IV. MATERIALS AND METHODS

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. *C. neoformans* isolates

Fifty clinical and 50 environmental isolates of *C. neoformans* serotype A (var. *grubii)* were collected in Chiang Mai, during the period of one year (from May 1999 to April 2000). Furthermore, two clinical isolates and ten clinical isolates were obtained from Japan and Khon Kaen, respectively. List of *C. neoformans* isolates is shown in Table 1.

Table 1. List of isolates of *C. neoformans* in the study

Isolates No.[a]	Isolate type[b]	Source	Geographical origin	Serotype[c]
Pt1	C	Cryptococcosis patient	Chiang Mai	A
Pt2	C	Cryptococcosis patient	Chiang Mai	A
Pt3	C	Cryptococcosis patient	Chiang Mai	A
Pt5	C	Cryptococcosis patient	Chiang Mai	A
Pt7	C	Cryptococcosis patient	Chiang Mai	A
Pt9	C	Cryptococcosis patient	Chiang Mai	A
Pt11	C	Cryptococcosis patient	Chiang Mai	A
Pt12	C	Cryptococcosis patient	Chiang Mai	A
Pt13	C	Cryptococcosis patient	Chiang Mai	A
Pt15	C	Cryptococcosis patient	Chiang Mai	A
Pt16	C	Cryptococcosis patient	Chiang Mai	A

Table 1. (Continued)

Isolates No.[a]	Isolate type[b]	Source	Geographical origin	Serotype[c]
Pt17	C	Cryptococcosis patient	Chiang Mai	A
Pt19	C	Cryptococcosis patient	Chiang Mai	A
Pt21	C	Cryptococcosis patient	Chiang Mai	A
Pt22	C	Cryptococcosis patient	Chiang Mai	A
Pt23	C	Cryptococcosis patient	Chiang Mai	A
Pt24	C	Cryptococcosis patient	Chiang Mai	A
Pt25	C	Cryptococcosis patient	Chiang Mai	A
Pt28	C	Cryptococcosis patient	Chiang Mai	A
Pt30	C	Cryptococcosis patient	Chiang Mai	A
Pt31	C	Cryptococcosis patient	Chiang Mai	A
Pt32	C	Cryptococcosis patient	Chiang Mai	A
Pt33	C	Cryptococcosis patient	Chiang Mai	A
Pt34	C	Cryptococcosis patient	Chiang Mai	A
Pt39	C	Cryptococcosis patient	Chiang Mai	A
C1	C	Cryptococcosis patient	Chiang Mai	A
C3	C	Cryptococcosis patient	Chiang Mai	A
C4	C	Cryptococcosis patient	Chiang Mai	A
C5	C	Cryptococcosis patient	Chiang Mai	A
C7	C	Cryptococcosis patient	Chiang Mai	A
C8	C	Cryptococcosis patient	Chiang Mai	A
C9	C	Cryptococcosis patient	Chiang Mai	A
C10	C	Cryptococcosis patient	Chiang Mai	A
C11	C	Cryptococcosis patient	Chiang Mai	A
C12	C	Cryptococcosis patient	Chiang Mai	A
C13	C	Cryptococcosis patient	Chiang Mai	A
C14	C	Cryptococcosis patient	Chiang Mai	A
C16	C	Cryptococcosis patient	Chiang Mai	A

Table 1. (Continued)

Isolates No.[a]	Isolate Type[b]	Source	Geographical origin	Serotype[c]
C17	C	Cryptococcosis patient	Chiang Mai	A
C18	C	Cryptococcosis patient	Chiang Mai	A
C19	C	Cryptococcosis patient	Chiang Mai	A
C20	C	Cryptococcosis patient	Chiang Mai	A
C22	C	Cryptococcosis patient	Chiang Mai	A
C23	C	Cryptococcosis patient	Chiang Mai	A
C26	C	Cryptococcosis patient	Chiang Mai	A
C27	C	Cryptococcosis patient	Chiang Mai	A
C30	C	Cryptococcosis patient	Chiang Mai	A
C31	C	Cryptococcosis patient	Chiang Mai	A
C33	C	Cryptococcosis patient	Chiang Mai	A
C34	C	Cryptococcosis patient	Chiang Mai	A
Pg1	E	Pigeon dropping	Chiang Mai	A
Pg2	E	Pigeon dropping	Chiang Mai	A
Pg3	E	Pigeon dropping	Chiang Mai	A
Pg21	E	Pigeon dropping	Chiang Mai	A
Pg26	E	Pigeon dropping	Chiang Mai	A
Pg32	E	Pigeon dropping	Chiang Mai	A
Pg37	E	Pigeon dropping	Chiang Mai	A
Pg46	E	Pigeon dropping	Chiang Mai	A
D1	E	Dove dropping	Chiang Mai	A
D2	E	Dove dropping	Chiang Mai	A
D3	E	Dove dropping	Chiang Mai	A
D4	E	Dove dropping	Chiang Mai	A
D5	E	Dove dropping	Chiang Mai	A
D6	E	Dove dropping	Chiang Mai	A
D9	E	Dove dropping	Chiang Mai	A

Table 1. (Continued)

Isolates No.[a]	Isolate type[b]	Source	Geographical origin	Serotype[c]
D12	E	Dove dropping	Chiang Mai	A
D13	E	Dove dropping	Chiang Mai	A
D14	E	Dove dropping	Chiang Mai	A
D15	E	Dove dropping	Chiang Mai	A
D16	E	Dove dropping	Chiang Mai	A
D17	E	Dove dropping	Chiang Mai	A
D18	E	Dove dropping	Chiang Mai	A
D19	E	Dove dropping	Chiang Mai	A
D22	E	Dove dropping	Chiang Mai	A
D24	E	Dove dropping	Chiang Mai	A
D25	E	Dove dropping	Chiang Mai	A
D26	E	Dove dropping	Chiang Mai	A
D27	E	Dove dropping	Chiang Mai	A
D28	E	Dove dropping	Chiang Mai	A
D30	E	Dove dropping	Chiang Mai	A
D31	E	Dove dropping	Chiang Mai	A
D33	E	Dove dropping	Chiang Mai	A
D34	E	Dove dropping	Chiang Mai	A
D35	E	Dove dropping	Chiang Mai	A
D36	E	Dove dropping	Chiang Mai	A
D41	E	Dove dropping	Chiang Mai	A
D42	E	Dove dropping	Chiang Mai	A
D43	E	Dove dropping	Chiang Mai	A
D44	E	Dove dropping	Chiang Mai	A
D45	E	Dove dropping	Chiang Mai	A
D46	E	Dove dropping	Chiang Mai	A
D64	E	Dove dropping	Chiang Mai	A

Table 1. (Continued)

Isolates No.[a]	Isolate type[b]	Source	Geographical origin	Serotype[c]
D69	E	Dove dropping	Chiang Mai	A
D71	E	Dove dropping	Chiang Mai	A
D73	E	Dove dropping	Chiang Mai	A
D76	E	Dove dropping	Chiang Mai	A
D79	E	Dove dropping	Chiang Mai	A
D82	E	Dove dropping	Chiang Mai	A
D83	E	Dove dropping	Chiang Mai	A
E24	E	Eucalyptus flower	Chiang Mai	A
K2	C	Cryptococcosis patient	Khon Kaen	A
K15	C	Cryptococcosis patient	Khon Kaen	A
K25	C	Cryptococcosis patient	Khon Kaen	untypeable
K31	C	Cryptococcosis patient	Khon Kaen	A
K36	C	Cryptococcosis patient	Khon Kaen	A
K38	C	Cryptococcosis patient	Khon Kaen	B
K39	C	Cryptococcosis patient	Khon Kaen	A
K45	C	Cryptococcosis patient	Khon Kaen	A
K67	C	Cryptococcosis patient	Khon Kaen	A
K97	C	Cryptococcosis patient	Khon Kaen	untypeable
J1	C	Cryptococcosis patient	Japan	A
J2	C	Cryptococcosis patient	Japan	A

[a] Pt, clinical isolates of *C. neoformans* obtained from The Office of Communicable Disease control (Region 10); C, clinical isolates of *C. neoformans* obtained from cryptococcosis patients who admitted to Maharaj Hospital, Faculty of Medicine, Chiang Mai University; Pg, Pigeon dropping; D, Dove dropping; K, Clinical isolates of *C. neoformans* obtained from Khon Kaen; J, Clinical isolates of *C. neoformans* obtained from Japan. [b] C, Clinical; E, Environmental. [c] Serotype determined by slide agglutination test with monoclonal antibodies specific to capsular polysaccharide (Crypto check, Iatron, Tokyo).

B. DNA extraction

Genomic DNA of *C. neoformans* was extracted by a method modified from those of Hynes et al., 1995 and Kersulyte et al., 1992. Each isolate was grown on SDA slants at 37°C for 72 hours. Three or four loopfuls of colonies from the slants were washed twice with phosphate-buffered saline, and resuspended in 500 µl of lysis buffer (50mM Tris-HCl pH 8.0-10 mM EDTA-150 mM NaCl-2% (w/v) Sodium dodecyl sulfate). Suspensions were incubated at 65°C for 1 hour, 100°C for 5 min, and then, were added with 500µl of phenol: chloroform: isoamyl alcohol (25:24:1). The microtubes were inverted about 5 minutes for mixing and centrifuged at 9,500xg for 15 min. The supernatants were transferred into a new tube. DNA was precipitated by adding 2 volume of cool isopropanol, and then gently mixed. After centrifugation at 9,500xg for 15 min, the DNA pellet was washed with 70% ethanol, air dried, resuspended in 50 µl of TE buffer, and then kept at –20°C. The DNA concentration was determined with a UV spectrophotometer.

C. RAPD analysis

The following three oligonucleotide primers were used for the RAPD analysis: 5'-ATGGATCCGC-3' (R28), 5'-TCGGACGTGA-3' (OPH-02), and 5'-GGGAGACATC-3' (OPH-20). Amplification reactions were performed in a 50 µl volume containing 25 ng of genomic DNA, 10X reaction buffer (100 mM Tris-HCl pH 8.3, 50 mM KCl, 0.001% gelatin), 25 mM $MgCl_2$, 10 mM dNTPs mix, 2.5 U *Taq* DNA polymerase and 25 µM (each) primer. Amplification was carried out in a Perkin Elmer Cetus DNA thermal cycle model 480, which was programmed for a first cycle of 4 min at 94 °C, 40 cycles of 45 sec at 92 °C, 60 sec at 30 °C, and 90 sec at 72 °C, and a final cycle of 10 min at 72 °C.

The amplification products were electrophoresed in agarose gels (2.0% Seakem ME and 0.1% Nusieve GTG) with 1X TAE buffer at 50 V for 1 h in *i*- Mupid (Cosmo Bio CO., LTD.) and the amplification products were visualized by UV light after staining with ethidium bromide. Band sizes were estimated from comigration of 1 Kb plus DNA Ladder (Gibco BRL) molecular size standard.

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

A. Patients and dwellings sampling.

Fifty-two clinical isolates of *C. neoformans* isolated from cryptococcosis patients in Chiang Mai were collected by Central Laboratory, Maharaj Hospital, Faculty of Medicine, Chiang Mai University, during the period of May to October 2002. Patients' home addresses were recorded. These clinical isolates were stored in distilled water at room temperature and in 20% glycerol at -20 °C.

B. Domiciliary visits

After a signed permission by the inhabitants, the patients' homes were visited. Presence of caged birds, chicken or ducks in domestic environment and in nearby surroundings were recorded on a questionnaire.

C. Environmental materials sampling

A total of 202 samples of avian droppings, including pigeon, dove, chicken and duck droppings (Fig. 6) were collected in clean plastic bags with metal spatula and forceps disinfected with 70% ethanol. Samples were labelled and brought to the laboratory for processing.

D. Material processing

Five-gram portion of each environmental sample was placed in a screw-capped test tube containing 30 ml of sterile phosphate buffer saline (PBS) with 4 mg/ml of sodium penicillin G and 2 mg/ml of streptomycin sulfate. The suspensions were vigorously shaken for 5 min and allowed to settle for approximately 15 min. The undiluted suspensions were plated directly by spreading 0.1 ml over the surface of both Sabouraud's Dextrose Agar (SDA) (plus 0.4 g/L chloramphenicol) and Littman's Oxgall Agar (LOA). All plates were incubated at 37°C and were observed daily for one week.

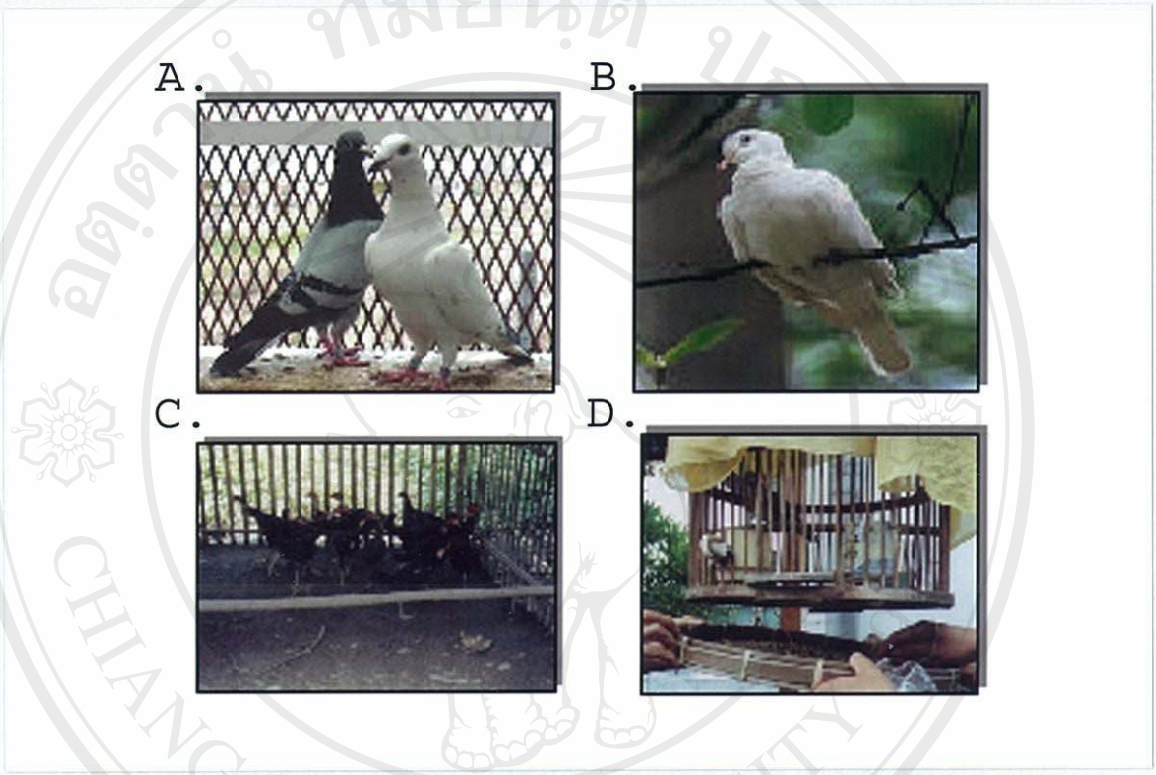

Figure 6. A. Picture of pigeon.
B. Picture of dove.
C. Picture of chicken.
D. Specimens of avian droppings were collected in clean plastic bags.

E. Identification

All suspected smooth yeast colonies were studied microscopically using India ink preparations. Globose, oval encapsulated, uni- or multi-budding yeast forms were streaked on SDA slants. The organisms were preliminary tested for urease production in urea agar slants and phenol oxidase production on caffeic acid agar slants, respectively The positive isolates, urease and brown- black colony production, were then identified by carbohydrate assimilation and fermentation tests. *C. neoformans* can assimilate glucose, galactose, sucrose, maltose and raffinose, but not for lactose (Table 2).

F. Serovariety typing

Serovarieties of *C. neoformans* were determined by slide agglutination test with monoclonal antibodies specific to capsular polysaccharide (Crypto check, Iatron, Tokyo). One loopful of each isolate was suspended in 200 µl 0.85% normal saline. The suspension was adjusted to Mac Farland No.1. Ten-microlitres suspensions were examined for agglutination reaction by mixing with 10 µl of antiserum on glass slide for 2 min. Suspensions were tested with all types of antiserum, including polyvalent antiserum, antiserum A, B, C, and D (Table 3). Known serotypes of *C. neoformans* (serotype A, strain MUMT01; serotype B, strain MUMT25; serotype C, strain RV45978 and serotype D, MUMT53) were used as control in the tests.

Table 2. Biological characteristics of *C. neoformans*

	C. neoformans
Culture	
- Sabouraud's Dextrose Agar (SDA)	cream colored, smooth, mucoid colonies
- Littman's Oxgall Agar (LOA)	blue colored, smooth, mucoid colonies
- Caffeic acid agar	brown-black colonies
- Urea agar	+
Fermentation	
- Glucose, galactose, sucrose, maltose raffinose, lactose	-
Assimilation	
- Glucose, galactose, sucrose, maltose raffinose	+
- Lactose	-

+, Positive

-, Negative

Table 3. Agglutination reaction between antiserum with *C. neoformans* serotypes

Antiserum	Serotype				
	A	B	C	D	AD
A	+	-	-	-	+
B	-	+	-	-	-
C	-	-	+	-	-
D	-	-	-	+	+
Polyvalent	+	+	+	+	+

+, Positive agglutination test

-, Negative agglutination test

G. Molecular typing by RAPD analysis

Environmental isolates of *C. neoformans* collected from cryptococcosis patient's house areas and their clinical isolates were extracted for genomic DNA. Molecular typing by RAPD analysis with three primers were performed as described above. RAPD patterns of environmental isolates that were isolated from avian droppings were compared with those of clinical isolates to determine their relations.

H. Biosafety consideration

All procedures were carefully performed following biosafety level 2 to minimize the creation of aerosols. All contaminated devices and wastes had to be autoclaved, incinerated or immersed in a germicide prior to disposal or reuse.