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RESEARCH ARTICLE

Cryptococcosis Serotypes Impact Outcome and Provide Evidence of *Cryptococcus neoformans* Speciation

Marie Desnos-Ollivier,[a,b] Sweta Patel,[a,b] Dorothée Raoux-Barbot,[a,b] Joseph Heitman,[c] Françoise Dromer,[a,b] The French Cryptococcosis Study Group

Institut Pasteur, Molecular Mycology Unit, National Reference Center for Invasive Mycoses & Antifungals, Paris, France[a]; CNRS URA3012, Paris, France[b]; Department of Molecular Genetics and Microbiology, Duke University, North Carolina, USA[c]

ABSTRACT ***Cryptococcus neoformans*** **is a human opportunistic fungal pathogen causing severe disseminated meningoencephalitis, mostly in patients with cellular immune defects. This species is divided into three serotypes: A, D, and the AD hybrid. Our objectives were to compare population structures of serotype A and D clinical isolates and to assess whether infections with AD hybrids differ from infections with the other serotypes. For this purpose, we analyzed 483 isolates and the corresponding clinical data from 234 patients enrolled during the CryptoA/D study or the nationwide survey on cryptococcosis in France. Isolates were characterized in terms of ploidy, serotype, mating type, and genotype, utilizing flow cytometry, serotype- and mating type-specific PCR amplifications, and multilocus sequence typing (MLST) methods. Our results suggest that *C. neoformans* serotypes A and D have different routes of multiplication (primarily clonal expansion versus recombination events for serotype A and serotype D, respectively) and important genomic differences. Cryptococcosis includes a high proportion of proven or probable infections (21.5%) due to a mixture of genotypes, serotypes, and/or ploidies. Multivariate analysis showed that parameters independently associated with failure to achieve cerebrospinal fluid (CSF) sterilization by week 2 were a high serum antigen titer, the lack of flucytosine during induction therapy, and the occurrence of mixed infection, while infections caused by AD hybrids were more likely to be associated with CSF sterilization. Our study provides additional evidence for the possible speciation of *C. neoformans* var. *neoformans* and *grubii* and highlights the importance of careful characterization of causative isolates.**

IMPORTANCE ***Cryptococcus neoformans*** **is an environmental fungus causing severe disease, estimated to be responsible for 600,000 deaths per year worldwide. This species is divided into serotypes A and D and an AD hybrid, and these could be considered two different species and an interspecies hybrid. The objectives of our study were to compare population structures of serotype A and serotype D and to assess whether infections with AD hybrids differ from infections with serotype A or D isolates in terms of clinical presentation and outcome. For this purpose, we used clinical data and strains from patients diagnosed with cryptococcosis in France. Our results suggest that, according to the serotype, isolates have different routes of multiplication and high genomic differences, confirming the possible speciation of serotypes A and D. Furthermore, we observed a better prognosis for infections caused by AD hybrid than those caused by serotype A or D, at least for those diagnosed in France.**

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Address correspondence to Françoise Dromer, dromer@pasteur.fr.

Cryptococcus neoformans is a life-threatening human fungal pathogen causing meningoencephalitis, mainly in patients with cellular immune defects, such as those with acquired immunodeficiency syndrome (AIDS). This yeast is estimated to cause 1 million annual cases globally and nearly 625,000 deaths/year (1). This species exists in two mating types (MAT**a** and MATα) (2) and two varieties, *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D), which were recently proposed as distinct species (3); most serotype D isolates are found in Europe (4, 5). The third serotype (AD hybrid) results from the fusion of serotypes A and D and in some cases has an apparent African origin (6–8). The proportion of AD hybrids varies worldwide (1.8% in Thailand, 1.3 to 5.9% in the Americas, and 3.4 to 45% in Europe) (4, 9). The allelic profiles for the mating types are also heterogeneous: a majority of αADα, fewer **a**ADα, and even fewer αAD**a** strains in the United States, a majority of αAD**a** strains in Spain, Portugal, and Germany, and a similar proportion of αAD**a** and **a**ADα strains in Italy (10). Of note, some AD hybrid isolates have only one mating type allele because of partial or complete chromosome deletion or chromosome loss and reduplication, suggesting genomic instability (11, 12). For serotype A, the vast majority of clinical and environmental isolates are MATα (0.1 to 2% MAT**a**) (13–15), except in sub-Saharan Africa (10% MAT**a**) (16). For serotype D, it is established that 15% of Dutch isolates are MAT**a** (17). αADα hybrids, which have two α type mating alleles, also occur and result from unisexual reproduction (18).

The *C. neoformans* population structure has been studied

mainly for serotype A using PCR fingerprinting, multilocus sequence typing (MLST), or variable-number tandem repeat (VNTR) methods (7, 17, 19–24). The majority of serotype A isolates exhibit clonal expansion with very few recombination events and low genetic diversity (2). These results show mitotic clonal expansion, inbreeding via unisexual reproduction of clonally related or identical isolates, or both. Geographic specificity has been observed so far only for isolates recovered in Botswana that exhibit higher genetic diversity and recombination events (25) and for environmental isolates in India that show evidence of recombination and extensive gene flow (26). The experimental design (sampling and geographical area) may contribute to differences in the extent of clonal expansion versus recombination events reported (6). For serotype D, a unique molecular type is usually described as VNIV or AFLP2 (27–30). Evidence of recombination was found after analysis of 58 environmental isolates from North Carolina (31) and 33 from other areas (11), which differs from results obtained with Dutch isolates (17).

In humans, serotype A is the more frequent serotype and is thought to be associated with more severe infections, at least in HIV-infected patients (5). In experimental animal models, most studies have established higher virulence for serotype A than serotype D and for MATα than MAT**a** (32, 33). Contradictory results have been published on AD hybrids. Their virulence may vary depending on the inoculation route and also on the chromosomal composition, with higher virulence for strains containing a majority of the serotype A genome (12, 34, 35). Overall, there are few clinical data on patients infected with AD hybrids.

Our objectives were thus to (i) analyze the population structure of serotype A versus serotype D using isolates recovered from patients diagnosed with cryptococcosis in France and (ii) analyze clinical presentations and outcomes in patients infected with AD hybrids compared to serotype A or D.

RESULTS

Molecular characterization of clinical isolates. Among the 400 CryptoA/D isolates recovered from the 181 patients, 61% (244) corresponded to serotype A, 19% (76) to serotype D, and 20% (80) to AD hybrids. Of note, the serotypes determined here by PCR matched those obtained previously using fluorescence microscopy (5) except for patients infected with AD hybrids that were previously considered infected with either serotype A (20/131, 15%) or D (11/50, 22%). Overall, diploid strains were found in 52 (29%) patients (37 hybrids, 6 serotype A, and 9 serotype D). None of the serotype A isolates were MAT**a**, whereas 13% (13/97) of the serotype D isolates were MAT**a**. Among the 37 (20%) patients infected with AD hybrids, 25 (67%) were infected with αAD**a**, 3 (8%) with αADα, and 6 (16%) with **a**ADα. For three hybrids, only one allele of the mating types was identified (1 of each **a**AD-, -AD**a**, and -ADα), probably due to partial or complete chromosome loss.

Among the 181 CryptoA/D patients, 26 (14.4%) had proven mixed infection with mixed serotypes (12 patients), ploidies (6), genotypes (2), or a combination of mixed serotypes/genotypes (2) or serotypes/ploidies (4) (Table 1), confirming data obtained for a subset of the patients (36). Patients with mixed genotypes of serotype A were infected with unrelated (patient 27) or related (patient 197) isolates. Patients with mixed ploidies were infected with haploid and diploid isolates sharing the same genetic profile, suggesting *in vivo* diploidization by endoreplication (36). Some patients were infected with unrelated A (or D) and AD isolates (differences in $\geq$3/5 loci), suggesting coinoculation. Finally, one patient was infected with potentially related D and AD isolates (differing at only 1 of 5 loci), suggesting possible *in vivo* hybridization even though the serotype A partner was never uncovered (36) (see Table S1 in the supplemental material). Mixed infections were suspected for 13 additional patients (PCR on the original haploid isolate suggested a mixture of A and D or the presence of AD, but the 10 single colonies analyzed for each patient were haploid and either A [9/13] or D [4/13]).

Comparison of genetic diversity and population structures of *C. neoformans*. Serotypes A and D differed in the number of genetic profiles (19 sequence types [STs] grouped in 3 major clusters and 3 singletons for the 121 serotype A isolates, versus 44 STs grouped in 7 clusters and 20 singletons for the 97 serotype D isolates) (Fig. 1; also, see Tables S2 and S3 in the supplemental material). For serotype D, the discriminatory power was 0.95, confirming the robustness of the MLST method for the serotype D population. ST121 was the major profile (17/97, 17.5%), but most STs corresponded to a single isolate. Isolates harboring MAT**a** belonged to 10 STs, including 2 STs shared with MATα isolates (ST116 and ST125) (Fig. 1). The genetic diversity was greater for serotype D than for serotype A, according to maximum-parsimony phylogenetic analysis (Fig. 2), gene diversity, and average number of alleles per locus (Table 2).

For both populations, the observed variance was significantly different from the expected variance ($P < 0.0001$), suggesting linkage disequilibrium among the 7 loci. Similarly, a significant test of congruence for 10/21 combinations for serotype D and 8/21 for serotype A suggested that some loci exhibited coevolution confirming linkage disequilibrium. However, based on the allelic compatibility test, compatibility was observed in 19/21 graphs for serotype D and in 6/21 graphs for serotype A (Fig. 3; also, see Fig. S1 in the supplemental material) serving as evidence of recombination in both but more recombination events among serotype D than serotype A populations. Furthermore, the index of association (I_A) was not significantly different from zero, suggesting that some recombination events could occur in the serotype D population, which contrasted with results obtained for serotype A. Recombination parameters (S, R, and Rm) differed between serotypes D and A (Table 2). Altogether, these results suggested recombination events in the serotype D and clonal expansion for the serotype A populations.

Finally, multiple alignment of the concatenated sequences of the 7 loci (>4,000 bp in length) showed that similarity ranged from 98.7% to 100% among serotype A isolates and from 99.2% to 100% among serotype D isolates. In contrast, 81 to 92% similarity was observed between sequences of A and D isolates. Multiple alignments revealed 7 gap positions corresponding to 20 (range, 1-7) bp for serotype A isolates, and 22 gap positions corresponding to 81 (range, 1-19) bp for serotype D isolates. These indels were distributed among all isolates and localized mainly in the IGS1 locus (5/7 positions for serotype A and 18/22 positions for serotype D), which is an untranslated region.

Influence of AD hybrids on clinical presentation and outcome of cryptococcosis. We compared the characteristics of the patients depending on the infecting serotype (A, D, and AD) for the 155 patients infected with a unique strain (Tables 3 and 4). Overall, the three populations did not differ in terms of sex or age. The proportion of patients infected with AD hybrids did not differ

TABLE 1 Molecular information for isolates recovered in the 26 proven mixed infections

Patient	Origin isolate	Serotype, mating type, and ploidy	Single-colony isolate	ST of serotype: A	ST of serotype: D	Type of mixed infection	Probable origin of mixed infection
5	AD5-87	Aα, *n*	AD11-71	104		Serotypes	Coinoculation
		Dα, *n*	AD11-72		129		
10	AD5-85	αAD**a**, 2*n*	AD5-35			Serotypes + genotypes	Coinoculation
	AD4-64	Aα, *n*	AD8-93	63			
		αAD**a**, 2*n*	AD2-86				
20[a]	AD3-28	Aα, *n*	AD9-61	63		Serotypes	Coinoculation
		Dα, 2*n*	AD1-75		114		
21	AD3-88	Aα, *n*	AD3-87	23		Serotypes	Coinoculation
		Dα, *n*	AD3-89		119		
23[a]	AD4-5	Aα, *n*	AD7-84	23		Serotypes	Coinoculation
		Dα, 2*n*	AD4-16		122		
27	AD4-44	Aα, *n*		23		Genotypes	Coinoculation
	AD4-45	Aα, *n*		77			
34	AD3-74	Aα, *n*	AD8-83	69		Serotypes	Coinoculation
		Dα, *n*	AD7-28		134		
35	AD3-37	Dα, *n*	AD11-22		108	Serotypes	Coinoculation
		Aα, *n*	AD5-71	45			
51	AD5-26	Dα, *n*	AD12-34		135	Ploidies	*In vivo* endoreplication
		Dα, 2*n*	AD12-35		135		
62	AD4-62	**a**ADα, 2*n*	AD3-35			Serotypes	Possible *in vivo* hybridization
		Dα, *n*	AD2-25		131		
71[a]	AD4-26	D**a**, *n*	AD10-73		132	Serotypes + ploidies	Coinoculation + *in vivo* endoreplication
		D**a**, 2*n*	AD10-72		132		
	AD4-27	Aα, *n*	AD7-68	32			
		D**a**, *n*	AD10-72		132		
80	AD3-91	Dα, *n*	AD3-91		121	Serotypes	Coinoculation
		aADα, 2*n*	AD7-25				
82	AD7-3	Dα, *n*	AD11-77		120	Ploidies	*In vivo* endoreplication
		Dα, 2*n*	AD11-79		120		
96	AD3-23	Aα, *n*	AD4-41	63		Ploidies	*In vivo* endoreplication
		Aα, 2*n*	AD4-34	63			
100	AD4-70	αADa, 2*n*	AD5-70			Serotypes	Coinoculation
		Aα, *n*	AD12-8	106			
119[a]	AD1-60	D**a**, *n*	AD6-82		130	Ploidies	*In vivo* endoreplication
		D**a**, 2*n*	AD10-75		130		
130	AD1-66	Aα, *n*	AD8-36	46		Serotypes	Coinoculation
		Dα, *n*	AD1-84		122		
139	AD4-77	Aα, *n*	AD3-64	71		Ploidies	*In vivo* endoreplication
		Aα, 2*n*	AD3-76				
140	AD4-80	Aα, *n*	AD2-77	63		Ploidies	*In vivo* endoreplication
		Aα, 2*n*	AD2-55				
161[a]	AD1-76	Aα, *n*	AD7-53	46		Serotypes + ploidies	Coinoculation + *in vivo* endoreplication
		Dα, *n*	AD1-70		121		
	AD1-77	Aα, *n*	AD7-99	46			
		Aα, 2*n*	AD8-18	46			

(Continued on following page)

TABLE 1 (Continued)

Patient	Origin isolate	Serotype, mating type, and ploidy	Single-colony isolate	ST of serotype: A	ST of serotype: D	Type of mixed infection	Probable origin of mixed infection
177[a]	AD4-20	Aα, *n*	AD9-73	63		Serotypes + ploidies	Coinoculation + *in vivo* endoreplication
		Dα, *n*	AD10-32		114		
	AD4-21	Dα, 2*n*			114		
188[a]	AD1-12	Aα, *n*		63		Serotypes + genotypes	Coinoculation
	AD1-36	Aα, *n*	AD8-34	32			
		Dα, *n*	AD2-78		121		
197	AD3-57	Aα, *n*		63		Genotypes	Coinoculation or microevolution
	AD3-58	Aα, *n*		58			
198[a]	AD4-58	αADa, 2*n*	AD10-66			Serotypes	Coinoculation
	AD5-14	Aα, *n*	AD10-49	46			
199	AD5-15	Aα, *n*	AD11-98	46		Serotypes + ploidies	Coinoculation + *in vivo* endoreplication
		Aα, 2*n*	AD11-99	46			
	AD5-16	αAD**a**, 2*n*	AD11-68				
217	AD3-17	Aα, *n*	AD11-42	40		Serotypes	Coinoculation
		Dα, *n*	AD11-43		133		

[a] Mixed infection that was described in our previous study (36).

according to the continent of birth, whereas there were no serotype D isolates in patients born in Africa. Of note, no association between geographical origin and cluster or ST was observed for serotype A or D isolates. There was a trend toward fewer serotype D and more AD hybrids among HIV-infected patients, whereas it was the opposite among HIV-negative patients. When four risk categories among the 33 HIV-negative patients ("malignancy," "solid organ transplantation," "others" [disease/treatment], and "none" [no known risk factor]) were considered, "malignancy" and "others" were the major risk factors for the 19 patients infected with A, "malignancy" and "none" were the major risk factors for the 10 infected with D, and all four categories were recorded for the 4 patients infected with AD. Clinical presentation and disease severity were similar despite a trend toward less disseminated infection and less frequent abnormal lung imaging with AD hybrids, lower serum antigen titers with serotype D and AD hybrids, and less frequent abnormal brain imaging with serotype D. Intracranial pressure was recorded for only 21 patients and was increased in 16 (14 infected with serotype A, 1 with D, and 1 with AD). Induction therapy with a combination of amphotericin B and flucytosine was prescribed with similar frequencies to patients infected with serotypes A and AD and significantly less frequently to patients infected with serotype D. Fluconazole induction therapy mirrored that of the amphotericin B-flucytosine combination. The proportion of mycological failure (nonsterilization at week 2 despite antifungal therapy) was lower in the case of the AD hybrid than in that of serotype A or D. This was significant when patients with meningoencephalitis were considered and near significant when all cases (meningeal and nonmeningeal cryptococcosis) were considered.

In a multivariate analysis on the entire database (unique and mixed infections), parameters independently associated with mycological failure (based on cerebrospinal fluid [CSF] sterilization) were a high serum antigen titer, lack of flucytosine during induction therapy, and mixed infection, while infection with AD hybrids was more often associated with mycological cure (Table 4). Overall survival at 3 months was not different in patients infected by hybrids compared to the others. Ten patients (1 with AD hybrids and 9 with serotype A) had neurological sequelae.

DISCUSSION

We used clinical data and isolates collected during the nationwide surveillance on cryptococcosis and the CryptoA/D study to further analyze *Cryptococcus* biology and the disease it causes. Parts of the data sets have been used in other studies (5, 36, 37). However, the discovery of AD hybrid isolates among those previously classified as serotype A or D allowed us to analyze here how the disease caused by AD hybrids in humans differed from that caused by A or D (5). Likewise, expanding our search for mixed infections to the entire data set of the CryptoA/D study allowed us to confirm the high incidence of mixed infections during cryptococcosis but also to include it with the data for AD hybrids in the model of multivariate analysis studying the parameters influencing outcome of infection. We thus confirmed previous findings but also extended our understanding of the biology of an important fungal pathogen and of parameters potentially useful for the management of cryptococcosis.

The diversity of the French *C. neoformans* clinical isolates was higher than previously reported (5), with a proportion of AD hybrids (20%) and a distribution of mating type profiles similar to European data (9). Cogliati et al. and Li et al. found that 71% of AD hybrids harbored the MAT**a** allele (73% here), suggesting that AD hybrids could be a reservoir preserving the MAT**a** mating type, rarely found outside Africa for serotype A (9, 11). The hypothetical African origin of AD hybrids (7) was further supported by the similar proportion of AD hybrids among patients born in Africa and in other continents in our study.

The difference in the population structures of serotypes A and D was striking. Serotype A is considered more virulent than serotype D, and its clonal expansion (at least for MATα) could con-

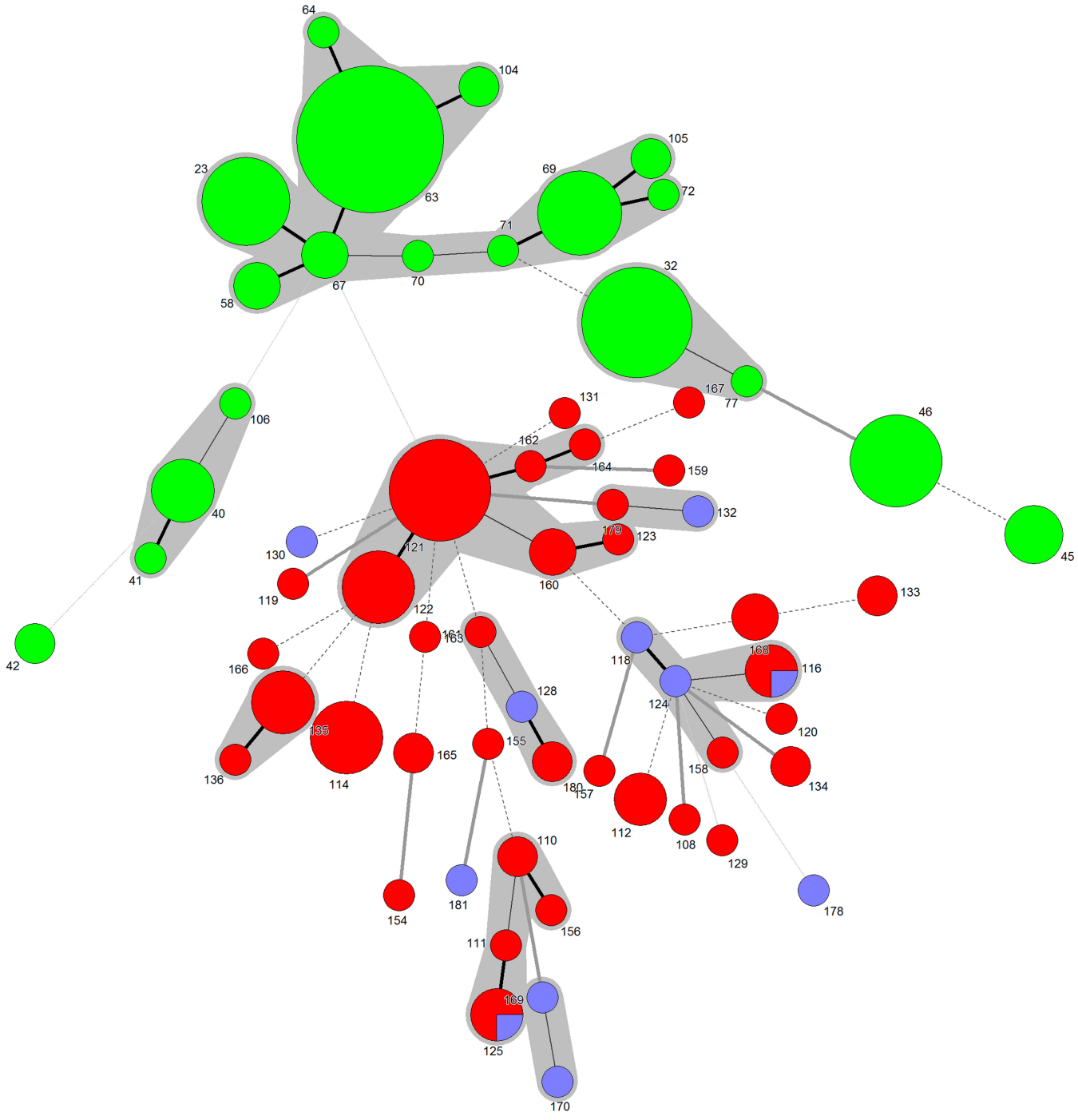

FIG 1 Minimum-spanning trees for isolates of serotype A and D. Minimum-spanning trees were constructed with the ST allelic profiles of the 7 MLST loci for the 97 isolates of serotype D and the 121 serotype A isolates. Green nodes, serotype A MATα; red nodes, serotype D MATα; blue nodes, serotype D MAT**a**. The denomination of the sequence type (ST) is indicated for each node. The size of the node is proportional to the number of isolates sharing the same ST, whereas the lines between STs indicate inferred phylogenetic relationships and are in bold black, plain black, discontinuous black, bold grey, or plain grey depending on the number of allelic mismatches between profiles (1, 2, 3, 4, or more than 4, respectively). Clusters are in grey and correspond to partition of nodes that differ by a maximum of two loci.

tribute to maintain genomic markers associated with virulence. Our data (low genetic diversity, lack of MAT**a**, linkage disequilibrium, values of recombination parameters, and low allelic compatibility) confirmed the hypothesis of clonal expansion for serotype A in France. The presence of both mating types can facilitate recombination in a population, and high genetic diversity within a population is often associated with sexual reproduction (11, 38). It is known that sexual reproduction of serotype D is robust and not strain specific (39), whereas 50% of clinical and environmental isolates of serotype A are fertile (40). Despite linkage disequi-

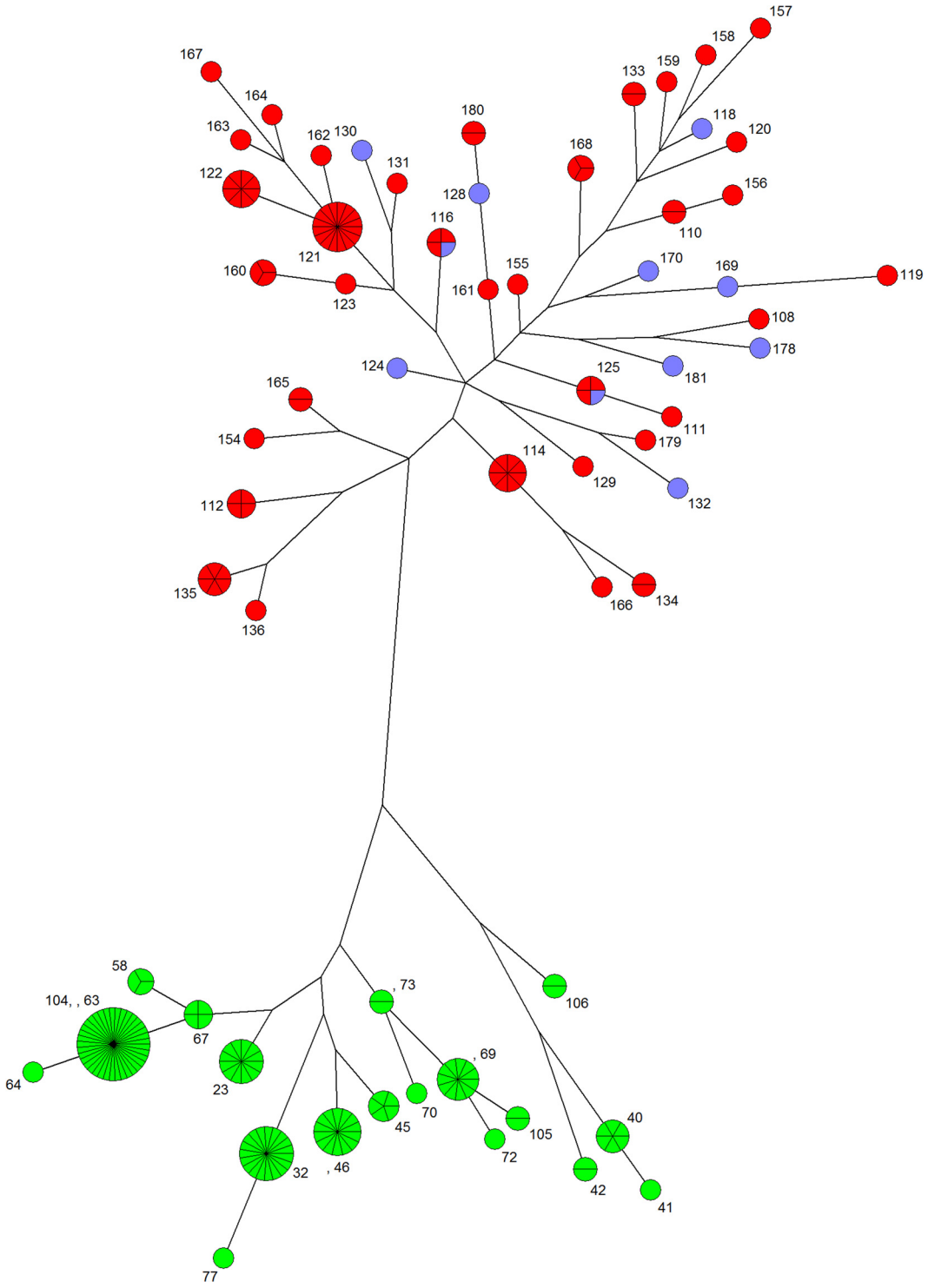

FIG 2 Maximum-parsimony trees for isolates of serotypes A and D. Maximum-parsimony trees were constructed with concatenate sequences of the 7 MLST loci for the 97 serotype D isolates and the 121 serotype A isolates. Green nodes, serotype A MATα; red nodes, serotype D MATα; blue nodes, serotype D MAT**a**. The denomination of the sequence type (ST) is indicated for each node. The size of the nodes increased with the number of isolates sharing similar sequences. The size of the lines between nodes increased with the number of differing nucleotides. Logarithmic scaling for branches was used.

librium, our results (high proportion of MAT**a** and diploid isolates, high genetic diversity, higher proportions of indels in coding regions, high allelic compatibility, and values of recombination tests) suggest that recombination events can occur among serotypes D isolates. Despite the possibility of recombination (31), no geographic specificity was observed, with the limitation that only clinical isolates were analyzed.

The possible speciation of serotypes A and D has been the sub-

TABLE 2 Comparison of population structure of *Cryptococcus neoformans* serotype D and serotype A

Parameter	Serotype D (97 patients)	Serotype A (118 patients)
% (no.) of patients infected with MAT**a** isolates	13.4 (13)	0 (0)
% (no.) of patients infected with diploid isolates	9.3 (9)	5.1 (6)
No. of STs	44	19
No. of combination with significant congruence/total of pairwise combinations	10/21	8/21
No. of graphs with allelic compatibility/total (Fig. 3 and Fig. S1)	19/21	6/21
Gene diversity (*H*)	0.95	0.86
Average no. of alleles per locus (*N*)	11.57	5.57
Index of association standardized (I_A)	0.00356 (44 STs); 0.1736 (97 isolates)	0.2537 (19 STs); 0.2958 (121 isolates)
No. of segregating sites (*S*)	76	81
No. of recombination events (*R*)	116	0.3
Minimum no. of recombination events (*Rm*)	16	8

ject of debate for many years (3, 6). Known differences between the two serotypes include geographical distribution (41), host-related susceptibility and type of disease produced (5), skin tropism (41), maximal growth temperature (42), proportion of MAT**a** (16), monokaryotic fruiting for MAT**a** (43), and overall genome differences (85 to 90% nucleotide sequence identity between JEC21 and H99 genomes [44, 45]). Here, we found major differences in population structure and 8 to 19% nucleotide difference over 4,000 bp. Reproductive isolation has been considered the main mechanism of speciation (46). Even if serotype A di-

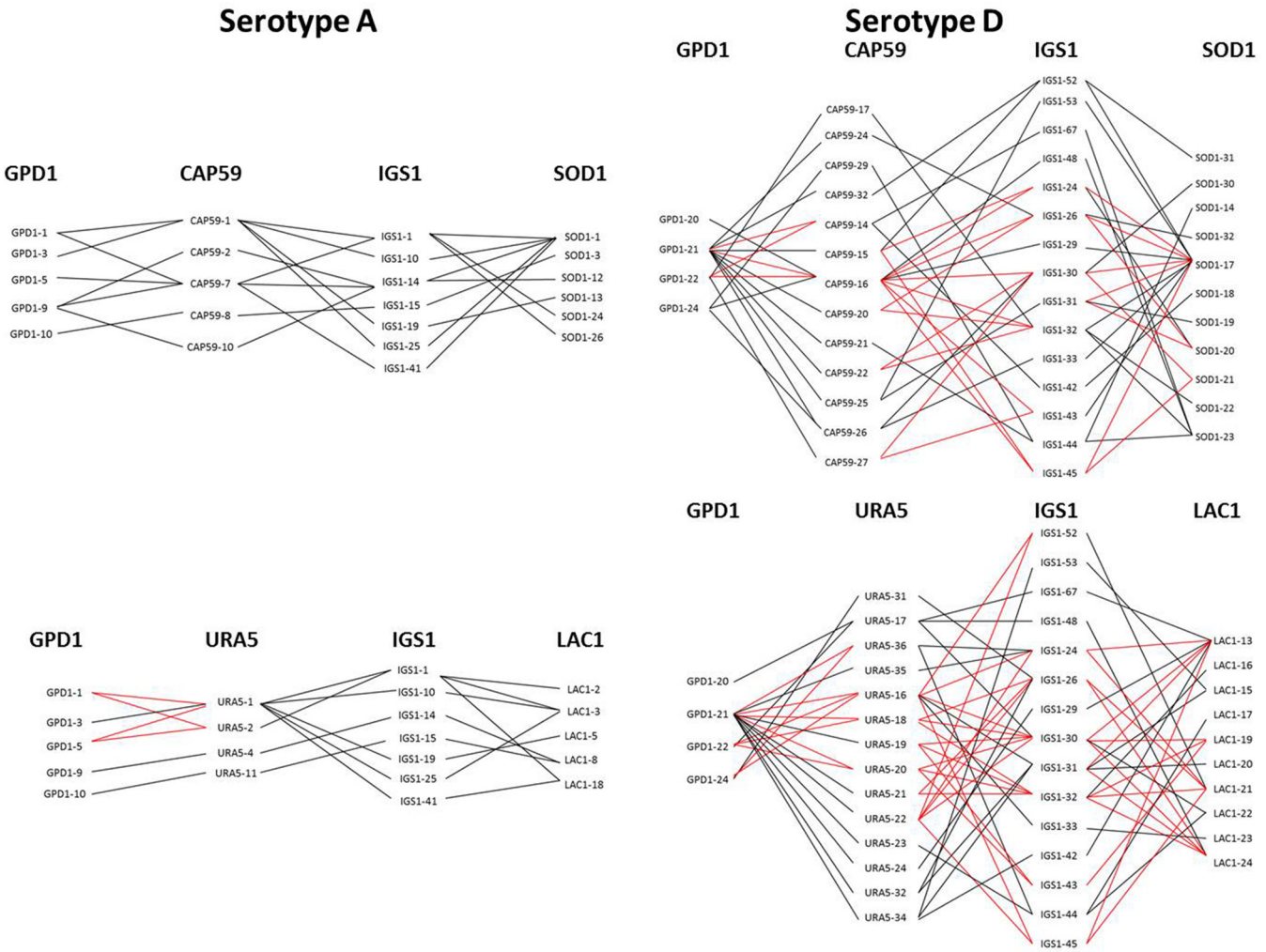

FIG 3 Comparison of allelic compatibility tests generated for serotype A and serotype D. An hourglass shape (red lines) indicates the presence of all four possible pairs of alleles and serves as evidence of recombination. A selection of 6 out of the 21 tests is shown, demonstrating allelic compatibility in 1/6 tests for serotype A and 6/6 tests for serotype D. All graphs are provided in Fig. S1 in the supplemental material.

TABLE 3 Characteristics of the 155 patients with single infection due to one of the three serotypes of *C. neoformans*

Characteristic	Serotype A ($n = 98.0$)	Serotype D ($n = 26.0$)	Serotype AD ($n = 31.0$)	*P*
No. of males/total (%)[a]	79/98 (80.6)	21/26 (80.8)	24/31 (77.4)	0.957
Age (yr) (mean ± SD)	41.2 ± 11.9	45.8 ± 12.5	42.7 ± 12.5	0.217
No. born in Africa/total (%)	31/98 (31.6)	0/26 (0)	6/31 (19.4)	0.001
No. HIV infected/total (%)	79/98 (80.6)	16/26 (61.5)	27/31 (87.1)	0.063
Mean CD4/mm^3 ± SD for HIV-infected patients	50 ± 82	44 ± 56	41 ± 54	0.866
No. with abnormal neurology/total (%)	40/98 (40.8)	10/26 (38.7)	15/31 (48.4)	0.733
No. with meningoencephalitis/total (%)	82/95 (86.3)	19/23 (82.6)	26/28 (92.9)	0.545
No. with fungemia/total (%)	42/94 (44.7)	11/25 (44.0)	11/28 (39.3)	0.888
No. with dissemination/total (%)	60/98 (61.2)	16/26 (61.5)	12/31 (38.7)	0.081
No. with high serum antigen titer (≥512)/total (%)	47/89 (52.8)	8/23 (34.8)	9/28 (32.1)	0.083
No. with high CSF antigen titer (≥512)/total (%)	40/85 (47.1)	6/17 (35.3)	8/24 (33.3)	0.398
No. with abnormal brain imaging/total (%)	28/80 (35.0)	2/18 (11.1)	11/27 (40.7)	0.084
No. with abnormal lung imaging/total (%)	48/97 (49.5)	11/23 (47.8)	8/30 (26.7)	0.088
No. with AMB + 5FC as induction therapy/total (%)[b]	54/98 (55.1)	7/26 (26.9)	14/31 (45.2)	0.031
No. with fluconazole as induction therapy/total (%)	24/92 (26.1)	11/24 (45.8)	12/29 (41.4)	0.086
No. with mycological failure at day 15/total (%)[c]	33/77 (42.9)	7/22 (31.8)	4/24 (16.7)	0.059
No. with CSF mycological failure at day 15/total (%)[d]	31/66 (47.0)	6/17 (35.3)	3/23 (13.0)	0.013
No. who died within 90 days after diagnosis/total (%)	21/82 (25.6)	7/22 (31.8)	7/25 (28.0)	0.812

[a] Total number of patients evaluated or for whom the information was available.
[b] AMB, amphotericin B; 5FC, flucytosine.
[c] Persistence of viable cryptococci in cultured samples.
[d] Persistence of viable cryptococci in cerebrospinal fluid samples.

verged from the serotype D lineage about 18.5 million years ago (8), incomplete intervarietal sexual cycles occurred at different times (0 to 3.2 million years ago), leading to introgression of *C. neoformans* var. *grubii* into *C. neoformans* var. *neoformans* and to the origins of AD hybrids (9, 44). AD hybrids seem to be locked in the diploid state because of genomic differences preventing progression through meiosis. Furthermore, basidiospores generated from intervarietal matings have a low viability and low propensity to germinate (39). On the basis of reproductive isolation already described and phylogenetic distance confirmed by our results, both varieties could be classified into different species (6, 47). In their new study, Hagen et al. (3) propose recognition of both varieties of *C. neoformans* as new species (*C. neoformans* for serotype A and the newly proposed *Cryptococcus deneoformans* for serotype D). This is based on the phylogenetic analysis of 11 genetic loci and on biochemical, physiological, and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry profiles. Our results are thus in agreement with this new taxonomic classification (3).

A fascinating discovery of the systematic screening of original isolates was the high proportion of mixed infections (21.5%), confirming and extending with 181 patients the results obtained with 49 (36). As reported, these mixed infection were the results of either coinoculation or *in vivo* evolution (transitions in ploidy or microevolution). However, we did not uncover hybridization that could explain the simultaneous presence of A, D, and AD hybrids in the same patient. It was important to expand the sample size to the entire population of the CryptoA/D study to ensure that our original finding was not due to some selection bias. This study also allowed us to determine by univariate analysis that patients infected with AD hybrids differed from those infected with serotype A or D (Table 4), especially in terms of type of infection (less frequent dissemination based on culture and serum antigen titers and less frequent lung involvement) and in terms of response to induction treatment (more CSF sterilization). Multivariate analysis showed that parameters independently associated with a lack of CSF sterilization at week 2 after initiation of treatment were a high serum antigen titer (>512), the lack of flucytosine during induction therapy (as reported before [5]), and the occurrence of mixed infection, while infection with AD hybrids was more often associated with CSF sterilization. Nevertheless, differences in CSF sterilization do not prove differences in virulence in humans. We should keep in mind that the AD hybrids identified in patients diagnosed in France are different from those found in Africa, and indeed, Wiesner et al. found no survivors among the 8 Ugandan patients infected by AD hybrids (48), whereas survival was similar

TABLE 4 Independent parameters associated with mycological failure at week 2 for 123 patients

	Univariate analysis			Multivariate analysis[a]	
Parameter	% with mycological failure (no./total)	% with mycological cure (no./total)	*P* value	OR (95% CI)	*P*
Male sex	92.2 (47/51)	77.8 (56/72)	0.046		
Disseminated infection	74.5 (38/51)	59.7 (43/72)	0.122		
Mixed infections	21.6 (11/51)	8.3 (6/72)	0.061	5.6 (1.4–22.6)	0.015
Lack of 5FC	54.9 (28/51)	34.7 (25/72)	0.029	5.8 (2.0–17.2)	0.001
Infection by AD hybrid	7.8 (4/51)	31.9 (23/72)	0.002	0.1 (0.02–0.46)	0.003
Serum antigen titer > 512	63.8 (30/47)	40.9 (27/66)	0.022	5.0 (1.7–14.4)	0.003

[a] OR, odds ratio; CI, confidence interval.

TABLE 5 New primers specific of the serotype and mating type used for amplification of *STE20*

Serotype/mating type	Primer	5′–3′ sequence	Amplicon size (bp)	Reference or source
Dα	JOHE21312	AGCACCAGCCTATGGAGTCCGTCT	668	60
	JOHE21322	TCAAAAGGTTGTCAGACTTGATGT		
D**a**	JOHE21313	CACATCTCAGATGCCATTTTACCA	526	60
	JOHE21323	TCATCACAATGATCTCATTCACAA		
A**a**	JOHE21314	CTAACTCTACTACACCTCACGGCA	457	11
	JOHE21324	CGCACTGCAAAATAGATAAGTCTG		
Aα	JOHE21691	AGCATCAGCTTTTGGAGTCTAC	413	Wenjun Li (Duke University)
	JOHE21692	AGCATCAGCTTTTGGAGTCTAC		

whatever the infecting serotype for the patients enrolled in the CryptoA/D study. Virulence of AD hybrids has been assessed in experimental infections using relatively few strains (12, 32, 34). The mating type, the inoculation route, and the presence of the Aα allele have been associated with enhanced virulence. A recent study, however, demonstrated a significantly lower virulence only for the **a**AD**a** hybrids, providing evidence for negative epistatic interactions between A**a** and D**a** alleles (49). This was not assessed here because of the small number of patients involved for some of the allele combinations. A higher DNA content does not explain differences in virulence for AD hybrids because genetically related haploid and diploid isolates exhibit similar virulence (36, 49). However, diploid cells are thought to be larger than haploid cells, and cell size can have an impact on phagocytosis (described for titan cells [50]). Chromosomal composition (proportion of serotype A or D genomes) in AD hybrids varies with partial and/or complete chromosome deletion and/or duplication (11, 12, 35), with an impact on capsule structure, as shown here by the difference in serotype assignment achieved with a monoclonal antibody (51).

Our study again highlights the importance of combining clinical and molecular data on original isolates for the study of cryptococcosis and most probably other fungal infections. This approach could be essential to identify putative species- and clade-specific risk factors and possible associations between particular strain types and host microenvironment and could lead to optimized recommendations for the management of the patients.

MATERIALS AND METHODS

Ethics statement. The CryptoA/D study is a prospective multicenter observational study that enrolled 230 patients with cryptococcosis in France between 1997 and 2001 (5). The CryptoA/D study was approved by the ethical committee and reported to the French Ministry of Health (registration number DGS970089). Patients enrolled in the CryptoA/D study gave their written informed consent for a systematic workup. Data were analyzed anonymously. Clinical data and isolates were collected. The cryptococcosis surveillance program is approved by the Institut Pasteur Internal Review Board (2009–14/IRB).

***Cryptococcus neoformans* isolates.** Molecular characterization was performed for 400 isolates obtained from 380 original isolates recovered from 181 patients (CryptoA/D isolates) and for 53 serotype D isolates collected from 53 patients during the surveillance program implemented at the National Reference Center for Invasive Mycoses and antifungals (CNRMA) (52). Reference strains JEC21 (serotype D, MATα), JEC20 (serotype D, MAT**a**), H99 (serotype A, MATα), KN99**a** (serotype, A MAT**a**), and KN99α (serotype A, MATα) were used.

Molecular characterization. (i) Determination of ploidy. Cells were prepared for flow cytometry (53). Data were acquired from 30,000 cells using the FL2 channel of a BD FACScan flow cytometer (Becton, Dickinson Company, Franklin Lakes, NJ, USA). Analysis was performed using CellQuest software version 3.3 (BD Biosciences, San Jose, CA, USA) using the profile of H99α as a reference for haploidy.

(ii) Determination of mating type and serotype. Yeasts were grown for 24 h at 28°C on solid YNB medium. DNA was extracted using the High-Pure PCR template preparation kit (Roche Applied Science, Indianapolis, IN). PCR were performed on an iCycler thermocycler (Bio-Rad, Hercules, CA) using primers specific for the serotype and the mating type (SXI1α/SXI2**a** and STE20α/**a**) (12). Serotype-specific primers for the *PAK1* and *GPA1* genes were also used for all original cultures and selected single colonies (12). For some isolates, other STE20 primers were used (Table 5) because of a deletion in the STE20 sequences.

(iii) MLST. Multilocus sequence typing (MLST) was performed on all serotype A and D isolates using the published scheme (*CAP59*, *URA5*, *GPD1*, *SOD1*, *LAC1*, *IGS1*, *PLB1*) (19) with slightly different conditions for serotype D (36). Of note, *LAC1* and *URA5* loci are both localized on chromosomes 8 and 7 for serotypes A and D, respectively, whereas the other five loci are on different chromosomes. Sequences were edited with Chromas Pro version 1.41 (Technelysium Pty. Ltd., Helensvale, Queensland, Australia) and Mega version 5.1 (54). Concatenate sequences of the 7 MLST loci were aligned to construct a similarity matrix using BioNumerics version 6.6 (Applied Maths, NV). The MLST allelic sequences and sequence type (ST) are available online (http://mlst.mycologylab.org/defaultinfo.aspx?Page=CN).

Discriminatory power was determined by using Hunter coefficient (55). Frequency of each ST was calculated by using START software version 2 and LIAN3.5 software (56). Gene diversity (H) was calculated as $[n/(n-1)](1-\Sigma p^2_i)$, where n is the number of samples and pi is the relative frequency of the ith allele. Average number of alleles per locus was determined as $(1/k)\Sigma n_i$, where k is the total number of loci and n_i is the number of alleles for one locus.

Genotypes for AD hybrid were determined for 5 loci (*PLB1*, *GPD1*, *SOD1*, *IGS1*, and *LAC1*) by using serotype-specific primers (11, 36). We were not able to design serotype-specific primers for the *CAP59* and *URA5* loci due to high sequence similarity between A and D alleles.

Phylogenetic analysis. For the population genetics analysis, only one isolate per patient was used except when different genotypes were observed in a given patient. A total of 97 isolates from 97 patients was studied for serotype D and 121 isolates from 118 patients for serotype A. Minimum-spanning trees were constructed with MLST alleles using BioNumerics. Clusters were defined as partitions of nodes having a maximum distance of two loci. Isolates located in the same cluster were considered related. Maximum-parsimony trees were constructed using BioNumerics, with concatenate sequences of the 7 MLST loci aligned using ClustalW.

An allele compatibility test was performed from ST global isolates by generation of 21 graphs. This test calculates the proportion of loci that show phylogenetic compatibility when compared in pairwise combinations. In the simplest case of phylogenetic compatibility, for two loci with two alleles each, if all four possible genotypes are found in the population, these two loci are called phylogenetically incompatible. An hourglass shape thus indicates the presence of all four possible pairs of alleles and serves as evidence for recombination (57).

The index of congruence (I_{cong}) was calculated using online calculation (58) (http://mobyle.pasteur.fr/cgi-bin/portal.py#welcome) for testing topological similarity between haplotypes trees by comparing pairwise combination (here, 21 combinations for 7 MLST loci) and calculating the *P* value for each combination. Congruence between trees can suggest coevolution of genes, i.e., no recombination events.

The index of association (I_A) and linkage disequilibrium were determined by using START software version 2 and LIAN3.5 software (56). I_A is the ratio of the observed variance in the association of alleles among loci to the corresponding expected variance based on random associations. Significant associations among alleles at different loci are inconsistent with random recombination but consistent with clonality.

DnaSP software version 5.10 was used to calculate recombination parameters, with *S* representing the number of segregating sites, *R* the number of recombination events, and *Rm* the number of recombination events that can be parsimoniously inferred from the sequences (59).

Statistical analysis. Correlations between clinical and molecular data were analyzed for the CryptoA/D patients (Stata 10.0; Stata Corporation, College Station, TX). Comparisons between groups were done using chi square or Fisher exact tests for categorical variables, and Student's *t* test or one-way analysis of variance for continuous variables. For the multivariate analysis, logistic regression was used to determine factors associated with mycological failure (lack of CSF sterilization) at week 2. Odds ratios (OR) and 95% confidence intervals (CI) were determined by means of logistic regression analysis. Variables that were clinically relevant with *P* values of 0.25 were entered simultaneously into the initial model. Variables were removed following a backward-stepwise selection procedure, leaving only variables with *P* values of 0.05 in the final model. We estimated overall survival (cumulative survival probabilities and their 95% CIs) by the Kaplan-Meier method, and comparison of survival between groups was performed by log rank tests.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00311-15/-/DCSupplemental.

Figure S1, PDF file, 0.05 MB.
Table S1, DOCX file, 0.02 MB.
Table S2, DOCX file, 0.02 MB.
Table S3, DOCX file, 0.02 MB.

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The French Cryptococcosis Study Group is composed of the following individuals, who actively participated in the data collection: J. Achard, D. Chabasse, J. P. Bouchara (Angers); S. Bland, J. P. Bru (Annecy); M. Pulik, F. Leturdu (Argenteuil); X. Lepeu, H. Lefrand, C. Jensen (Avignon); M. Ferrand, M. Larrouy (Bayonne); M. Bentata, C. Bouges-Michel, J. Camuset, L. Guillevin, B. Jarrousse, M. Robineau, J. J. Rousset (Bobigny); B. Couprie, M. Dupon, H. Dutronc, J. Y. Lacut, J. L. Pellegrin, J. M. Ragnaud, J. F. Viallard, F. X. Weill (Bordeaux); M. E. Bougnoux, X. Montreal, S. Morelon, E. Rouveix, (Boulogne); P. Granier, H. de Montclos (Bourg-en-Bresse); A. Desveaux, M. Gavignet, A. S. Labussiere, M. Mornet (Bourges); L. De Saint-Martin, E. Moalic (Brest); J. Roucoules, J. F. Loriferne, G. Otterbein (Bry-sur-Marne); J. F. Desson, M. Leporrier, C. Duhamel, J. Bonhomme (Caen); J. M. Korach (Chalons en Champagne); B. Salles, C. Sire (Chalon/Saône); V. Herve, B. Souleau, (Clamart); J. Beytout, M. Cambon, D. Pons, P. Poirier (Clermont-Ferrand); Y. Boussougant, D. Dreyfuss, X. Michon, P. Vinceneux (Colombes); G. Belkacem-Belkaki, S. Bretagne, M. Chousterman, P. Grimberg, A. S. Lascaux, A. Schaeffer, A. Sobel (Créteil); J. L. Bacri, G. Berthelot (Dieppe); A. Bonnin, M. Duong, J. Lopez, H. Portier, F. Dalle (Dijon); M. Gauthier, O. Salmon (Évry); J. Bizet (Fresnes); J. L. Gaillard, C. Perronne (Garches); M. A. Desailly, H. Maisonneuve (La Roche-sur-Yon); J. P. Bedos, J. Doll, O. Eloy, J. C. Ghnassia, S. Roussin-Bretagne (Le Chesnay); C. Brocard, P. Guiffault, A. Layet, A. Morel (Le Havre); F. Botterel, P. Bouree, J. F. Delfraissy, Y. Kertaimont, A. Angoulvant, P. Lozeron, K. Rérat, G. Saïd (Le Kremlin-Bicêtre); X. Cricks (Les Mureaux); M. L. Darde, A. Jaccard, B. Bouteille, D. Azjenberg (Limoges); D. Bouhour, E. Dannaoui, X. Mallet, D. Peyramond, M. A. Piens, C. Trepo (Lyon); L. Berardi, F. Tremolieres (Mantes-la-Jolie); Y. Berland, A. Blancard, L. Collet, J. Delmont, H. Gallais, X. Gamby, A. Michel Nguyen, J. Moreau, N. Petit, J. M. Sainty, J. Sampol-Roubicek (Marseille); M. Bietrix, M. Nezri (Martigues); A. Fiacre, S. Levy (Meaux); C. Chandesris, X. La Torre (Montargis); P. Andres, E. Billaud, F. Boiffin, M. Hamidou, O. Morin, B. Planchon, P. Poirier, F. Raffi, D. Villers, F. Morio (Nantes); P. Clevenbergh, F. De Salvador, P. Dellamonica, X. Durand, M. Gari-Toussaint (Nice); A. Romaru, M. Texereau (Niort); L. Bret, T. Prazuk, D. Poisson (Orléans); X. Bernard, Y. Pacheco (Pierre-Bénite); B. Becq-Giraudon, C. Kauffmann-Lacroix, J. C. Meurice, T. Pasdeloup (Poitiers); J. Deville, D. Toubas (Reims); C. Arvieux, F. Cartier, S. Chevrier, B. Degeilh, T. Frouget, C. Guiguen, P. Le Cavorzin, J. P. Gangneux, C. Michelet, V. Noyon (Rennes); P. Abboud, P. Brasseur, J. Leroy, J. F. Muir, L. Favennec (Rouen); P. Babinet, F. Fraisse, N. Godineau, S. Hamane, P. Margent, D. Mechali, M. Thuong (Saint-Denis); C. Soler, (Saint-Mandé); B. Hery, J. Y. Leberre (Saint-Nazaire); A. Gregory, O. Prevot (Saint-Julien-en-Genevois); D. Christmann, J. Waller, V. Letscher-Bru (Strasbourg); O. Bletry, P. Cahen, D. Zucman (Suresnes); B. Fortier (Toul); X. Aubert, S. Chadapaud, X. Delbeck, A. Lafeuillade, X. Raoult (Toulon); E. Bonnet, S. Cassaing, A. Gadroy, M. D. Linas, J. F. Magnaval, P. Massip, L. Prudhomme, L. Sailler, (Toulouse); V. Baclet, C. Coignard, Y. Mouton, I. Ravaux (Tourcoing); C. Eloy, A. Fur, L. Rezzouk (Troyes); C. Fontier, E. Mazards (Valenciennes); M. F. Biava, P. Canton, L. Kures, C. Rabaud, M. Machouart (Vandœuvre-lès-Nancy); D. Vittecocq, E. Chachaty (Villejuif); S. Dellion, O. Patey (Villeneuve-St.-Georges); J. P. Bedos, O. Benveniste, C. Bouchard, S. Belaich, C. Carbon, C. Chochillon, J. P. Coulaud, V. Descamps, X. Duval, C. Leport, F. Lheriteau, P. Longuet, H. Mouas, F. Vachon, J. L. Vilde, P. Yeni (Hôpital Bichat-Claude Bernard, Paris); V. Lavarde, C. Piketty (Hôpital Broussais, Paris); B. Christoforov, J. Dupouy-Camet, J. P. Luton, A. Paugam, M. T. Baixench (Hôpital Cochin, Paris); N. Desplaces, G. Raguin (Hôpital de La Croix-Saint-Simon, Paris); P. Chevalier, M. Kazatchkine, V. Lavarde, A. Meyrier (Hôpital Européen Georges Pompidou, Paris); A. Bernadou, M. Cornet, J. P. Marie, S. Oudart (Hôpital de l'Hôtel-Dieu, Paris); M. Gayraud, Y. Pean (Institut Mutualiste Montsouris, Paris); C. Aznar, B. Dupont, H. Poncelet, Amaury de Gouvelho, Clarisse Loyer, Odile Launay (Hôpital de l'Institut Pasteur); P. Berche, B. Dupont, V. Mathé, (Hôpital Necker-Enfants Malades, Paris); L. Baril, P. Bossi, F. Bricaire, J. Carrière, A. Datry, S. Herson, M. Jouan, M. Levy-Soussan, C. Mouquet, B. Orcel, M. M. Thiebaut (Hôpital Pitié-Salpétrière, Paris); J. Frottier, J. B. Guiard-Schmidt, B. Lebeau, J. L. Meynard, M. C. Meyohas, J. L. Poirot, P. Roux, X. Urban (Hôpital Saint-Antoine, Paris); F. Daniel, J. Gilquin, J. F. Timsit (Hôpital Saint-Joseph, Paris); C. Lacroix, JC Brouet, J. M. Decazes, F. Derouin, B. 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