

Genetic relatedness of *Candida* strains isolated from women with vaginal candidiasis in Malaysia

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The aims of this study were to compare the genetic relatedness of: (i) sequential and single isolates of *Candida* strains from women with recurrent vaginal candidiasis (RVC); and (ii) *Candida* strains from women who had only one episode of infection within a 1-year period. In total, 87 isolates from 71 patients were cultured, speciated and genotyped by random amplification of polymorphic DNA (RAPD) analysis. Patients were categorized into three groups, namely those with: (i) a history of RVC from whom two or more yeast isolates were obtained (group A); (ii) a history of RVC from whom only a single isolate was obtained (group B); and (iii) a single episode of vaginal candidiasis within a 1-year period (group C). Six yeast species were detected: *Candida albicans*, *Candida glabrata*, *Candida lusitanae*, *Candida famata*, *Candida krusei* and *Candida parapsilosis*. Interestingly, the prevalence of non-*albicans* species was higher in group A patients (50 %) than in patients in groups B (36 %) or C (18.9 %). Eighty RAPD profiles were observed, with a total of 61 polymorphic PCR fragments of distinct sizes. Clustering analysis showed that, overall, the majority of patients in group A had recurrent infections caused by highly similar, but not identical, sequential strains [mean pairwise similarity coefficient (S_{AB}) = 0.721 ± 0.308]. The range of mean S_{AB} values for intergroup comparisons for *C. albicans* isolates alone was 0.50–0.56, suggesting that there was no significant relatedness between strains from different groups. Genetic similarity of *C. albicans* isolates from patients in group A was lower than that of *C. albicans* isolates from patients in group C (mean S_{AB} = 0.532 ± 0.249 and 0.636 ± 0.206, respectively); this difference was statistically significant (P = 0.036). These results demonstrate that the cause of recurrent infections varies among individuals and ranges between strain maintenance, strain microevolution and strain replacement; the major scenario is strain maintenance with microevolution. They also show that *C. albicans* strains that cause recurrent infections are less similar to each other than strains that cause one-off infections, suggesting that the former may represent more virulent subtypes.

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INTRODUCTION

Opportunistic yeasts that belong to the genus *Candida* are causative agents of vaginal candidiasis. About 75 % of women will have at least one episode of vaginal infection during their lifetime (Lanchares & Hernandez, 2000); 15–20 % of women without any specific predisposing factor will experience repeated infections within 3 months of treatment (Mercure *et al.*, 1993). *Candida albicans* is the dominant species that causes vaginitis, with a prevalence of 70–90 % in various reports; other *Candida* species, such as *Candida glabrata* (approx. 5–10 %), *Candida tropicalis* (5–10 %),

Candida parapsilosis and *Candida krusei* (2–5 %), are less common pathogens in vaginitis. However, a significant increase in non-*albicans* species (up to 20–30 % in some cases) has been reported to be associated with recurrent candidiasis (Nyirjesy *et al.*, 1995).

Recurrent vaginal candidiasis (RVC) can be defined as the occurrence of at least four mycologically proven symptomatic episodes within 12 months (Sobel, 1992), or at least three episodes unrelated to antibiotic therapy within 1 year (Ringdahl, 2000). Two main hypotheses have been proposed for the cause of recurrent vaginitis: (i) reinfection through sexual transmission or from reservoirs in other organ systems (e.g. gastrointestinal or urinary tracts) (Fidel & Sobel, 1996); (ii) relapse due to incomplete eradication of *Candida* species

Abbreviations: RAPD, random amplification of polymorphic DNA; RVC, recurrent vaginal candidiasis; S_{AB} , pairwise similarity coefficient.

that have increased virulence or are drug-resistant (Sobel, 1993; Faro, 1996). Decreased cell-mediated local immunity is also a risk factor for RVC (Fidel & Sobel, 1996).

In a study on 18 patients with RVC, Lockhart *et al.* (1996) used three probes to evaluate the genetic relatedness of *Candida* strains; they reported that the predominant scenario was strain maintenance with minor genetic variations, a process that they termed 'substrain shuffling'. Vazquez *et al.* (1994) found that eight of ten patients maintained a single strain of *C. albicans* through sequential infectious episodes but, in two patients, the strain had undergone replacement by another strain. In a similar study, Mercure *et al.* (1993) demonstrated that the majority of patients had maintenance of a single strain, whereas a few patients had strain replacement. These studies were focused only on *C. albicans*, whereas patients with non-*albicans* infections were excluded. Therefore, the present study aimed to identify the different *Candida* species that give rise to recurrent infections in a representative Malaysian population and to assess their genetic relatedness by using random amplification of polymorphic DNA (RAPD).

METHODS

Patients and vaginal swab specimens. In total, 87 high vaginal swabs were collected from 71 patients who visited the Obstetric and Gynaecology Clinic in Hospital Kuala Lumpur, the main government hospital in the capital city of Malaysia; the University Malaya Medical Centre, a teaching hospital; and also Hospital Seremban, a district hospital in another state. The common criterion of patients included in this study was the complaint of vaginal itch and discharge. The patients comprised women from different races and a wide age range (19–49 years). Specimens were collected using sterile Dacron swabs and placed in Amies Transport Medium with Charcoal (Difco) and sent on the same day to the laboratory for culture. Informed consent was obtained from each patient that participated in the study. Patients were categorized into three groups: those with a history of RVC from whom two or more *Candida* isolates were obtained within a 1-year period (designated group A); those with a history of RVC from whom only one isolate was obtained within the study period (group B); and those with a single episode of vaginal candidiasis in a 1-year period (group C).

Vaginal secretions were cultured for 2–7 days at 37 °C on Sabouraud's dextrose agar (Difco) alone, and also supplemented with chloramphenicol (final concentration, 50 µg ml⁻¹). Morphologically distinct colonies from each culture were subcultured and stored on Sabouraud's dextrose slant for species differentiation, DNA fingerprinting and storage. *C. albicans* was identified by using the germ-tube test (Rippon, 1974). *Candida* species were also identified by using the biochemical sugar assimilation test (Rippon, 1974) as well as the API 20C AUX yeast identification kit (bioMérieux).

DNA extraction and RAPD-PCR. Genomic DNA was isolated from each *Candida* strain for genotyping. A loopful of a single colony of *Candida* was added to 2.0 ml Sabouraud's dextrose broth and incubated with shaking at 37 °C for 48 h. Yeast cells were collected by centrifugation, resuspended in 1.0 ml PBS and washed twice with PBS. The cell pellet was resuspended in 500 µl lysis buffer [50 mM Tris/HCl (pH 7.5), 10 mM EDTA, 0.5 % β-mercaptoethanol and 0.2 mg lyticase ml⁻¹ (Sigma)] and incubated at 37 °C for 30 min on a rotary shaker (150 r.p.m.). Lysed cells were treated with 25 µl 20 % (v/v) SDS and 2.5 µl proteinase K (20 mg ml⁻¹), incubated at 56 °C for 60 min and then boiled for 5 min. DNA was

extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1, pH 8.0; Pierce Biotechnology). DNA was then precipitated with 0.1 vol 3 M sodium acetate (pH 5.2) and an equal volume of 2-propanol; the resultant pellet was washed with cold 70 % (v/v) ethanol and finally resuspended in 20 µl sterile distilled water.

An arbitrary oligonucleotide primer, PA03 (5'-AGTCAGCCAC-3'; custom-made by Operon Technologies) was used for DNA fingerprinting, using the conditions described by Xu *et al.* (1999) with slight modifications. DNA amplifications were performed in 25 µl volumes that contained 10–40 ng DNA template, 2.5 µl 10× PCR buffer, 1.5 µl 25 mM MgCl₂, 20 pmol PA03 primer, 2.5 µl 0.5 mM dNTP mixture and 0.5 U SuperTherm DNA polymerase (Bertec Enterprise). For the negative control, sterile distilled water was used as substitute for the DNA template. RAPD-PCR was carried out by using a thermal cycler (Biometra) with an initial denaturation step of 97 °C for 3 min, followed by 45 cycles of 60 s at 93 °C, 60 s at 36 °C and 120 s at 72 °C, and a final extension cycle of 5 min at 72 °C. Amplicons were separated by electrophoresis in 1.5 % agarose gel with added ethidium bromide (final concn, 0.5 µg ml⁻¹) in 1× TBE buffer for 4 h at 2 V cm⁻¹, using the Maxicell Primo horizontal gel electrophoresis system (EC Apparatus). Bands were visualized by using a UV-light transilluminator and pictures were captured digitally with the Gene Genius BioImaging system (Syngene).

Analysis of RAPD profiles and statistical test. The RAPD technique produced a mixture of fragments from each DNA sample that was compared. When these fragments were separated by gel electrophoresis in an immobile gel, a characteristic pattern of bands was obtained from each sample. When fragment mixtures from two or more samples were compared, the total number of unique bands produced by a particular DNA sample compared to all other samples was determined. To compare DNA samples that were run in different gels or in non-adjacent lanes of the same gel, the molecular sizes of all bands were computed by using the GeneTool software (Syngene); bands with highly similar or identical sizes were deemed to be homologous. Subsequently, the presence or absence of every band in each pattern was recorded in a chart. Relatedness of the DNA samples was assessed using the RAP-Distance program (Armstrong *et al.*, 1994). The Dice metric (Dice, 1945) was the statistical method chosen to calculate the similarity coefficients (S_{AB}) of sample pairs. S_{AB} was calculated as follows: $S_{AB} = 2n_{11}/(2n_{11} + n_{01} + n_{10})$, where n_{11} represents the number of bands shared by samples A and B, n_{01} represents the number of bands present in sample A but not in sample B and n_{10} represents the number of bands present in sample B but absent in sample A.

Dendrograms based on a pairwise distance ($1 - S_{AB}$) matrix of the DNA samples (which determines within- and between-group similarities) were generated by UPGMA. The MEGA software package, version 2.1 (Kumar *et al.*, 2001) was used to generate the UPGMA phenograms. A paired *t*-test was used to compare the mean S_{AB} values among defined groups.

In order to compare the distribution of species among different groups of patients, species diversity for each group was determined. Species diversity was calculated as $(1 - \sum p_i^2)$, where p_i represents the frequency of a particular species (Xu *et al.*, 1999). The species diversity ranges from 0, where all isolates are of the same species, to 1, where every isolate is of a different species.

RESULTS

Species distribution and diversity

In total, 120 clinical specimens were collected from 180 patients during the course of this study, but only 87 isolates were used for the analysis as the remaining specimens were

either culture-negative or were cases of bacterial or *Trichomonas* vaginosis. In group A, isolates from two or more consecutive episodes of *Candida* vaginitis were obtained from each of the 12 patients who had a history of RVC ($n = 28$); one isolate was obtained from each of the 22 patients in group B ($n = 22$). Group C comprised patients ($n = 37$) who had only a single episode of vaginal candidiasis over a 1-year period. Among the 87 *Candida* isolates obtained, *C. albicans* was the predominant species detected (70.1 %), followed by *C. glabrata* (14.9 %), *Candida lusitanae* (5.7 %), *Candida famata* (4.6 %), *C. krusei* (2.3 %) and *C. parapsilosis* (2.3 %). Table 1 summarizes the strains of

Table 1. *Candida* species isolated and analysed from each specimen

Patient code	Pregnancy status*	No. isolates	Strain designation	<i>Candida</i> species
Group A				
pLML	N	3	LML1	<i>C. glabrata</i>
			LML2	<i>C. glabrata</i>
			LML3	<i>C. glabrata</i>
pELZ	N	2	ELZ1	<i>C. glabrata</i>
			ELZ2	<i>C. glabrata</i>
pHRY	Y	2	HRY1	<i>C. albicans</i>
			HRY2	<i>C. albicans</i>
pZKH	Y	2	ZKH1	<i>C. albicans</i>
			ZKH2	<i>C. albicans</i>
pLSL	N	2	LSL1	<i>C. lusitanae</i>
			LSL2	<i>C. lusitanae</i>
pNFZ	Y	2	NFZ1	<i>C. albicans</i>
			NFZ2	<i>C. albicans</i>
pLSY	N	2	LSY1	<i>C. albicans</i>
			LSY2	<i>C. albicans</i>
pMHR	N	2	MHR1	<i>C. albicans</i>
			MHR2	<i>C. albicans</i>
pMHS	N	2	MHS1	<i>C. glabrata</i>
			MHS2	<i>C. glabrata</i>
pNML	N	2	NML1	<i>C. albicans</i>
			NML2	<i>C. albicans</i>
pGCL	N	2	GCL1	<i>C. albicans</i>
			GCL2	<i>C. krusei</i>
pTHN	Y	5	THN1	<i>C. albicans</i>
			THN2	<i>C. albicans</i>
			THN3	<i>C. albicans</i>
			THN4	<i>C. albicans</i>
	N		THN5	<i>C. lusitanae</i>
Group B				
pHNS	N	1	HNS	<i>C. glabrata</i>
pDVK	N	1	DVK	<i>C. glabrata</i>
pMZL	N	1	MZL	<i>C. glabrata</i>
pKTK	N	1	KTK	<i>C. albicans</i>
pHMG	N	1	HMG	<i>C. albicans</i>
pLLC	Y	1	LLC	<i>C. albicans</i>
pVJR	Y	1	VJR	<i>C. albicans</i>
pLBY	N	1	LBY	<i>C. albicans</i>

Table 1. cont.

Patient code	Pregnancy status*	No. isolates	Strain designation	<i>Candida</i> species
pFRD	Y	1	FRD	<i>C. lusitanae</i>
pCSY	N	1	CSY	<i>C. glabrata</i>
pTVM	N	1	TVM	<i>C. krusei</i>
pZRD	Y	1	ZRD	<i>C. albicans</i>
pDNP	Y	1	DNP	<i>C. albicans</i>
pFZL	Y	1	FZL	<i>C. famata</i>
pALM	Y	1	ALM	<i>C. albicans</i>
pJSY	Y	1	JSY	<i>C. albicans</i>
pSTY	N	1	STY	<i>C. albicans</i>
pPNT	N	1	PNT	<i>C. albicans</i>
pAMN	N	1	AMN	<i>C. albicans</i>
pRJS	N	1	RJS	<i>C. glabrata</i>
pLCM	N	1	LCM	<i>C. albicans</i>
pVML	N	1	VML	<i>C. albicans</i>
Group C				
pKGV	Y	1	KGV	<i>C. albicans</i>
pYSY	Y	1	YSY	<i>C. albicans</i>
pTBL	N	1	TBL	<i>C. parapsilosis</i>
pVSN	N	1	VSN	<i>C. albicans</i>
pRMW	Y	1	RMW	<i>C. glabrata</i>
pSRP	Y	1	SRP	<i>C. albicans</i>
pTCL	Y	1	TCL	<i>C. albicans</i>
pMHW	N	1	MHW	<i>C. albicans</i>
pNHZ	N	1	NHZ	<i>C. albicans</i>
pALG	Y	1	ALG	<i>C. albicans</i>
pRNH	Y	1	RNH	<i>C. albicans</i>
pNAZ	Y	1	NAZ	<i>C. albicans</i>
pLSY	Y	1	LSY	<i>C. albicans</i>
pANM	N	1	ANM	<i>C. albicans</i>
pRNJ	N	1	RNJ	<i>C. parapsilosis</i>
pPTI	Y	1	PTI	<i>C. albicans</i>
pMRZ	N	1	MRZ	<i>C. albicans</i>
pZTN	N	1	ZTN	<i>C. albicans</i>
pJVM	N	1	JVM	<i>C. albicans</i>
pNLH	N	1	NLH	<i>C. albicans</i>
pASB	N	1	ASB	<i>C. albicans</i>
pNSR	N	1	NSR	<i>C. albicans</i>
pSTF	Y	1	STF	<i>C. albicans</i>
pPAH	N	1	PAH	<i>C. lusitanae</i>
pSYR	Y	1	SYR	<i>C. albicans</i>
pSHR	N	1	SHR	<i>C. albicans</i>
pMGW	Y	1	MGW	<i>C. albicans</i>
pARK	N	1	ARK	<i>C. famata</i>
pZLH	N	1	ZLH	<i>C. albicans</i>
pRKH	Y	1	RKH	<i>C. albicans</i>
pVST	N	1	VST	<i>C. famata</i>
pSRA	Y	1	SRA	<i>C. albicans</i>
pGNK	Y	1	GNK	<i>C. famata</i>
pMLV	Y	1	MLV	<i>C. albicans</i>
pSZN	Y	1	SZN	<i>C. albicans</i>
pWRH	Y	1	WRH	<i>C. albicans</i>
pRJG	N	1	RJG	<i>C. albicans</i>

*Y, Pregnant; N, not pregnant.

Candida species that were isolated and analysed from each patient; Table 2 shows the distribution of each *Candida* species within the different patient groups. Species diversities for each group of patients are presented in Table 3. Thirty-one patients were pregnant at the time of specimen collection and there was a high prevalence ($n = 27$, 87.1%) of *C. albicans* isolated from these patients. Non-*albicans* *Candida* species were isolated from only four of these pregnant patients, with *C. famata* in two patients and *C. glabrata* and *C. lusitaniae* in one patient each (3.2%).

Interspecies RAPD profiles

For 87 isolates from 71 patients, 61 polymorphic PCR fragments of distinct sizes were detected and scored. However, only 80 RAPD profiles were obtained (seven of the isolates failed to produce any PCR amplicons, despite repeated attempts). Fig. 1 parts (a), (b) and (c) are representative of the electrophoresis of PCR products obtained from isolates of patients in groups A, B and C, respectively.

Substantial interspecies differences in RAPD profiles were observed when isolates of *C. albicans*, *C. glabrata*, *C. lusitaniae*, *C. famata*, *C. krusei* and *C. parapsilosis* were compared, as evident from Fig. 1. The number and size of DNA fragments generated were fairly characteristic for each species, especially *C. glabrata*, *C. lusitaniae* and *C. krusei*. However, *C. albicans*, *C. famata* and *C. parapsilosis* had similar patterns to each other. Despite the relatively similar array of species-specific DNA fragments, distinct profiles generated by RAPD analysis were observed for *C. albicans* isolates, as shown by that of *HRY2* compared with that of *ZKH1* (Fig. 1a). The former generated bands that ranged from 200 to 1400 bp, whereas the latter produced bands that ranged from 200 to 1700 bp. Most *C. albicans* isolates

produced bands that resembled the pattern of *HRY2*. However, some *C. albicans* isolates only produced between one and three bands. *C. krusei* isolates also produced two different profiles, as seen in those of *GCL2* and *TVM*.

Genetic similarity of *Candida* species within patients in group A

Among the 12 patients in group A, five sequential *Candida* isolates were obtained from one patient (pTHN; *THN1*–5), three isolates from another patient (pLML; *LML1*–3) and two isolates from each of the remaining ten patients. To assess the genetic similarity of sequential isolates within an individual, S_{AB} values between each pair of isolates were computed and tabulated in Table 4.

As a quantitative measure of the level of unrelatedness of isolates, a collection of unrelated isolates (each obtained from different individuals) must be used as a control collection for determination of the threshold of unrelatedness (Marco *et al.*, 1999). For the present study, 25 unrelated *C. albicans* isolates (each collected from a different patient in group C) were used as the control collection. The mean S_{AB} value for this control collection was 0.60 ± 0.20 , which represents an estimate of unrelatedness for vaginal isolates that were used in this study. Based on the empirical estimation of unrelatedness derived from the control group, an S_{AB} value of 1.00 represents identical strains, an S_{AB} value of 0.80–0.99 represents highly similar (but non-identical) strains and usually reflects microevolution of a single strain, and an S_{AB} value of < 0.80 represents unrelated strains.

Pairwise similarities of only *C. albicans* isolates ($n = 17$) in group A patients were compared. It was found that 12.5% of *C. albicans* isolates were genotypically identical, 56.25% were highly similar (but non-identical) while 31.25% of the isolates were unrelated. Fig. 2 presents the genetic similarity of all 28 isolates of different *Candida* species from group A patients in a UPGMA phenogram. This figure shows that isolates of the same species clustered together, with one exception: isolate *ZKH1*, which was identified morphologically and biochemically as *C. albicans*, was more closely related to *C. glabrata* and *C. krusei* in the phenogram.

The three isolates obtained from patient pLML were genotypically identical to each other, with an S_{AB} value of 1.00. The isolates were also clustered together on the phylogenetic tree (Fig. 2). The most closely related but non-identical strains from two different isolates of a single patient were from patients pELZ, pLSL and pMHS, as both of their strains of *Candida* species clustered together on the phylogenetic tree. The two different isolates of patients pNFZ, pLSY and pNML were also related to each other, although not as closely related as the isolates from pELZ, pLSL and pMHS.

For patient pTHN, who had five consecutive isolates, the first four isolates (*THN1*, *THN2*, *THN3* and *THN4*) were fairly closely related to each other, whereas the fifth isolate, *THN5*, was totally unrelated to the first four isolates ($S_{AB} < 0.8$). This was because this patient was colonized by a different

Table 2. Prevalence of *Candida* species isolated from each group

Species: 1, *C. albicans*; 2, *C. glabrata*; 3, *C. lusitaniae*; 4, *C. famata*; 5, *C. krusei*; 6, *C. parapsilosis*. Values are percentages of each species.

Group	1	2	3	4	5	6
A ($n = 28$)	60.7	25.0	10.7	0.0	3.6	0.0
B ($n = 22$)	63.6	22.7	4.5	4.5	4.5	0.0
C ($n = 37$)	81.1	2.7	2.7	8.1	0.0	5.4

Table 3. Species diversity for each group of patients

Group	Species diversity*
A ($n = 28$)	0.5676
B ($n = 22$)	0.5370
C ($n = 37$)	0.3815

*Species diversity is calculated as $1 - \sum p_s^2$, where p_s represents the frequency of a particular species (Xu *et al.*, 1999).

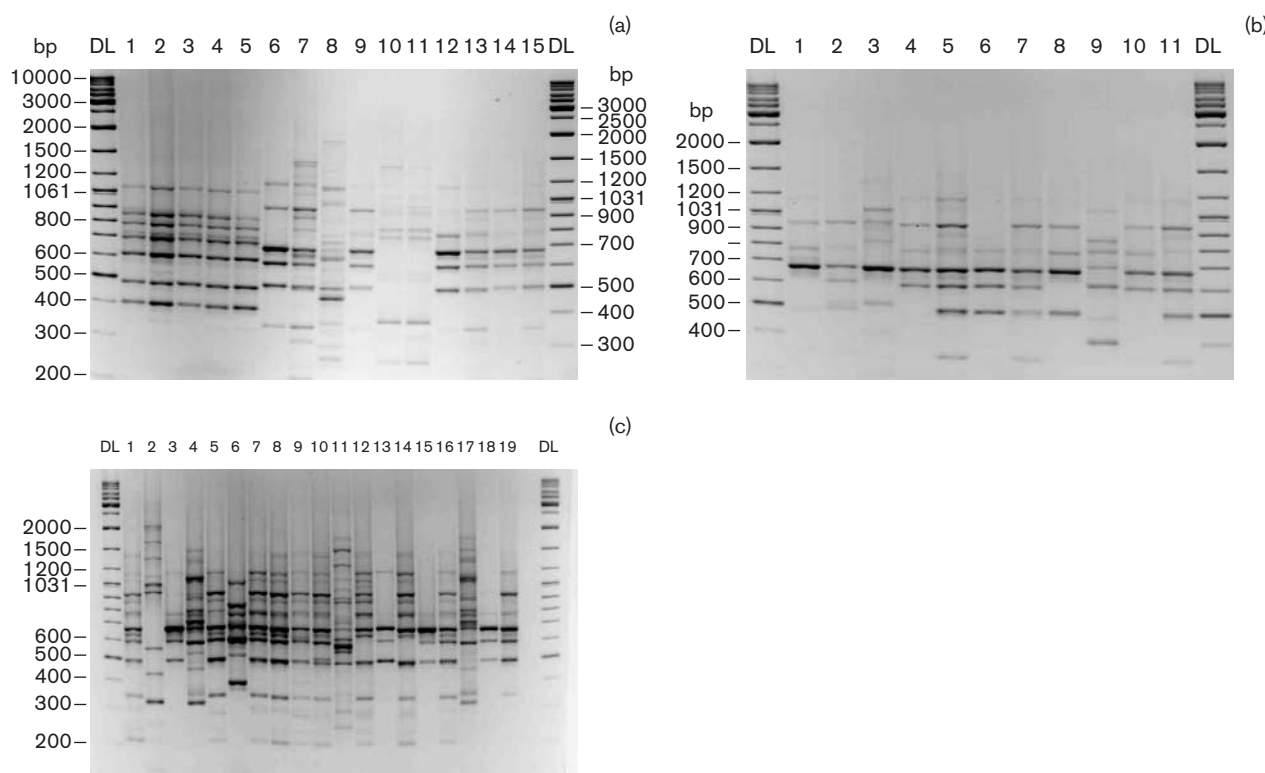


Fig. 1. (a) Electrophoretic separation of RAPD-PCR products obtained with primer PA03 from vaginal *Candida* isolates of selected patients in group A. Molecular size markers (bp) are presented at both sides of the photograph. Lanes: DL, GeneRuler DNA Ladder Mix (Fermentas); 1–3, profiles of *C. glabrata* isolates LML1, LML2 and LML3 from patient pLML; 4–5, *C. glabrata* isolates ELZ1 and ELZ2 from patient pELZ; 6–9, *C. albicans* isolates HRY1, HRY2, ZKH1 and ZKH2 from patients pHRY and pZKH; 10–11, *C. lusitanae* isolates LSL1 and LSL2 from patient pLSL; 12–14, *C. albicans* isolates NFZ1, LSY1 and LSY2 from patients pNFZ and pLSY. (b) Electrophoretic separation of RAPD-PCR products obtained with primer PA03 from vaginal *Candida* isolates of selected patients in group B. Molecular size markers (bp) are presented at to the left of the photograph. Lanes: DL, GeneLadder DNA Ladder Mix (Fermentas); 1, 2, 4–8, 10 and 11, profiles of *C. albicans* isolates from patients pZRD, pDNP, pALM, pJSY, pSTY, pPNT, pAMN, pLTC and pVMR, respectively; 3, *C. famata* isolate from patient pFZL; 9, *C. glabrata* isolate from patient pRJS. (c) Electrophoretic separation of RAPD-PCR products obtained with primer PA03 from vaginal *Candida* isolates of selected patients in group C. Molecular size markers (bp) are presented to the left of the photograph. Lanes: DL, GeneRuler DNA Ladder Mix (Fermentas); 1, profile from patient pKGV (*C. albicans*); 2, unidentifiable fungal species from a patient excluded from the analysis; 3, 5 and 7–16, *C. albicans* isolates from patients pYSY, pVSN, pSRP, pTCL, pMHW, pNHZ, pRNV, pALG, pRNH, pNAZ, pLSY, pANM, respectively; 4 and 17, *C. parapsilosis* isolates from patients pTBL and pRNJ, respectively; 6, *C. glabrata* isolate from patient pRMW; 18 and 19, *C. albicans* isolates from patients pPTI and pMRZ, respectively.

Candida species during the fifth episode of vaginitis compared with the first four episodes of infection. For patient pGCL, the second isolate obtained was of a different *Candida* species (*C. krusei*) from the first isolate (*C. albicans*).

Although conventional methods for the identification of *Candida* species showed that some of the sequential isolates were phenotypically identical, RAPD analysis revealed them to be genotypically different, as observed for ZKH1 vs ZKH2, HRY1 vs HRY2 and MHR1 vs MHR2. Analysis of *C. albicans* isolates gave rise to the observation of two distinct groups in the phenogram. The first group consisted of ZKH1, the only isolate that was on its own and did not cluster with any other isolate. The second group comprised two subgroups, with MHR1 and MHR2 in one subgroup and all remaining *C. albicans* isolates in the other.

In summary, for five patients (pLML, pELZ, pMHS, pLSL and pMHR), subsequent infection was caused by identical or very similar strains, whereas, for the remaining seven patients (pTHN, pZKH, pGCL, pNML, pLSY, pNFZ and pHRY), a different *Candida* strain or species caused subsequent infections.

Comparison of between-group and within-group genetic relatedness of isolates

Table 5 summarizes the genetic similarity between pairs of isolates for the within- and between-group comparisons. A composite UPGMA dendrogram for all 87 isolates from patients in groups A, B and C was generated, as shown in Fig. 3. Scrutiny of the dendrogram revealed that all isolates that belong to the same *Candida* species were grouped together

Table 4. Genetic similarity between sequential isolates of the same individual in group A

Patient code	Strain-pair	S_{AB}
1. pLML	LML1, LML2	1.000
	LML2, LML3	1.000
2. pELZ	ELZ1, ELZ2	0.889
3. pHRY	HRY1, HRY2	0.545
4. pZKH	ZKH1, ZKH2	0.211
5. pLSL	LSL1, LSL2	0.923
6. pNFZ	NFZ1, NFZ2	0.833
7. pLSY	LSY1, LSY2	0.899
8. pMHR	MHR1, MHR2	0.667
9. pMHS	MHS1, MHS2	0.933
10. pNML	NML1, NML2	0.800
11. pGCL	GCL1, GCL2	0.105
12. pTHN	THN1, THN2	0.857
	THN2, THN3	0.941
	THN3, THN4	0.800
	THN4, THN5	0.125

Mean S_{AB} = 0.721 ± 0.308 .

within the same cluster, except for the *C. famata* isolates; there is a greater tendency for group B and group C isolates to cluster within their own groups than to cluster with the other groups.

Analysis of the results also indicated that the pregnancy status of a patient had no correlation with higher genetic similarity of the *Candida* isolates.

(i) Comparison of isolates from patients in groups A and C.

For comparison of *Candida* isolates between patients with recurrent vaginitis (groups A and B) or single-episode vaginitis (group C), only isolates from the species *C. albicans* were used, so that an additional variable factor of different species would not be introduced. The S_{AB} values of isolates from these groups were calculated; the mean S_{AB} was found to be 0.56 ± 0.21 , which represented the unrelatedness between the strains from patients in groups A and C. There were no genotypically identical pairs of strains when these two groups were compared.

For the within-group (intragroup) genetic relatedness of *C. albicans* isolates in the two groups, the mean S_{AB} for group A was 0.532 ± 0.25 , whereas that for group C was 0.636 ± 0.206 (Table 5). A paired *t*-test showed that this difference was significant ($P = 0.036$). Thus, it can be deduced that the *C. albicans* isolates in group C were more closely related to each other than to the *C. albicans* isolates in group A.

(ii) Comparison of isolates from patients in groups B and C.

When the *C. albicans* isolates of patients in group B and group C were compared, two pairs of isolates were found to be genotypically identical. Isolate KTK from group B was

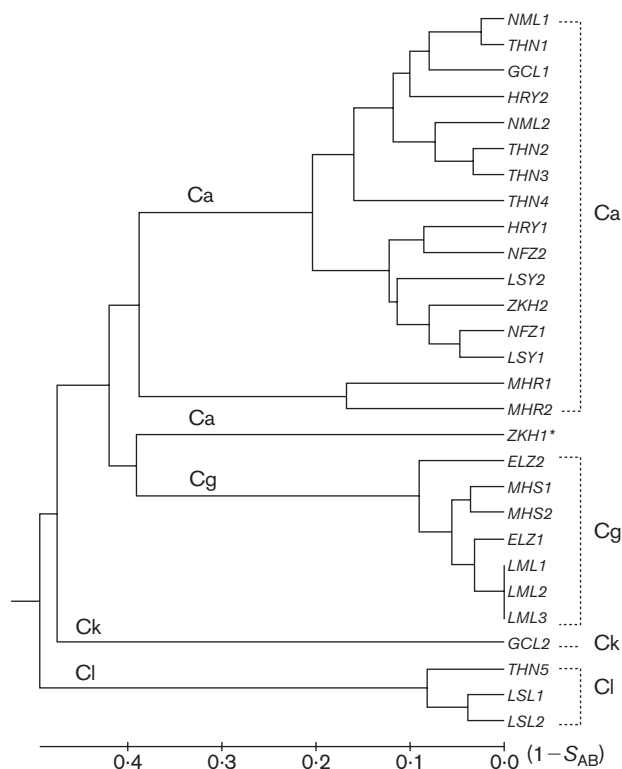


Fig. 2. Dendrogram for all 28 isolates from patients in group A, generated from the DNA fingerprinting patterns obtained with primer PA03, which were used to calculate S_{AB} values. Lengths of horizontal lines in each cluster are proportional to genetic distances ($1 - S_{AB}$) and are drawn to the scale shown beneath the phylogenetic tree. Ca, *C. albicans*; Cg, *C. glabrata*; Ck, *C. krusei*; Cl, *C. lusitanae*. *Isolate ZKH1, identified as *C. albicans*, was in a separate cluster from other *C. albicans* isolates.

genotypically identical to PTI and RKH from group C ($S_{AB} = 1.00$). Of all possible pairs of strains that were compared, only 8.9 % of the strain-pairs from groups B and C were closely related but non-identical to each other. The rest of the strain-pairs (91.1 %) were unrelated to each other. This finding was consistent with the mean S_{AB} of 0.55 ± 0.17 , derived from pairwise comparison of group B isolates versus group C isolates, which reflected unrelatedness between strains from these two groups. This suggested that intragroup relatedness was higher than intergroup relatedness. The mean S_{AB} value for group B, calculated from *C. albicans* isolates ($n = 14$), was 0.561 ± 0.173 . This contrasts with the mean S_{AB} value for group C, which was 0.636 ± 0.206 for *C. albicans* isolates only. However, this difference was not statistically significant ($P = 0.325$).

DISCUSSION

Prevalence of different *Candida* species

This study compared the genetic relatedness of *Candida* species from patients with RVC or single episodes of *Candida*

Table 5. Similarity coefficients between pairs of isolates from the same group or different groups

Sample type	A	B	C	A–B	B–C	A–C
<i>C. albicans</i> only	0.602 ± 0.206	0.561 ± 0.173	0.636 ± 0.206	0.509 ± 0.165	0.543 ± 0.175	0.559 ± 0.208
All <i>Candida</i> species	0.305 ± 0.304	0.316 ± 0.281	0.482 ± 0.254	0.307 ± 0.263	0.362 ± 0.255	0.355 ± 0.274

vaginitis. In the present study, the most common species isolated from the vagina was *C. albicans* (70.1 %), followed by *C. glabrata* (14.9 %), a result that is consistent with previous studies. This result also correlated with that of a local study (Ng *et al.*, 1999), in which *C. albicans* and *C. glabrata* were the most common *Candida* species isolated. An observation was made, based on this study, on the increased emergence of non-*albicans* species, particularly *C. glabrata* and *C. lusitanae*, in recurrent vaginitis; this is evident from the high prevalence of these species in isolates from patients in groups A and B. This finding implies that the pattern of mycological shift from *C. albicans* to non-*albicans* *Candida* species is universal in both the western (Fidel *et al.*, 1999) and Asian populations. There was only one strain of *C. krusei* isolated in this study; this species is isolated infrequently from vaginitis patient populations, as reported in earlier studies (Samaranayake & Samaranayake, 1994), but is emerging as an important cause of candidaemia in immunocompromised hosts that receive azole antifungal prophylaxis. Both *C. glabrata* and *C. krusei* are known to be inherently less resistant to azole drugs; another ongoing study in our laboratory is focused on the *in vitro* drug susceptibilities of these isolates.

In addition, the results of this study showed that a wider spectrum of *Candida* species is involved in causing recurrent vaginitis than one-episode vaginitis. The lower species diversity in patients in group C, compared with those in groups A and B, was because the majority of cases of one-episode vaginitis were caused by *C. albicans*, except for a few isolated cases that were attributed to *C. glabrata*, *C. lusitanae*, *C. parapsilosis* and *C. famata*. Likewise, the higher number of non-*albicans* species contributed to the higher values obtained for the species diversities of patients with recurrent vaginitis.

RAPD profiles of *C. albicans* isolates

It was interesting to note that isolates that were typed as *C. albicans* by using conventional methods and the API 20C AUX kit exhibited three main types of distinct RAPD profiles. Moreover, there exist at least three groups of clusters among the *C. albicans* isolates when all isolates from groups A, B and C were looked at, as shown in the dendrogram in Fig. 3. This could be due to several causes. Firstly, it could be that some of these isolates were not actually *C. albicans* strains but belonged to *Candida dubliniensis*, a newly delineated species that is phenotypically highly similar to *C. albicans*. However, this possibility could be ruled out as the API 20C AUX kit has the capacity to distinguish between *C. albicans* and *C.*

dubliniensis, based on differences in growth on xylose and methyl α -D-glucose. The second possibility is that some of these isolates were of type I *Candida stellatoidea*, a species that shares high DNA homology with *C. albicans*. However, opinions still differ among taxonomists on whether or not to classify *C. stellatoidea* as a separate species from *C. albicans*. A recent finding by Biswas *et al.* (2001) reported the successful use of cytochrome *b* gene sequence analysis to differentiate between *C. stellatoidea* and *C. albicans*; hence, future work could include the use of this technique to genotype ambiguous strains. Nonetheless, there exists the third possibility that the *C. albicans* isolates that showed distinct genotypes were in fact due to subgroups or variants within the species, as it has been noted that a genotyping method that used RFLP analysis of rRNA genes could differentiate four genotypic groups of *C. albicans* (McCullough *et al.*, 1999).

Genetic relatedness of sequential *Candida* isolates

Results from previous studies have led to the suggestion of three basic scenarios for the genetic relatedness of isolates obtained from patients with sequential episodes of RVC, best described by Lockhart *et al.* (1996). These are: (i) strain maintenance with no genetic variation; (ii) strain maintenance with minor genetic variation; and (iii) strain replacement by a different strain. Overall, it was observed from this study that all three scenarios were occurring, differing from patient to patient and from one episode to another. However, the majority of patients had recurrent infections caused by highly similar strains with minor genetic variations that can be ascribed to microevolution. This result is in agreement with the study of Lockhart *et al.* (1996), which reported that the main scenarios for the relatedness of sequential isolates from 18 patients were strain maintenance with and without minor variation.

From our study, an interesting observation was noted for patient pTHN, from whom five sequential isolates were obtained within a period of 1 year. The genetic similarity (measured by S_{AB}) between the first and subsequent isolates seemed to be in descending order compared with the similarity between the first and second isolates, as more episodes of vaginitis occurred (shown by progressive decrease of computed S_{AB} values from 0.857 to 0.8, 0.632 and finally to 0.000, for comparison of *THN1* to *THN2*, *THN3*, *THN4* and *THN5*). This seemed to suggest that a particular strain has undergone gradual genetic variation or microevolution each time it recolonized patient pTHN, probably due to drug pressure selection; by the fifth episode, it was replaced by a different species. In contrast, patient pLML

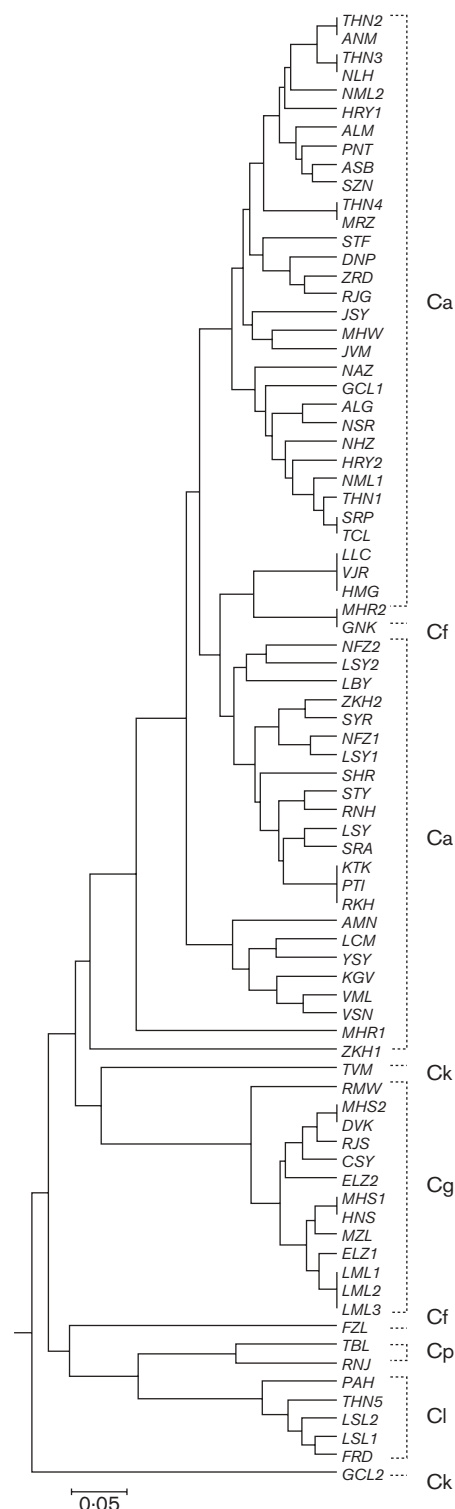


Fig. 3. Dendrogram based on S_{AB} values for all 87 isolates from patients in groups A, B and C, generated from RAPD analysis that used primer PA03. There is no significant clustering of strains on the basis of groups of patients, although isolates of the same *Candida* species were clustered together. A *C. famata* isolate, GNK, clustered with *C. albicans* isolates. Detailed descriptions and group designations for the isolates are listed in Table 1.

seemed to exhibit strain maintenance without genetic variation, in which all three sequential isolates were genotypically identical with an S_{AB} value of 1. For patient pGCL, the third scenario of strain replacement seemed to have occurred, as the second isolate was of a different species from the first. The remaining patients appeared to display strain maintenance with minor genetic variation.

Prevalence of strain replacement

The present study reports 14 % occurrence of strain replacement by an unrelated strain or species (4 of 28). Two patients (pHRY and pZKH) were subsequently colonized by a strain of *C. albicans* that was unrelated from the first episode. Patients pGCL and pTHN had subsequent vaginitis episodes caused by a totally different *Candida* species. Nonetheless, Lockhart *et al.* (1996) did not find any instances of strain replacement among the sequential isolates of 18 patients studied by using DNA fingerprinting probes. Mercure *et al.* (1993) found that 14 % of recurrent cases were due to colonization by a different strain, by using a Southern hybridization method. By using karyotyping resolved by PFGE, Vazquez *et al.* (1994) found that as many as 20 % of sequential isolates involved strain replacement.

Intragroup versus intergroup relatedness

Mean S_{AB} values derived from statistical analysis suggested that intragroup relatedness for group C isolates was higher than groups B vs C and A vs C intergroup relatedness. In contrast, the intragroup relatedness of both group A and group B isolates did not differ significantly from the intergroup comparison with group C isolates. This suggests that strains that cause recurrent vaginitis (from groups A and B) do not possess any significant difference in their genotypes from strains that cause one-off episodes of vaginitis (group C).

From the outset, we had hoped to identify specific genotypes that correlated with recurrent vaginitis infections, based on our premise that strains that cause RVC are more virulent and would display a common genotype not found in the strains that cause occasional infections. However, we found that even strains of the same species isolated from the same patient did not share an identical phylogenetic profile. Hence, RVC in these patients could be due to a myriad of other factors, such as non-compliance with therapy, reinfection with a new strain of endogenous or exogenous origin or infection with a drug-resistant strain.

Reproducibility of RAPD analysis and comparison of results to earlier reports

To determine the reproducibility of the RAPD analysis procedure, RAPD-PCR was repeated at least three times for each DNA sample, so that the amplification profiles for each attempt could be compared. We found the profiles for a given strain to be highly reproducible, with very little variation from one RAPD analysis to another. On average, the mean S_{AB} values reported here for each group are much lower

(0.5–0.6) than those reported by Xu *et al.* (1999), which ranged from 0.6 to 0.9. However, the study by Xu *et al.* (1999) compared pairs of isolates obtained from different body sites within the same patients, whereas this study mainly compared pairs of vaginal isolates from different patients from either the same or different groups. In addition to using the same primer as in the current study (PA03), the study by Xu *et al.* (1999) also used another primer (the M13 core sequence primer); this could explain the discrepancy between the computed S_{AB} values. Another reason could be the different algorithms used by those authors and the current study. A total of 51 polymorphic PCR fragments were obtained in the study by Xu *et al.* (1999), whereas the total number of polymorphic fragments obtained in the present study was 61.

Lockhart *et al.* (1996) employed a different method from this study, namely Southern probing with Ca3, C1 and CARE2 probes for fingerprinting. As well as the band positions of the fragments, signal intensities were also considered in computation of S_{AB} values by Lockhart *et al.* (1996). Band intensities were not taken into account in this study, as PCR is known to produce fragments of varying intensities. As the PA03 primer is a random arbitrary oligonucleotide, it generated a vast range of S_{AB} values, from as low as 0 to the maximum value of 1. In contrast, the range of S_{AB} values obtained by Lockhart *et al.* (1996) was 0.69–1.00. When a different algorithm, the simple matching (Apostol) metric with the formula $S_{AB} = (n_{11} + n_{00})/n$, was used to calculate S_{AB} values in the present study, higher values were obtained than those obtained by using the Dice metric (Dice, 1945).

Another recent study in our laboratory involved genotyping of the *Candida* isolates from this study by using different primer sets, namely primers ERIC1 and ERIC2 (S. R. Abdul Hadi, P. P. Chong, B. C. Tan & K. P. Ng, unpublished data). The resultant genotypic clusters and subgroups correlated well with results obtained by using the PA03 primer, although only 68 unique RAPD profiles and 39 distinct polymorphic fragments were generated, which is comparatively fewer than the number obtained with the PA03 primer. Metzgar *et al.* (1998) also used these ERIC primers to perform RAPD analysis of *Candida* strains isolated from HIV-infected patients.

In summary, results from this study demonstrated that genotyping of *Candida* species by using the PA03 primer and RAPD-PCR is feasible for differentiation of various species and strains. The results also strongly suggest that recurrent vaginal candidiasis patients in Malaysia are most frequently colonized by a single persistent *Candida* strain over an extended period of time, with minor microevolution due to accumulated genetic changes.

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