V. RESULTS

1. To study genetic diversity and epidemiology of clinical and environmental isolates of *C. neoformans* in Chiang Mai, clinical isolates from Khon Kaen and Japan by RAPD analysis with three arbitrary primers (R28, OPH-02 and OPH-20).

A. RAPD analysis of *C. neoformans*

The RAPD patterns of 112 isolates of *C. neoformans* obtained with three different 10-mer primers (R28, OPH-02 and OPH-20) were analysed. Primer R28 generated two profiles (profiles a and b) among clinical and environmental isolates. One hundred and ten isolates were of profile a (Fig. 7) and 2 clinical isolates obtained from Khon Kaen (K25 and K38) were of profile b (Fig 8). Primer OPH-02 generated two profiles (i and ii). One hundred and ten isolates were of profile i (Fig 7) and 2 clinical isolates obtained from Khon Kaen (K25 and K38) were of profile ii (Fig.8). Primer OPH-20 generated five profiles (1, 2, 3, 4 and 5). Forty-three clinical and 43 environmental isolates were of profile 1 (Fig. 9). Sixteen clinical and 7 environmental isolates were of profile 2 (Fig. 10). Profiles 3, 4 and 5 were found in 3 clinical isolates (Fig. 11, 10 and 12, respectively). Table 4 and Table 5 summarized the RAPD profiles of all isolates . The RAPD profiles obtained with the three primers revealed five patterns (pattern I, II, III, IV and V) among all isolates. For isolates belonged to *C. neoformans* serotype A, three patterns (pattern I, II and III) were found among 50 clinical Chiang Mai isolates, two patterns (pattern I and II) among 50 environmental Chiang Mai isolates, one pattern (pattern I) among 7 clinical Khon Kaen isolates and one pattern (pattern II) among 2 clinical Japanese isolates. One isolate of serotype B from Khon Kaen (K38) produced pattern IV, two untypeable isolates of the same province (K25 and K97) produced patterns V and I, respectively. The most common pattern was pattern I, which was produced by 35 clinical, 43 environmental Chiang Mai isolates and 8 Khon Kaen isolates.

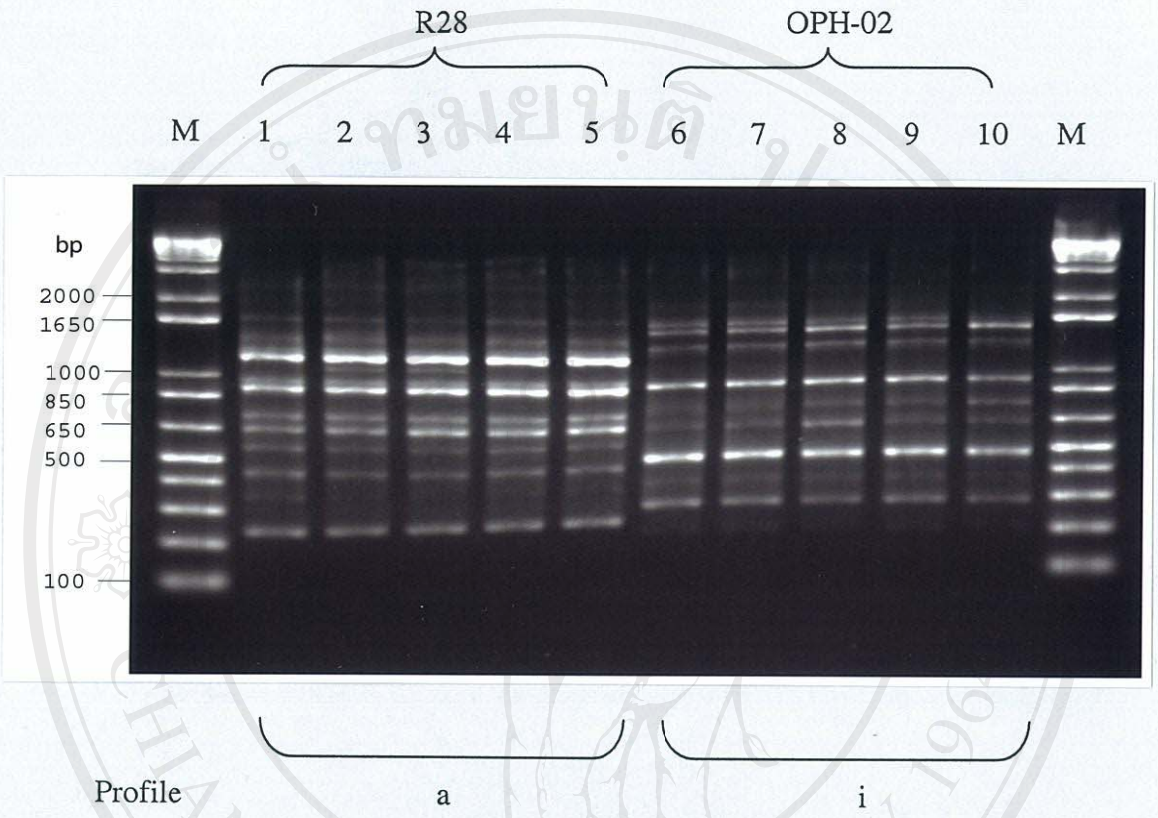

Figure 7. RAPD profiles of five isolates of *C. neoformans* (lane 1,6 : K67 ; lane 2,7 : K97 ; lane 3,8 : Pt1 ; lane 4,9 : Pt2 ; lane 5,10 : Pt3). Lane1-5 : patterns generated with primer R28. Lane 6-10 : patterns generated with primer OPH-02. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Figure 8. RAPD profiles of five isolates of *C. neoformans* (lane 1,6 : K31 ; lane 2,7 : K36 ; lane 3,8 : K38 ; lane 4,9 : K39 ; lane 5,10 : K45). Lane1-5 : patterns generated with primer R28. Lane 6-10 : patterns generated with primer OPH-02. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Figure 9. RAPD profiles of five isolates of *C. neoformans* (lane 1 : K67 ; lane 2 : K97 ; lane 3 : Pt1 ; lane 4 : Pt2 ; lane 5 : Pt3). Lane1-5 : patterns generated with primer OPH-20. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Figure 10. RAPD profiles of five isolates of *C. neoformans* (lane 1 : K2 ; lane 2 : K15 ; lane 3 : K25 ; lane 4 : J1 ; lane 5 : J2). Lane1-5 : patterns generated with primer OPH-20. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Figure 11. RAPD profiles of five isolates of *C. neoformans* (lane 1 : K31 ; lane 2 : K36 ; lane 3 : K38 ; lane 4 : K39 ; lane 5 : K45). Lane1-5 : patterns generated with primer OPH-20. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Figure 12. RAPD profiles of five isolates of *C. neoformans* (lane 1 : Pt13 ; lane 2 : Pt15 ; lane 3 : Pt16 ; lane 4 : Pt17 ; lane 5 : Pt19). Lane1-5 : patterns generated with primer OPH-20. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Table 4. RAPD profile of *C. neoformans* isolates

Isolates No.	RAPD profile			Pattern	Serotype
	Primer R28	Primer OPH-02	Primer OPH-20		
Clinical isolates					
Pt1	a	i	1	I	A
Pt2	a	i	1	I	A
Pt3	a	i	1	I	A
Pt5	a	i	2	II	A
Pt7	a	i	2	II	A
Pt9	a	i	1	I	A
Pt11	a	i	1	I	A
Pt12	a	i	2	II	A
Pt13	a	i	1	I	A
Pt15	a	i	1	I	A
Pt16	a	i	5	III	A
Pt17	a	i	1	I	A
Pt19	a	i	1	I	A
Pt21	a	i	1	I	A
Pt22	a	i	2	II	A
Pt23	a	i	2	II	A
Pt24	a	i	1	I	A
Pt25	a	i	1	I	A
Pt28	a	i	1	I	A
Pt30	a	i	1	I	A
Pt31	a	i	1	I	A
Pt32	a	i	1	I	A
Pt33	a	i	1	I	A
Pt34	a	i	1	I	A
Pt39	a	i	2	II	A

Table 4. (Continued)

Isolates No.	RAPD profile			Pattern	Serotype
	Primer R28	Primer OPH-02	Primer OPH-20		
Clinical isolates					
C1	a	i	1	I	A
C3	a	i	2	II	A
C4	a	i	1	I	A
C5	a	i	1	I	A
C7	a	i	2	II	A
C8	a	i	1	I	A
C9	a	i	1	I	A
C10	a	i	1	I	A
C11	a	i	1	I	A
C12	a	i	1	I	A
C13	a	i	1	I	A
C14	a	i	2	II	A
C16	a	i	1	I	A
C17	a	i	2	II	A
C18	a	i	1	I	A
C19	a	i	2	II	A
C20	a	i	1	I	A
C22	a	i	1	I	A
C23	a	i	2	II	A
C26	a	i	2	II	A
C27	a	i	1	I	A
C30	a	i	1	I	A
C31	a	i	1	I	A
C33	a	i	1	I	A
C34	a	i	2	II	A

Table 4. (Continued)

Isolates No.	RAPD profile			Pattern	Serotype
	Primer R28	Primer OPH-02	Primer OPH-20		
Environmental isolates					
Pg1	a	i	1	I	A
Pg2	a	i	1	I	A
Pg3	a	i	1	I	A
Pg21	a	i	1	I	A
Pg26	a	i	1	I	A
Pg32	a	i	1	I	A
Pg37	a	i	2	II	A
Pg46	a	i	1	I	A
D1	a	i	1	I	A
D2	a	i	1	I	A
D3	a	i	1	I	A
D4	a	i	2	II	A
D5	a	i	2	II	A
D6	a	i	1	I	A
D9	a	i	1	I	A
D12	a	i	1	I	A
D13	a	i	2	II	A
D14	a	i	1	I	A
D15	a	i	1	I	A
D16	a	i	2	II	A
D17	a	i	1	I	A
D18	a	i	1	I	A
D19	a	i	2	II	A
D22	a	i	1	I	A
D24	a	i	1	I	A

Table 4. (Continued)

Isolates No.	RAPD profile Primer R28	Primer OPH-02	Primer OPH-20	Pattern	Serotype
Environmental isolates					
D25	a	i	1	I	A
D26	a	i	1	I	A
D27	a	i	1	I	A
D28	a	i	1	I	A
D30	a	i	1	I	A
D31	a	i	1	I	A
D33	a	i	1	I	A
D34	a	i	1	I	A
D35	a	i	1	I	A
D36	a	i	1	I	A
D41	a	i	1	I	A
D42	a	i	1	I	A
D43	a	i	1	I	A
D44	a	i	2	II	A
D45	a	i	1	I	A
D46	a	i	1	I	A
D64	a	i	1	I	A
D69	a	i	1	I	A
D71	a	i	1	I	A
D73	a	i	1	I	A
D76	a	i	1	I	A
D79	a	i	1	I	A
D82	a	i	1	I	A
D83	a	i	1	I	A

Table 4. (Continued)

Isolates No.	RAPD profile			Pattern	Serotype
	Primer R28	Primer OPH-02	Primer OPH-20		
Environmental isolates					
E24	a	i	1	I	A
Clinical isolates					
K2	a	i	1	I	A
K15	a	i	1	I	A
K25	b	ii	4	V	untypeable
K31	a	i	1	I	A
K36	a	i	1	I	A
K38	b	ii	3	IV	B
K39	a	i	1	I	A
K45	a	i	1	I	A
K67	a	i	1	I	A
K97	a	i	1	I	untypeable
J1	a	i	2	II	A
J2	a	i	2	II	A

Table 5. RAPD profiles of 112 isolates of *C. neoformans* generated with three arbitrary primers

R28		OPH-02		OPH-20	
Profile	no. of isolates	Profile	no. of isolates	Profile	no. of isolates
a	110	**i**	110	**1**	86
b	2	**ii**	2	**2**	23
-	-	-	-	**3**	1
-	-	-	-	**4**	1
-	-	-	-	**5**	1
	Total 112		Total 112		Total 112

B. Reproducibility of RAPD profiles

Reproducibility of the method was confirmed by subculturing the original isolate three times and repeating the DNA isolation and PCR amplification. The identical profiles are shown in Fig. 13.

Figure 13. Reproducibility of RAPD profiles of the isolate Pg1. Lane 1, 4, 7 : pattern generated with primer R28. Lane 2, 5, 8 : pattern generated with primer OPH-02. Lane 3, 6, 9 : pattern generated with primer OPH-20. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left. Lane 1-3 : (Pg 1) original isolate ; Lane 4-6 : (Pg 1) original isolate, PCR repeated testing ; Lane 7-9 : (Pg 1) isolate after three-time subcultured.

2. To study sources of cryptococcosis from patients' homes in Chiang Mai.

A. Sources of cryptococcosis in the domestic environment or nearby the homes of cryptococcosis patients (6-month case, 52 patients' homes)

A total of 202 of avian droppings, including pigeon, dove, chicken, duck, goose, Hill Myna, Budgerigars droppings, were collected from 52 patients' homes from various locations in Chiang Mai (Fig. 14). Two of 202 samples (0.99 %) were positive for *C. neoformans*. Of these 2 isolates, one was recovered from dove dropping at the home of a patient in Amphoe Hang Dong and the other was recovered from pigeon dropping at the home of a patient in Amphoe Doi Saket (Table 6, 7).

B. Serovariety typing

Two environmental isolates of *C. neoformans* (Do 3/2 and Pge 12/2) and their two clinical isolates (Cne 3 and Cne 12) were determined serovariety by slide agglutination test with monoclonal antibody specific to capsular polysaccharide (Crypto check, Iatron, Tokyo, Japan). The results revealed that all belonged to serotype A (*C. neoformans* var. *grubii*) (Table 7).

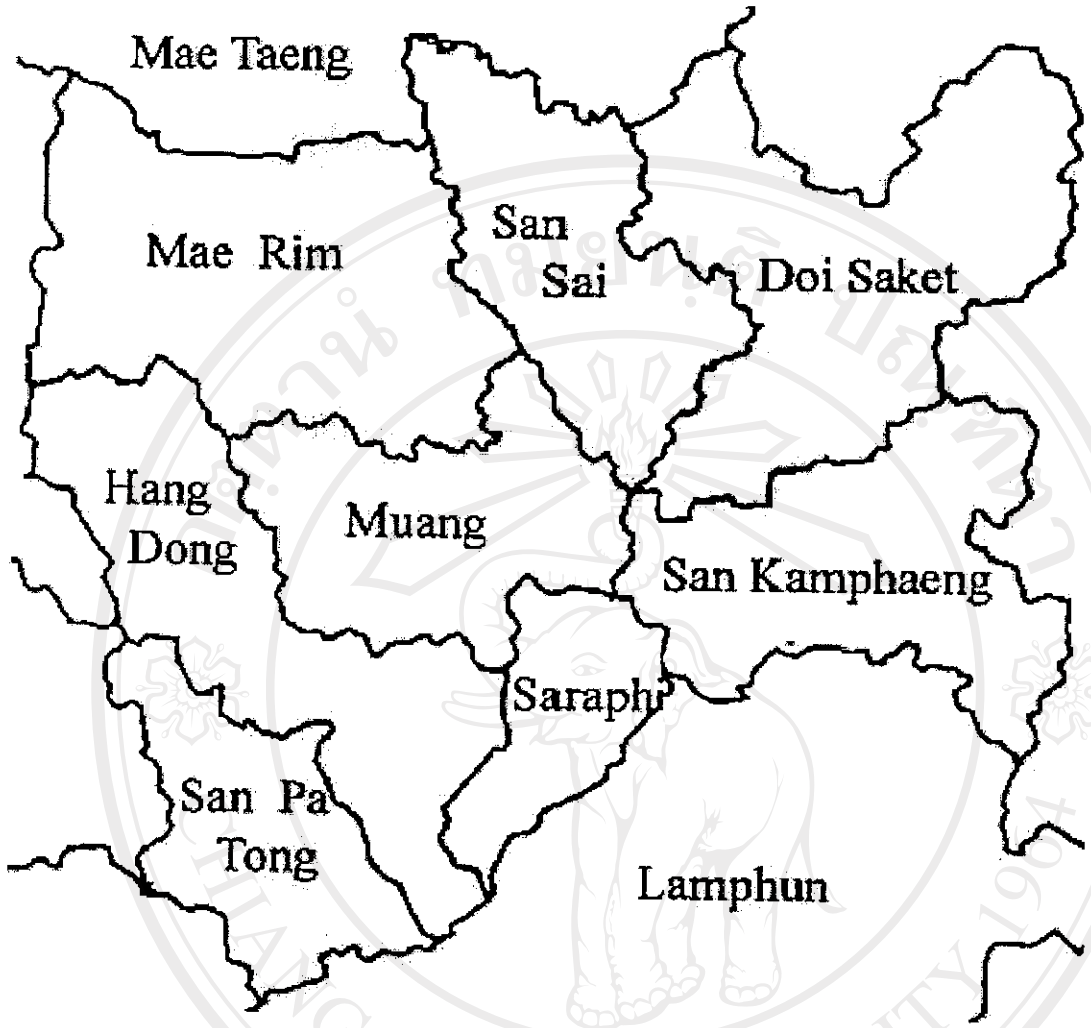

Figure 14. The map of studied Amphoes in Chiang Mai.

Table 6. Isolation of *C. neoformans* from avian droppings from patients' homes from various locations in Chiang Mai.

Avian type	Number of samples collected	Positive samples	
		No.	%
Pigeon (*Columba livia*)	6	1	16.7
Dove (Spotted dove, *Streptopelia chinensis* ; Zebra dove, *Geopelia striata* and Collared dove, *Streptopelia decaocta*)	18	1	5.6
Chicken (*Gallus domesticus*)	152	0	0
Duck (*Anas domesticus*)	21	0	0
Goose (*Anser indicus*)	3	0	0
Hill Myna (*Gracula religiosa*)	1	0	0
Budgerigars (*Melopsittacus undulatus*)	1	0	0
Total	202	2	0.99

Table 7. *C. neoformans* in avian droppings collected from fifty-two dwellings in Chiang Mai.

Code of clinical isolates	Area (Amphoe)	Avian dropping	No.	Total number	No. of Positive samples	Serovarieties
Cne 1	Hang Dong	Chicken	3	8	-	-
		Duck	3		-	-
		Hill Myna	1		-	-
		Budgerigars	1		-	-
Cne 2	Hang Dong	Chicken	3	7	-	-
		Duck	3		-	-
		Dove	1		-	-
Cne 3*	Hang Dong	Chicken	3	8	-	-
		Duck	3		-	-
		Dove	2		1 (Do3/2)[a]	A (var. *grubii*)
Cne 4	Hang Dong	Chicken	3	3	-	-
Cne 5	Saraphi	Chicken	3	3	-	-
Cne 6	Saraphi	Chicken	3	3	-	-
Cne 7	San Sai	Chicken	3	3	-	-
Cne 8	San Sai	Chicken	3	3	-	-
Cne 9	San Sai	Chicken	3	3	-	-
Cne 10	Doi Saket	Chicken	3	6	-	-
		Duck	3		-	-
Cne 11	San Kamphaeng	Chicken	3	3	-	-
Cne12*	Doi Saket	Chicken	3	9	-	-
		Dove	3		-	-
		Pigeon	3		1 (Pge12/2)[b]	A (var. *grubii*)

Table 7. (Continued)

Code of clinical isolates	Area (Amphoe)	Avian dropping	No.	Total Number	Positive samples	Serovarieties
Cne 13	Hang Dong	Chicken	3	3	-	-
Cne 14	Hang Dong	Chicken	3	3	-	-
Cne 15	Lumphun	Chicken	3	3	-	-
Cne 16	Lumphun	Chicken	3	3	-	-
Cne 17	Lumphun	Chicken	3	3	-	-
Cne 18	Lumphun	Chicken	3	3	-	-
Cne 19	Lumphun	Chicken	3	3	-	-
Cne 20	Mae Rim	Chicken	3	3	-	-
Cne 21	San Sai	Chicken	3	3	-	-
Cne 22	Mueang	Chicken	3	6	-	-
		Pigeon	3		-	-
Cne 23	San Kamphaeng	Chicken	3	3	-	-
Cne 24	Mueang	Chicken	2	2	-	-
Cne 25	Mueang	Chicken	3	6	-	-
		Duck	3		-	-
Cne 26	Mueang	Chicken	3	6	-	-
		Goose	3		-	-
Cne 27	Mueang	Chicken	3	3	-	-
Cne 28	Mueang	Chicken	3	3	-	-
Cne 29	San Kamphaeng	Chicken	3	3	-	-
Cne 30	Doi Saket	Chicken	3	3	-	-
Cne 31	Doi Saket	Chicken	3	3	-	-
Cne 32	Mueang	Chicken	3	3	-	-
Cne 33	Mueang	Chicken	3	3	-	-

Table 7. (Continued)

Code of clinical isolates	Area (Amphoe)	Avian dropping	No.	Total number	Positive Samples	Serovarieties
Cne 34	Mueang	Chicken	3	3	-	-
Cne 35	Mueang	Chicken	3	3	-	-
Cne 36	Mueang	Dove	3	3	-	-
Cne 37	Mueang	Chicken	3	3	-	-
Cne 38	Mueang	Chicken	3	6	-	-
		Dove	3		-	-
Cne 39	Mueang	Chicken	3	3	-	-
Cne 40	Mueang	Chicken	3	3	-	-
Cne 41	San Kamphaeng	Chicken	3	3	-	-
Cne 42	Doi Saket	Chicken	3	6	-	-
		Duck	3		-	-
Cne 43	Mueang	Chicken	3	3	-	-
Cne 44	San Kamphaeng	Chicken	3	6	-	-
		Dove	3		-	-
Cne 45	Mueang	Chicken	3	6	-	-
		Dove	3		-	-
Cne 46	Saraphi	Chicken	3	3	-	-
Cne 47	Mueang	Chicken	3	3	-	-
Cne 48	San Kamphaeng	Chicken	3	3	-	-
Cne 49	Hang Dong	Chicken	3	3	-	-
Cne 50	Mueang	Chicken	3	3	-	-
Cne 51	Hang Dong	Chicken	3	3	-	-
Cne 52	San Kamphaeng	Chicken	3	3	-	-

* Clinical isolates (Cne 3 and Cne 12) were identified as *C. neoformans* var. *grubii* (Serotype A).

[a] *C. neoformans* isolated from dove dropping (Do 3/2); Do = Dove.

[b] *C. neoformans* isolated from pigeon dropping (Pge 12/2); Pge = Pigeon.

C. RAPD analysis of *C. neoformans* (domestic isolates and their clinical isolates)

Two environmental (Do 3/2 and Pge 12/2) and two clinical (Cne 3 and Cne 12) isolates of *C. neoformans* serotype A (var. *grubii*) were analyzed by RAPD with three primers (R28, OPH-02 and OPH-20). Primer R28 generated two profiles (a and c) among two environmental and two clinical isolates. Two clinical and one environmental (Do 3/2) isolates were of profile a (Fig. 15) and the other one (Pge 12/2) were of profile c (Fig. 17). Primer OPH-02 generated two profiles (i and iii) among two environmental and two clinical isolates. Two clinical and one environmental (Do 3/2) isolates were of profile i (Fig. 15) and the other one (Pge 12/2) were of profile iii (Fig. 17). Primer OPH-20 generated three profiles (1, 2 and 6) among isolates. One clinical (Cne 3) and one environmental (Do 3/2) isolate were of profile 2 (Fig. 16). One clinical (Cne 12) and one environmental (Pge 12/2) isolate were of profile 1 (Fig. 16) and 6 (Fig. 17), respectively. RAPD profiles with these three primers revealed three patterns (I, II and VI) among isolates. Table 8 summarized the RAPD profiles for all isolates. One clinical (Cne 3) and one environmental (Do 3/2) isolate were of pattern II. One clinical (Cne 12) and one environmental (Pge 12/2) isolate were of pattern I and VI, respectively.

Figure 15. RAPD profiles of five isolates of *C. neoformans* (lane 1,6 : Cne 3 ; lane 2,7 : Cne 12 ; lane 3,8 : Do 3/2 ; lane 4,9 : Pge 12/2 ; lane 5,10 : Pt 39). Lane1-5 : patterns generated with primer R28. Lane 6-10 : patterns generated with primer OPH-02. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Figure 16. RAPD profiles of five isolates of *C. neoformans* (lane 1 : Cne 3 ; lane 2 : Cne 12 ; lane 3 : Do 3/2 ; lane 4 : Pge 12/2 ; lane 5 : Pt 39). Lane1-5 : patterns generated with primer OPH-20. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Figure 17. RAPD profiles of environmental isolates of *C. neoformans* (Pge 12/2). Lane 1 : pattern generated with primer R28. Lane 2 : pattern generated with primer OPH-02. Lane 3: pattern generated with primer OPH-20. Lane M : 1 kb plus DNA Ladder. Molecular size markers (in base pairs) are indicated on the left.

Table 8. RAPD profile of clinical and environmental isolates.

Isolates No.	RAPD profile			Pattern
	Primer R28	Primer OPH-02	Primer OPH-20	
Cne 3	a	i	2	II
Cne 12	a	i	1	I
Do 3/2	a	i	2	II
Pge 12/2	c	iii	6	VI

Primer R28

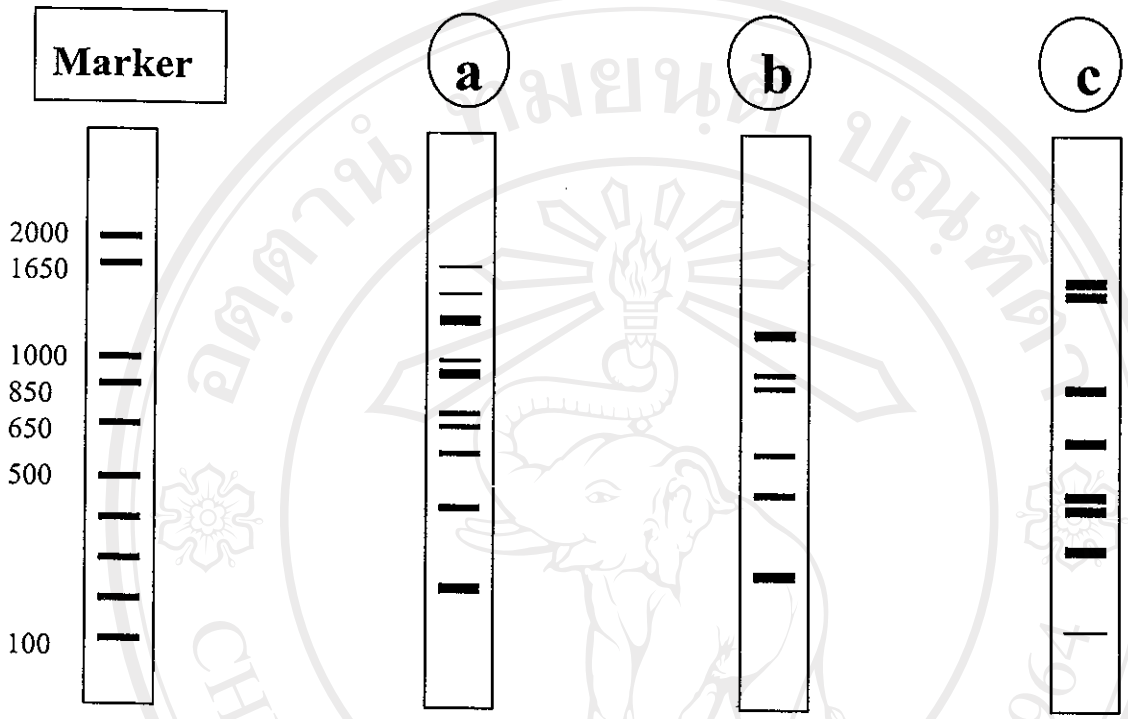

Figure 18. Schematic representation of the three distinct RAPD profiles of *C. neoformans* isolates generated with primer R28.

Primer OPH-02

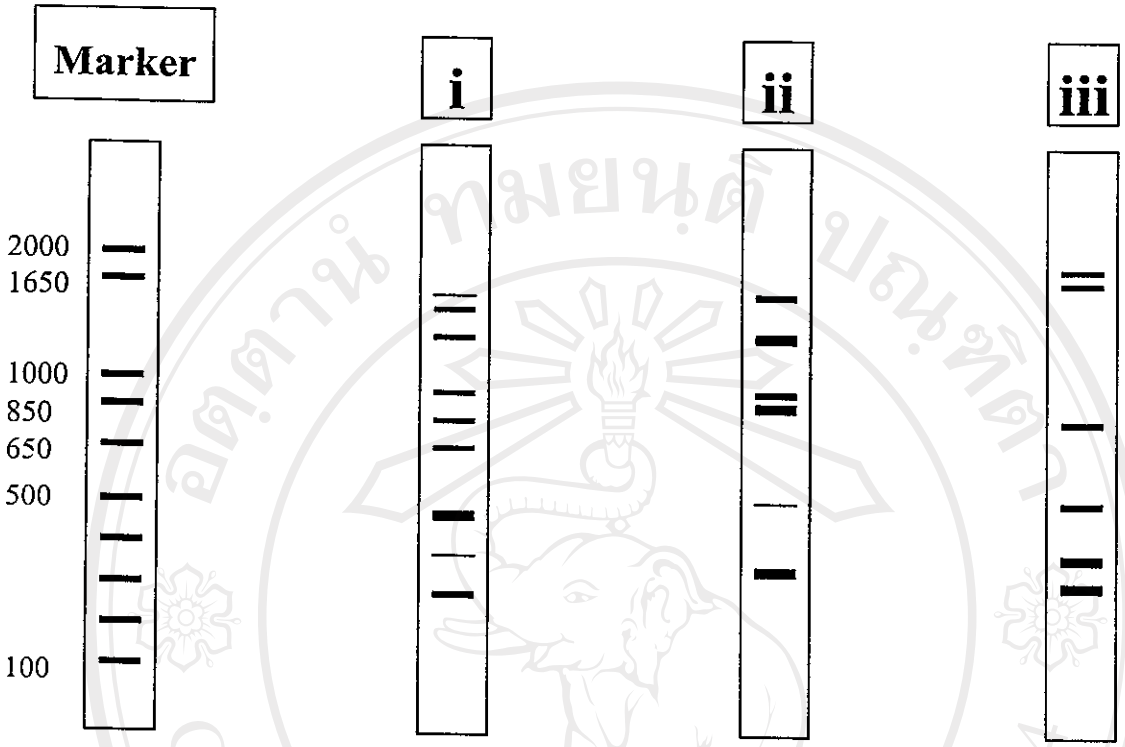

Figure 19. Schematic representation of the three distinct RAPD profiles of *C. neoformans* isolates generated with primer OPH-02.

Primer OPH-20

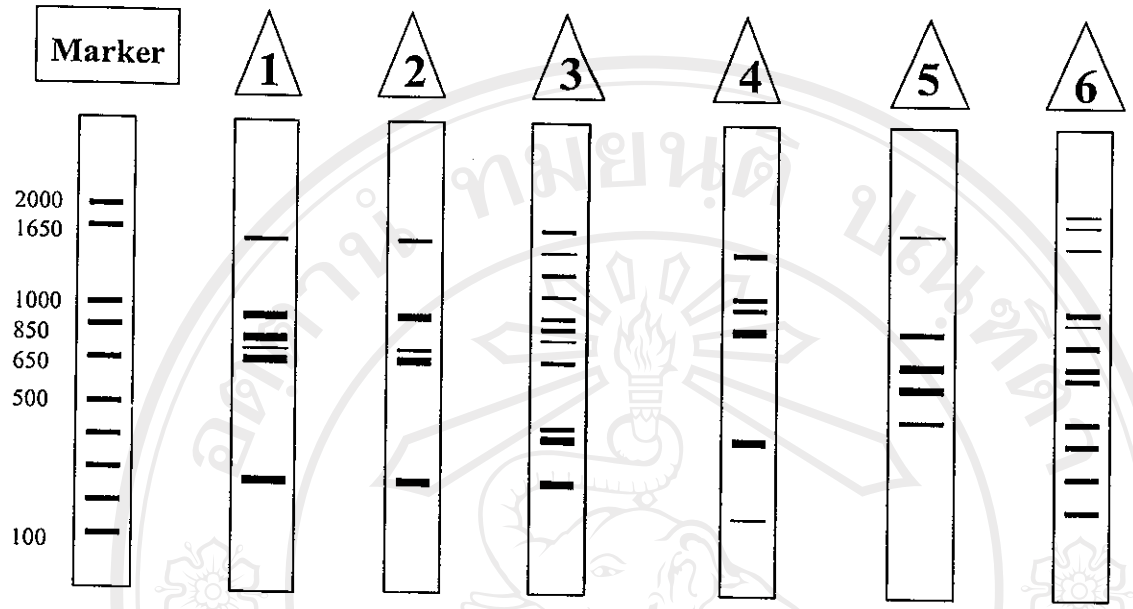

Figure 20. Schematic representation of the six distinct RAPD profiles of *C. neoformans* isolates generated with primer OPH-20.