

## Comparison of PCR fingerprinting, by random amplification of polymorphic DNA, with other molecular typing methods for *Candida albicans*

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Fingerprinting by random amplification of polymorphic DNA (RAPD) was compared with existing molecular typing systems for *Candida albicans*. Fifteen isolates were chosen, including three from the same patient; these gave 14 distinct karyotypes by pulsed-field gel electrophoresis (PFGE) and 7 different DNA types by *EcoRI*-generated restriction fragment length polymorphisms (RFLPs). RAPD with primer I (5' GCTGGTGG3') gave 5 types, whereas primer II (5' GCGCACGG3') yielded 11 types. Combining the results from both primers, all isolates were unique by RAPD with the exception of the three from the same patient. RAPD provided a fast, economical and reproducible means of typing *C. albicans* with a level of discrimination approaching that of PFGE.

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### Introduction

The polymerase chain reaction (PCR) can be adapted for fingerprinting micro-organisms by using paired primers derived from previously characterized sequences for PCR amplification. This has been applied to the human fungal pathogen *Cryptococcus* (Vilgalys & Hester, 1990). Recently, a new DNA polymorphism assay has been developed based on PCR amplification of random DNA fragments with single short primers of arbitrary nucleotide sequence (Welsh & McClelland, 1990; Williams *et al.*, 1990). This method has the advantage that no prior sequence information is required. These polymorphisms have been termed random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990). RAPD has been applied to two fungal plant pathogens, *Fusarium solari* (Crowhurst *et al.*, 1991) and *Leptosphaeria maculans* (Goodwin & Annis, 1991).

This paper describes the application of RAPD to the human fungal pathogen *Candida albicans*. *C. albicans* is a common cause of oral and vaginal infections and is

increasingly the cause of severe systemic infections in immunocompromised patients (Odds, 1988; Edwards, 1991). To facilitate studies on the epidemiology of *Candida*, numerous phenotypic and genotypic typing systems have been developed. These have been reviewed by Merz (1990) who emphasized the potential of DNA typing systems such as restriction fragment length polymorphisms (RFLPs) and electrophoretic karyotypes. Digestion with a single restriction enzyme like *EcoRI* yields several fragments present in high enough concentrations to give distinct bands on a stained electrophoretic gel (Scherer & Stevens, 1987). These bands are derived from mitochondrial and ribosomal DNA and they vary sufficiently in size to permit the development of an epidemiologically useful typing system. This has been applied to the identification of outbreaks of candidosis (Matthews & Burnie, 1989; Lee *et al.*, 1991). Although RFLPs have the advantage of being easy and rapid to perform, they have the disadvantage that the number of possible types is limited, with certain patterns appearing very commonly so that sometimes geographically distinct strains appear identical (Lee *et al.*, 1991; Magee *et al.*, 1992). Greater resolving power can be achieved by using a moderately repeated DNA sequence as a probe (Olivo *et al.*, 1987; Scherer & Stevens, 1988; Soll *et al.*, 1988) or by electrophoretic karyotyping by pulsed-field gel electrophoresis (PFGE) (Magee & Magee, 1987; Snell *et al.*,

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Abbreviations: PFGE, pulsed-field gel electrophoresis; RAPD, random amplification of polymorphic DNA; RFLP, restriction fragment length polymorphism.

1987; Merz, 1990; Vazquez *et al.*, 1991; Magee *et al.*, 1992). The disadvantages of both of these procedures are the greater cost and time required compared to RFLP. We therefore investigated the use of RAPD to see whether this technique, which is very convenient, quick and economical to perform, could match the resolving power of electrophoretic karyotyping. It was applied to 14 isolates which appeared distinct by PFGE, and compared to *EcoRI*-generated RFLPs.

## Methods

**Strains.** Fifteen isolates of *C. albicans* were examined, including 13 from 11 patients with disseminated candidosis. The isolates were identified as *C. albicans* by their assimilation pattern with the API 20 C system (Bueschug *et al.*, 1979) and by chlamydospore formation. Isolates 4, 6 and 7 were from the same patient. Isolate 1 was the reference strain NCTC 3153 (serotype A) and isolate 48 was the reference strain NCTC 3156 (serotype B).

**Preparation of yeast DNA for RFLP and RAPD.** After overnight growth in 2% (w/v) glucose nutrient broth, the yeast was harvested by centrifugation, washed in 1 M-sorbitol and incubated at 37 °C for 40 min in 1 ml SE buffer (1.2 M-sorbitol, 0.1 M-EDTA pH 7.5) containing 1 µl 2-mercaptoethanol and 0.3 mg yeast lytic enzyme (Zymolyase 100 T, ICN Biochemicals). The resulting spheroplasts were harvested by centrifugation (2500 r.p.m. for 5 min), washed in SE buffer and resuspended in 0.5 ml 0.15 M-NaCl 0.1 M-EDTA pH 7.5. They were lysed by the addition of proteinase K (final concentration 400 µg ml<sup>-1</sup>) and SDS (1% (w/v) final concentration). RNAase was added (20 µl of a 20 mg ml<sup>-1</sup> stock solution) prior to incubation at 55 °C for 30 min. After centrifugation, the supernatants were extracted twice with phenol/chloroform and once with chloroform. Then the DNA was precipitated with 2-propanol before dissolving in 50 µl TE buffer (10 mM-Tris, 0.1 mM-EDTA pH 8.0).

**Generation of RFLP.** A portion (45 µl) of each sample of *Candida* DNA in TE was digested to completion with *EcoRI*. Digests were analysed on a 20 × 20 cm, 300 ml horizontal 1.2% (w/v) agarose gel in 1 × TBE buffer (89 mM-Tris, 89 mM-boric acid, 2.5 mM-EDTA, pH 8.3) containing ethidium bromide (0.5 µg ml<sup>-1</sup>) and run at 35 V for 24–30 h with *HindIII-EcoRI* digested phage lambda as size markers. It was photographed under UV illumination.

**RAPD.** To the remaining 5 µl of *Candida* DNA was added an equal volume of TE buffer. Then 2.5 µl aliquots were used for the PCR, in a final reaction volume of 100 µl 10 mM-Tris/HCl pH 8.8, 50 mM-KCl, 1.5 mM-MgCl<sub>2</sub> containing 1 µg of primer and 200 µM of each dNTP. Magnesium titration indicated an optimal concentration of 1.5 mM-MgCl<sub>2</sub>. The reaction mix was overlaid with mineral oil and subjected to an initial denaturation at 95 °C for 10 min. Then 2.5 U *Taq* Polymerase (Northumbria Biologicals) were added and PCR was done on a Techne Mark 2 thermal cycler as follows: 1 min at 96 °C, 2 min at 30 °C, 2 min at 72 °C. After completion of 45 cycles, the reaction was held at 72 °C for 7 min. Control tubes with no template DNA were included in each run.

Amplified products were resolved by gel electrophoresis on a horizontal, 10 × 10 cm, 100 ml 1.3% agarose gel in TBE buffer, containing 0.5 µg ethidium bromide ml<sup>-1</sup>. *HindIII-EcoRI* digested phage lambda and *HaeIII* digested  $\phi$ X174 DNA were used as molecular mass markers. The PCR products were resolved by electrophoresis at 50 V for 3 h.

**Primer synthesis.** Oligodeoxynucleotide primers were synthesized by standard phosphoramidite chemistry on an Applied Biosystems PCR

MATE DNA synthesizer. After removal of protecting groups in ammonium hydroxide at 55 °C overnight, the samples were dried under vacuum, dissolved in TE and used without further purification. Primer sequences (5' to 3') were as follows: no. I, GCTGGTGG and no. II, GCGCACGG (Caetano-Anolles *et al.*, 1991).

**PFGE.** Cell samples were prepared as described previously by Khattak *et al.* (1992). Briefly, cells were washed twice in 50 mM-EDTA, pH 7.5 and resuspended in the same buffer to make spheroplasts by incubating at 37 °C for 20 min with 200 µl 2 mg yeast lytic enzyme ml<sup>-1</sup> per 0.3 ml of cell suspension. The cell-enzyme suspension was mixed with 0.9 ml 2% low-temperature-gelling agarose and allowed to solidify in moulds at 20 °C for 5 min. The agarose plugs were then transferred to buffer [0.5 M-EDTA pH 8.0, 0.01 M-Tris/HCl pH 7.5, 7.5% (v/v) 2-mercaptoethanol] and incubated overnight at 37 °C. The plugs were washed three times in 50 mM-EDTA pH 8.0 and cell lysis was completed by adding 0.01 M-Tris/HCl pH 7.5, 0.5 M-EDTA pH 8.0, 1% (w/v) laurylsarcosine, 1 mg Proteinase K ml<sup>-1</sup>. This was incubated for 2 d with gentle shaking. Plugs were washed three times with 50 mM-EDTA pH 8.0 and loaded onto 1% (w/v) agarose gels in 0.5% TBE. Electrophoresis was done in a Bio-Rad CHEF-DRII system for 40 h at 12 °C, with an electric field of 4.2 V cm<sup>-1</sup> for 24 h with a pulse time of 300 s and at 2.4 V cm<sup>-1</sup> for 48 h with a pulse time of 1200 s. Gels were stained with ethidium bromide (0.5 µg ml<sup>-1</sup> in distilled water) for 15 min and destained in distilled water for 3 h. DNA bands were visualized under UV light and photographed.

## Results

### RAPD

Both primers successfully typed all isolates examined (Tables 1 and 2, Figs 1 and 2). Discrimination was greatest with primer II, generating 11 types out of 15 isolates, whereas primer I produced 6 types. With both primers the three isolates from the same patient (isolates

Table 1. RAPD fingerprinting of *C. albicans* with primer no. I

(+), Faint band; DB, double band.

Isolate no.	Bands present				RAPD type
	1	2	3	4	
4	DB	DB	(+)	+	1
111	DB	DB	(+)	+	1
232	DB	—	+	+	2
1	DB	(+)	+	+	3
11	DB	+	(+)	+	3
8	DB	—	+	+	2
22	DB	DB	(+)	+	1
21	DB	+	+	+	3
48	DB	DB	(+)	+	1
617	DB	+	—	+	4
214	DB	—	—	+	5
373	DB	+	(+)	+	3
77	DB	DB	—	+	6
6	DB	DB	(+)	+	1
7	DB	DB	(+)	+	1

\* Extra band between bands no. 3 and 4.

Table 2. RAPD fingerprinting of *C. albicans* with primer no. II

( + ), Faint band; DB, double band.

Isolate no.	Bands present								RAPD type
	1	2	3	4	5	6	7	8	
4	-	+	+	+	-	DB	-	-	1
111	+	(+)	+	+	-	+	-	-	2
232	+	(+)	+	DB	+	DB	+	-	3
1	-	+	+	DB	+	DB	+	-	4
11	+	(+)	+	+	+	+	+	-	5
8	+	+	+	+	-	DB	(+)	-	6
22	+	+	+	+	+	+	+	-	7
21	-	+	+	+	-	DB	-	-	1
48	+	(+)	+	+	(+)	+	-	-	8
617	(+)	(+)	+	(+)	-	DB	-	+	9
214	-	(+)	+	(+)	-	DB	-	-	1
373	+	-	+	+	+	+	(+)	+	10
77	+	(+)	+	(+)	-	-	-	-	11
6	-	+	+	+	-	DB	-	-	1
7	-	+	+	+	-	DB	-	-	1

4, 6 and 7) were indistinguishable from each other. Reproducibility was assessed by repeating the preparation of DNA from the same isolate and amplifying by PCR on two different occasions. Sometimes the bands produced were less intense, but their overall position and whether they were present or absent was highly consistent.

Multiple bands were produced with primer I, but greatest variation occurred among two bands (numbers 2 and 3) just below the constant double band (number 1) at 564 bp (Fig. 1). These two bands were present, absent

or of double width (Table 1). Band 4 was consistently present in all isolates. In the case of isolate 617 an extra band appeared just above band 4.

With primer II, fingerprints were identified by differences in 7 of the 8 relatively intense bands in the size range between the 831 bp and the 3530 bp markers (Fig. 2, Table 2). Band 3 was constant. Bands 1, 2 and 4-8 were variable, being present, absent or, in the case of isolates 4 and 6, of single or double width.

### PFGE

The 14 different PFGE karyotypes (Figs 3 and 4) were compared with a well-characterized reference strain, *C. albicans* SGY126 (Khattak *et al.*, 1992), for estimation of the sizes of each band (Table 3). Bands at 3000 kbp, and 1700 kbp were generally conserved and therefore unhelpful for typing. Bands at 1400 kbp were sometimes replaced by a slightly higher band (isolates 4, 21, 617 and 214) or an extra band occurred just below 1400 kbp (isolates 6 and 7). Similar anomalies occurred with bands at 2200 kbp. The band at 2600 kbp was often absent (isolates 4, 111, 232, 22, 214, 373 and 77). The bands at 1200 kbp, 1070 kbp and 1020 kbp were reproducibly unresolvable for some isolates (numbers 4, 6, 7, 21 and 373). Consistent extra bands occurred below 1020 kbp for isolates 8, 22, 48, 617 or above 1200 kbp for isolate 373. The bands at 1070 kbp and 1020 kbp did not resolve well and only in isolates 617 and 214 could they be clearly distinguished. Of the three isolates from the same patient (Fig. 4), two were indistinguishable by PFGE (isolates 6 and 7) but one was clearly different (isolate 4).

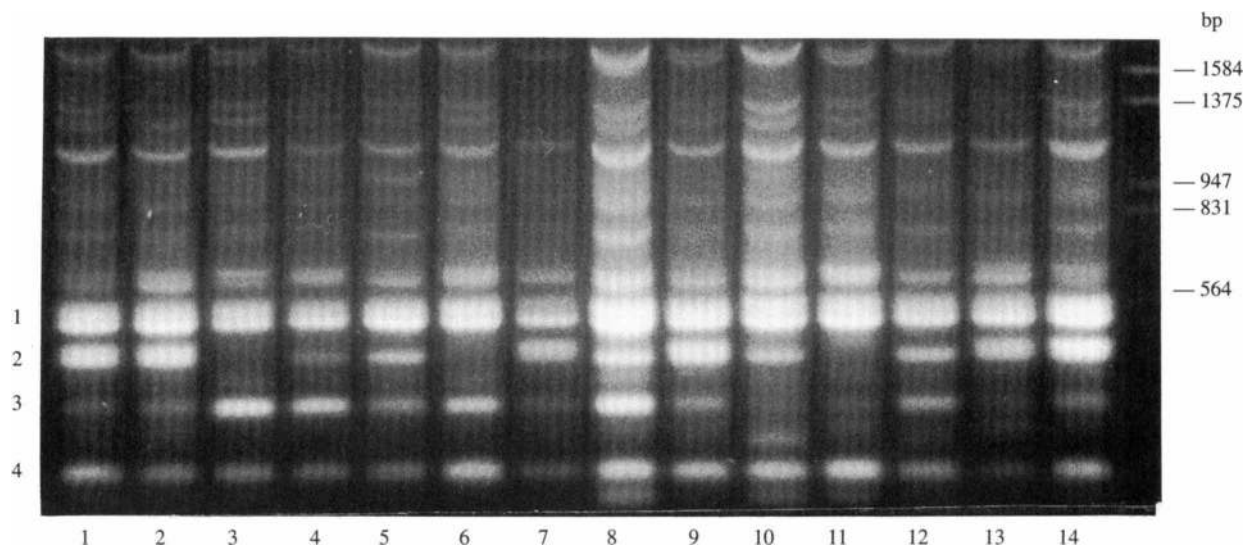


Fig. 1. RAPD fingerprinting with primer I (5' GCTGGTGG 3') showing isolates 4 (lane 1), 111 (2), 232 (3), 1 (4), 11 (5), 8 (6), 22 (7), 21 (8), 48 (9), 617 (10), 214 (11), 373 (12), 77 (13) and 6 (14). Size markers are shown on the right-hand side and band numbers on the left.

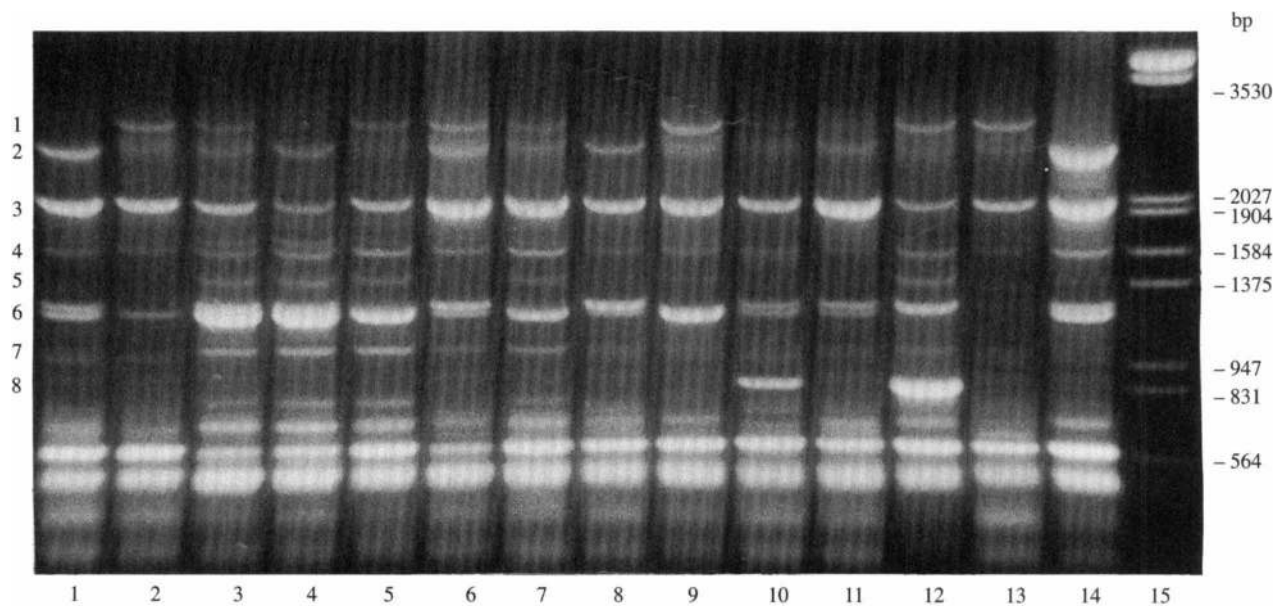


Fig. 2. RAPD fingerprinting with primer II (5'GCGCACGG3') showing isolates 4 (lane 1), 111 (2), 232 (3), 1 (4), 11 (5), 8 (6), 22 (7), 21 (8), 48 (9), 617 (10), 214 (11), 373 (12), 77 (13) and 6 (14). Lane 15, *EcoRI*-*HindIII* cut phage lambda with sizes given on the right. Band numbers are shown on the left.

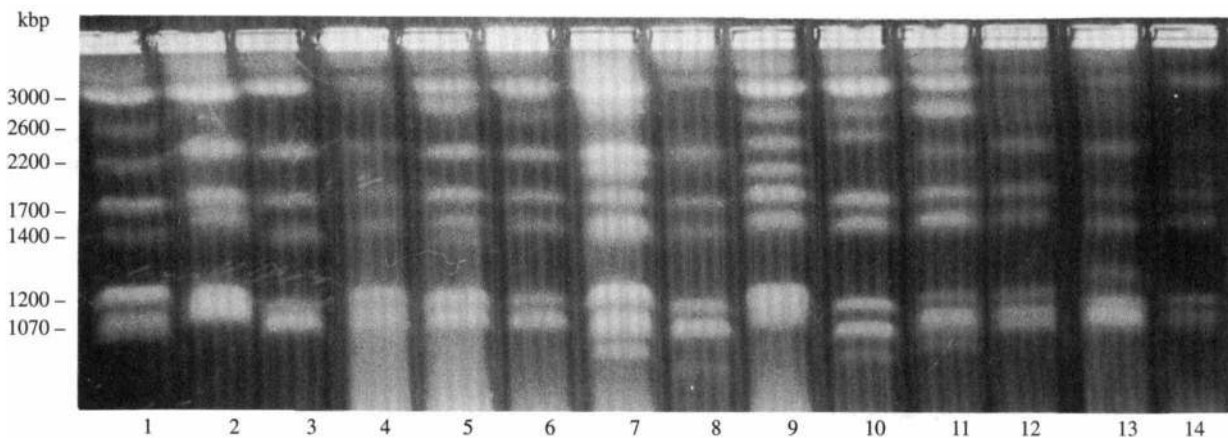


Fig. 3. PFGE karyotyping with isolates 4 (lane 2), 111 (3), 232 (4), 1 (5), 11 (6), 8 (7), 22 (8), 21 (9), 48 (10), 617 (11), 214 (12), 373 (13) and 77 (14). *C. albicans* reference strain SGY 126 is shown in lane 1 and sizes are given on the left.

### RFLP

*EcoRI*-generated RFLP gave rise to 7 different types (Table 4), designated according to previously published criteria (Matthews & Burnie, 1989; Lee *et al.*, 1991).

### Discussion

RAPD compared very favourably with other DNA typing systems for *C. albicans*. With 15 isolates representing 14 different PFGE karyotypes, RAPD with primer II generated 11 different types. Isolates 4, 6 and 7 were from the same patient, with systemic candidosis,

number 4 being an antemortem blood culture isolate and numbers 6 and 7 being postmortem isolates collected 2 d later from the heart and meninges. The latter two isolates were indistinguishable from each other by PFGE but clearly different from the earlier isolate (Fig. 4). In contrast, all three isolates were indistinguishable by RAPD, even with the highly discriminatory primer II (Fig. 2, lanes 1 and 14). This raises the question as to whether, because of the propensity of *C. albicans* to genetic switching (Scherer & Magee, 1990; Soll, 1992), typing by PFGE carries the risk of two genetically similar isolates appearing to be different because of a genetic rearrangement. Alternatively, two different

Table 3. PFGE karyotypes of *C. albicans* isolates

2200 EB, extra band between 1700 and 2200 kbp; 1400 EB, extra band just below 1400 kbp; 1200 EB, extra band just above 1200 kbp; 1020 EB, extra band running below 1020 kbp; 1020 DB, double band at 1020 kbp. UR, bands at 1200 and 1070 and 1020 kbp unresolvable.

Isolate no.	Size of bands present (kbp)							1070/1020	PFGE type
	3000	2600	2200	1700	1400	1200			
4	+	-	+	+	+	UR	UR	1	
111	+	-	+	+	+	+	+	2	
232	+	-	+	+	+	+	+	3	
1	+	+	+	+	+	+	+	4	
11	+	+	+	+	+	+	+	5	
8	+	+	EB	+	+	+	EB	6	
22	+	-	+	+	+	+	EB	7	
21	+	+	EB	+	+	UR	UR	8	
48	+	+	+	+	+	+	EB	9	
617	+	+	+	+	+	+	DB/EB	10	
214	+	-	+	+	+	+	DB	11	
373	+	-	+	+	+	EB/UR	UR	12	
77	+	-	+	+	+	+	+	13	
6	+	+	EB	+	EB	UR	UR	14	
7	+	+	EB	+	EB	UR	UR	14	

\* Band runs high. † Band runs low.

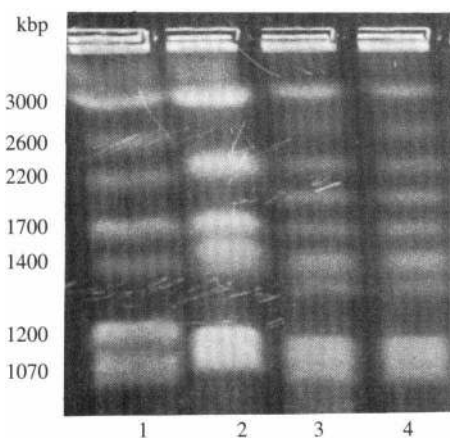


Fig. 4. PFGE karyotyping with three isolates from the same patient: isolates 4 (lane 2), 6 (3) and 7 (4). *C. albicans* SGY 126 (lane 1). Sizes are given on the left.

strains co-existed in this patient, which were differentiated by PFGE but not by RAPD or *EcoRI*-generated RFLP.

RAPD with primer I was less discriminatory than primer II, yielding only 5 types, but it was useful in increasing still further the discriminatory powers of primer II. Isolates 21 and 214 were indistinguishable from isolates 4, 6 and 7 by RAPD with primer II, but clearly different with primer I. By combining the results obtained with both primers, we could identify each of the isolates as unique with the exception of the three isolates (numbers 4, 6 and 7) from the same patient. With the

Table 4. Comparison of molecular typing techniques

Isolate no.	PFGE karyotype	RAPD		RFLP type
		Primer I	Primer II	
4	1	1	1	14
111	2	1	2	1
232	3	2	3	2
1	4	3	4	1
11	5	3	5	2
8	6	2	6	2
22	7	1	7	6
21	8	3	1	14
48	9	1	8	4
617	10	4	9	10
214	11	5	1	2
373	12	3	10	9
77	13	1	11	2
6	14	1	1	14
7	14	1	1	14
No. of types...	14	5	11	7

same isolates, *EcoRI*-generated RFLP generated only 7 types. Although discrimination might be improved with multiple restriction enzymes, experience has shown that the number of possible types generated is limited and some patterns occur very commonly so that geographically distinct strains can appear identical (Vazquez *et al.*, 1991; Magee *et al.*, 1992). Greater discrimination can be achieved by Southern blotting against a suitable probe such as ribosomal DNA but this is relatively time-consuming (Magee *et al.*, 1987).

RAPD has the advantage of being the least labour-intensive of these typing methods, requiring only small amounts of DNA and being relatively easy to standardize between laboratories. It requires little knowledge of the molecular biology of the species being examined and no sequence information is necessary. Unlike PCR typing performed with pairs of primers from a known sequence, it is not confined to changes occurring in only a small part of the genome. Each primer gives a different pattern of PCR products, and a primer with great potential for detecting polymorphisms within one species may be poorer at discriminating between the strains of another species. Sources of polymorphisms include deletions of a priming site and insertions that either increase the size of the PCR product or render the priming sites too distant to support amplification.

If a single DNA typing system is to be adopted, RAPD is probably the first choice for large-scale epidemiological studies of *C. albicans*. It is a quick, economical technique, with high reproducibility and typability, and provides a degree of discrimination approaching that of PFGE. The value of such epidemiological studies lies in the recent evolution of *C. albicans* as a major fungal pathogen in

hospitals, including nosocomial outbreaks, as well as in the community and among patients with AIDS (Edwards, 1991).

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