

Genetic Structure of Typical and Atypical Populations of *Candida albicans* from Africa

Anja Forche,^{*,1} Gabriele Schönian,[†] Yvonne Gräser,[†] Rytas Vilgalys,[‡] and Thomas G. Mitchell^{*}

^{*}Department of Microbiology, Duke University Medical Center, Durham, North Carolina 27710; [†]Institute for Microbiology and Hygiene, Humboldt-University, Charité Hospital, Dorotheenstrasse 96, 10119 Berlin, Germany; and [‡]Department of Botany, Duke University, Durham, North Carolina 27706

Accepted for publication July 9, 1999

Forche, A., Schönian, G., Gräser, Y., Vilgalys, R., and Mitchell, T. G. Genetic Structure of Typical and Atypical Populations of *Candida albicans* from Africa. *Fungal Genetics and Biology* 28, 107–125. Atypical isolates of the pathogenic yeast *Candida albicans* have been reported with increasing frequency. To investigate the origin of a set of atypical isolates and their relationship to typical isolates, we employed a combination of molecular phylogenetic and population genetic analyses using rDNA sequencing, PCR fingerprinting, and analysis of co-dominant DNA nucleotide polymorphisms to characterize the population structure of one typical and two atypical populations of *C. albicans* from Angola and Madagascar. The extent of clonality and recombination was assessed in each population. The analyses revealed that the structure of all three populations of *C. albicans* was predominantly clonal but, as in previous studies, there was also evidence for recombination. Allele frequencies differed significantly between the typical and the atypical populations, suggesting very low levels of gene flow between them. However, allele frequencies were quite similar in the two atypical *C. albicans* populations, suggesting that they are closely related. Phylogenetic analysis of partial sequences encoding the nuclear 26S rDNA demonstrated that all three populations belong

to a single monophyletic group, which includes the type strain of *C. albicans*.

© 1999 Academic Press

Index Descriptors: sscp; phylogenetic analysis; genetic variation; cryptic species; atypical strains; population structure; clonality; recombination; index of association.

A member of the normal mammalian flora, *Candida albicans* is the predominant pathogenic fungus of humans, in whom it may cause mucosal, mucocutaneous, and systemic infections. The spectrum of candidiasis includes superficial infections in immunocompetent individuals, as well as systemic, life-threatening infections in immunocompromised patients (Odds *et al.*, 1992). In culture, *C. albicans* is distinguished from other yeast species by the rapid formation of germ tubes in serum at 37°C, the production of chlamydospores on nutritionally deficient media, and the pattern of assimilation of a battery of small organic molecules as substrates (Kurtzman and Fell, 1998).

Since the advent of accurate methods to identify individual strains, atypical isolates of *C. albicans* have been reported with increasing frequency (Díaz-Guerra *et al.*, 1997; Pla *et al.*, 1996; Redkar *et al.*, 1996; Thanos *et al.*, 1996). Atypical strains differ from most typical *C. albicans* in their expression of one or more phenotypes, and they are often difficult to identify by routine methods. Atypical isolates may represent variants of *C. albicans* and can even sometimes represent new species of *Candida*. In one series of studies, similar atypical strains were isolated from the

¹ To whom correspondence should be addressed. Fax: (919) 681-8911. E-mail: forche@abacus.mc.duke.edu.

oral cavities of patients infected with the human immunodeficiency virus (HIV) (Sullivan *et al.*, 1993; Boerlin *et al.*, 1995; McCoullough *et al.*, 1995; Pujol *et al.*, 1997). Similar to *C. albicans*, these isolates produced germ tubes and chlamyospores. However, testing with the ID 32 C Identification System (Biomeri ux) revealed assimilation profiles that were atypical for *C. albicans*. Extensive genetic characterization, based on multilocus enzyme electrophoresis, DNA fingerprinting, and karyotyping, demonstrated the uniqueness of this atypical group and led to its designation as a new species, *Candida dubliniensis* (Sullivan *et al.*, 1995; Sullivan and Coleman, 1998). Additional isolates were subsequently assigned to this new species (Boerlin *et al.*, 1995; McCoullough *et al.*, 1995; Pujol *et al.*, 1997). In another study of atypical *C. albicans* strains, vaginal yeast isolates from HIV-negative women in Africa produced germ tubes but failed to develop chlamyospores, grew very slowly at 37°C, and were unable to assimilate glucosamine and *N*-acetylglucosamine (Tietz *et al.*, 1995). PCR fingerprint patterns confirmed that these atypical African strains were genetically distinct from reference strains of *C. albicans*; they also differed from closely related species, including *Candida sake*, *Candida stellatoidea*, and *Candida tropicalis* (Tietz *et al.*, 1995).

Recently, studies have focused on the genetic structure of populations in *C. albicans* as an approach to understanding epidemiology and pathogenicity. Although a predominantly clonal mode of reproduction has been reported for most populations of *C. albicans*, evidence for recombination has also been recently documented (Pujol *et al.*, 1993; Boerlin *et al.*, 1996; Gr aser *et al.*, 1996; Xu *et al.*, 1999). Atypical populations of *C. albicans* have not been subjected to similar population genetic analyses. The aim of this study was to compare the population structure of several African population samples of *C. albicans*, which included both typical and atypical strains. Phylogenetic analysis of ribosomal DNA (rDNA) confirmed that the African populations sampled are variants of *C. albicans*.

MATERIALS AND METHODS

Candida Isolates and DNA Extraction

Most strains used in this study were described previously (Tietz *et al.*, 1995). Briefly, 45 typical and 11 atypical strains of *C. albicans* (including 3 double isolates) were isolated from women in the Gynecology Clinic at the Medical University of Luanda, Angola. A second group of

14 atypical isolates of *C. albicans* (1 isolate per patient) was obtained from women attending several sexually transmitted diseases outpatient clinics in Madagascar. All strains were tested for their ability to produce germ tubes and chlamyospores, and their biochemical patterns were characterized using ATB ID 32 C strips (BioMeri ux SA, March-l'Ettoile, France). All the atypical strains grew more slowly at 37°C and failed to produce chlamyospores (Tietz *et al.*, 1995). All strains were grown on Sabouraud glucose agar for 1 to 7 (atypical strains) days, and DNA was isolated with CTAB buffer (Gardes and Bruns, 1993) and stored at -20°C until use.

Co-Dominant DNA Markers

Anonymous co-dominant DNA markers were developed by screening randomly amplified DNA fragments for sequence polymorphisms as previously described (Burt *et al.*, 1994; Gr aser *et al.*, 1996). Briefly, pairs of 10-mer oligonucleotides (Operon Technologies; Kits B, C, and F) were used to screen a panel of reference strains for amplification products. Monomorphic amplicons (present in all reference strains) were sequenced either by cloning/sequencing or by direct sequencing in order to design locus-specific primers that amplified DNA regions of 175 to 1000 bp in size (Table 1). Each primer pair was tested, and conditions were optimized to amplify the corresponding PCR fragment (Cobb and Clarkson, 1994). Conventional PCRs were performed in a total volume of 25 µl with 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 100 µM each of dATP, dCTP, dGTP, and dTTP (Pharmacia), 1.0 unit AmpliTaq DNA polymerase (Perkin-Elmer), 10 or 20 ng DNA template, and 7.5 or 15 mM each primer. The samples were overlaid with sterile light mineral oil (Sigma, St. Louis, MO), and PCR was carried out for 34 cycles as follows: Initial denaturation for 3 min at 95°C, denaturation step for 1 min at 94°C, 30 s at specific primer annealing temperature, extension step for 1 min at 72°C, and final extension for 5 min at 72°C. Sequencing was performed by dsDNA Cycle Sequencing (Gibco BRL, USA) using γ-33P dATP as label.

Polymorphisms in PCR fragments less than 700 bp in length were screened by surveying single-strand conformation polymorphisms (SSCP; Hayashi, 1991). Ten to 15 µl PCR product were mixed with 2 ml 1% sodium dodecylsulfate (SDS), 10 mM EDTA, pH 8.0, and 2 ml stop solution (Gibco BRL), denatured for 15 min at 98°C, and immediately placed on ice. Samples were run under non-denaturing conditions on sequencing gels (0.5× TBE, 6% MDE hydrolink gel solution; FMC Bioproducts) at 10 W for

12–16 h (fragment size <500 bp) or 30 W for 14–17 h (fragment size 1500–700 bp) at 4°C. Gels were silver stained and dried on a gel dryer. To confirm polymorphisms, DNA fragments from 2–4 strains representing each detectable SSCP pattern were sequenced using standard dye-terminator sequencing kits (Applied Biosystems Inc., Foster City, CA) with an automated sequencer (ABI Model 377 or 373; Perkin–Elmer) following manufacturer's instructions. Sequence data were assembled and analyzed using Sequencher 3.0 software (Gene Codes Corp.).

To detect polymorphisms in PCR fragments larger than 750 bp in size, fragments were first screened for restriction fragment length polymorphism (RFLP). Four-, five-, and six-base cutting restriction endonucleases were tested for their ability to detect polymorphisms in the PCR fragments amplified with the specific primers. Twenty microliters of PCR product were digested for 2 h at 37 or 65°C using buffer conditions recommended by the supplier (Promega). Restriction fragments were resolved by gel electrophoresis on 3% Nusieve agarose gels (FMC Bio-Products) with 0.5× TBE buffer and visualized by staining with ethidium bromide.

Hybridization of Markers to Chromosomes

To determine linkage among DNA markers, chromosomes of *C. albicans* were separated by pulsed-field gel electrophoresis (PFGE) and each marker was allowed to hybridize to a blot of the resulting karyogram. Plugs for PFGE were prepared as described previously (Wickes *et al.*, 1991). To adequately separate the chromosomes, two different sets of electrophoretic conditions were employed (R. Swoboda, personal communication). For smaller chromosomes, melted samples were gently loaded into the wells of an agarose gel (Seakem Gold, FMC, 0.8%; 0.5× TBE), and the wells were sealed with low-melting agarose (Pharmacia). Electrophoresis was performed in a CHEF-DR III system (Bio-Rad) at 180 V (5.4 V/cm) for 12 h with a pulse time of 120 s, followed by a pulse time of 180 s for 12 h. Larger chromosomes were separated by running the melted samples in 1.0% agarose (SeaKem Gold, FMC) in 0.5× TBE at 150 V (4.5 V/cm) for 24 h with a pulse time of 120 s, followed by 36 h with a pulse time of 240 s. A standard yeast chromosome size marker (Boehringer Mannheim, Germany) was used. Both runs were performed with a pulse angle of 120° at 14°C in 2 L 0.5× TBE buffer. Gels were stained in ethidium bromide (1 mg/ml) for 15 min, destained for 15 min in distilled water, and photographed. Chromosomes were transferred from the gel to nylon

membranes (GeneScreen) by capillary transfer under alkaline conditions, and Southern hybridization was performed as described elsewhere (Sambrook *et al.*, 1989). Radiolabeled DNA fragment probes were prepared by random labeling using α -³²P dCTP (Amersham, USA). Hybridization was detected by autoradiography, and hybridized fragments were assigned manually to appropriate chromosomes using the established chromosomal nomenclature for *C. albicans* (Wickes *et al.*, 1991).

PCR Fingerprinting

For PCR fingerprinting, primer T3B (5'-AGG TCG GGG GTT CGA ATC C-3' [McClelland *et al.*, 1992]) was used as a single primer for arbitrary amplification of polymorphic DNA (Schönian *et al.*, 1996; Thanos *et al.*, 1996). PCRs were performed in 50- μ l volumes containing 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 3 mM magnesium acetate, 200 μ M each of dATP, dCTP, dGTP, and dTTP (Pharmacia), 1.5 units AmpliTaq DNA polymerase (Perkin–Elmer), 50 ng genomic DNA, and a final primer concentration of 25 mM. Samples were overlaid with sterile light mineral oil (Sigma) and amplified for 32 cycles as follows: initial denaturing for 5 min at 95°C; denaturation, for 15 s at 95°C; annealing, 30 s at 52°C; extension, 1 min 20 s at 72°C; and a final extension step for 6 min at 72°C. PCR products were concentrated to a final loading volume of 20 μ l (Speed Vac; Savant, Hicksville, NY), electrophoresed in agarose (1.2%, 0.5× TBE) for 5 h at 3 V/cm in 0.5× TBE buffer, stained with ethidium bromide, and photographed. DNA fragments were sized and compared using scanner hardware and software (RFLPscan, version 2.01; Scanalytics CSP Inc., Billerica, MA).

rDNA Sequencing and Phylogenetic Analysis

Genomic DNA samples representing atypical and typical populations (one strain each) were selected for rDNA sequencing. A portion of the nuclear-encoded large subunit 28S rDNA gene was amplified using primers NL1–NL4 (O'Donnell, 1993), sequenced using fluorescent dye terminator chemistry, and run on an ABI 373 or 377 Automated Sequencer (Perkin–Elmer Applied Biosystems, Foster City, CA) using the manufacturer's protocols. Both strands from each PCR product were sequenced. Sequence contigs were assembled and edited using Sequencher 3.0 software (Gene Codes Corp., Ann Arbor,

MI). Sequences were aligned with other 26S rDNA sequences that were obtained from authentic and type strains of diverse *Candida* species (Kurtzman and Robnett, 1997).

Phylogenetic analyses were carried out using the PAUP* computer package (Swofford, 1999). Heuristic searches were conducted using the maximum parsimony method with the following settings: random addition sequence (100 replicates), tree-bisection-reconnection (TBR) branch-swapping, MULPARS option in effect, steepest descent option not in effect, MAXTREES setting unlimited, and branches having minimum length zero were allowed to collapse to yield polytomies. Branch robustness was evaluated using 300 bootstrap (Felsenstein, 1985) random-addition replicates with other settings as described above.

The sequences used from this study were deposited with GenBank under Accession Nos. AF156536, AF156537, and AF156538.

RESULTS

Three Populations of C. albicans in Africa

The morphology and assimilation patterns of 56 strains from Angola and 14 from Madagascar were examined. Forty-five Angolan isolates were typical strains of *C. albicans*. The other 11 isolates from Angola were described as atypical strains of *C. albicans*: they grow slowly, fail to produce chlamydospores, and do not assimilate glucosamine or *N*-acetylglucosamine (Tietz *et al.*, 1995). All 14 isolates from Madagascar were atypical strains of *C. albicans*, sharing the same phenotypic profile as the 11 atypical strains from Angola. In the subsequent analyses, these three populations are referred to as typical Angola, atypical Angola, and atypical Madagascar populations.

Polymorphic Markers in C. albicans

To develop DNA markers, genomic DNA samples from several typical strains of *C. albicans* were amplified with pairs of commercial arbitrary PCR primers to identify monomorphic bands, which were subsequently screened for polymorphisms. Fourteen anonymous DNA fragments were consistently amplified from this subset of polymorphisms. Fourteen anonymous DNA fragments were consistently amplified from this subset of typical strains and further tested for their potential as DNA markers. PCR primer pairs were designed to amplify each fragment, and

10 of these primer pairs reliably amplified a single PCR fragment from all strains. These amplicons from the three populations revealed SSCPs in 8 of the 10 fragments (Fig. 1, Tables 1 and 2). In addition, 5 of 6 previously described PCR fragments (Gräser *et al.*, 1996) also showed polymorphisms among typical strains of *C. albicans* from Africa. Subsequent analyses of the African populations were therefore based on these 13 polymorphic DNA fragments (Table 1).

The three population samples of *C. albicans* differed in the presence of polymorphisms for the 13 DNA fragments. SSCPs were detected in all 13 fragments of the typical Angola population. In contrast, for both atypical populations, SSCPs were found in only 10 of the 13 fragments; 2 fragments showed no polymorphisms, while a third fragment could not be amplified from any atypical strain.

DNA products representing unique SSCP for 12 polymorphic DNA fragments were sequenced, and all differed by one or more point mutations. The 13th amplicon was too large for direct sequencing and was therefore analyzed separately by RFLP as described below. Isolates of *C. albicans* are diploid, and both SSCP gels and sequence data detected heterozygous individuals that possessed both alleles for the same locus (Figs. 1 and 2).

For typical Angola strains, SSCPs revealed a total of 51 polymorphic nucleotide sites distributed among 12 polymorphic DNA fragments. For the atypical populations from Angola and Madagascar, 28 and 30 polymorphic sites, respectively, were detected among only 9 polymorphic fragments (Table 2). The largest PCR fragment, B13B19,

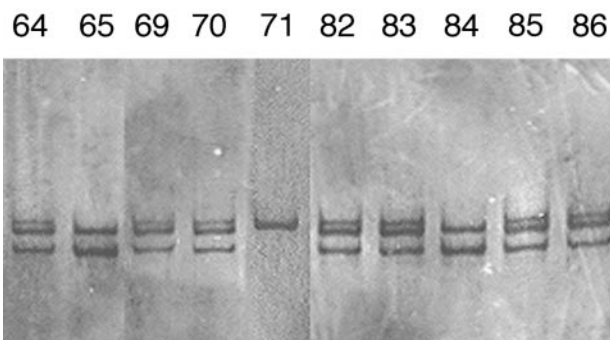


FIG. 1. DNA polymorphisms in *Candida albicans* detected by single-strand conformation polymorphisms (SSCP). SSCP patterns are shown for marker B15B20 from several typical *C. albicans* strains from Angola; strains 65 and 84 are homozygous for one allele; strain 71 is homozygous for the other allele; and strains 64, 69, 70, 82, 83, 85, and 86 represent the heterozygous genotype.

TABLE 1
Primer Pairs Used for Detection of Co-Dominant Markers in *C. albicans*

Primer pair	Fragment size (bp)	Primer sequence	GenBank Accession no.	Chromosome	Annealing (in °C) in PCR
C2F10 ^{1,2,4}	264	f: 5' TTGCTACTACAAATAGTCG 3' r: 5' GCTTAACATTTACCTGCTTC 3'	Y07666	1	50
C12F10 ^{1,2,4}	288	f: 5' ACGTAATAAGGGTATTGTTG 3' r: 5' GCAATTTGTCACCTCATCCAG 3'	Y07664	1	50
C15F2 ^{1,2,4}	332	f: 5' TAGTTAGTTTGCCTTGTTCC 3' r: 5' GAGAGCTACGTGAGCTCGTG 3'	Y07668	n.d.	58
C2F7 ^{1,2,4}	285	f: 5' GTTTGATCTGGAACGATCTC 3' r: 5' AGAAACCAACCAGCGTGTG 3'	Y07669	6	50
C2F17 ^{1,2,4}	294	f: 5' ACTAATCTATCGAGAGAACG 3' r: 5' GTCAGATGGTACGGACAAG 3'	Y07665	3	50
B5B7 ¹	742	f: 5' CAGAACACAGACTAT 3' r: 5' ATGTATGAGCTGAAGTGG 3'	AF064537	n.d.	53
B15B20 ^{1,2}	282	f: 5' GGAATTGGAAGAAGTCA 3' r: 5' GCATATAGTCTACCCAGTG 3'	AF064530	n.d.	56
B7B20 ^{1,2}	217	f: 5' TTATCGCCAAAACCGTC 3' r: 5' CATCCAACACACCAAACC 3'	AF064536	n.d.	54
B4B16 ¹	175	f: 5' CTCTGACTCTTCGGTATCGT 3' r: 5' TTTCATATTTATGTCGTGG 3'	AF064529	4	56
B13B19 ^{1,2}	1000	f: 5' TGCCCAAATGTCTTCCGAT 3' r: 5' GAGGTAAGGGTCAAGTCCA 3'	AF064532	n.d.	58
B8B12 ^{1,2}	223	f: 5' CTCCATCTACCCATTTC 3' r: 5' GGTCAGAAGGGTATGGTA 3'	AF064531	n.d.	50
B8B16 ¹	189	f: 5' CCAGTGTAAGGGTATTG 3' r: 5' CCCGGACAAATATGGAAT 3'	AF064533	n.d.	52
B5B20 ^{1,2}	757	f: 5' CTCTCTTTGTCGTCTTTGGTC 3' r: 5' TGTCTGGATTGGTATG 3'	AF064534	n.d.	56
C13F10 ^{3,4}	340	f: 5' TGCTATCTTCGTACCGTATC 3' r: 5' ATCTCGTCTCTACATCATC 3'	Y07667	n.d.	50
B4B17 ³	246	f: 5' TGAGCCACAAGAGCAAG 3' r: 5' GGAACGAGCAGCAAAC 3'	AF064535	R, 5	51
B8B19 ³	233	f: 5' GGACCTAAAGTGTGTGCT 3' r: 5' TCAAAGGACTCACGCAATG 3'	AF064538	R, 7	53

Note. Marker names consist of both primer designations, fragment size in bp, primer sequences for forward (f) and reverse primer (5' to 3' end), GenBank Accession No., the chromosomal location, and the specific annealing temperature for PCRs; n.d., not determined.

¹ Polymorphic markers for the typical *C. albicans* population.

² Polymorphic markers for both atypical *C. albicans* populations.

³ Markers are not polymorphic.

⁴ Markers included from a previous study (Gräser *et al.*, 1996).

was analyzed by RFLP, and five restriction enzymes yielded polymorphisms among typical Angola strains. In atypical populations, digestion with three restriction enzymes detected 1 polymorphic site for each enzyme (Table 2).

From the three populations of *C. albicans*, a total of 76 scoreable polymorphisms were obtained (see Appendix 1). Unique polymorphisms (alleles) were observed within all three populations. Of the 76 polymorphic sites, 42 were present only in the typical Angola strains, while 1 and 3 unique alleles were observed for the atypical isolates from

Angola and Madagascar, respectively. In addition, both atypical populations shared 17 alleles that were absent from the typical Angola isolates.

Sequence diversity. Sequence diversity was estimated as the proportion of polymorphic nucleotides in each population. For the typical Angola population, 56 of 5047 bp were variable, yielding a diversity estimate of 1.1%. Of 3941 bp that were sequenced in both atypical populations, 31 polymorphisms were detected from Angola and 33 from Madagascar, yielding sequence diversity estimates of 0.79 and 0.84%, respectively.

TABLE 2
Genotypic Frequencies of Polymorphic Nucleotides from Three *C. albicans* Populations

Locus	Primer pair	Fragment size	Position	Genotypic frequencies				
				Typical population from Angola	Atypical population from Angola	Atypical population from Madagascar		
1	C12F10	288	168	GG(45); GT(0); TT(0)	GG(9); GT(2); TT(0)	GG(14); GT(0); TT(0)		
2			218	GG(40); GT(5); TT(0)	GG(10); GT(0); TT(1)	GG(13); GT(0); TT(1)		
3			246	CC(43); CT(2); TT(0)	CC(11); CT(0); TT(0)	CC(14); CT(0); TT(0)		
4	C2F10	264	150	AA(9); AG(3); GG(33)	AA(10); AG(1); GG(0)	AA(13); AG(1); GG(0)		
5			225	CC(45); CT(0); TT(0)	CC(1); CT(0); TT(10)	CC(1); CT(0); TT(13)		
6	C2F7	285	50	GG(5); GT(0); TT(40)	GG(1); GT(0); TT(10)	GG(1); GT(0); TT(13)		
7			68	AA(1); AG(0); GG(44)	AA(0); AG(0); GG(11)	AA(0); AG(0); GG(14)		
8			83	AA(45); AT(0); TT(0)	AA(10); AT(0); TT(1)	AA(13); AT(0); TT(1)		
9			95	CC(6); CT(1); TT(38)	CC(1); CT(0); TT(10)	CC(1); CT(0); TT(13)		
10			107	AA(4); AG(0); GG(41)	AA(0); AG(0); GG(11)	AA(0); AG(0); GG(14)		
11			110	AA(41); AG(4); GG(0)	AA(11); AG(0); GG(0)	AA(14); AG(0); GG(0)		
12			119	AA(40); AG(4); GG(1)	AA(10); AG(0); GG(1)	AA(13); AG(0); GG(1)		
13			167	AA(0); AG(0); GG(45)	AA(1); AG(0); GG(10)	AA(1); AG(0); GG(13)		
14			248	CC(42); CT(2); TT(1)	CC(11); CT(0); TT(0)	CC(14); CT(0); TT(0)		
15			263	AA(6); AG(0); GG(39)	AA(0); AG(0); GG(11)	AA(0); AG(0); GG(14)		
16	C2F17	293	97	CC(6); CT(2); TT(37)	CC(10); CT(0); TT(1)	CC(13); CT(0); TT(1)		
17			206	CC(3); CT(0); TT(43)	CC(11); CT(0); TT(0)	CC(14); CT(0); TT(0)		
18			215	AA(14); AG(25); GG(6)	AA(11); AG(0); GG(0)	AA(14); AG(0); GG(0)		
19	C15F2	332	104	CC(45); CT(0); TT(0)	CC(1); CT(10); TT(0)	CC(1); CT(13); TT(0)		
20			174	AA(2); AG(30); GG(11)	AA(0); AG(0); GG(11)	AA(0); AG(0); GG(14)		
21			207	AA(26); AG(15); GG(4)	AA(0); AG(0); GG(11)	AA(0); AG(0); GG(14)		
22			286	AA(1); AG(38); GG(6)	AA(0); AG(0); GG(11)	AA(0); AG(0); GG(14)		
23			B7B20	217	79	AA(42); AC(0); CC(3)	AA(1); AC(0); CC(10)	AA(1); AC(0); CC(13)
24	184	AA(42); AC(0); CC(3)			AA(11); AC(0); CC(0)	AA(14); AC(0); CC(0)		
25	B4B16	175	109	AA(2); AG(0); GG(43)	n.p.	n.p.		
26	B8B16	189	154	AA(5); AG(4); GG(36)	n.p.	n.p.		
27	B8B12	223	24	CC(1); CT(0); TT(44)	CC(11); CT(0); TT(0)	CC(14); CT(0); TT(0)		
28			57	CC(4); CT(0); TT(41)	CC(0); CT(0); TT(11)	CC(0); CT(0); TT(14)		
29			58	AA(43); AT(0); TT(2)	AA(0); AT(0); TT(11)	AA(0); AT(0); TT(14)		
30			60	AA(43); AG(0); GG(2)	AA(0); AG(0); GG(11)	AA(0); AG(0); GG(14)		
31			70	AA(2); AG(0); GG(43)	AA(11); AG(0); GG(0)	AA(14); AG(0); GG(0)		
32			83	AA(39); AT(0); TT(6)	AA(0); AT(0); TT(11)	AA(0); AT(0); TT(14)		
33			89	AA(43); AG(0); GG(2)	AA(0); AG(0); GG(11)	AA(0); AG(0); GG(14)		
34			98	AA(2); AC(0); CC(43)	AA(11); AC(0); CC(0)	AA(14); AC(0); CC(0)		
35			101	CC(0); CT(0); TT(45)	CC(10); CT(1); TT(0)	CC(13); CT(1); TT(0)		
36			124	CC(43); CT(2); TT(0)	CC(11); CT(0); TT(0)	CC(14); CT(0); TT(0)		
37			171	AA(0); AC(0); CC(45)	AA(10); AC(0); CC(1)	AA(13); AC(0); CC(1)		
38			B5B20	757	185	CC(2); CT(39); TT(4)	CC(11); CT(0); TT(0)	CC(14); CT(0); TT(0)
39					265	CC(0); CT(0); TT(45)	CC(10); CT(0); TT(1)	CC(13); CT(0); TT(1)
40					281	CC(39); CT(20); TT(4)	CC(11); CT(0); TT(0)	CC(14); CT(0); TT(0)
41					379	CC(45); CT(0); TT(0)	CC(1); CT(10); TT(0)	CC(1); CT(13); TT(0)
42					388	AA(4); AG(39); GG(2)	AA(1); AG(10); GG(0)	AA(1); AG(13); GG(0)
43					395	CC(0); CT(0); TT(45)	CC(10); CT(0); TT(1)	CC(13); CT(0); TT(1)
44	399	CC(45); CG(0); GG(0)			CC(1); CG(10); GG(0)	CC(1); CG(13); GG(0)		
45	400	AA(45); AG(0); GG(0)	AA(10); AG(0); GG(1)	AA(13); AG(0); GG(1)				
46	535	AA(4); AG(2); GG(39)	AA(1); AG(0); GG(10)	AA(1); AG(0); GG(13)				
47	621	AA(39); AG(2); GG(4)	AA(11); AG(0); GG(0)	AA(14); AG(0); GG(0)				
48	B13B19	1000	Bfa I	AA(7); AB(35); BB(3)	AA(11); AB(0); BB(0)	AA(14); AB(0); BB(0)		
49			Ase I	AA(11); AB(32); BB(2)	AA(11); AB(0); BB(0)	AA(14); AB(0); BB(0)		
50			Tsp509 I	AA(12); AB(33); BB(0)	AA(10); AB(1); BB(0)	AA(13); AB(1); BB(0)		
51			Taq I	AA(8); AB(36); BB(1)	AA(1); AB(10); BB(0)	AA(1); AB(13); BB(0)		
52			Mse I	AA(11); AB(32); BB(2)	AA(11); AB(0); BB(0)	AA(14); AB(0); BB(0)		
53	Rsa I	AA(45); AB(0); BB(0)	AA(10); AB(1); BB(0)	AA(13); AB(1); BB(0)				
54	B5B7	742	137	AA(4); AG(0); GG(41)	n.a.	n.a.		

TABLE 2—Continued

Locus	Primer pair	Fragment size	Position	Genotypic frequencies		
				Typical population from Angola	Atypical population from Angola	Atypical population from Madagascar
55			216	CC(0); CT(4); TT(41)		
56			283	CC(0); CT(1); TT(44)		
57			351	CC(44); CT(1); TT(0)		
58			417	CC(33); CT(0); TT(12)		
59			441	AA(0); AG(4); GG(41)		
60			464	AA(38); AG(7); GG(0)		
61			609	CC(41); CT(4); TT(0)		
62			656	AA(9); AG(0); GG(36)		
63			713	CC(41); CT(4); TT(0)		
64	B15B20	282	46	CC(0); CT(0); TT(45)	CC(1); CT(0); TT(10)	CC(1); CT(0); TT(13)
65			52	CC(31); CT(0); TT(14)	CC(0); CT(0); TT(11)	CC(0); CT(0); TT(14)
66			53	CC(45); CT(0); TT(0)	CC(1); CT(10); TT(0)	CC(1); CT(13); TT(0)
67			69	AA(12); AG(31); GG(2)	AA(11); AG(0); GG(0)	AA(14); AG(0); GG(0)
68			79	CC(45); CT(0); TT(0)	CC(11); CT(0); TT(0)	CC(13); CT(0); TT(1)
69			84	AA(45); AG(0); GG(0)	AA(10); AG(0); GG(1)	AA(13); AG(0); GG(1)
70			109	AA(4); AG(31); GG(10)	AA(0); AG(9); GG(2)	AA(0); AG(12); GG(2)
71			148	AA(0); AG(0); GG(45)	AA(0); AG(0); GG(11)	AA(1); AG(0); GG(13)
72			198	AA(45); AG(0); GG(0)	AA(10); AG(1); GG(0)	AA(13); AG(1); GG(0)
73			205	AA(0); AG(0); GG(45)	AA(7); AG(0); GG(4)	AA(1); AG(0); GG(13)
74			211	AA(12); AG(31); GG(2)	AA(11); AG(0); GG(0)	AA(13); AG(1); GG(0)
75			239	CC(3); CT(31); TT(11)	CC(11); CT(0); TT(0)	CC(14); CT(0); TT(0)
76			260	CC(43); CT(0); TT(2)	CC(9); CT(1); TT(1)	CC(11); CT(1); TT(2)

Note. The number of the locus and the name and the fragment size of each marker is provided; the position of the polymorphism from the 5'-end of the forward primer is given followed by the genotypic frequencies of the alleles at this polymorphic locus; n.a., fragment could not be amplified; n.p., no polymorphism detected.

Multilocus genotypes and heterozygosity. Since all 76 polymorphisms were scored as co-dominant markers, each strain was assigned a multilocus genotype (MLG) (see Appendix 1). Each polymorphic site was scored for the presence of both allelic states to obtain evidence for heterozygosity. For the typical Angola population, 35 of 56 (62.5%) polymorphic sites exhibited heterozygosity, showing both allelic states in at least one individual. For the atypical populations from Angola and Madagascar, 15 of 31 (48.4%) and 14 of 33 (42.4%) of the respective polymorphic sites were heterozygous.

Evidence for Clonality and Recombination

Clonal population structure in microorganisms may be indicated by overrepresented genotypes, fixed heterozygosity, and deviation from random expectations for both intralocus (Hardy-Weinberg equilibrium) and interlocus (linkage disequilibrium) genotypic associations (Tibayrenc *et al.*, 1990, 1991; Avise, 1994). These tests were therefore

used to assess the extent of clonality and recombination in each population.

Overrepresented MLGs. The first indication of clonality is the repetition of identical MLGs within populations. Overrepresented MLGs were detected in all three populations. The typical Angola population of 45 strains was composed of 27 unique MLGs, with the most common MLG represented by 15 strains, and 4 MLGs were represented by 2 strains each. The remaining 22 strains each had a unique MLG. The atypical Angola population of 11 strains was composed of 6 unique MLGs, and the most common MLG was shared by 6 strains. The atypical Madagascar population of 14 strains was composed of 4 distinct MLGs; the most common MLG was represented by 11 strains. No MLG was shared among the three populations, suggesting that these populations are not recombining (sharing genes) with each other.

Heterozygosity. Another indicator of clonal structure in diploid populations is evidence for fixed heterozygosity. In both atypical populations, isolates showed nearly fixed

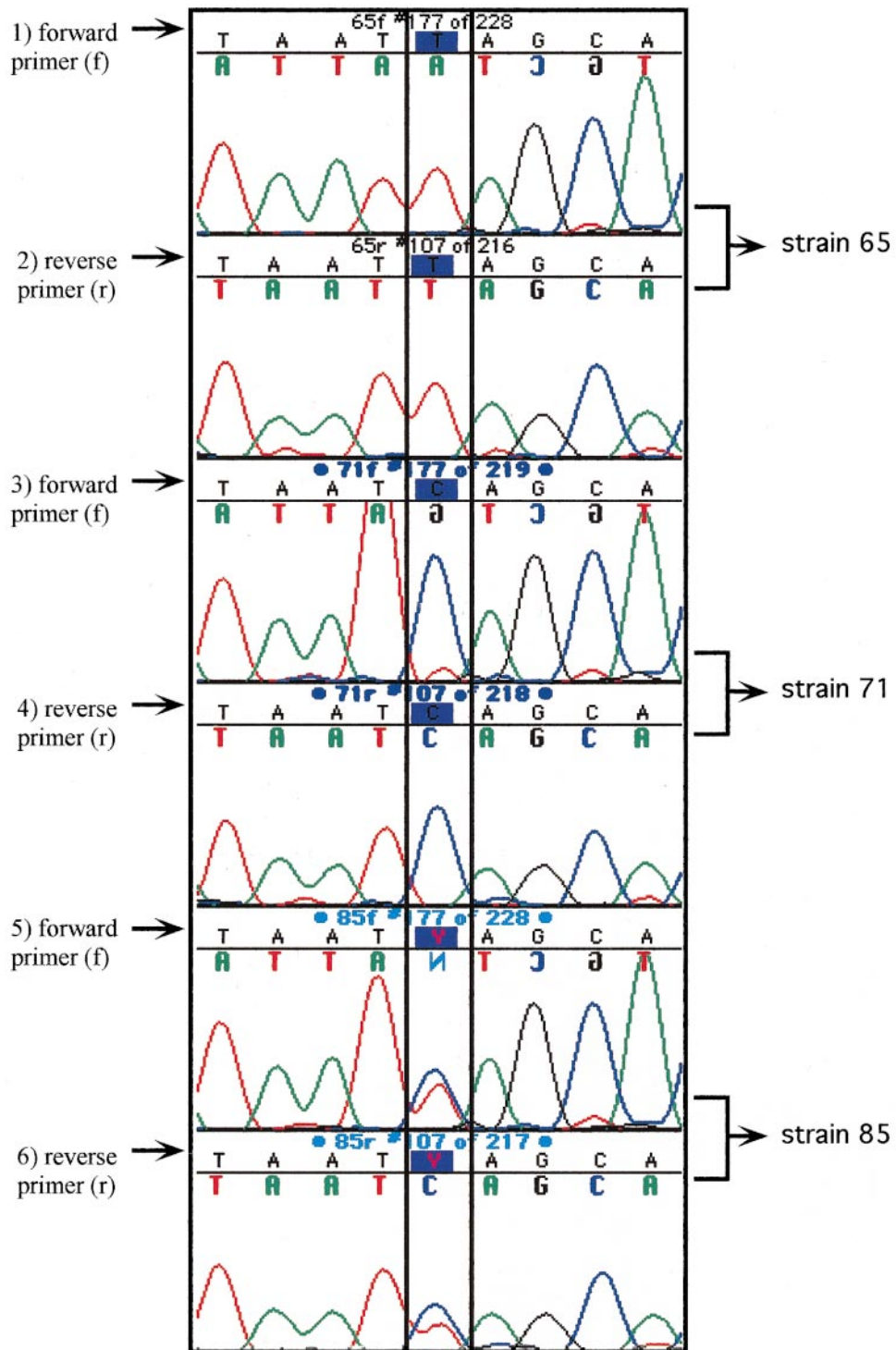


FIG. 2. Detection of heterozygosity by automated sequencing. Example shows sequences obtained with primer pair B15B20 (forward and reverse) for 3 *C. albicans* strains from Angola; rows 1, 3, and 5 represent sequences obtained by the forward primer; rows 2, 4, and 6 represent sequences detected by the reverse primer. The detected polymorphic locus is marked by a frame; at this site strain 71 is homozygous with "cc", strain 85 is heterozygous with "ct", and strain 65 is homozygous with "tt".

heterozygosity for 7 polymorphic sites. Among the atypical populations from Angola and Madagascar, 91 and 93% of strains exhibited both allelic states, respectively. In the typical Angola population, fixed heterozygosity was not observed at any single site; however, 16 of 56 polymorphic sites exhibited excessive heterozygosity when tested against Hardy–Weinberg expectations. Comparing polymorphic loci in all populations, fragments C15F2 and B5B20 each displayed excess heterozygosity.

Intralocus associations. Scoring each polymorphic site as an individual locus, we tested for random association within and among sites. Random segregation within polymorphic sites was examined using goodness of fit tests for Hardy–Weinberg expectations. Levels of significance were calculated using χ^2 tests. In the typical Angola population, 41 of 56 (73%) sites deviated significantly from Hardy–Weinberg equilibrium. The remaining 15 sites did not differ significantly from Hardy–Weinberg equilibrium, which may be the result of recombination within these sites. For the atypical populations from Angola and Madagascar, respectively, 25 of 31 (81%) and 27 of 33 (82%) of the polymorphic sites deviated significantly from Hardy–Weinberg expectations.

In recombining populations, all three possible genotypes would be expected to occur at every locus with two alleles. Genotypic counts at most sites deviate significantly from random expectations, although genotypic variation was reduced at individual sites in all three populations. Within the typical population, one of the three possible genotypes was absent at 33 of 56 (59%) polymorphic sites. In both atypical populations, 97% of polymorphic sites lacked one of the three expected genotypes.

Interlocus recombination. In randomly recombining populations, interlocus associations are expected to be in linkage equilibrium, while in clonal populations (without recombination), most pairs of loci will exhibit significant linkage disequilibrium. To test for random association of alleles across sites between and within loci, two different analyses of linkage disequilibrium were applied. One analysis of linkage employed the “Gamete” software developed by Paul O. Lewis (personal communication). In this test, for the typical population, 39% of all pairwise comparisons were found to be significantly in linkage disequilibrium. Since this frequency is greater than expected by chance for a freely recombining population (for random mating populations, only 5% of disequilibrium estimates are attributable to chance events), the population of typical Angola isolates is predominantly clonal. However, the majority of pairwise comparisons were not significant,

which suggests that some of these sites may still be recombining.

In contrast, nearly all polymorphic sites in both atypical populations were found to be in linkage disequilibrium; pairwise comparisons among loci within Angola and Madagascar were 93 and 83%, respectively. When the linkage analyses were repeated but with only one polymorphic site (the most polymorphic) per locus, the observed linkage disequilibrium values that were significant increased for each population by 5 to 20%.

The Index of Association (I_A) is a general measure of linkage disequilibrium that assesses the extent of clonality in microbial populations (Maynard-Smith *et al.*, 1993). This test was applied to the MLG data sets for all three African populations (software for performing these analysis with diploid populations was kindly provided by Austin Burt). This analysis was applied twice: first, all MLGs were analyzed; in the second analysis, repeated MLGs were excluded (clone corrected). I_A values differed significantly from zero ($P < 0.001$), indicating a strong clonal structure in all three populations (Table 3). For both the original and the clone-corrected samples, values of I_A for both atypical populations were each about two times greater than that of the atypical population, indicating that the extent of clonality in atypical populations is also greater.

Finally, we examined each population for direct evidence of recombinant genotypes existing within populations. For recombining populations, all possible combinations of genotypes will be expected among MLG for two or more loci (Gräser *et al.*, 1996). Inspection of pairwise comparisons among polymorphic sites revealed interlocus recombination among many loci for the typical population, and all nine possible recombinant genotypes were detected for at least two polymorphic sites (Table 4). Although recombinant genotypes were identifiable in both atypical

TABLE 3

Average Rescaled Indices of Association (I_A) for the Three Populations of *C. albicans* from Africa

Population	<i>P</i>	Index of association (I_A)	
		All MLGs	Unique MLGs only (clone-corrected sample)
Typical Angola	<0.001	10.85	5.80
Atypical Angola	<0.001	25.24	24.67
Atypical Madagascar	<0.001	21.82	22.36

TABLE 4

Counts of Recombinant Genotypes between Two Sets of Unlinked Nucleotide Sites Sampled from the Typical *C. albicans* Population from Angola

	C2F17-3		
	aa	ag	gg
B15B29-1			
aa	2	1	2
ag	5	23	2
gg	7	1	2
C15F2-2			
aa	6	18	2
ag	7	5	3
gg	1	2	1

Note. Genotype counts given for C2-F17-3 with B15B20-1 and C2-F17-3 with C15F2-2, respectively.

populations (not shown), complete combinations of genotypes were lacking for all pairs of polymorphic sites. This last observation is also consistent with a higher level of clonality in the atypical populations.

Linkage Relationships

Nonrandom genotypic associations may arise when markers are physically linked within a single PCR fragment or exist on the same chromosome. To determine physical linkage among markers, chromosomes of *C. albicans* were separated by pulsed-field gel electrophoresis, blotted to membranes, and probed with PCR fragments. With one exception, each PCR fragment hybridized to a different chromosome (Table 1). PCR fragments C12F10 and C2F10 are located on chromosome 1. Consequently, 11 of 13 marker fragments used to analyze the typical population and 8 of 10 markers applied to both atypical populations were not physically linked. To test the independence of polymorphic sites within fragments, polymorphic sites were treated as loci and subjected to linkage analysis. Among typical isolates, only 48.5% of these sites displayed significant linkage disequilibrium, which suggests that the remaining sites are truly independent. For the atypical populations from Angola and Madagascar, 93 and 91% of their respective polymorphic sites were in significant linkage disequilibrium, as might be expected if these populations were largely clonal.

Analysis of PCR Fingerprints

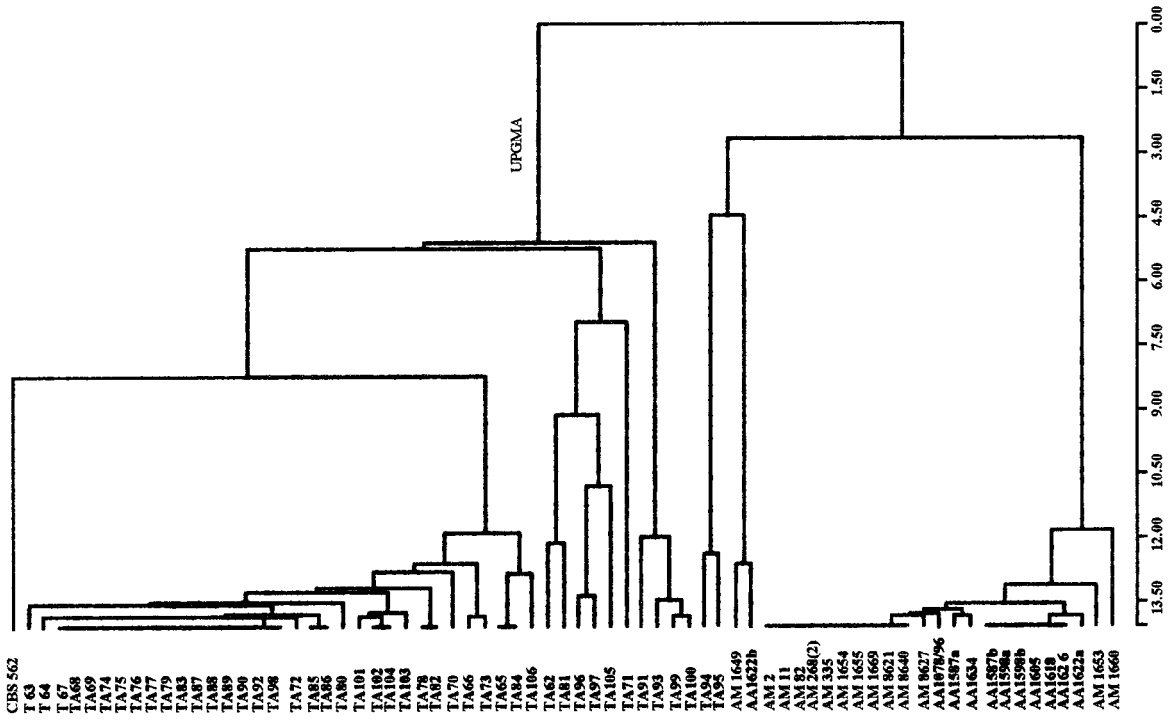
To provide an independent set of markers to compare with MLG, we analyzed PCR fingerprint patterns among the three African populations based on arbitrarily amplified DNA banding patterns reported in a previous paper (Tietz *et al.*, 1995). Using scanner-associated computer hardware and software programs (RFLPscan, version 2.01; Scanalytics CSP Inc., Billerica, MA), 39 scoreable bands were detected among the three populations (see Appendix 2). Each strain was assigned a PCR fingerprint genotype (GT) by scoring bands as present (1) or absent (0). The typical population revealed nine individual GTs: two GTs were overrepresented and found in 18 and 13 strains, respectively; two GTs were represented by 5 and 4 strains, respectively; and five GTs were each specific to 1 strain. Among the atypical Angola isolates, only three GTs were detected: one GT was represented by 9 strains, and two GTs were represented by 1 strain each. The atypical Madagascar isolates had five GTs: one GT represented 10 strains, and four GTs each represented a single strain. Two GTs were shared between the atypical populations, and one of the shared GTs was overrepresented in each population.

The high similarity of the GTs detected in both atypical populations is consistent with a clonal population structure and suggests that they are closely related. In contrast, DNA fingerprint patterns for the typical population revealed greater differences among strains, which suggests greater variability in this population (Tietz *et al.*, 1995).

Comparison of MLGs and PCR Fingerprints

The availability of two independent sets of markers (MLG and PCR fingerprints) for the three African populations provided another method for assessing genetic isolation and clonality. To compare both data sets, UPGMA distance trees were generated for the MLG and GT data for all three populations, using the distance option in PAUP* (Swofford, 1999). The UPGMA trees generated by each data set divided the isolates into two major clusters representing the typical population (cluster I) and both atypical populations (cluster II) (Fig. 3). The MLG data

FIG. 3. UPGMA dendrograms obtained for the PCR fingerprint data (left tree) and for the multilocus genotype data (right tree) for the *C. albicans* populations from Africa. Cluster I represents the typical *C. albicans* populations from Angola and cluster II represents both atypical *C. albicans* populations from Angola and Madagascar.



cluster I | cluster II



yielded greater resolution of tree topology within each cluster, including somewhat better separation of the two atypical populations in cluster II (see Fig. 3). With the GT or PCR fingerprint data, both atypical populations were mixed together and one atypical strain (AM1660) clustered with the typical isolates.

The pattern of relationships inferred based on either MLG or GT data for cluster I also shows evidence of both clonality and recombination for the typical Angola population. Certain strains, such as TA91, TA93, TA99, and TA100, are always identical in both trees, which is consistent with their being clonally related with each other. In contrast, many other groups of strains clustered together in one tree (e.g., strains TA94–TA98 of the GT denodrogram) but were separated in the MLG tree and vice versa, which is consistent with the possibility of recombination.

Genetic Isolation between Populations

F statistics were used to evaluate genetic diversity that might be due to genetic isolation or geographic structure among the three populations (Wright, 1969; Weir and Cockerham, 1984). Wright's F statistics (Table 5) comparing the typical and atypical populations of *C. albicans* from Angola revealed a high degree of subdivision, with an average F_{ST} of 0.311. In contrast, average F_{ST} values calculated between the atypical populations from Angola and Madagascar were much lower (0.063), indicating less isolation between these populations.

Genetic similarity based on Nei's genetic distance was also calculated using the GDA software package (Lewis and Zaykin, 1998) (Table 5). Both atypical populations were genetically nearly identical, with an estimated identity of 0.996. The typical Angola isolates differed genetically from both atypical populations with estimates of 0.5998 (typical Angola vs atypical Madagascar population) and 0.60 (typical vs atypical Angola population).

TABLE 5
Wright's F_{ST} (above Diagonal) and Nei's Genetic Identity (below Diagonal) among Three African Populations of *C. albicans*

	Atypical population (Angola)	Atypical population (Madagascar)	Typical population (Angola)
Typical population (Angola)	0.317	0.311	—
Atypical population (Madagascar)	0.063	—	0.5998
Atypical population (Angola)	—	0.9960	0.6000

Phylogenetic Relationships of African Strains to Other *Candida* Species

To determine whether typical and atypical populations are related with other *C. albicans* strains, a phylogenetic analysis was performed using sequence data for a portion of the large subunit 26S ribosomal DNA gene. The region sequenced contains a hypervariable region (divergent domain D2) that has been shown to be diagnostic for many different yeast species (Kurtzman and Robnett, 1997). Sequences representing all three populations were aligned against partial 26S rDNA sequences of several other *Candida* species, including several type strains belonging to the *C. albicans* clade (Kurtzman and Robnett, 1997). The aligned 26S rDNA sequence data set consisted of 343 bases from 31 taxa (the PAUP data set is available upon request from the first and last authors). Forty-nine positions within this alignment were considered to have ambiguous alignment due to the presence of small insertions or deletions and were therefore excluded from the analysis. Of the remaining 294 positions, 56 bases were variable, and of these, 38 positions were parsimony informative. Parsimony analysis using 100 random addition sequences all resulted in a single most-parsimonious tree with a length of 103 steps and a consistency index (excluding uninformative characters) of 0.612 (Fig. 4). All sequences from this study belong within a single group that includes the type strain (NRRL Y-12983) as well as other authentic strains of *C. albicans*. This *C. albicans* group is strongly supported by a bootstrap value of 95% and is distinct from all other species, including *C. dublinensis* (91% bootstrap support), which is identified here as a sister group to *C. albicans* (with 88% support).

DISCUSSION

Genetic structure of both typical and atypical populations was compared using population genetic analyses and PCR fingerprinting. Analyses of population genetics involved co-dominant single-locus markers that were developed in this or a previous study (Gräser *et al.*, 1996). Co-dominant markers are preferable because they detect heterozygosity when it is present in the diploid genome of *C. albicans* (Milgroom, 1996).

Fourteen primer pairs from this study and 6 primer pairs previously reported were initially screened for DNA polymorphisms. Thirteen of 20 primer pairs (65%) were polymorphic (Table 2), yielding a success rate that was

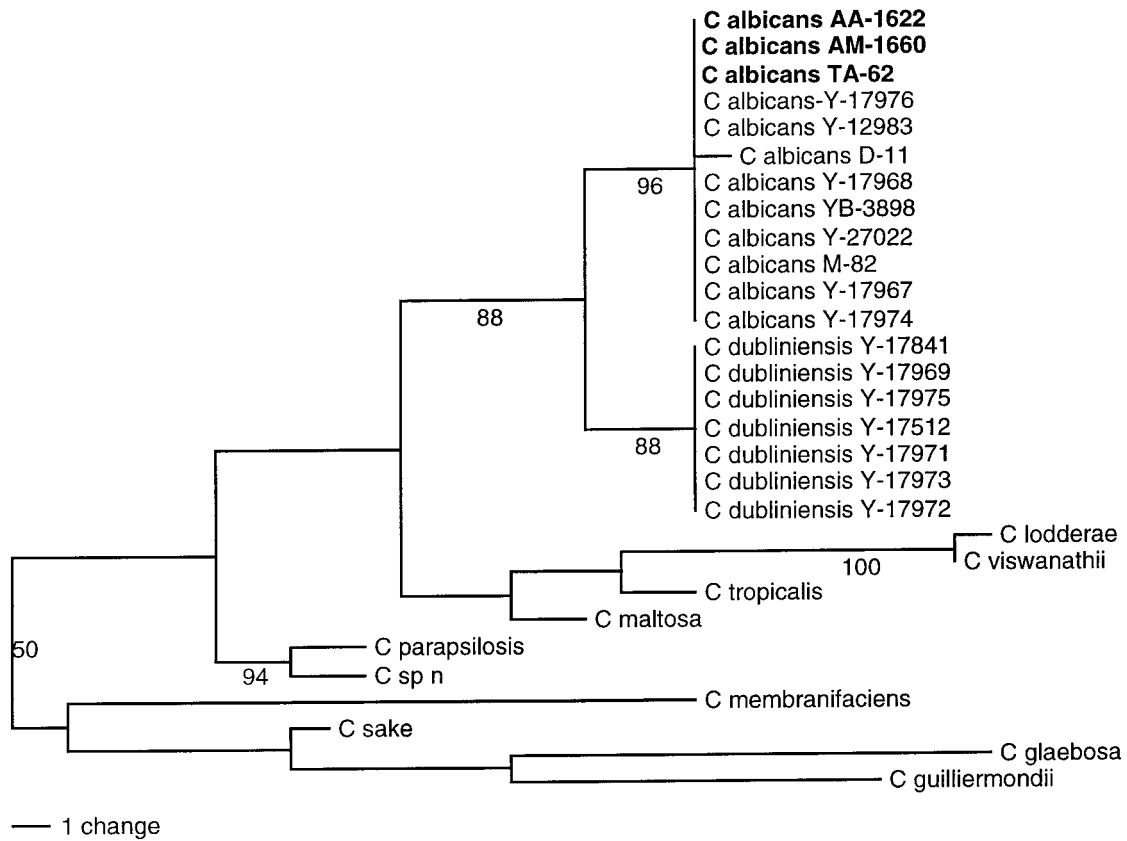


FIG. 4. Phylogenetic relationships among typical and atypical *Candida* isolates based on partial 26S rDNA sequences. Shown is the single most parsimonious tree (length = 103 steps, CI = 0.612) for sequences from representative strains of African *C. albicans* isolates (in boldface) with authentic and type strains of other *Candida* species. Bootstrap support values are given for branches with greater than 50% support (based on 300 replicates).

relatively high compared to other studies in which the rates of polymorphisms ranged from 43 to 55% (Gräser *et al.*, 1996; Karl *et al.*, 1992). Polymorphisms in the PCR products were first detected by screening all strains of *C. albicans* for SSCPs, after which strains were selected for subsequent evaluation by RFLP and direct sequencing. All SSCP polymorphisms were resolved at the molecular level by DNA sequencing (Table 2 and Fig. 2). No aneuploid or polyploid strains were detected, supporting the consensus that *C. albicans* is diploid (Gräser *et al.*, 1996; Pujol *et al.*, 1993; Whelan and Magee, 1981).

The population genetic structure of each African population was characterized using Tibayrenc's criteria for a clonal mode of reproduction (Tibayrenc *et al.*, 1990, 1991). All three populations from this study have a predominantly clonal population structure, confirming the results of previous population studies on *C. albicans* (Boerlin *et al.*, 1996; Gräser *et al.*, 1996; Pujol *et al.*, 1993). However, recombination was detected to some degree in all three populations.

Typical *C. albicans*

Analysis of the typical Angola population revealed evidence for both clonal and recombinant population structures. Clonality was supported by the absence of segregation and an excess of heterozygosity at 28% of the polymorphic sites. One process that generates deviation from panmictic population structure, self-fertilization, could be excluded since fixed heterozygosity was present in this population (Pujol *et al.*, 1993). Fixed heterozygosity is incompatible with biparental reproduction (Tibayrenc *et al.*, 1991). In addition, since one of the three possible genotypes was absent at 59% of the polymorphic sites and since the majority of polymorphic sites showed significant deviation from Hardy-Weinberg equilibrium, clonal reproduction is the most parsimonious explanation for the results of the segregation tests.

Analysis of genotypic variation for the typical Angolan population revealed departure from panmictic expectations but also suggested recombination in this population.

From a total of 27 observed MLGs, 1 was represented by 15 strains and 4 MLGs were represented by 2 strains each. Therefore, some of the genotypes appear to be overrepresented, which further supports a clonal population structure. This result is consistent with the observations of other studies that used enzyme electrophoresis (Boerlin *et al.*, 1996; Pujol *et al.*, 1993) and DNA sequence polymorphism (Gräser *et al.*, 1996) to study the population structure of *C. albicans*. Yet, linkage disequilibrium analysis suggests evidence of recombination: the findings that 61% of all pairwise comparisons of loci are not significantly in linkage disequilibrium and that 50% of the polymorphic sites within loci are independent (not significantly linked) indicates that the majority of loci are recombining.

Similar results were obtained with the linkage analyses when only one polymorphic site per locus was analyzed (clone correction). Furthermore, the presence of all nine possible recombinant genotypes in two pairs of loci for the typical population is consistent with sexual reproduction (Table 4). Population genetic data obtained for the typical Angola population are in good agreement with those reported recently for a "natural" population of *C. albicans* from the United States (Gräser *et al.*, 1996).

In contrast, the Index of Association of the typical Angola population revealed values that were significantly different from zero, supporting a strong clonal population structure. However, even I_A values that differ significantly from zero do not exclude the possibility of rare recombination (Maynard-Smith *et al.*, 1993).

Atypical Populations

As expected from the highly similar PCR fingerprint patterns, our initial hypothesis of clonal population structure for both atypical *C. albicans* populations from Angola and Madagascar (Tietz *et al.*, 1995) was supported by the population genetic analysis. There was little evidence of recombination. Most polymorphic sites showed significant deviation from Hardy-Weinberg expectation, and most pairwise comparisons of loci were significantly in linkage disequilibrium. These results for the atypical populations are consistent with several studies, in which a primarily clonal mode of reproduction was proposed (Boerlin *et al.*, 1996; Gräser *et al.*, 1996; Lockart *et al.*, 1995; Pujol *et al.*, 1993).

Several lines of evidence suggest that both atypical population samples from this study are genetically divergent from typical populations (Figs. 3 and 4, Table 5). Both multilocus genotypes and PCR fingerprints (GT) showed genetic differences between typical and atypical populations, as well as a higher degree of clonality within both

atypical populations (Fig. 3). The typical and atypical populations of *C. albicans* differed in the amount of DNA polymorphisms that were detected. The typical Angola population showed 33% more polymorphic sites than either atypical population from Angola or Madagascar. In both atypical populations, no polymorphisms could be detected for markers B8B16 and B4B16, while another marker, which showed as many as 10 polymorphic sites in typical strains (B5B7), could not be amplified from any strains in the atypical populations (Table 2). This reduction in the amount of genetic polymorphism observed in typical and atypical populations may be attributable to several factors, including genetic isolation between populations as well as differences in their genetic structure. All the markers used to assess MLGs in this study were derived using typical population isolates, and so it is likely that loci may be found in atypical populations which are polymorphic in atypical but not in typical populations as a result of genetic divergence (Taylor *et al.*, 1999).

Are Atypical and Typical Populations the Same Species?

It is not possible to tell whether the three populations represent one, two, or even three species. Both atypical populations lack the ability to assimilate glucosamine and *N*-acetylglucosamine and to produce chlamydo spores (Tietz *et al.*, 1995). As such, they potentially represent different varieties, even species, of *Candida*. Phylogenetic analysis of rDNA sequences from this study show that isolates from different locations in Africa are all closely related to other strains of *C. albicans* (Fig. 4). Within the portion of the 26S rDNA region that was employed for this study, most sequences belonging to the *C. albicans* group were identical or nearly so. In contrast, rDNA sequences from the next closest species, *C. dublinensis*, differed significantly from *C. albicans* strains by at least six nucleotide substitutions (Fig. 4). Thus, based on molecular systematics evidence, both atypical population samples in this study are very closely related to other "typical" populations of *C. albicans*.

Peterson and Kurtzman (1991) first proposed that rDNA sequence divergence can be used as a criterion for recognizing separate species. For many yeasts, they observed that more than 1% nucleotide substitution within the variable region of the large subunit ribosomal DNA usually denotes differences at the species level. Based on this criterion, atypical strains from this study probably do not represent new species of *Candida*. However, defining species based on rDNA sequence divergence alone is not advisable, since rDNA evidence may not be sufficient to

determine whether or not atypical populations are conspecific with *C. albicans*. Evidence from both multilocus genotyping and PCR fingerprinting suggests that both atypical populations are genetically divergent from the typical population included in this study (Fig. 3, Table 5), and additional genetic differences may be found if other regions of DNA are sequenced (e.g., the rDNA ITS region). These differences are also evident in the contrasting population structure of the atypical and typical populations, as well as by genetic differences revealed through *F* statistics and genetic distance.

Recent precedent exists for recognizing certain atypical strains of *Candida* as different species. Clinical populations of *Candida* were described that produce abundant chlamydospores and are unable to grow at 42°C, unlike typical isolates of *C. albicans*, which grow well at this temperature (Sullivan *et al.*, 1993). Those strains were further examined with molecular methods, including karyotyping, multilocus enzyme electrophoresis, hybridization with *C. albicans*-specific DNA fingerprinting probes, and rDNA sequencing. All methods confirmed that these atypical populations differed genetically from *C. albicans* and other closely related *Candida* species. Consequently, this group of atypical isolates was placed in the new species, *Candida dubliniensis* (Sullivan *et al.*, 1995; Sullivan and Coleman, 1998). Similar examples of cryptic species are quite common among other groups of fungi, including *Coccidioides immitis*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (Taylor *et al.*, 1999). In this regard, both atypical populations from this study can be regarded as incipient species that are very closely related to a larger typical population of *C. albicans* and therefore possibly of very recent origin.

Initial studies of PCR fingerprint patterns led to the assumption that the atypical strains from this study might represent a subtype of *C. albicans* or even a new species (Tietz *et al.*, 1995). Phylogenetic analysis of sequences from the D2 region of the large subunit rDNA locus reveals only minimal divergence of the three African populations from other *C. albicans* strains. African strains from either the atypical or the typical populations differ by only one base pair but are otherwise not phylogenetically distinct from other typical strains of *C. albicans*. In contrast, isolates of *C. dubliniensis* belong to a distinct lineage that differs from the *C. albicans* clade (Fig. 3).

Most other evidence from this study and the previous one (Tietz *et al.*, 1995) suggest that all three African populations from this study are genetically isolated and in the process of genetically diverging. Both atypical populations, with their apparently higher level of clonal structure and small amount of

genetic divergence from typical *C. albicans*, might represent a good example of what Maynard-Smith *et al.* (1993) has described as an "epidemic" population structure, in which new lineages evolve via clonal expansion from a larger and more variable recombining parental population.

CONCLUSIONS

Our results describe different patterns of genetic structure for African populations of *C. albicans*. Several differences between typical and atypical strains are apparent. Two atypical populations from this study are both closely related. One came from the west coast of South Africa (Angola) and the other from the island of Madagascar, which is located off the east coast of South Africa. They are separated by a distance of more than 1000 km. For these isolates, the average F_{ST} value (6.5%) was quite low, which indicates that the atypical populations are highly similar. *F* statistics also demonstrated significant genetic difference between the typical and the atypical populations from the same hospital in Luanda (Angola), with an average F_{ST} value of 31.4%.

Our group has initiated a global study of the population genetics of *C. albicans*. Since *C. albicans* has become an increasingly important pathogen, population genetic analyses are of great relevance in this species. Strategies for the development of vaccines and antifungal drugs are strongly affected by the mode of reproduction of the target microorganism (Tibayrenc *et al.*, 1991). Recombination favors combinations of advantageous genes and therefore enhances their adaptation to new environments and to antifungal drugs. Conversely, under stable environmental conditions, selection is more efficient if one genotype is overrepresented. Favorable gene combinations would not be disrupted by recombination (Milgroom, 1996). As indicated earlier, all population genetic analyses in *C. albicans* have detected a basically clonal mode of reproduction, as well as some evidence of recombination. Therefore, it is important to investigate the distribution of clonal reproduction and the amount of recombination occurring in natural populations of *C. albicans*.

The broad goals of this study are not only to address questions of distribution of the different mechanisms of reproduction. We also want to investigate global correlations between genetic diversity and specific biological properties in *C. albicans*, the amount of genetic variability, and the differences between populations regarding allelic and genotypic frequencies. Answering these questions may discover the evolutionary processes leading to high variability among isolates of this important medical yeast.

APPENDIX 1
MLGs for All Polymorphic Sites and All Strains of *C. albicans*

MLG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
AM2 (10)*	gg	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AM1649	gg	tt	cc	ag	cc	gg	gg	tt	cc	gg	aa	gg	aa	cc	gg	tt	cc	aa	cc	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	ct	cc	cc	
AM1653	gg	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AM1660	gg	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AM8627	gg	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AA1078/96	gg	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AA1587a	gg	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AA1587b (6)	gg	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AA1622a	gl	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AA1622b	gl	tt	cc	ag	cc	gg	gg	tt	cc	gg	aa	gg	aa	cc	gg	tt	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
AA1634	gg	gg	cc	aa	tt	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	ct	gg	gg	gg	cc	aa	gg	aa	cc	tt	tt	gg	aa	tt	gg	aa	cc	cc	aa	
TA62	gg	gg	cc	aa	cc	tt	gg	aa	cc	gg	aa	aa	gg	cc	aa	cc	tt	gg	cc	gg	ag	ag	cc	cc	gg	aa	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc	
TA63	gg	gg	cc	gg	cc	tt	gg	aa	cc	gg	aa	aa	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA64	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	ag	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA65 (2)	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	ag	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA66	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	aa	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA70	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	ag	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA71	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	ag	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA72	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	ct	tt	ag	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA73	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	ag	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA78 (2)	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA80	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA81	gg	gl	cc	aa	cc	tt	gg	aa	cc	gg	aa	aa	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA85 (2)	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA91	gg	gg	cc	aa	cc	gg	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA93	gg	gg	cc	aa	cc	gg	gg	aa	tt	aa	ag	ag	gg	cc	gg	cc	tt	aa	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA94	gg	gl	cc	ag	cc	gl	gg	aa	tt	gg	aa	aa	gg	cc	gg	cc	cc	aa	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA95	gg	gl	cc	ag	cc	gl	gg	aa	ct	gg	aa	aa	gg	cc	aa	cc	cc	aa	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA96	gg	gg	cc	aa	cc	tt	gg	aa	cc	gg	aa	aa	gg	cc	aa	cc	cc	aa	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA97	gg	gl	cc	aa	cc	tt	gg	aa	cc	gg	aa	aa	gg	cc	aa	cc	tt	aa	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA99	gg	gg	cc	aa	cc	gg	gg	aa	tt	aa	ag	ag	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA100	gg	gg	cc	aa	cc	gg	gg	aa	tt	aa	ag	ag	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA101	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA102 (2)	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA103	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA105	gg	gl	cc	aa	cc	tt	gg	aa	cc	gg	aa	aa	gg	cc	gg	cc	tt	aa	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc
TA106	gg	gg	cc	gg	cc	tt	gg	aa	tt	gg	aa	aa	gg	cc	gg	tt	tt	gg	cc	gg	cc	gg	cc	aa	gg	aa	cc	tt	tt	aa	aa	gg	aa	aa	cc	tt	cc	cc

APPENDIX 2

Fingerprint Genotypes for the Three African Populations of *C. albicans*

	1	11	21	31
TA62 (14 strains)	11000001010010100100010110001010010010			
TA63	11000001010010100000010100100010010010			
TA71	1100000100101010100100110110001010010010			
TA78 (18 strains)	11000001010010100000010100101100110010			
TA81	11000001001010100100010100001010010010			
TA91 (4 strains)	110000010010111000000101101011100010010			
TA94 (5 strains)	11000001001010100100010101001001110010			
TA105	11000001010010100000010110000100100010			
AA1622a	11000001010010100101100010000100011010			
AA1622b	11000001010010100101011010001100010110			
AM2 (19 strains)	11000001010010100101101010000100011010			
AM335	11000001010010100101011010001000011010			
AM1649	11000001010010100101011010001000011010			
AM1660	11000001100010100100010100000100010000			

ACKNOWLEDGMENTS

We thank Dr. M. P. Andrade for collecting strains of *C. albicans* and Dr. R. Swoboda for providing the *C. albicans* fosmid library for chromosome localization. We also thank Dr. C. P. Kurtzman for kindly providing the 26S rDNA sequence data set for ascomycetous yeasts and Dr. A. Burt for providing the software for calculating the Index of Association. Dr. J. Xu and two anonymous reviewers provided many helpful comments and suggestions on the manuscript. This research was supported by a Public Health Service Grant (AI 28836) and two Grants from the Deutsche Forschungsgemeinschaft (Scho 448/3-1, 448/3-2). This is a publication of the Duke University Mycology Research Unit.

REFERENCES

- Awise, J. C. 1994. *Molecular Markers, Natural History and Evolution*. Chapman and Hall, New York.
- Boerlin, P., Boerlin-Petzold, F., Durussel, C., Addo, M., Pagani, J.-L., Chave, J.-P., and Bille, J. 1995. Cluster of oral atypical *Candida albicans* isolates in a group of human immunodeficiency virus-positive drug users. *J. Clin. Microbiol.* **33**: 1129–1135.
- Boerlin, P., Boerlin-Petzold, F., Goudet, J., Durussel, C., Pagani, J.-L., Chave, J.-P., and Bille, J. 1996. Typing *Candida albicans* oral isolates from human immunodeficiency virus-infected patients by multilocus enzyme electrophoresis and DNA fingerprinting. *J. Clin. Microbiol.* **34**: 1235–1248.
- Burt, A., Carter, D. A., White, T. J., and Taylor, J. W. 1994. DNA sequencing with arbitrary primer pairs. *Mol. Ecol.* **3**: 523–525.
- Cobb, B. D., and Clarkson, J. M. 1994. A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucleic Acids Res.* **22**: 3801–3805.
- Diáz-Guerra, T. M., Martínez-Suárez, J. V., Labuna, F., and Rodríguez-Tudela, J. L. 1997. Comparison of four molecular typing methods for evaluating genetic diversity among *Candida albicans* isolates from human immunodeficiency virus-positive patients with oral Candidiasis. *J. Clin. Microbiol.* **35**: 856–861.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783–791.
- Gardes, M., and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes—Applications to the identification of *mycorrhizae* and rusts. *Mol. Evol.* **2**: 113–118.
- Gräser, Y., Volovsek, M., Arrington, J., Schöni, G., Presber, W., Mitchell, T. G., and Vilgalys, R. 1996. Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. *Proc. Natl. Acad. Sci. USA* **93**: 12473–12477.
- Hayashi, K. 1991. PCR-SSCP: A simple and sensitive method for detection of mutations in the genomic DNA. *PCR Meth. Appl.* **1**: 34–38.
- Karl, S. A., Bowen, B. W., and Awise, J. C. 1992. Global population genetic structure and male-mediated gene flow in the green Turtle (*Chelonia mydas*): RFLP analysis of anonymous nuclear loci. *Genetics* **131**: 163–173.
- Kurtzman, C. P., and Fell, J. W. 1998. *The Yeasts: A Taxonomic Study*, 4th ed. Elsevier, Amsterdam.
- Kurtzman, C. P., and Robnett, C. J. 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J. Clin. Microbiol.* **35**: 1216–1223.
- Lewis, P. O., and Zaykin, D. 1993. Genetic Data Analysis: software for the analysis of discrete genetic data. Sinauer, Sunderland, MA.
- Lockhart, S. R., Fritch, J. J., Meier, A. S., Schröppel, K., Srikantha, T., Galask, R., and Soll, D. R. 1995. Colonizing populations of *Candida albicans* are clonal in origin but undergo microevolution through C1 fragment reorganization as demonstrated by DNA fingerprinting and C1 sequencing. *J. Clin. Microbiol.* **33**: 1501–1509.
- Maynard-Smith, J., Smith, N. H., O'Rourke, M., and Spratt, B. G. 1993. How clonal are bacteria? *Proc. Natl. Acad. Sci. USA* **90**: 4384–4388.

- McClelland, M., Petersen, C., and Welsh, J. 1992. Length polymorphisms in tRNA intergenic spacer detected by using the polymerase chain reaction can distinguish streptococcal strains and species. *J. Clin. Microbiol.* **30**: 1499–1504.
- McCoullough, M., Ross, B., and Reade, P. 1995. Characterization of genetically distinct subgroup of *Candida albicans* strains isolated from oral cavities of patients infected with human immunodeficiency virus. *J. Clin. Microbiol.* **33**: 696–700.
- Milgroom, M. G. 1996. Recombination and the multilocus structure of fungal populations. *Annu. Rev. Phytopathol.* **34**: 457–477.
- Peterson, S. W., and Kurtzman, C. P. 1991. Ribosomal RNA sequence divergence among sibling species of yeasts. *Syst. Appl. Microbiol.* **14**: 124–129.
- Odds, F. C., Brawner, D. L., Staudinger, J., Magee, P. T., Soll, D. R. 1992. Typing of *Candida albicans* strains. *J. Med. Vet. Mycol.* **30**: 87–94.
- Pla, J., Gil, C., Monteoliva, L., Navarro-García, F., Sánchez, M., and Nombela, C. 1996. Understanding *Candida albicans* at the molecular level. *Yeast* **12**: 1677–1702.
- Pujol, C., Renaud, F., Mallié, M., Meeüs, T. D., and Bastide, J.-M. 1997. Atypical strains of *Candida albicans* recovered from AIDS patients. *J. Med. Vet. Mycol.* **35**: 115–121.
- Pujol, C., Reynes, J., Renaud, F., Raymond, M., Tibayrenc, M., Ayala, F., Janbon, F., Mallié, M., and Bastide, J.-M. 1993. The yeast *Candida albicans* has a clonal mode of reproduction in a population of infected human immunodeficiency virus-positive patients. *Proc. Natl. Acad. Sci. USA* **90**: 9456–9459.
- Redkar, R. J., Dubé, M. P., McCleskey, F. K., Rinaldi, M. G., and DelVecchio, V. G. 1996. DNA fingerprinting of *Candida rugosa* via repetitive sequence-based PCR. *J. Clin. Microbiol.* **34**: 1677–1681.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schönian, G., Schweynoch, C., Slateva, K., Oskam, L., Kroon, N., Gräser, Y., and Presber, W. 1996. Identification and determination of the relationships of species and strains within the genus *Leishmania* using single primers in the polymerase chain reaction. *Mol. Biochem. Parasitol.* **77**: 19–29.
- Sullivan, D., Bennett, D., Henman, M., Harwood, P., Flint, S., Mulahy, F., Shanley, D., and Coleman, D. 1993. Oligonucleotide fingerprinting of isolates of *Candida* species other than *Candida albicans* and of atypical *Candida* species from human immunodeficiency virus-positive and AIDS patients. *J. Clin. Microbiol.* **31**: 2124–2133.
- Sullivan, D., and Coleman, D. 1998. *Candida dubliniensis*: Characteristics and identification. *J. Clin. Microbiol.* **36**, No. 2: 329–334.
- Sullivan, D. J., Westerneng, T. J., Bennett, D. E., and Coleman, D. C. 1995. *Candida dubliniensis* sp. nov.: Phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141**: 1507–1521.
- Swofford, D. L. 1999. PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4. Sinauer, Sunderland, MA.
- Taylor, J. W., Geiser, D. M., Burt, A., and Koufopanou, V. 1999. The evolutionary biology and population genetics underlying fungal strain typing. *Clin. Microbiol. Rev.* **12**: 126–146.
- Thanos, M., Schönian, G., Meyer, W., Schweynoch, C., Gräser, Y., Mitchell, T. G., Presber, W., and Tietz, H.-J. 1996. Rapid identification of *Candida* species by DNA fingerprinting with PCR. *J. Clin. Microbiol.* **34**: 615–621.
- Tibayrenc, M., Kjellberg, F., Arnaud, J., Oury, B., Brenière, S. F., Dardé, M.-L., and Ayala, F. J. 1991. Are eucaryotic microorganisms clonal or sexual? A population genetics vantage. *Proc. Natl. Acad. Sci. USA* **88**: 5129–5133.
- Tibayrenc, M., Kjellberg, F., and Ayala, F. J. 1990. A clonal theory of parasitic protozoa: The population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. *Proc. Natl. Acad. Sci. USA* **87**: 2414–2418.
- Tietz, H.-J., Kuessner, A., Thanos, M., Andrade, M. P. D., Presber, W., and Schönian, G. 1995. Phenotypic and genotypic characterization of unusual vaginal isolates of *Candida albicans* from Africa. *J. Clin. Microbiol.* **33**: 2462–2465.
- Whelan, W. L., and Magee, P. T. 1981. Natural heterozygosity in *Candida albicans*. *J. Bacteriol.* **145**: 896–903.
- Weir, B. S., and Cockerham, C. C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.
- Wickes, B., Staudinger, J., Magee, B. B., Kwon-Chung, K.-J., Magee, P. T., and Scherer, S. 1991. Physical and genetic mapping of *Candida albicans*: Several genes previously assigned to chromosome 1 map to chromosome R, the rDNA-containing linkage group. *Infection Immunity* **59**: 2480–2484.
- Wright, S. 1969. *Evolution and Genetics of Populations*, Vol. 2. Univ. of Chicago Press, Chicago.
- Xu, J., Mitchell, T. G., and Vilgalys, R. 1999. PCR-restriction fragment length polymorphisms (RFLP) analyses reveal both extensive clonality and local genetic differences in *Candida albicans*. *Mol. Ecol.* **8**: 59–73.