DNA fingerprinting patterns of *Candida* species using *Hinf*I endonuclease

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Strain delineation was performed by means of restriction endonuclease analysis (REA) of genomic DNA with the restriction enzyme *Hinf*I followed by conventional electrophoresis. A total of 337 yeast isolates representing 21 *Candida* species and five non-*Candida* yeast species was evaluated. A survey of isolates showed that *Candida albicans* and non-*albicans* species could be divided into mutually exclusive groups, and that subgroups could be created. Individual REA patterns for 111 *C. albicans* isolates, four *Candida krusei* isolates and 35 *Candida glabrata* isolates varied greatly, whereas 11 *Candida dubliniensis* isolates, 48 *Candida tropicalis* isolates and 41 *Candida guilliermondii* isolates could be divided into two, nine and nine groups, respectively. REA of the 49 *Candida parapsilosis* isolates with *Hinf*I, however, showed that 47 (95%) of them belonged to one group. REA patterns of the other yeast isolates, representing 19 species, were also quite different at the species level. These results showed that REA with *Hinf*I may be useful for the identification and strain delineation of common and emerging *Candida* species.

Keywords: epidemiology, restriction endonuclease analysis, *Candida*, DNA

INTRODUCTION

The opportunistic *Candida* species cause mucosal and systemic infections in patients with immunosuppressed or neutropenic cancer and in patients with indwelling intravenous catheters. The frequency of infections has prompted several epidemiological studies regarding *Candida* infections (Clemons *et al.*, 1997; Sullivan *et al.*, 1996).

Over the last few years, molecular methods of studying strain relatedness have proven useful, allowing investigators to assess the epidemiological aspects of outbreaks of candidiasis and to identify *Candida* strains. The DNA-based methods used to differentiate *Candida* strains involve a variety of techniques including karyotyping, restriction fragment length polymorphism analysis by pulsed-field gel electrophoresis (PFGE), Southern blot hybridization with probes that hybridize to repetitive genetic elements, PCR fingerprinting and randomly amplified polymorphic DNA (RAPD) fingerprinting (Clemons *et al.*, 1997; Diaz-

Guerra *et al.*, 1997; Stefan *et al.*, 1997; Thanos *et al.*, 1996; Vazquez *et al.*, 1991).

Restriction endonuclease analysis (REA) requires a rather large amount of DNA (i.e. >10 µg) and generates much more complex patterns, but Scherer & Stevens (1987) have clearly demonstrated the ease of performance, reproducibility and stability of REA by conventional electrophoresis for differentiating several *Candida* species. More recently, REA with *Hinf*I digestion of genomic DNA has been shown to be a useful tool for the differentiation of *Candida glabrata* (Arif *et al.*, 1996) and *Candida krusei* (Carlotti *et al.*, 1994). However, the REA epidemiological and taxonomic studies reported so far have used relatively few *Candida* species and a small number of *Candida* strains. In addition, the enzymes used to discriminate *Candida* strains by REA differed among investigators (Magee *et al.*, 1987; Vazquez *et al.*, 1991). These results prompted us to examine a variety of *Candida* and non-*Candida* species to see whether common and uncommon yeast isolates could be reliably distinguished.

METHODS

Strains. A total of 273 isolates (given the prefix K) recovered from clinical materials of different patients were used in this

Abbreviations: PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; REA, restriction endonuclease analysis.

study. These isolates were *Candida albicans* ($n = 96$), *Candida dubliniensis* ($n = 11$), *C. glabrata* ($n = 34$), *Candida guilliermondii* ($n = 28$), *C. krusei* ($n = 3$), *Candida lipolytica*, ($n = 1$), *Candida lusitanae* ($n = 4$), *Candida parapsilosis* ($n = 48$) and *Candida tropicalis* ($n = 48$). Presumptive identification of these isolates was achieved by means of colony colour on CHROMagar Candida and the isolates were definitely identified with API kits such as API 20C and ID 32C (bioMérieux). If necessary, the ability to produce germ tubes and chlamydospores and the ability to grow at 42 °C were tested. Sixty-four isolates that were originally identified at species level were also examined in this study (Table 1). These organisms were obtained from the Institute for Fermentation, Osaka, Japan (IFO), Research Center for Medical Mycology, Teikyo University, Tokyo, Japan (TIMM) and Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Wako, Japan (JCM).

DNA extraction. Genomic DNAs were prepared with the method of Scherer & Stevens (1987) with minor modifications. In brief, each strain was grown on the CHROMagar *Candida* at 35 °C for 1–2 d. One loopful (2 mm in diameter) of the culture was suspended in 2 ml SE medium (1.2 M sorbitol and 0.1 M EDTA, pH 7.5), and centrifuged. The sediments were then resuspended in 0.5 ml SE medium containing 1–2 mg Zymolyase 100T (Seikagaku) and 0.01 ml 2-mercaptoethanol, after which the solution was incubated for 30 min at room temperature. The resultant spheroplasts were packed by centrifugation and suspended in 0.5 ml lysis solution (10 mM Tris, 50 mM EDTA, pH 7.5) with 0.5% SDS to which 0.01 ml proteinase K solution (20 mg ml⁻¹) was added. After 15 min incubation at 55 °C, 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1) was added and the resultant solution was mixed thoroughly with a vortex mixer. After further centrifugation, the aqueous supernatant was transferred to a new microcentrifuge tube. The supernatant was then mixed with 0.05 ml 3 M sodium acetate and 0.55 ml 2-propanol and centrifuged to precipitate the nucleic acids. The sediment was suspended in 0.5 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was treated with 1 µl RNase A (10 mg ml⁻¹) for 5 min at room temperature, after which 5 µl proteinase K solution was added to the DNA sample. After 10 min incubation at 55 °C, DNA was extracted by phenol/chloroform treatment. The purified DNA was resuspended in 0.1 ml TE buffer and an aliquot was removed to determine nucleic acid purity and concentration by spectrophotometry at 260 and 280 nm.

Restriction endonuclease analysis. DNA (15 µg) was incubated with 50 U *HinfI* enzyme for 2 h at 37 °C. The DNA fragments were separated on 1% agarose gels immersed in 0.5 × TBE buffer (45 mM Tris-borate, pH 8.4, 1 mM EDTA) at 1.5 V cm⁻¹ for 4.5 h. DNA was then stained with SYBR Gold (Molecular Probes) for 30 min, and the results were recorded photographically. Differences of two or more intense and sharp bands (major bands) among the isolates were used as sufficient criterion for the designation of different strains. The fingerprints of all the strains were established at least twice. Selected isolates were also digested with a battery of other enzymes, including *HindIII*, *MspI*, *EcoRI* and *HaeIII*. These enzymes were chosen on the basis of the literature (Carloti *et al.*, 1994; Magee *et al.*, 1987; Vazquez *et al.*, 1993). In our preliminary study, digestion patterns with *HindIII*, *MspI*, *EcoRI* and *HaeIII* showed less discriminatory capability and a stronger background compared to those obtained with *HinfI* digestion. Therefore, *HinfI* restriction enzyme was used in the present study.

Computerized analysis. The computer program Diversity Database (PDI) was used to calculate the molecular sizes. The *HinfI* patterns of three strains, *C. albicans* TIMM3169, *C. albicans* K6692 and *C. krusei* K1128, were analysed ten times, following DNA extraction on different cultures. The standard deviations of calculated molecular sizes were ± 0.01 kb for fragments in the range of <3.0 kb, ± 0.02 kb for fragments in the range of 3.0–5.0 kb and ± 0.04 kb for fragments in the range of >5.0 kb by the computer-assisted method. Fragments with molecular sizes that differed by more than two standard deviations were considered different. Pattern reproducibility was ensured by comparing the *HinfI* patterns of five randomly selected strains every three months (over two years) and these controls gave satisfactory results for the patterns.

RESULTS

Sizes of *HinfI*-digested major bands and distribution of DNA groups of *C. albicans* isolates and other yeast strains examined in this study are shown in Table 1. The *HinfI* patterns of *C. albicans* contained two to six major bands of variable sizes in the ranges 7.5–3.3 kb and 2.8–2.0 kb. The patterns of low-molecular-mass fragments (<2.0 kb) were the same among the *C. albicans* strains tested, and a 1.86 kb band was common among these isolates (Fig. 1). As a result, a total of 111 isolates could be separated into 48 groups. Group al34 contained ten isolates, group al37 contained nine isolates, group al36 contained seven isolates, groups al17, al27 and al43 contained five isolates each, and the remaining 42 groups contained one to four isolates. REA patterns of eleven *C. dubliniensis* isolates were quite different from those of *C. albicans*. Two groups were recognized among these isolates: group du1 (nine isolates) and group du2 (two isolates). Two to three intense bands with a size >10 kb (about 15 kb) were characteristic of this species and there were four major bands between 2.9 and 2.3 kb (Fig. 1).

For the 48 *C. tropicalis* strains, the *HinfI* patterns were divided into nine groups and REA patterns of representatives from seven of these groups are shown in Fig. 2. A 2.61 kb band, which was less intense, was demonstrated among all *C. tropicalis* strains tested. Group tr7 contained 18 strains, group tr8 contained eight strains, group tr3 contained five strains, and groups tr1 and tr2 contained four strains each. Further discrimination of *C. tropicalis* strains could not be obtained by digestion with other restriction enzymes or combinations of enzymes. Two groups were recognized by REA with *HinfI* among 49 *C. parapsilosis* strains obtained from 49 epidemiologically unrelated patients (Fig. 2); group pa1 had two isolates and group pa2 had 47 isolates. Group pa2 isolates all had similar REA patterns and were found to have three intense bands of 2.56, 2.14 and 1.79 kb in common. REA of the seven *C. lusitanae* strains revealed four groups: lu1 (one strain), lu2 (one strain), lu3 (two strains) and lu4 (three strains). A 1.83 kb fragment was common to *C. lusitanae* strains (Fig. 2).

Table 1. Distribution of DNA fingerprinting patterns of yeast isolates

Species	Strain*	Synonym	Size of <i>Hinf</i> I-digested major fragments (kbp)†	DNA type	Total no. of strains
<i>C. albicans</i>	K1136		3·34 2·80 2·48 2·37 2·03	al1	1
	IFO 1398	<i>C. stellatoidea</i>	4·21 4·00 2·12	al2	1
	IFO 0692	<i>C. stellatoidea</i>	4·36 4·23 4·01 2·13	al3	1
	K7241		4·58 2·34 2·23	al4	1
	K877		4·58 2·46	al5	2
	IFO 1974 (ATCC 22972)	<i>C. langeronii</i>	4·59 4·43 2·41	al6	1
	TIMM3169 (ATCC 90028)		4·69 2·29	al7	6
	K9080		4·73 2·38	al8	2
	K5789		4·93 4·60 2·38	al9	1
	IFO 0583		4·94 4·89 4·37 2·42	al10	1
	K13019		5·00 4·69 4·47 2·32	al11	1
	IFO 1061 (ATCC 10261)		5·09 4·37 2·29	al12	1
	K1068		5·12 4·46 2·34	al13	3
	IFO 10108		5·19 2·41	al14	4
	K1045		5·20 4·87 4·46 2·29	al15	2
	K5		5·22 5·03 2·38	al16	1
	K999		5·30 2·34 2·28	al17	3
	IFO 1390	<i>Oidiomyces unguium</i>	5·37 2·30	al18	4
	K6692		5·37 4·68 4·13 2·30	al19	1
	K13488		5·41 4·61 2·58 2·41	al20	1
	170		5·41 5·04 4·65 2·33	al21	1
	K952		5·42 2·40	al22	2
	IFO 1385 ^T (ATCC 18804 ^T)		5·46 2·35 2·29	al23	1
	K866		5·47 2·42 2·30	al24	1
	IFO 1594 (ATCC 10231)		5·47 4·88 2·40	al25	3
	K4804		5·51 2·39	al26	2
	K4698		5·58 2·36 2·31	al27	5
	K12255		5·59 2·33	al28	2
	K1094		5·72 4·92 2·38	al29	2
	IFO 1393		5·75 2·38	al30	3
	K1120		5·78 4·90 4·51 2·42	al31	1
	K959		5·79 4·99 2·41	al32	4
	K20299		5·81 5·60 4·40 2·38	al33	1
	K10420		5·82 4·98 2·52	al34	10
	K899		5·82 5·24 2·41	al35	1
	K585		5·83 2·41	al36	7
	K1135		5·89 2·30	al37	9
	K1057		5·89 4·76 2·29 2·14	al38	2
	K1342		5·92 5·60 2·38 2·26	al39	1
	K460		5·96 5·19 2·37	al40	2
	K6774		5·97 5·25 2·47	al41	2
	K343		6·03 5·63 4·59 4·46 2·37 2·26	al42	1
	K1685		6·11 4·50 2·35	al43	5
	K1501		6·57 4·59 2·52 2·40	al44	1
	IFO 0197	<i>Monilia pylori</i>	6·63 5·42 2·30	al45	1
	IFO 1397 (ATCC 11006)	<i>C. stellatoidea</i>	7·11 6·34 5·61 4·98 4·58 2·12	al46	1

Table 1 (cont.)

Species	Strain*	Synonym	Size of <i>Hinf</i> I-digested major fragments (kbp)†	DNA type	Total no. of strains
<i>C. catenulata</i>	IFO 1856 (ATCC 32032)	<i>Syringospora albicans</i>	7·51 5·90 5·49 5·10 2·41	al47	1
	K14773		7·51 6·60 5·87 2·38	al48	1
	IFO 0745 (ATCC 10565)		7·22 6·75 5·13 3·95 3·76 3·45	cat1	1
<i>C. dubliniensis</i>	K745		2·83 2·78 2·46 2·35	du1	9
	K20238		2·88 2·78 2·47 2·35	du2	2
<i>C. famata</i>	IFO 0664	<i>Torulopsis famata</i>	5·66 3·63 2·15 1·75 1·62 1·57	fa1	1
	IFO 856	<i>Torulopsis famata</i>	5·84 3·69 2·20 1·79 1·66 1·56	fa2	1
<i>C. glabrata</i>	K719		2·72 1·51	gl1	6
	K818		2·72 2·51 2·09 1·74	gl2	1
	K16963		2·72 2·54 1·56 1·50	gl3	13
	K1065		2·72 2·66 1·57	gl4	2
	TIMM3171 (ATCC 90030)		2·72 2·66 2·45 1·56	gl4	11
	K8016		2·72 2·66 2·52 1·50	gl5	1
	K867		2·78 2·70 2·49 1·59	gl6	1
<i>C. guilliermondii</i>	IFO 10279 (ATCC 6260)		2·56 2·40 2·27	gu1	2
	IFO 1972	<i>Torulopsis carpophila</i>	2·75 2·43 2·16 2·06 1·91 1·83	gu2	2
	IFO 0566 (ATCC 9058)		2·84 2·40	gu3	6
	IFO 1913 (ATCC 42715)	<i>C. mamillae</i>	2·86 2·57 2·40 2·28	gu1	16
	IFO 0679 (CBS2022)	<i>Torula fermentati</i>	2·99 2·47 2·20 2·02 1·81	gu4	6
	K961		3·12 2·82 2·29	gu5	1
	K111		3·32 2·10 2·02	gu6	1
	JCM 5932		4·25 2·43	gu7	4
	K6793		4·68 2·40	gu8	2
	IFO 0437	<i>Torulopsis miso</i> α	5·96 2·81 2·45 1·97	gu9	1
<i>C. holmii</i>	IFO 1629		7·63 4·19 3·76 3·59 3·22 3·04 2·92 2·69	ho1	1
<i>C. inconspicua</i>	IFO 0621 (ATCC 16783)	<i>Torulopsis inconspicua</i>	7·63 4·19 3·76 3·59 3·22 3·04 2·92 2·69	inc1	1
<i>C. intermedia</i>	IFO 0761 ^T (ATCC 14439 ^T)		1·96 1·89 1·55 1·49	int1	1
<i>C. kefyri</i>	IFO 0586	<i>C. pseudotropicalis</i>	3·87 3·70 2·91 2·14 1·95	ke1	1
	IFO 10287 (ATCC 42266 ^T)		3·86 3·47 2·91 2·60 2·30 2·07 1·85 1·73	ke2	2
	IFO 1065 (ATCC 9767)	<i>C. pseudotropicalis</i>	3·87 3·71 2·92 1·97 1·76	ke3	1
	IFO 0942	<i>Torula cremoris</i>	3·91 3·58 2·92 2·58 1·94 1·90	ke4	3
	IFO 0706 (ATCC 748)	<i>C. macedoniensis</i>	4·71 4·45 3·33 3·15 2·63 2·26 1·91	ke5	1
<i>C. krusei</i>	IFO 0584		5·94 5·75 3·99 3·54 3·06 2·98 2·71	kr1	1
	K987		6·22 5·86 4·46 3·60 3·52 3·35 3·22 3·03 2·70	kr2	1
	K950		6·22 6·09 4·55 4·15 3·69 2·80 2·71	kr3	1
	K1128		6·27 5·99 5·88 4·49 4·06 3·89 3·71 3·63 3·07 2·70	kr4	1
<i>C. lambica</i>	IFO 10289 (ATCC 24750)		2·80 2·30 2·22 2·07 1·95 1·86 1·42	la1	11

Table 1 (cont.)

Species	Strain*	Synonym	Size of <i>Hinf</i> I-digested major fragments (kbp)†	DNA type	Total no. of strains
<i>C. lipolytica</i>	IFO 1549 (ATCC 18945)		2·34 1·95 1·92 1·86 1·78 1·74	li1	2
	IFO 1548 (ATCC 18942)		2·37 1·95 1·90 1·79	li2	2
<i>C. lusitanae</i>	K18948		2·30 1·82	lu1	1
	IFO 10059 ^T (ATCC 38533 ^T)		2·46 1·83	lu2	1
	K6793		2·47 1·94 1·84 1·60 1·49	lu3	2
	IFO 10058 (ATCC 24009)	<i>C. obtusa</i>	2·56 1·83	lu4	3
<i>C. norvegensis</i>	IFO 1020 (ATCC 22977)		3·29 2·70 2·15 1·95 1·70 1·66 1·49	no1	1
<i>C. parapsilosis</i>	K6865		2·02 1·75 1·70 1·57	pa1	2
	IFO 1396 ^T (ATCC 22019 ^T)		2·55 2·13 1·79	pa2	47
<i>C. rugosa</i>	IFO 1364		2·61 2·01 1·95 1·63 1·53 1·46	ru1	1
<i>C. saitoana</i>	IFO 0768	<i>Torula candida</i>	4·75 4·50 4·20 3·37 3·14 2·97 2·12	sa1	1
<i>C. tropicalis</i>	K1910		2·02	tr1	4
	K937		2·01 1·93 1·81	tr2	4
	K887		2·08 1·95 1·91	tr3	5
	K9065		2·13 1·93	tr4	2
	IFO 1400 ^T (ATCC 750 ^T)	<i>Oidium tropicalis</i>	2·13 2·09 1·99 1·95	tr5	3
	IFO 10241 (ATCC 42678)	<i>C. paratropicalis</i>	2·40 2·13 1·93	tr6	4
	K9683		2·47 2·06 1·93	tr7	18
	K765		2·88 2·41 1·95	tr8	8
	K1032		2·96 2·05 1·80	tr9	2
<i>C. utilis</i>	IFO 10707 ^T (ATCC 22023 ^T)	<i>Torula utilis</i>	2·67 2·38 2·32 2·08 2·01 1·80 1·64	ut1	1
<i>C. zeylanoides</i>	IFO 10326 (ATCC 28871 ^T)	<i>Trichosporon piscium</i>	3·30 2·80 2·66 1·88	ze1	1
<i>Dipodascus capitatus</i>	IFO 1197 (ATCC 10663 ^T)	<i>Trichosporon capitatum</i>	2·36 2·22 2·09 2·01 1·53	cap1	1
<i>Pichia anomala</i>	IFO 10213 ^T (ATCC 8169 ^T)	<i>Hansenula anomala</i>	4·13 2·64 2·26 1·85	an1	1
<i>Pichia chambardii</i>	IFO 1274 ^T (ATCC 24239 ^T)		2·26 1·91	ch1	1
<i>Saccharomyces cerevisiae</i>	IFO 0565 (ATCC 9080)		3·52 3·34 2·85 2·67 2·52 2·32 2·22 1·61	ce1	1
<i>Stephanoascus ciferrii</i>	IFO 1854 ^T (ATCC 58443 ^T)		1·91 1·53	ci1	1

* ATCC, American Type Culture Collection, Manassas, VA, USA; IFO, Institute for Fermentation, Osaka, Japan; TIMM, Research Center for Medical Mycology, Teikyo University, Tokyo, Japan; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Wako, Japan; T, type strain.

† All patterns were established at least twice in different gels; mean sizes of fragments are shown.

Representative REA patterns of 41 *C. guilliermondii* isolates are shown in Fig. 3. Nine groups were identified according to two to six intense bands. Group gu1 was the most frequently encountered pattern (18 strains), followed by groups gu3 and gu4 (six strains each), and gu7 (four strains). Two isolates each

belonged to groups gu2 and gu8, and one each belonged to groups gu5, gu6 and gu9.

REA with *Hinf*I divided 35 *C. glabrata* isolates into six groups on the basis of two to four intense bands of size 2·8–1·5 kb; groups gl1 (six strains), gl2 (one strain), gl3

Fig. 1. Representative REA patterns of *Hin*fi-digested DNA from *C. albicans* and *C. dubliniensis* isolates. Lanes 1–10, *C. albicans*; lane 1, TIMM3169; lane 2, IFO 10108; lane 3, IFO 1390; lane 4, IFO 1393; lane 5, K1094; lane 6, K10420; lane 7, K1135; lane 8, IFO 1397; lane 9, IFO 0692; lane 10, IFO 1398. Lanes 11–12, *C. dubliniensis*; lane 11, K745; lane 12, K20238; lane M, 1 kb DNA ladder. Arrows indicate common bands to *C. albicans* (1.86 kb) and *C. dubliniensis* (2.78, 2.46, 2.35, 2.26 and 2.04 kb).

Fig. 2. Representative REA patterns of *Hin*fi-digested DNA from *C. tropicalis*, *C. parapsilosis* and *C. lusitanae* isolates. Lanes 1–7, *C. tropicalis*; lane 1, K937; lane 2, K1910; lane 3, K887; lane 4, K9065; lane 5, K765; lane 6, IFO 10241; lane 7, K9683. Lanes 8–11, *C. parapsilosis*; lane 8, IFO 1396; lane 9, TIMM3172; lane 10, K6865; lane 11, K2797. Lanes 12–15, *C. lusitanae*; lane 12, K18948; lane 13, IFO 10058; lane 14, IFO 10059; lane 15, K6793; lane M, 1 kb DNA ladder. Arrows indicate common bands to *C. tropicalis* (2.61 kb) and *C. lusitanae* (1.83 kb).

(13 strains), gl4 (13 strains), gl5 (one strain) and gl6 (one strain). In addition, several less intense bands made strain discrimination possible for each group of isolates. As a result, REA patterns for all *C. glabrata* isolates were found to differ from strain to strain. Four *C. krusei* isolates demonstrated several intense well-separated bands in the range 6.3–1.7 kb, and three of

these bands (about 2.71, 2.12 and 1.73 kb in size) were found to be common. Eight *Candida kefyr* isolates showed five to eight intense bands of size 4.8–1.7 kb and five groups were recognized. *Hin*fi digestion of 16 strains belonging to 14 other *Candida* and non-*Candida* species that are rarely isolated from clinical materials revealed a unique pattern for each species.

Fig. 3. Representative REA patterns of *Hinf*I-digested DNA from *C. guilliermondii* isolates. Lane 1, IFO 1913; lane 2, IFO 0566; lane 3, IFO 10279; lane 4, JCM 5932; lane 5, K6793; lane 6, IFO 0679; lane 7, IFO 1972; lane 8, K111; lane 9, K961; lane M, 1 kb DNA ladder.

DISCUSSION

The prompt and accurate identification of yeasts is becoming increasingly important because new anti-fungal agents with different activities against various species are being developed (Hazen, 1995) and because *Candida* species may also differ in their virulence characteristics (Wingard, 1995). The Iatron serological *Candida* check kit (Candida Check) permitted rapid (results in 10 min to 5 h) and reliable identification of *Candida* isolates representing eight medically important species (Shinoda *et al.*, 1981). More recently, the RapID Yeast system, which can identify yeast isolates in 4 h incubation, has been evaluated and good agreement was obtained with identifications with the RapID Yeast system and the API 20C kit (results in 72 h) for the eight most common *Candida* species as well as *Cryptococcus neoformans* (Espinel-Ingroff *et al.*, 1998). In contrast, the agreement was lower when emerging *Candida* species and other yeast-like pathogens were tested (Espinel-Ingroff *et al.*, 1998).

Molecular technology for identification of yeast isolates has been reviewed by Sullivan *et al.* (1996), who found that RAPD analysis has many advantages, including simplicity and speed, over other molecular procedures for typing isolates of *Candida*. RAPD analysis takes about 3 h for PCR and 1–2 h for gel electrophoresis (Lehmann *et al.*, 1992; Stefan *et al.*, 1997). Thus, it takes a maximum of only 5 h to identify a species of *Candida* by RAPD fingerprinting after purified DNA is obtained. Latouche *et al.* (1997) also reported the usefulness of PCR fingerprinting for identification of yeast isolates. However, the repro-

ducibility of these PCR-based analyses depends on many factors, each of which must be controlled carefully if accurate and consistent results are to be obtained. Furthermore, the RAPD primer that yields informative fingerprint patterns for strain delineation of *Candida* isolates from clinical specimens has not yet been identified (Reagan *et al.*, 1990).

As for the methodology, Arif *et al.* (1996) reported that their REA required 4 h for enzyme digestion and 16 h for gel electrophoresis. However, in our preliminary study, excellent REA patterns were obtained with 2 h digestion with *Hinf*I, 4.5 h gel electrophoresis and 0.5 h for gel staining, which makes it possible to identify *Candida* isolates to species level on the same day as their isolation. Washing of the gel in water is not necessary after gel staining with SYBR gold instead of ethidium bromide. All the species examined in this study with the REA method have proved to be reasonably homogeneous internally and quite distinct from other species. Although further studies are needed with additional yeast isolates from clinical materials including control strains, results obtained suggest that REA is useful for the identification of *Candida* isolates at species level.

REA has been successfully applied to various micro-organisms for epidemiological studies (Arbeit, 1995). These studies have demonstrated that different M serotypes of *Streptococcus pyogenes* isolates were associated with specific *Hind*III digests (Cleary *et al.*, 1988), and that REA and PFGE had comparable discriminatory powers for epidemiological typing of *Clostridium difficile* (Kristjansson *et al.*, 1994). REA for *Candida* species was initially used by Scherer & Stevens (1987), who predicted that this method, because of its stability and reproducibility, would be useful for large-scale epidemiological studies of *C. albicans*. The most frequently used restriction enzyme for REA is *Eco*RI (Magee *et al.*, 1987; Pearce & Howell, 1991; Reagan *et al.*, 1990; Scherer & Stevens, 1987); *Hinf*I, *Bst*NI, *Hind*III and *Msp*I have also been used by some investigators (Arif *et al.*, 1996; Cutler *et al.*, 1988; Doebbeling *et al.*, 1991; Magee *et al.*, 1987; Reagan *et al.*, 1990), but the discriminatory power of these enzymes is controversial. Our preliminary observation that the *Hinf*I enzyme was more useful for strain differentiation among various *Candida* isolates than *Eco*RI or *Msp*I supports the findings of previous studies by Magee *et al.* (1992) and Carlotti *et al.* (1994).

High-molecular-mass *Hinf*I fragments, previously shown to be characteristic of *C. dubliniensis* (Sullivan *et al.*, 1995), were evident for each of the ten isolates tested but not for the other *Candida* species. *C. dubliniensis* isolates have been shown to form a homogeneous cluster (100% similarity) based on the comparison of 500 bp of the V3 variable region of the large ribosomal subunit genes (Sullivan *et al.*, 1996). As noted in a previous report (Thanos *et al.*, 1996), the *Hinf*I-digested patterns of ten *C. dubliniensis* isolates

studied were very similar to each other, so that only two groups were recognized.

Although *EcoRI*, *BstNI* and *HinfI* have been used for REA (Doebbeling *et al.*, 1991; Reagan *et al.*, 1990; Scherer & Stevens, 1987; Vazquez *et al.*, 1993), the discriminatory capability of this method for *C. tropicalis* strains is not known because of the small number of isolates tested. In our study, about 30% of the *C. tropicalis* isolates clustered into one group. Thus, further strain differentiation by means of PFGE (Vazquez *et al.*, 1993) or by fingerprinting analysis with a cloned repeat sequence probe (Joly *et al.*, 1996) is required for epidemiological studies. These two methods, however, are not suitable for routine application to clinical specimens because of their complexity.

Three distinct groups among *C. parapsilosis* isolates were reported on the basis of REA with *EcoRI* (Scherer & Stevens, 1987) and RAPD profiles (Latouche *et al.*, 1997), whereas seven karyotypes were identified by contour clamped homogeneous field electrophoresis (Carruba *et al.*, 1991). It was surprising that most *C. parapsilosis* isolates (95%) examined by us showed the same REA pattern, suggesting less heterogeneity than other *Candida* species. Lin *et al.* (1995) showed the existence of three distinct genotypes, groups I, II and III, on the basis of isoenzyme profiles and DNA sequences of internally transcribed spacer sequences flanking the 5-8S RNA gene. Because two of these groups (groups II and III) showed significant genetic dissimilarity from the type strain of *C. parapsilosis* (Roy & Meyer, 1998), it is strongly suggested that they represent distinct species or subspecies. Two isolates of group pa1 in our study seemed to belong to group III, because the REA pattern obtained by double digestion with *HaeIII* and *HindIII* was similar to that of group III presented by Roy & Meyer (1998). Among 37 *C. lusitaniae* isolates, 14 electrophoretic karyotypes were reportedly found compared to only eight different types by REA (Vazquez *et al.*, 1993). King *et al.* (1995) reported that 11 epidemiologically unrelated strains had distinct patterns identified by four DNA-based fingerprinting analyses, which did not include REA. These previous reports indicate a high degree of heterogeneity among *C. lusitaniae* strains. Although only six *C. lusitaniae* strains were examined, three distinct patterns were shown in our study.

Few data are available regarding the molecular typing of *C. guilliermondii* (Magee *et al.*, 1987). However, San Millan *et al.* (1997) recently showed five distinct groups among clinical isolates of *C. guilliermondii* on the basis of isoenzyme and RAPD analyses. They demonstrated that the *Candida fermentati* CBS 2022 isolate belonged to one of these groups. In the present study, a wide degree of genetic heterogeneity was found in *C. guilliermondii* and nine groups were established among the 41 isolates. For the differentiation of *C. glabrata* isolates by REA, Vazquez *et al.* (1993) classified 34 isolates into 14 groups with *EcoRI* and further

subdivided these isolates into four subgroups with *MspI*. However, Arif *et al.* (1996) demonstrated that *HinfI* was a more discriminatory enzyme than *EcoRI* or *MspI*, and managed to distinguish 13 strains from 14 sources. Similar results were obtained in this study; each of the 30 *C. glabrata* isolates showed a separate and distinct REA pattern. Moreover, the results of REA with *HinfI* confirmed the observations of previous investigators regarding the usefulness of REA for strain delineation of *C. krusei* (Berrouane *et al.*, 1996; Carlotti *et al.*, 1994). In addition, this typing method enabled an excellent identification of *C. krusei* isolates because of the characteristic REA patterns typical of this species.

In a review of typing methods for *Candida* species, Arbeit (1995) listed five criteria for an effective typing method, namely, typeability, reproducibility, discriminatory power, ease of interpretation and ease of performance. Other factors to consider are cost and rapidity. In this study, well-defined bands of >1.5 kb in size were obtained from yeast isolates tested by REA with *HinfI*, showing unique REA patterns for each *Candida* species. The disadvantage of using REA for the initial typing system is the limited number of reference fingerprintings. Comparison of unknown isolates requires cross-reference to a known standard, which at this stage has been established for only 22 *Candida* species. Therefore, further study of REA patterns with common opportunistic yeasts as well as uncommon yeast pathogens is necessary to overcome this problem. Another problem with molecular typing of *Candida* species is the lack of standardization in terms of methodology, interpretation and reporting of results. Therefore, establishment of reference strains for the REA method and a computerized analysis system may be necessary.

In conclusion, REA with *HinfI* enzyme followed by conventional electrophoresis was found to be useful for the identification and strain delineation of *Candida* species.

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