CHAPTER IV

MATERIALS AND METHODS

1. Microbial isolates

1.1 Yeast Reference strains

C. albicans	ATCC 90029*
C. parapsilosis	ATCC 90018*
C. krusei	ATCC 6258*
C. tropicalis	ATCC 750*
C. guilliermondii	ATCC 9058*
C. glabrata	TIMM 1063*
C. dubliniensis	16F*

ATCC = American Type Culture Collection.

TIMM = Teikyo University's Institute of Medical Mycology.

* From Laboratory of Medical Mycology, Nagoya University School of Medicine, Japan.

1.2 Yeast isolates

One hundred and twenty *Candida* clinical isolates were collected from blood, pus, urine, skin and other specimens from January to October 2002 in Mycology Unit, King Chulalongkorn Memorial Hospital.

2. Culture methods

All clinical specimens, such as blood, pus, urine, and sputum, were plated onto Sabouraud dextrose agar (SDA) and then incubated at 30 °C for 1-2 day before discard. After the cultures grow, a single colony was collected. All selected colony were cultured

on SDA slant. All cultures were identified by morphological and biochemical examinations and were kept at -70 °C by used.

3. Identification methods

3.1 Conventional methods

The morphology of yeast isolates was initially confirmed by microscopy. After that, yeast isolates was identified by conventional methods, both morphological and biochemical studies. There were germ tube test, chlamydospore production, carbohydrates assimilation and carbohydrates fermentation.

3.1.1 Morphological examination

A. Germ tube test

The strains to be tested for germ tube formation were grown on SDA at room temperature, 24 - 28 °C for 24 hrs. The yeast colonies were inoculated into 0.5 ml serum and incubated at 37 °C for 3 hrs. The cells with germ tube were determined microscopically at the end of the inoculation period. Most of the isolates are likely to be *C. albicans* or *C. dubliniensis* if germ tubes form. However, several other species are also able to form germ tube: *C. stellatoidea*, *C. utilis*, *C. tropicalis*, and *Schizosaccharomyces fragilis*. Some exceptional strains of *C. albicans* will not form germ tube (Beneke and Roger) [162].

B. Chlamydospore formation

Chlamydospore formation was determined at room temperature on glutinous rice agar supplemented with 1% (v/v) Tween 80. The tests were performed in accordance with standard procedures (Beneke and Roger) [162]. The small amount of yeast colony on SDA was transferred onto the surface of the glutinous rice agars and covered by a flamed cover

slip over the streak and incubated at 25 °C for 48-72 hrs. *C. albicans* produces the chlamydospores. Occasionally, *C. stellatoidea* and *C. tropicalis* produce chlamydospores.

3.1.2 Biochemical examinations

A. Carbohydrate assimilation

A yeast strain was grown in Sabouraud dextrose broth (SDB) for 18-24 hrs. at 30 °C under shaking at 150 cycles/minute. After that, the cells were harvested by centrifugation at 1,500xg and wash twice with sterile distilled water. For inoculation, a suspension of yeast cell is prepared equivalent to a McFarland Standard number 4 - 7 using sterile distilled water (DW). This yeast suspension was spread onto assimilation media by a swab. Disks impregnated with 10% solutions of the following carbohydrates; glucose, maltose, sucrose, lactose, galactose, trehalose, inositol, melibiose, cellibiose, raffinose, dulcitol and xylose, were placed onto the plates. All of them were incubated at 24 - 30 °C for 24 - 72 hrs. Growth around the disk indicates the utilization of the carbohydrate contained in the disk (positive result). The disk without any carbohydrate but DW was included as negative control [162].

B. Carbohydrate fermentation test

The yeast suspension was prepared as same as the inoculums suspension used for assimilation test. Then, 0.2 ml of the suspension was added to each 16x125 mm tube containing 10 ml of broth with 1% sugars; glucose, sucrose, lactose, maltose, galactose, and trehalose with Durham tube and covered the surface of medium with 2 ml paraffin oil. All the tubes were incubated at 37 °C for 48 hrs. The indicator of bromcresal purple changed from purple to yellow indicated the acid production. Acid or acid with gas was a positive reaction [162].

To determine the species of *Candida*, the patterns of carbohydrates assimilation and fermentation were comparing with Table 8. For result unidentified the species of *Candida*, the carbohydrates assimilation with commercial kit (API 20C AUX, bioMerieux, France) was performed. Also, this kit verified some of the identified yeast with conventional method.

Table 8. Physiological criteria for identification of *Candida* species [162]

Candida species	Chlamydospores	Germ tube	Pseudo hyphae	Carbohydrate assimilation												Carbohydrate fermentation					
				g	*m*	*s*	*l*	*ga*	*me*	*c*	*i*	*x*	*r*	*t*	*d*	*g*	*m*	*s*	*l*	*ga*	*t*
C. albicans	+	+	+	+	+	+	-	+	-	-	-	+	-	+	-	AG	AG	A	-	AG/A	AG/A
C. tropicalis	±	-	+	+	+	+	-	+	-	+	-	+	-	+	-	AG	AG	AG	-	AG	AG
C. glabrata	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	AG	-	-	-	-	A
C. parapsilosis	-	-	+	+	+	+	-	+	-	-	-	+	-	+	-	AG	-	-	-	AG	AG/A
C. krusei	-	-	+/-	+	-	-	-	-	-	-	-	-	-	-	-	AG	-	-	-	-	-
C. dubliniensis	+	+	+	+	+	+	-	+	-	-	-	+	-	+	-	AG	AG	A	-	AG/A	AG/A
C. guilliermondii	-	-	+	+	+	+	-	+	+	+	-	+	+	+	+	AG	-	AG	-	AG/A	AG/A

g= glucose, m= maltose, s= sucrose, l= lactose, ga= galactose, me= melibiose, c= cellibiose, i=inositol, x=xylose, r= raffinose, t=trehalose, d=ducitol, A=acid, G=gas, AG=Acid with gas

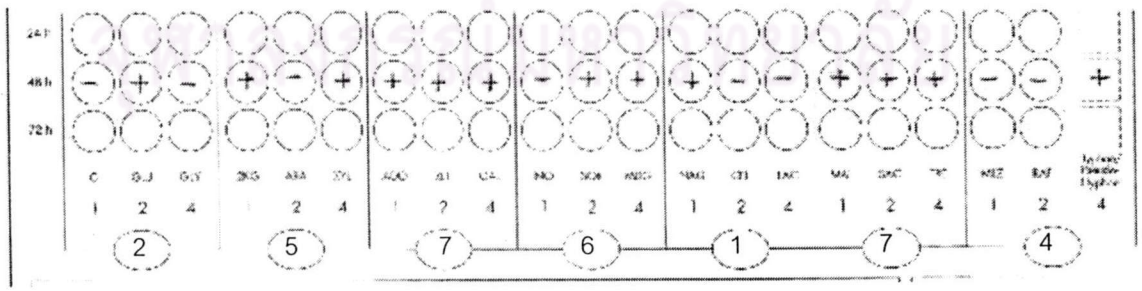
3.2 API 20C AUX Yeast identification system (bioM'erieux, France)

API 20C AUX is a commercial kit for the precise identification of the most frequently encountered yeasts in clinical microbiology. The API 20 C AUX strip consists of 20 cupules containing dehydrated substrates which enable the performance of semi-solid minimal medium and the yeasts will only grow if they are capable of utilizing each substrate as the sole carbon source. The reactions are read by comparing them to growth controls and identification is obtained by referring to the Analytical Profile Index (API) or using the identification software.

Test names

GLU	Glucose	MDG	alpha-Methyl-D-Glucoside
GLY	Glycerol	NAG	N-Acetyl-D-Glucosamine
2KG	2-Keto-D-Gluconate	CEL	Cellibiose
ARA	L-arabinose	LAC	Lactose
XYL	D-xylose	MAL	Maltose
ADO	Adonitol	SAC	Saccharose/Sucrose
XLT	Xylitol	TRE	Trehalose
GAL	Galactose	MLZ	Melezitose
INO	Inositol	RAF	Raffinose
SOR	Sorbitol		Hyphae/pseudohyphae

Figure 4. The API 20C AUX (bioM'erieux, France) **sheet results**



2 576 174 *C. albicans*

Methods

Preparation of the strip

- Prepare the incubation box (tray and lid) and distribute about 5 ml of distilled water into the honeycombed wells of the tray to create a humid atmosphere.
- Record the strain reference on the elongated flap of the tray.
- Remove the strip from its individual packaging and place it in the incubation tray.

Preparation of the inoculums

- Using a loop for pick up a portion of yeast colony, suspension in to SDB, shaking overnight at 30 °C for 18 - 28 hrs. After that washing 3x in DW and make a suspension in DW with a turbidity equal to 2 McFarland.
- Open an ampoule of C Medium and transfer 100 µl of the previous suspension into it. Gently homogenize with the pipette, avoiding the formation of bubbles.

Inoculation of the strip

- Fill the cupules with the suspension obtained in the ampoule of C medium. Avoid the formation of bubbles by placing the tip of the pipette against the side of the cupules. Care should be taken not to overfill or underfill the cupules (the surface should be flat or slightly convex, but never concave), otherwise incorrect results may be obtained.
- Place the lid on the tray and incubate at 30 °C for 24 - 72 hrs.

Reading the strip

- After 24, 48, and 72 hrs. of incubation compare growth in each cupule to the zero cupule, which is used as a negative control. A cupule more turbid than the control indicates a positive reaction to be recorded on the result sheet.

Identification

- Using the Analytical Profile Index: the pattern of the reactions obtained must be coded into a numerical profile.
- On the result sheet, (Fig. 4) the tests are separated into groups of 3 and a number 1, 2 or 4 indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit number is obtained which constitutes the numerical profile.
- Using the identification software by manually entering the 7-digit numerical profile via the keyboard.

3.3 Molecular methods

3.3.1 Genomic DNA preparation by Wizard ™ Genomic DNA Purification Kits (Promega, USA)

Candida strains were grown overnight in 8 ml SDB at 30°C on rotary shaker. Of each cell suspension, 0.5 ml was pellet, washed three times in DW and resuspended in 300 µl cell lysis buffer with glass beads and then incubated at room temperature for 50 min on a shaker. After incubation, the mixture was centrifuged at 5,000 rpm for 0.5 min. The supernatant was transferred to a new tube and was centrifuged at 13,000 rpm for 3 min. After the supernatant was removed, 300 µl of nucleic acid lysis buffer was added to the sample and mixed. Protein precipitate buffer was added to the suspension at 100 µl, mixed, and centrifuged at 13,000 for 3 min.

The supernatant was transferred to a new tube, 500 µl of isopropanol was added, and the suspension was incubated at –20 °C for 30 min. The supernatant was then decanted, the extracted DNA was rinsed with 70% ethanol, dried at room temperature, and dissolved in 50 µl of sterile distilled water.

3.3.2 Polymerase chain reaction (PCR) amplification

PCR amplification was performed on total DNA with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by Williams *et al.* [146]. The DNA fragment amplified with these primers includes the internal transcribed spacer 1 and 2 (ITS1 and ITS2, respectively) regions and the intervening 5.8S rDNA of ribosomal DNA. Reaction volume of 50 µl contained 5 µl of 10xPCR buffer, 5 µl of a deoxynucleoside triphosphate mixture (2 mM each dNTP), 1 µl of each primer (10 pmol of each primer), 0.2 µl (5.0U) of Taq DNA polymerase (Promega, USA), and 0.5 µg of template DNA, with the remaining volume consisting of distilled water. Negative controls were performed with sterile distilled water in place of the template DNA. Amplification reaction were carried out in a Perkin Elmer Cetus DNA thermal cycler (Perkin Elmer Corporation, USA) with initial denaturation for 1 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final extension for 5 min at 72 °C. The size and purity of the PCR products were confirmed by 3% agarose gel electrophoresis.

3.3.3 Sensitivity studies

To determine the limit of sensitivity of this PCR assay, DNA of each *Candida* reference strains was diluted in distilled water and PCRs were performed with 400 ng, 40 ng, 4 ng, 400 pg, 40 pg, 4 pg, 400 fg, 40 fg and 4 fg of genomic DNA. The experiment was performed at least three times in difference round.

3.3.4 Purification of PCR products by QIA quick PCR Purification Kit

A QIAquick spin column (QIAGEN, Hilden, Germany) was used to purify the PCR products and the manufacturer's reagents and protocol used throughout. Buffer PB was added in a ratio of 5:1 to the PCR reaction and mixed. The sample was applied to a QIAquick column and centrifuged at 13,000 rpm for 1 min. The column was washed by centrifugation with 750 µl Buffer PE and the purified PCR products were eluted with 50 µl distilled water.

3.3.5 Restriction Fragment Length Polymorphism (RFLP)

After purification as above, the amplified fragments were digested individually with the restriction enzymes *Dde* I, *Hae* III, *Tru9* I, and *Mbo* I (Promega, USA) Restriction conditions were as follows: 2 µl of 10x reaction buffer, 0.2 µl of BSA (Bovine Serum Albumin), 0.5 µl of one restriction endonuclease in each reaction, and 5 µl of purified PCR products. The solution was incubated at 37 °C (*Dde* I, *Hae* III, and *Mbo* I) and 65 °C (*Tru9* I) for overnight (Table 9). After incubation period, the mixture was electrophoresed through 3% agarose gel.

Table 9. The cleavage site of *Hae* III, *Dde* I, *Tru9* I and *Mbo* I restriction enzymes

Enzyme name	Restriction site	Active Temperature
Hae III	GG▼CC CC▲GG	37 °C
Dde I	C▼TNA G G ANT▲C	37 °C
Tru9 I	T▼TA A A AT▲T	65 °C
Mbo I	G▼A T C C T A▲G	37 °C

3.3.6 Agarose gel electrophoresis for PCR and restricted endonucleases digestion products

Gel electrophoresis with 3% agarose gels was conducted with 1x TBE buffer (0.1 M Tris, 0.09 M boric acid, 1mM EDTA) at 100 V for 2 hrs. Two DNA markers; 100 base pair DNA ladder (Pacific science, USA) and ϕX174 DNA/*Hae* III (Amersham pharmacia biotech, USA) were run concurrently with amplicons for sizing of the bands. Gel were stained with ethidium bromide solution for 30 min, destained with distilled water for 30 min, and then visualized by UV transillumination on the Gel Doc image analysis system (Bio-Rad, USA).

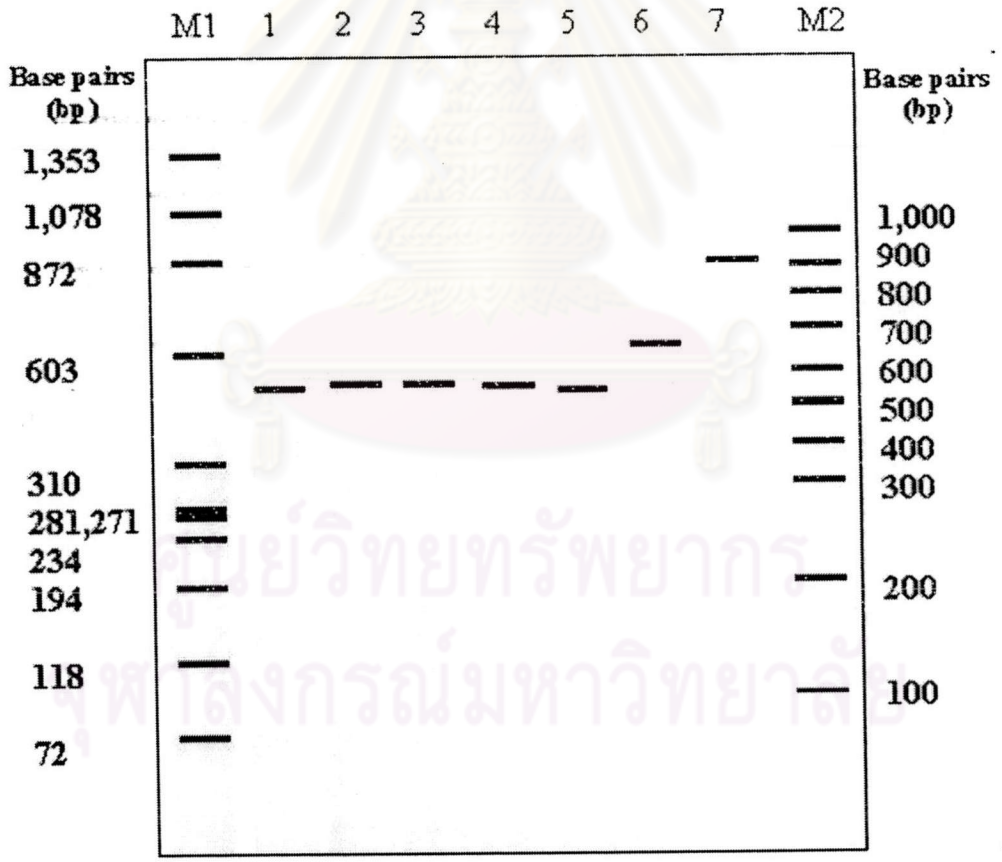
3.3.7 Interpretation of PCR and RFLP patterns

The DNA patterns of *Candida* species from PCR were compared with reference strains. The primers ITS1 and ITS4 were designed to amplify the rDNA gene ranged between interspacer 1 and interspacer 4 of each *Candida* species. The PCR products of *C. glabrata* and *C. guilliermondii* yielded product sizes approximately 800 and 600 bp, respectively (Fig. 5, Table 10). A product of approximately 520 bp was obtained with *C. albicans, C. dubliniensis, C. tropicalis, C. parapsilosis and C. krusei* (Fig. 5, Table 10). These PCR products were studied further by RFLP analysis following digestion of the amplicons by the restriction enzymes *Dde* I, *Hae* III, and *Tru9* I. The restriction profiles of these seven *Candida* species are shown schematically in Fig. 6, 7, 8, Table 11, 12 and 13. All atypical *Tru9* I restriction profiles of *C. albicans* were confirmed with *Mbo* I enzyme and some were further analyzed by the DNA sequencing. The *Mbo* I RFLP patterns of *C. albicans* showed the restricted fragment at 217, 161 and 138 bp (Bioedit program).

Table 10. PCR products size of *Candida* species using ITS1 and ITS4 primers

Organisms	GenBank Accession no.	PCR Products (base pairs)
C. albicans	AF217609	536
C. tropicalis	AF321539	523
C. glabrata	AF218966	875
C. parapsilosis	L47109	521
C. krusei	L47113	510
C. dubliniensis	AF405231	541
C. guilliermondii	AY168784	608

Figure 5. Diagram of PCR products size of *Candida* species using ITS1 and ITS4 primers.

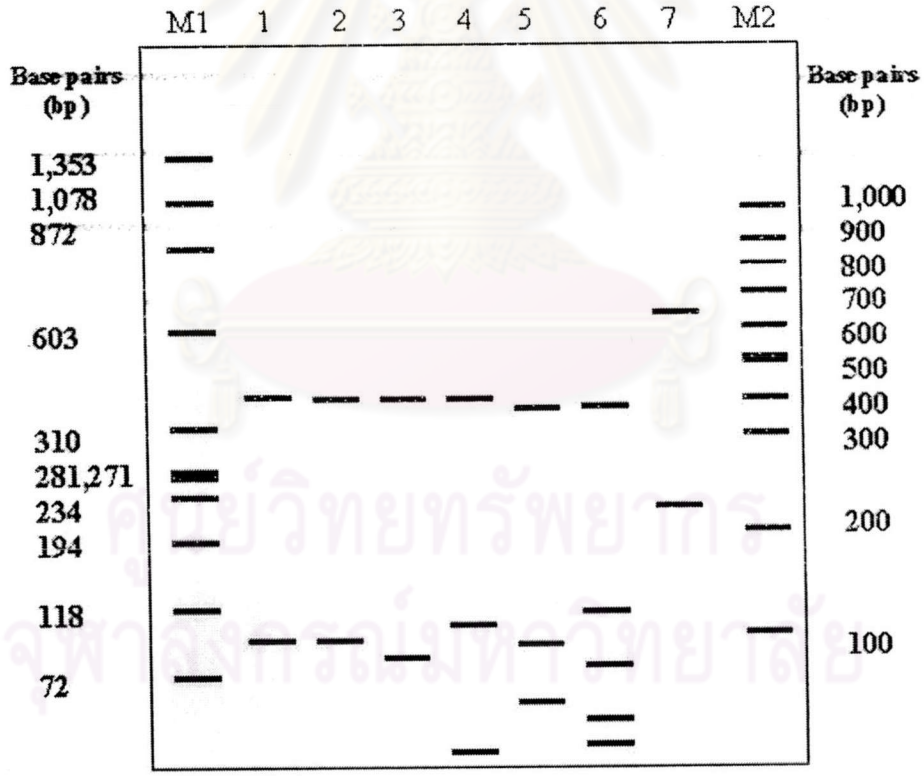


Lane No. M1= Φx174/Hae III, 1 = *C. albicans*, 2 = *C. dubliniensis*, 3 = *C. tropicalis*, 4= *C. parapsilosis*, 5 = *C. krusei*, 6 = *C. guilliermondii*, 7 = *C. glabrata*, M2 = 100 bp ladder

Table 11. RFLP patterns of *Candida* species using *Hae* III enzyme

Organisms	GenBank Accession no.	RFLP patterns of *Hae* III enzyme (base pairs)
C. albicans	AF217609	445,91
C. tropicalis	AF321539	445,78
C. glabrata	AF218966	652,223
C. parapsilosis	L47109	402,105,14,3
C. krusei	L47113	382,90,38
C. dubliniensis	AF405231	451,90
C. guilliermondii	AB105435	390, 117, 79, 17, 5

Figure 6. Diagram of PCR-RFLP patterns of *Candida* species using *Hae* III enzyme.

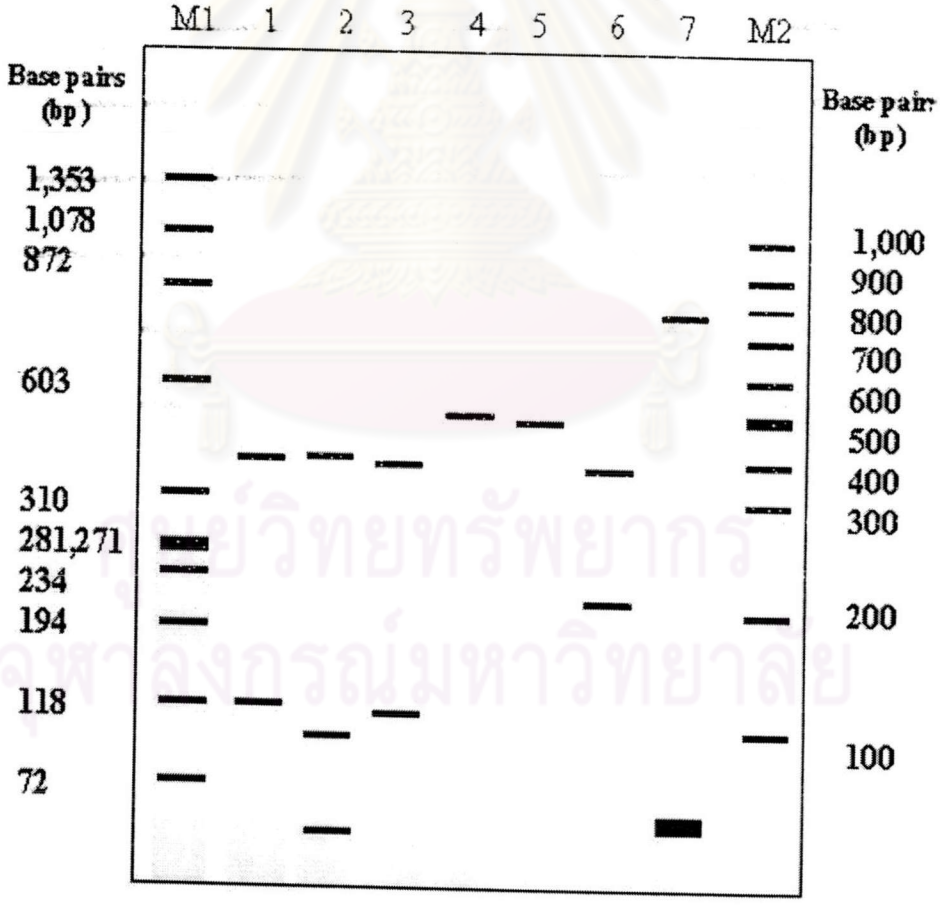


Lane No. M1= Φx174/Hae III, 1 = *C. albicans*, 2 = *C. dubliniensis*, 3 = *C. tropicalis*, 4 = *C. parapsilosis*, 5 = *C. krusei*, 6 = *C. guilliermondii*, 7 = *C. glabrata*, M2 = 100 bp ladder

Table 12. RFLP patterns of *Candida* species using *Dde* I enzyme

Organisms	GenBank Accession no.	RFLP patterns of *Dde* I enzyme (base pairs)
C. albicans	AF217609	418,118
C. tropicalis	AF321539	410,113
C. glabrata	AF218966	782,49,44
C. parapsilosis	L47109	521
C. krusei	L47113	510
C. dubliniensis	AF405231	420,98,23
C. guilliermondii	AY168784	383,213,12

Figure 7. Diagram of PCR-RFLP patterns of *Candida* species using *Dde* I enzyme

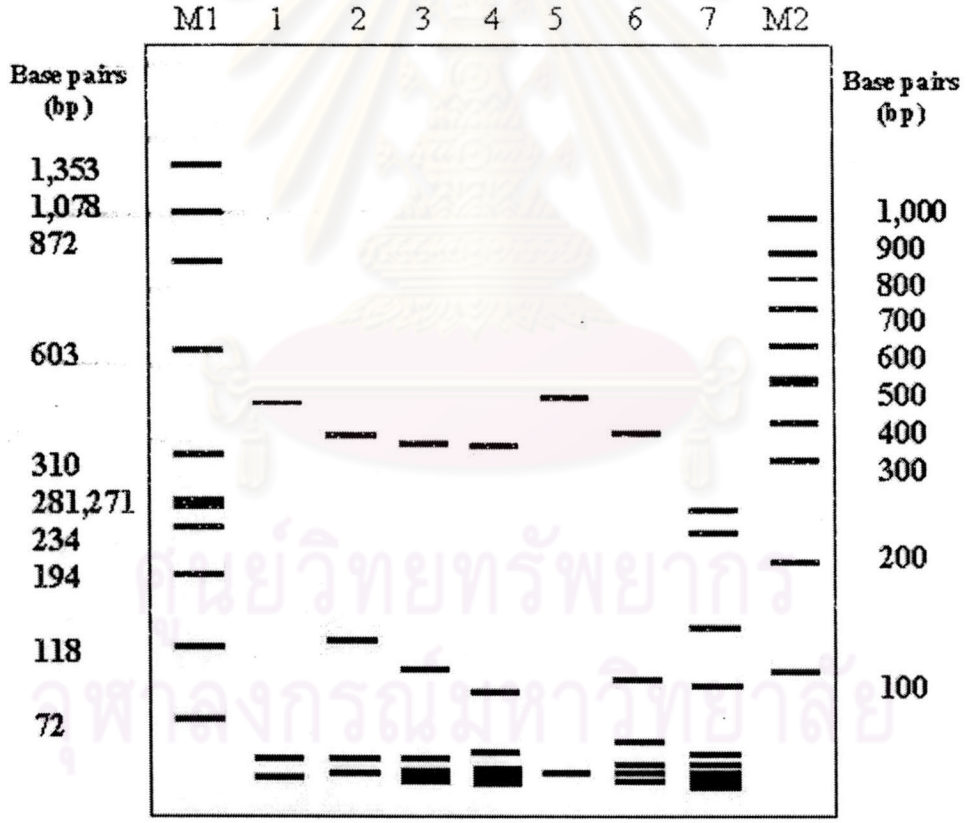


Lane No. M1= Φx174/Hae III, 1 = *C. albicans*, 2 = *C. dubliniensis*, 3 = *C. tropicalis*, 4 = *C. parapsilosis*, 5 = *C. krusei*, 6 = *C. guilliermondii*, 7 = *C. glabrata*, M2 = 100 bp ladder

Table 13. RFLP patterns of *Candida* species using *Tru9* I enzyme

Organisms	GenBank Accession no.	RFLP patterns of *Tru9* I enzyme (base pairs)
C. albicans	AF217609	473,40,23
C. tropicalis	AF321539	344,101,40,20,18
C. glabrata	AF218966	253,231,122,87,56,40,34,28,23
C. parapsilosis	L47109	327,85, 46,24,21,18
C. krusei	L47113	487,23
C. dubliniensis	AF405231	359,119,40,23
C. guilliermondii	AY168784	395,92,51,32,23,15

Figure 8. Diagram of PCR-RFLP patterns of *Candida* species using *Tru9* I enzyme



Lane No. M1 = *Φx174/Hae* III, 1 = *C. albicans*, 2 = *C. dubliniensis*, 3 = *C. tropicalis*, 4 = *C. parapsilosis*, 5 = *C. krusei*, 6 = *C. guilliermondii*, 7 = *C. glabrata*, M2 = 100 bp ladder

3.3.8 Sequencing for ITS region of *Candida albicans*

A. Purification of PCR products

The PCR products of *C. albicans* were purified by QIAquick PCR purification kit as described by the manufacturer (QIAGEN, Germany). The QIAquick system is a combination of spin column technology with the selective binding properties of a uniquely designed silica-gel membrane. DNA was absorbed to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities were efficiently washed away and the pure DNA was then eluted with Tris buffer. The concentration of DNA was measured by spectrophotometer (Bio-Rad, U.S.A) and approximately adjusted to 100 ng /μl for preparation of sequencing reaction.

B. Sequencing reaction preparation

Approximately 100-150 ng of DNA sample was sequenced using ITS1 primer (5'-TCCGTAGGTGAACCTGCGG-3') with ABI prism Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer Corporation, USA). The sequence reaction required 4 μl of BigDye terminator as described by manufacturer, with 3.2 pmole of primer and 100 ng of purified PCR product template in a total volume of 10 μl. The sequencing cycle was performed using 25 cycles of denaturing step: 10 sec at 96 °C, annealing step: 5 sec at 50 °C, and extension step: 4 min at 60 °C.

C. Ethanol-sodium acetate precipitation

The PCR products were precipitated using 2 μl of 3 M sodium acetate (NaOAc), pH 4.6 and 50 μl of 95% ethanol (EtOH) for each sequencing reaction as described by manufacturer. The precipitated DNA was stored at -20 °C until used. The precipitated DNA was subjected to automated sequence analysis on ABI prism 310 automated sequencer (Perkin Elmer Corporation, USA).

D. Analysis

The nucleotide sequences were compared with *C. albicans* reference strain by using Clustal W, version 1.74 program.