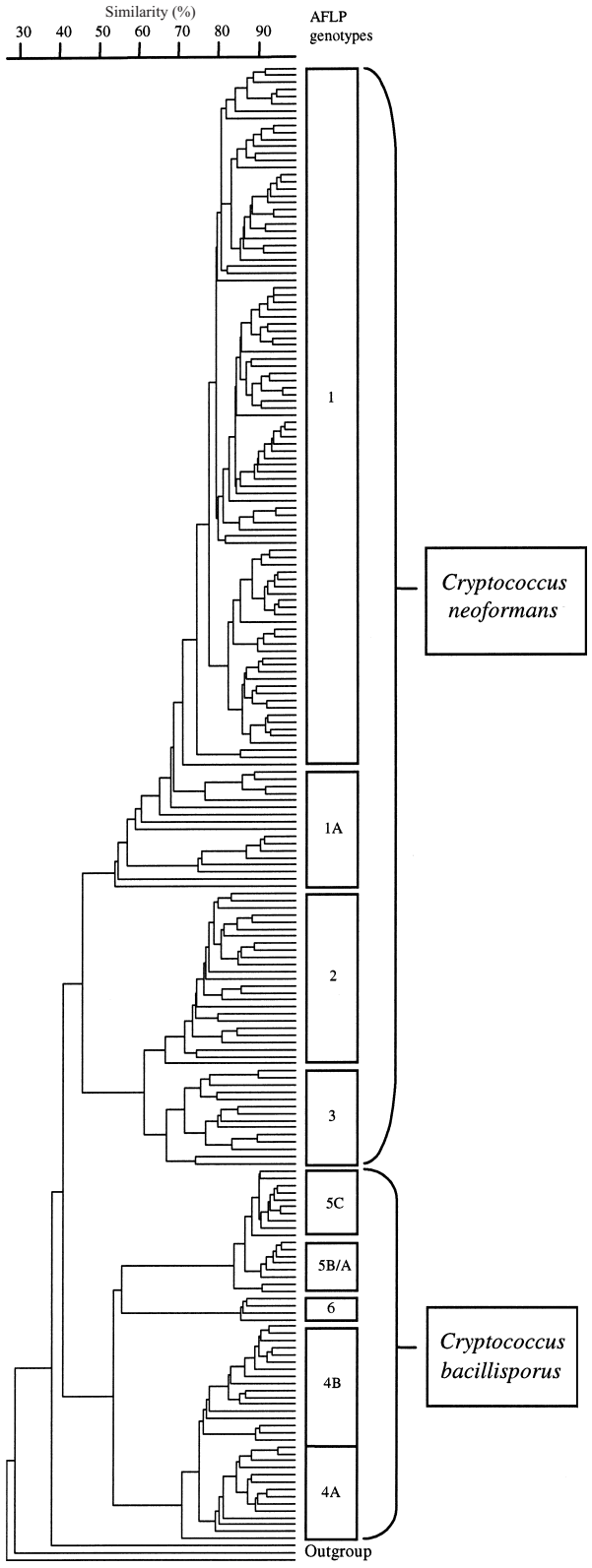

Fig. 1. Clustering of AFLP banding patterns of isolates of *C. neoformans* and *C. bacillisporus* (= *C. neoformans* var. *gattii*) by UPGMA. Two main branches, each with three genotypes, can be distinguished. Genotypes 3 and 6 represent hybrid genotypes.

was recently recognized for serotype A isolates based on molecular data such as DNA fingerprints and *URA5* sequences (Franzot *et al*., 1999). This variety is encountered in nearly all of the AIDS-related infections in the USA (Casadevall & Perfect, 1998; Kovacs *et al*., 1985).

The varieties *neoformans* and *grubii* differ from variety *gattii* in their electrophoretic karyotypes (Boekhout *et al*., 1997; Wickes *et al*., 1994), random amplified polymorphic DNA (RAPD) (Boekhout *et al*., 1997), DNA fingerprints (Varma *et al*., 1995), PCR fingerprints (Meyer & Mitchell, 1995; Meyer *et al*., 1993), intergenic spacer (IGS) sequences of the rDNA (Diaz *et al*., 2001), a number of physiological and biochemical characteristics (Bennett *et al*., 1978; Cherniak & Sundstrom, 1994; Dufait *et al*., 1987; Kwon-Chung *et al*., 1987; Mukaramangwa *et al*., 1995; Polacheck & Kwon-Chung, 1980), susceptibility to killer toxins of *C. laurentii* CBS 139 (Boekhout & Scorzetti, 1997), geographical distribution and habitat (Casadevall & Perfect, 1998; Kwon-Chung & Bennett, 1984), and clinical manifestation (Kwon-Chung *et al*., 1988; Speed & Dunt, 1995). Current views based on different genetic typing methods, such as RAPD, DNA and PCR fingerprints, and *URA5* sequences, suggest that serotypes A and D form distinct genetic lineages (Boekhout *et al*., 1997; Franzot *et al*., 1998a, 1999; Meyer & Mitchell, 1995; Varma *et al*., 1995).

In this study we used amplified fragment length polymorphism (AFLP) (Blears *et al*., 1998; Janssen *et al*., 1996; Savelkoul *et al*., 1999; Zabeau & Vos, 1993) to investigate the genetic structure and epidemiological relationships of a range of cryptococcal isolates collected worldwide.

METHODS

Strains, media and serotyping. The strains studied listed in Table 1 (see also http://www.cbs.knaw.nl/publications/online/hybrids_cryptococcus_neoformans), were maintained on YPGA (1% yeast extract, 0·5% peptone, 4% glucose agar) at 10 °C and stored at −80 °C. One hundred and fifty-three isolates of *C. neoformans* var. *neoformans* and 54 of *C. neoformans* var. *gattii* were studied from AIDS and non-AIDS patients, the environment and animals, and from all continents (except Antarctica). Isolates were obtained from CBS (Utrecht, The Netherlands), the Prince Leopold Institute for Tropical Medicine (Antwerp, Belgium), the National Institutes of Health (Bethesda, MD, USA), the University of Sydney at Westmead Hospital (Sydney, Australia), and from individual researchers from Colombia, Spain, USA, Brazil and France. Data on the serotypes were taken from the literature (Boekhout *et al*., 1997) or provided by the depositors of the isolates. In the case of anomalous results, serotypes were redetermined using immunofluorescence with monoclonal antibody (Dromer *et al*., 1993) and agglutination with the IatronO kit (Ikeda *et al*., 1982). These are indicated with an asterisk in Table 1. *Filobasidiella depauperata* (Petch) Samson *et al*. CBS 7844, *Cryptococcus podzolicus* (Bab'eva & Reshetova) Golubev CBS 6819 and *Mrakia frigida* (Fell *et al*.) Yamada & Komagata CBS 5917 were used as outgroups.

Table 2. Percentages of isolates in the different serotype (a), clinical/environmental origin (b) and geographical origin (c) classes within the six AFLP genotypes of *C. neoformans* and *C. bacillisporus*

Figures are rounded off; figures in parentheses indicate the percentage of the total number of isolates.

(a) Genotype	A	D	AD	B	C		
1 ($n = 112$)	99 (55)	–	1 (0·5)	–	–		
2 ($n = 22$)	–	100 (11)	–	–	–		
3 ($n = 14$)	7 (0·5)	57 (4)	36 (2·5)	–	–		
4 ($n = 30$)	–	–	–	100 (15)	–		
5 ($n = 18$)	–	–	–	33 (3)	67 (6)		
6 ($n = 4$)	–	–	–	100 (2)	–		
(b) Genotype	**AIDS**	**Non-AIDS**	**Animal**	**Environmental**	**?**		
1 ($n = 112$)	39 (22)	13 (7·5)	5 (2)	39 (22)	4 (2·5)		
2 ($n = 22$)	17 (2)	21 (2·5)	17 (2)	29 (3·5)	16 (1·5)		
3 ($n = 14$)	46 (3)	15 (1)	–	23 (15)	15 (1)		
4 ($n = 30$)	–	45 (7)	23 (3·5)	29 (4·5)	3 (0·5)		
5 ($n = 18$)	–	50 (4·5)	–	28 (2·5)	22 (2)		
6 ($n = 4$)	–	25 (0·5)	25 (0·5)	50 (1)	–		
(c) Genotype	**NAm.**	**SAm.**	**Eur.**	**Afr.**	**Asia**	**Austr.**	**?**
1 ($n = 112$)	16 (9)	15 (8·5)	26 (14·5)	17 (8·5)	10 (5·5)	5 (3)	11 (6)
2 ($n = 22$)	26 (3)	–	57 (6·5)	–	4 (0·5)	–	13 (1·5)
3 ($n = 14$)	15 (1)	–	69 (4·5)	–	–	–	15 (1)
4 ($n = 30$)	19 (3)	7 (1)	19 (3)	13 (2)	19 (3)	16 (2·5)	7 (1)
5 ($n = 18$)	78 (7)	16 (1)	–	–	–	–	16 (1)
6 ($n = 4$)	50 (1)	50 (1)	–	–	–	–	–

DNA isolation and AFLP. For DNA isolation, cells were harvested from 2–3-d-old cultures and lyophilized. DNA was isolated by the CTAB method (O'Donnell *et al*., 1997). The AFLP procedure was performed according to the 'AFLP Microbial Fingerprinting Protocol' of the manufacturer (PE Biosystems), with some modifications. Restriction–ligation was performed simultaneously on 10 ng genomic DNA, using 1 unit *Mse*I, 5 units *Eco*RI and 3 units T4 DNA Ligase (Biolabs). The reaction took place in a total volume of 5·5 µl with 0·36 µM *Eco*RI adaptor and 3·64 µM *Mse*I adaptor from the AFLP Microbial Fingerprinting Kit (PE Biosystems), 0·1 M NaCl, 0·91 mM Tris/HCl (pH 7·8), 0·18 mM $MgCl_2$, 0·18 mM dithiothreitol, 18 µM ATP and 91·36 µg BSA ml^{-1}. The restriction–ligation mixture was incubated for 2 h at 37 °C and diluted by adding 25 µl sterile bidistilled water. The first PCR was performed with the two preselective primers (*Eco*RI core sequence and *Mse*I core sequence) and 'AFLP Amplification Core Mix' from the 'AFLP Microbial Fingerprinting Kit' according to the manual, under the following conditions: 2 min at 72 °C, followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C. The PCR product was diluted by adding 25 µl sterile bidistilled water. A second PCR used more selective primers: *Eco*RI-AC FAM and *Mse*I-G. The conditions were: 2 min at 94 °C, followed by 10 cycles consisting of 20 s at 94 °C, 30 s at 66 °C decreasing 1 °C every step of the cycle, and 2 min at 72 °C, followed by 25 cycles consisting of 20 s at 94 °C, 30 s at 56 °C and 2 min at 72 °C. The samples were prepared for acrylamide electrophoresis with the following loading mix: 1·0 µl selective amplification product, 1·25 µl deionized formamide, 0·25 µl blue dextran in 50 mM EDTA and 0·5 µl GeneScan-500 [ROX] size standard. After incubation for 3 min at 95 °C, 1·5 µl mix was loaded and run for 3 h on a 5% polyacrylamide gel on the ABI 377 sequencer (PE Biosystems) using 1× TBE running buffer. Data were analysed with the Bionumerics software package (version 1.01, Applied Maths, Kortrijk, Belgium), using (a) Pearson correlation based on similarities of the densitometric curves, and (b) the unweighted pair group method using arithmetic means (UPGMA) analysis with the Dice coefficient and the fuzzy logic option. Statistical significance of the clusters was tested by cophenetic correlation and bootstrap analysis. Statistical analysis of the resulting genotypes used chi-squared analysis by calculating exact *P*-values (SPSS 9.0 for Windows, SPSS Inc.).

RESULTS

AFLP genotyping

The AFLP genotyping resulted in a clear separation between *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* (serotypes A, D and AD) versus

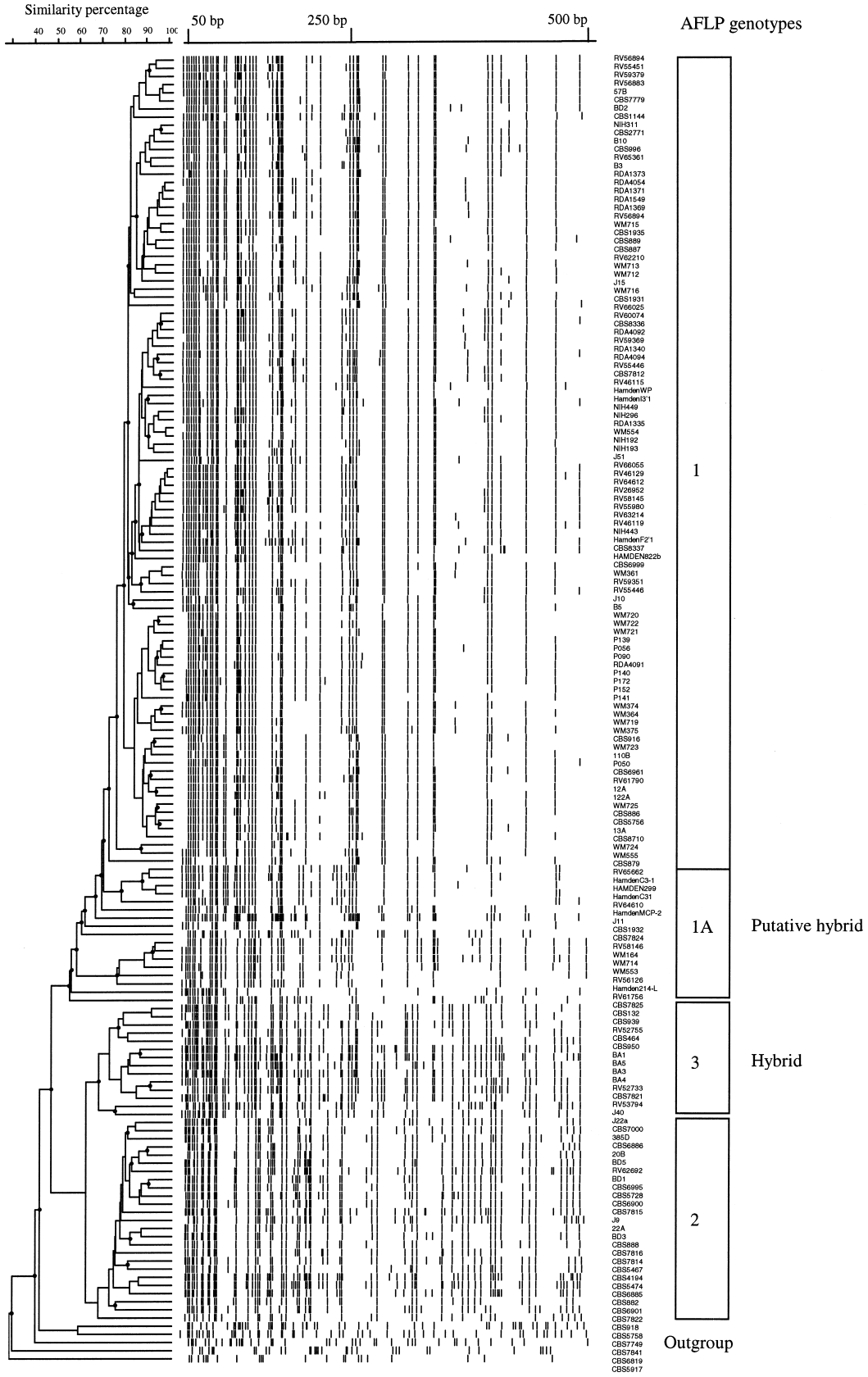

Fig. 2. Clustering of AFLP banding patterns of isolates of *C. neoformans* by UPGMA. Dots indicate well-supported clusters (CCV $\geqslant$ 85 %). Note the hybrid pattern of genotype 3, containing bands occurring in both genotypes 1 and 2.

C. neoformans var. *gattii* (serotypes B and C), thus supporting the genetic separation between *C. neoformans*/*F. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (= *C. bacillisporus*)/*F. neoformans* var. *bacillispora* (Fig. 1, Table 2). Almost all isolates possessed unique AFLP banding patterns. Six major genotypic clusters were found to be supported by high cophenetic correlation values (CCV). Three clusters (numbered 1–3) represented *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*, and three clusters (numbered 4–6) *C. neoformans* var. *gattii* (Figs 1, 2 and 3). Both the terminal and deeper branches were strongly supported, but the intermediate branches usually lacked strong support (Figs 2 and 3). The majority (56%) of the isolates belonged to cluster 1; 11·5% belonged to cluster 2, 7·0% to cluster 3; 15% to cluster 4; 9% to cluster 5 and 2% to cluster 6. All isolates from AIDS patients were found in clusters 1–3. In the rest of this paper we have, for convenience, used the name *C. neoformans* for *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*, and *C. bacillisporus* for *C. neoformans* var. *gattii*. The arguments for this species separation are given in the Discussion.

For both *C. neoformans* and *C. bacillisporus* the genotypes did not significantly differ in their ecological origin (viz. AIDS patients, non-AIDS patients, animals and environmental sources), with respective overall *P*-values of 0·186 and 0·162. However, within *C. neoformans* the geographical and serotype results were found to be highly significantly associated with the genotypes, with respective *P*-values of 0·002 and $<0{\cdot}001$. Regarding geography and serotype distributions, the African population of *C. neoformans* was significantly different from the North American and European populations, with the Asian, Australian and South American populations intermediate (Fig. 4a). For *C. bacillisporus* the geographical and serotype outcomes were significantly associated with the genotypes, with overall *P*-values of 0·001 and $<0{\cdot}001$, respectively. The North American population of *C. bacillisporus* is distinct from the African, Australian, European and Asian populations, with the South American population intermediate (Fig. 4b). We did not find a significant association between geographical origin and serotypes in *C. bacillisporus* ($P = 0{\cdot}095$); this is in contrast to *C. neoformans*, where this association was significant ($P \leqslant 0{\cdot}001$).

C. neoformans

Cluster 1 (= genotype 1) agreed with variety *grubii*, as all isolates were of serotype A (Table 2). Identical numbers of isolates were from environmental sources and AIDS patients (both 39%), whereas lower numbers came from non-AIDS patients (13%), animals (5%) or from unknown origins (4%). Members of this cluster were found to be present worldwide without an apparent geographical substructure (Table 2). No genetic differences were observed between the isolates from AIDS patients, non-AIDS patients, animals and the environment. The statistically supported subclusters contained isolates from different origins and localities. The isolates of genotype 1A (Table 1), representing isolates of AIDS patients from Brazil, Rwanda, USA and Belgium, and from the environment in Brazil, Zaire and Australia, seem genetically different from isolates of genotype 1 because of the presence of additional bands (Fig. 2).

Cluster 2 (= genotype 2) contained only serotype D isolates (Table 2). Within our collection of isolates this cluster was represented in Europe (57%), North America (26%), Asia (4%) and of unknown origin (13%). The majority of isolates came from environmental sources (29%) and non-AIDS patients (21%), and fewer from AIDS patients (17%), animals (17%) or unknown origin (16%) (Table 2). All isolates of unknown origin were probably made prior to the AIDS era. Therefore, we assume that they originated from non-AIDS patients. Both mating strains of *F. neoformans* (NIH 12 = CBS 6885 and NIH 430 = CBS 6886) clustered here.

Some of the bands in cluster 3 isolates (= genotype 3) corresponded in size with bands in either cluster 1 or 2 (Fig. 2). Twenty-three and 21 bands agreed in size with bands of clusters 1 and 2, respectively. Thirty-two bands occurred in genotypes 1, 2 and 3, and three bands were found to be unique for genotype 3. Genotype 3 represented isolates from Europe (69%), North America (15%) and from unknown origins (15%). This distribution pattern is similar to that of genotype 2. Forty-six per cent of the isolates came from AIDS patients, 15 from non-AIDS patients, 23% from environmental sources, and 15% is from unknown origin (Table 2). Serotype D accounted for 57%, serotype AD for 36% and serotype A isolates for 7%. The type strain CBS 132 of *C. neoformans* was in this genotype.

C. bacillisporus

Cluster 4 (= genotype 4) contained serotype B isolates, including those from *Eucalyptus*, and occurred in Australia, the Americas, Africa, Asia and Southern Europe (Table 2). Forty-five per cent came from non-AIDS patients, 23% from animals, 29% from the environment, and 3% were of unknown origin. The two subclusters (4A and 4B, Figs 1 and 3) corresponded geographically with Africa/USA/Europe/Asia/S. America (cluster 4A) and with Australia/Asia/America (cluster 4B). Isolates from Spanish goats (Baró *et al.*, 1998) occurred in cluster 4A together with a number of clinical isolates, and one isolate from *Eucalyptus* debris from a Californian zoo. The type strain RV 20186 (= CBS 6289) of *C. neoformans* var. *gattii* belonged here. Most of the *Eucalyptus* isolates from Australia and the USA formed a well-supported cluster in genotype 4B, together with clinical isolates from India, Honduras and China.

All cluster 5 (= genotype 5) isolates originated from the Americas, with 45% from clinical non-AIDS samples, 29% from the environment, and 23% from unknown

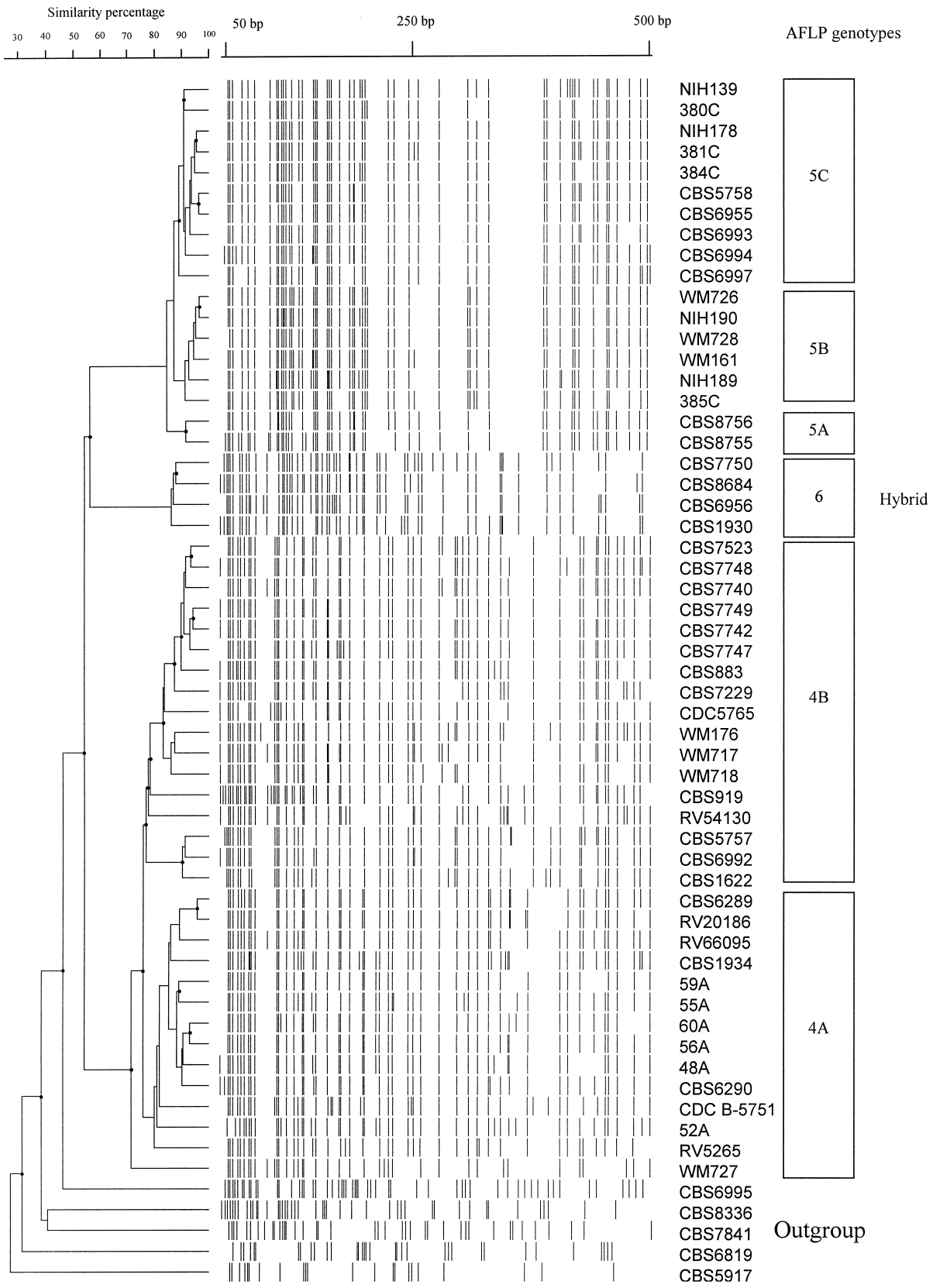

Fig. 3. Clustering of AFLP banding patterns of isolates of *C. bacillisporus* (= *C. neoformans* var. *gattii*) by UPGMA. Dots indicate well-supported clusters (CCV $\geqslant$ 85 %). Note the hybrid pattern of genotype 6, containing bands occurring in both genotypes 4 and 5.

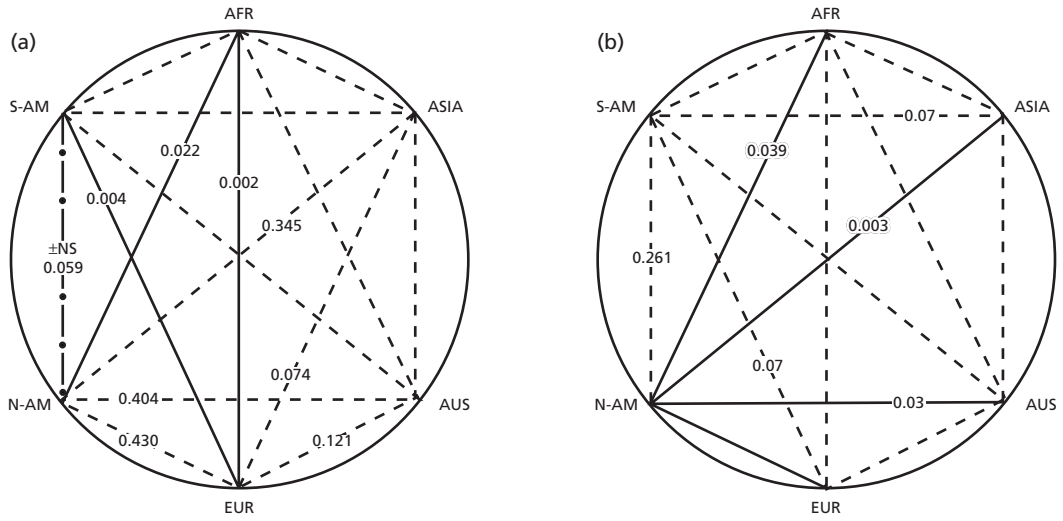

Fig. 4. Schematic representation of geographical distributions of AFLP genotypes of *Cryptococcus neoformans* (a) and *C. bacillisporus* (b). The numbers are exact *P*-values (chi-squared statistics). Full lines indicate statistically significant distributions (exact *P*-values $< 0{\cdot}05$); dashed lines indicate statistically non-significant distributions (exact *P*-values $> 0{\cdot}05$); dash-dotted lines indicate a near-significant (NS) distribution (exact *P*-value 0·059). In *C. neoformans* (a) the North American (N-AM) and European (EUR) populations differ from the African (AFR) and South American (S-AM) populations, with the latter being nearly significantly different from the North American population (exact *P*-value 0·059). The Asian and Australian (AUS) populations are not significantly different from the others. In *C. bacillisporus* (b) the North American population is significantly different from those of Africa, Asia, Australia and Europe, but not from the South Amrican one. The South American, African, Asian and European populations do not differ. Consequently the South American population has an intermediate position.

origin. None was AIDS related (Table 2). This cluster contained both serotype C (67 %) and serotype B (33 %) isolates. Three subclusters were apparent (5A–C) (Figs 1 and 3). The two environmental isolates from almond trees isolated in Colombia (Callejas *et al*., 1998) were in cluster 5A. Cluster 5B contained serotype B isolates and one serotype C isolate, and originated from non-AIDS patients, *Eucalyptus* trees and related sources. All isolates from cluster 5C were serotype C, and, as far as known, all came from non-AIDS patients. The type strain of *F. bacillispora* (NIH 191 = CBS 6955) clustered here.

The sixth cluster (= genotype 6) contained only four serotype B isolates, CBS 1930, CBS 7750, CBS 8684 and CBS 6956, which originated from the Americas (Table 2). Two of the isolates were from environmental sources (a wasp nest, and *Eucalyptus* debris), one came from a sick goat, and the fourth was isolated from human sputum. This latter isolate was one of the mating strains of *F. bacillispora* (Kwon-Chung, 1998). The banding patterns of isolates belonging to this genotype were characterized by fewer AFLP bands, which in part agreed in size with bands from both genotypes 4 and 5 (Fig. 3). At least 12 bands corresponded in size with those of cluster 4, 11 agreed with bands of cluster 5, 14 were unique for this genotype, and 14 occurred in both genotypes 4 and 5.

Mating strains of *F. bacillispora* clustered in all genotypes of the species, as follows: CBS 6992 (serotype B, genotype 4) × 6993 (serotype C, genotype 5), and CBS 6956 (serotype B, genotype 6) × 6955 (serotype C, genotype 5).

DISCUSSION

AFLP as a genotypic tool for the *C. neoformans* and *C. bacillisporus* species complex

AFLP is a multilocus genotyping method combining universal applicability, high discriminative power and reproducibility for which only small amounts of DNA are required (Blears *et al*., 1998; Savelkoul *et al*., 1999; Vos *et al*., 1995). The method has been used for the genotyping of bacteria, plants, fungi and animals, and also for the construction of genetic maps (Blears *et al*., 1998; Breyne *et al*., 1999; de Barros Lopes *et al*., 1999; Janssen *et al*., 1996; Leissner *et al*., 1997; Otsen *et al*., 1996; Qi & Lindhout, 1997; Savelkoul *et al*., 1999; Van der Lee *et al*., 1997). A large number of strain-specific genetic characters is available, because of the large number of bands generated using AFLP compared with RAPD and PCR fingerprinting (Boekhout *et al*., 1997; Meyer & Mitchell, 1995; Meyer *et al*., 1993). This renders AFLP a sensitive tool allowing differentiation of genetically related strains.

To generalize the results of genotype studies, the collection of isolates studied should be representative of the genetic variation within the species. The collections of *C. neoformans* strains in culture collections are

biased towards clinical isolates. However, if the ecological and geographical distribution of genotypes within a species is random, one may expect that the distributions of the respective genotypes would not widely differ from that of the species as a whole. With respect to geographical and serotype distribution this is not the case in *C. neoformans*, as can be seen in Table 2 and the chi-squared statistics applied. This strongly suggests that the genotypes differ in geographical distribution, which is clinically relevant as the pathogen may be acquired from the environment (Casadevall & Perfect, 1998).

The species problem in *C. neoformans*

The recognition of species is an important, but controversial, problem in biology. Proper recognition of species is important because it may relate to differences in e.g. pathogenicity, resistance and virulence. In the *C. neoformans* complex the biological species concept has been used since the observation of matings (Kwon-Chung, 1975, 1976). Initially, two distinct sexual species were distinguished within *C. neoformans*, namely *F. neoformans* and *F. bacillispora*. However, based on the observation of an interspecific mating between the strains CBS 6991 (*F. neoformans*, *MAT*a, serotype D) and CBS 6956 (*F. bacillispora*, *MAT*α, serotype B), which produced 30% viable basidiospores, the two species were considered as conspecific and both taxa were recognized as varieties (e.g. Kwon-Chung, 1998). Unfortunately, a genetic analysis of the basidiospores was not performed, and it has been noted that many interspecies crossings did not produce a fertile progeny. In addition, the sexual state has not been observed in nature. In time, growing evidence accumulated that the two varieties differ in many aspects, including phenotypic, biochemical, molecular, serological, ecological, epidemiological and clinical differences (Aulakh *et al*., 1981; Bennett *et al*., 1978; Boekhout & Scorzetti, 1997; Boekhout *et al*., 1997; Cherniak & Sundstrom, 1994; Dufait *et al*., 1987; Ellis & Pfeiffer, 1992; Howard & Kwon-Chung, 1995; Kwon-Chung *et al*., 1978, 1982a, 1987, 1988; Meyer & Mitchell, 1995; Meyer *et al*., 1993; Mukaramangwa *et al*., 1995; Pfeiffer & Ellis, 1993; Polacheck & Kwon-Chung, 1980; Speed & Dunt, 1995; Swinne, 1984; Wickes *et al*., 1994). Aulakh *et al*. (1981) observed only 55–63% DNA relatedness between the varieties, and the chromosomal organization was found to be different as well (Boekhout *et al*., 1997; Wickes *et al*., 1994).

In our opinion, all these aspects weaken the suggested conspecificity of the taxa. Our AFLP results indicate the presence of two main genetic lineages within the pathogen, which seem to correspond with reproductively isolated groups of populations. The observation of hybrid genotypes within each lineage strongly suggests the presence of hybridization within the lineages, which therefore may be interpreted as biological species. Phylogenetic trees based on AFLP and the IGS of the rDNA (Diaz *et al*., 2001) strongly suggest that both these biological species are monophyletic as well. Therefore, the proposed species concept in the *C. neoformans*/*C.bacillisporus* complex is based on monophyletic and interbreeding groups of populations. It also supports the notion of Avise & Wollenberg (1997) that the distinction between the biological species concept and the phylogenetic species concept, which is based on the concept of monophyly, is not always a sharp line.

We propose to name AFLP genotypes 1–3 as *Filobasidiella neoformans* Kwon-Chung with the anamorph *Cryptococcus neoformans* (Sanfelice) Vuillemin and AFLP genotypes 4–6 as *Filobasidiella bacillispora* Kwon-Chung with the anamorph *Cryptococcus bacillisporus* Kwon-Chung & Bennett (= *C. neoformans* var. *gattii* Vanbreuseghem & Takashio).

C. neoformans

The teleomorph *F. neoformans* and the anamorphs *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* originated from AIDS and non-AIDS patients, veterinary sources, bird droppings, and occasionally from substrates such as fermenting fruit juice, drinking water, wood, soil and air (Casadevall & Perfect, 1998; Levitz, 1991; Mitchell & Perfect, 1995; Swinne-Desgain, 1975; www.cbs.knaw.nl/search_ydb.html). All isolates obtained from AIDS patients belonged to this group. Recently, *C. neoformans* var. *neoformans* was also isolated from decaying wood of trees in Brazil (Lazera *et al*., 1993, 1996), and two of these isolates (CBS 8336, 8337) occurred in cluster 1. Trees may be the primary niche of the pathogen (Swinne, 1988). This is in agreement with the phylogenetic position in the order Tremellales (Fell *et al*., 2000) and the presence of the laccase enzyme system (Petter *et al*., 1996; Williamson, 1994). Because of the presence of haustorial branches in the *Filobasidiella* states, the hyphal state may occur as a mycoparasite (Bandoni, 1995).

Genotype 1 is identical with *C. neoformans* var. *grubii* (= serotype A). Incidentally, a serotype A isolate clustered in genotype 3 (isolate CBS 464). AFLP (this work) and IGS sequence data (Diaz *et al*., 2001) largely support the existence of *C. neoformans* var. *grubii*, but also showed that the resulting genotypic clusters do not entirely correspond with the serotype boundaries. This lack of concordancy can also be concluded from the *URA5* sequence and CNRE-1 fingerprints presented by Franzot *et al*. (1998a), as in their data the serotype D isolate J22 clustered among serotype A isolates.

Global distribution of serotype A, which occurs mainly as the α mating type, may be related to the global expansion of one of its hosts, the common dove (Franzot *et al*., 1997). In this scenario, the original distribution of var. *grubii* was the same as that of this bird, namely North Africa and Europe. So far, no genetic differences have been observed between Old and New World isolates using a variety of molecular typing techniques such as RAPD, restriction analysis, electrophoretic karyotyping (PFGE), IGS sequences and AFLP patterns

(Boekhout *et al*., 1997; Diaz *et al*., 2001; Meyer & Mitchell, 1995; Varma *et al*., 1995). These observations were interpreted to support a clonal expansion model for this genotype, probably with the pigeon as vector (Franzot *et al*., 1997). However, the clonal expansion model seems to be contradicted by the observed inter-strain variation in electrophoretic karyotypes, which are mitotically stable but meiotically unstable (Boekhout & van Belkum, 1997; Boekhout *et al*., 1997), and the variation we observed in AFLP banding patterns. The observed geographical differences between genotypes 1 and 2 seem to contradict the 'pigeon as the primary ecological niche' hypothesis, as both genotypes commonly occur in bird excreta (Table 2).

The banding patterns of isolates in cluster 1A differed from those of other cluster 1 isolates by the presence of a number of bands in common with genotype 2. We presume that these isolates may represent hybrids between genotypes 1 and 2, but further genetic studies using additional markers are needed to confirm the genetic nature of these isolates.

Genotype 2 agrees with *C. neoformans* var. *neoformans* because it contains most serotype D isolates. However, a nomenclatural problem exists because the type strain of *C. neoformans* CBS 132 belongs to the hybrid genotype 3. Genotype 2 has a more limited geographical distribution than genotype 1. Its dominance in Europe (57 %) is in agreement with earlier observations on the dominance of serotype D isolates in Europe (Dromer *et al*., 1994, 1996). The environmental isolates mainly came from bird droppings, which were previously considered a source of pathogenic serotype D strains (Garçia-Hermoso *et al*., 1997).

The observed difference between the African versus the North American and European populations of *C. neoformans* seems clinically relevant, because of the severe problem of AIDS-related cryptococcosis in sub-Saharan Africa. Probable differences in virulence to AIDS patients between the three genotypes need further study in infection experiments using animal models, and analysis of virulence-related factors, such as capsule thickness, growth rates at 37 °C, melanin formation, protease activity and phospholipase activity (Franzot *et al*., 1998b; Fries & Casadevall, 1998).

C. bacillisporus (= *C. neoformans* var. *gattii*)

This species, usually referred to as *C. neoformans* var. *gattii*, represents a distinct genetic lineage. The observed AFLP clustering of isolates of *F. bacillispora* was almost identical with that based on IGS sequences of the rDNA (Diaz *et al*., 2001), thus supporting the reliability of both methods for microbial typing. The naming of the taxon is complicated by the existence of older names which have priority, namely *Cryptococcus hondurianus* Castellani at the species level and *Torulopsis neoformans* var. *sheppei* Giordano at the variety level.

C. bacillisporus is limited to the tropics, the Southern hemisphere and Southern Europe, where it usually occurs in non-AIDS patients, animals, or saprobically associated with *Eucalyptus* and almond trees and bat guano (Ellis & Pfeiffer, 1990, 1992; Howard & Kwon-Chung, 1995; Pfeiffer & Ellis, 1993; Sorrell *et al*., 1996; Speed & Dunt, 1995; www.cbs.knaw.nl/search_ydb.html). The almost complete absence of this species in AIDS patients suggests a difference in virulence mechanisms as compared with *C. neoformans* (Kwon-Chung *et al*., 1988; Speed & Dunt, 1995).

Within this species three AFLP clusters occurred (numbered 4–6), thus suggesting a considerable genetic divergence. This divergence did not coincide with serotype boundaries, but rather followed geographical borders. Genotypes 5 and 6 occurred only in the Americas, and did not correspond with environmental versus clinical isolates. The North American population differed from the Asian, African, Australian and European populations. However, the South American population was not different from either of these groups of populations. Few isolates from our study were included in a recent published paper in which RAPD fingerprints and ITS sequences were used to investigate the geographical structure of the species (Imai *et al*., 2000). Our AFLP genotype 4 may coincide with their Asia-1 group, genotype 5 may be similar to their America-1 group, whereas genotype 6 may represent the America-2 group. However, more isolates from this study need to be investigated by AFLP to analyse the geographical relationships between the two types of data.

Hybrid strains

AFLP genotype 3 of *C. neoformans* contained a large number of bands corresponding in size with bands from either cluster 1 or cluster 2 (Fig. 2). Because of this mixed nature we postulate that genotype 3 represents a hybrid between AFLP genotypes 1 and 2. This hybrid, which formed a distinct cluster from another group of isolates representing a putative hybrid (genotype 1A), comprises about 9 % of the *C. neoformans* isolates studied. The hybridization hypothesis is supported by the geographical distribution of the hybrid genotype, which is the same as that of one of the supposed hybridization partners (genotype 2). In our hybrid hypothesis, genetic material may be transferred between genotypes 1 (serotype A *MAT*α) and 2 (serotype D *MAT*a). Because genotype 3 occurs in relatively high proportion in AIDS patients (46 %) and in Europe (69 %), we assume that the hybridization events may contribute to the high incidence of serotype D isolates (either genotype 2 and 3) in AIDS-related cryptococcosis on this continent.

A number of isolates included in this study have been part of other genotyping studies as well. Using CNRE-1 fingerprints, serotype D isolate J9 (clustering in AFLP genotype 2) clustered with other serotype D isolates, including NIH 3501 (= CBS 6900) and NIH 3502 (= CBS 6902). However, serotype D isolate J22 (our hybrid AFLP genotype 3) clustered among serotype A isolates, rather distant from serotype D isolates (Franzot *et al*.,

1997, 1998a). When *URA5* sequences were used, the isolate J22 clustered clearly distinct from serotype A isolates, and occurred with serotype D isolate B3501 (= CBS 6900), which in our AFLP analysis belonged to genotype 2. This lack of concordancy can be explained as a result of hybridization, as this may have caused separation of genetic markers. Genome sizes estimated from electrophoretic karyotypes ranged from 20 to 27 Mb in the hybrid isolates, and from 15 to 19 Mb in the parental isolates (Boekhout *et al*., 1997).

Genotype 6, representing about 8 % of *C. bacillisporus* isolates, may represent a hybrid between isolates of genotypes 4 and 5 of this species. Although only four isolates of this genotype were studied, it is notable that only one of the hybrid strains came from clinical sources, as compared with about 45 % and 50 % for genotypes 4 and 5, respectively. [The recent discovery of another four isolates belonging to this hybrid genotype, which all came from trees in South America (M. Lazera & T. Boekhout, unpublished) seems to support our observation that this genotype mainly occurs in the environment.] The estimated genome sizes for the putative hybrid isolates (14–15 Mb) in *C. bacillisporus* are intermediate as compared with the parental isolates (12–18 Mb) (data from Boekhout *et al*., 1997).

As in *C. neoformans*, the geographical distribution of the putative hybrid of *C. bacillisporus* was limited to one of the hybridization partners, in this case genotype 5. Possibly the observed hybridization in *C. bacillisporus* can be explained by the introduction of genotype 4 isolates together with *Eucalyptus* trees into the Americas. This hypothesis is supported by the observed isolation of the North American population as compared with those from other parts of the world, and the intermediate position of the South American population. One hybrid isolate came from a *Eucalyptus* tree from California, and about 28 % of the American genotype 5 isolates were isolated from *Eucalyptus*-related substrates. An alternative explanation is that both hybridization partners occur on *Eucalyptus*, and that genotype 5 and 6 isolates have not yet been found in other parts of the world.

The mechanism of hybridization in both species is not clear, and may be due to either sexual or parasexual processes. In the case of sexual hybridization in *C. neoformans*, we presume that the hybrids are due to hybridization between serotype A *MAT*α and serotype D *MAT*a isolates, because the serotype A population is dominated by mating type α (Kwon-Chung & Bennett, 1978). Heterozygosity was demonstrated to occur in putative hybrids by sequence analysis of PCR amplicons (Cogliati *et al*., 1999b), and diploid isolates were found in most cases to be serotype AD (Cogliati *et al*., 1999a; Tanaka *et al*., 1999). In a reanalysis of isozyme data generated by Brandt *et al*. (1993), Taylor *et al*. (1999) rejected the presence of recombination based on the assumption that the serotypes represented a single species. However, hybridization could not be rejected if serotypes A and D represented different species, and shuffling of alleles occurred only within a serotype. These authors suggested that serotypes of *C. neoformans* var. *neoformans* are undergoing cryptic speciation, and that recombination occurs within the A and D serotypes. The observed genetic divergence between serotypes A and D (Franzot *et al*., 1997, 1998a) was considered to support this hypothesis. In contrast, our results indicate that hybridization may occur between either the A and D, or the B and C serotypes. The natural niche(s) of the fungus, be it trees, other fungi or another substrate, may be a good choice for investigating the occurrence of hybridization and the sexual states in nature.

Based on our AFLP results, as well as the literature discussed, we propose the presence of hybridization in *C. neoformans* and *C. bacillisporus*. Therefore, we favour the scenario that the fungus uses both (para)sexual and asexual reproduction strategies. Consequently, genetic material can be transferred between isolates of different genetic background, which may result in strains with an altered virulence and/or resistence to antifungal agents. Using subsequent clonal expansion, these strains may disseminate. Our hypothesis on the reproduction biology of the pathogen differs from the earlier proposed clonal reproduction of *C. neoformans*, which was based on linkage disequilibrium studies (Brandt *et al*., 1995, 1996), the observed concordance between molecular parameters (e.g. *URA5* sequences and *CNRE1* hybridization patterns) from geographically separated populations, and the dominance of a few genotypes in the population (Franzot *et al*., 1997). We propose a complex life cycle for the pathogen comprising both recombination and clonal expansion, and we suggest a role for the sexual *Filobasidiella* state in this process *in vivo*.

Conclusions

To summarize our data, we propose the following. (1) At least two pathogenic cryptococcal species exist, namely (a) *C. neoformans* with variety *neoformans* and variety *grubii*, and a sexual *F. neoformans* state, and (b) *C. bacillisporus* (synonym *C. neoformans* var. *gattii*) with the sexual state *F. bacillispora*. (2) In both species three AFLP genotypes occur, which differ in geographical and ecological behaviour. In each species one of these three genotypes has a hybrid nature. (3) Both sexual and asexual reproduction strategies are part of the reproduction biology of the pathogen. (4) Newly generated genotypes may disseminate clonally. (5) Serotype boundaries do not fully coincide with the genotypic groups, and therefore serotyping is not a reliable characteristic to differentiate infraspecific taxa.

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