GENOTYPING OF *CRYPTOCOCCUS NEOFORMANS* SPECIES COMPLEX THAI ISOLATES

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รูปแบบทางพันธุกรรมของ *Cryptococcus neoformans* species complex ในประเทศไทย

นางสาวศิรดา ขาวเจริญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	Genotyping of *Cryptococcus neoformans* species complex Thai isolates
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ศิรดา ขาวเจริญ : รูปแบบทางพันธุกรรมของ *Cryptococcus neoformans* species complex ในประเทศไทย. (Genotyping of *Cryptococcus neoformans* species complex Thai isolates) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร. อริยา จินดามพร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: Associate Professor Wieland Meyer, Ph.D. , 222 หน้า.

วิธี Multilocus Sequence Typing (MLST) และ Multilocus Microsatellite Typing (MLMT) ใช้ในการตรวจสอบความหลากหลายทางพันธุกรรมของเชื้อ *Cryptococcus neoformans* species complex ที่แยกได้จากแหล่งต่างๆได้แก่ ผู้ป่วย สัตว์และสิ่งแวดล้อมในประเทศไทย ระหว่างช่วงปี พ.ศ. 2546 ถึง พ.ศ. 2550 การศึกษาคัดกรองรูปแบบทางพันธุกรรมระดับ variety ของเชื้อ *C. neoformans* species complex จำนวน 481 สายพันธุ์ ด้วยวิธี *URA5*-RFLP พบว่า เชื้อทั้งหมดเป็น *C. neoformans* var. *grubii* (serotype A) molecular type VNI (98%) และ VNII (2%) และมี mating type ชนิด mating type α การแยกรูปแบบพันธุกรรมระหว่างสายพันธุ์เบื้องต้นด้วยวิธี M13-fingerprinting ผลพบเชื้อ *Cryptococcus* มีรูปแบบของ fingerprinting ทั้งหมดจำนวน 9 subtypes ได้แก่ subtype A ถึง I โดยที่ subtype A, B, C, D, E และ F จัดอยู่ใน molecular type VNI และ subtype G, H และ Iจัดอยู่ใน molecular type VNII โดย subtype ที่พบมากที่สุด คือ subtype A (88.25%) จากนั้นได้ทำการสุ่ม *C. neoformans* var. *grubii* จำนวน 31 สายพันธุ์ที่เป็นตัวแทนแต่ละ subtype มาวิเคราะห์ต่อด้วยวิธี MLST และ MLMT ซึ่งวิธีนี้เป็นการศึกษารูปแบบความแตกต่างในลำดับเบสรวม (Sequence Type; ST) ของ housekeeping gene และ virulence gene รวมทั้งหมด 8 ยีนในเชื้อแต่ละสายพันธุ์ โดย ST เป็นการรวมรูปแบบความแตกต่างในลำดับเบสของแต่ละยีน (Allele Type; AT) ของเชื้อแต่ละสายพันธุ์เข้าด้วยกัน จากการศึกษา MLST พบรูปแบบทางพันธุกรรม 12 STs และเมื่อทำการเปรียบเทียบกับฐานข้อมูล MLST พบว่ามี AT ใหม่ที่ค้นพบทั้งหมด 13 ATs ส่วนวิธี MLMT จะศึกษาในส่วนของลำดับเบสซ้ำบริเวณต่างๆ (Microsatellite allele; MA) และนำผลของแต่ละบริเวณมาวิเคราะห์ร่วมกัน (Microsatellite type; MT) โดยศึกษารวม 5 Loci และพบ Microsatellite Type จำนวน 13 MTs เมื่อเปรียบเทียบผลการศึกษารูปแบบทางพันธุกรรมทั้งสามวิธีนี้พบว่าวิธี MLST และ MLMT มีค่าความสามารถในการจำแนกรูปแบบทางพันธุกรรมสูงกว่าวิธี M13-fingerprinting โดยมีค่าเป็น 0.86, 0.82 และ 0.75 ตามลำดับจากข้อมูล MLST เมื่อนำไปศึกษาด้วยการสร้าง Phylogenetic tree พบว่าเชื้อ *C. neoformans* var. *grubii* ทั้ง 31 สายพันธุ์มีลักษณะทางพันธุกรรมที่ใกล้เคียงกันและสามารถจัดอยู่ในกลุ่มเดียวกัน นอกจากนี้เมื่อเปรียบเทียบกับเชื้อที่แยกได้แหล่งต่างๆทั่วโลกพบว่ามีรูปแบบทางพันธุกรรมที่คล้ายคลึงกัน ในการศึกษานี้กล่าวโดยสรุปได้ว่า เชื้อ *C. neoformans* var. *grubii* เป็นเชื้อที่พบได้มากที่สุดในประเทศไทยซึ่งสอดคล้องกับรายงานก่อนหน้า และมีรูปแบบทางพันธุกรรมทั้งหมด 12 STs และ 13 MTs โดยเป็นวิธีที่เหมาะสมในการศึกษาระบาดวิทยาและผลการศึกษาที่ได้สามารถนำไปเปรียบเทียบกับข้อมูลจากห้องปฏิบัติการต่างๆและสามารถเผยแพร่ข้อมูลผ่านสื่อออนไลน์ได้

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2552 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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SIRADA KAOCHAROEN: GENOTYPING OF *CRYPTOCOCCUS NEOFORMANS* SPECIES COMPLEX THAI
THESIS ADVISOR: ASSOC. PROF. ARIYA CHINDAMPORN, Ph.D.,
THESIS CO-ADVISOR : ASSOC. PROF. WIELAND MEYER, Ph.D., 222 pp.

To investigate the genetic polymorphism of human, animal, and environmental isolates of *Cryptococcus neoformans* in Thailand during 2003 – 2005, Multilocus Sequencing Typing (MLST) and Multilocus Microsatellite Typing (MLMT) techniques were used. 481 isolates of *C. neoformans* species complex from clinical and environmental sources were screened for their variety using *URA5*-RFLP, mating type. All the isolates were identified as *C. neoformans* var. *grubii* serotype A, molecular type VNI (98%) and molecular type VNII (2%) with mating type α. Intra-species level were studied by M13-fingerprinting technique. Nine subtypes, subtype A to I, were observed. Subtype A, B, C, D, E, and F were grouped in molecular type VNI while subtype G, H and I were molecular type VNII. The most frequency subtype was subtype A (88.25%). Totally 31 isolates from all individual subtype was recruited for MLST and MLMT study. For MLST, the analytical fragments (Sequence Type, ST) of each individual isolate were obtained by PCR reaction using primers from eight of housekeeping and virulence genes. ST is composed of the combination of allele types (AT) or allele profiles from any distinct genes. The genetic variation of 12 STs was demonstrated. Comparing our STs with MLST database website, new 13 ATs were revealed. For MLMT method, the allele of each DNA locus (microsatellite allele; MA) were analyzed in the combination of locus; resulting Microsatellite type (MT). In this study, five loci showed polymorphisms, totally 13 MTs found in the study. The concordance of M13-fingerprinting, MLST and MLMT method was found with the discriminatory power of 0.75 and 0.86 and 0.82 respectively. Thus, the genetic information among Thai isolates was classified in the same genetic cluster shared and grouped. In addition, the genetic relationship with global isolates was also demonstrated. In conclusion, *C. neoformans* var. *grubii* was the majority variety in Thailand according with the previous report. Genetic polymorphism revealed 12 STs and 13 MTs. MLST is one of the promising typing methods for epidemiological study and the result can be comparable and also applicable in the e-network.

Field of Study: Medical Microbiology Student's Signature .....
Academic year: 2009 Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I

Introduction

Recently, *Cryptococcus neoformans* species complex; the causing agent of cryptococcosis, comprise with two species and 5 varieties of the organisms; *C. grubii* (serotype A), *C. neoformans* var. *neoformans* (serotype D), *C. neoforamns* AD hybrid (serotype AD) and *C. gattii* (serotype B and C). The distinguish properties between varities are polysaccharide capsule composition, environmental source, illness severity, distribution and also the teleomorph morphology (1). The nature habitat of this yeast is hypothesized, however there has been successfully isolated this encapsulated yeast from Eucalyptus tree, Big tree, pigeon dropping and contaminated soil (2). The various kinds of animal are also suspected as the source of *C. neoformans* species complex such as canine, feline, horse and birds (3). However, the actual infected source of the organism and the ecological nitch are still unclear.

Cryptococcosis is one majority of the life threatening mycoses in both immunocompromised and immunocompetent host, especially in patients with AIDS (2). This disease has distributed worldwide and its rate of infection has markedly increased in recent years (4, 5). This situation has been also found in Thailand and this infection was ranked the second opportunistic infection in HIV infected patient (6). It is evidented that the patient inhaled the small basidiospore (sexual spore) and/or blastospore (budding yeast cell) into the lung. In immunocompetent host, this yeast cells are eliminated by immune response, resulting the relief of the infection. On the other hand, in the immunocompromised host, the infected propagules are unable to be

killed by the immune system, and then the primary symptom will occur in the lower respiratory tract, called cryptococcal pneumonia. This infected yeast can spread the target organ, the central nervous system prior disseminate through other organs including skin, causing fatal meningitis, finally (7). In general, *C. gatti*i is common isolated from the immunocompetent hosts whereas others varieties of *Cryptococcus* cause the disease in the immunocompromised hosts (2). The reason of this different favors infection is still unknown. Not only the host condition factor but virulence factors of the organism are also affected the severity of the disease. Its significant virulence factors are capsular composition, phenoloxidase, human body temperature growth ability (35-37 C), and phospholipase (8).

As mentioned before, difference species of this encapsulated yeast is able to cause distinct manifestation. The reason or their mechanism is still vague even though some genetic information has been reported (9). If we have the genetic information of *C. grubii* and *C. neoformans* isolated from patients together with their demography and their clinical manifestations, the reasons and the mechanism could be revealed. Actually it is difficult to collect all the data and then analyze. One more factor is that the polymorphism among species has been evidenced. To analyze the diversity, several molecular typing techniques have been developed, such as Multilocus Enzyme Electrokaryotype (MLEE), DNA hybridization, PCR-fingerprinting (10-13). Amplify Fragment Length Polymorphisms (AFLP) analysis (14), Restriction Fragment Length Polymorphisms (RFLP) analysis (15), However, all of these typing methods still have drawbacks such as inadequate discrimination power, inability to estimate the genetic relationships among the isolates, low reproducibility and the most important is the difficulty in comparing the result between laboratories. To overcome all these points,

two new approaches, Multilocus Sequence Typing (MLST) and Multilocus Microsatellite Typing (MLMT) were established (16-18).

Multilocus sequence typing (MLST) is a technique that was for the first time recommended by Maiden *et al* (18). MLST technique is based on sequencing of ten or more genes to identify the genetic diversity of organisms such as bacteria and fungi (16, 18-20). For each gene, the different sequences are assigned as alleles. All alleles of each gene are called allele profiles. Finally the alleles of all sequenced genes are assigned as sequence type (STs) for each isolate (16, 18-20).

While MLMT technique was also reported and used for typing many kinds of fungus such as *Penicillium maneffeii, Neurospora sp., Aspergillus fumigatus* and *C. neoformans* var. *grubii* (19, 21-23). This technique is based on the different of repeating unit length; single tandem repeat (STR); of the genome of organisms since the length and the position of the unit will differ between the strains. Similar with MLST, many loci of repeating unit were study for each locus the different sequences are assigned as microsatellite type (MT). All MT of each locus are analyzed in the profiles. These new approach methods can overcome the drawback of the old fashion typing methods easily to transport data leading to data highly portable, no isolates exchange between laboratory and infection source tracking and most important they can reveals the genetic evolution and phylogenetic relation (16, 18-20).

In Thailand, the first environmental isolates from cuckoo droppings and a sample from pigeon nest was reported in 1968 (16). This report was also the first time of discovery of *C. neoformans* from natural habitat in Southeast Asia. The serotyping of 187 *C. neoformans* clinical isolates was reported in 1996 by using commercial agglutination test (24). From the results, the author suggested that during the pre

AIDS-era in Thailand, serotype B was a common pathogen of cryptococcosis. This pattern changed drastically after the AIDS epidemic, with serotype A becoming the predominant serotype, which is similar with the predominant serotype reported worldwide.

Poonwan and co-workers reported the serotype of 97 isolates of *C. neoformans* Thai isolate and showed that serotype A was a predominate type according to the previous study (25). The molecular type was also examined in the same study by using RAPD technique. The result showed the different patterns between *C. neoformans* and *C. gattii*. 87 isolates of *C. neoformans* was typed by using M13-fingerprinting and serotyping.

From retrospective review of the molecular study in Thailand showed that the selected molecular method of each study was different and the result was not useful since it can not compare with the global study because the limitation of the typing technique. And more over, the isolated from the other animal, except bird, has been not reported. In this study, we study the epidemiology using M13-fingerpritning, *URA5*-RFLP, MLST and MLMT technique to reveal the genetic diversity of *C. neoformans* species complex Thai isolates. The sources of the isolates were clinical, environment and veterinary isolates. We aim to establish the cryptococcal epidemiology from Thai isolates with the global network since the Cryptococcus are one of recently worldwide; including Thailand; major morbidity causing in AIDS patient. The benefit of the study is to understand the molecular pattern of this yeast that can use for prevent the outbreak crisis in Thailand and also to more understand the genetic distribution.

CHAPTER II

Review Literature

1. Discovery of *Cryptococcus neoformans*

In 1894, *C. neoformans* was for the first time identified as a human pathogen. Two German physicians, Otto Busse and Abraham Buschke, isolated the yeast from a tebial of a 31 years old infected patient and the organism was thought to be a blastomycete. In the following year Sanfelice, an Italian scientist also isolated a similar yeast from peach juice and called it *Saccheromyces neoformans*. In addtion Sanfelice demonstrated that this yeast was pathogenic in laboratory animals (26). In the 1960s Busse isolated the yeast cell from the lesion and observed that the round yeast cell had a capsule. He called the organism *Saccharomyces hominis* (1).

In 1895, Curtis, a French pathologist, isolated a similar organism from an ulcerated lesion and named it *Saccharomyces subcutaneous tumefacens* (2).

In 1901, Vuillemin, a French botanist, found that this yeast did not produce ascospores and was also unable to consume the carbon sources during fermentation. He changed the name of this yeast to *Cryptococcus hominis*. In 1905, von Hansemann reported the first case of crypococcal meningtitis (2).

In 1916, this organism was renamed to *Torula histolytica*, since Stoddard and Cutler incorrectly reported that the clearing that appeared surrounding the yeast cells in host tissue was caused by the digestion of cellular matter and believed that it was a different organism. The name *Torula histolytica* remained for over fifty years, in part

due to Stoddard and Cutler's published literature being widely cited. However, in 1931 Freeman described that the clearing around the yeast cells actually was due to the capsule. The same report proposed for the first time the infection pathway, the respiratory tract was hypothesized as a primary site of infection (2).

In 1935, Benham re-examined the yeasts that had been classified into various genera such as *Torula, Cryptococcus* and *Saccharomyces*. The results of his studies differed from the previous studies, in the fact that he showed that morphology alone was not enough to differentiate these organisms. Benham also for first time reported a classification based on the physiological characteristics, such as fermentation, serological studies of the capsule and pathogenesis in parallel with colony and cell morphology. He subdivided the yeast into four groups by the serological analysis, based on colony characteristic and agglutination testes, and named it *Cryptococcus hominis* (27). The serotype is determined by structural variation of the capsular polysaccharides (28). The name *Cryptococcus neoformans* was introduced for this pathogenic yeast and has been accepted by mycologist around the world (1).

In 1970, Shadomy identified "clamp connections" in the hyphae of *C. neoformans* and suggested that it was a basidiomycete. This report was associated with Sanfelice, since he observed both yeast cells and hyphae from the peach juice. The discovery of the sexual reproductive cycle was reported by Kwon-Chung (29). Mating experiments were performed and these determined that there were two different sexual states, which produce different of spores. The two sexual states were named *Filobasiodiella neoformans* and *Filobasidiella bacillispora*.

2. Classification

The genus *Cryptococcus* contains forty species. *C. neoformans* is only pathogenic species in this genus. In addition a few cases of infection caused by *C. albidus* and *C. laurentii* have been reported (30).

The members of the genus *Cryptococcus* are anamophic basidiomycetous yeasts, which are unable to assimilate inositol and are able to secrete the enzyme urease (Barnett et al 2002). In the anamorphic state, this organisms does not produce either true hyphae or pseudohypahe.

C. neoformans was originally classified in to two varieties: *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. Recently these were raised to species based on molecular findings: *C. neoformans* and *C. gattii*. *C. neoformans* contains two varieties: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D), as well as a hybrid between serotype A and D.

The diverges of the serotypes A and D which have been estimated to have diverged 37 million years ago, compared with the serotypes B and C was estimated to have been occurred approximately 9.5 million years ago.

Kingdom Fungi
Phylum Basidiomycota
Class Heterobasidiomycetes*
Order *Filobasidiales* (*Tremellales*)*
Family *Filobasidiaceae* (*Tremellacaeae*)*
Genus *Filobasidiella* Kwong-Chung
Species *Filobasidiella depauperata* (Petch)

Cryptococcus aerius (Saito) Nannizzi (pollacci and Nannizzi)
Cryptococcus albidismilis Vashniac and Kurtzman
Cryptococcus albidus (Saito) CE Skinner
Cryptococcus amylolentus (van der Walt et al.) Golubev
Cryptococcus antracticus Vishniac and Kurtzman
Cryptococcus aquaticus (Jones and Sloof) Rodrigues de Miranda and Weijman
Cryptococcus ater (Castellani ex. WB Cooke) Phaff and Fell
Cryptococcus bhutanensis Goto and Sugiyama
Cryptococcus cellulolyticus Nakase
Cryptococcus consortionis Vishniac
Cryptococcus curiosus (Komagata and Nagase) Rodrigues de Miranda and Weijman
Cryptococcus curvatus (Diddens and Lodder) Golubev
Cryptococcus diffluens (Zach) Lodder and Krejer-van Rij
Cryptococcus dimennae Fell and Phaff
Cryptococcus feraegula Saez and Rodrigues de Miranda
Cryptococcus flavus (Saito) Phaff and Fell
Cryptococcus friedmannii Vishniac
Cryptococcus fuscescens Golubev
Cryptococcus gastricus Reiersol and di Menna
Cryptococcus gilvescens Chernov and Bab'eva
Cryptococcus heveanensis (Groenewege) Baptis and Kurtzman
Cryptococcus huempii (Ramirez and Gonzalez) Roeijmans et al.
Cryptococcus humicola (Daszewka) Golubev
Cryptococcus hungaticus (Zsolt) Phaff and Fell
Cryptococcus infrimo-miniatum (Okunuki) Phaff and Fell
Cryptococcus kuetzingii Fell and Phuff
Cryptococcus luteolus (Saito) Skinner
Cryptococcus masverans (Frederiksen) Phaff and Fell
Cryptococcus magnus (Lodder and Kregger-van Rij) Baptis and Kurtzman
Cryptococcus matinus (van Uden and Zobell) Golubev
Filobasidiella neoformans Kwon-Chung — *Cryptocccus neoformans* (Sanfilice)Vuillmin
variety *bacillispora* Kwon-Chung — variety *gattii* Vanbreuseghem and Takashio = *C. gattii* (Kwon-Chung, Boekhout, Fell and Diaz)
variety *neoformans* Kwon-Chung — variety *grubii* Franzot, Salkin and Casadevell
variety *neoformans* (Sanfelice) Vuillmin
Cryptococcus podzolicus (Bab'eva and Roshetova) Golubev
Cryptococcus skinneri Phaff and do Carmo-Sousa
Cryptococcus terreus di Menna
Cryptococcus terricola TA Pedersen
Cryptococcus uniguttulatus
Cryptococcus vishniacii Vishniac and Hempfling
variety *vishniacii* Vishniac and Hempfing
variety *asocialis* Vishniac and Hempfing
variety *vladimiri* Vishniac and Hempfing
variety *wolfi* Vishniac and Hempfing
Cryptococcus wrigtensis Vishniac and Hempfing
Cryptococcus yarrowii A. Fonseca and Uden

Figure1. Current classification of *Cryptococcus neoformans* and *Filobasidiells neoformans* in relation to other *Cryptoccoccus* and *Filobasidiella* species (data from (31))* Molecular based relationship of the order Filobasidiales to the Tremallales, and the family Filobasidiaceae to the Tremellaceae (32).

The sexual state or teleomoph of *Cryptococcus* is classified in the genus *Filobasidiella*, in family *Filobasidiaceae*. This genus contains four species: *F*. *neoformans* Kwon-chung (32), *F*. *depauparata* (33), *F*. *lutea* P. Roberts (34) and *F*. *xianghuijun* Zang (35).

The classification of genus *Cryptococcus* and *Filobasidiella* is shown in Figure 1.

3. Identification of *Cryptococcus neoformans*

3.1 India ink preparation

C. neoformans is an encapsulated yeast. The polysaccharide capsule can be observed under the microscope using an India ink preparation (36). The ink would not stain the yeast cell, but instead provides a dark background and the capsule would be seen as a likely clear zone surrounding the cell.

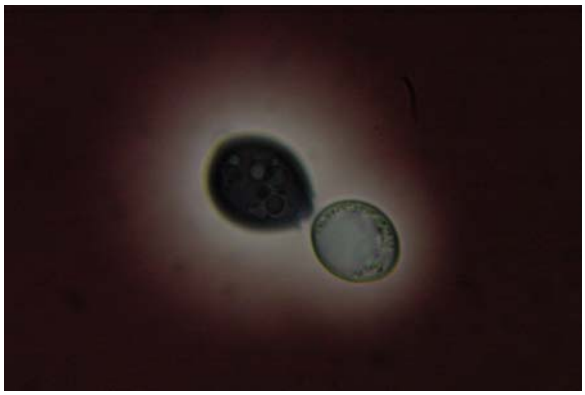


Figure 2. *C. neoformans* cell morphology by India ink preparation; a hollow around the budding yeast cells is caused by the capsule.

3.2 Growth at 37ºC

C. neoformans can be distinguished from the other cryptococcal species by using the ability to grow at 37ºC. However, *C. albidus* and *C. laurentii* are also grow at this temperature.

3.3 Colony morphology

Cell and colony morphology can be used for distinguishing among the members of the genus *Cryptococcus*. However, it should never be used as the sole identification method (36). If *C. neoformans* is grown on Sabouraud‘s Dextrose Agar (SDA) at 30-37 ºC for 48 to 72 hours the colonies are white to cream colored, shiny and muciod with entire margin. *C. neoformans* has a different colony morphology from *C. gattii*. The later one is often more mucoid and dents to have a bigger colony size. In addition *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* cells are globose to spherical while *C. gattii* cells are oval to lemon shaped and have a thicker capsule (37), Barnett et al 2000). However, the growth conditions can affect the characteristic of the colony.

3.4 Melanin Production

The melanin production is a typical property of *C. neoformans*, this mechanism is using the enzyme laccase. The enzyme consumes diphenolic compound as a substrate (more detail of the mechanism in the *Virulence factors* section).

When *C. neoformans* is cultured on media such as birdseed agar, which contains niger seed extraction (*Guizotia abyssnica*) or caffeic acid agar, the yeast cells

will be colored dark brown. These media are very useful when isolating *C. neoformans* from the environment or clinical samples, since it can separate *C. neoformans* from other yeasts (38). However, false positives and negatives are observed when the colonies are pale dark (Wickes et al, 1997). Moreover, green colonies can be observed in some strains of *C. neoformans* var. *gattii* and *C. laurentii* (9-10)

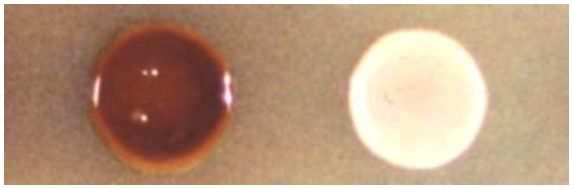


Figure3. Melanin production of *C. neoformans*; melanin synthesis resulting in a dark colony (left) and the white colony (right) is a colony unable to produce melanin.

3.5 Biochemical test

Biochemical tests an be used to differentiate *C. neoformans* from other yeasts. However, either *C. neoformans* or *C. gattii* show the same results for fermentation and assimilation.

Sugar fermentation, including: Glucose, Sucrose, Lactose, Galactose, Maltose and Trehalose, is negative. However, there could be various assimilation results for each substrate. The result are positive for glucose, galactose, maltose, sucrose, trehalose, D-Xylose, melezitose. Weak positive results are found for raffinose, cellobiose, inositol, L-Rhamnose, D-arabinose and D-ribose. For L-arabinose, galactitol, D-Mannitol and D-glucitol the results can be delayed. The variable results are also found for soluble starch, glycerol, succinic acid, ribitol, L-sorbose, salicin, citric acid and DL-lactic acid.
The assimilation of potassium nitrate, lactose, melibiose and erythritol is negative.

There are two commercial biochemical identification kits available; Vitek Yeast Biochemical card and API ID32C system (both from BioMérieux, France). These kits comprise miniaturized and standardized biochemical tests sufficiently specific to identify any yeast contained in the respective database provided with the kits.

3.6 Growth on L-canavanine glycine bromthymol blue (CGB) media

The CGB agar can distinguish *C. neoformans* from *C. gattii* based on the ability to use the chemical substances. *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* are unable to use glycine as a carbon or nitrogen source and also

susceptible to canavanine. Whereas *C. neoformans* var. *gattii* are able to use glycine and resistant to canavanine. When *C. neoformans* var. *gattii* is incoulated on the CGB agar, it can grow since it resistant to canavanine and furthermore the media will turn alkaline, because of the ammonia product from the glycine utilization, the media indicator color changes to blue. In contrast *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are susceptible to canavanine, so they can not grow on CGB media. In addition, they are unable to use glycine, so the media will remain yellow (37).

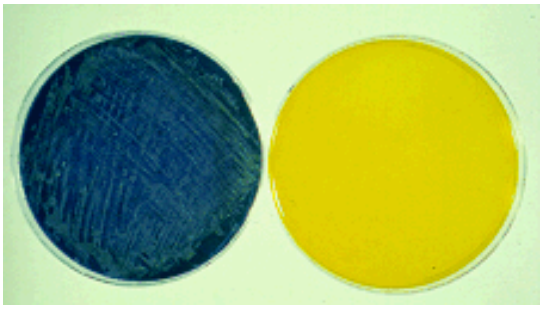


Figure 4. CGB media to differentiate *C. gattii* from *C. neoformans*; blue plate means positive result for *C. gatii* and yellow plate means negative result for *C. neoformans***.**

3.7 Serotyping by latex agglutination

Serotyping is based on the ability of agglutination with monoclonal antibody specific with different antigenic epitopes presented by the polysaccharide capsule. The capsule is primarily made up of higher molecular weight polysaccharide referred as Glucuronoxylomannan (GXM), composed of an alpha 1,3-D-mannopyranose

backbone, with beta-D-xylopyranosyl and beta-D-glucuronopyranosyl. The different degree of xylopyranosyl and O-acetyl group substitutions distinguishes *C. neoformans* into 4 serotypes and 1 hybrid, serotype A and D, produced by *C. neoformans* var. *grubii* and *C. neformans* var. *neoformans*, respectively. And serotype B and C, produced by *C. gattii* (39). These two serotypes, B and C, are closely related and can not be separated by all molecular typing techniques currently used. In addition to the 4 major serotypes, the less common serotype, AD exists, which is a hybrid between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*.

These serotypes can be determined using the commercial CryptoCheck kit (Iatron, Tokyo, Japan) based on the detection of the capsule polysaccharide (40). However, acapsular strains can not be identified using serotyping and there are a number of strains which have a changing or unclear serotype reaction or are untypable.

4. Ecology of *C. neoformans*

In the 1950s, the major risk group for cryptococcosis correlated was associated with persons with the underlying diseases of Hodgkin's lymphoma, leukemia, and sarcoma. A dramatic increase of cryptococcal infections was observed in last 50 years., with immunocompromisation being the highest risk factor in the era of AIDS.

Cryptococcal infection is likely gender specific, since the infection rate in males, 62-90%, is much higher than in females. This result may be affected by the different immunity of males and females (2, 44). In HIV infected children, the incidence rate of *Cryptococcus* infection is lower than in adults. The prevalence is approximately 0.85% compared with the prevalence in HIV-infected adult, 5-10% (41).

Kwon-Chung and co-workers (42) studied the world-wide distribution of the two cryptococcal species, by analyzing 628 clinical *C. neoformans* isolates and additional data of 97 isolates from other laboratories, Hundred percent of the isolates from Austria, Belgium, Denmark, France, Germany, Holland, Italy, Switzerland, and Japan were classified as *C. neoformans*. Whereas, 85 percent of the isolates from Argentina, Canada, the United Kingdom and United States, except Southern California, were classified as *C. neoformans*, while the remain isolates belonged to *C. gattii*. Among the totally *C. neoformans* isolates studied, ,91 percent were serotype A, while 9 percent were serotype D, which were common in Europe but also found in other countries. *C. gattii* were found in higher prevalence in Australia, Brazil, Cambodia, Hawaii, Southern California, Mexico, Paraguay, Thailand, Vietnam, Nepal and countries in central Africa, 35-100 percent. The prevalence of serotype B was 4.5

times higher than serotype C. The author concluded that *C. gattii* is prevalent only in tropical and subtropical regions while *C. neoformans* has world-wide distribution.

This report showed that the distribution of the two species is different, that is why the ecology of these encapsulated yeast species will be described separately.

4.1 *C. neoformans var. grubii* and *C. neoformans var. neoformans*

C. neoformans var. *grubii* and *C. neoformans* var. *neoformans* can be world-wide isolated. *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* are associated in nature with avian guano feces accumulation (2). Chee and co-workers (43) collected pigeon excreta from 26 different places in Korea, and *C. neoformans* var. *grubii* serotype A was found from 8 places. Another independent study reported from another part of the world, where pigeon droppings and air samples were collected from 10 churches and close areas for 1 year. The data showed *C. neoformans* was cultured from all 10 churches. The yeast has also been isolated from soil, insects, eggs, nests, feather and air samples around the churches.

C. neoformans var. *grubii* and *C. neoformans* var. *neoformans* is a common pathogen in immunocompromised hosts, particularly HIV-infected patients. The association of HIV-infection and cryptococcosis has been found world-wide (44, 45). A higher risk for disseminated disease is decreased CD4+ T cells, to 100-200cell/mm3 blood (46).

C. neoformans var. *grubii* (serotype A) is the world-wide majority serotype.

4.2 *C. gattii*

Recently, a large global study of *C. gattii* was reported. *C. gattii* has been believed to be restricted geographical to tropical and subtropical areas (2). However, the outbreak of cryptococcosis on Vancouver Island, Canada, indicated a climate change and a new ecological niche for *C. gattii* (11).

On Vancouver Island 38 cases of human cryptoccoosis was diagnosed between January 1999 to December 2001, with all of them being caused by *C. gattii*. The incidence of infection, 8.5 to 37 cases per million residents per year, on Vancouver Island was much higher then the one reported in Australia, 0.94 cases per million residents per year, where *C. gattii* is endemic (47). Molecular typing using two different methods, PCR-fingerprinting and Amplified Fragment Length Polymorphisms (AFLP) analysis showed tat this outbreak was caused by a specific sub-genotype of VGII/AFLP6 (11). Recently, the evidence of a spread of the Vancouver Island outbreak strain was reported by Upton and co-workers (2007), who found that the isolate obtained from the Puget Sound, Washington, USA was similar to the Vancouver Island strains using PCR-fingerprinting assuming that the outbreak strain has been distribute to this nearby area.

As *C. gattii* infects mainly healthy, immunocompetent hosts it is a primary pathogen. It is unclear why *C. gatti* is less frequently reported from immunocompromised hosts (48).

4.3 The role of animals in cryptococcal ecology

Cryptococcal yeast cells can be isolated from old dry bird excreta, suggesting that *C. neoformans* consumes the nutrient substance from dry bird feces. It is hypothesized that the urine in bird feces is associate with the growth of the yeasts cells on bird excreta. *C. noeformans* is an osmophilic yeast. It can utilize the high concentration of such substances as purines, urea and creatinine contained in urine. In addition, *C. neoformans* can extremely well utilized creatinine, compared with the other species of this genus An additional benefit for growth on dry excreta may be the

reduced competition with a smaller number of other organisms (49). The soil 'animalization', meaning that enrichment of soil with hair, skin scales, crusts, feces and other organic matter is another suitable habitat for *C. neoformans* growth

Cafarchia and co-workers (50) examined the role of birds as carriers and spreaders of *C. neoformans* and other zoonotic yeasts. They collected 454 samples from three different sample groups: Group I: cloacae swab samples from several bird species; Group II: feces samples from bird in habitat places; Group III: digestive tract swabs from dead birds. Three of the cloacae samples from *Falco tinnunclus* and one sample from *Buteo buteo* were positive for *C. neforomans* var. *grubii*. Positive cultures were also observed from the aviaries samples from these birds. The researchers concluded that birds of prey may play a role as carriers and spreaders of *C. neoformans* in the environment.

Since the pigeon was also concerned to be a major natural source of *C. neofromans* cultivation of pigeon samples from beak, crop, feet and rectal swab was carried out. The results showed only low concentrations of yeast cells. The reason for this my be the high body temperature of birds, 42 ºC, and high ammonia concentration in fresh excreta which are inhibiting the yeast cells growth (2) suggested that the soil itself maybe the original habitat of *C. neoformans*, which is enhanced after contamination with bird feces, which create favored conditions for the yeast to grow. On the other hand, the birds maybe the original source of a few yeast cells and the yeast can than multiply more in the environment than inside a bird.

Domestic animals, such as dogs and cats, may also be a source of *Cryptococcus* in the environment. Those animals are normally nasal colonized with cryptococcal

cells and are mainly asymptomatic. However, no animal-animal and animal-human transmission of cryptococcosis has ever been reported (49).

4.4 The role of trees cryptococcal ecology

Recent evidence suggests, that *C. neoformans* and *C. gattii* have separate natural tree habitats.

4.4.1 C. neoformans

Trees were reported as habitat for *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* from Brazil. They were isolated from decaying wood debris hollows of various kinds of trees including: *Syzygium jambolana*, *Cassia grandis*, *Senna multijuga*, *Ficus microcarpa* and *Caesalpinia peltophoroides* (51). Both varieties of *C. neoformans* have also been isolated from fruits and vegetables, including various types of citrus, strawberries, apples, guava, avocado, cauliflower, cabbages and tomatoes (52).

4.4.2 C. gatii

Eucalyptus camaldulensis, an Australian native tree, was reported as a major natural source of *C. gattii* after a search for it based on the fact that Australia is considered as an endemic area of *C. gattii*. Further studies on *E. tereticornis*, *E. rudis*, *E. gomphocephala* also revealed positive cultures for *C. gattii*. Eucalypts in California and Italy are also positive for *C. gattii* (2).

Recently, many kinds of trees are reported as a natural source of *C. gattii*, these include: *Syncarpia glomurifera*, *E. microcorys, and E. grandis* in Australia, *Terminalia catappa*, *E. Globulus*, *Ficus soatensis*, *Croton bogotanus*, *C. funckianus*, *Coussapoa* sp., *Cupressus lusitanica*, *Pinus radiata* and *Acacia decurrens* in

Columbia. *C. gattii* was also isolated from the hollow of *Marlierea tomentosa* and *Guettarda acreana* in Brazil.

C. gattii was cultured from a large number of different kind of trees on Vancouver Island as part of the investigation of an outbreak of cryptococcosis on Vancouver Island. Previously, *C. gattii* was though to be geographically restricted and Australia was an endemic area for the molecular type VGII. However, Kidd *et al* (11) described the same molecular type VGII from those tree species in British Columbia, Canada. This observation has changed the view on the distribution of *C. gattii*, which was previously thought to be restricted to tropical or subtropical areas

Equally it has been shown now, that the molecular types VGIII and VGIV, which were though to be also restricted to certain parts of the world, can be found in the other part of the world (53).

In 2000, Lazera and co-workers (54) for the first time reported the isolation of *C. neoformans* and *C. gattii* from the same tree hollow. The result indicated that two cryptococcal species can share the same natural source, which opens up studies to better understand the life cycle and the environmental habitat of the two species.

Sorrell and Ellis (2) suggested that the decaying wood and tree hollow are beneficial for cryptococcal growth as woody debris offer polyphenol compounds and lignin to yeast. Moreover, wood hollows also protected the yeast cells from the sunlight and dryness, which could be lethal to fungi.

C. neoformans was ever isolated from plant seed, as had been shown to be a source of *Cryptococcus albidus* or *C. laurentii.* This fact excludes the possibility that birds could have picked up *C. neoformans* by consuming infected plant seeds.

5. The *C. neoformans* life cycle

Although *C. neoformans* is isolated from patients and environment as a budding yeast, the filamentous form of this fungus can be observed by mating and monokaryotic fruiting Both mating and monokaryotic fruiting are induced by similar environmental conditions including nitrogen starvation, desiccation, darkness and the presence of mating pheromones (Ref). The life cycle of this encapsulated yeast has been reviewed by Idnurm and co-workers and is described below (9).

5.1 Mating

Mating results in the sexual reproduction cycle of *C. neoformans*. It occur by the fusion of haploid cells of the opposite mating type, **a** and **α**, which results in dikaryotic filaments (Idnurum et al, 2005). Mating is induced by secretion of the two pheromones MF α and MFa from the yeast cells of the opposite mating type. Intervarietal mating can result in the production of basidiospores, which loose their viability. In addition, this kind of mating also generates many diploid and aneupliod progeny, which indicates that the genomic divergence impairs meiosis during the mating. The cryptococcal mating type locus is >100 kb in size, and comprises at least twenty genes In the laboratory, mating crosses of *C. neoformans* generate equal numbers of an **a** and **α** progeny. However, in clinical and environmental strains the α mating type outnumbers the **a** mating type by 30:1 and 40:1. This lead to the observation, that mating type **α** is the one of virulence factors of *C. neoformans* (28).

After the opposite mating type yeast cells secrete peptide pheromones that trigger cell-cell fusion, nuclear fusion occurs, resulting in a dikaryon, which initiates

filamentous growth. The two nuclei from each mating type migrate co-ordinary in the dikaryotic hyphae, and the cells are separated by septum formation. One nucleus is transferred to the penultimate hyphal cell through the clamp connection. The termini of the hyphae differentiate to form basidia in which nuclear fusion occurs. The two parent nuclei fuse and undergo meiosis to produce four meiotic products in the stage of basiospore development. Then the four meiotic spores undergo mitosis and bud from the surface of the basidium to produce a chain of basidiospores. The four chains of basidiospores comprise of a random mixture of meiotic products (28).

The teleomorphic states of *C. neoformans* and *C. gattii* have been designated *Filobasidiella neoformans* and *Filobasidiella bacillispora* respectively.

5.2 **Monokaryotic fruiting (Haploid fruiting)**

Monokaryotic fruiting and mating have similar morphological features. During monokaryotic fruiting the nuclei in the filaments are diploid by endoduplication or nuclear fusion in the early stage of the filamentation. The diploid monokaryotic hyphae form unfused rudimentary clamps connection. In the stage of basidium development, meiosis occurs and haploid basidiospores are produced in four chains. The advantage for the yeast to undergo meiosis with out the opposite mating type might be to remove transposable elements, enable large-scale genome rearrangements or promote the repair of genome damage (28).

The *C. neoformans* life cycle including mating and monokaryotic fruiting is shown in Figure 5.

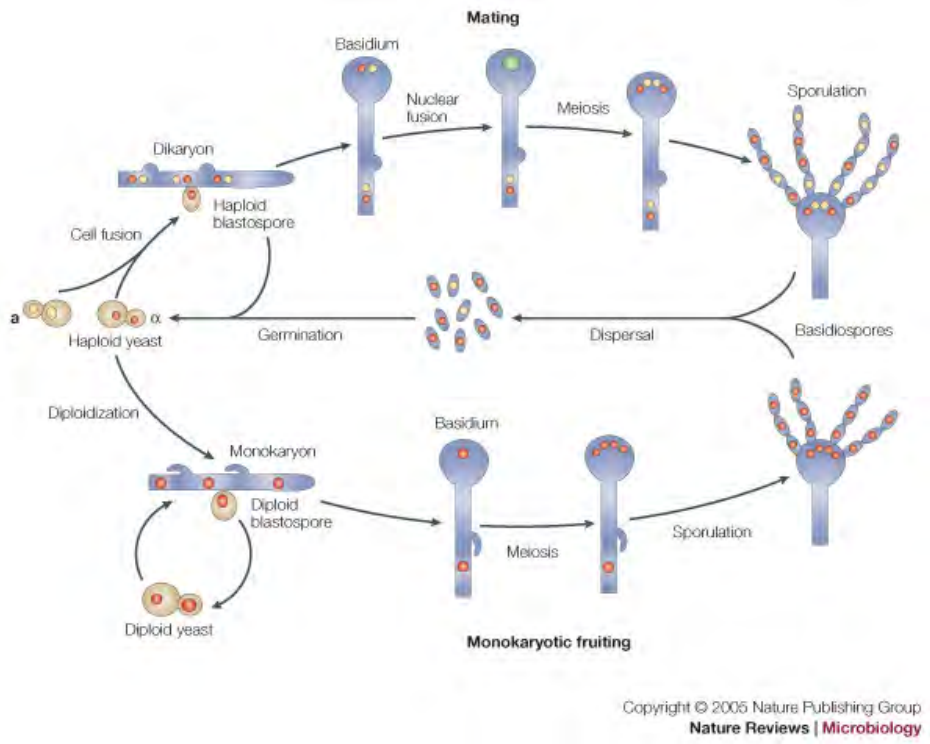


Figure5. The life cycle of *C. neoformans* (28).

6. The *Cryptococcus neoformans* Infection Pathway

Cryptococcus neoformans normally is an opportunistic pathogen in the immunocompromised host, frequently AIDS patients. However, it also can be a pathogen in the immunocompetent host. *C. neoformans* can be commonly isolated from the environment such as pigeon droppings, soil, and hollows of the many kinds of tree. It is hypothesized, that the infection in humans occurs via their exposure to the basidiospores and/or dessicated yeast cells from the environment and the inhalation of those infectious particle through the respiratory tract. The role spores lay during infection is subject of many investigations. The cilairy action of the lung epithelium removes particles larger than 5 µm, such as yeast cells, but spores, which are less than

2 µm can penetrate and lodge in the alveoli of the lung. Desiccated yeast cells can be detected in soil and other environmental sources and are also viable and infectious. However, the nature of the infectious propagules remains a central question (28).

A major question is, why *C. neoformans* maintains its virulence when mammals are not required for the *C. neoformans* life cycle. Infectious propagules of *C. neoformans* are in the environmental fully virulent. Their environmental source however, is unknown. In soil, *C. neoformans* is in contact with many other soil microorganisms and therefore virulence factors might have developed as defense mechanisms against predators as is reflected in the fact, that many *C. neoformans* genes that are required for human disease are also required for infection, survival and killing of other soil organisms, amoeba, nematodes and insects (28).

Moreover, *C. neoformans* infections are also encountered in animals. Virulence genes required for the infection of small rodents or other mammals are also maintained. After the death of those animal hosts, the virulent strains are reintroduced to the environment. In laboratory experiments the infected animal sheds the fungus most likely by exhalation and contaminates its surroundings. This might be another route to maintained virulent strains in the environment (28).

It is hypothesized that the infectious particles pass through the host respiratory tract early in life, after that the immune responses will eliminate the infectious propagules, a latent infection can develop, or establish an asymptomatic infection. In the immune defect host, the fungus can spread from a local lung infection to other organs, including skin, eyes, bones, joints or the central nervous system. Infection of the lungs with *C.neoformans/C. gattii* can cause pneumonia. In the case of an latent infection the infectious yeasts can be reactivated in response to compromised

immunity. The organism multiplies in the lung and spread though the bloodstream or lymphatic system to other organs, including the brain, where it causes meningoencelphalitis and can lead to fatal complications (28). The infection pathway was shown below in Figure 6.

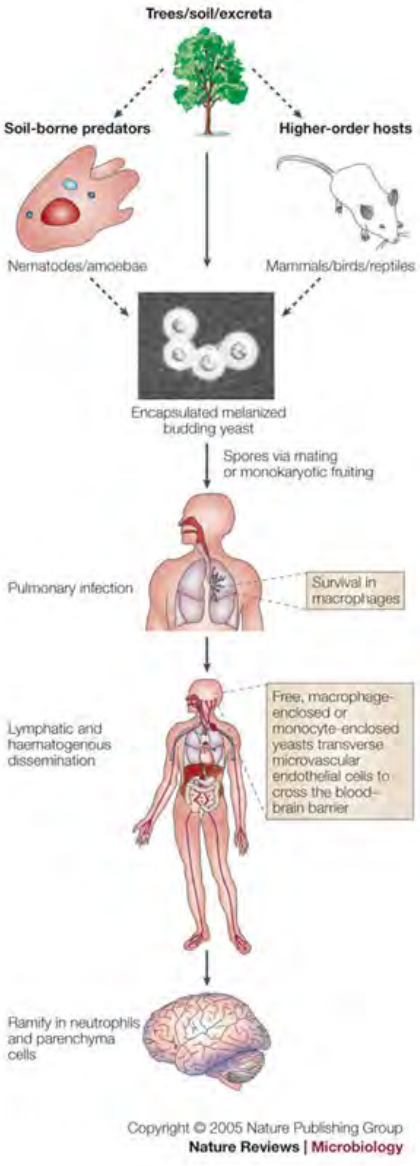


Figure6. Model of the infectious pathway of *C. neoformans* (28).

7. Cryptococcosis

Cryptococcosis is a disease caused by the encapsulated yeasts *C. neoformans* and *C. gattii* in humans and various animals. The main route of infection is via inhalation of the infectious agents However, accidental infection through the skin damage was also suggested.

7.1 Human Cryptococcosis

Cryptococcosis in humans can reach from latent or asymptomatic infection to pneumonia, meningitis and other systemic organ infections.

7.1.1 *Latent or acute or asymptomatic infection*

Cryptococcus can colonize the human respiratory tract without developing disease symptoms. The host immune response can clear the fungus or the fungus can become latent in the lungs.

Reactivation or relapse of the disease was hypothesized from the study of serial C. *neoformans* isolates from AIDS patients. The result showed a similar genotype of the yeast at any studied time point. In addition, *C. neoformans* isolated from patients in France who lived in Africa for 10 years before they had moved to Europe, showed similar genotypic patterns, suggesting they carried an dormant asymptomatic infection which they had acquired in Africa and was reactivated in France. The same study showed specific genotype patterns corresponding to European, American and Asian isolates

In immunocompromised hosts, the yeast can be reactivated and disseminate to other organs such as the CNS through the blood vessels.

7.1.2 *Cryptococcal Meningitis*

According to Lin and Heitman (49) there are three possible hypotheses could explain the migration of *C. neoformans* to the brain.

First; cathecholamine and diphenol compounds are rich substances in the brain that are suitable for cryptococcal growth. These compounds are the substrate for melenogenesis, a potential virulence factor. An other site these factors may also be involved in neutropism of *Cryptococcus,* since these compounds are also found in adrenal grand but there is no evidence of colonization during the infection in this organ. Further more, in the primary infection site, the lung, the yeast can also produce melanin.

Second; the immune privilege site in brain protects cryptococcal cells from the host immune response. *C. neoformans* is capable of crossing the blood brain barrier and can be recovered from the peripheral blood either as free cells or intracellular mononuclear cells, which are transferred to microcapillary beds and interact with the blood brain barrier. *C. neoformans* can cross endothelail cells either directly by transcytosis or carrier mononuclear phagocytes such as neutrophils or macrophages.

Third; only he brain may presents fungal specific receptors more than the other organs. There is a study showing that after *in vivo* intravenous injection, cryptococcal cells binding soon to brain endothelial cells.

7.1.3 *Cryptococcosis in other organs*

Cryptococcosis can also be observed from the several organs including: skin, and ocular lesions.

Skin lesions observed alone in healthy patients represent a localized infection. If they are observed in immunodeficient patients skin lesion indicate the dissemination of the disease.

Human-human transmission is virtually unknown. Possibly transmissions have been occurred by needle stick injury and similar accidents (Dromer et al, 1996a). In one case, a corneal transplant receiver acquired the infection from an infected donor. Recently, the transmission between HIV infected mothers to their children's have been reported.

7.2 Aminal cryptococcosis

Cryptococcosis has been reported in dogs, cats, ferrets, guinea pigs, horses, sheeps, goats, pigs, llamas, foxes, minks, cheetahs, gazelles, koalas, wallabies, porpoises and non-human primates.

7.2.1 Cryptococcosis in Cats and Dogs

Cryptococcosis in cats and dogs was studied retrospectively in Australia. The study reports on 155 cats and 40 dogs over a period of 20 years, from 1981 until 2001 (55). The common form of cryptococcosis in cats and dogs was upper respiratory infection. Skin lesion especially on face, CNS disseminates infection, ocular lesion,

osteomyelitis also found. In dogs, the severe CNS infection is common but also ocular lesions

7.2.2 Cryptococcosis in ***Avians***

Although bird feces are considered as being a major source of *C. neoformans* in the environment case of cryptococcosis in birds are very rarely reported.

One report about avian cryptococcsis in Australia has been reported by Malik and co-workers (56), *C. gattii* was reported as a causative agent parrots, indicating that exposure to eucalypts may be a predisposing factor.

The explanation of the infrequency of cryptococcosis in birds may be ue to their body temperature, 42 ºC, which does not favor the growth of *C. neoformans*. In a temperature regulated experiment the yeast was isolated from bird feces after short time incubation at high temperature, whereas no growth was reported after longer incubation at high temperature. The authors suggested that the birds may have an effective immunity to eliminate the yeast cells from the body. The normal body flora ma also be involved in the clearance of the fungus since the bacteria isolated from the intestinal tract of birds inhibited yeast growth in *in vitro* experiments (57). The actual role of birds as an environmental source of *C. neoformans* still needs further studies.

7.2.3 Other animal

Various animals can be infected by *Cryptococcus neoformans/C. gattii*. In cattle,cryptococcal mastitis has been observed. Mastitis has also described in sheeps and goats in addition to pulmonary infectionr. Upper respiratory, pulmonary disease

and CNS complication have been observed in infected horse. *C. gattii* was also isolated from porpoises in relations with the outbreak of cryptococcosis on Vancouver Island, Canada (11).

8. Virulence Factors of *C. neoformans*

Although a number of virulence factors of *C. neoformans* have been reported, only three factors have been established, including: the polysaccharide capsule, melanin production and the ability to growth body temperature.

8.1 The polysaccharide capsule as a virulence factor of *C. neoformans*

The role of the *C. neoformans* capsule is to protect the yeast cell from dehydration in the environment. The capsule consists of polysaccharides (see above, 3.7). Capsule synthesis is induced by a number of factors. In a *in vitro* study, the capsule size was controlled by varying amounts of sugar in the cultivation media. Encapsulation was suppressed at high (16%) sugar concentration, while low, 1%, sugar concentration supported maximal encapsulation. High osmolar medium also produced thin capsuled yeast cells. However, in animal experiments, the death rate of mice is not affected by the size of the capsule, indicating that the size of the capsules is not involved in the virulence (Dykstra et al 1977). Serum induction, iron limitation and physiological CO_2 levels are other factors involved I capsule production (49).

The first reported capsule gene was *CAP59*. The relation of capsule formation and the pathogenesis was investigated by Chang and Kwon-Chung in 1994 (58). The CAP^+ plasmid complementation of the acapsular cryptococcal mutated strains was

performed by transformation. The pathogenecity was restored when tested after mice inoculation. This study demonstrated that the capsule is playing a role in virulence and pathogenesis of *C. neoformans*.

Recently, more capsule genes were reported, *CAP10, CAP60* and *CAP64* and have been examined for their essentiality for virulence and capsule production. In the same manner, as for *CAP59* the mutants cannot produce the capsule and also diminished the virulence in mice. The reconstituted strains can form encapsulated yeasts and also produced fatal infections in mice (59)In terms of immune response the capsule plays a significant role in many ways in the host.

Encapsulated cryptococcal cells are the potential activators of the alternative complement system. The incubation of *C. neoformamns* with normal human serum leads to C3 binding and generats a number of chemotactic factors. The C3 binding rate is higher in cells with a large capsule than in ones which have a thin capsule. In addition, the C3 binding ability different in all four serotypes, C>B>A>D. The complement system is encouraging the phagocyte opsonisation of encapsulated *C. neoformans*. In addition of the C3 proteins being involved in complement fixing, C5a is also a powerful chematactic factor that contributes to increased vascular permeability, chemotaxis, neutrophil adherence and degranulation, which are leading factors in the inflammation response and fixation (60).

On the other hand, the *C. neoformans* capsule also plays a key role as an antophagocytosis factor (61). It can inhibit leukocyte migration and modulation of the host immune responses by complement activation resulting in depletion and unresponsiveness of antibodies. The capsule also protects the yeast cell from soil

amoeba and dessication (62). The shedding of cryptococcal capsule polysaccharide by intracellular cryptococci may also contribute to cell destruction (63).

The increasing of the capsule size during the infection leads to a larger cell and subsequently giant cell formation, however, the role of this phenomenon is still unknown (64). The size of the capsule of cryptococci infecting the lung is thicker than that infecting the brain (65). The exact mechanism has not yet been elucidated but it may be due to the difference of the organ-related nutrition or the enhancement of GXM shedding.

8.2 Melanin as a virulence factor of *C. neoformans*

Melanin is a black or dark brown pigment that is produced by several neutropic fungi (66). The melanogenesis biochemical assay proposed that the synthesis pathway start when 3,4 dihydroxyphenylalanine (DOPA), the member of dihydroxy phenol compound family, were converted to dopaquinone by phenoloxidases. The spontaneously dopaquinone rearrangement would generated dopachrome which transforms to melanin by autooxidation. This phenomenon is a rate-limiting step, like the other enzyme reactions (8). The melanin synthesis pathway is shown in Figure 7. Cathecholamine such as DOPA are mainly located in human brain tissues that is suitable for cryptococcal growth and may lead to the organism migrating to the brain. However, cathecholamine cannot be used as a carbon source by the organism (8, 49).

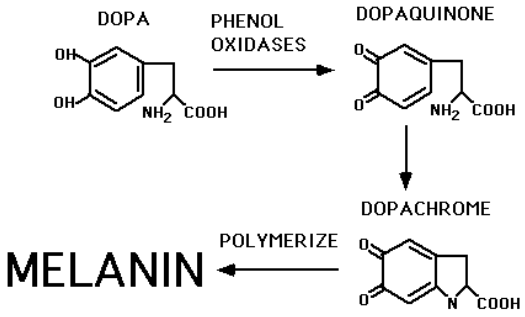


Figure 7. Proposed pathway for melanin synthesis by *C. neoformans* (8).

Recently, two melanin genes were reported, *LAC1* and *LAC2*, both can synthesis the melanin, but *LAC1* expresses the dominant activity of the enzyme more than *LAC2* (49, 67). In addition of the two major melanin synthesis genes, there have also been other LAC related gene clusters discovered. These clusters associated to the organism's colonization such as genes encoding the primary metabolism proteins and cell structure changing proteins (49).

A melanin lacking mutant was constructed by UV radiation. The mutant lacked the active transport systems for diphenolic compounds and the phenoloxidase enzyme, indicated defects in melanogenesis. The melanin production was recovered after mating the mutant with the wild type (68). This study indicated that cryptococcal melanin is produced by the phenoloxidase enzyme and consumes diphenolic compounds as substrates.

Further independent studies identified the association of melanin pigment production and virulence. Antiphagocytosis and antifungal drug resistance properties of melanin were discovered. Melanized *C. neoformans* cells exerted antiphagocytosis

activity to mice-macrophage when compared with non melanized yeast cells. The effect of melanin to antifungal drug resistance also showed in same trend (67, 69) examined the effect of melanin on the host immune response. Melanized *C. neoformans* cells and non melanized cells were intratracheal inoculated to mice to identify the pulmonary immune response. The investigation showed higher fungal burden, Interleukin 4 and MCP-1 level, and also higher number of infiltrating leukocyte were observed from melanized infected mice.

In summary, the cyptococcal melanin synthesis may be an effective virulence factor, melanin play a major role as an antioxidant, antiphogocytosis, drug resistant, suppress innate immunity cytokine production and finally lead to inhibit the specific immune response. However at 37 ºC, human body temperature, phenoloxidase is produced at a low level as compared to the enzyme level at 25 ºC (70).

8.3 Growth in mammalian cells as a virulence factor of *C. neoformans*

For disease development, *C. neoformans* must be able to survive and grow in the host body, 5% CO_2, alkaline pH and mammalian body temperature, 37-39 ºC. Thermotolerance is considered to be a major virulence factor (71). Cultivation of *C. albidus,* a closely related nonpathogenic species, at 37 ºC showed growth inhibition of this yeast compared to *C. neoformans* which growth fine at this temperature. Growth inhibition in C. *albidus* corresponded with a decrease in DNA synthesis (72). *C. neoformans* has a serine threonine specific phosphatase, calcineurin, which plays a significant role in the survival of stress conditions. Odom and co-workers (73) constructed a calcineurin mutant strain that can not grow at host body temperature, but

growth normally at 24 ºC. The pathogenecity of calcineurin mutant yeasts also diminished in immunosuppressed rabbits

Kraus and coworkers (74) identified *C. neoformans* temperature regulated gene expression when cultured at 37 ºC using a genomic-DNA microarray. They found an increased expression of the transcription factor homolog *Mga2,* and targeting enzymes involved in fatty acid biosynthesis. The authors suggested that membrane remodeling is important to *C. neoformans* for growth at higher temperature.

Serial analysis of gene expression (SAGE) of *C. neoformans* var. *neoformans* and *var grubii* has been carried out. At 25 ºC, both strains created higher transcription levels of several genes including a set of histone genes. However, growth at 37 ºC, elevated transcripts levels of heat shock proteins and translation machinery related enzymes was also observed. Among those genes, some genes may play a key role in the formation of two kinds of phenotypes influenced by temperature, including growth in the host and dimorphic transition (71).

8.4 Superoxide Dismutase as a virulence factor of *C. neoformans*

Superoxide dismutase (SODs) are metalloenzymes that convert and detoxify superoxide radicals to hydrogen peroxide and oxygen (75).

In 1994, Jacobson and colleges investigated the relationship between SOD and melanin in *C. neoformans,* since melanin has a low activity at 37 ºC (76). Tested *C. neoformans* strains showed a higher level of SOD production in 37 ºC than 25 ºC, whereas the melanin producing enzyme was temperature regulated at 37 ºC. In addition, the melanin mutant did not show a defect in SOD production. Cryptococcal SOD is not secreted, it may protect the cells from intracellular oxidants, while melanin

is deposited out side the cell wall, playing a key role in protecting the cells from extracellular oxidants. At body temperature, SOD may have a complementary antioxidant activity to melanin.

The superoxide dismutase encoding genes from *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii* and *C. gattii* have been characterized. The *SOD1* mutant of *Saccharomyces cerevisiae* was complemented with an expression library from two varieties of *C. neoformans and C. gattii*. The phylogenetic analysis showed a significant variation between *C. neoformans* var. *grubii* and *C. gattii* and *CNSOD1* distinct classified from other eukaryote SODs (77).

The enrolment of *SOD1* in virulence was reported by COX and co-workers (75). *In vitro* studies have shown that the *SOD1* mutant of *C. neoformans* strains had a decreased SOD activity and attenuated growth compared with the *SOD1* wild type strains. *In vivo* experiments confirmed this finding and showed that the mutant strains had a significantly diminished virulence compared to the wild type strains. The complemented *SOD1* strains also decreased the enzyme producing level in macrophages.

8.5 Phospholipase B as a virulence factor of *C. neoformans*

Phospholipase B (PLB), but no phospholipase C or phospholipase D or other lipases, was detected from cryptococcal culture supernatants. PLB is a secreted enzyme with lysophospholipase hydrolase and lysophospholipase transacylase activities.

Noverr and co-workers (78) investigated the role of *PLB1* in the evasion of the host immune responses. Mice were inoculated with the wild-type *C. neoformans* H99 strain, the *PLB1* mutant and the *PLB1* reconstitute via the respiratory tract. A non-protective inflammatory response was observed in mice infected with the wild-type and the reconstituted strains. While mice infected with the mutant stain generated a protective immune response. Fungal eicosanoid production has been involved in the survival of yeast cells in macrophages and host eicosanoid have been shown to down modulate macrophage functions. When the *PLB1* reconstituted strain was co-cultured with macrophages, a decreased survival rate was observed, which was due to the fact that the eicosanoid production was defect in this strain. The authors suggested that *PLB1* was required for the releases of arachiodonic acid from phospholipids, assumed that *PLB1* act as a virulence factor of *C. neoformans* by enhancing the ability to survive macrophage antifungal defense mechanisms, possibly by facilitating fungal eicosanoid production.

The role of *PLB1 in vivo* was also tested by Steenburgen and co-workers (79). *C. neoformans* was phagocytosed by amoeba. The phospholipase mutanted strains had a decreased replication rate in amoeba compare with the wild-type.

8.6 Mannitol as a virulence factor of *C. neoformans*

The evidence of mannitol being involved in pathogenesis was reported by Wong and coworkers (80). They suggested hexal-D mannitol production as a virulence factor of *C. neoformans*. In a *in vivo* model, intracisternally with *C. neoformans* inoculated rabbits, compared with the control group of uninfected rabbits treated with cortisone or infected rabbit untreated, produced low levels of mannitol in

spinal cord fluid (CSF). The mannitol level in the CSF was higher in the *C. neoformans* infected rabbits treated with cortisone. The level of mannitol in the CSF correlated well with the numbers of *C. neoformans* cells and cryptococcal antigen titer. The result showed that mannitol maybe an effective indicator of cryptococcal brain infection.

Chatuvadi and colleges (77) examined the role of mannitol in the protection of *C. neoformans* against reactive oxygen intermediates. The *C. neoformans* mannitol mutant was killed by polymorphonuclear neutrophils significantly higher than the *C. neoformans* wild-type. The PMN killing was inhibited by mannitol at the same level as superoxide dismutase and dimethyl sulfoxide.

They suggested two key roles of mannitol in pathogenesis: First**,** brain edema may be caused by the high concentrations of D-mannitol and second, D-mannitol may protect *C. neformans* by scavenging reactive oxygens (80, 81).

Mannitol is a potent virulence factor of *C. neoformans* since it protects the yeast from reactive oxygen intermediates damage, heat and osmotic pressure stress (8).

8.7 Urease as a virulence factor of *C. neoformans*

The role of urease as a virulence factor of *C. neoformans* was described by Olszewski and co-workers (82). *C. neoformans* H99 wild-type, urease the mutant strains and the reconstitute strains were characterized. Mice infected with wild-type H99 had an extremely high load of *C. neoformans* cells in brain at the time of death, whereas only two out of the six died mice infected with the mutant strains had detectable *C. neoformans* cells in the brain. Urease me contribute to the dissemination of cryptococcosis to the CNS, by enhancing yeast sequestration within micropilary

beds during blood vessel spread, thereby facilitating the blood to brain invasion. However, *C. neoformans* urease was not required to grow in the host brain.

8.8 Protease as a virulence factor of *C. neoformans*

Only a low amount of proteases is produced by *C. neoformans*. However, proteases may be one of the cryptococcal virulence factors by digesting immunological proteins and complement at the site of infection and also degrading host tissue (8) *C. neoformans* proteases may act as a virulence factor by initiating invasion of the host tissues . Proteases may play a role as an extracellular nutritional supplier since in *in vitro* studies they enable the digestion of human plasma protein and casein (83) Furthermore, proteases may possibly be involved in the development of skin lesions by digesting the collagen in the skin (84).

9. Genetic Diversity in Fungi

In order to understand the population genetics of *C. neoformans* in the mechanisms, which contribute to the genetic variation have to be introduced. There are a number of mechanisms involve in genetic variation. The role of each of these mechanisms will be discussed below.

9.1 Mutation

Mutation is the major mechanisms of all genetic variation. DNA mutation include all heritable changes in a single replicating genome include substitution by DNA replication errors named point mutation, rearrangement, frame-shifts, inversions and duplications. A mutation maybe classified as synonymous mutation if the mutation does not result in an amino acid change, or non-synonymous if the amino acid change occurs. Amino acid substitutions may be classified as two types, conservative and non-conservative. An amino acid change showing a similar amino acid function is classified as conservative, whereas an amino acid substitution change resulting in a changed amino acid function is classified as non-conservative. The amino acid alteration is deleterious and eliminated by natural selection. The rate of mutation in various organism can vary, not among only different species but also among difference genes of the same species and in the same gene. The mutation rate at different time points can also vary.

Transposons and the genetic mobile elements are also believed to be a cause of mutations by causing insertions or deletions. Rearrangements are involving insertions or deletions of a DNA region, larger than a point mutation. Asexual organisms show a

low level genetic diversity because the mutation rate is limited. The most spontaneous mutations for individual loci occur at a very low frequency, approximately 1 in every 10^6 DNA replications. In hypervariable regions such as microsatellites, the frequency of mutations can be much higher, occurring up to 1 in every 10^2 DNA replications (85).

9.2 Genetic Recombination

Gene recombination refers to the DNA rearrangements that affect the level of variation as well as the level of expression of genes by the combination of genes present in a given genome. The role of recombination in the increase the variation level can be explained as follows: Somehow, if the alleles are not randomly distributed among the populations and are found in different individuals more often than expected by chance, recombination can increase the genetic variance. In some condition, genetic recombination can decrease the genetic variation level (86). This increases the chances that a proportion of the mating population will survive after an environmental change.

C. neoformans can reproduce both asexually and sexually. In the case of sexual reproduction, the parent conjugation necessarily involves genetic recombination resulting in different genes combinations in the progeny. This phenomenon is also found in meiosis by homologous chromosome crossing-over or independent assortment of chromosomes (87).

9.3 Natural selection

The role of natural selection is also important for genetic variation as is mutation and recombination. It refers to the differential reproduction of genetically distinct individuals or genotypes within a population.

For instance in the case of negative selection, if mutation or recombination create a new genotype that has a reduced fitness of the carrier, these mutant will be selected against and removed from the population and no genetic variation will be observed. This also prevents the accumulation of genetic variation between independently evolving populations (86). Selectively neutral mutations are not acted upon by the selection. If the mutation that generates a new advantageous genotype, these mutation will be spread thorough the population and finally will be fixed in the population by positive selection, however, this rarely occurs (88). In some cases, the allele loci that are closely linked to a high selective value allele will possibly be fixed and persist among the population (87). Natural selection works upon two factors: the effect on fitness and population size (86).

9.4 Genetic Drift

Random genetics drift refers to the chance of allelic frequencies changing in a given population. Random genetics drift of selectively natural mutant alleles is the basis of the majority of evolutionary changes and the variation at the molecular level. Notably, the direction of changing is random at any point in time when allelic frequencies change from generation to generation (88). In very small size populations, changes in allelic frequencies due to genetic drift will be very small from one generation to next, however accumulated effects over many generations may be highly significant.

The founder effect is a phenomenon that random genetics drift extremely occurs when a new population is established by only a few individuals. From generation to generation, the gene frequencies at various loci of the progeny are likely to be different from the parent. Environmental changes causing unfavoring conditions, which lead to an extreme decrease in the population size and may leave the population close to extinction, are referred to as Bottlenecking. Over the time the population may recover the size but the allelic frequencies would be altered during this event, even in the next generation.

9.5 Tandemly repeated DNA

In the eukaryote genome, repetitive DNA can be observed as an integral part and may exist in an interspersed arrangement or in a tandemly repeated configuration. Tandem repeats are classified according to the length and copy number of the repeated DNA as satellite, minisatellite and microsatellite (85).

Microsatellites are also called simple sequence repeats (SSRs) or short tandem repeats (STRs) (86). Microsatellite sequences are characterized by a repeat size of 2-6 base pair, short DNA segments, repeated up to 100 times at multiple sites in the genome. These sequences are distributed with high frequency throughout the eukaryotic genome (89). Microsatellites are observed in the euchromatin (86).

Minisatellites, comprise repeat arrays of approximately 10-100 base pairs which range in size from 0.5 to 30 kilobase but still lesser than the satellite repetition degree. Minisatellites often form families of repeat arrays with related sequence and may occur at thousands of site in the genome (89). The region of genome in which minisatellites can be observed is euchromatin. Minisatellites are highly

variable in repeat size (86). The variation of the minisatellite repeat size may be caused by homologous recombination (90).

Satellites, are characterized be a very high copy number, typically between 1,000 and 100,000 copies of the repetitive element, which can reach a size of up to 100 megabases. The repeating unit size ranging from 5 - 100 base pair (86) but 100 to 300 nucleotides being the most commonly found repeats (Weising,1995). Satellites are found in the heterochromatin near chromosome centromeres and telomeres. The sizes are not variable within population (86).

The tandem repeat unit occurs as a result of slippage of the DNA polymerase during replication and repair, resulting in the addition or deletion of the repeat array (91). The slipped-strand misspairing was summarized by (21) when replication occurs, the nascent strand may dissociate from the template strand and, due to the repetitive nature of the microsatellite DNA, the strand may reanneal incorrectly, or "out of register". The paring of misaligned repeat causes one to several repeats to loop out from either the nascent or the template strand. This slipped-strand miss pairing results in the nascent strand having a different number of repeats from the template strand once DNA replication is complete. This mutation process allows the same microsatellite allele to arise multiple times, thus generating size homoplasy. Three mutation models were propose for this homoplasy; *firstly*: the stepwise mutational model (SMM) which assumes all mutational events involve a change in a single repeat only, *secondly*: the two phase model (TPM) assumes a proportion of mutations to involve changes greater than single repeats; *thirdly*: the infinite-allele model (IAM), which dose not allow for homoplasy and assumes that every mutation results in the creation of a new allele. The mutation rate in the repeating unit ranges from 1 in 102 to

1 in 105 DNA replication, which is higher than normal DNA mutations, which occur 1 in 106 DNA replications (85). The mutation must happen sufficiently often to maintain a high degree of allelic polymorphism within a given population but not so often that there is polymorphism between successive generations (91).

The function of the repeatitive unit is not well understood at present. Initially it was though, that repeat units are associated with gene regulation or could form a recombination hot spot (80).

Micro- and Minisatellites are potent makers for genetic studies, including genome analysis, allelic imbalance analysis, analysis of genetics related disease, genetic mapping, epidemiology studies, population biology, evolutionary stuies and forensic applications (92).

10. Typing techniques used for the analysis of the *C. neoformans* species complex

10.1 Serotyping

Serotyping is based on the ability of agglutination with monoclonal antibody specific with different antigenic epitopes presented by the polysaccharide capsule (see above, 3.7).

The five different serotypes differ in their ecology, epidemiology, pathogenecity and geographic distribution, as well as their molecular and morphological characteristics.

Serotyping has been used for epidemiology studies of *Cryptococcus* species complex and is widely used as the preliminary diagnostic test in routine fungal laboratories. The disadvantages of this method are: the limited discriminatory power,

which does not allow for a further subdivision of different strains and changing typing results, especially for the hybrid serotype AD.

10.2 Multilocus enzyme electrophoresis

Multilocus Enzyme Electrophoresis (MLEE) is a commonly used method in epidemiological studies of bacteria and fungi. The concept of this method is the mobility variants of enzymes can be equated with alleles at the corresponding structural gene loci, hence electrophoretic types can be represented with genotypes.

MLEE was used to distinguish *C. neoformans* isolates of each variety and serotype (10). *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* were distinguished by varied mobility of the enzymes: 6-phosphoglucinate dehydrogenase (6PD), malate dehydeogenase (MDH), phosphoglucose isomerase (PGI), and phosphoglyceromutase (PGM), while *C. gattii* was distinguished from *C. neoformans* by the enzymes: MDH, alcohol dehydrogenase (ADH), PGM and glutamate dehydrogenase (GD2) (10) Four subtypes could be distinguished within serotype A, while five subtypes were distinguished among isolates of serotype D. Enzyme 6PD mobility can separate serotype D from AD in all studied isolated. MLEE profile of the *C. gattii* isolates revealed genetic diversity. However, MLEE failed to separate *C. neoformans* from the closely related species *C. laurentii* (10).

In 1995, Brandt and coworkers used MLEE to verify the correlation between genotype and geographic origin. The results showed that the MLEE profiles of the Californian isolates were distinct from those ones isolated from the South East of United States (93).

Another study determined the relationship between genotype and serotype , as well as between genotype and strain origin of 107 *C. neoformans* isolates. Twelve out of 14 enzyme loci studied were polymorphic, with each comprising between three and six alleles, which revealed 48 different electrophoresis types classifying the studied

isolates into three clusters. Cluster I consisted of one strain of *C. laurentii*, indicating a close relationship between the two species. Cluster II included *C. neoformans* isolates, while *C. neoformans* and *C. laulentii* were grouped in Cluster III (94).

An advantage of the MLEE technique is, that all alleles are recovered, so that alleles are rarely missing. In addition it can determine of a locus is co-dominant in a diploid cell. MLEE has a low discriminatory power in separating strains of the same molecular type hence there is a degree of difficulty in separate closely related strains. Another drawback of this method is, that it is time consuming because of multiple enzymes must be tested to provide variability among isolates. Additional criticisms of MLEE are: (1) that this method detects the genotype indirectly, so the variation that is not leading to changes in the amino acid composition would be not detected; (2) changes in the amino acid composition do not necessary change the electrophoretic mobility of the protein, meaning alleles that are considered to be the same protein alleles for different individuals may represent different gene alleles, and (3) the genetic selection may be acting on the polymorphisms (95).

10.3 Electrophoretic Karyotyping

Electrophoretic karyotyping (EK) is a technique that detects different numbers and sizes of chromosomes by Pulse Filed Gel Electrophoresis (PFGE). EK is a very sensitive method often used in epidemiological studies to determine strain relatedness or diversity via the detection of polymorphic variation in chromosomal size, which uses an electric field of alternating orientation to move intact chromosomes through the agarose gel matrix (95, 96).

C. neoformans and *C. gattii* were studied by EK for the first time in 1989 by Polecheck and Labens (1989), who show three major karyotype patterns. *C. neoformans* serotype A gave eight groups of the chromosome pattern, while serotype D revealed the various patterns among the studied isolates. Serotype B and C showed similar patterns of chromosomes. In 1993, Perfect et al studied the chromosomes of *C. neoformans* and *C. gattii* by PFGE (97). They showed that *C. neoformans* has 8-12 chromosomes, ranging in size from 770 kb to 2.2 mega base pairs, and *C. gattii* has between 11-14 chromosomes, ranging in size from 400 kb to 2.7 Mb.

EK has been used for tracking the source of an infection, taking into account the different chromosome patterns of the *C. neoformans* strains, which had been isolated from different sources. Wicker and co-workers (98) used EK to study the EK profiles of *C. neoformans* clinical isolates obtained from the USA and compared them with the isolates obtained from Eucalyptus trees from Australia and California.

EK has been used o visualize the chromosome patterns of *C. neoformans* isolated from different body sites of 30 patients (99). The results showed 23 distinct EK patterns among 83 typed isolates. Identical EK chromosome patterns were found in 24 patients. However, the serial isolates of 6 patients, two isolates from each patient possessed distinct profiles in at least.

Karyotyping can be a useful tool in epidemiological studies to identify variations between strains even so there are reservations for this technique, because of reported chromosomal instability in some strains, which reduces the reproducibility of the obtained EK patterns and its time consuming nature.

10.4 Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphisms (RFLP) analysis assays the DNA sequence variation of the genome by using restriction endonucleases. Restriction endonucleases recognize specific DNA sequence, usually 4 to 6 nucleotide bases in length and cut the DNA in or near the recognition site. The alteration of the recognition sequence by nucleotide substitution, insertion or deletion, methylation of the nucleotides can prevent the restriction endonucleases from acting and can lead to variation in fragment patterns (95, 96).

Any region of the DNA can be used for RFLP analysis if variation can be visualized using multi copies DNA molecules, such as mitochondrial DNA or ribosomal DNA. rDNA-RFLP patterns of the four major serotypes wthin the *Cryptococcus* species complex: A, B, C and D could not be distinguished, due to a lack of variation in this region (100). The further study showed that the rDNA gene complex of the Cryptococcus spcies complex is highly conserved (101)..

RFLP of the *URA5* gene, the gene that encoding orotidine monophosphate pyrophosphorylase in the xylose metabolisms cycle, using the endonuclease *HhaI* and *Sau96I* in a double digestion, resulted in eight *URA5*-RFLP patterns for the *Cryptococcus* species complex, which correlate with the eight major molecular types observed by PCR fingerprinting (14). In addition, *URA5*-RFLP of the AD hybrid strains revealed multiple genotypes, which combined the RFLP patterns of either VNI (serotype A) with VNIV (serotype D) or VNII (serotype A) and VNIV (serotype D). Both of those serotypes have been known to be the parental origin of the serotype AD hybrid strains (102)

PLB1-RFLP analysis using *Ava*I or *Hind*III has resulted in eight or five distinct RFLP patterns, respectively, corresponding to the molecular types defined by PCR-fingerprinting for all investigated cryptococcal strains (103, 104). RFLPs were the first DNA markers used for fungal evolutionary biology. Critics of the RFLP analyses, involve the fact that the restriction sites in different individuals are mostly identical by descent, in some case the restriction endonuclease site can be lost by substitution or the site can suffer length mutations (95, 96). In addition this typing method is very expensive, time-consuming and needs large amount of DNA.

10.5 Random amplified polymorphic DNA or Arbitrary Primed PCR analysis

Random amplified polymorphic DNA (RAPD) is the typing method, which assays DNA sequence variation via the amplification of short DNA segments. In the PCR priming region, 3' end nucleotide substitution can prevent amplification by mismatch with the primer annealing sequence. RAPD analysis uses short PCR primers and a low annealing temperature to generate several random fragments (95, 96).

RAPD analysis of Australian *C. gattii* strains, using 12 to 22mer primer pairs, grouped all studied isolates into three major molecular groups, VGI-VGIII, these groups did not correspond to the serotypes (105). The result obtained in this study also showed a high level of genetics concordance between the majority of clinical and environmental isolates. This finding is consistent with the hypothesis that human disease can be acquired from exposure to an environmental source, such as eucalyptus trees. RAPD analysis carried out on clinical, environmental and veterinary *C. neoformans* and *C. gattii* isolates demonstrated genetic differences between their

RAPD patterns (106).. RAPD analysis has been used for epidemiological studies within the *Cryptococcus* species complex, in order to differentiate between the two species *C. neoformans* and *C. gatii*, the geographical locations and for tracking the source of infections. However, the reproducibility of RAPD analysis is a major problem. Different profiles are frequently obtained in different laboratories due to the use of different Taq polymerases, thermal cyclers and other chemicals. An additional concern is, that equal RAPD bands and missing bands may not be homologous, due to the fact that bands can be lost by nucleotide substitution or by length mutations (95, 96).

10.6 Amplified Fragment Length Polymorphisms

Amplified Fragment Length Polmorphisms (AFLP) is a method, which analysis a PCR-amplified library of restriction fragments representative of the entire fungal genome. The analysis depends on the restriction endonucleases site and the adaptors used for the amplification of a subset of the restriction products obtained (107).

Boekhout and co-workers (108) used AFLP to study the genetics structure and epidemiology of *C. neoformans*. They showed a clear separation of *C. neoformans* var. *grubii*, *C. neoformans* var. *neoformans* and *C. gattii.* Almost all isolates revealed unique AFLP patterns, and six major genotype clusters were found: Cluster 1 (93.4%) and 3 (6.6%) represented *C. neoformans* var. *grubii* (serotype A), while Cluster 2 (63.7%) and Cluster 3 (36.3%) represented *C. neoformans* var. *neoformans* (serotype D). For *C. gattii* serotype B, was observed among Cluster 4A (8.3%), 4B (6.8%), Cluster 5B (2.9%), 5C (0.5%) and Cluster 6 (1.9%), while serotype C was observed among Cluster 5A (1.0%) and 5C (4.4%). The AD hybrid was observed among

Cluster 1 (2.7%) and 3 (97.3%). The results of this AFLP study supported the proposals of the AD hybrid serotype being a recombinant of the two paternal serotypes, A and D. Based on the distinct genetic lineages revealed by AFLP, the authors recommend recognition of *C. neoformans* var.*grubii* and *C. neoformans* var. *neoformans* as one species separate from *C. gattii*, formally *C. bacillispora.*

Halliday and Carter (109) studied the AFLP patterns of *C. gattii* isolates to investigate the mode of reproduction using 5 primer combinations. The AFLP patterns generated between 4 to 19 bands. One pattern was unique present only among α mating type isolates, while three of the AFLP polymorphic loci were observed among only **a** mating type isolates. AFLP analysis was also performed on Brazilian environmental isoaltes. The obtained AFLP pattern of isolated from the same tree were observed to be more closely related than those obtained isolates from different trees. For cluster 1 (95.0%), a majority pattern was observed among *C. neoformans* var. *grubii* (serotype A), mating type α isolates. *C. neoformans* var. *neoformans* (serotype D) and mating type α isolates belonged to Cluster 2 (4%), while AD hybrid isolates belonged to Cluster 3 (1%). All *C. gattii* isolates grouped into one cluster (110). AFLP analysis may be useful for medical important fungi that are difficult to cultivate, because AFLP analysis uses a genomic restriction fragment library as a template, which can be generated from very small amounts of DNA. AFLP analysis reveals more polymorphism between isolates, since it produces many bands more fragments than RFLP and RAPD analysis and it is more reproducibile than RAPD analysis.

10.7 DNA fingerprinting with Repetitive DNA Sequence

DNA-fingerprinting is a technique that detects hypervariable, repetitive DNA sequences, such as minisatellites or microsatellites via the hybridization of specific DNA probes to restriction endonuclease digested genomic DNA (111).

The genetic differentiation of *C. neoformans* was studied by Spitzer and Spitzer, (112) and Chen et al (113), using DNA fingerprinting. The CNRE-1 probe allowed recognition of multiple profiles among clinical isolates of *C. neoformans*. CNRE-1 hybridises to a polymorphic family of repetitive elements containing 10 to 20 members, which are minimum of 5 to 10 kb in length. The genetic variation detected is the result of chromosome rearrangements, sexual recombination or genetics drift.

Varma and Kwon-Chung (114) identified 26 isolates of *C. neoformans* and *C. gattii* by using 7-kb linear plasmid UT-4p of *URA5*- gene as a hybridization probe. Twenty one unique fingerprint patterns were produced, that represented the varieties and serotype A, D, B and C. The UT-4p probe was also used to characterize the genetic profile of 156 *C. neoformans* and *C. gattii* isolates from different geographical areas. Nine and 12 distinct DNA fingerprint patterns were observed for isolates of *C. neoformans* and *C. gattii*, respectively (115). Another study using the UT-4p probe demonstrated a correction between isolates from a fingerprint group designated I, and patients of a high-risk group, such as drug addict and homosexuals (45). The fingerprint patterns were found to be reproducible despite repeated sub-culturing of the isolates and growth on different media. Furthermore, the reproducibility of the fingerprints was unaffected by murine passage (96).

In comparisons of different strain-typing methods, the fingerprinting methods are usually the most discriminatory ones. Fingerprints are useful for identifying

individual fungi but problematic for estimating genetic or phylogenetic relationships among individuals.

10.8 PCR-fingerprinting

PCR fingerprinting is a technique based on the PCR amplification of inter-repeat sequences with single primers specific to microsatellite or mimisatellite sequence, that were originally designed and used as a hybridization probes for DNA fingerprinting (12). It generates unique multilocus patterns that are specific and distinguish species and strains.

Meyer and co-workers (11) were the first ones to report the application of PCR fingerprinting with the microsatellite specific sequence of the phage M13 core sequence to differentiate strains of six fungal genera, including: *Penicillium, Trichoderma, Leptosphaeria, Sacchromyces, Candida a*nd *Cryptococcus.* In further study PCR-fingerprinting, using the minisatellite specific core sequence of the phage M13 or the microsatellite specific primers: $(GTG)_5$ and $(GACA)_4$, was utilized to type 42 clinical and environmental *C. neoformans* isolates. The results showed hat PCR-fingerprinting can separate the *C. neoformans* varieties and *C. gattii*. All three primers were highly reproducible and separated the out group species of the genus *Cryptococcus* including *C. albidus, C. laurentii* and *Rhodotorula rubra* (11). Viviani and co-workers (116) discriminated *C. neoformans* from other yeasts using $(GACA)_4$ PCR fingerprinting. In the same study, all four serotypes and the AD hybrid strain were distinguished. Using PCR-fingerprinting eight major molecular genotypes/molecular type of *C. neoformans* and *C. gattii* can be disdingusihed. The molecular types VNI and VNII are correlated to *C. neoformans* var. *grubii* (serotype

A), VNIII is correlated to the hybrid between *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* (serotype AD) and VNIV is correlated to *C. neoformans* var. *neoformans* (serotype D). While the four remains molecular patterns VGI to VGIV are correlated to *C. gattii* (serotypes B and C), which assume no correlation between these two serotypes and molecular types (111) . The molecular type VNI is a predominat molecular type around the world in clinical and environmental isolates, especially in patients with HIV and AIDS. In contrast VGI is the most common molecular type of *C. gattii*, causing infections in immunocompetent patients (13).

11. New epidemiological typing tools for *C. neoformans*

11.1 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is a technique that was for the first time recommended by Maiden et al in 1988 (18) when he used this method to investigate Neisiarhea meningitis. This technique is based on sequencing of ten or more genes to identify the genetic diversity of organisms such as bacteria and fungi (19). Urwin and Maiden (16) reviewed three elements that needed to be present to design of a new MLST system.

First: the number of isolates used in the initial developmental step should be around 100 to ensure that the designed primers can amplify as many isolates as possible and the genetic diversity level of each locus could be detected. Moreover, the isolates should be ideally representative of the population under investigation rather than only be a subset of the population.

Second: good candidate genes or genetic loci that are to be used as markers for an MLST scheme are house-keeping genes. The appropriate length of the sequence should be the length at could be determined with one sequencing reaction in each direction. House keeping genes were chosen based on the basis of its predicted function in genome annotations. Other genes might be experiencing unexpected levels of recombination or selection and therefore prove unsuitable.

Third: based on the above mentioned characteristics primers should be designed. Nested primers that lay within the longer sequence, that was initially amplified are recommended for final sequencing.

For each gene, the different sequences are assigned as alleles. All alleles of each gene are called allele profiles. Finally the alleles of all sequenced genes are assigned as sequence type (STs) for each isolate (17).

MLST is a very useful typing method, since the currently used typing methods have many drawbacks, including inappropriate discrimination power, available agent limitation, poor reproducibility within and between laboratories (18).

For example, MLEE is hiding variation or requires increasing numbers oftested enzymes to differentiate among known genotypes as new genotypes are added. DNA-DNA hybridization, Electrophoretic Karyotyping and RAPDs are all sensitive to small changes between different laboratories (19). The most important limitation of current typing methods is the difficultly of comparing the obtained results between different laboratories (18). MLST also exerts a beneficial point over single nucleotide substitution (SNPs), because the new SNPs can be detected by MLST (18).

A big advantage of MLST is that new data or new SNPs can added to all previously obtain data to form a database that is available for all researchers via the internet network (18).

MLST has been used for bacterial typing of such organisms as *Streptococcus pneuminiae*, *S. pyogenes* and *Staphylococcus aureus* (17) For fungal typing, Koufopanou and co-workers (117) used MLST to study *Coccidioides immitis* and used it for the first time for specie recognition. Five genes, including *CHS1*, *pyrG*, *tcrP*, serine proteinase and *CTS2*, with a total of 2,384 nucleotides were sequenced from seventeen clinical isolates of *C. immitis*. The results showed the highest discrimination power either when analyzing each gene separately or combine all genes together. The studied isolates were divided into two strongly support groups. Two further independent studies separated the two groups into a old species, *C. immitis* and new specie *C. posadasii* (118, 119).

MLST has also been used for *Candida albicans* typing in order to epidermiologically characterie related or unrelated *C. albicans* strains of various clinical origins (120). Six housekeeping genes were sequenced from forty *C. albicans* isolates. Sixty-eight polymorphic sites were identified, with at least one isolate was found to be heterozygous at the sixty-five sites. Is this correct? For each locus, ten to twenty four different genotypes were detected that resulted in thirty-nine unique genotype combinations. When related and unrelated isolates were compared with related isolated isolates from the same hospital the related isolates showed genetically very close combined sequence types, while the unrelated isolates gave independent sequence types.

MLST was also applied to the *Cryptococcus* species complex. In 2005, Fraser and coworkers used MLST to investigate the origin of Vancouver Island *C. gattii* outbreak strains (121). Nine loci were sequenced from 202 environmental and clinical isolates, using a total of 4,376 nucleotides. The result showed 273 informative polymorphic sites that represent 78 genotypes. Among the studied strains, 75 strains were VGII, a molecular type which is closely related to the outbreak strains. The MLST results of those *C. gattii* VGII isolates corresponded with PCR-fingerprinting and AFLP analysis, which grouped the outbreak isolates into two VGII discrete genotypes: a major VGIIa and minor VGIIb genotype, which corresponds to two independent clonal populations which caused the outbreak. To further characterize the genetic variation MLST of twenty-two additional loci as performed on a subset of those world-wide collected VGII strains. The result suggested that the major genotype had been present in the Pacific Northwest and minor genotype was related to Australia isolates. The authors concluded that these two major genotypes were the product of same-sex mating between the minor genotype and unknown mating partner.

Litvinseka and colleges reported the use of MLST in connection with the use of AFLP as prescreening method to study 1085 *C. neoformans* var. *grubii* isolates (122). AFLP analysis was performed on all 1085 isolates which detected forty-seven AELP genotypes. Isolates representing each of the 47 genotypes were selected for further MLST analysis, using twelve loci. Both typing techniques revealed three corresponding genetically distinct subpopulations, which were named as group VNI, VNII and VNB. Whereas group VNI and VNII were worldwide spread, group VNB was a unique group of isolates from Botswana. The MLST data generated in this study

formed the basis for the *C. neoformans* MLST database hosted at the Imperial College in London (www.mlst.net).

Beside all the advantages of MLST in certain cases there could still be problems. For example, if a species or population has insufficient genetic variation to differentiated individual isolates, in this case even MLST would be an inappropriate typing method (18).

11.2 Multilocus Microsatellite Typing

Due to the fact that Multilocus Microsatellite Typing (MLST) is becoming the established method for discrimination of individuals in many infectious organisms, insufficient genetic variation in eukaryotic species can pose a problem. In this case, loci with increased variability are required (Fisher et al, 2004). Recently, analysis of the length polymorphisms of microsatellite containing regions has become an important typing tool for population genetics studies of many organisms (123).

Microsatellites are tandemly repeated stretches of short nucleotide motifs of 1 to 6 bp ubiquitously distributed throughout the genome of eukaryotic organnisms (123). The mutation rate is five to six orders of magnitude higher than that of the bulk pf DNA (124). Microsatellite loci present high variability mainly due to allelic repeat length variation, which can be observed between isolates of the same species (122, 123). The molecular mechanism for producing differences in allele's size is primarily due to polymerase slippage errors as discussed above (122).

The length variation of individual loci can easily be screened after amplification with primers that anneal specifically to their flanking regions (122, 123)

The advantages of MLMT are its high reproducibility and exchangeability between laboratories (123) and that microsatellite loci are codominant markers, which evolve rapidly in a genome and may be able to distinguish between isolates of microorganism with a low degree of sequence variation (20).

Fisher and co-workers (125) reported an MLMT study addressing broad scale patterns of genetic variation in *Penicillium maneffei.* The study analyzed two separate populations one isolates from the Centraalbureau voor Schimmelcultures, The Netherland and the other contained isolates from the Chiang Mai University, Thailand with twenty three microsatellite loci. The MLMT results showed a high genetic diversity and extensive spatial structure among the clinical isolates. Two clades of the isolate were observed which correlated with the geographical origin. One clade contained isolates from endemic areas of *P. marneffei*, such as China, Hongkong, Indonesia and Vietnam (eastern part) and the other clade contained isolates from Thailand and India (western part). Multilocus linkage association was highly significant within each clade and the authors suggested that these two clades are due to either two genetically differentiated populations or by are generated by an exclusively clonal reproductive mode or both reason.

Candida parapsilosis is an one more example for fungal MLMT study. In 2006, Lasker and co-workers (20) using MLMT analyzed forty-two *C. prapsilosis* Group I isolates. Seven loci were typed and the MLMT results were compared with Cp3-13 DNA hybridization. The allele size range from 5 to 14 and a high degree of discrimination power was observed, 0.971. Thirty different microsatellite types were assigned among these types. Twenty-four types were found represented by a single isolate. The result of both MLMT and Cp3-13 DNA hybridization were similar.

MLMT also showed its potential to detect potential outbreak isolates as well as to investigate the relatedness between isolates obtained from different body sides from the same patient. MLMT detected size variation at a single locus from six isolates, which had been collected after an outbreak and others which were collected from a different body site. The authors concluded, that the size variation was caused by microevolution. The reproducible of MLMT is a highly reproducible technique as identical results had been observed from two patients by both typing methods. Moreover, MLMT found two alleles per locus in some strains, which may be indicative of an aneuploid strain. MLMT as a robust and efficient genotyping method, because of its advantages in terms of high through put, reproducibility and discrimination power.

MLMT studies in the *Cryptococcus* species complex has not yet been published. However, in 2003, Karaoglu designed microsatellite specific primers for discrimination of cryptococcal isolatesas part of his PhD project (PhD. Theses of University of Sydney 2003). Only 12% of all designed primers amplified isolates of all major molecular types of the *Cryptococcus* species complex, since the primers had been designed based on the *C. neoformans* var. *neoformans*, serotype D genome. Eleven polymorphic SSR markers were characterized. These 11 markers detected 82 alleles with a mean number 7.5 per locus. The discrimination power of the polymorphic loci ranged from 0.42 to 0.93. Among seven of the most polymorphic loci, the discrimination power was 0.989 when combine together. Moreover, the primers were also able accurately identify the eight molecular types previously observed by PCR fingerprinting. In the same thesis, the application of SSR typing in tracking the infection source of cryptococcsis had also been reported. The researcher

suggested that SSR typing methods for *Cryptococcus* species complex showed at least the same or better discriminatory power than PCR-fingerprinting and should be included as an additional typing method for the ongoing epidemiological survey of this species complex.

12. Cryptococcosis in Thailand

The first case of cryptococcosis in Thailand was reported in 1960 (6) Before the AIDS-epidemic period in Thailand, between 1988 and 1991, the most common mycoses were aspergillosis, candidiasis, and cryptococcsis. During the aera of AIDS, cryptococcosis increased dramatically from 30 cases of cryptococcosis recorded in 1992, from which 27 having been isolated from HIV-infected patients to 57 cases, with 49 AIDS-related cases. The most common mycoses in HIV-infected patients, penicillosis marnefii, had also been reported in 8 cases. Even though this report was recorded from only one hospital, Siriraj Hospital, this increasing rate of cryptococcsis might be representative for the situation of the disease throughout Thailand. In addition, the increasing rate is correlated with the greater number of HIV-infections in Thailand. Among AIDS patients in Thailand, cryptococcsis is the second most common opportunistic infection after tuberculosis (6).

Cryptococcosis was also examined in children. Between January 1994 and December 2001, 21-HIV-infeted children were diagnosed with cryptococcosis, but only 19 cases had a medical record. Among these group, 16 cases had meningitis and two relapse cases were observed (126). Another study of cryptococcosis in children was reported from the King Chulalongkorn Memorial Hospital, by Pancharoen and co-workers (127). This study reported 8 cases from 1991 to 2000. 5 cases were AIDS-

related and the remainder suffered from systemic lupus erythematosus. The reasons for the lower rat of cryptococcosis in children than adults are unclear.

Cryptococcosis in HIV-seronegative humans in Thailand was reported (128). Between 1987 and 2003, 40 cases were recorded; only 37 patients had a medical record. Among these cases, 24 patients had as underlying disease: immunosuppressive drug treatment, systemic lupus erythematosus, malignancies and diabetes mellitus. 23 cases had disseminated disease. Meningitis was found in 14 patients and six cases were asymtompmatic. However, this study did not report the serotype, any molecular data or the infection species or variety.

Unfortunately, there are no impact data for cryptococcosis in Thailand, since all the studies carried out have been restricted to a specific hospital or institute. That is why, no general overview of cryptococcosis in Thailands possible. However, the rate of cryptococcal infections has increased in parallel with the HIV-infection rate in a similar way as has been reported worldwide.

13. Studies of the *Cryptococcus* species complex in Thailand

In 1968, Taylor and Duangmani reported the positive culture of *C. neoformans* from environmental sample in Thailand. Among 43 samples, which were collected from bird habitat, 8 strains of *C. neforomans* were observed. The yeast was isolated from soil contaminated with pigeon droppings, soil contaminated with cuckoo droppings and a sample from a pigeon nest. This study was the first report of the discovery of *C. neoformans* from natural substrates in Southeast Asia (129).

Imwidthaya and coworkers (1989) reported cryptococcsis case in Thailand. From 13 patients, include seven females and six males, *C. neoformans* was isolated. Among thirteen cases, eight cases had meningitis and five cases suffered from disseminated disease. In three cases, the underlying disease was systemic lupus erythematosus. In the same study *C. neoformans* was also observed from bird feces (5).

One hundred and eighty seven strains of *C. neoformans* isolated from patients in Thailand were characterized and reported by Sukroongreung and co-workers in 1996 (130). The variety was identified by using canavanine-glycine-bromthymolblue agar. The serotype was identified using the Iatron slide agglutination test from Japan. Among the studied *C. neoformans* strains, which were isolated before AIDS-era in Thailand, fifty-five percent (10 out of 18) were serotype B, 28 percent were serotype A, and 11 percent were serotype C. In contrast to this results isolates observed during the AIDS epidemic period, January 1993 to March 1995, predominantly serotype A, 93 percent (157 out of 169), while serotype B was 3.6 percent and serotype D and AD hybrid were both 1.8 percent. The authors suggested that during the pre AIDS-era in Thailand, *C. neoformans* var. *gattii* serotype B or C were the common pathogen of

cryptococcosis. This pattern changed drastically after the AIDS epidemic, with serotype A becoming the predominant serotype, which is similar with the predominant serotype reported worldwide.

Serotyping results were also reported by Poonwan and co-workers in there 1997 study (25). In this study isolates were collected from the Northern, Central, Northeastern and Southern parts of Thailand, including 97 isolates from AIDS patients. The serotype A was the predominate serotype as also reported in Sukroongreung's study, 133 out of 139 isolates, while only six isolates were serotype B. There was no correlation between serotypes and regional distribution. The molecular epidemiology was examined in this study by using random amplified polymorphic DNA (RAPD) analysis. The results showed different patterns between *C. neoformans* var. *neoformans* and *C. gattii*. The serotype B isolates showed a clonal pattern.

Another molecular study of *C. neoformans* isolated from Thailand was reported in 1999 (6). Eighty-seven isolates of *C. neoformans* from patients were collected from January 1996 to December 1997, for all isolates the serotype was identified and PCR fingerprinting was carried out. From the M13-PCR fingerprinting, eighty six isolates showed the PCR fingerprinting pattern of serotype A and the remainder showed the serotype B pattern. The MICs were examined using the macrodilution method. The mean MICs of amphotericin C, flucytosine, fluconazole and itraconazole against all studied *C. neoformans* were 0.55 μg/ml, 9.5 μg/ml, 6.9 μg/ml and 0.36 μg/ml, respectively.

In 2004, Kuroki and colleges (131) reported the positive isolation of *C. neoformans* from environmental samples in Thailand. Chicken feces samples were

collected from the Chaing Muan district, Pon District and Chaing Kham District in Phayao Province, Thailand. Clinical isolates from the Chaing Kham Hospital were also included in this study. From 150 chicken feces isolates, 36 samples (24.0%) were positive for *C. neoformans* in the dry season and 6 samples (4.0%) were positive in the rainy season. All isolates were identified by Auxacolor system and serotyping was tested by the Iatron latex agglutination test. All environmental isolates were serotype A, similar ly all of clinical isolates were serotype A. These findings suggested that *C. neoformans* in the natural environment could be the cause of cryptococcal meningitis. The researchers tried to determine the relation between environmental and clinical strains. However, they concluded that the RAPD analysis did not reveal any clear relationships between environmental and clinical isolates, which may be due to its poor resolution.

The lasted report of *C. neoformans* from Thailand was in 2005 by Ngamwongsatit and co-workers (132) using RAPD, PCR fingerprinting and PFGE methods. Thirty-seven *C. neoformans* isolates were examined; all of them were serotype A and isolated from AIDS patients. By using two different RAPD primers, all of the studied isolates had the pattern similar to the serotype A reference strain, *C. neoformans* ATCC 34871, while the pattern of the serotype D reference strain, *C. neoformans* ATCC45981 showed a different pattern. PCR-fingerprinting using the $(GACA)_4$ primer gave a similar result as the RAPD analysis, whereas the M13+1 primer result showed that two of the clinical isolates showed the typical patterns, while the remainder showed the fingerprinting pattern similar with the serotype A reference strain. PFGE analysis gave 17 different karyotypes, which can be grouped into 4 groups; EKA (1-6), EKB (1-5), EKC (1-5) and EKD (1). Among the clinical isolates,

the EKA group was the predominant karyotype (20 out of 47) and among this group subtype EKA1 was the dominated pattern (16 out of 20), while the four remaining isolates were typed as EKA2-EKA5, each isolate exhibiting a separate type. The karyotype EKB was observed from 12 isolates, with the EKB1 being the predominant type (8 out of 12) and only one isolate of each of the types EKB2 to EKB5. Four isolates were typed into group EKC and one isolate was typed into EKD. The predominant karyotype identified from the studied isolates may represent the originally common clone of *C. neoformans*.

In conclusion, *C. neoformans* is present in Thailand and a number of epidemiological typing studies have been carried out so far. None of them are representative for the whole of Thailand and none of the results can be easily compered due to the fact that all conducted studies use different typing methods and different standard strains. Since, the new molecular typing techniques are now available, which are potentially more standardized, the subject of this PhD thesis was to develop and apply those new techniques to the Thai cryptococcal isolates. And compare their discriminatory power und usefulness within an epidemiological setting.

Chapter III

Materials and Methods

1. *Cryptococcus neoformans* species complex strains

1.1 Clinical strains

The 367 clinical *C. neoformans* Thai isolates in the study were collected from the hospital/institute/university in Thailand following this:

Mycology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University

Mycology Unit, Department of Microbiology, Faculty of Medical Technology, Siriraj Hospital, Mahidol University

Mycology Unit, Department of Microbiology, Faculty of Medicine, Chiangmai University

Microbiology Unit, Bamrasnaradura Infectious Institute

Microbiology Unit, Bhumibhol Hospital

The strains from Mycology unit, Department of Microbiology, Faculty of medicine, Chulalongkorn University was subculture from -70 ºC stock culture. The stock cultures were grown on Saboraud dextrose agar for 48-72 hours at 37 ºC. A single colony was picked up and grown again on the media for DNA extraction in the same growth condition.

C. neoformans strains from Microbiology Unit, Bamrasnaradura Infectious Institute were stored in distill-water culture stock and keep at the ambient temperature. For include to the study, the culture stocks were cultured on Saboraud dextrose agar for 48-72 hours at 37 ºC. A single colony was picked up and grown again on the media as the same condition for DNA extraction.

For another source of clinical strains, the yeast was grown on the Saboraud dextrose agar and transferred to Mycology unit, Department of Microbiology, Faculty of medicine, Chulalongkorn University. The yeast was subculture for DNA extraction from a single colony on Saboraud dextrose agar for 48-72 hours at 37 ºC.

1.2 Environmental isolates

The 52 environmental isolates were collected as a part of the Master degree thesis in 2004. All these isolates were isolated and identified from the pigeon dropping collected from 21 districts of Bangkok, Thailand in 2003.

Additional, the 28 environmental strains were collected from the pigeon dropping from the Trimitr Temple and Borpitpimuk Temple in Sampantawong District, Bangkok, Thailand in 2005. And 2 strains isolated from pigeon received from Chaing-mai University also included in the study.

The pigeon dropping was sampling into sterile zip-lock bag from 21 districts around Bangkok and transferred to the Mycology Unit laboratory for isolated and identification. Figure 8 showed the pigeon dropping on the floor and on the window of the study places.

Figure 8. Pigeon dropping at Trimitr Temple, Sampantawong district, Bangkok.

The pigeon dropping was dissolved in 0.85% normal saline in ratio 1g of dropping: 10 ml of 0.85% normal saline as showed in Figure 9. The solutions were vortexed vigorously for 15 minutes then allow to settling the residue at least for 5 minutes. The upper solution phase was transferred to new sterile 0.85% normal saline tube for ten-times and one-hundred times dilution (1: 10 and 1: 100).

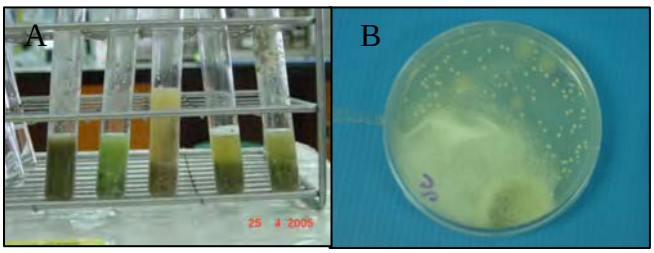


Figure 9. Environmental isolates identification; (A): pigeon dropping dissolved in 0.85% normal saline; and (B): the organisms grown on SDA only yeast colony was picked

The 100 µl of each solution was spread to Saboraud Dextrose Agar plate and incubated 37 ºC and checked for a yeast colony everyday. The grown yeast colony was picked up and identified by these methods:

India-ink preparation: the colony was pepared for India-ink preparation and the positive result was marked when the encapsulated yeast was observed

Urease test: the colony was inoculated on Christensen's urea medium, incubated 37 ºC for 24 hours. Positive test was recorded when the medium turn to pink.

Phenoloxidase production test: the colony was cultured on DOPA medium, incubated 30 ºC for 3 -14 days. The dark brown colonies were scored as positive.

Each selected colony showed the positive for all these three tests (as show in figure 10) were preliminary identified as *Cryptococcus neoformans* species complex and was a subjected for the further study.

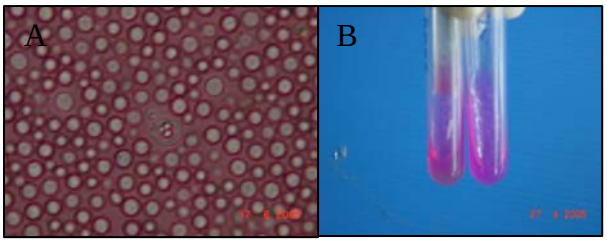


Figure 10. The positive result of three identification criteria; (A) encapsulated yeast in India ink preparation; (B) media turn to color pink in Urease test; (C) dark brown colony on DOPA plate.

1.3 Veterinary strains

The cryptococcal isolates were collected from the cats that were brought to Small Animal Hospital, Faculty of Veterinary, Chulalongkorn University. A cats had the lesion on face (nose, neck and eye) were suspected infection with *C. neoformans*. When the Giemsa or PAS staining of lesion biopsy was positive for encapsulated yeast, the specimens would be collected and transferred to Mycology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University. The lesion and the staining showed below in Figure 11.

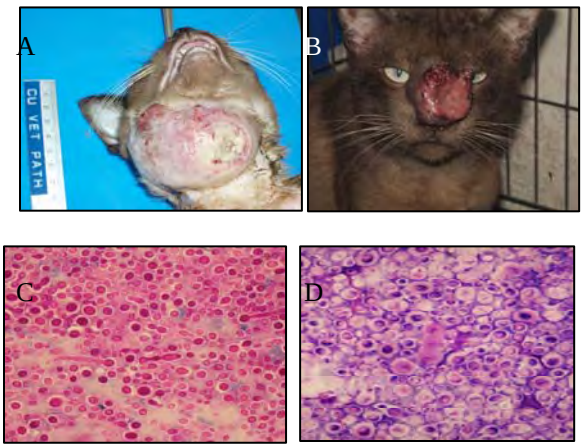


Figure 11. Microscopy of *C. neoformans*; (A) and (B) showed the lesion on suspected *Cryptococcus* infected cats' face; (C) and (D) showed the Geimsa and PAS staining, respectively.

The specimens were streaked on the Sabouroad dextrose agar plate and incubated at 37 ºC for 42-72 hours. The yeast colonies, four colonies were randomly collected based on the colony characterization, for example the mucoid and rough colony. The identification methods as described above for environmental strains identification.

The total summarize of *C. neoformans* isolates in the study showed in table 1 and table 2 described the demographic data of the studied isolates.

Table 1. Source of the studied *C. neoformans* isolates in the study

Source	Place	Total (strains)
Clinical isolates	Chulalongkorn Univerity	160
	Siriraj Hospital	84
	Chiang mai University	18
	Bhumibhol Adulyadej Hospital	19
	Bamrasnaradua Infectious Institute	86
Animal (Cat) isolates	Chulalongkorn University (Faculty of Veterinary Science)	32
Environmental isolates	Pigeon droppings (2003)	52
	Pigeon droppings (2005)	28
	Chiangmai University	2
	Total	**481**

Table 2. List of *C. neoformans* strains studied, including lab numbers , number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
1	C87	1	Clinical	CSF	2003	M	-	-	M D.	-	-	-
2	C135	2	Clinical	CSF	2003	M	-	-	‘”	-	-	-
3	C91	1	Clinical	CSF	2003	M	HIV+	active	S S.	38	1/4/2511	Nonthaburi
4	C93	2	Clinical	-	2003	M	HIV+	active	“	38	1/4/2511	Nonthaburi
5	C114	3	Clinical	-	2003	M	HIV+	active	“	38	1/4/2511	Nonthaburi
6	C97	1	Clinical	CSF	2003	M	HIV+	dead	J J.	41	5/7/2507	Bangkok
7	C133	2	Clinical	CSF	2003	M	HIV+	dead	“		5/7/2507	Bangkok
8	C101	1	Clinical	CSF	2003	M	HIV+	active	S C.	34	29/6/2514	Bangkok
9	C106	2	Clinical	-	2003	M	HIV+	active	“	34	29/6/2514	Bangkok
10	C102	1	Clinical	CSF	2003	M	HIV+	dead	E S.	35	5/5/2513	Srisaketh
11	C119	2	Clinical	-	2003	M	HIV+	dead	“	35	5/5/2513	Srisaketh
12	C120	3	Clinical	-	2003	M	HIV+	dead	“	35	5/5/2513	Srisaketh
13	C104	1	Clinical	SERUM	2003	F	-	dead	U S.	31	22/11/1906	Bangkok
14	C117	2	Clinical	-	2003	F	-	dead	“	31	22/11/1906	Bangkok
15	C105	1	Clinical	-	2003	M	-	dead	P H.	30	10/8/2518	Samutprakarn
16	C203	2	Clinical	CSF	2003	M	HIV+	dead	“	30	10/8/2518	Samutprakarn
17	C107	1	Clinical	CSF	2003	F	HIV+	active	H	32	2517	Bangkok
18	C115	2	Clinical	-	2003	F	-	active	“	32	2517	Bangkok
19	C108	1	Clinical	SERUM	2003	F	-	dead	M S.	13	-	Bangkok
20	C111	2	Clinical	CSF	2003	F	-	dead	“	13	-	Bangkok
21	C113	3	Clinical	-	2003	F	-	dead	“	13	-	Bangkok

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
22	C134	4	Clinical	CSF	2003	F	HIV+	dead	-	13	-	Bangkok
23	C110	1	Clinical	CSF	2003	M	-	active	M S. 1	38	22/1/2511	Khonkhean
24	C121	1	Clinical	CSF	2003	M	-	-	N P.	-	-	-
25	C140	2	Clinical	CSF	2003	M	-	-	"	-	-	-
26	C136	1	Clinical	CSF	2003	F	-	active	M I.	35	12/9/2513	Narn
27	C137	2	Clinical	CSF	2003	F	-	active	"	35	13/9/2513	Narn
28	C200	3	Clinical	CSF	2003	F	-	active	"	35	12/9/2513	Narn
29	C109	1	Clinical	CSF	2003	M	-	Active	M S 1.	38	22/1/2511	Khonkhean
30	C110	1	Clinical	CSF	2003	M	-	active	M S. 1	38	22/1/2511	Khonkhean
31	C138	1	Clinical	CSF	2003	M	-	active	S B.	21	11/6/2527	Bangkok
32	C139	2	Clinical	SERUM	2003	M	-	active	"	21	11/6/2527	Bangkok
33	C142	1	Clinical	CSF	2003	M	-	dead	B R.	51	2498	Bangkok
34	C143	2	Clinical	-	2003	M	-	dead	"	51	2498	Bangkok
35	C150	3	Clinical	-	2003	M	-	dead	"	51	2498	Bangkok
36	C144	1	Clinical	CSF	2003	M	-	active	S S 1.	33	12/7/2515	Bangkok
37	C145	2	Clinical	CSF	2003	M	-	active	"	33	12/7/2515	Bangkok
38	C146	3	Clinical	CSF	2003	M	-	active	"	33	12/7/2515	Bangkok
39	C148	4	Clinical	-	2003	M	-	active	"	33	12/7/2515	Bangkok
40	C157	1	Clinical	CSF	2003	F	-	active	T W.	43	2506	Bangkok
41	C159	2	Clinical	CSF	2003	F	-	active	"	43	2506	Bangkok
42	C26	1	Clinical	CSF	2004	F	-	dead	K.	44	30/8/2504	Bangkok

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
43	C30	2	Clinical	CSF	2004	F	-	dead	K.	44	30/8/2504	Bangkok
44	C165	1	Clinical	HEMO	2003	M	-	active	K K.	34	24/8/2514	Samutprakarn
45	C167	2	Clinical	CSF	2003	M	-	active	"	34	24/8/2514	Samutprakarn
46	C169	3	Clinical	-	2003	M	-	active	"	34	24/8/2514	Samutprakarn
47	C180	1	Clinical	CSF	2003	M	-	active	C S.	42	30/1/2507	Bangkok
48	C181	2	Clinical	CSF	2003	M	-	active	"	42	30/1/2507	Bangkok
49	C201	3	Clinical	CSF	2003	M	-	active	"	42	30/1/2507	Bangkok
50	C211	4	Clinical	-	2003	M	-	active	"	42	30/1/2507	Bangkok
51	C182	1	Clinical	CSF	2003	M	-	active	S J.	37	20/3/2512	Bangkok
52	C183	2	Clinical	CSF	2003	M	-	active	"	37	20/3/2512	Bangkok
53	C209	1	Clinical	-	2003	F	-	active	P J.	35	18/9/2513	Chonburi
54	C79	2	Clinical	CSF	2003	F	-	active	"	35	18/9/2513	Chonburi
55	C3	1	Clinical	-	-	F	-	-	K P.	-	-	-
56	C4	2	Clinical	-	-	F	-	-	"	-	-	-
57	C5	3	Clinical	-	-	F	-	-	"	-	-	-
58	C24	1	Clinical	CSF	2004	M	-	-	S C. 1	-	-	-
59	C34	2	Clinical	CSF	2004	M	-	-	"	-	-	-
60	C45	3	Clinical	CSF	2004	-	-	-	"	-	-	-
61	C6	1	Clinical	CSF	2004	F	-	dead	S N.	49	2500	Samutprakarn
62	C7	2	Clinical	CSF	2004	F	-	dead	"	49	2500	Samutprakarn
63	C8	3	Clinical	-	2004	F	-	dead	"	49	2500	Samutprakarn

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation,source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
64	C15	4	Clinical	CSF	2004	F	-	dead	“	49	2500	Samutprakarn
65	C12	1	Clinical	CSF	2004	F	-	active	A W.	29	6/2/2520	Bangkok
66	C13	1	Clinical	CSF	2004	M	-	active	W S.	37	3/9/2511	Samutsongklarm
67	C14	2	Clinical	CSF	2004	M	-	active	“	37	3/9/2511	Samutsongklarm
68	C16	1	Clinical	CSF	2004	F	-	active	S P.	31	11/3/2518	Bangkok
69	C25	2	Clinical	-	2004	F	-	active	“	31	11/3/2518	Bangkok
70	C31	3	Clinical	HEMO	2004	F	-	active	“	31	11/3/2518	Bangkok
71	C32	4	Clinical	CSF	2004	F	-	active	“	31	11/3/2518	Bangkok
72	C33	5	Clinical	CSF	2004	F	-	active	“	31	11/3/2518	Bangkok
73	C17	1	Clinical	CSF	2004	M	-	active	S M.	44	30/5/2504	Nonthaburi
74	C196	2	Clinical	CSF	2004	M	-	active	“	44	30/5/2504	Nonthaburi
75	C20	1	Clinical	HEMO	2004	M	-	dead	P P.	34	27/1/2515	Ubolratchathani
76	C21	2	Clinical	CSF	2004	M	-	dead	“	34	27/1/2515	Ubolratchathani
77	C22	3	Clinical	CSF	2004	M	-	dead	“	34	27/1/2515	Ubolratchathani
78	C23	4	Clinical	CSF	2004	M	-	dead	“	34	27/1/2515	Ubolratchathani
79	C28	5	Clinical	HEMO	2004	M	-	dead	“	34	27/1/2515	Ubonratchathani
80	C40	6	Clinical	CSF	2004	M	-	dead	“	34	27/1/2515	Ubonratchathani
81	C41	7	Clinical	CSF	2004	M	-	dead	“	34	27/1/2515	Ubonratchathani
82	C67	8	Clinical	CSF	2004	M	-	dead	“	34	27/1/2515	Ubonratchathani
83	C71	9	Clinical	CSF	2004	M	-	dead	“	34	27/1/2515	Ubonratchathani
84	C72	10	Clinical	CSF	2004	M	-	dead	“	34	27/1/2515	Ubonratchathani

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
85	C55	1	Clinical	CSF	2004	M	-	active	P N.	43	31/8/2505	Chaingrai
86	C56	2	Clinical	CSF	2004	M	-	active	“	43	31/8/2505	Chaingrai
87	C58	3	Clinical	-	2004	M	-	active	“	43	31/8/2505	Chaingrai
88	C29	1	Clinical	CSF	2004	M	-	dead	N T.	40	4/6/2508	Suphanburi
89	C42	1	Clinical	SERUM	2004	M	-	active	Y J.	45	2/7/2503	Nakornphanom
01	C43	2	Clinical	CSF	2004	M	-	active	“	45	2/7/2503	Nakornphanom
91	C44	3	Clinical	BLOOD	2004	M	-	active	“	45	2/7/2503	Nakornphanom
92	C46	4	Clinical	CSF	2004	M	-	active	“	45	2/7/2503	Nakornphanom
93	C47	5	Clinical	CSF	2004	M	-	active	“	45	2/7/2503	Nakornphanom
94	C54	6	Clinical	CSF	2004	M	-	active	“	45	2/7/2503	Nakornphanom
95	C195	7	Clinical	CSF	2004	M	-	active	“	45	2/7/2503	Nakornphanom
96	C57	1	Clinical	CSF	2004	F	-	active	S B. 1	33	28/12/2515	Khonkhean
97	C59	2	Clinical	CSF	2004	F	-	active	“	33	28/12/2515	Khonkhean
98	C62	1	Clinical	CSF	2004	F	-	active	A T.	33	16/1/2516	Bangkok
99	C64	2	Clinical	-	2004	F	-	active	“	33	16/1/2516	Bangkok
100	C65	1	Clinical	-	2004	M	-	active	P S.	47	12/5/2501	Bangkok
101	C66	2	Clinical	-	2004	M	-	active	“	47	12/5/2501	Bangkok
102	C70	3	Clinical	CSF	2004	M	-	active	“	47	12/5/2501	Bangkok
103	C68	1	Clinical	CSF	2004	M	-	active	B S.	38	13/1/2511	Pichit
104	C69	2	Clinical	-	2004	M	-	active	“	38	13/1/2511	Pichit
105	C74	1	Clinical	HEMO	2004	M	-	active	S S. 2	38	27/2/2511	Nakornnayok

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
106	C77	2	Clinical	CSF	2004	M	-	active	"	38	27/2/2511	Nakornnayok
107	C78	3	Clinical	CSF	2004	M	-	active	"	38	27/2/2511	Nakornnayok
108	C75	1	Clinical	HEMO	2004	F	-	active	P S. 1	26	17/1/2523	Bangkok
109	C76	2	Clinical	SERUM	2004	F	-	active	"	26	17/1/2523	Bangkok
110	C80	1	Clinical	CSF	2004	M	-	active	S S. 3	40	2/10/2508	Umnajcharoen
111	C82	2	Clinical	CSF	2004	M	-	active	"	40	2/10/2508	Umnajcharoen
112	C88	1	Clinical	-	2004	M	-	active	S.	33	10/11/2515	Pathumthani
113	C89	1	Clinical	CSF	2004	F	-	active	N K.	37	7/10/2511	Pathumthani
114	C95	1	Clinical	HEMO	2004	M	-	active	B S. 1	36	5/1/2513	Nonthabuti
115	C100	1	Clinical	CSF	2004	F	-	active	C P.	62	2487	Bangkok
116	C116	1	Clinical	CSF	2004	M	-	dead	A S.	36	18/12/2512	Bangkok
117	C118	1	Clinical	-	2004	F	-	active	J.	28	5/5/2520	Phayao
118	C149	1	Clinical	CSF	2004	M	-	active	S N.1	52	2497	Nakornpathom
119	C151	1	Clinical	CSF	2004	M	-	active	W K.	38	9/7/2510	Bangkok
120	C153	1	Clinical	CSF	2004	M	-	active	T A.	36	9/7/2512	Bangkok
121	C154	1	Clinical	CSF	2004	M	-		B W.	-	-	-
122	C156	1	Clinical	CSF	2004	-	-		T T.	-	-	-
123	C161	1	Clinical	CSF	2004	M	-	active	B D.	61	5/4/2488	Bangkok
124	C164	1	Clinical	PUS	2004	F	-		S S.4	-	-	-
125	C166	1	Clinical	CSF	2004	M	-	active	S S.5	76	2473	Bangkok
126	C171	1	Clinical	CSF	2004	M	-	active	P P.1	38	5/12/2510	Bangkok

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
127	C173	1	Clinical	CSF	2004	M	-	-	S A.	30	-	-
128	C175	1	Clinical	CSF	2004	M	-	-	A T.1	-	-	-
129	C176	1	Clinical	CSF	2004	M	-	active	S R.	52	5/1/2497	Nakornnayok
130	C178	1	Clinical	CSF	2004	F	-	dead	J J. 1	31	2518	Chaingmai
131	C179	1	Clinical	-	2004	-	-	-	S.	-	-	-
132	C192	1	Clinical	SERUM	2004	F	-	active	T K.	49	15/5/2499	Nakornpathom
133	C205	1	Clinical	CSF	2004	M	-	active	T T. 1	37	1/5/2512	Bangkok
134	C207	1	Clinical	HEMO	2004	M	-	active	P S. 1	44	15/11/2504	Prajinburi
135	C208	1	Clinical	CSF	2004	-	-	-	O S.	-	-	-
136	C73	1	Clinical	SINUS	2004	-	-	-	S W.	-	-	-
137	C86	1	Clinical	CSF	2004	F	-	dead	W C.	31	25/4/2518	Bangkok
138	C81	1	Clinical	-	2004	F	-	active	L.	36	4/11/2512	Bangkok
139	C63	1	Clinical	CSF	2004	M	-	dead	P J.1	49	8/5/2499	Bangkok
140	C61	1	Clinical	HEMO	2004	M	-	active	C P.1	39	7/5/2509	Samutprakarn
141	C132	1	Clinical	CSF	2003	M	HIV+	Active	C W.	29	23/9/2519	-
142	C83	1	Clinical	CSF	2003	M	HIV+	Active	C S.1	45	10/5/2503	-
143	C206	1	Clinical	SERUM	2004	M	-	dead	D T.	24	14/1/2525	Bangkok
144	C1	1	Clinical	-	2004	M	-	-	W W.	-	-	-
145	C2	1	Clinical	-	2004	F	-	-	A P.	-	-	-
146	C10	1	Clinical	CSF	2004	F	-	active	P K.	36	17/7/2512	Samutprakarn
147	C11	1	Clinical	CSF	2004	M	-	active	P K.1	40	11/5/2508	Khalasint

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
148	C19	1	Clinical	CSF	2004	M	-	active	S T.	63	14/3/2486	Bangkok
149	C27	1	Clinical	HEMO	2004	M	-	dead	S L.	39	17/4/2510	Bangkok
150	C35	1	Clinical	CSF	2004	M	-	dead	D J.	29	22/10/2519	Bangkok
151	C36	1	Clinical	CSF	2004	F	-	active	K J.	29	2520	Samutprakarn
152	C37	1	Clinical	SERUM	2004	M	-	active	P K.2	34	20/3/2515	Bangkok
153	C38	1	Clinical	CSF	2004	M	-	active	W K.1	31	24/7/2517	Yasothorn
154	C39	1	Clinical	SERUM	2004	M	-	active	S L.1	37	22/9/2511	Bangkok
155	C48	1	Clinical	CSF	2004	M	-	active	A C.	28	28/10/2520	Bangkok
156	C50	1	Clinical	CSF	2004	M	-	-	A I.	-	-	-
157	C51	1	Clinical	CSF	2004	M	-	-	U M.	-	-	-
158	C52	1	Clinical	HEMO	2004	M	-	active	S S.6	6	16/12/2542	Bangkok
159	C60	1	Clinical	-	2004	-	-	active	C T.		28/12/2515	Khonkhean
160	C194	1	Clinical	CSF	2004	M	-	dead	J S.	60	2489	Bangkok
161	P2	1	Clinical	CSF	-	M	HIV+	-	M K.	36	-	-
162	P3	1	Clinical	Blood	-	F	-	-	S J.1	31	-	-
163	P4	1	Clinical	-	-	-	-	-	-	-	-	-
164	P6	1	Clinical	-	-	F	-	-	S1	-	-	-
165	P7	1	Clinical	-	-	-	-	-	-	-	-	-
166	P8	1	Clinical	-	-	F	-	-	W	-	-	-
167	P9	1	Clinical	-	-	-	-	-	-	-	-	-
168	P10	1	Clinical	-	-	M	-	-	A.	-	-	-
169	P11	1	Clinical	CSF	-	M	HIV+	-	T N.	32	-	-
170	P12	1	Clinical	-	-	-	-	-	-	-	-	-

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
171	P13	1	Clinical	CSF	-	M	HIV+	-	K K.1	27	-	-
172	P14	-	Clinical	-	-	-	-	-	-	-	-	-
173	P15	1	Clinical	blood	-	F	-	-	K J.1	56	-	-
174	P16	2	Clinical	CSF	-	M	HIV+	-	K K.1	27	-	-
175	P17	1	Clinical	CSF	-	M	HIV+	-	W S.1	35	-	-
176	P18	-	Clinical	-	-	-	-	-	-	-	-	-
177	P19	1	Clinical	blood	-	F	HIV+	-	B C.	36	-	-
178	P20	1	Clinical	CSF	-	M	HIV+	-	S C.1	35	-	-
179	P21	1	Clinical	CSF	-	F	HIV+	-	J1.	-	-	-
180	CM1	-	Clinical	-	-	-	-	-	-	-	-	-
181	CM2	-	Clinical	-	-	-	-	-	-	-	-	-
182	CM3	-	Clinical	-	-	-	-	-	-	-	-	-
183	CM4	-	Clinical	-	-	-	-	-	-	-	-	-
184	CM5	-	Clinical	-	-	-	-	-	-	-	-	-
185	CM6	-	Clinical	-	-	-	-	-	-	-	-	-
186	CM7	1	Clinical	-	2004	F	-	-	J K.	36	-	Chiangmai
187	CM8	1	Clinical	-	2004	M	-	-	P S.2	41	-	Chiangmai
188	CM9	1	Clinical	-	2004	M	-	-	S T.1	33	-	Mae-Hogsorn
189	CM10	1	Clinical	-	2004	M	-	-	C P.2	30	-	Lopburi
190	CM11	1	Clinical	-	2004	M	-	-	T M.	59	-	Payao
191	CM12	2	Clinical	-	2004	M	-	-	C P.2	30	-	Lopburi
192	CM13	2	Clinical	-	2004	M	-	-	T M.	59	-	Payao
193	CM14	1	Clinical	-	2004	M	-	-	J B.	35	-	Chiangmai

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
194	CM15	3	Clinical	-	2004	M	-	-	C P.3	30	-	Lopburi
195	CM16	2	Clinical	-	2004	M	-	-	J B.	35	-	Chiangmai
196	CM17	1	Clinical	-	2004	F	-	-	M P.	32	-	Chiangmai
197	CM18	1	Clinical	-	2004	M	-	-	W S.2	33	-	Chiangmai
198	CM19	-	Environment	-	-	-	-	-	-	-	-	-
199	CM20	-	Environment	-	-	-		-	-	-	-	-
200	S1	-	Clinical	-	-	-	-	-	-	-	-	-
201	S2	-	Clinical	-	-	-	-	-	-	-	-	-
202	S4	-	Clinical	-	-	-	-	-	-	-	-	-
203	S5	-	Clinical	-	-	-	-	-	-	-	-	-
204	S6	-	Clinical	-	-	-	-	-	-	-	-	-
205	S7	-	Clinical	-	-	-	-	-	-	-	-	-
206	S8	-	Clinical	-	-	-	-	-	-	-	-	-
207	S9	-	Clinical	-	-	-	-	-	-	-	-	-
208	S10	-	Clinical	-	-	-	-	-	-	-	-	-
209	S11	-	Clinical	-	-	-	-	-	-	-	-	-
210	S12	-	Clinical	-	-	-	-	-	-	-	-	-
211	S13	-	Clinical	-	-	-	-	-	-	-	-	-
212	S14	-	Clinical	-	-	-	-	-	-	-	-	-
213	S15	-	Clinical	-	-	-	-	-	-	-	-	-
214	S16	-	Clinical	-	-	-	-	-	-	-	-	-

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
215	S18	-	Clinical	-	-	-	-	-	-	-	-	-
216	S19	-	Clinical	-	-	-	-	-	-	-	-	-
217	S20	-	Clinical	-	-	-	-	-	-	-	-	-
218	S21	-	Clinical	-	-	-	-	-	-	-	-	-
219	S23	-	Clinical	-	-	-	-	-	-	-	-	-
220	S24	-	Clinical	-	-	-	-	-	-	-	-	-
221	S25	-	Clinical	-	-	-	-	-	-	-	-	-
222	S26	-	Clinical	-	-	-	-	-	-	-	-	-
223	S27	-	Clinical	-	-	-	-	-	-	-	-	-
224	S28	-	Clinical	-	-	-	-	-	-	-	-	-
225	S29	-	Clinical	-	-	-	-	-	-	-	-	-
226	S30	-	Clinical	-	-	-	-	-	-	-	-	-
227	S31	-	Clinical	-	-	-	-	-	-	-	-	-
228	S33	-	Clinical	-	-	-	-	-	-	-	-	-
229	S34	-	Clinical	-	-	-	-	-	-	-	-	-
230	S35	-	Clinical	-	-	-	-	-	-	-	-	-
231	S36	-	Clinical	-	-	-	-	-	-	-	-	-
232	S37	-	Clinical	-	-	-	-	-	-	-	-	-
233	S38	-	Clinical	-	-	-	-	-	-	-	-	-
234	S40	-	Clinical	-	-	-	-	-	-	-	-	-
235	S42	-	Clinical	-	-	-	-	-	-	-	-	-

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation , sources, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab numberS45	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
236	S43	-	Clinical	-	-	-	-	-	-	-	-	-
237	S44	-	Clinical	-	-	-	-	-	-	-	-	-
238	S45	-	Clinical	-	-	-	-	-	-	-	-	-
239	S46	-	Clinical	-	-	-	-	-	-	-	-	-
240	S47	-	Clinical	-	-	-	-	-	-	-	-	-
241	S48	-	Clinical	-	-	-	-	-	-	-	-	-
242	S49	-	Clinical	-	-	-	-	-	-	-	-	-
243	S50	-	Clinical	-	-	-	-	-	-	-	-	-
244	S51	-	Clinical	-	-	-	-	-	-	-	-	-
245	S54	-	Clinical	-	-	-	-	-	-	-	-	-
246	S55	-	Clinical	-	-	-	-	-	-	-	-	-
247	S56	-	Clinical	-	-	-	-	-	-	-	-	-
248	S57	-	Clinical	-	-	-	-	-	-	-	-	-
249	S59	-	Clinical	-	-	-	-	-	-	-	-	-
250	S60	-	Clinical	-	-	-	-	-	-	-	-	-
251	S61	-	Clinical	-	-	-	-	-	-	-	-	-
252	S62	-	Clinical	-	-	-	-	-	-	-	-	-
253	S63	-	Clinical	-	-	-	-	-	-	-	-	-
254	S64	-	Clinical	-	-	-	-	-	-	-	-	-
255	S65	-	Clinical	-	-	-	-	-	-	-	-	-
256	S66	-	Clinical	-	-	-	-	-	-	-	-	-

Table2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, \isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
257	S68	-	Clinical	-	-	-	-	-	-	-	-	-
258	S69	-	Clinical	-	-	-	-	-	-	-	-	-
259	S70	-	Clinical	-	-	-	-	-	-	-	-	-
260	S71	-	Clinical	-	-	-	-	-	-	-	-	-
261	S72	-	Clinical	-	-	-	-	-	-	-	-	-
262	S73	-	Clinical	-	-	-	-	-	-	-	-	-
263	S75	-	Clinical	-	-	-	-	-	-	-	-	-
264	S76	-	Clinical	-	-	-	-	-	-	-	-	-
265	S77	-	Clinical	-	-	-	-	-	-	-	-	-
266	S78	-	Clinical	-	-	-	-	-	-	-	-	-
267	S79	-	Clinical	-	-	-	-	-	-	-	-	-
268	S80	-	Clinical	-	-	-	-	-	-	-	-	-
269	S81	-	Clinical	-	-	-	-	-	-	-	-	-
270	S82	-	Clinical	-	-	-	-	-	-	-	-	-
271	S83	-	Clinical	-	-	-	-	-	-	-	-	-
272	S84	-	Clinical	-	-	-	-	-	-	-	-	-
273	S85	-	Clinical	-	-	-	-	-	-	-	-	-
274	S86	-	Clinical	-	-	-	-	-	-	-	-	-
275	S87	-	Clinical	-	-	-	-	-	-	-	-	-
276	S88	-	Clinical	-	-	-	-	-	-	-	-	-
277	S89	-	Clinical	-	-	-	-	-	-	-	-	-

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
278	S90	-	Clinical	-	-	-	-	-	-	-	-	-
279	S91	-	Clinical	-	-	-	-	-	-	-	-	-
280	S92	-	Clinical	-	-	-	-	-	-	-	-	-
281	S93	-	Clinical	-	-	-	-	-	-	-	-	-
282	S94	-	Clinical	-	-	-	-	-	-	-	-	-
283	S95	-	Clinical	-	-	-	-	-	-	-	-	-
284	B1	-	Clinical	-	-	-	-	-	-	-	-	-
285	B2	-	Clinical	-	-	-	-	-	-	-	-	-
286	B3	-	Clinical	-	-	-	-	-	-	-	-	-
287	B5	-	Clinical	-	-	-	-	-	-	-	-	-
288	B6	-	Clinical	-	-	-	-	-	-	-	-	-
289	B7	-	Clinical	-	-	-	-	-	-	-	-	-
290	B9	-	Clinical	-	-	-	-	-	-	-	-	-
291	B10	-	Clinical	-	-	-	-	-	-	-	-	-
292	B11	-	Clinical	-	-	-	-	-	-	-	-	-
293	B12	-	Clinical	-	-	-	-	-	-	-	-	-
294	B13	-	Clinical	-	-	-	-	-	-	-	-	-
295	B14	-	Clinical	-	-	-	-	-	-	-	-	-
296	B15	-	Clinical	-	-	-	-	-	-	-	-	-
297	B16	-	Clinical	-	-	-	-	-	-	-	-	-
298	B17	-	Clinical	-	-	-	-	-	-	-	-	-

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
299	B18	-	Clinical	-	-	-	-	-	-	-	-	-
300	B19	-	Clinical	-	-	-	-	-	-	-	-	-
301	B20	-	Clinical	-	-	-	-	-	-	-	-	-
302	B21	-	Clinical	-	-	-	-	-	-	-	-	-
303	B22	-	Clinical	-	-	-	-	-	-	-	-	-
304	B23	-	Clinical	-	-	-	-	-	-	-	-	-
305	B25	-	Clinical	-	-	-	-	-	-	-	-	-
306	B26	-	Clinical	-	-	-	-	-	-	-	-	-
307	B27	-	Clinical	-	-	-	-	-	-	-	-	-
308	B28	-	Clinical	-	-	-	-	-	-	-	-	-
309	B29	-	Clinical	-	-	-	-	-	-	-	-	-
310	B30	-	Clinical	-	-	-	-	-	-	-	-	-
311	B31	-	Clinical	-	-	-	-	-	-	-	-	-
312	B32	-	Clinical	-	-	-	-	-	-	-	-	-
313	B33	-	Clinical	-	-	-	-	-	-	-	-	-
314	B34	-	Clinical	-	-	-	-	-	-	-	-	-
315	B35	-	Clinical	-	-	-	-	-	-	-	-	-
316	B36	-	Clinical	-	-	-	-	-	-	-	-	-
317	B37	-	Clinical	-	-	-	-	-	-	-	-	-
318	B38	-	Clinical	-	-	-	-	-	-	-	-	-
319	B39	-	Clinical	-	-	-	-	-	-	-	-	-

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
320	B40	-	Clinical	-	-	-	-	-	-	-	-	-
321	B41	-	Clinical	-	-	-	-	-	-	-	-	-
322	B42	-	Clinical	-	-	-	-	-	-	-	-	-
323	B43	-	Clinical	-	-	-	-	-	-	-	-	-
324	B44	-	Clinical	-	-	-	-	-	-	-	-	-
325	B45	-	Clinical	-	-	-	-	-	-	-	-	-
326	B46	-	Clinical	-	-	-	-	-	-	-	-	-
327	B47	-	Clinical	-	-	-	-	-	-	-	-	-
328	B48	-	Clinical	-	-	-	-	-	-	-	-	-
329	B49	-	Clinical	-	-	-	-	-	-	-	-	-
330	B50	-	Clinical	-	-	-	-	-	-	-	-	-
331	B53	-	Clinical	-	-	-	-	-	-	-	-	-
332	B55	-	Clinical	-	-	-	-	-	-	-	-	-
333	B56	-	Clinical	-	-	-	-	-	-	-	-	-
334	B57	-	Clinical	-	-	-	-	-	-	-	-	-
335	B58	-	Clinical	-	-	-	-	-	-	-	-	-
336	B59	-	Clinical	-	-	-	-	-	-	-	-	-
337	B61	-	Clinical	-	-	-	-	-	-	-	-	-
338	B62	-	Clinical	-	-	-	-	-	-	-	-	-
339	B63	-	Clinical	-	-	-	-	-	-	-	-	-
340	B64	-	Clinical	-	-	-	-	-	-	-	-	-

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
341	B65	-	Clinical	-	-	-	-	-	-	-	-	-
342	B66	-	Clinical	-	-	-	-	-	-	-	-	-
343	B67	-	Clinical	-	-	-	-	-	-	-	-	-
344	B68	-	Clinical	-	-	-	-	-	-	-	-	-
345	B69	-	Clinical	-	-	-	-	-	-	-	-	-
346	B70	-	Clinical	-	-	-	-	-	-	-	-	-
347	B71	-	Clinical	-	-	-	-	-	-	-	-	-
348	B72	-	Clinical	-	-	-	-	-	-	-	-	-
349	B73	-	Clinical	-	-	-	-	-	-	-	-	-
350	B74	-	Clinical	-	-	-	-	-	-	-	-	-
351	B75	-	Clinical	-	-	-	-	-	-	-	-	-
352	B76	-	Clinical	-	-	-	-	-	-	-	-	-
353	B77	-	Clinical	-	-	-	-	-	-	-	-	-
354	B78	-	Clinical	-	-	-	-	-	-	-	-	-
355	B79	-	Clinical	-	-	-	-	-	-	-	-	-
356	B80	-	Clinical	-	-	-	-	-	-	-	-	-
357	B81	-	Clinical	-	-	-	-	-	-	-	-	-
358	B82	-	Clinical	-	-	-	-	-	-	-	-	-
359	B83	-	Clinical	-	-	-	-	-	-	-	-	-
360	B84	-	Clinical	-	-	-	-	-	-	-	-	-
361	B85	-	Clinical	-	-	-	-	-	-	-	-	-

Table 2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolations,source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
362	B86	-	Clinical	-	-	-	-	-	-	-	-	-
363	B87	-	Clinical	-	-	-	-	-	-	-	-	-
364	B88	-	Clinical	-	-	-	-	-	-	-	-	-
365	B89	-	Clinical	-	-	-	-	-	-	-	-	-
366	B90	-	Clinical	-	-	-	-	-	-	-	-	-
367	B91	-	Clinical	-	-	-	-	-	-	-	-	-
368	B92	-	Clinical	-	-	-	-	-	-	-	-	-
369	B95	-	Clinical	-	-	-	-	-	-	-	-	-
370	A1	1	Animal	nose	2005	-	-	-	-	-	-	-
371	A2	2	Animal	nose	2005	-	-	-	-	-	-	-
372	A3	3	Animal	nose	2005	-	-	-	-	-	-	-
373	A4	4	Animal	nose	2005	-	-	-	-	-	-	-
374	A5	1	Animal	nose	2005	-	-	-	-	-	-	-
375	A6	2	Animal	nose	2005	-	-	-	-	-	-	-
376	A7	3	Animal	nose	2005	-	-	-	-	-	-	-
377	A8	4	Animal	nose	2005	-	-	-	-	-	-	-
378	A9	1	Animal	nose	2005	-	-	-	-	-	-	-
379	A10	2	Animal	nose	2005	-	-	-	-	-	-	-
380	A11	3	Animal	nose	2005	-	-	-	-	-	-	-
381	A12	4	Animal	nose	2005	-	-	-	-	-	-	-
382	A13	1	Animal	nose	2005	-	-	-	-	-	-	-

Table2.(cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
383	A14	2	Animal	nose	2005	-	-	-	-	-	-	-
384	A15	3	Animal	nose	2005	-	-	-	-	-	-	-
385	A16	4	Animal	nose	2005	-	-	-	-	-	-	-
386	A17	1	Animal	eye	2005	-	-	-	-	-	-	-
387	A18	2	Animal	eye	2005	-	-	-	-	-	-	-
388	A19	3	Animal	eye	2005	-	-	-	-	-	-	-
389	A20	4	Animal	eye	2005	-	-	-	-	-	-	-
390	A21	1	Animal	nose	2005	-	-	-	-	-	-	-
391	A22	2	Animal	nose	2005	-	-	-	-	-	-	-
392	A23	3	Animal	nose	2005	-	-	-	-	-	-	-
393	A24	4	Animal	nose	2005	-	-	-	-	-	-	-
394	A25	1	Animal	nose	2005	-	-	-	-	-	-	-
395	A26	2	Animal	nose	2005	-	-	-	-	-	-	-
396	A27	3	Animal	nose	2005	-	-	-	-	-	-	-
397	A28	4	Animal	nose	2005	-	-	-	-	-	-	-
398	A29	1	Animal	blood	2005	-	-	-	-	-	-	-
399	A30	2	Animal	lung	2005	-	-	-	-	-	-	-
400	A31	3	Animal	kidney	2005	-	-	-	-	-	-	-
401	A32	4	Animal	blood	2005	-	-	-	-	-	-	-
402	E1	1	Environment	Dropping	2003	-	-	-	-	-	-	Ladprao
403	E2	2	Environment	Dropping	2003	-	-	-	-	-	-	Ladprao

Table2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
404	E3	3	Environment	Dropping	2003	-	-	-	-	-	-	Ladprao
405	E4	4	Environment	Dropping	2003	-	-	-	-	-	-	Ladprao
406	E5	5	Environment	Dropping	2003	-	-	-	-	-	-	Ladprao
407	E6	6	Environment	Dropping	2003	-	-	-	-	-	-	Ladprao
408	E7	1	Environment	Dropping	2003	-	-	-	-	-	-	Yannawa
409	E8	2	Environment	Dropping	2003	-	-	-	-	-	-	Yannawa
410	E9	3	Environment	Dropping	2003	-	-	-	-	-	-	Yannawa
411	E45	1	Environment	Dropping	2003	-	-	-	-	-	-	Pasicharoen
412	E46	2	Environment	Dropping	2003	-	-	-	-	-	-	Pasicharoen
413	E38	1	Environment	Dropping	2003	-	-	-	-	-	-	Thonburi
414	E39	2	Environment	Dropping	2003	-	-	-	-	-	-	Thonburi
415	E40	3	Environment	Dropping	2003	-	-	-	-	-	-	Thonburi
416	E41	4	Environment	Dropping	2003	-	-	-	-	-	-	Thonburi
417	E42	1	Environment	Dropping	2003	-	-	-	-	-	-	Bangplad
418	E43	2	Environment	Dropping	2003	-	-	-	-	-	-	Bangplad
419	E44	1	Environment	Dropping	2003	-	-	-	-	-	-	Phayathai
420	E47	1	Environment	Dropping	2003	-	-	-	-	-	-	Pranakorn
421	E48	2	Environment	Dropping	2003	-	-	-	-	-	-	Pranakorn
422	E49	1	Environment	Dropping	2003	-	-	-	-	-	-	Minburi
423	E50	2	Environment	Dropping	2003	-	-	-	-	-	-	Minburi
424	E51	3	Environment	Dropping	2003	-	-	-	-	-	-	Minburi

Table2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
425	E10	1	Environment	Dropping	2003	-	-	-	-	-	-	Pomprab
426	E11	1	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
427	E12	2	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
428	E13	3	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
429	E14	4	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
430	E15	5	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
431	E16	6	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
432	E17	7	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
433	E18	8	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
434	E19	9	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
435	E20	10	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
436	E21	11	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
437	E22	12	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
438	E23	13	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
439	E24	14	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
440	E25	15	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
441	E26	16	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
442	E27	17	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
443	E29	18	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
444	E30	19	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
445	E31	20	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
446	E32	21	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong

Table2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
447	E33	22	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
448	E34	23	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
449	E35	24	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
450	E36	25	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
451	E37	26	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
452	E52	27	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
453	E53	28	Environment	Dropping	2003	-	-	-	-	-	-	Sampuntawong
454	T1	1	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
455	T2	2	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
456	T3	3	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
457	T4	4	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
458	T5	1	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
459	T6	2	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
460	T8	4	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
461	T9	5	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
462	T11	6	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
463	T12	1	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
464	T13	2	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
465	T14	3	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
466	T15	4	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
467	T17	2	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong

Table2. (cont.) List of *C. neoformans* strains studied, including lab numbers, number of isolates, isolation source, isolation site, isolation date, gender, underlining disease, patient name, age, date of birth and place.

No.	Lab number	No. of Isolates	Isolation source	Isolation site	Isolation date	Gender	Underlying disease	Alive/Dead	Patient name	Age	Date of birth	Place
468	T18	3	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
469	T19	1	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
470	T20	2	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
471	T21	1	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
472	T22	2	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
473	T23	3	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
474	T24	4	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
475	T25	5	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
476	T26	6	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
477	T31	7	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
478	T27	8	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
479	T28	9	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
480	T30	10	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong
481	T32	11	Environment	Dropping	2005	-	-	-	-	-	-	Sampuntawong

2. DNA extraction

High molecular weight genomic DNA was extracted from the strains, the using method was previous described by Meyer (111). A loopful of the culture was transferred to a 1.5 microcentrifuge tube containing 500 µl of sterile distilled water. The cells were settled down by centrifugation for 15 minutes at 13,000xg, and the supernatant discarded. The pellet was snap-shot frozen in liquid nitrogen and grinded to the fine homogenoues powder using a sterile miniature pestle. The powder was kept at -20 C immediately to prevent the DNases activity induced the DNA damage. The lysis solution was prepared and 500 µl of lysis solution was added to each frozen tube and vortex vigorously for 5 minutes. 500 µl of Phenol was added, the solutions were mixed by gently inverted tube for 2 minutes. The aqueous and organic phase of solution was separated by centrifugation 13,000xg at 4 degree Celsius for 30 minutes. The upper (aqueous) supernatant was transferred to the new sterile 1.5 microcentrifuge tube, the organic phase discarded. Chloroform: isoamyl alcohol (24:1) 500 µl was added to the microcrntrifuge tube contained the aqueous solution and mixed gently. The DNA solution was centrifuged at 13,000xg at the temperature 4 degree Celsius for 30 minutes. The supernatant was carefully transferred to a new sterile microcentrifuge tube again. DNA was pelleted by added 1 time of volume Isopropanol, mixed gently by inverted tube. The tube was transferred to -20 degree Celsius freeze for as least 30 minutes, frozen overnight can increase the DNA yield. The DNA was precipitated at 13,000xg at 4 degree Celsius for 30 minutes. The pellet was gentle washed by 70% cold absolute ethanol and centrifuge at 13,000 xg for 15 minutes. The ethanol was decanted and the air-dried pellet at room temperature, and stored thereafter at -20 C as a stock DNA. The DNA was re-dissolved in sterile distilled water 50 µl standing the

tube overnight. The DNA concentration and quality was determined by spectrophotometer using the absorbance wavelength at lambda = 260 nm. The 10 ng DNA of each strain was prepared for molecular study.

3. M13-PCR fingerprinting

PCR fingerprinting was determined according to a method previously reported (14) using the minisatellite specific core sequence of the wild type phage M13 (5' GAG GGT GGG GGT TCT 3') used as single primer.

The PCR reaction was performed in 50 µl volumes , containing 50 ng of high molecular weight genomic DNA, with 1X Taq polymerase PCR buffer (Applied Biosystem), 1.5 mM Magnesium acetate, 0.2 mM each of dATP, dTTP, dCTP, dGTP, 50 pmol M13 primer, 0.25 U Amplitaq DNA polymerase (Applied Biosystem, USA). The PCR reactions were amplified in a Thermal Hybaid PCR Express thermal cycler (Hybaid, USA). M13- fingerprinting reaction using the following program:

94 ºC Initial denaturation	3 min
94 ºC Denaturation	45 s
62 ºC Annealing	40 s
72 ºC Extension	2 min

For 35 cycles then follow with 72 ºC Final Extension for 10 min

All of M13-PCR fingerprints products were amplified at Mycology Unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand and separated on 1.4% agarose gels at the Molecular Mycology Research Laboratory, Centre of Infecious Disease, Westmead Hospital, University of Sydney, Sydney, Australia. The protocol was perfomed following Meyer, et al (14).

M13-PCR products were separated on 1.4% agarose gel using 1X Tris-Borate-EDTA (TBE) electrophoresis buffer. The gel was added with 20 ug of ethidium bromide for each 100 ml of agarose gel. The standard ladder, 1 kb ladder (New England Biolabs, USA) was loaded every 8-10 lanes to enable normalization within and between each gel.

The gel electrophoresis was performed in a Gel-O-submarine system electrophoresis chamber (model JSB-120, Jordan Scientific), at 1.6 V/cm (70 V) to a length of 14 cm according to the lowest molecular weight front of loading dye, approximately 9 hours. The bands were visualized on a UV transluminator and photographed. The pictures were analyzed using the BioloMICS software program (Bio*Aware*, Hannut, Belgium). The molecular weights of the PCR bands were identified, by comparing their migration with the migration of the 1 Kb plus marker. The result was uploaded to the BioloMICS data base of the Molecular Mycology Research Laboratory, Centre of Infectious Disease, Westmead Hospital, University of Sydney, Sydney, Australia.

The subtypes were defined by the difference of one additional or deleted PCR band (14).

4. *URA5*-Restrcition Fragment Length Polymorphisms (*URA5*-RFLP)

URA5 gene was universal used for molecular typing since this gene shown the specific pattern for each molecular type of *C. neoformans* (14). RFLP analysis of the *URA5* gene was performed for all 481 studied isolates. The *URA5* gene was amplified

with the primers: *URA5* (5’ ATG TCC TCC CAA GCC CTC GAC TCC G 3’) and *SJ01* (5’ TTA AGA CCT CTG AAC ACC GTA CTC 3’).

The PCR reaction were done in a 50 µl volume using 0.5 mL thin-walled reaction tube containing 50 ng of DNA, 1X Taq polymerase PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dCTP, dGTPs, 50 ng of each primer, 0.25 U BioTaq taq DNA polymerase (BIoLine, USA). The PCR reactions were amplified in a Hybaid PCR Express thermal cycler (Hybaid, USA) using the following program:

94 ºC Initial denaturation	3 min
94 ºC Denaturation	45 s
62 ºC Annealing	40 s
72 ºC Extension	2 min

For 35 cycles then follow with 72 ºC Final Extension for 10 min

The *URA5*-PCR products were electrophoresed on 1.4% agarose gel added 20 µg ethidiumbromide for 100 mL of gel solution. The gel electrophoresesed with 1X TBE to detected the product if the PCR band showed (777 bp) the products were subjected to RFLP step. The double-restriction endonuclease digest reaction was performed , for total 5 µl in each reaction containing 2.5 µl of 5X NEBuffer 4, 0.25 BSA, 0.67 U of *Hha*I and 3U of *Sau96*I (New England Biolabs, USA). The mixed digestion solution was 5 µl added to perform the double-restriction endonuclease digest reaction and incubated 37 degree Celsius for 24 hours. The *URA5*-RFLP bands were detected on 3% agarose gel with 1X TBE supplemented with 0.2 ug/mL ethidium bromide to 100 ml.The gel was electrophoresis at 100 V for 3 hours. The 100 bp mass Ruler was used as a marker standard size (Fermentas, USA). The RFLP patterns were

analyzed by compared with the standard strains *C. neoformans* and *C. gattii* to identify the *URA5*-RFLP molecular type.

5. Mating type Analysis

As we know that the mating type of *C. neoformans* plays a role as one major of virulence factor. We determined the mating type of the strains following this protocol.

The mating type was determined by co-amplification of primer specific for mating type **a** and mating type α. PCR reaction 25µl volume contained 25 ng of genomic DNA, 1X PCR buffer (Bioline, USA), 0.2 mM each of dNTP, 0.2 µM each of primers and 1.25 U Bioline Taq DNA polymerase (Bioline, USA). The reactions were performed in a Hybaid PCR Express thermal cycler (Hybaid, USA) using the following program:

94 ºC Initial denaturation	3 min
94 ºC Denaturation	45 s
62 ºC Annealing	40 s
72 ºC Extension	2 min

For 35 cycles then follow with 72 ºC Final Extension for 10 min

PCR products were visualised on a 1.5% agarose-TBE gel supplemented with 0.2 µg/ml ethidium bromide and the agarose gel was electrophoresed at 100 V for 30 minutes.

6. Multilocus Sequence Typing (MLST)

To establish a more reproducible strain typing method MLST analysis of the above selected isolates was carried out. The selected strains criteria based on the differentiation of the M13-fingerprinting pattern.

Initially the following five genes have been selected from Fraser et al (121) including:

- Capsular associated protein gene (*CAP59*) gene
- Orotidine monopyrophosphate (*URA5*) gene
- Laccase (*LAC1*) gene
- Actin (*ACT1*) gene
- Phospholipases B (*PLB*1) gene

The name and primer sequence showed in table 3:

Table 3. Primer and primer sequence used in the MLST study

Locus	**Primer Sequence**	**Product size (bp)**	**PCR conditions**
CAP59	5’ TCC GCT GCA CAA GTG ATA CCC 5’ CTC TAC GTC GAG GTC AAG	606	35 cycles 61 ºC
PLB1	5’ CTT CAG GCG GAG AGA GGT TT 5’ GAT TTG GCG TTG GTT TCA GT	674	35cycles 61 ºC
URA5	5’ ATG TCC TCC CAA GCC CTC GAC TCC G 3’ 5’ TTA AGA CCT CTG AAC ACC GTA CTC 3’	777	35 cycles 61ºC
ACT1	5’ AAAT CTC GCC CAA CAT GT 5’ TTA GAA ACA CTT TCG GTG GAC G	1,371	35 cycles 48ºC
LAC1	5’ GGC GAT ACT ATT ATC GTA 5’ TTC TGG AGT GGC TAG AGC	586	35 cycles 58 ºC

The PCR condition for each gene are the same manner; pre-denature 94 ºC followed by 35 cycles of 94 ºC denature 3 minutes , and 72 ºC 1 minute for the extension stage the annealing temperature are shown in table 3. After that followed with final extension at 72ºC 10 minutes.

The amplified genes were checked on 1.4% agarose gels and purified by Purification kit (GE Healthcare, USA) before sequence. The purified PCR products were commercially sequenced at Macrogen Inc, South Korea. The sequences were manually edited using the program Sequencer 4.6 (Genes Code, USA). Alignments were generated using the program **ClustalX (<ftp:ftp-igbmc.u-strasbg. fr/pub/ ClustalX/>).**

In order to increase the discriminative power of the MLST system for the studied Thai isolates, four more genes were selected for the MLST analysis based on the study of Litvintseka et al. (122) including:

- Translation Elongation Factor alpha (*TEF1*),
- Cu, Zn superoxide dismutase (*SOD1*),
- Ribosomal RNA intergenic spacer (*IGS1*)
- Other segment gene of *CAP59*

For all new primers the PCR condition were similar; The PCR condition for each gene are the same manner; pre-denature 94 ºC followed by 35 cycles of 94 ºC denature 3 minutes , 60ºC annealing temperature, 72 ºC for 1 minute extension stage, followed with post extension at 72ºC 10 minutes as shown in table 4.

Table 4. Additional primers and primer sequence used in the MLST study.

Location	Primer Sequence	Product size (bp)	PCR conditions
Cap59CN	5’ GAT TTG GCA GAG TAG GAG ACA GA 5’ ATA TCC CAG ACT TTT CGG TCG TA	594	35 cycles 60 ºC
IGS1	5’ ATC CTT TGC AGA CGA CTT GA 5’ GTG ATC AGT GCA TTG CAT GA	790	35 cycles 60 ºC
SOD1	5’ TCT AAT CGA AAT GGT CAA GG 5’ CGC AGC TGT TCG TCT GGA TA	680	35 cycles 60 ºC
TEF1	5’ AAT CGT CAA GGA GAC CAA CG 5’ CGT CAC CAG ACT TGA CGA AC	844	35 cycles 60 ºC

The sequence bases were corrected and edited by manual by Sequencer 4.6 (Gene Code Corp, USA). The phylogenetic tree was generated by PAUP version 10.0.

The tree will be generated follow these:

The tree of each gene generated from all selected strains 31 strains were used.

The tree of each gene generated from all selected strains 31 strains and all sequence type (ST) from www.mlst.net/ cgrubii, the reference database

The combine gene tree from all selected strains 31 strains.

The combine gene tree from all selected strains 31 strains with the reference data.

7. Multilocus Microsatellite Typing (MLMT)

Taking into account the low variability detected in the PCR-fingerprinting analysis and the MLST analysis, it was decided to test a normally highly discriminatory typing system MLMT on the set of 31 selected strains.

Eleven MLMT loci were tested based on the preliminary results conducted in the Molecular Mycology research Laboratory at Westmead Hospital (Karaoglu & Meyer, unpublished data). The loci amplification was determined in a 96 well low profile reaction microtitre plate (Astral scientific, USA) using a T3 Thermocycler (Biometra®, USA). The microsatellites were amplified in a volume of 25 µl; 10 pM of each primer, 0.2 mM of each dNTP, 0.25 mM $MgCl_2$, 2 ng of genomic DNA and 2.5 unit of Bioline Taq polymerase (Bioline, USA). The reactions were performed using the T1 Thermal cycler (Biometra®, USA), under the following PCR conditions: initial denaturation at 94˚C for 4 minutes, followed by 30 cycles of denaturing at 94˚C for 1 min, annealing at 58˚C for 30 seconds, and extension at 72˚C for 30 seconds, followed by a final extension at 72˚C for 5 minutes. For the forward primer of each locus, the primer was 5' labeled with incorporate either of the fluorophre IRD-700 or 800 dyes (MWG Biotech, Germany).

The PCR product was detected by polyacrylamide gel electrophoresis in LI-COR®-IR2 sequencer (LI-COR Bioscience, USA). A 8.0% polyacrylamide (7M urea) solution was prepared as composing of 20 ml polyacrylamide gel of 40% acrylamide solution (Amresco, USA), 10X Tris-Borate-EDTA buffer and 2.5% of Urea (see appendix). For all loci primers were use 8% polyacrylamide gel, except only one primer, CAA8, a 5% polyacrylamide gel was recommend for the amplicon detection

The polyacrylamide gel dimension was 25 cm long glass plates and 0.25 mm thick rubber spacers were set up according to the manufacturer's protocols (LI-COR Bioscience, USA).

The 64 wells comb was used for lane formation. Before the sample loading, each sample was mixed with 2X buffer loading dye (see appendix) in ratio 25 µl of sample per 5 µl of loading dye. The loading dye-mixed PCR was denatured at 94 ºC for 4 minutes and transferred to ice bath immediately. 1 µl of each sample was loaded with a single barrel Hamilton sample loading syringe. A 50-350 bp or 50-700 bp concentrated sizing standard IRD700 or IRD 800 dye (LI-COR Bioscience, USA) were used to determine the length of the microsatellite alleles and were loaded every 8-10 lanes. The running buffer was 1XTBE, pH 8.3. The polyacrylamide gel was run at 1,500 V constant voltages, 40 Watts, 40 mA for 1.30 hours and the gel temperature was maintained at 45 ºC.

The IRD labeled marker for each primer and primer sequences were shown in table 5. For the locus $(CAA)_8$, the 50-700 bp IRD700 sizing standard was used. Signals were read on an automatic sequencer LI-COR IR^2 (LI-COR, USA). The raw PCR result data was presented as image and stored in TIFF format using the image software package (LI-COR Bioscience, USA). Band sizes were determined automatically using SAGA GT ^{TM} analysis software (LI-COR Bioscience, USA). Alleles were designed according to the size of PCR products.

The microsatellite products were also amplified and determined the repeating unit by DNA sequencing using an automatic sequencer (Commercial Company; Macrogen Inc., Korea). The repeating unit were analyzed and compared with the microsatellite allele type.

The population genetic was determined using GenAlex6 program

8. Discriminatory power (D) of the studied molecular typing techniques

The ability of the microsatellite to discriminate between the isolates of each typing technique was calculated with Simpson's index of diversity (133) The index measures the probability that any two isolates will have different genotypes. The formula showed as below:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j-1)$$

Where ***N*** is the total number of strains in the sample population, ***S*** is the total number of types described, and $\boldsymbol{n_j}$ is the number of strains belonging to the ***j*** type.

Table 5.. List of MLMT loci and primers

Loci	Size	IRD dye	Primer
$(GA)_9$	161	700	F 5’ ATT CAT GGG AAG ATC GCT TGG R 5’ GCG GCT CGG CCT TAC CAA TA
$(TG)_{12}G(TG)_{12}$	166	700	F 5’AGA GAC GGG CAA ATC AGA GT R 5’GAC TGG ATT CTT GAA CGG CT
$(TCCTTT)_6$	187	700	F 5’ TCG CTG TTG AGC GTG TTA TC R 5’ AGA TGG ATG GGA AAG GGA AG
$(TTA)_{10}$	198	700	F 5’ TGA TGT TTG GAG GAC AGC TTC TG R 5’TCT GTC TCC CTC CGA TAT GTT GT
$(AC)_{12}$	283	700	F 5’ TCC TTG AAA TCC GGT CGC TAA R 5’ CTC TTC TCC TCC GCC GAA AC
$(CAA)_8$	455	700	F 5’ AGA GCC CAA GGC AAA GAA GA R 5’ GCC TTA CCC ATG TAC TGA GC
$(GT)_{12}$	165	800	F 5’ CGG TGC TGG AAG GTC ATA R 5’ TTG AAA TCC GGT CGC TAA A
$(TATTT)_9$	174	800	F 5’ TAT TGG ATA TGC TGG ATC TGA C R 5’ GGA GTA TAA GGC TAC CGT GTC T
$(GCC)_8$	193	800	F 5’ CGC TTT CTT GCA GTC TGA TGT C R 5’ TAC TTT GCC CTC CAC ATA CTG A
$(GTT)_9$	208	800	F 5’ CCG ACA CCT GGA GAA TAA A R 5’TGA GGA CGG GAG TCG AAA AG
$(GGAT)_{33}$	306	800	F 5’ GGA TTG ATG CAG GAT GCG TAA G R 5’ CAT CGG TAG CCT CGC CCA GA

Chapter IV

Results

1. Molecular typing of *C. neoformans* species complex using *URA5*-Restriction fragment length polymorphisms (*URA5*-RFLP)

URA5 gene of 481 isolates of *C. neoformans* and the eight molecular type standard strains of *C. neoformans* species complex were amplified and double digested with restriction enzymes, *Sau*96I and *Hha*I. The RFLP pattern was interpreted by comparing with those of the eight standard strains. All *C. neoformans* included in this study are classified as ***C. neoformans*** var**.** ***grubii***. Overall, the most common molecular type was VNI, comparable with *C.neoformas var. grubii* WM148 standard strain molecular type VNI, accounting for 98.5% (n=474) of the isolates which was classified as *C. neoformans var. grubii* serotype A. Only 7 isolates (1.5%, n=7) were classified as *C. neoformans var. grubii* molecular type VNII since they gave the similar *URA5*-RFLP pattern comparable with *C. neoformans var. grubii* WM626 standard strain molecular type VNII. Moreover, all of these VNII isolates was particularly observed from the veterinary source in the study. None of the studied isolates classified as *C. neoformans* AD hybrid VNIII, *C. neoformans* var. *neoformans* VNIV, or *C. gatti* VGI-VGIV (Table 1)

Regarding the most commonly found molecular type, VNI, there was no significant association between the molecular type and sources of the isolates. However, due to *C. neoformans var. grubii* molecular type VNII was only observed in veterinary isolates, it might be possible that *C. grubii* molecular type VNII in Thailand

were preferentially colonized in animal rather than in environmental and clinical source.

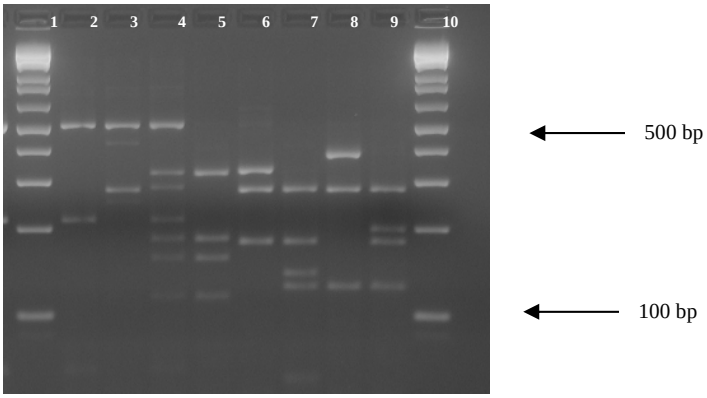


Figure12. *URA5*-RFLP patterns of *C. neoformans/ C. gattii species complex* standard strains: Lane (L) 2 = WM148 (VNI); L3 = WM626 (VNII); L4 = WM628 (VNIII); L5 = WM629 (VNIV); L6= WM179 (VGI); L7 = WM 178(VGII), L8 = WM175 (VGIII), L9 = WM779 (VGIV); (L) 1 and L10 = 1 kb plus marker (Fermentas, USA)

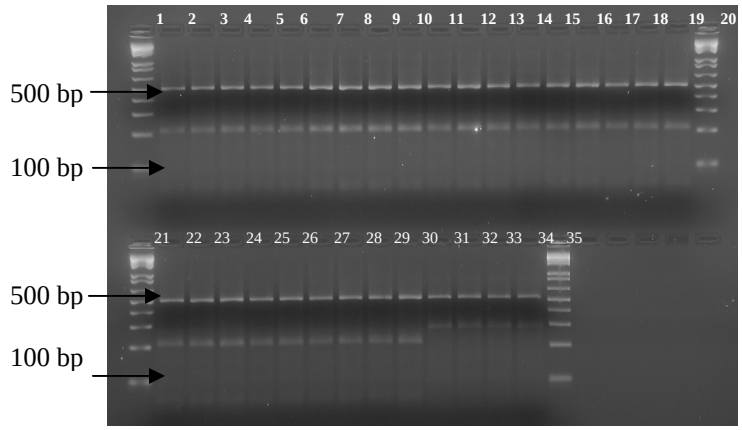


Figure13. Example of the *URA5*-RFLP patterns of *C. neoformans* strains: Lane (L)2 = C121, L3 = C146; L4 = S24; L5 = CM1;L6 = CM19; L7 = B1; L8 = B59; L9 = P4; L10 = P17; L11 = A13; L12 = A25; L13 = E1; L14 = E11; L15 = T1; L16 = T28; L17 = A19; L18 = B7; L19 = A17; L22 = A18; L23 = C110; L24 = S87; L25 = E12; L26 = B31; L27 = C140; L28 = CM17; L29 = B2; L30 = E38; L31 = A5; L32 = A10; L33 = A11; L34 = A6 (for strain information see Table1); L1, L20, L21 and L35 = 1kb plus marker (Fermentas, USA).

Table 6. Summary of the results of the *C. neoformans* species complex *URA5*-RFLP patterns

Source	**Place**	***URA5*-RFLP Molecular type (strain)**	
		VNI	**VNII**
Clinical Isolate	Chulalongkorn University	160	0
	Sirisaj Hospital	84	0
	Chiangmai University	18	0
	Bamrasnadura Infectious Institue	86	0
	Bhubibhol Adulyadej Hospital	19	0
Animal Isolate	Chulalongkorn University	25	7
Environmental Isolate	Bangkok (2003)	52	0
	Bangkogk (2005)	28	0
	Chiangmai University	2	0
	Total	474	7

2. **Mating type analysis**

The mating type was also determined for all 481 studied isolates of *C. neoformans* var. *grubii* by PCR co-amplification of fragments from *MAT***α** and *MAT***a** loci. As an example, the mating type-specific fragments amplified from some isolates are shown in figure 14. All 481 isolates, regardless of their sources, were *C. grubii* mating type **α** (100%, n=481). This was also confirmed by no PCR products from amplification using mating type **a** specific primers. *C. neoformans* JEC 20 (mating type **a)** and JEC21 (mating type **α**) were used as control strains.

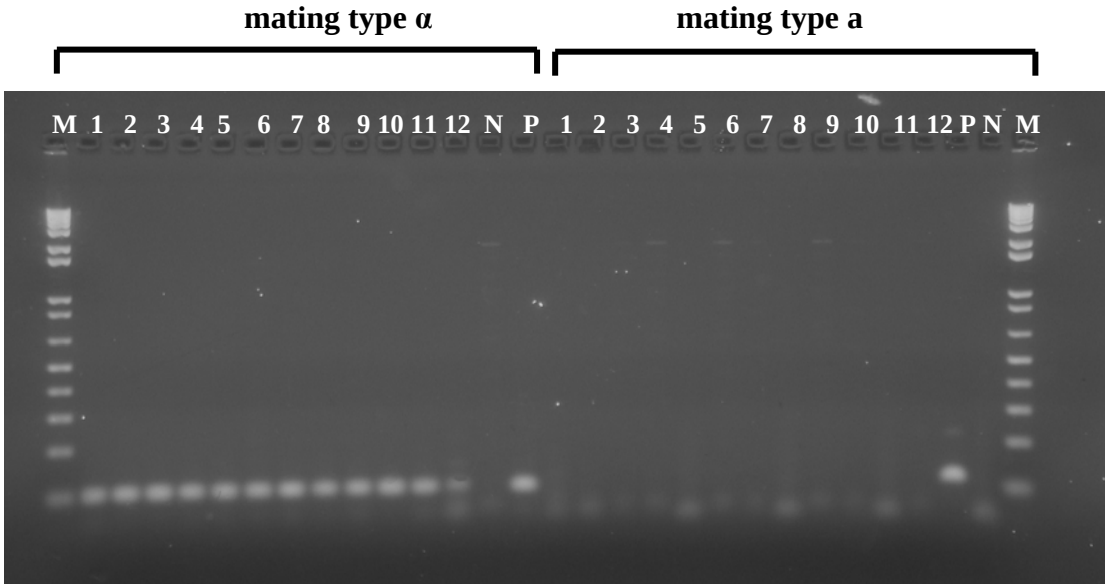


Figure14. Examples of the mating type PCR showing the mating type alpha specific PCR band.; Lane (L) 1=S1, L2=S2, L3=S4, L5=S6, L6=S7, L7=S8, L8=S9, L9=S10, L10=S11, L11=C23, L12=C27, L13=C28,N= Negative control, P= Positive control; M= 1 Kb plus Marker (Invitrogen, USA)

3 .M13-fingerprinting analyses

481 isolates of *C. neoformans* var. *grubii*, and the eight molecular type standard strains, were performed the PCR fingerprinting using M13 core specific primer. The M13-fingerprinting patterns were analyzed using the BioloMICS software program (Bio*Aware*, Hannut, Belgium). Bands were defined manually since the automated band designation options included in the software occasionally detected the inappropriate band sizes. The molecular weights of the PCR bands were identified, by comparing their migratory distances with those of the 1 Kb plus marker (Invitrogen, USA). In The M13-fingerprinting analysis, all bands within the size range of 550-2850 bp were included in the analysis regardless of intensity. the presence of bands outside of this range have been excluded since they were less reproducible (Kidd, 2003, Ph.D. Thesis University of Sydney). The result was uploaded to the BioloMICS data base of the Molecular Mycology Research Laboratory, Westmead Hospital, University of Sydney. The M13-fingerprinting gel electrophoresis was showed in figure 15, 16 and 17. The cladogram of M13-fingerprinting showed in figure 18.

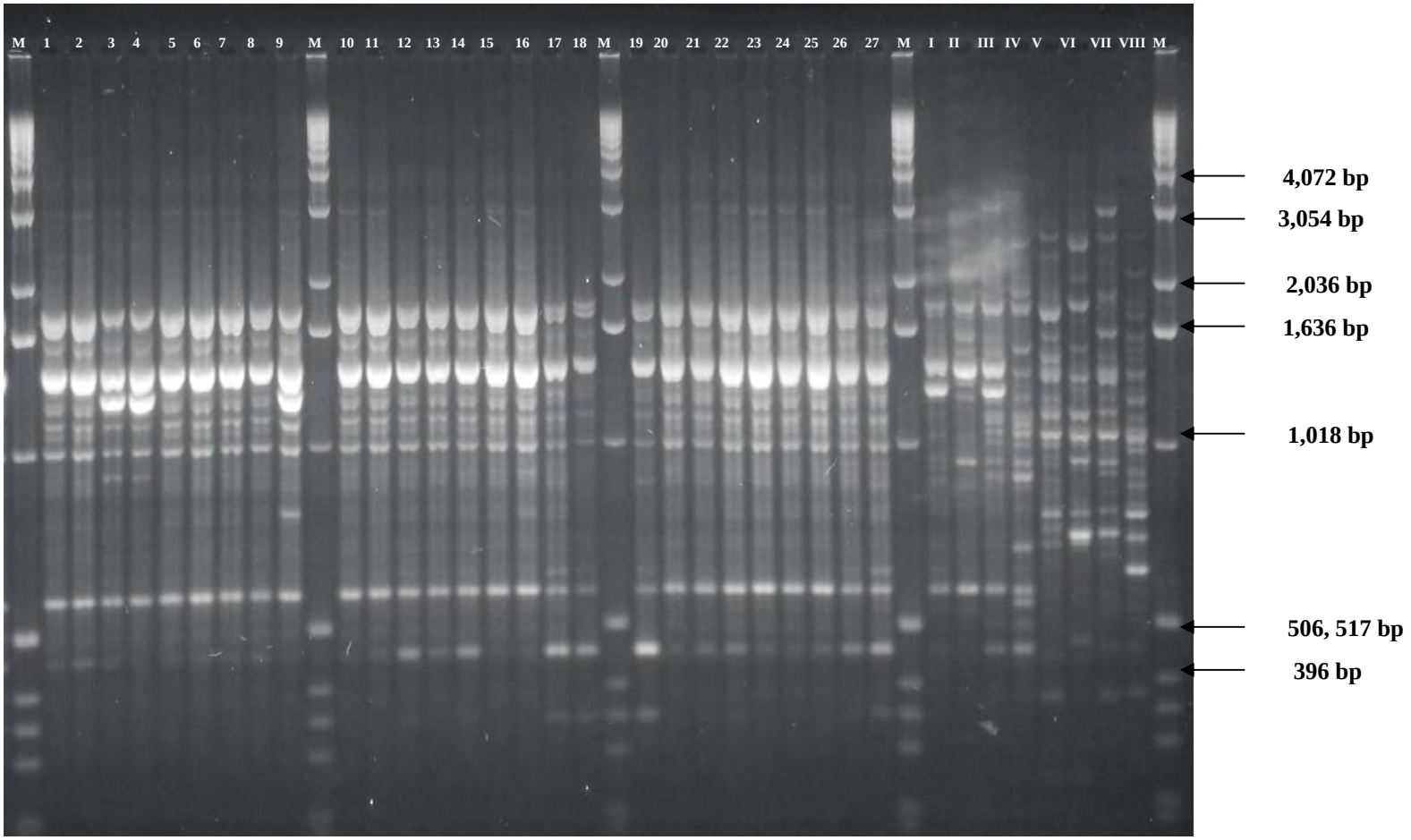


Figure15.1 M13-fingerprinting of clinical isolates ; Lane(L)1=C97; L2= C133; L3= C101; L4=C106; L5=C102; L6= C119; L7= C120; L8; = C121; L9=C140;L10=C136; L11=C137; L12=C200; L13=C105; L14= 203; L15=C107; L16=C115; L17=C55; L28=C56; L19=C58; L20=C80; L21=C82; L22=C180; L23=C181; L24=201; L25=C211; L26=C57; L27=59; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179;VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Intrivigen, USA)

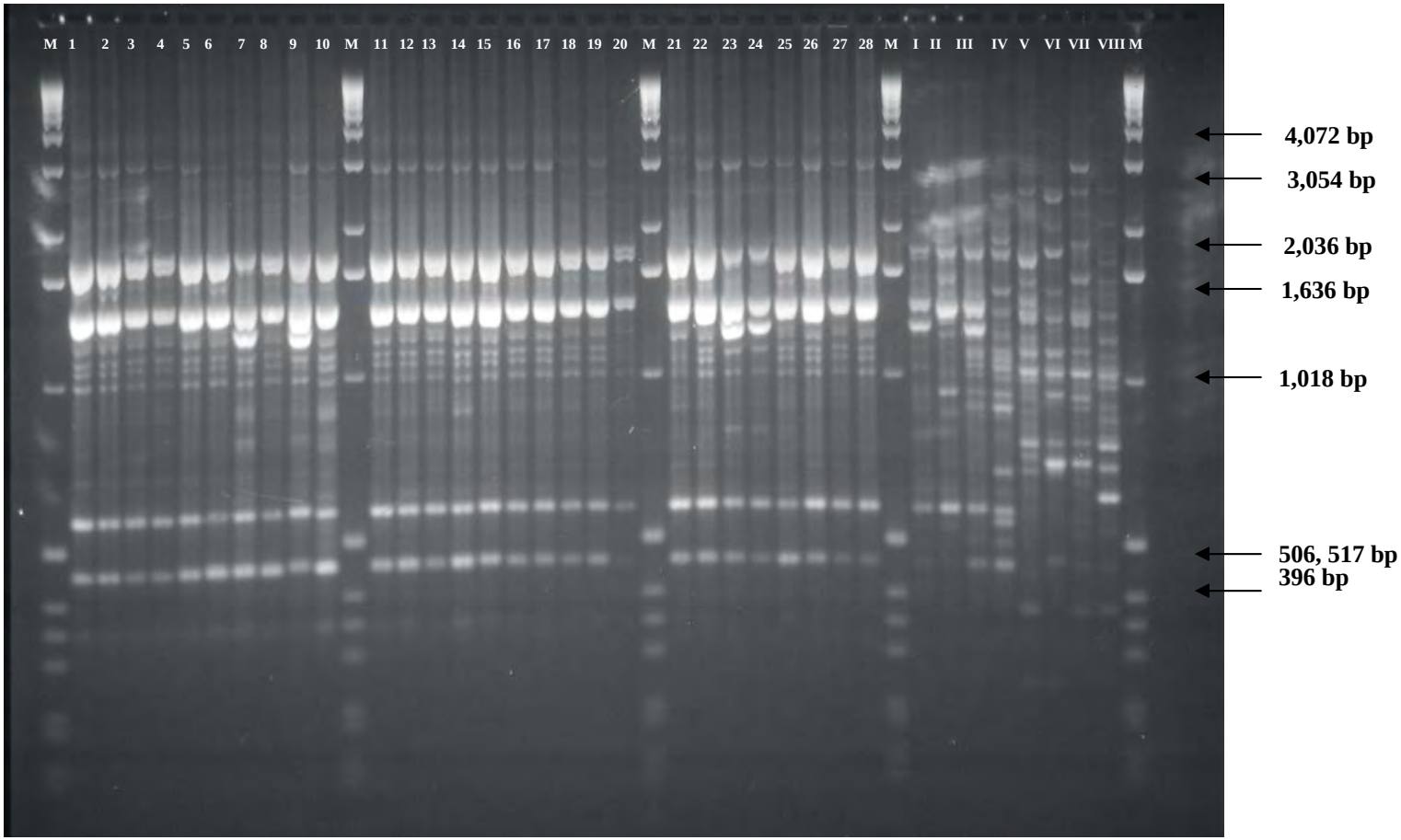


Figure15.2 M13-fingerprinting of clinical isolates ; Lane(L)1=C87; L2= C135; L3= C182; L4=C183; L5=C209; L6= C79; L7= C3; L8; = C4; L9=C5;L10=C24; L11=C34; L12=C45; L13=C12; L14= C14; L15=C13; L16=C16; L17=C25; L28=C31; L19=C32; L20=C33; L21=C17; L22=C196; L23=C65; L24=66; L25=C70; L26=C74; L27=C77; L28=C78; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Introgen, USA)

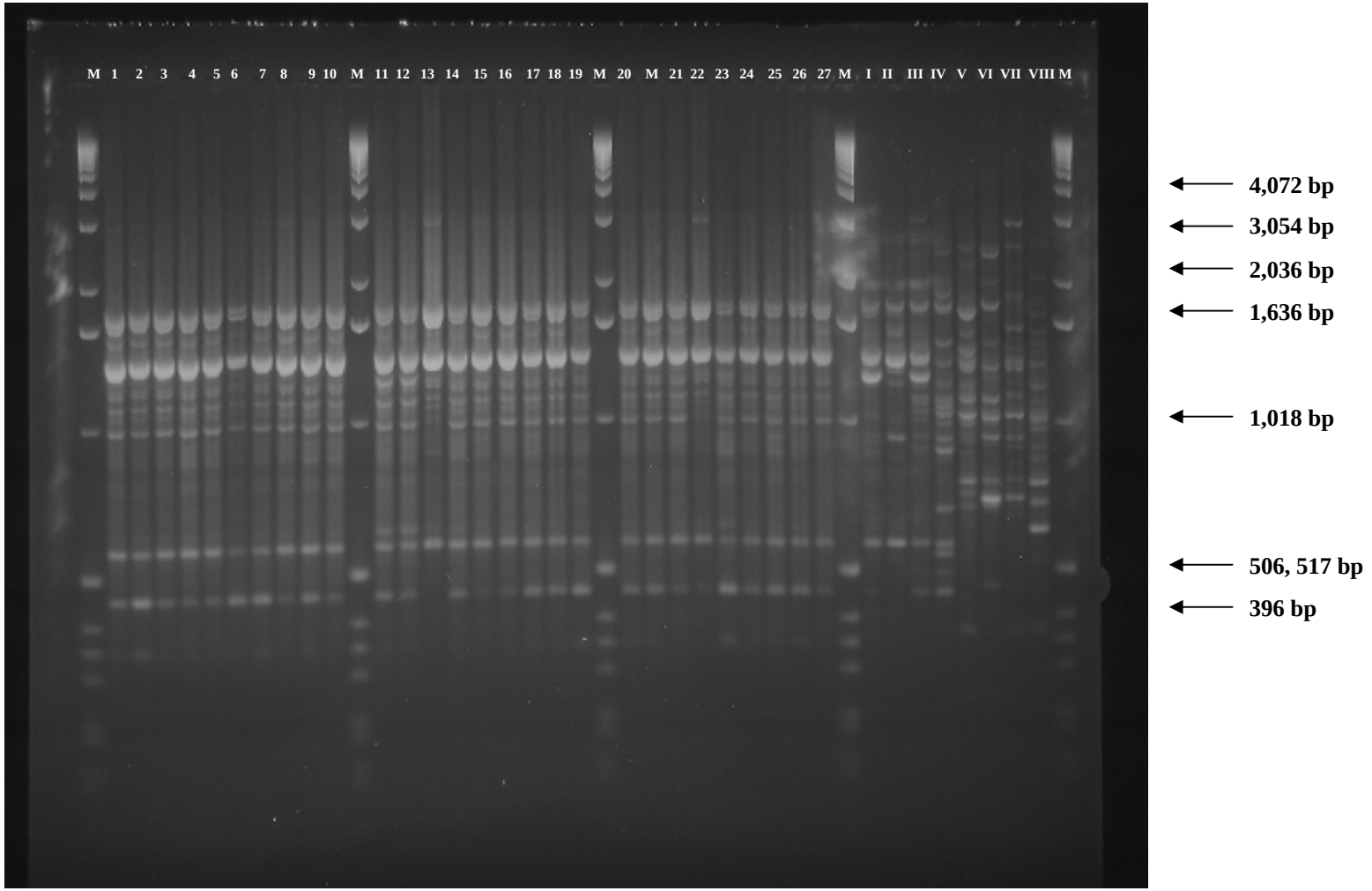


Figure15.3 M13-fingerprinting of clinical isolates ; Lane(L)1=C6; L2= C6; L3= C8; L4=C15; L5=C20; L6= C21; L7= C22; L8; = C23; L9=C28; L10=C40; L11=C41; L12=C67; L13=C71; L14= 72; L15=C29; L16=C42; L17=C43; L28=C44; L19=C46; L20=C47; L21=C54; L22=C195; L23=C62; L24=64; L25=C68; L26=C69; L27=75; L28=C76; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

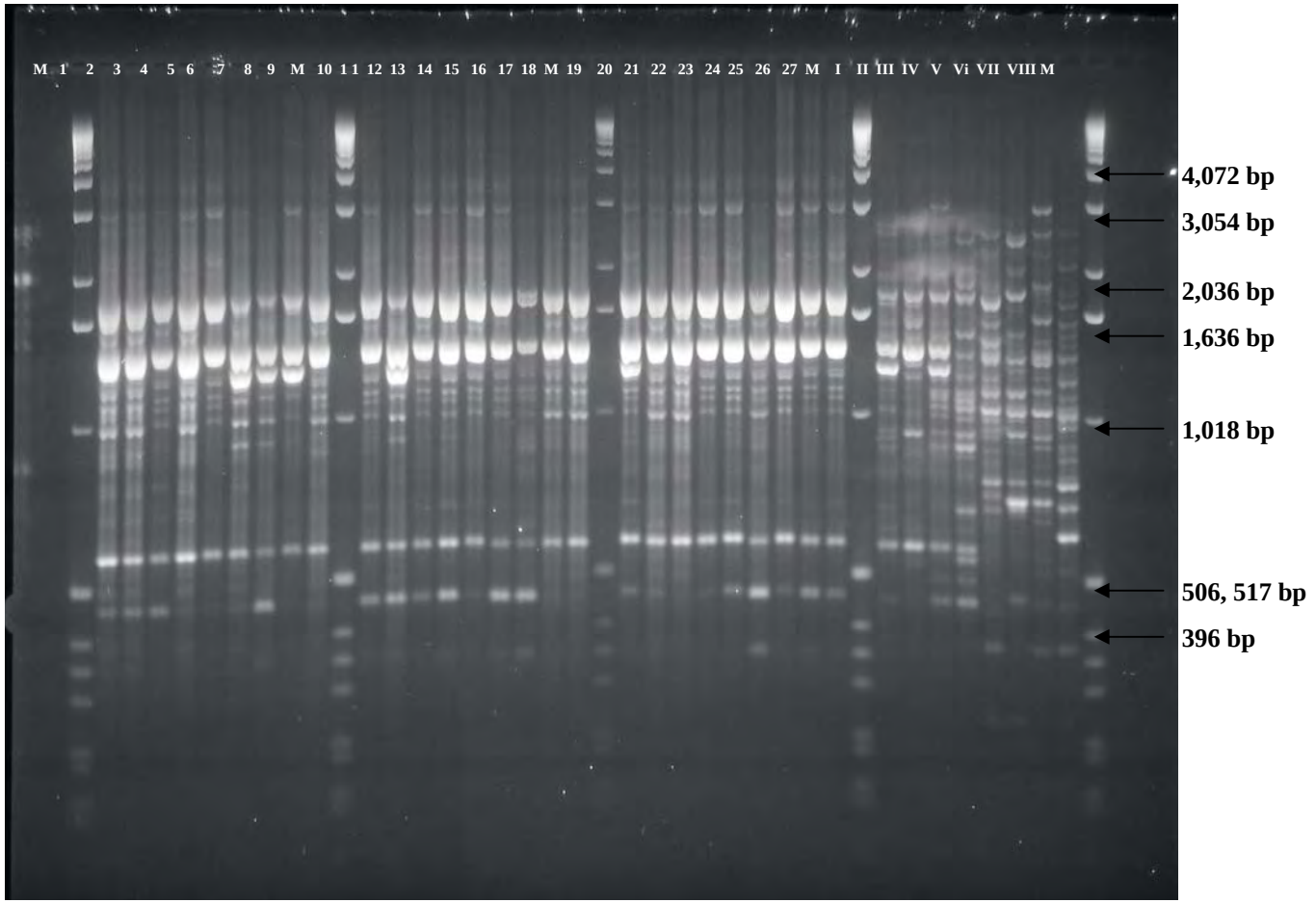


Figure15.4 M13-fingerprinting of clinical isolates ; Lane(L)1=C91; L2= C93; L3= C114; L4=C104; L5=C117; L6= C108; L7= C111; L8; = C113; L9=C134;L10=C109; L11=C110; L12=C138; L13=C139; L14= C142; L15=C143; L16=C150; L17=C144; L28=C145; L19=C146; L20=C157; L21=C159; L22=C168; L23=C26; L24=30; L25=C165; L26=C167; L27= 169; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

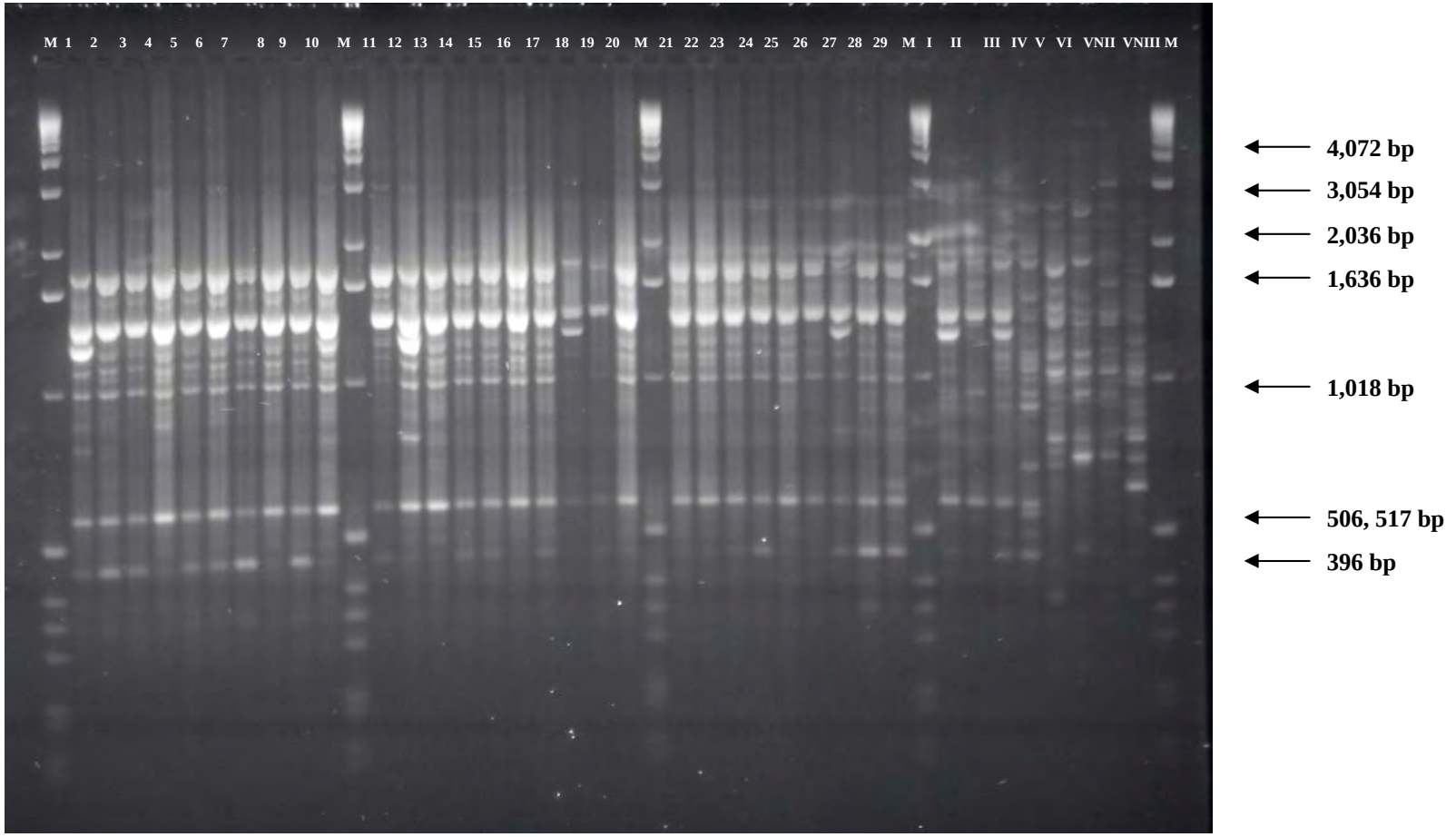


Figure15.5 M13-fingerprinting of clinical isolates ; Lane(L)1=C81; L2= C86; L3= C88; L4=C89; L5=C95; L6= C100; L7= C116; L8; = C118; L9=C149; L10=C151; L11=C153; L12=C154; L13=C156; L14= C161; L15=C164; L16=C166; L17=C171; L28=C173; L19=C175; L20=C176; L21=C178; L22=C179; L23=C192; L24=205; L25=C207; L26=C208; L27=C73; L28=C61; L29=C63; ; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

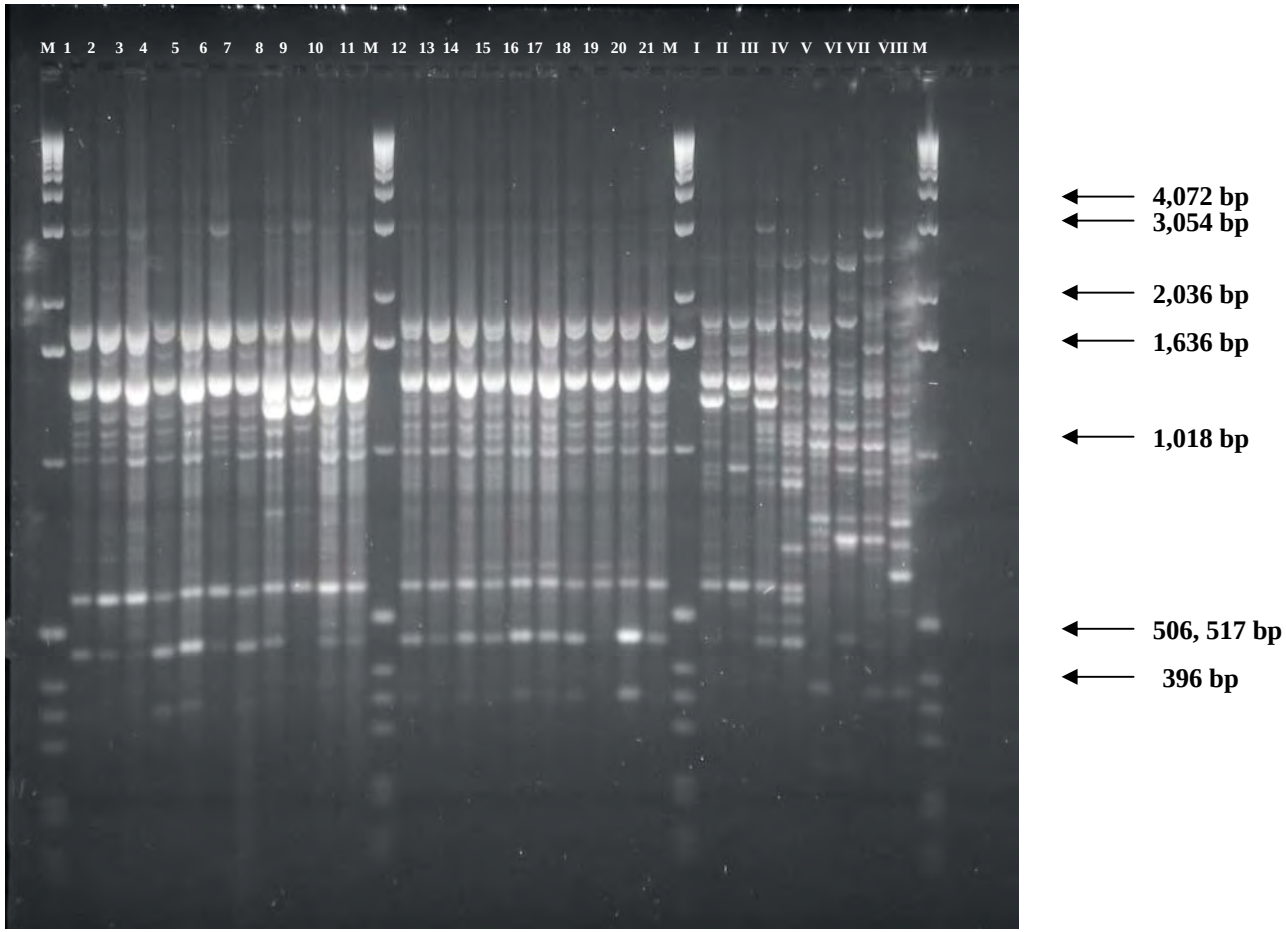


Figure15.6 M13-fingerprinting of clinical isolates ; Lane(L)1=C132; L2= C83; L3= C206; L4=C1; L5=C2; L6= C9; L7= C19; L8; = C11; L9=C16;L10=C27; L11=C35; L12=C36; L13=C37; L14= C38; L15=C39; L16=C48; L17=C50; L28=C51; L19=C52; L20=C60; L21=C194; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179;VI=WM178; VII=WM175; VIII=WM779; M=1 kb marke (Invitrogen, USA)

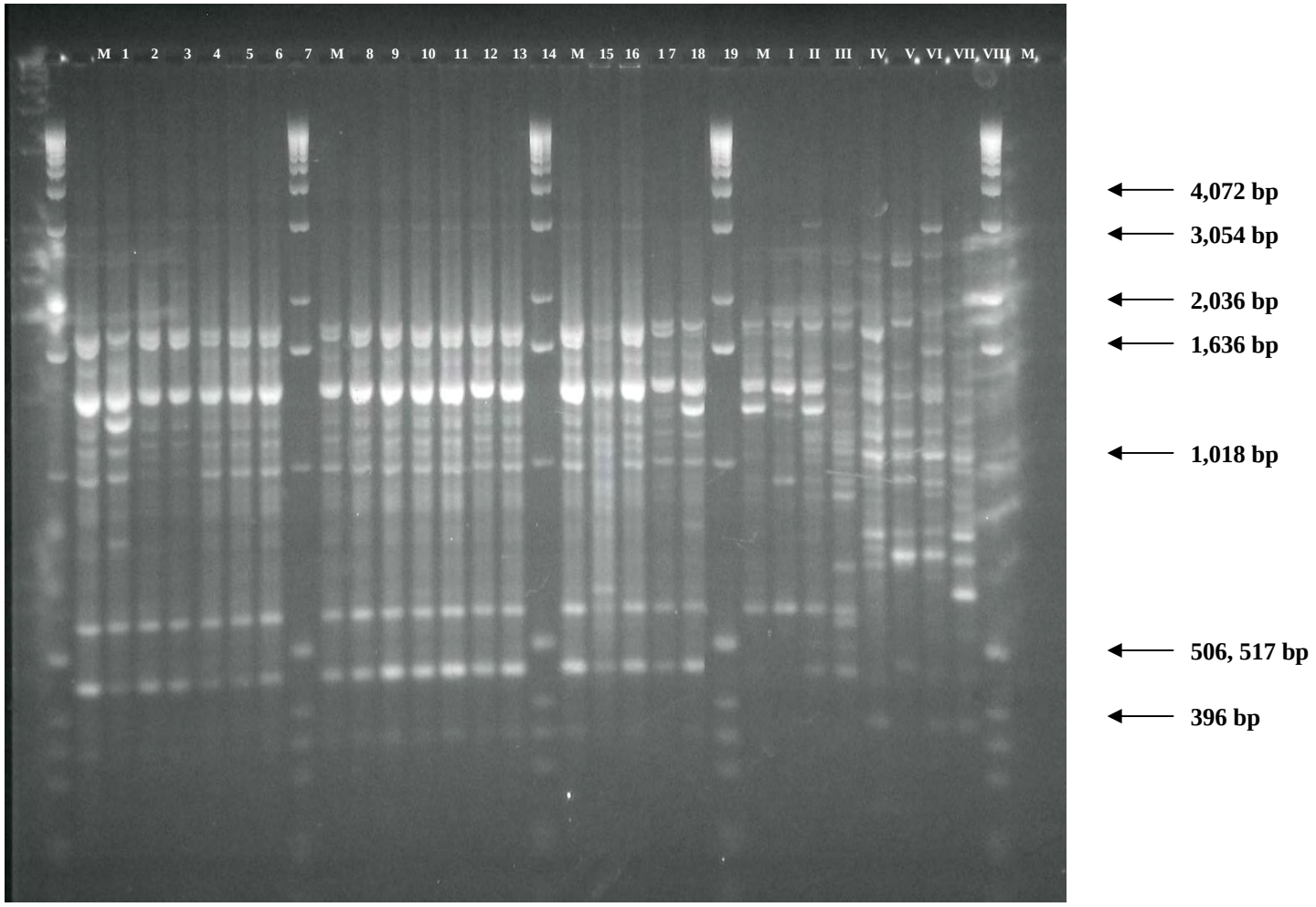


Figure15.7 M13-fingerprinting of clinical isolates; Lane(L)1=P2; L2= P3; L3= P4; L4=P6; L5=P7; L6= P8; L7= P9; L8; = P10; L9=P11;L10=P12; L11=P13; L12=P14; L13=P15; L14= P16; L15=P17; L16=P18; L17=P19; L28=P20; L19=P21; L20; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

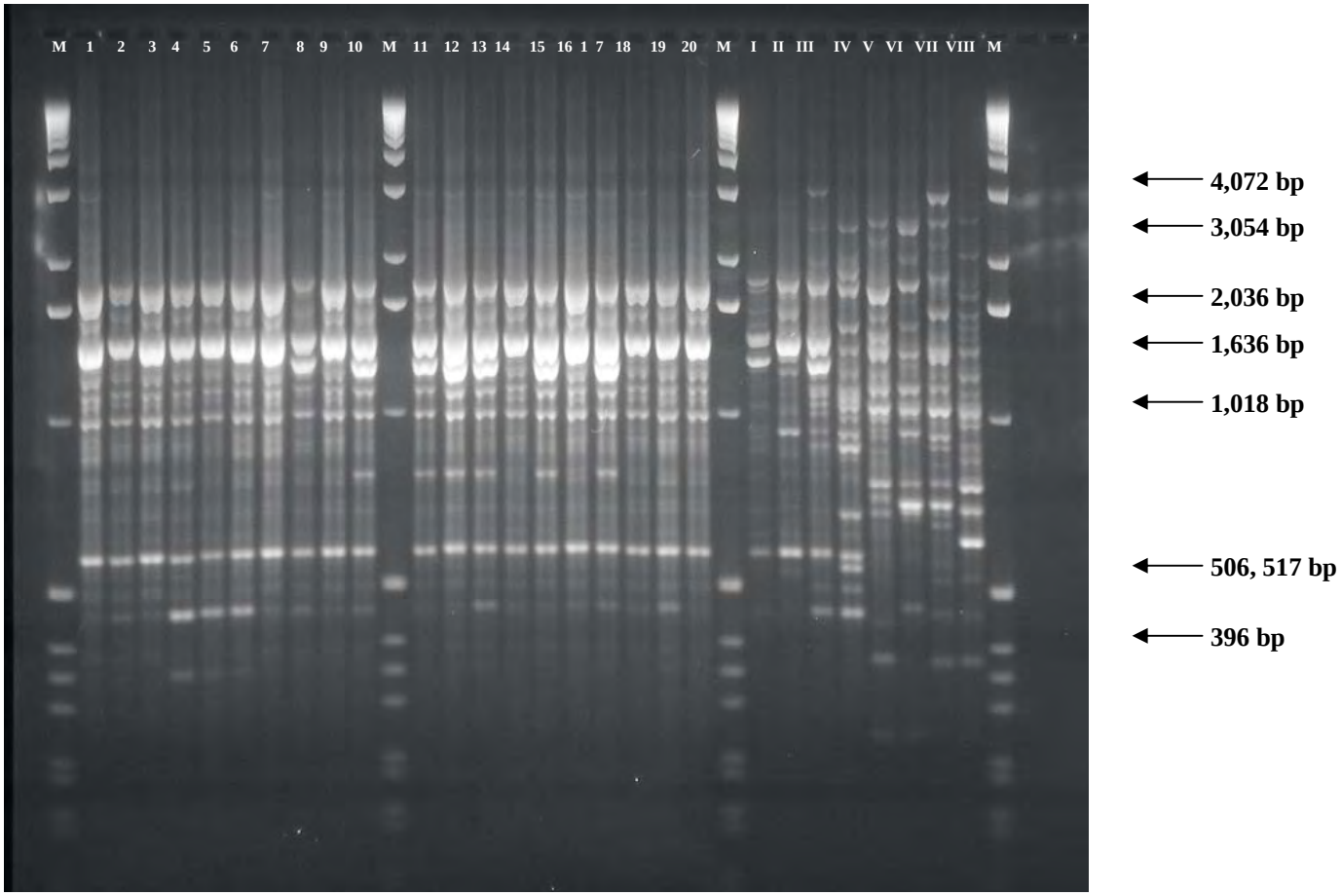


Figure15.8 M13-fingerprinting of clinical isolates ; Lane(L)1=CM1; L2= CM2; L3= CM3; L4=CM4; L5=CM5; L6= CM6; L7= CM7; L8; = CM8; L9=CM9;L10=CM10; L11=CM11; L12=CM12; L13=CM13; L14= CM14; L15=CM15; L16=CM16; L17=CM17; L28=CM18; L19=CM19; L20=CM20; L21=C82; L22=C180; L23=C181; L24=201; L25=C211; L26=C57; L27=59; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

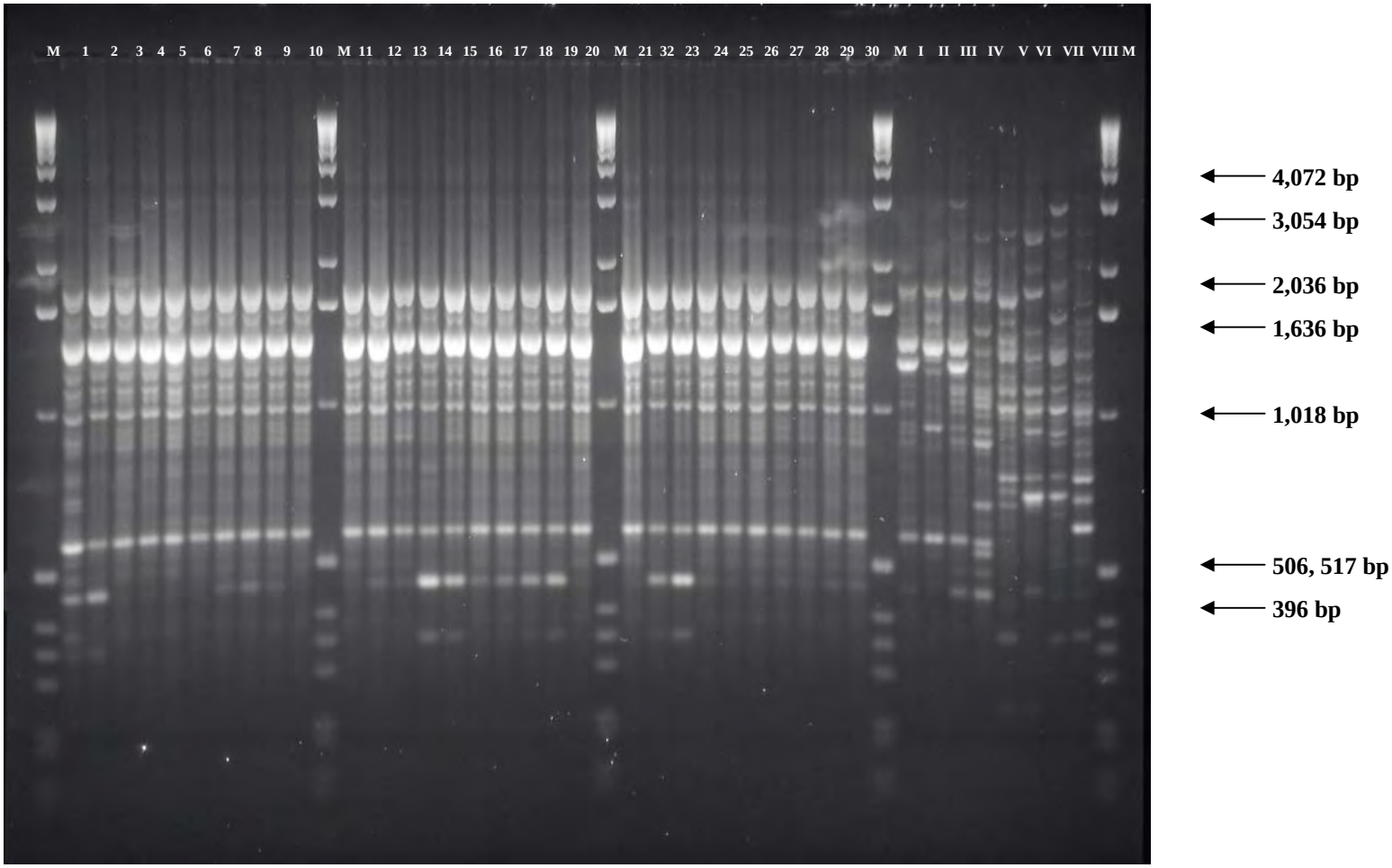


Figure15.9 M13-fingerprinting of clinical isolates ; Lane(L)1=S1; L2= S2; L3= S4; L4=S5; L5=S6; L6= S7; L7= S8; L8; = S9; L9=S10;L10=S11; L11=S12; L12=S13; L13=S14; L14= S15; L15=S16; L16=S18; L17= S19; L18=S20; L19=S22; L20=S23; L21=S24; L22=S24; L23=S26; L24=S27; L25=S28; L26=S29; L27=S30; L28=S31; L29=33; L30=S34; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

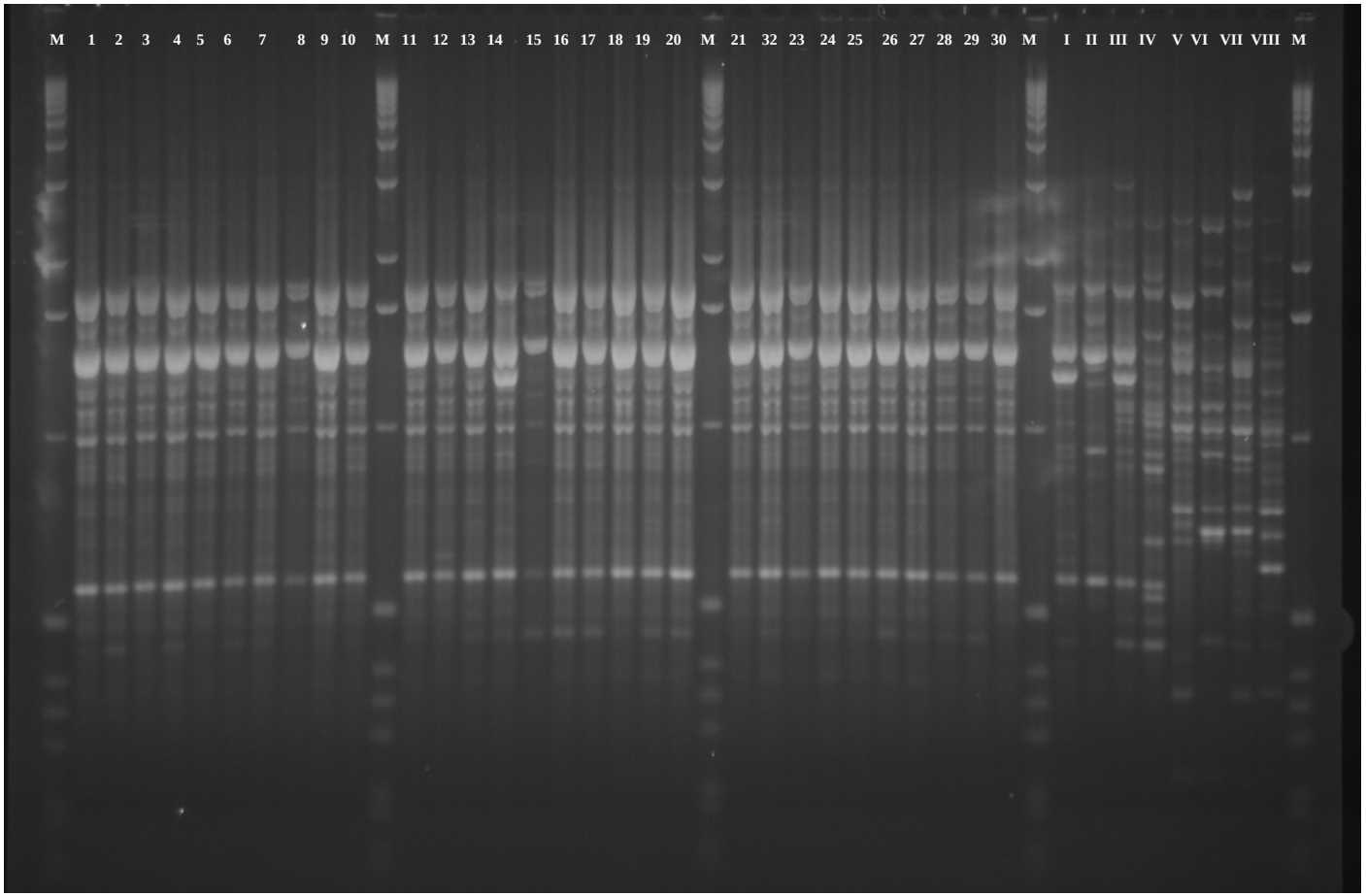


Figure15.10 M13-fingerprinting of clinical isolates ; Lane(L)1=S35; L2= S36; L3= S37; L4=S38; L5=S40; L6= S42; L7= S43; L8; = S44; L9=S45;L10=S46; L11=S47; L12=S48; L13=S49; L14= S50; L15=S51; L16=S54; L17=S55; L28=S56; L19=S57; L20=S59;L21=S60; L22=S61; L23=S62; L24=S63; L25=S64; L26=S65; L27=S66; L28=S68; L29=S69; L30=S70; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

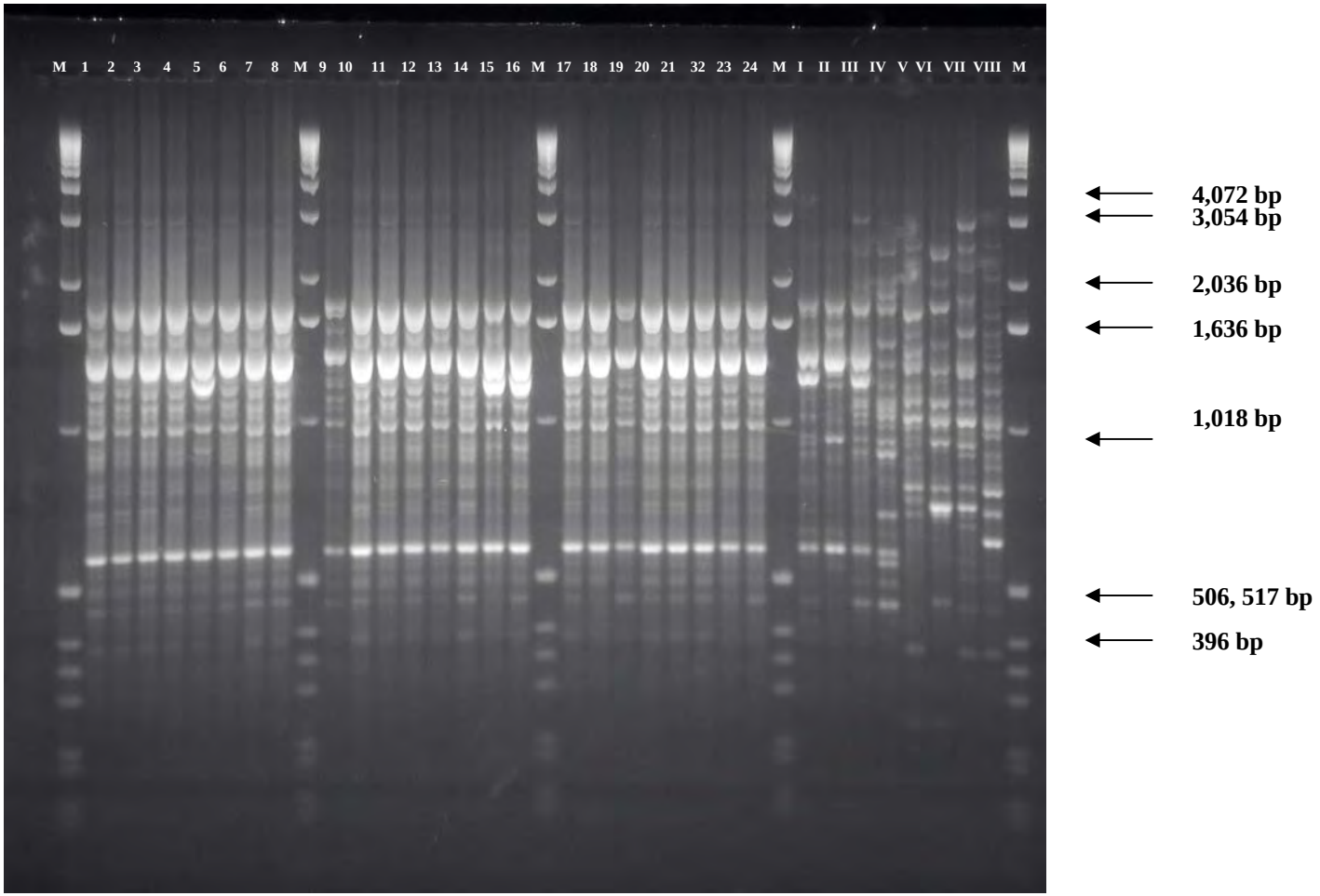


Figure15.11 M13-fingerprinting of clinical isolates ; Lane(L)1=S71; L2= S72; L3= S73; L4=S74; L5=S75; L6= S76; L7= S77; L8; = S78; L9=S79; L10=S80; L11=S81; L12=S82; L13=S83; L14= S84; L15=S85; L16=S86; L17=S87; L28=S88; L19=S89; L20=S90; L21=S91; L22=S92; L23=S93; L24=S94; L25=S95; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen USA)

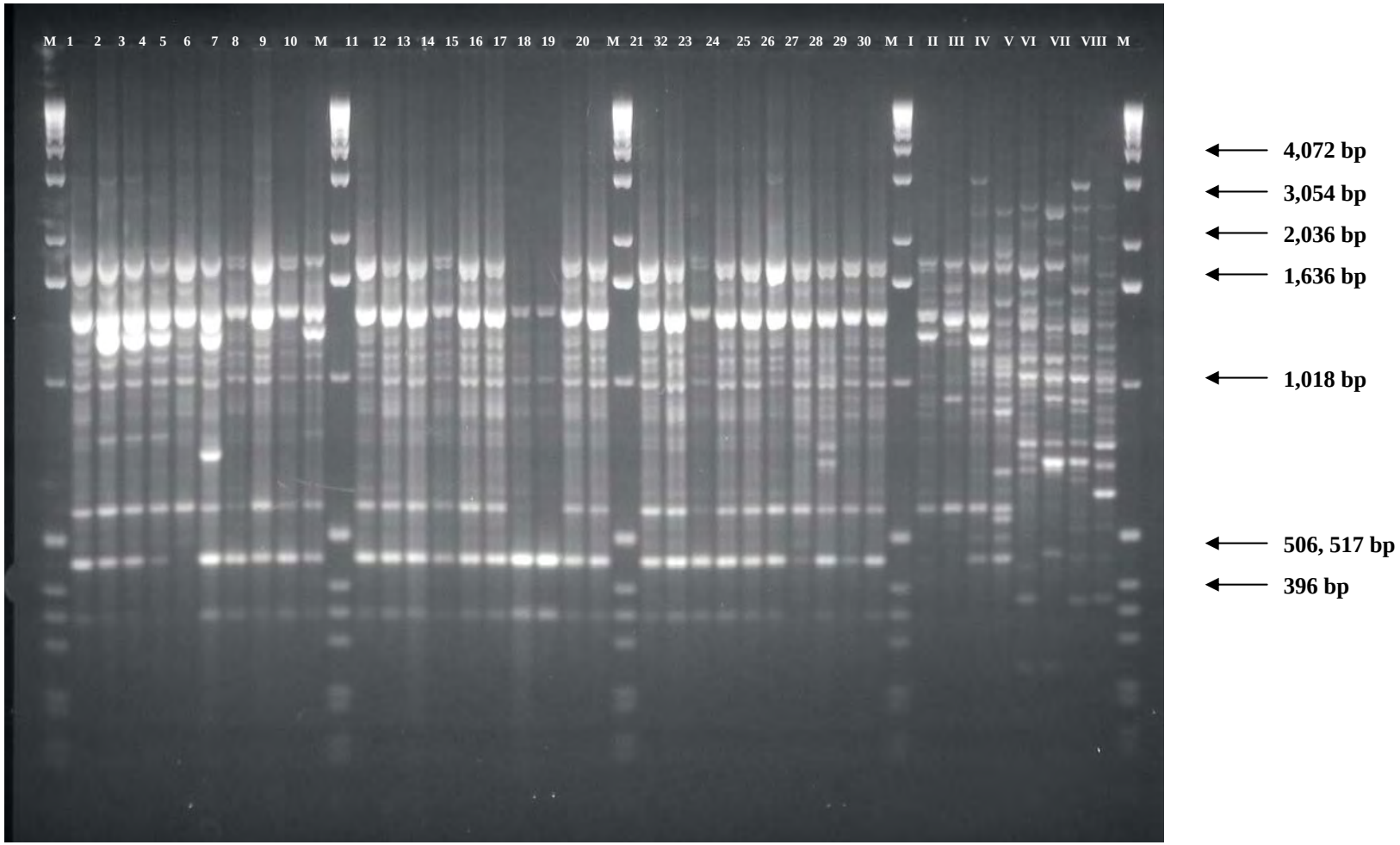


Figure15.12 M13-fingerprinting of clinical isolates ; Lane(L)1=B1; L2= B2; L3= B3; L4=B5; L5=B6; L6= B7; L7= B9; L8; =B10; L9=B11;L10=B12; L11=B13; L12=B14; L13=B15; L14= B16; L15=B17; L16=B18; L17=B19; L28=B20; L19=B21; L20=B22; L21=B23; L22=B25; L23=B26; L24=B27; L25=B28; L26=B29; L27=B30; L28= B31; L29=B32; L30=B33; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

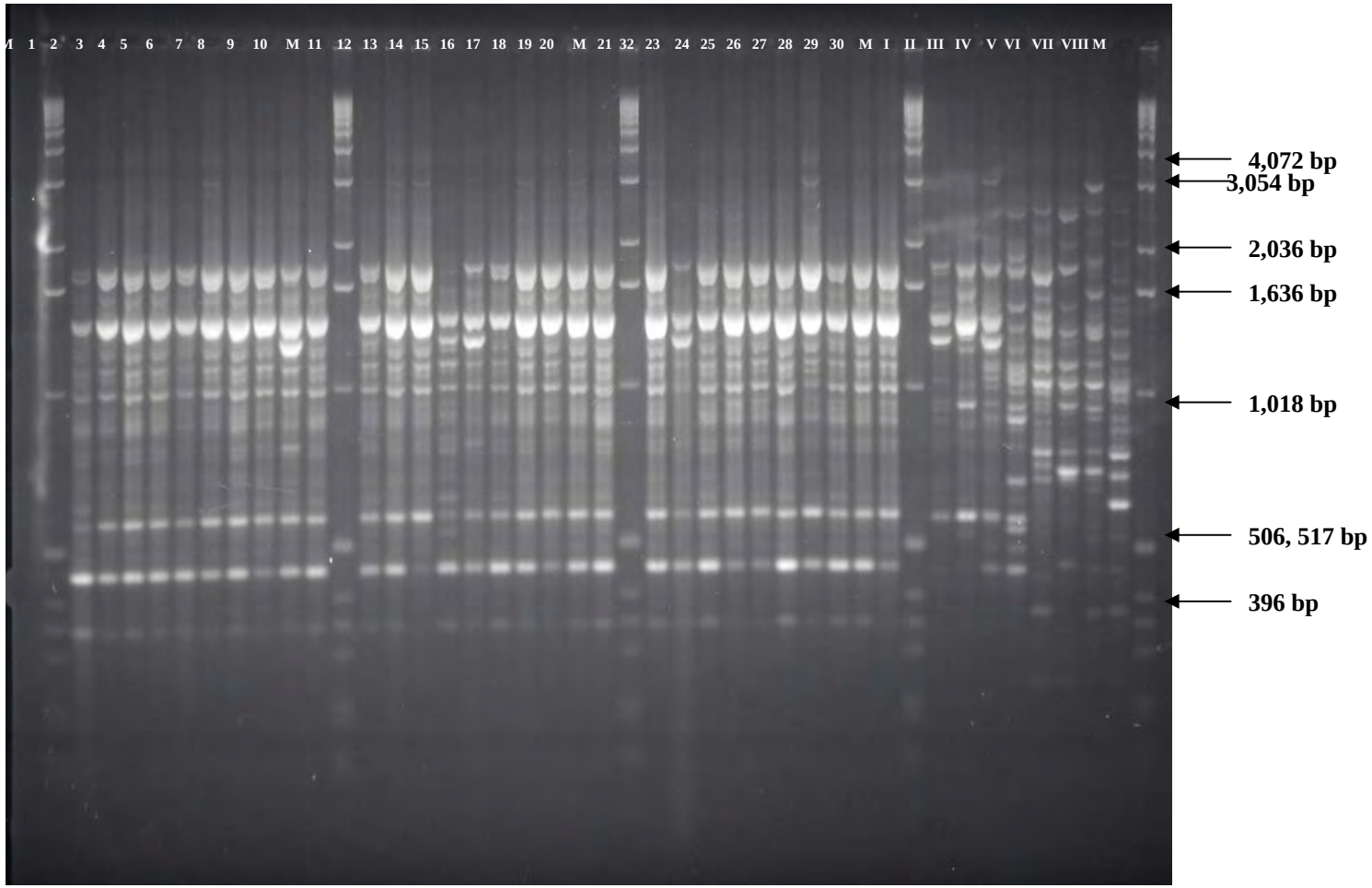


Figure15.13 M13-fingerprinting of clinical isolates; Lane(L)1=B34; L2= B35; L3= B36; L4=B37; L5=B38; L6=B39; L7= B40; L8; = B41; L9=B42; L10=B43; L11=B44; L12=B45; L13=B46; L14= B47; L15=B48; L16=B49; L17=B50; L28=B53; L19=B55; L20=B56;L21=B57; L22=B58; L23=B59; L24=B61; L25=B62; L26=B63; L27=B64; L28=B65; L29=B66; L30=B67; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

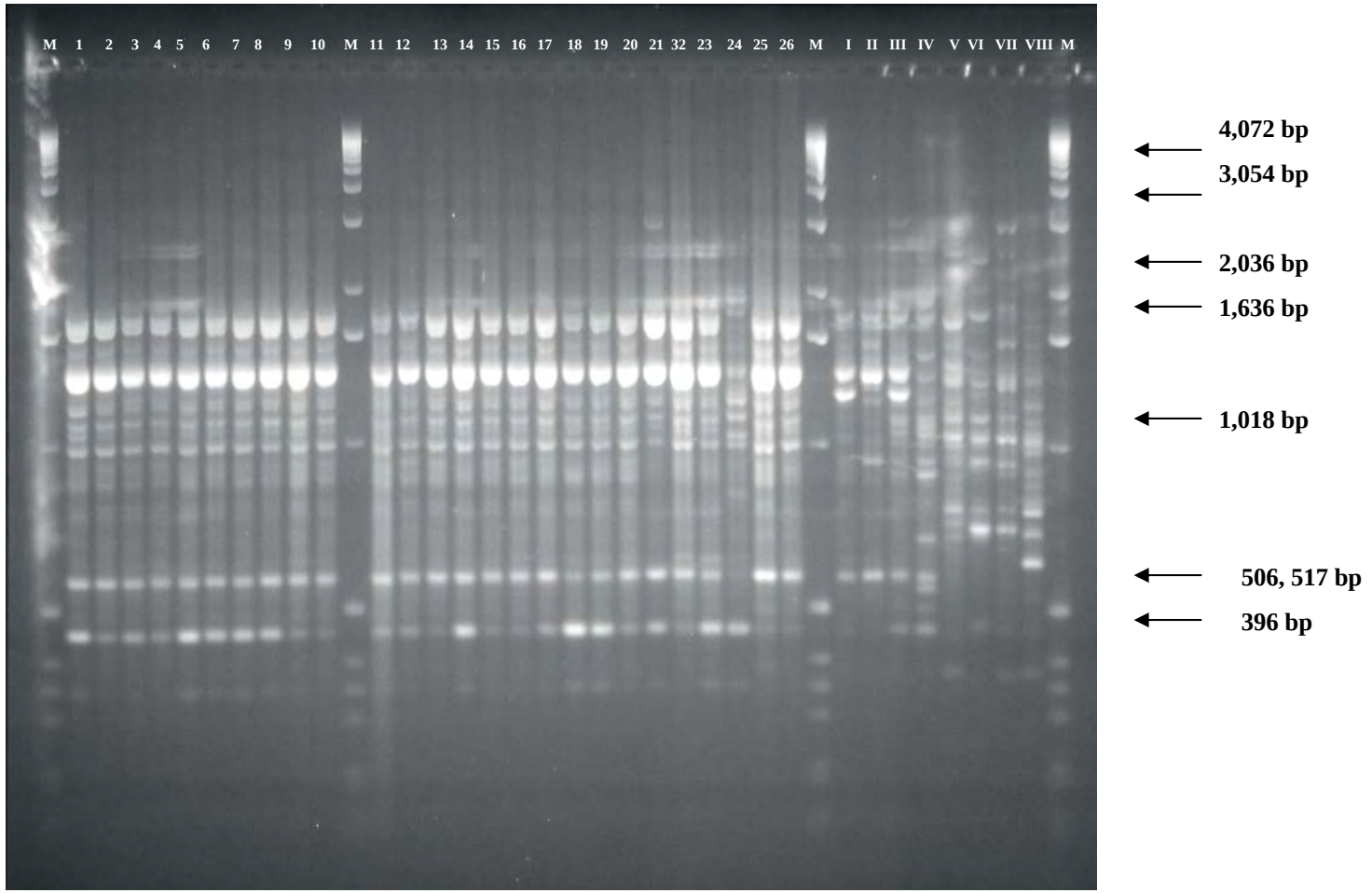


Figure15.14 M13-fingerprinting of clinical isolates ; Lane(L)1=B68; L2= B69; L3= B70; L4=B71; L5=B72; L6= B73; L7= B74; L8; = B75; L9=B76;L10=B77; L11=B78; L12=B79; L13=B80; L14= B81; L15=B82; L16=B83; L17=B84; L28=B85; L19=B86; L20=B87; L21=B88; L22=B89; L23=B90; L24=B91; L25=B92; L26=B95; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

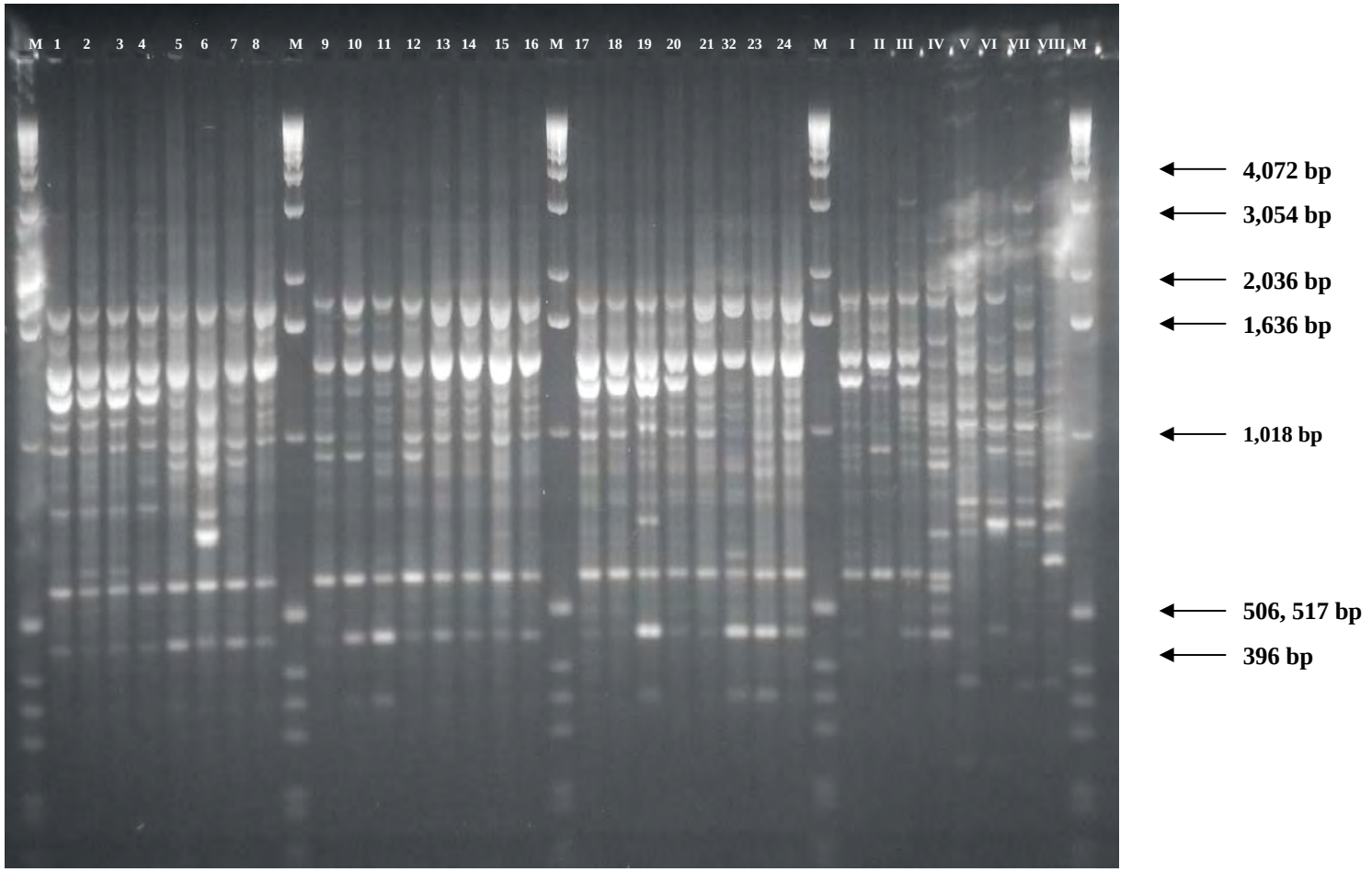


Figure16.1 M13-fingerprinting of veterinary isolates ; Lane(L)1=A1; L2= A2; L3= A3; L4=A4; L5=A5; L6= A6; L7= A7; L8; = A8; L9=A9;L10=A10; L11=A11; L12=A12; L13=A13; L14= A14; L15=A15; L16=A16; L17=A17; L28=A18; L19=A19; L20=A20; L21=A21; L22=A22; L23=A23; L24=A24; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

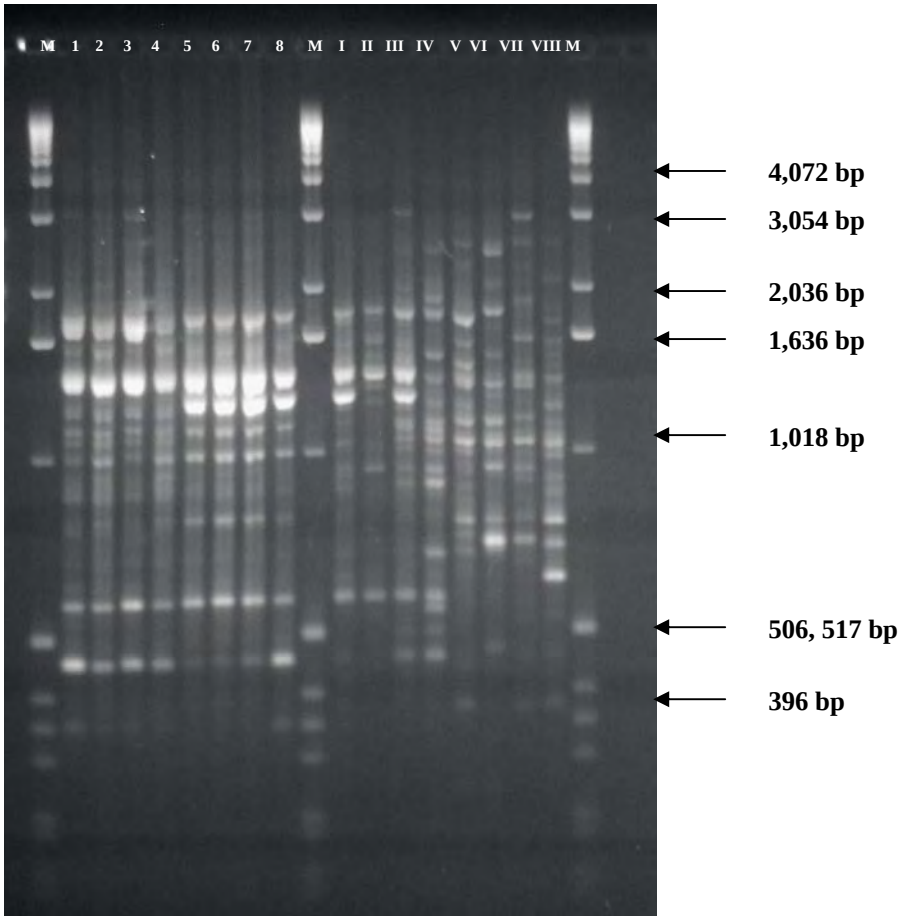


Figure16.2 M13-fingerprinting of veterinary isolate ; Lane(L)1=A25; L2= A26; L3= A27; L4=A28; L5=A29; L6= A30; L7= A31; L8; = A32; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

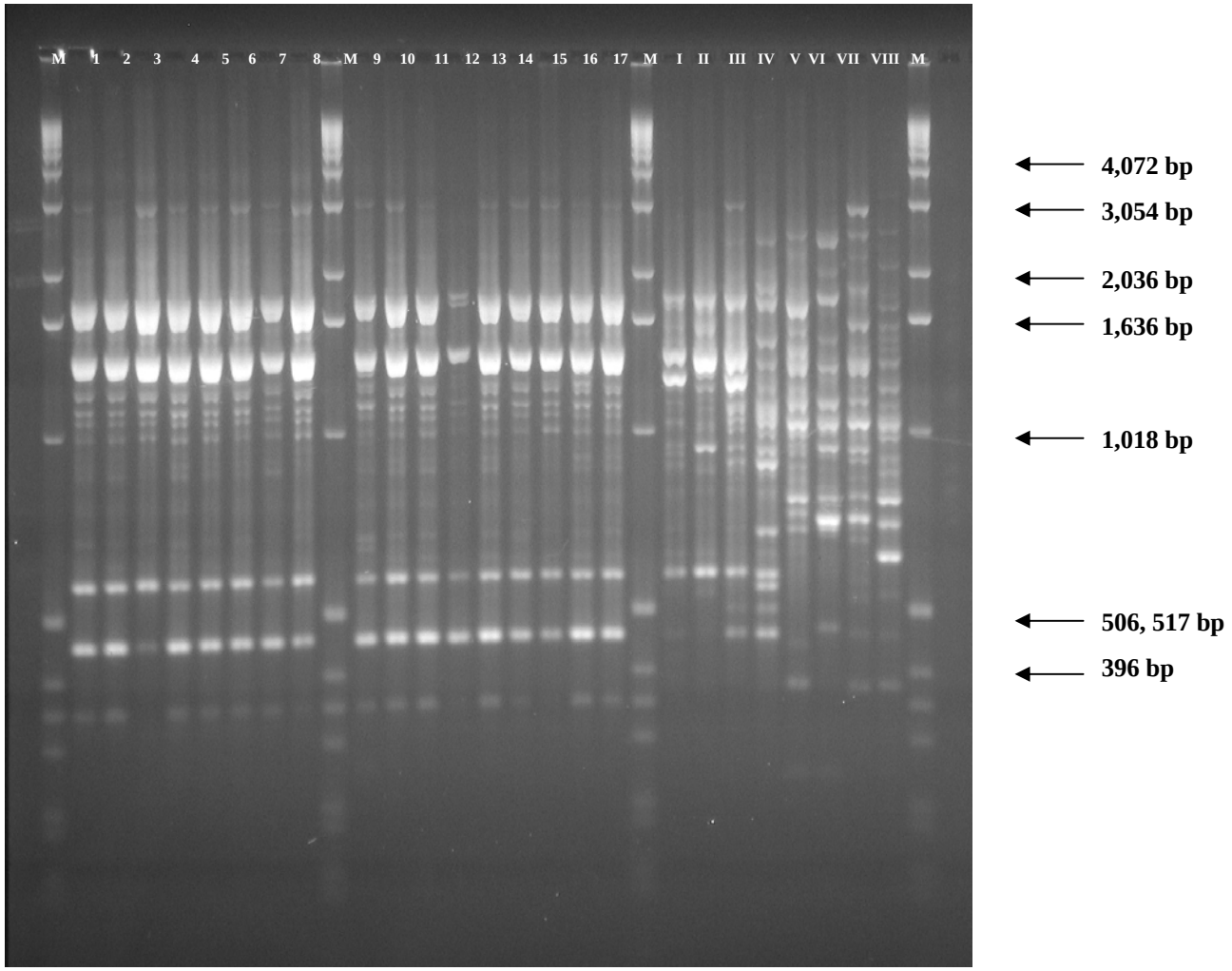


Figure17.1 M13-fingerprinting of environmental isolates ; Lane(L)1=T1; L2= T2; L3= T3; L4=T4; L5=T5; L6= T6; L7= T8; L8; = T9; L9=T12; L10=T13; L11=T14; L12=T15; L13=T16; L14=T18; L15=T17; L16=T19; L17=T20; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

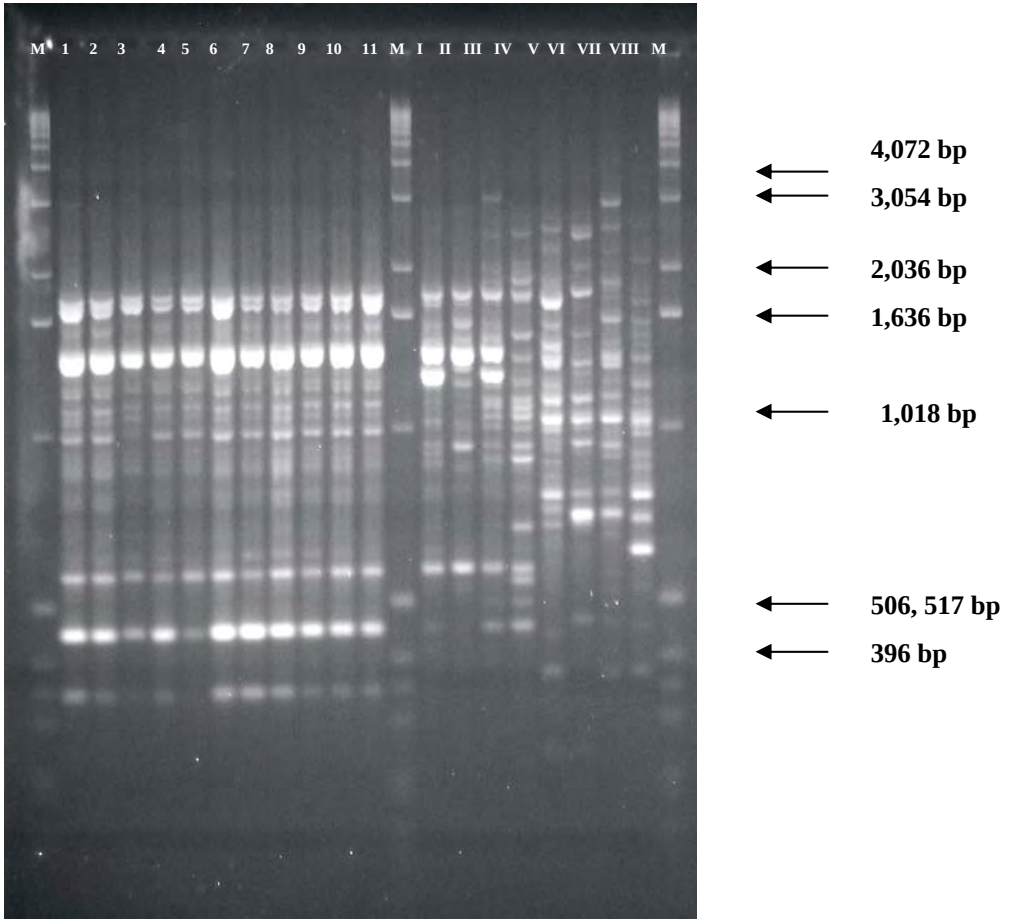


Figure17.2 M13-fingerprinting of environmental isolates; Lane (L) 1=T21; L2= T22; L3= T23; L4=T24; L5=T25; L6= T26; L7= T27; L8; =T28; L9=T30; L10=T31; L11=T32;; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179;VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

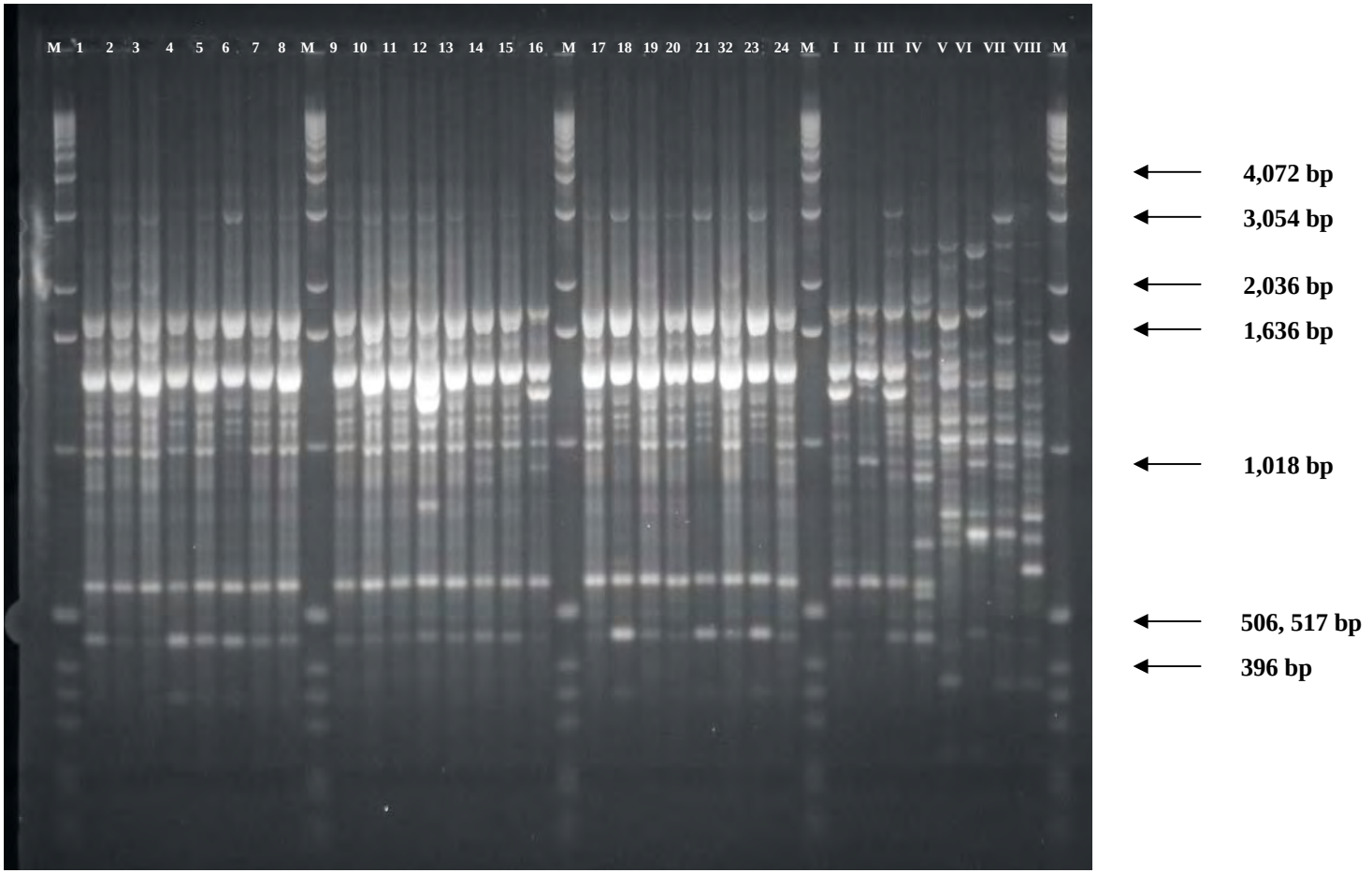


Figure17.3 M13-fingerprinting of environmental isolates ; Lane(L)1=E1; L2= E2; L3= E3; L4=E4; L5=E5; L6= E6; L7= E7; L8; = E8; L9=E9 ; L10=E45; L11=E46; L12=E38; L13=E39; L14= E40; L15=E41; L16=E42; L17=E43; L28=E44; L19=E47; L20=E48; L21=E49; L22=E50; L23=E51; L24=E10; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

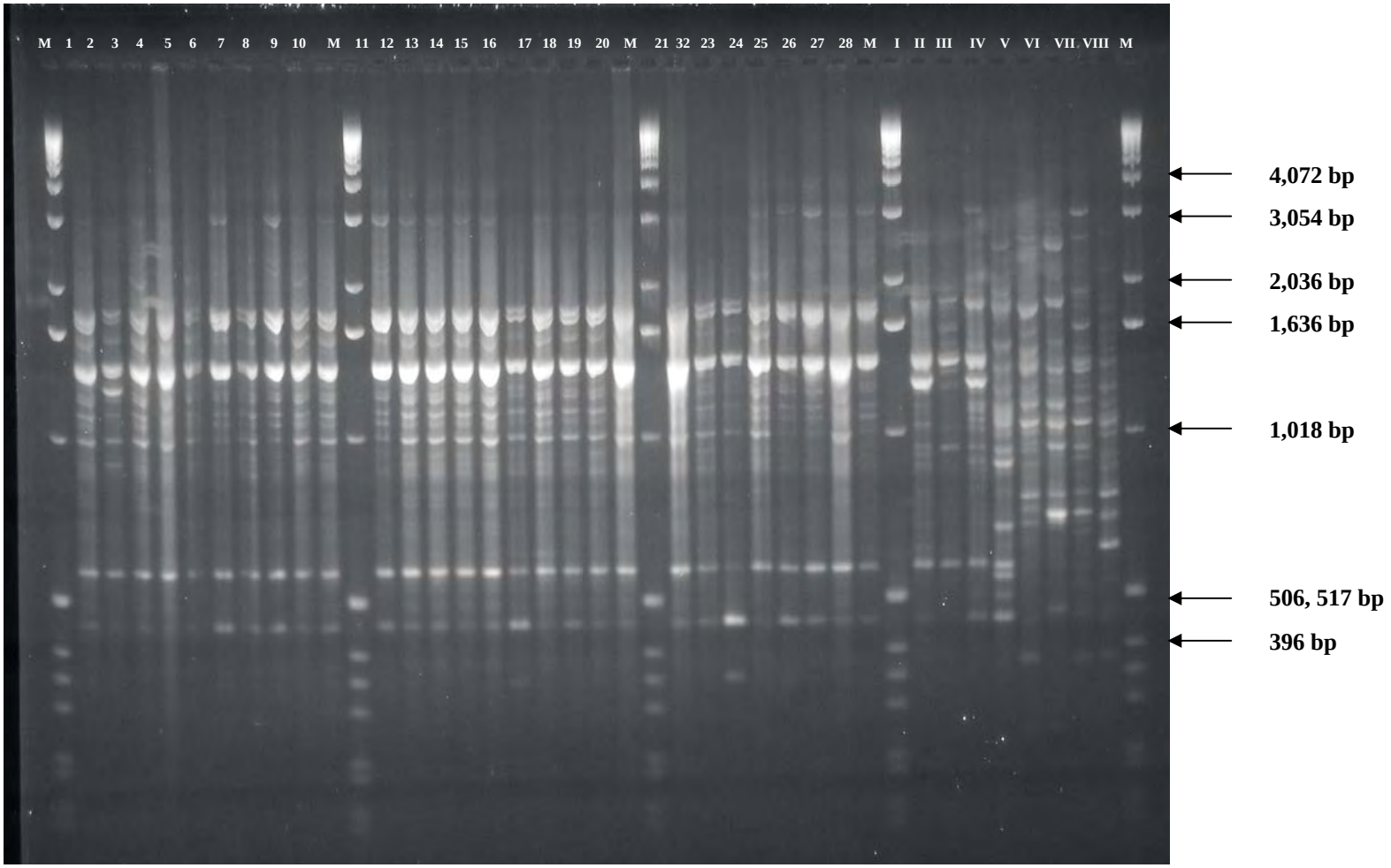


Figure17.4 M13-fingerprinting of environmetnal isolates ; Lane(L)1=E11; L2= E12; L3= E13; L4=E14; L5=E15; L6= E16; L7= E17; L8; = E18; L9=E19;L10=E20; L11=E21; L12=E22; L13=E23; L14= E24; L15=E25; L16=E26; L17=E27; L28=E29; L19=E30; L20=E31; L21=E32; L22=E33; L23=E34; L24=E35; L25=E36; L26=E37; L27=E52; E28= E53; I=WM148; II=WM626; III=WM628; IV=WM629; V=WM179; VI=WM178; VII=WM175; VIII=WM779; M=1 kb marker (Invitrogen, USA)

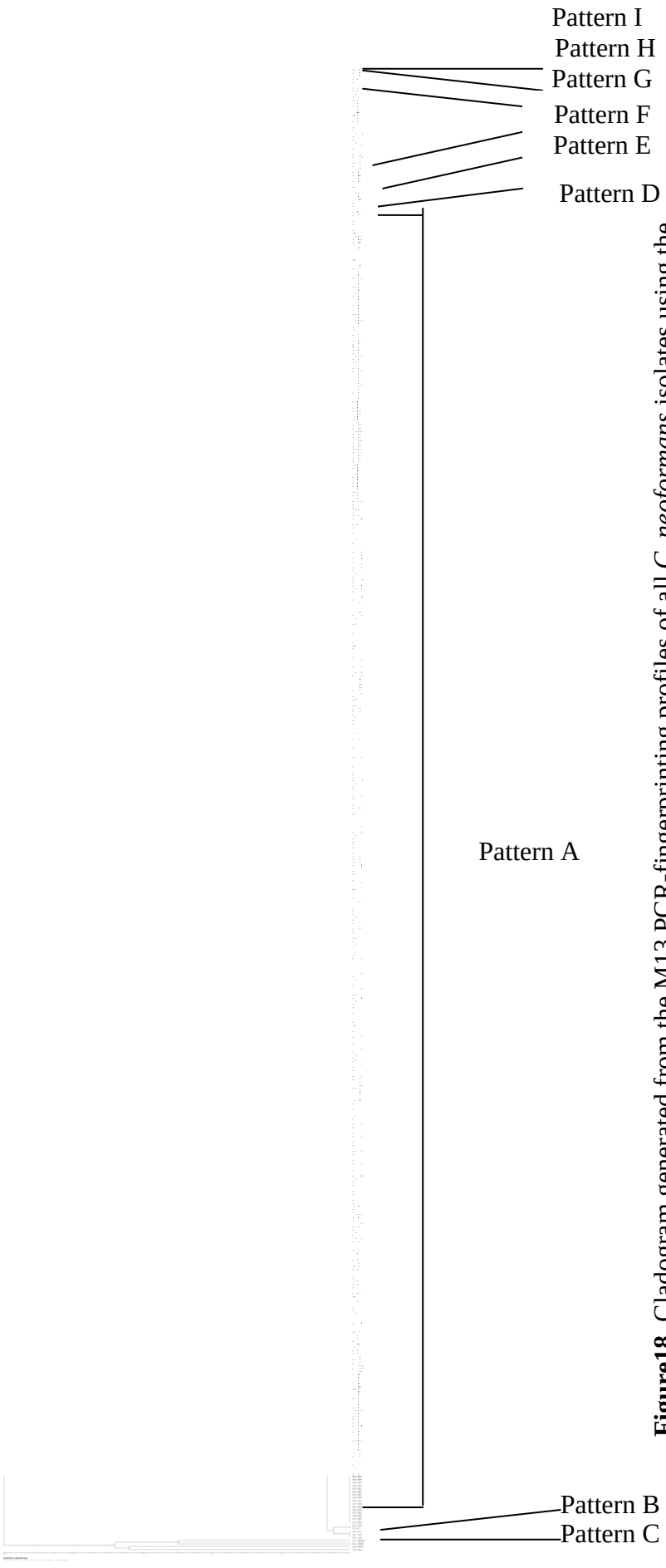


Figure18. Cladogram generated from the M13 PCR-fingerprinting profiles of all *C. neoformans* isolates using the program BioloMICS

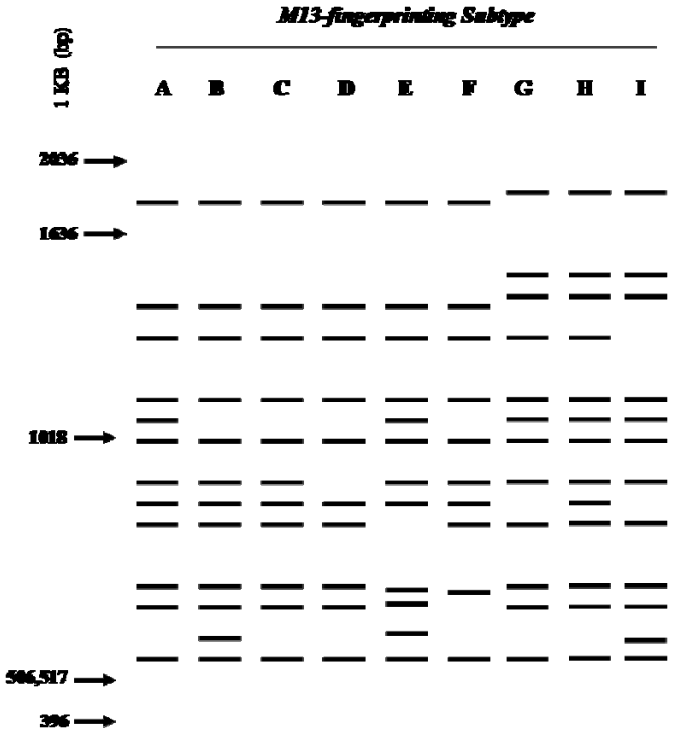
There were 9 M13-subgroup patterns observed among *C. grubii* Thai isolates and each pattern was named as A, B, C, D, E, F, G, H and I. Among the 9 patterns, the M13-fingerprinting pattern A was the most common since 425 out of 481 (88.35%) isolates showed this fingerprinting pattern. The 8 minor patterns were found in the rest of the isolates; 32 isolates gave pattern F; 11 isolates gave pattern D; 5 isolates gave pattern G; 3 and 2 isolates gave the C and B pattern, respectively. Only one isolate showed in G, H and I pattern. The band sizes and total isolate number of each pattern were showed in figure 18.

3.1 M13-fingerprinting patterns were a variety specific

Similar to the previous study, the M13-fingerprinting pattern was molecular type (serotype) specific. In this study out of 9 patterns, 6 patterns (A to F) were specific for only *C. grubii* molecular type VNI while 3 patterns, G, H and I, were specific for *C. grubii* molecular type VNII.

Among the molecular type VNI specific pattern, pattern A and pattern F can be found in all three sources of the isolates while pattern B can be found in (the) clinical and veterinary isolates. Moreover, pattern C and E can be observed in only one veterinary and one clinical source, respectively (Table 7). However, the association of the isolate(s) source with the M13-pattern cannot be observed in this study. The rare patterns such as C or E pattern were found only in a few isolates. Therefore, the pattern might be observed in these sources when the greater numbers of isolates are typed. Pattern G, H and I were found only in veterinary isolates, which were of molecular type VNII (*URA5*-RFLP) isolate.

Hence the results concordance with the previous studies that M13-fingerprinting show the molecular type specific pattern.



Subgroups (Pattern)	*Molecular weights of the PCR-fingerprinting bands*	*No. of PCR fingerprinting band*	*Number of strains per subgroup*
A	*601.45/ 806.85/ 841.5/ 908.14/ 936.95/ 965.9/ 1017.68/ 1095.16/ 1154.5/ 1250.39/ 1385.73/ 1722.99*	*12*	*425*
B	*601.45/ 765/ 806.85/ 841.5/ 908.14/ 936.95/ 965.9/ 1017.68/ 1154.5/ 1250.39/ 1385.73/ 1722.99*	*12*	*2*
C	*601.45/ 806.85/ 841.5/ 908.14/ 936.95/ 965.9/ 1017.68/ 1154.5/ 1250.39/ 1385.73/ 1722.99*	*11*	*3*
D	*601.45/ 806.85/ 841.5/ 908.14/ 936.95/ 1017.68/ 1154.5/ 1250.39/ 1385.73/ 1722.99*	*10*	*11*
E	*601.45/767.06/ 878.89/ 907.7/ 936.95/ 965.9, 1017.68/ 1095.16/ 1154.5/ 1250.39/ 1385.73/ 1722.99*	*12*	*1*
F	*601.45/ 836.1/ 908.14/ 936.95/ 965.9/1017.68/ 1154.5/ 1250.39/ 1385.73/ 1722.99*	*10*	*32*
G	*601.45/ 806.95/ 841.6/ 908.24/ 965.49/ 1017.78/ 1095.26/ 1154.6/ 1250.49/ 1351/ 1375/ 1723.09*	*12*	*5*
H	*601.45/ 806.85/ 841.5/ 908.14/ 936.9/ 965.29/ 1017.58/ 1095.06/ 1154.4/ 1250.29/ 1351/ 1375/ 1722.89*	*13*	*1*
I	*601.45/ 775/ 806.95/ 841.6/ 908.24/ 964.94/ 1017.78/ 1095.26/ 1154.05/ 1351/ 1375/ 1722.54*	*12*	*1*
	Totally		*481*

Figure19. M13-fingerprinting profile of *C. neoformans* var. *grubii* Thai isolates (A) Schematic diagram of M13-fingerpritning typing; (B) M13 fingerprinting band sizes details.

3.2 Association of M13-PCR fingerprinting pattern and the isolates source

From the M13 fingerprinting result, there is no association between the source of the isolates and the M13-fingerprinting patterns since almost all of the M13-patterns can be found in isolates from clinical, vetrinary and environmental sources (Table 7). Though the pattern E seemed to be clinical-specific, the fact that only one isolates were found undermined the proposal. In addition, the isolate from veterinary and environmental which were only 80 and 32 isolates might be unable to cover M13-fingerpritning pattern E in this study since among 361 clinical isolates, pattern E was found only one isolates (0.28%). Thus, further epidemiological survey is indispensable before drawing such conclusion. Table 7 showed the association M13-fingerprinting of *C. neoformans* var. *grubii* from each source.

Table7. The association between M13-fingerprinting and the isolates source.

Source	M13-fingerprinting pattern								
	VNI *URA5* RFLP						VNII *URA5*-RFLP		
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
C	335	1	-	9	1	23	-	-	-
E	77	-	-	2	-	1	-	-	-
V	13	1	3	-	-	8	5	1	1
Total	**425 (88.35%)**	**2 (0.4%)**	**3 (0.62%)**	**11 (2.23%)**	**1 (0.2%)**	**32 (6.65%)**	**5 (1.04%)**	**1 (0.2%)**	**1 (0.2%)**

C= clinical isolates

E=Environmental isolates

V= Veterinary isolates

3.3 The M13-fingerprinting of serial isolates

The different pattern of M13-fingerprinting among the serial *C. neoformans* var. *grubii* studied isolates can be observed. For the clinical isolates, there were three cases that showed the different M13 pattern, case MS, NP and PS.

In the MS case, 4 *C. grubii* isolates, three clinical isolates including C108, C111 and C113, were collected in January while isolate C134 was collected in February 2003. The isolate C108 was collected from serum, C111 and C134 were collected from CSF and, unfortunately, there was no specimen recording for C113

In the NP case, the clinical isolates C121 and C140 showed pattern differences since C121 gave pattern A and C140 gave pattern F. Both of the isolates were derived from CSF in the two different occasions; C121 was the first to collect in January, 2003 while C140 was collected later in February.

Finally, in the PS case, all of three clinical *C. grubii* isolates were isolated in March, 2003. The isolates C65 and C66 showed pattern F while the isolate C70 showed pattern A. However, the origin of the isolates C65 and C66 were not recorded whereas C70 was isolated from CSF.

Similarly to the clinical isolates, the environmental isolates also found the differences in patterns from the dropping samples that collected in the same time and same area. For Bangplad district, there were two isolates of *C. grubii*, E42 and E43. The M13-pattern of E42 was pattern D while E43 isolates gave pattern A. The same result also discovered in *C. grubii* isolated from Sampantawong district, among the isolates collected in this area there was only E12 that showed the pattern D while the rest showed pattern A.

In addition, the veterinary isolates from the same infected cat case also exert the M13-pattern differentiation. Based on the morphological differences, mucoid and non-mucoid, 4 isolates were collected from each case. In case number 2, two subtype of *C. neoformans var. grubii* molecular type VNI (1 isolate) and VNII (3 isolates) was found and among three of VNII isolates the different patterns were detected when the isolates A5 and A7 gave pattern G while A6 gave the unique pattern I. The isolates from cat case number 3 also found the different pattern between the isolates when A11 showed pattern H whereas the isolates A9, A10, A12 gave the similar pattern, pattern G. And in case number 5, pattern B was observed from isolate A19 while pattern A17, A18 and A20 showed pattern C.

There is a possibility that the difference of M13-patterns caused by the mix subtype infection or the microevolution phenomenon.

4. Multilocus Sequence Typing (MLST) analysis

4.1 Phylogenetic Analysis

The sequences from 8 genes were obtained based on amplification from 9 pairs of primer. *CAP59* gene was sequenced by two different primers which recognized the different fragment of *CAP59* gene. The sequences were edited and assembled manually by Sequencher4.6 (Gene Code Corp., USA). DNA sequences were aligned by clustalW, and manually edited with Bioedit. The orthologous sequences from *C. grubii* WM148 and WM626 were included into the phylogeny as standard strains of *C. grubii.* In addition, *C. neoformans* serotype D molecular type VNIV standard strains and *C. gattii* serotype B and C molecular type VGI were also included and defined as an outgroup.

Maximum parsimony was conducted with PAUP with the following conditions; heuristic search with TBR branch swapping. Gaps were treated as missing character. Trees were obtained by stepwise addition of 50 random replicates. Bootstrap supports for branches were based on 500 replications with random taxon additions. The details demonstrated as below.

4.1.1 Actin gene (*ACT1*)

All actin gene sequences were 1,069 nucleotides in length of the analysis. Overall, the actin sequences were highly conserved among the 31 selected *C. grubii*. Sixteen variation positions were observed among the 1,069 nucleotides sequence length with 1.10 % sequence diversity.

Among the studied isolates, *C.neoformans* var. *grubii* molecular type VNI and VNII isolates showed differences in nucleotide base at 11 positions as shows in figure 20. Moreover, molecular type VNII revealed an adenosine (A) nucleotide insertion at position 34. The insertion of nucleotide cluster (CCCT) at position 996 to 970 can be observed in all VNII molecular type including a selected isolate of VNI molecular type, S87. Finally, 3 allele types (ATs) were assigned as show in table 8.

The phylogenetic tree of actin gene was generated to estimate the relationships of isolates within the 31 selected strains. 1160 bases are constant and 10 bases are parsimony informative.. The most parsimonious tree was generated with a length of 93 steps. The tree had a consistency index (CI) of 98.9, a retention index (RI) of 0.987, and a homoplasy index (RC) of 1.000.

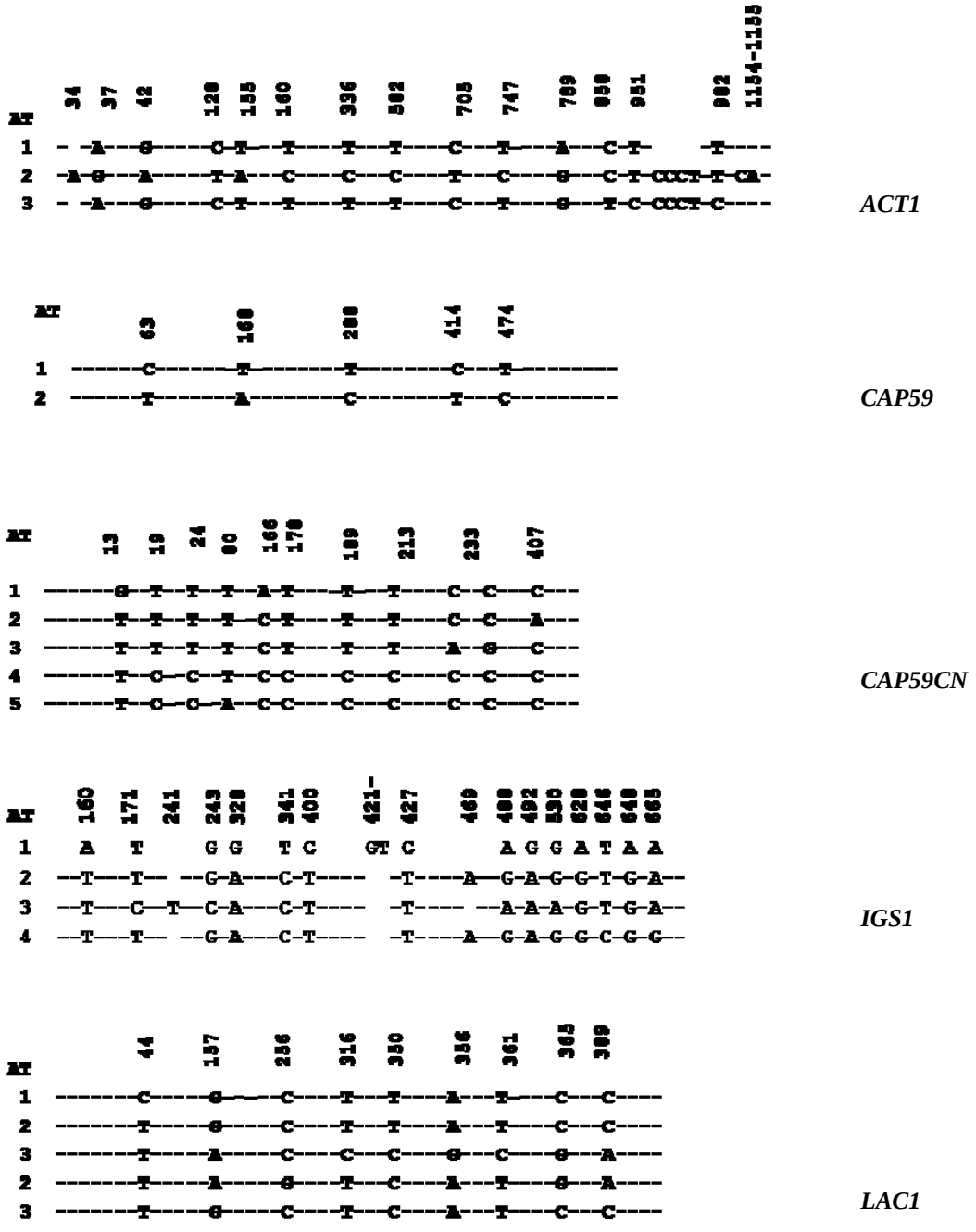


Figure20. Allele types (AT) and polymorphic nucleotide sites (numbering on each column) of nine gene fragments

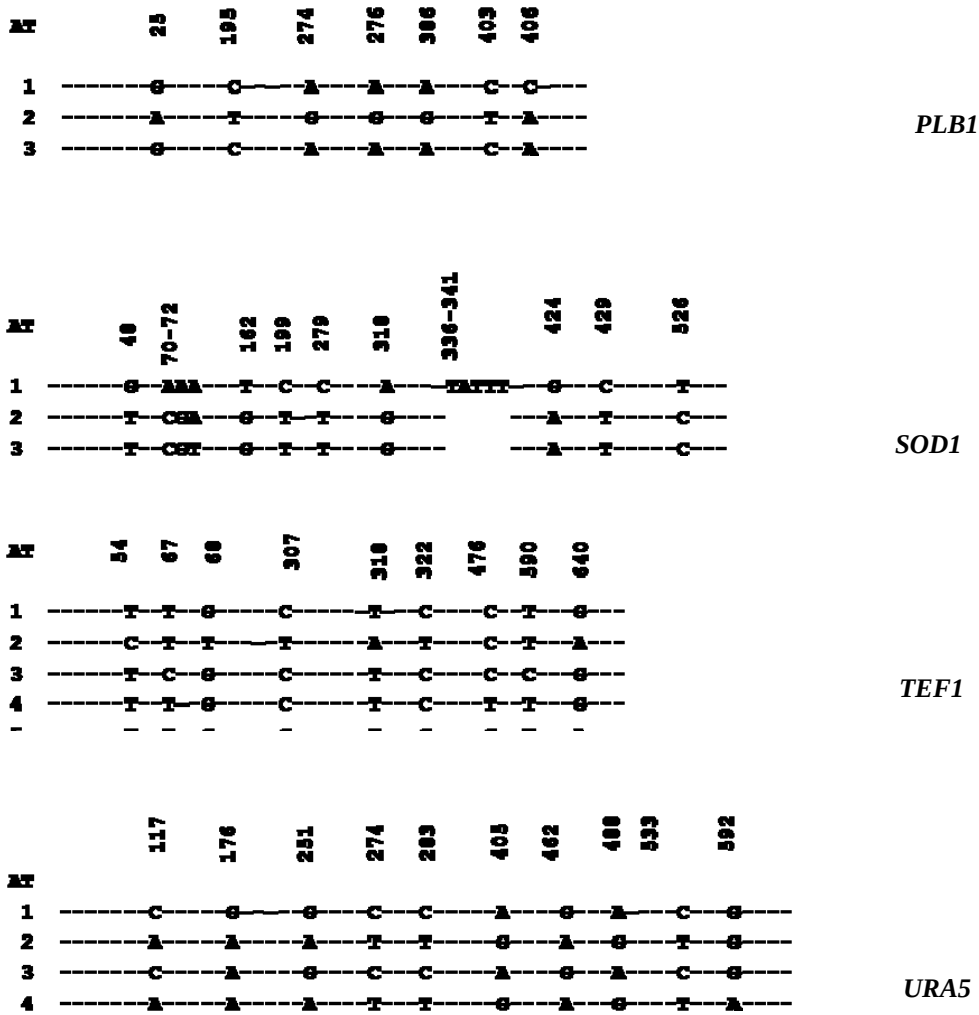


Figure20. (cont.) Allele types (AT) and polymorphic nucleotide sites (numbering on each column) of nine gene fragments

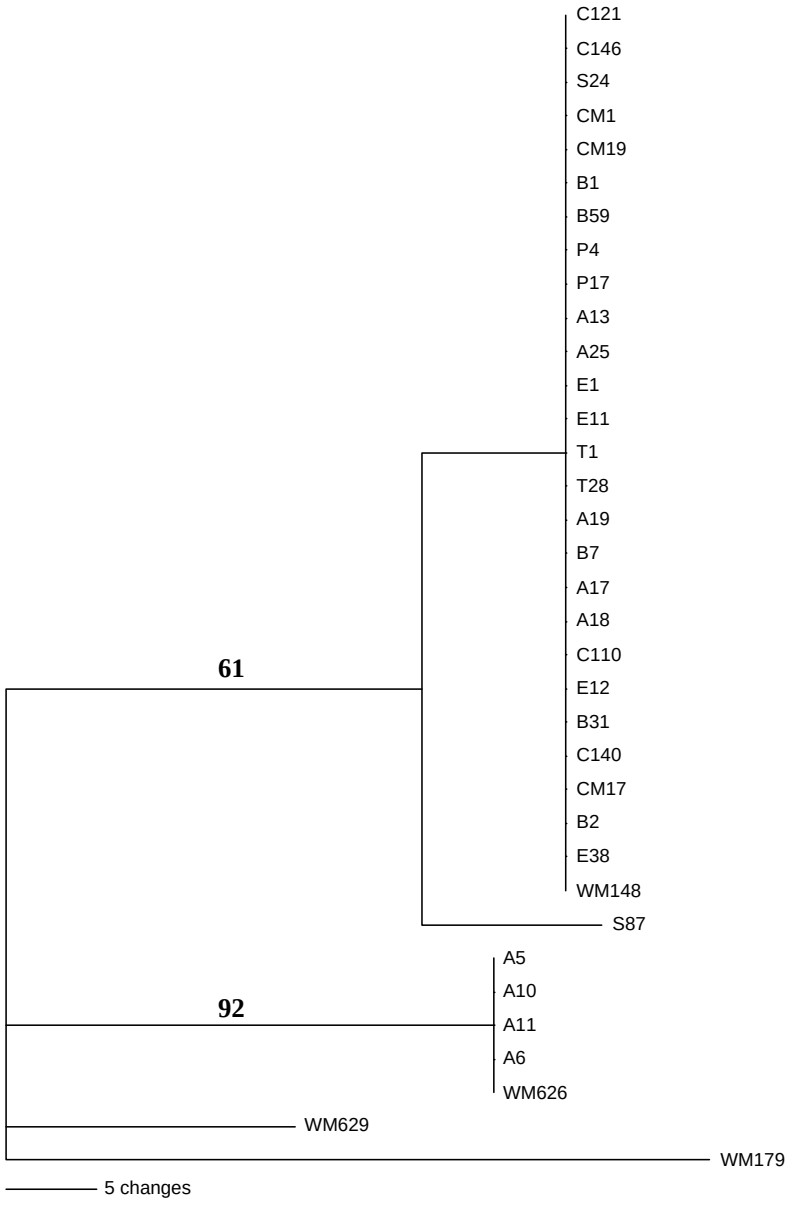


Figure21. Phylogenetic tree of *ACT1* sequence. Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

The 31 *C. grubii* isolates were divided into 2 major groups. The first group was a group of molecular type VNI which received 61% bootstrap support. The second group was a group of all 4 isolates of molecular types VNII and the bootstrap support was 92%. (Figure21)

4.1.2 Capsular associated protein (*CAP59*) gene

Two set of primers used to amplify *CAP59* gene was CAP59 were used. Results from each primer were described below.

For CAP59 primers, *CAP59* gene sequences were 566 nucleotides in length. Over all the *CAP59* sequences amplified by these primers were highly conserved. Only 5 positions were variable with 0.8 % sequence diversity. However, each nucleotide of the 5 position could differentiate between molecular type VNI and VNII including position 63 (‘A’ in VNI and ‘G’ in VNII) and position 168, 288, 414 and 467 (‘G’ in VNI and ‘A’ in VNII), as shown in Figure20. According to the result, CAP59 primer unable to distinguish the diversity among 31 selected isolates since it discriminated only the differences between molecular type VNI and VNII. This suggests the *C. grubii* Thai isolates are clonal.

The phylogenetic tree is also generated to estimate the relationships of isolates within the 31 selected strains, 561 bases were constant, and 5 bases were the parsimony informative. The most parsimonious tree was generated with a length of 42 steps. The tree had a consistency index (CI) of 1.000, a retention index (RI) of 1.000, and a homoplasy index (RC) of 1.000. The tree is shown in figure 22.

As describe above the selected isolated were separated to two groups, one was a *C. grubii* molecular type VNI and the remaining group was molecular type VNII supported by bootstrap value 95% and 86%, respectively. .

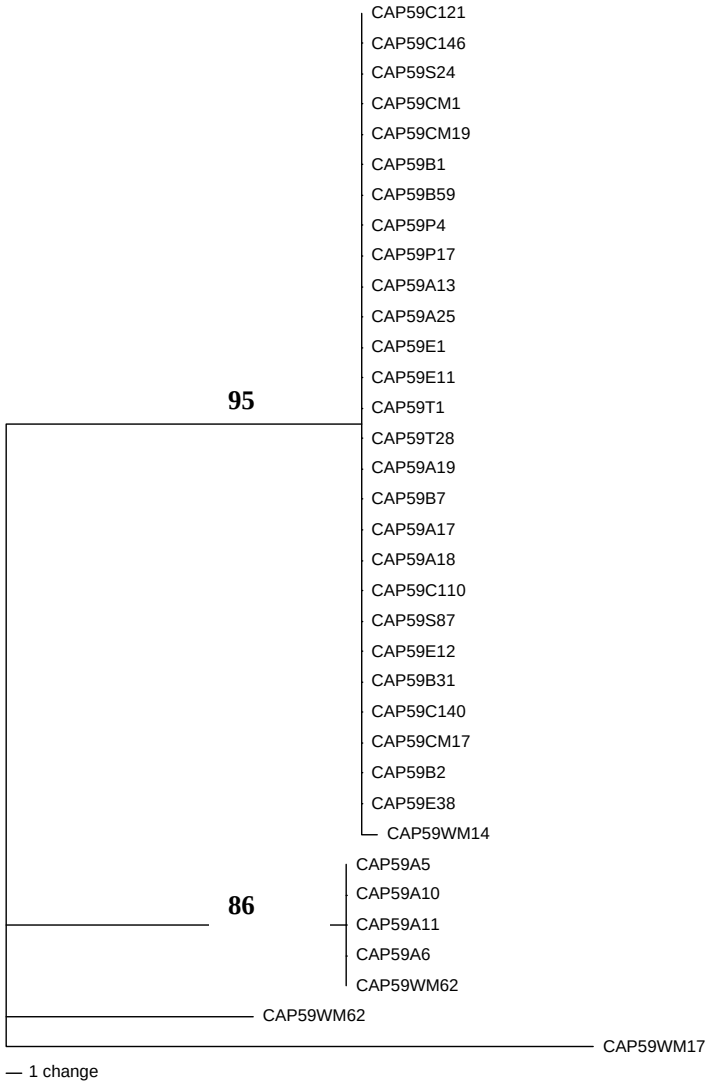


Figure22. Phylogenetic tree of *CAP59* sequence. Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

The nucleotide difference among studied isolates can be observed when *CAP59* gene was amplified by *CAP59CN* primer.

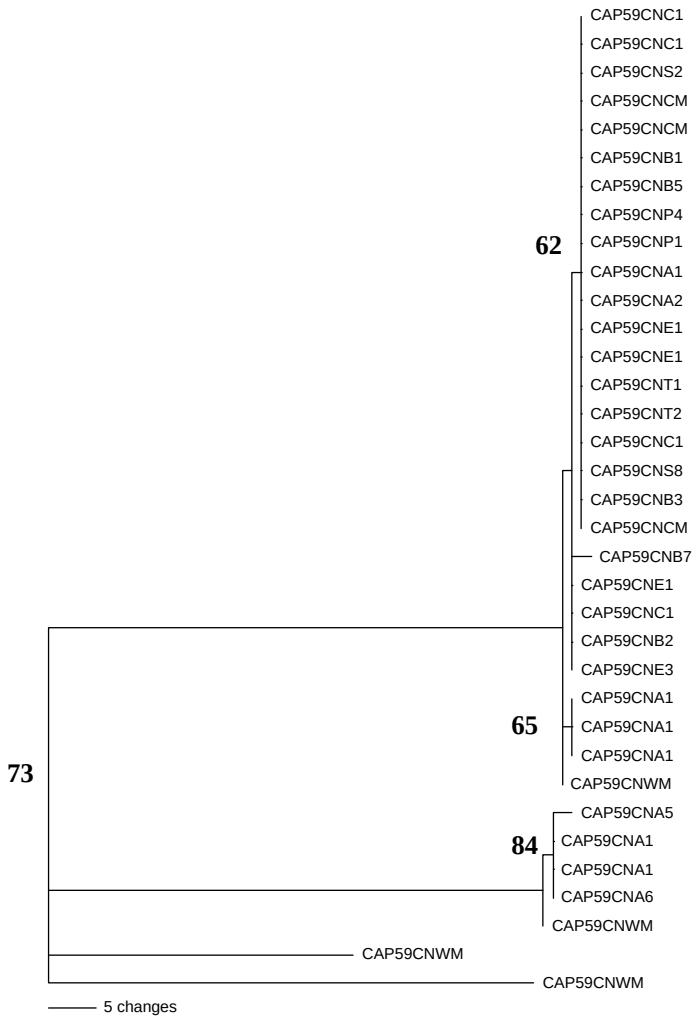


Figure23. Phylogenetic tree of *CAP59* sequence using *CAP59CN* primer**.** Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

Eleven variation positions were observed among the 443 nucleotides sequence of *CAP59* using *CAP59CN* primer, length with 2.26% sequence diversity. Molecular type VNI showed three allele type that AT 1 showed the variation at position 13 and 166 (“G’ and ‘A’). For AT2, the variation found in position 407 (‘A’) and AT 3 found the variation at position 233 ‘A’). The remaining two AT was observed in molecular type VNII. AT4 and AT5 showed the different base at position 80 (‘T’ for AT4 and ‘A’ for AT5). As shown in figure 20.

The phylogenetic tree is also generated to estimate the relationships of isolates within the 31 selected strains, 374 bases were constant, and 7 bases were the parsimony informative. The most parsimonious tree was generated with a length of 73 steps. The tree had a consistency index (CI) of 1.000, a retention index (RI) of 1.000, and a homoplasy index (RC) of 1.000. The tree is shown in Figure 23.

The molecular type VNI and VNII was clustered separately according to the sequences. Among molecular type VNI isolates two clusters were observed support by bootstrap value over 60%

4.1.3 Laccase (*LAC1*) gene

Nine variable positions were found in 471 bp of *LAC1* gene as shown in figure 20. Five allele types were given to each pattern of the gene sequences. Three types (type 1, 2 and 5) were given for all *C. grubii* molecular type VNI while there were 2 types among molecular type VNII (type3 and 4); the details showed in table 8.

For *C. grubii* molecular type VNI, they were discriminated to 3 types, type 1, 2 and 5. While type 1 shows the different nucleotide base from type 2 and 5 at position 44 (‘C’ in type 1 and ‘T’ in type 2 and 5), type 2 shows the different base from type 3 at position 350 (‘T’ in type 2 and ‘C’ in type 3. For molecular type VNII, there were 2 types. The differences among this molecular type were found in 3 position, position 256 (‘C’ in type 3 while ‘G’ in type 4), 316 (‘C’ in type3 and ‘T’ in type 4) and 356 (‘G’ in type 3 and ‘A’ in type 4).

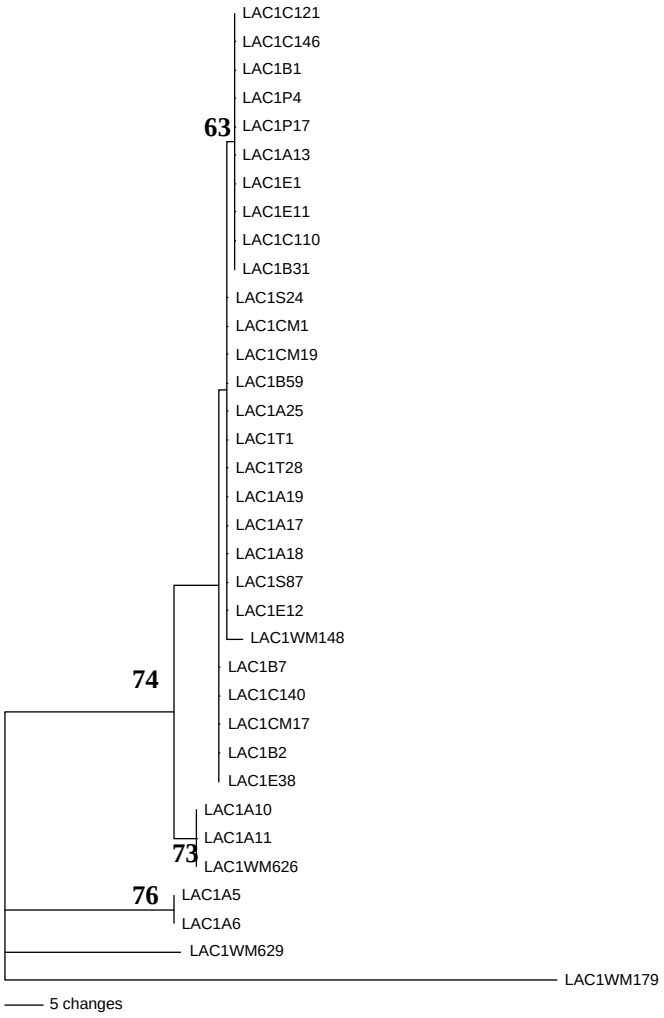


Figure24. Phylogenetic tree of *LAC1* sequence. Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

The *LAC1* gene phylogenetic tree was generated with 31 strains *C. grubii*. For the gene length 478 bp, 429 bases were constant and 40 were parsimony informative bases. The most parsimonious tree was generated with a length of 50 steps. The tree had a consistency index (CI) of 0.980, a retention index (RI) of 0.976,

and a homoplasy index (RC) of 0.956. Figure24 shows the *LAC1*- gene phylogenetic tree.

Four cluster were observed among the tested strains and very interestingly, because 2 isolates of molecular type VNII was grouped with molecular type VNI isolates instead of molecular type VNII.

4.1.4 Ribosomal RNA Spacer (*IGS1*) gene

Among 31 selected strains, *IGS1* gene was 736 bases long and 4 types were detected. While molecular type VNI was separated to type 1, 3 and 4, all 4 strains of molecular type VNII could be separated to only 1 type and type 2. The nucleotide substitution among VNI is highly polymorphisms since upto13 variation sites are detected. The details of all these 13 variation sites are reported following: position 160 (‘A’ in type 1 and ‘T’ in type 3 and 4), 171 (‘T’ in type 1 and 4 while ‘C’ in type 3), 328 (‘G’ in type 1 and ‘A’ in type 3 and 4), 341 (‘T’ in type 1 and ‘C’ in type 3 and 4), 400 and 427 (‘C’ in type 1 and ‘T’ in type 3 and 4), 488 and 665 (‘A’ in type 1 and 3 while in type 2 ‘G’), 492 (‘G’ in type 1 and ‘A’ in type 3 and 4), 628 (‘A’ in type 1 while ‘G’ in type 3 and 4), 646 (‘T’ in type 1 and 3 while in type 4 ‘C’) and position 648 (‘A’ in type 1 and ‘G’ in type 3 and 4). In addition, three insertion sites are found among VNI isolates, position 241 (‘T’ insertion in type 3), 421-422 (‘GT’ insertion in type 1) and 469 (‘A’ insertion in type 4 as well as type 2; molecular type VNII). The variation and insertion sites are shown in figure 20.

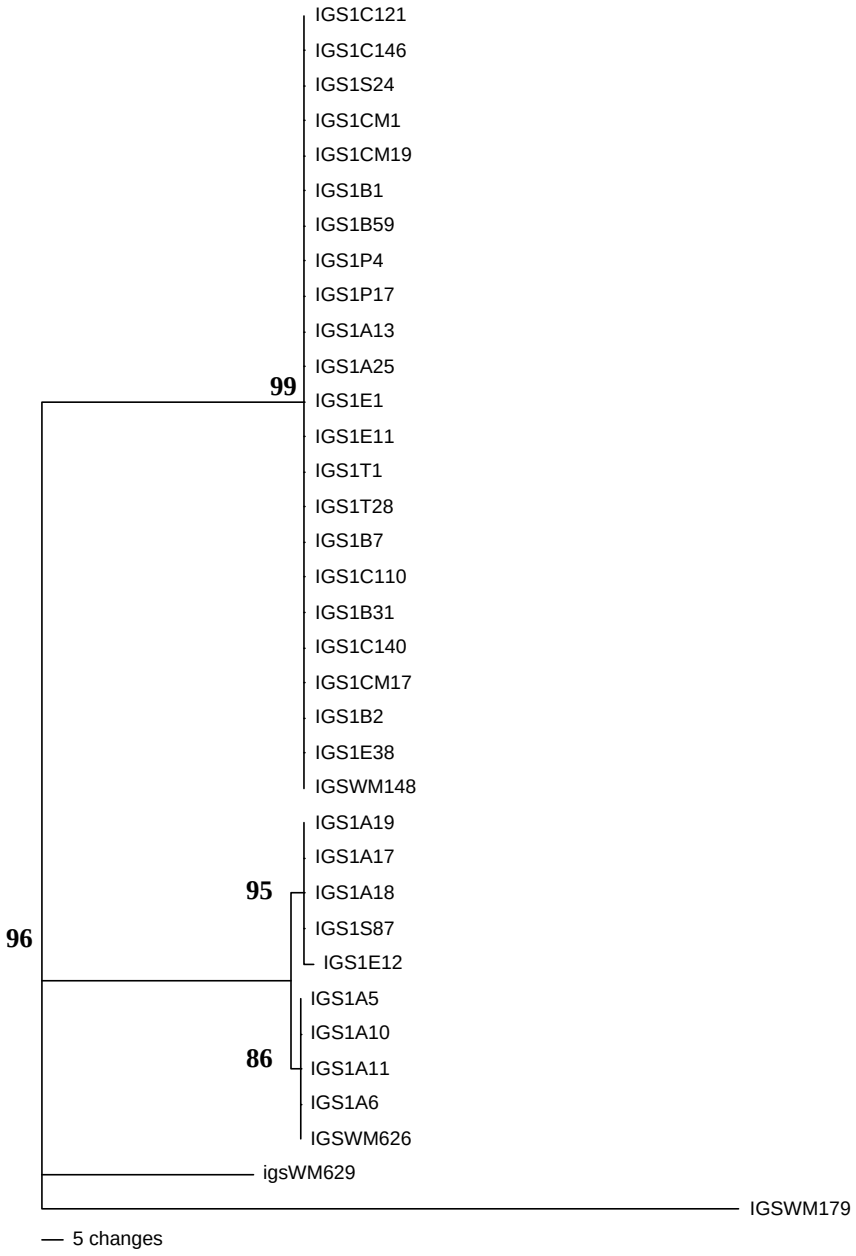


Figure 25. Phylogenetic tree of *IGS1* sequence. Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

716 constant bases and 12 parsimony informative bases are reported when analyzed with phylogenetic analysis. The most parsimonious tree was generated with a length of 97 steps.The tree had a consistency index (CI) of 1.000, a retention index (RI) of 1.000, and a homoplasy index (RC) of 1.000. Figure 25 showed the *IGS1* gene phylogenetic tree.

Three major cluster types were observed. One cluster belonged to molecular type VNII. The remaing two clusters showed the major AT of the isolates including

4.1.5 Phospholipase B (*PLB1*) gene

Three *PLB1* allele types are detected among 31 selected strains. There are two types among molecular type VNI (type 1 and type 3) and only one variation site is found, position 406 (‘C’ in type 1 and ‘A’ in type 3) whereas molecular types VNII isolates can not be separated (Figure26).

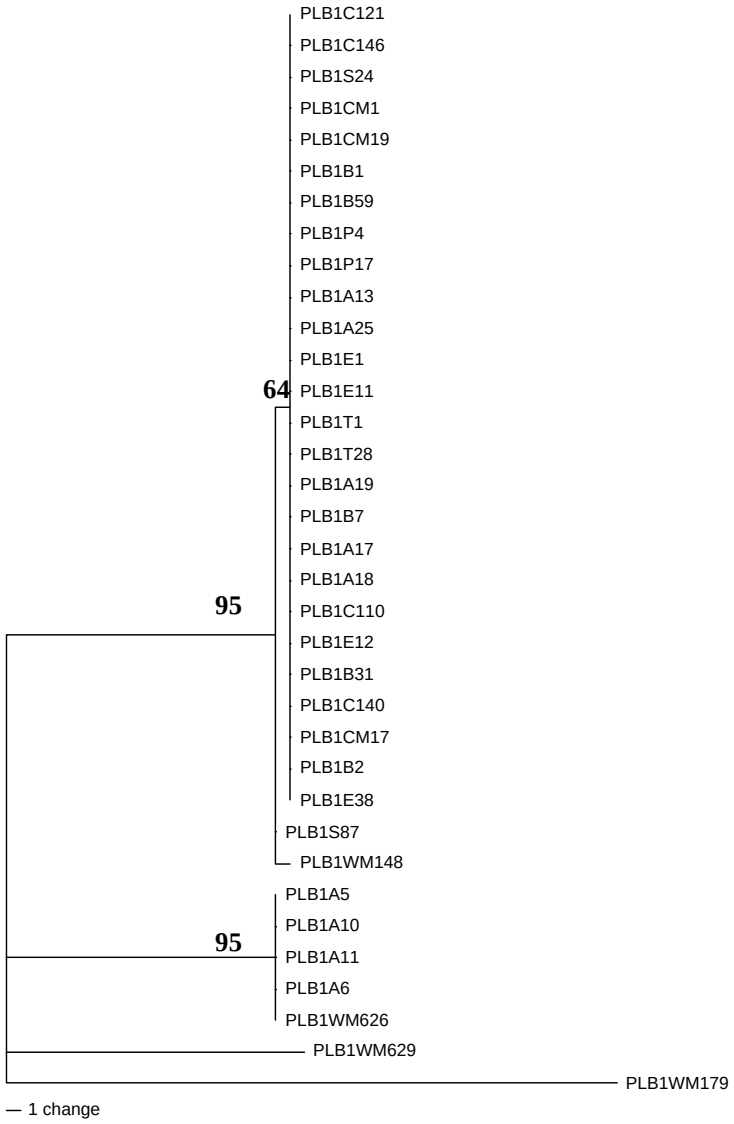


Figure26. Phylogenetic tree of *PLB1* sequence. Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

526 bases and 7 bases are reported as a constant base and parsimony informative base respectively for 533 bases in length when the sequences are analyzed by phylogeny tree. The most parsimonious tree was generated with a length of 40 steps. The tree had a consistency index (CI) of 1.000, a retention index (RI) of 1.000, and a homoplasy index (RC) of 1.000. Figure 26 show the *PLB1*- gene phylogenetic tree. Only two clusters were generated among the tested strains.

4.1.6 Superoxide dismutase (*SOD1*) gene

In length 537 bases, there are three types of *SOD1* gene among selected strains, one type for molecular type VNI (type 1) and two types for molecular type VNII (type 2 and type 3). Among molecular type VNII, only one variation site at position 73 is found (‘A’ in type 2 and ‘T’ in type 3) as show in figure 20.

The phylogenic tree is generated and 525 bases are constant base and 12 bases are a parsimony informative base. The most parsimonious tree was generated with a length of 70 steps.The tree had a consistency index (CI), a retention index (RI), and a homoplasy index (RC) of 1.000. Figure27 showed the *SOD1*- gene phylogenetic tree. Only the two clusters separated the molecular type were observed.

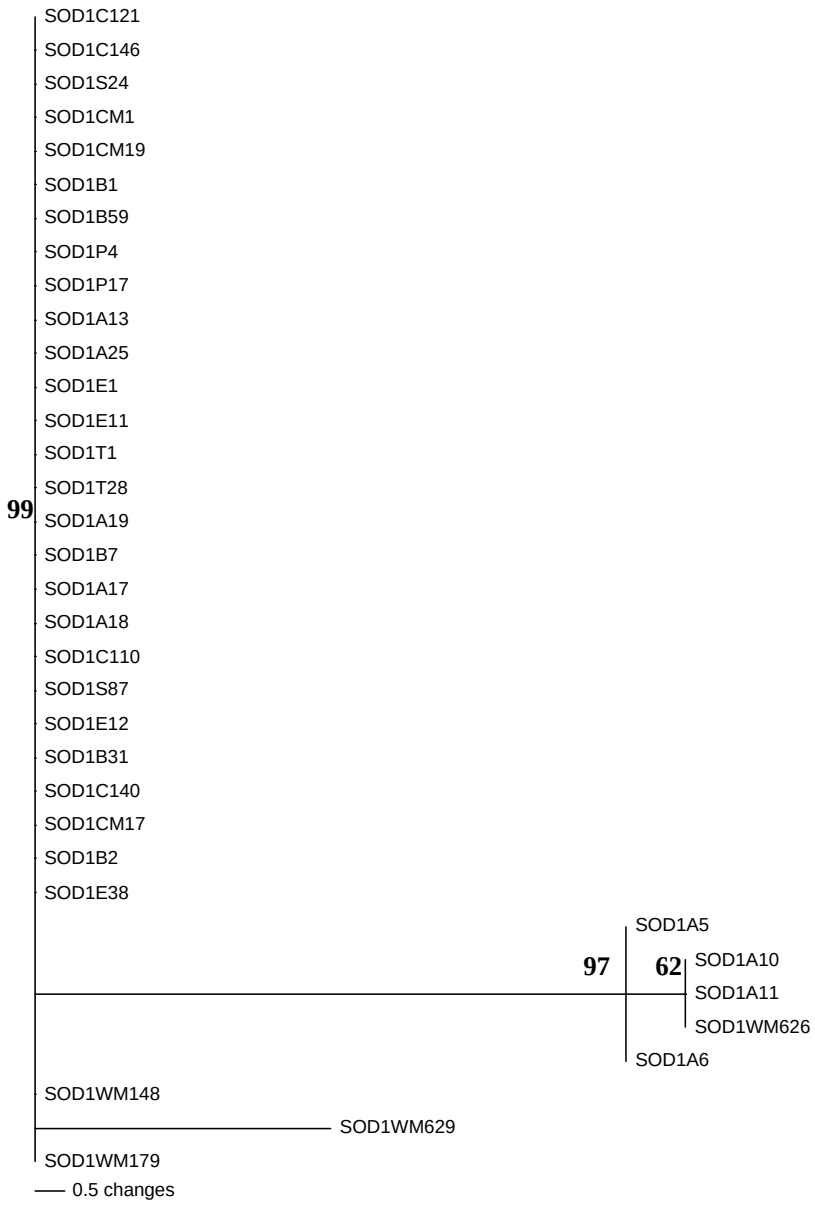


Figure27. Phylogenetic tree of *SOD1* sequence. Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

4.1.7 Translation Elongation Factor1α (*TEF1*) gene

All *TEF1* gene sequences were 653 nucleotides in length of the analysis. Over all the *TEF1* sequences were polymorphic among the 31 selected *C. grubii*. Nine variation positions were observed among the 653 nucleotides sequence length, 1.38 % sequence diversity.

Among molecular type VNII 4 isolates (type 2), they con not be separated while among 27 isolates of molecular type VNI, 4 type are found (Type 1, 3, 4 and 5) and three variation sites are found. The three variation sites are found in position 476 (‘C’ in type 1, 3 and 5 while ‘T’ in type 4), 590 (‘T’ in type 1, 4 and 5 while ‘C’ in type 3) and 640 (‘G’ in type 1, 3 and 4 while ‘A’ in type 5). Figure 20 shows the details of the variation bases and sites.

The phylogenetic tree is generated, when the length of analysis is 653 bases, 644 bases are constant base and 6 bases are parsimony informative base. The most parsimonious tree was generated with a length of 38 steps.As the previous genes the phylogenetic tree had a consistency index (CI), a retention index (RI), and a homoplasy index (RC) of 1.000. Figure28 showed the *TEF 1*- gene phylogenetic tree.

Three of clusters were found. Two clustered belonged to molecular type VNI and one last cluster was VNII group.

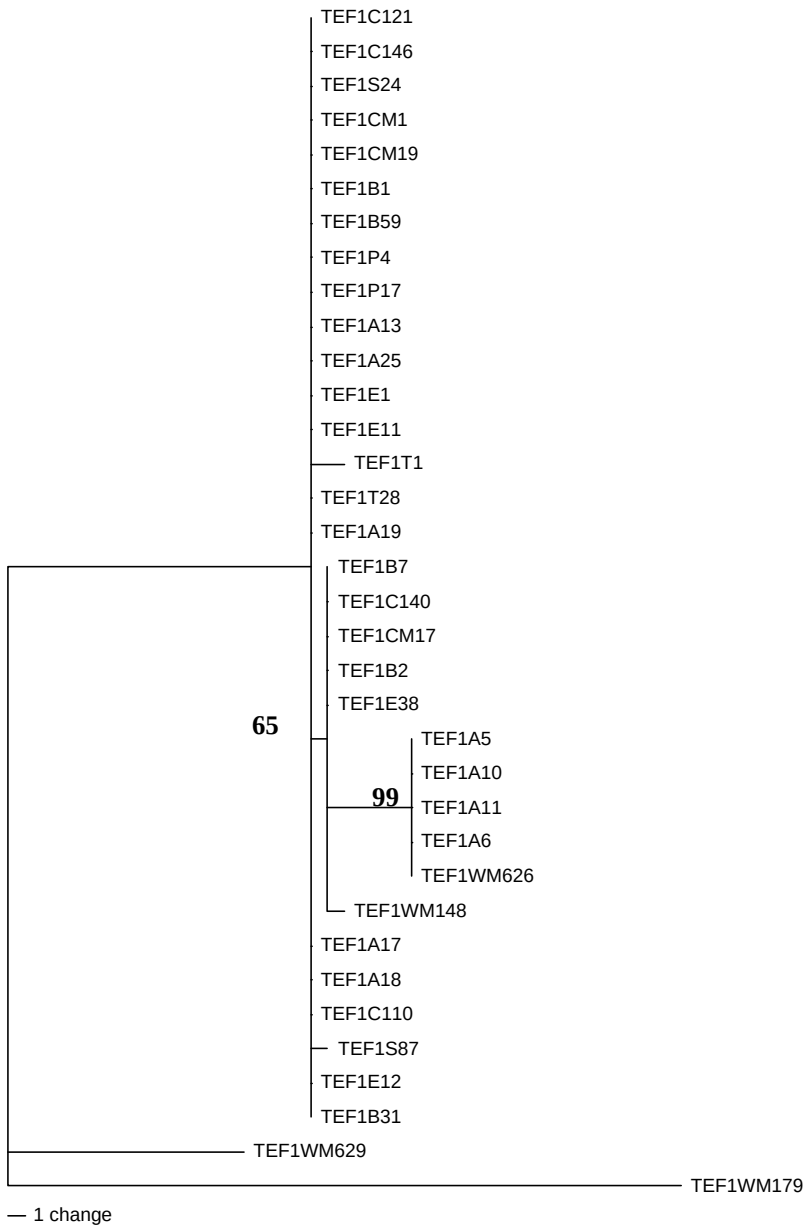


Figure28. Phylogenetic tree of *TEF1* sequence. Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

4.1.8 Orotidine monopyrophosphatase (*URA5*) gene

In length of analysis, 602 bases, ten polymorphic sites are found in all 31 selected isolates. Among both molecular type VNI and VNII isolates they are separated to two types (type 1 and 3 for VNI; type 2 and 4 for VNII) in totally 4 types is reported for *URA5* gene. Only one polymorphic site is observed in each molecular type. For molecular type VNI, there is a polymorphic site at position 174 ('G' in type 1 and 'A' in type 3) while molecular type 2 the polymorphic site is found in position 592 (G' in type 2 and 'A' in type 4) as shown in figure 20.

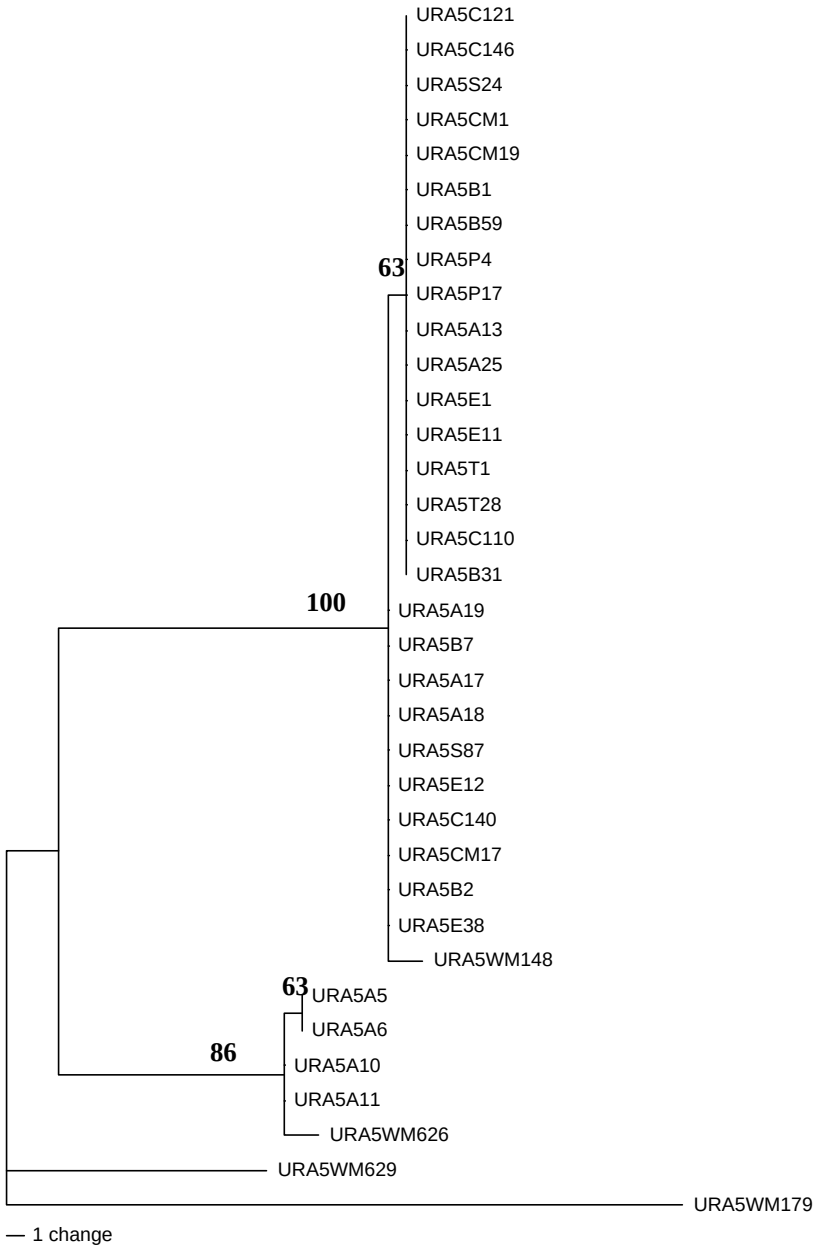


Figure29. Phylogenetic tree of *URA5* sequence. Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups.

The length of phylogenetic tree analysis is 602 bases, 592 bases are constant base and 10 bases are parsimony informative base. The most parsimonious tree was generated with a length of 38 steps. As all previous studied genes, the phylogenetic tree had a consistency index (CI), a retention index (RI), and a homoplasy index (RC) of 1.000. Figure 29 showed the *URA5*- gene phylogenetic tree.

4.3 Combine genes Analysis

Phylogenetic tree of 9 combine sequence fragments were generated to observe the genetic evolution among the tested strains using PAUP 4.0 (Figure30). Totally, 3,411 nucleotides were analyzed from all 31 tested isolates revealed 228 parsimony informative characters to be compared. The phylogenetic tree showed that the molecular type VNI and VNII were clearly distinguished. Among the molecular type VNI group, 5 clusters were observed (bootstrap value more than 60%) and, in molecular type VNII group, two clusters were presented. In molecular type VNI, cluster VNI-I included the STth2 while cluster VNI-II included STth1. Cluster VNI-III consisted of STth4, 7 and 8. Other two clusters, cluster VNI-IV and VNI-V, were composed of STth3 and STth 5, 6, repectively. Regarding the molecular type VNII, cluster VNII-I was composed of STth9 and 12 while cluster VNII-II included STth10 and 11.

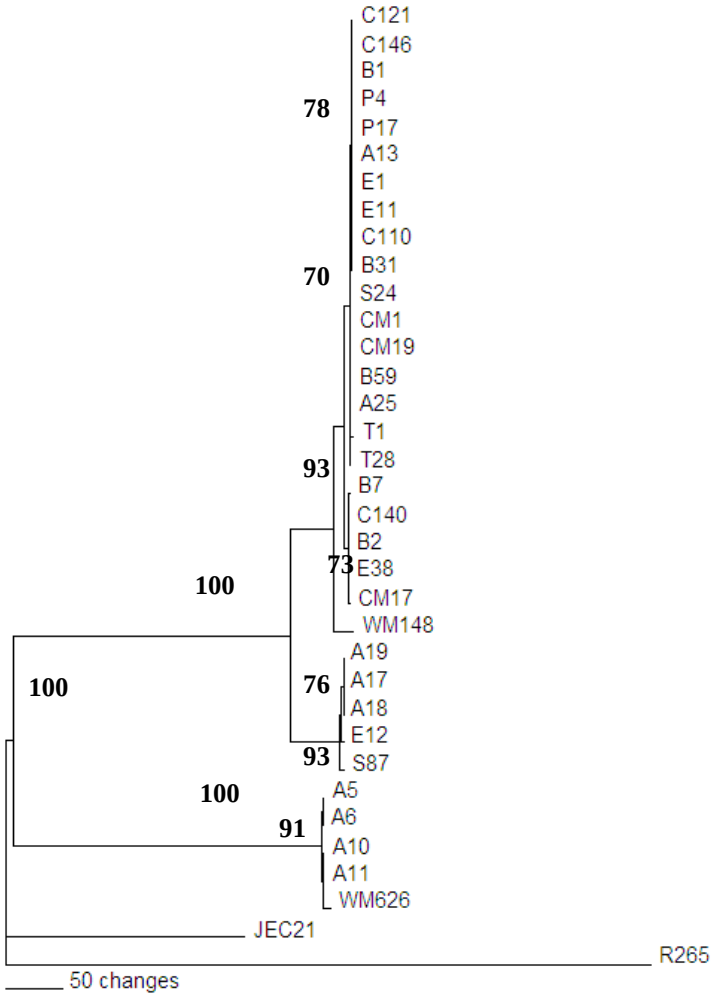


Figure30. Phylogenectic tree of MLST 9 combine gene sequences of 31 *C. neoformans* var. *grubii* Thai isolate**.** Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups

4.3 Sequence Type (ST) Analysis

The PCR products from 8 chosen genes with 9 studied fragments were sequenced**.** Of the 3,441 nucleotides, 92 polymorphic sites and 12 Sequence Type (STths) were classified. The variable sites ranged from 5-17 positions. The fragments in *ACT1* showed the most variable sites whereas those of *CAP59* (amplified by *CAP59* primer) were the least polymorphic sites (Table8)

Regarding the Allele Type (AT), *CAP59CN* showed six ATs, the highest alleles in this study whereas *CAP59* primer showed only 2 ATs which was the lowest one (Table 8). The new ATs, 13 ATs, in addition to the report from Litvintseva *et al*, (122) was found. Verification of these new ATs fragments was performed by repeating PCR amplification and sequencing.

Table8. *URA5*-RFLP and M13-fingerprinting results of the studied *C. neoformans* var. *brubii*

M13-fingerprinting Subtype	Name	Source	*URA5*-RFLP Pattern	Allele tpe (AT) from MLST studied genes									Sequence type (STths)
				ACT1	*CAP59*	*CAP59CN*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*TEF1*	*URA5*	
A	**C121**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**1**
A	**C146**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**1**
A	**S24**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**2**	**2**	**15**	**4**	**1**	**2**
A	**Cm1**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**2**	**2**	**15**	**4**	**1**	**1**
A	**Cm19**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**2**	**2**	**15**	**4**	**1**	**1**
A	**B1**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**2**
A	**B59**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**2**	**2**	**15**	**4**	**1**	**1**
A	**P4**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**2**
A	**P17**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**2**
A	**A13**	**Animal**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**2**
A	**A25**	**Animal**	**VNI**	**1**	**1**	**28**	**19***	**2**	**2**	**15**	**4**	**1**	**1**
A	**E1**	**Dropping**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**2**

Table8.(cont) *URA5*-RFLP and M13-fingerprinting results of the studied *C.neoformans* var. *brubii*

M13-fingerprinting Subtype	Name	Source	*URA5*-RFLP Pattern	Allele tpe (AT) from MLST studied genes									Sequence type (STths)
				ACT1	*CAP59*	*CAP59CN*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*TEF1*	*URA5*	
A	**T28**	**Dropping**	**VNI**	**1**	**1**	**28**	**19***	**2**	**2**	**15**	**4**	**1**	**1**
B	**A19**	**Animal**	**VNI**	**1**	**1**	**30***	**20***	**2**	**2**	**15**	**4**	**3**	**3**
B	**B7**	**Clinical**	**VNI**	**1**	**1**	**29***	**19***	**5**	**2**	**15**	**5**	**3**	**4**
C	**A17**	**Animal**	**VNI**	**1**	**1**	**30***	**20***	**2**	**2**	**15**	**4**	**3**	**3**
C	**A18**	**Animal**	**VNI**	**1**	**1**	**30***	**20***	**2**	**2**	**15**	**4**	**3**	**3**
D	**C110**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**1**
D	**S87**	**Clinical**	**VNI**	**3**	**1**	**28**	**19***	**2**	**4**	**15**	**3**	**3**	**5**
D	**E12**	**Dropping**	**VNI**	**1**	**1**	**31***	**22***	**2**	**2**	**15**	**4**	**3**	**6**
E	**B31**	**Clinical**	**VNI**	**1**	**1**	**28**	**19***	**4**	**2**	**15**	**4**	**1**	**1**
F	**C140**	**Clinical**	**VNI**	**1**	**1**	**31**	**31**	**5**	**2**	**15**	**5**	**3**	**7**
F	**Cm17**	**Clinical**	**VNI**	**1**	**1**	**28**	**19**	**5**	**2**	**15**	**5**	**3**	**8**
F	**B2**	**Clinical**	**VNI**	**1**	**1**	**31**	**31**	**5**	**2**	**15**	**5**	**3**	**7**
F	**E38**	**Dropping**	**VNI**	**1**	**1**	**31**	**31**	**5**	**2**	**15**	**5**	**3**	**7**

Table8. (cont) *URA5*-RFLP and M13-fingerprinting results of the studied *C. neoformans* var. *grubii*

M13-fingerprinting Subtype	Name	Source	*URA5*-RFLP Pattern	Allele tpe (AT) from MLST studied genes									Sequence type (STths)
				ACT1	*CAP59*	*CAP59CN*	*IGS1*	*LAC1*	*PLB1*	*SOD1*	*TEF1*	*URA5*	
G	**A5**	**Animal**	**VNII**	**2**	**2**	**32**	**21**	**9***	**11**	**13**	**18**	**4**	**9**
G	**A10**	**Animal**	**VNII**	**2**	**2**	**33**	**21**	**8**	**11**	**14**	**18**	**1**	**10**
H	**A11**	**Animal**	**VNII**	**2**	**2**	**33**	**21**	**8**	**11**	**14**	**18**	**2**	**11**
I	**A6**	**Animal**	**VNII**	**2**	**2**	**33**	**21**	**9**	**11**	**13**	**18**	**4**	**12**

The asterisk showed the new AT

4.4 Genetic relationship with the global isolates

C. neoformans var. *grubii* Thai isolates share the genetic information with worldwide isolates. Total 3,441 nucleotdies length were analyzed. 3,295 bases were constant, and 112 bases were the parsimony informative. The most parsimonious tree was generated with a length of 516 steps (CI= 0.556, RI=0.898, RC= 0.499). STth1 and STth2 (represented by C121 isolate and S24 isolate, respectively, (Table 8) were grouping together with ST2 and ST3 which included other Thai isolates from the previous study. Other Thai isolates in our study were clustered with the isolates from various countries such as USA, Malawi, Australia, Brazil and Japan. However, all of *C. neoformans* var. *grubii* Thai isolates in this study were grouped together within the group I of the phylogenetic tree (Figure 28). Then *C. grubii* Thai isolates in this study showed the genetic sharing with the other *C. grubii* global isolates. Moreover, the genetic information within Thai population was closely since they were clustered with Cluster I.

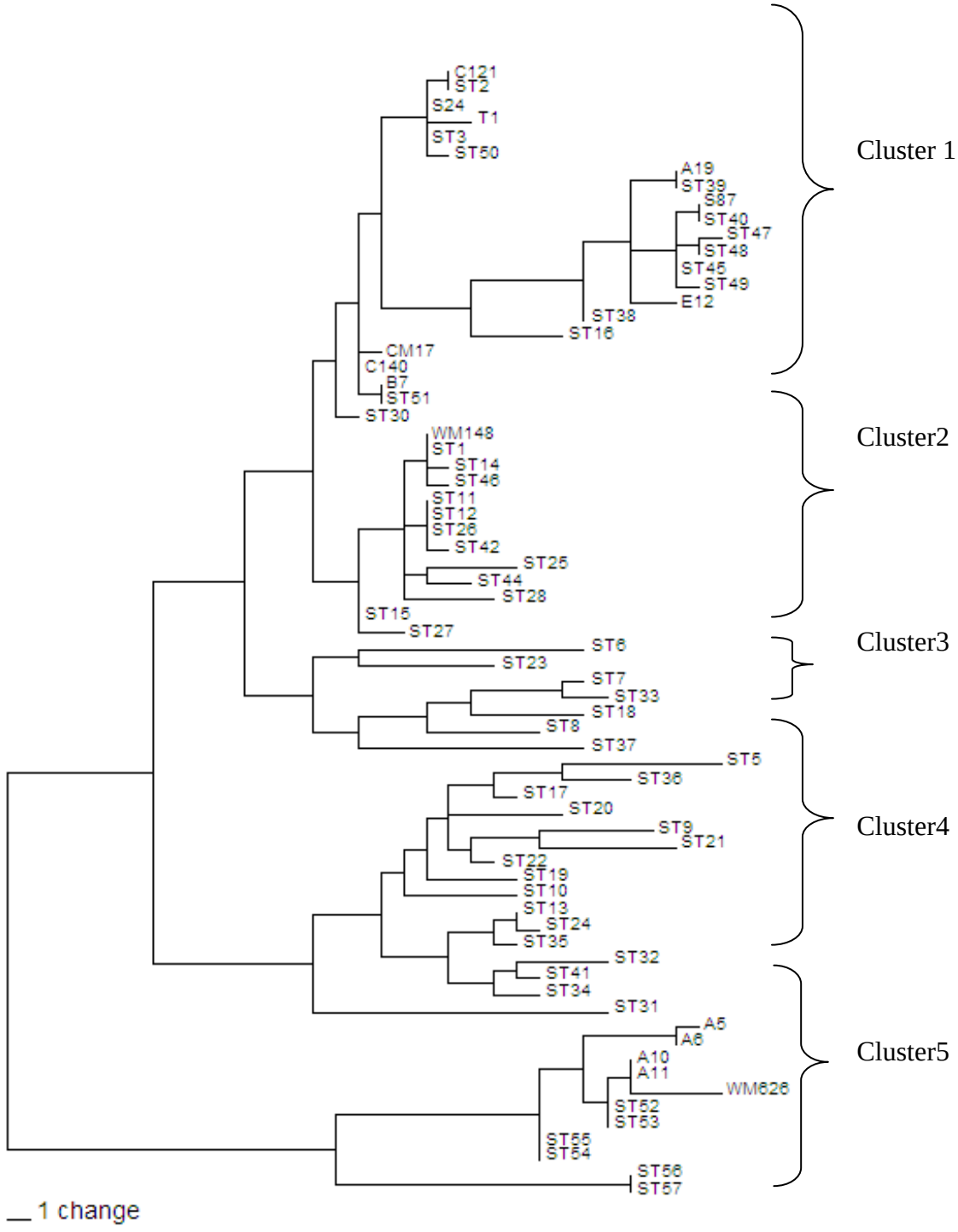


Figure31. The genetic evolution of *C. neoformans* var. *grubii* Thai isolates compare with worldwide isolates; ST indicates the Sequence Type of *C. neoformans* var. *grubii* from the MLST database**.** Parsimony bootstrap support above 60 is indicated. The phylogenetic tree is rooted using *C. neoformans* var. *neoformans* (WM629) and *C. gattii* (WM179) as outgroups**.**

5. Multilocus Microsatellite Typing (MLMT) Analysis

The eleven microsatellite loci were tested on 31 individuals of *C. grubii* from clinical environmental and veterinary sources. Six loci including GTT9, GA9, TATTT9, TG12GTG2, GCC8 and TTA10 were not the polymorphic among Thai isolates. All of 31 isolates shown the same allele size 192, 152, 145, 147, 156 and 175 base pairs, respectively. The remaining 5 loci were the polymorphic loci, with the number of alleles ranging from 3 to 7 per locus (mean = 2). These 5 loci including GT_{12}, $TCCTTT_6$, $GGAT_{33}$, AC_{12} and CAA_8.

Among 31 strains, 3 microsatellite types (allele type) were observed in two loci, $TCCTTT_6$ and CAA_8. The range size was 197 to 209 base pairs and 474 to 496 base pairs, respectively. The common size of $TCCTTT_6$ locus was 197 base pairs and the common size of CAA_8 locus was 474 base pairs. Four allele types were observed in locus $GGAT_{33}$, range from 211-220 base pairs and the common size was 220 base pairs. Locus GT_{12} showed 5 allele types, allele size range were 130 to 150 base pairs and the most common size was 132 base pairs. The most polymorphic locus was locus AC_{12}, the allele size was range from 247 to 269 base pairs and the common size was 253 base pairs (Table 9).

Table9. The summary of allele size of MLMT polymorphic loci.

Loci	No. Allele	Range size (bp)	Common size
GT12	5	130-150	132
TCCTTT6	3	197-209	197
CAA8	3	474-496	474
GGAT33	4	211-220	220
AC12	7	247-269	253

5.1 MLMT pattern

Five polymorphic MLMT loci were combining together, 13 allele types were observed. The majority of microsatellite type was microsatellite type 1. The minor microsatellite type was type 2, 10 and 11which included 3, 2 and 2 strains, respectively. The remaining microsatellite type only one isolates was typed in each microsatellite type. The microsatellite allele and designed microsatellite type (MT) showed in table10.

Table10. List of *C. neoformans* var. *grubii* strains used and their source, microsatellite allele and designed microsatellite type (MT)

Name	Source	Microsatellite allele					Microsatellite type (MT)
		GT_{12}	$TCCTTT_6$	$GGAT_{33}$	AC_{12}	CAA_8	
C121	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
C146	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
S24	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
Cm1	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
Cm19	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
B1	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
B59	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
P4	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
P17	**Clinical**	**2**	**1**	**4**	**4**	**1**	**1**
A13	**Animal**	**2**	**1**	**4**	**4**	**1**	**1**
A25	**Animal**	**2**	**1**	**4**	**4**	**1**	**1**
E1	**Dropping**	**2**	**1**	**4**	**4**	**1**	**1**
E11	**Dropping**	**2**	**1**	**4**	**4**	**1**	**1**
T1	**Dropping**	**2**	**1**	**4**	**4**	**1**	**1**
T28	**Dropping**	**2**	**1**	**4**	**4**	**1**	**1**
A19	**Animal**	**3**	**2**	**3**	**5**	**1**	**2**
B7	**Clinical**	**4**	**2**	**3**	**7**	**3**	**3**
A17	**Animal**	**3**	**2**	**3**	**5**	**1**	**2**
A18	**Animal**	**3**	**2**	**3**	**5**	**1**	**2**
C110	**Clinical**	**2**	**1**	**4**	**3**	**1**	**4**
S87	**Clinical**	**2**	**2**	**2**	**3**	**1**	**5**
E12	**Dropping**	**2**	**1**	**4**	**3**	**1**	**6**

Table10. (cont.) List of *C. neoformans* var. *grubii* strains used and their source, microsatellite allele and designed microsatellite type (MT)

Name	Source	Microsatellite allele					Microsatellite type (MT)
		GT_{12}	$TCCTTT_{6}$	$GGAT_{33}$	AC_{12}	CAA_{8}	
B31	**Clinical**	**5**	**1**	**4**	**3**	**1**	**7**
C140	**Clinical**	**4**	**2**	**2**	**7**	**2**	**8**
Cm17	**Clinical**	**4**	**2**	**2**	**5**	**2**	**9**
B2	**Clinical**	**4**	**2**	**2**	**6**	**2**	**10**
E38	**Dropping**	**4**	**2**	**2**	**6**	**2**	**10**
A5	**Animal**	**1**	**1**	**1**	**1**	**2**	**11**
A10	**Animal**	**1**	**3**	**1**	**1**	**2**	**12**
A11	**Animal**	**1**	**3**	**1**	**2**	**2**	**13**
A6	**Animal**	**1**	**1**	**1**	**1**	**2**	**11**

5.2 MLMT repeating unit sequence

Sixteen isolates from each M13-fingerpritning pattern were selected as a representative of each fingerprinting patterns for sequencing. The repeating unit of each isolates was analyzed. The number of repeating unit of 5 polymorphic loci was shown in table 11. The nucleotide sequence of GT_{12} locus showed that microsatellite allele 1, 2 and 3 had the repeating number of GT as 4, 5 and 7 respectively. However, allele type 4 showed two length of GT repeat, 14 and 13 unit. Microsatellite allele 5 showed 5 of repeat units same as microsatellite allele 2. In $TCCTTT_6$ locus, microsatellite allele type 1 and 2 showed the same number of repeating unit, 2 units while microsatellite allele 3 had only 1 repeating unit. $GGAT_{33}$ gave 3 allele sizes in this study, however, the repeating unit of all allele showed the same number of repeating unit, 1 unit. In AC_{12} locus, microsatellite allele 1 and 2 showed the same number of repeating unit, 5 units. While allele type 3 and 4 showed six repeating units. Moreover, allele type 1 had two length of repeating both 5 and 6 units. The remaining allele, allele type 5, 6 and 7 had repeating unit 8, 14 and 15 respectively. In this study, only CAA_8 loci showed the relative of repeating unit number and the allele size. Microsatellite allele 1 had the repeating unit number 2/4, allele 2 and allele 3 had the repeating unit number 2/3/4 and 2/3/3/4, respectively.

Table 11. Repeating unit of 16 *C. neoformans* var. *grubii* isolates represent for MLMT allele type

Name	Microsatellite type (Repeating Unit Number)				
	GT_{12}	**$TCCTTT_{6}$**	**$GGAT_{33}$**	**AC_{12}**	**CAA_{8}**
C121	2(5)	1(4)	2(1)	4(13)	1(2/4)
T1	2(5)	1(4)	2(1)	4(6)	1(2/4)
A19	3(7)	2(4)	3(1)	5(8)	1(2/4)
B7	4(14)	2(4)	3(1)	7(15)	3(2/3/3/4)
A17	3(7)	2(4)	3(1)	5(8)	1(2/4)
C110	2(5)	1(4)	4(1)	3(6)	1(2/4)
S87	2(5)	2(4)	2(1)	3(6)	1(2/4)
B31	5(5)	1(4)	4(1)	3(6)	1(2/4)
C140	4(14)	2(4)	2(1)	7(15)	2(2/3/4)
Cm17	4(14)	2(4)	2(1)	5(6)	2(2/3/4)
B2	4(13)	2(4)	2(1)	6(14)	2(2/3/4)
E38	4(13)	2(4)	2(1)	6(14)	2(2/3/4)
A5	1(4)	1(4)	1(2)	1(6)	2(3/3/4)
A10	1(4)	3(1)	1(2)	1(5)	2(3/3/4)
A11	1(4)	3(1)	1(2)	2(5)	2(3/3/4)
A6	1(4)	1(4)	1(2)	1(6)	2(3/3/4)

5.3 Allele frequency

The frequency of microsatellite alleles was calculated by GeneAlex6 program. As shown in figure 32 microsatellite variety-allele specific *C. neoformans* var.*grubii* molecular type VNII were observed including allele 1 of GT_{12} (Locus1), allele 1 of $GGAT_{33}$ (Locus 3), and also allele 1 of AC_{12} (Locus 4). The shared-allele between *C. neoformans* var. *grubii* VNI and VNII were detected in 2 loci; allele 1 (197 base pairs) of locus$TCCTTT_6$ and allele 1 (474 base pair) of locus AC_{12}. The unique microsatellite allele in single strains also found. Strain B7 had three unique allele; allele 4 (150 base pair) and allele 7 (269 base pair), and allele 3 (496 base pair) of locus GT_{12}, AC_{12} and CAA_8, respectively. Strain B31 also showed the unique allele type, allele 5 (150 base pair) and allele 7 (496 base pair) of GT_{12} and AC_{12} locus. This strain-specific allele resulting in unique microsatellite type in both strains.

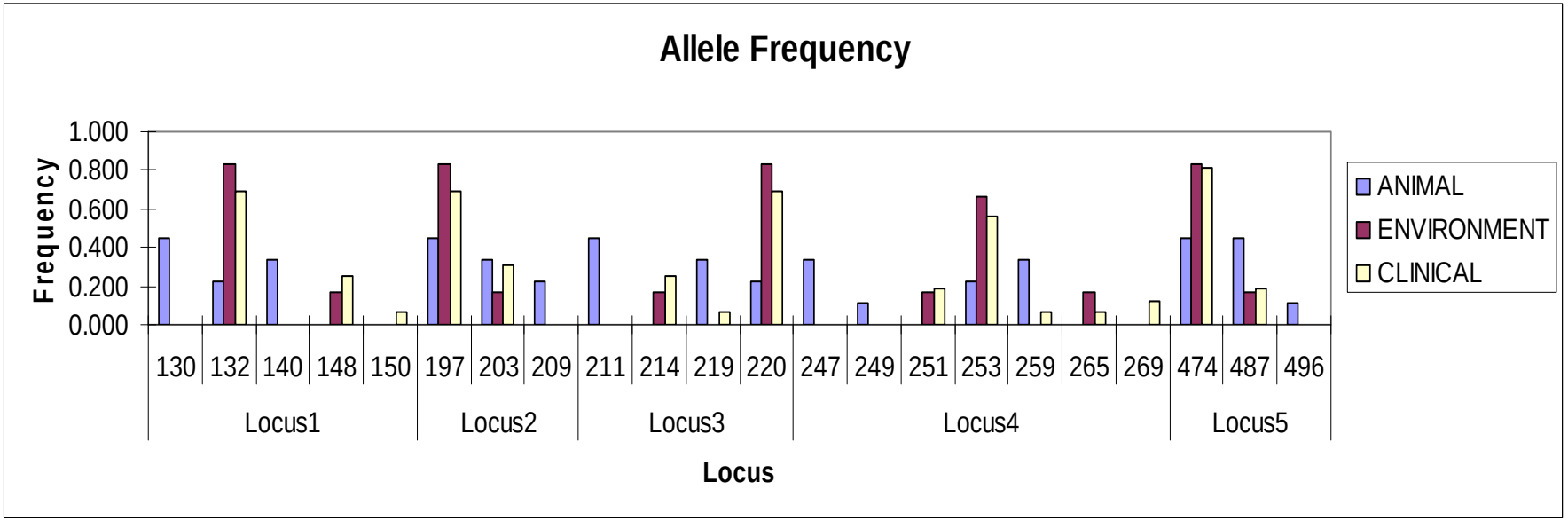


Figure32. Allele size distribution at the polymorphic microsatellite loci of 31 *C. neoformans* var. *grubii* Thai isolates; Locus1=GT_{12}, Locus2=$TCCTTT_{6,}$Locus3=$GGAT_{33}$,Locus4=AC_{12}andLocus5=CAA_{8}.

5.4 Genetic distance analysis of *C. neoformanas* var. *grubii* Thai isolates

Evidence of an epidemiology link between natural habitat of *C. neoformans* species complex and human infection remains circumstantial. The population genetic was used for genetic relatedness among Thai isolate.

For population genetic study, three populations were assigning according to the source of the isolates; clinical, veterinary and environmental source. GenALeX 6 program were used for the genetic statistic calculation. The *C. neoformasns* var. *grubii* molecular type VNII were exclude from the analysis since the molecular type different may affect the distance instead of the evolution of the strains.

Pairwise Population Matrix of Nei Unbiased Genetic Distance was calculated. The genetic distance of animal population is different from clinical and environment with genetic distance of 0.333 and 0.408, respectively. While the genetic distance between clinical and environmental population is 0.019, suggesting they might have identical genetic information.

6. Discriminatory power and the concordance of molecular typing methods

The highest degree of discriminatory power (D) was observed for Multilocus Microsatellite Typing (D= 0.82). Discriminatory powers for Multilocus Sequence Typing and M13-fingerprintingwere D= 0.86 and D= 0.75, respectively.

The grouping by these three methods was similar. Nine M13-fingerprinting subtypes, 12 STs by MLST and 13 MTs by MLMT were demonstrated. M13-fingerprinting subtype A amd MLMT 1 were divided into two MLST-groups, STth1 and STth2. Figure 30 and Figure 31 demonstarted the comparison clustering of C.

neoformans var, grubii between M13-fingerpriniting with MLST and M13-fingerprinting with MLMT, respectively.

From the topology of Figure33 there were thress inconsistent results between two typing methods. Isolates A19 and, B7 were grouped together in M13-fingerprinting subtype B but isolate A19 was classified as STth3 while B7 was classified as STth4. Moreover, isolate C110 and B31 which were classified in difference subtypes, subtype D and E, respectively, were classified into one ST, that was STth1. Comparison between MLMT technique and M13-fingerpritning, only two inconsistent results was observed (Figure34). Isolates S87 was classified as M13-fingerprinting subtype D but by MLMT typing this isolates was separate from the other subtype D isolates. Isolates B7 and A19 which were subtype B isolates in M13-fingerprinting but from the MLMT results this two isolates were separated.

The remaing isolates of three methods showed the clustering concordance between the methods.

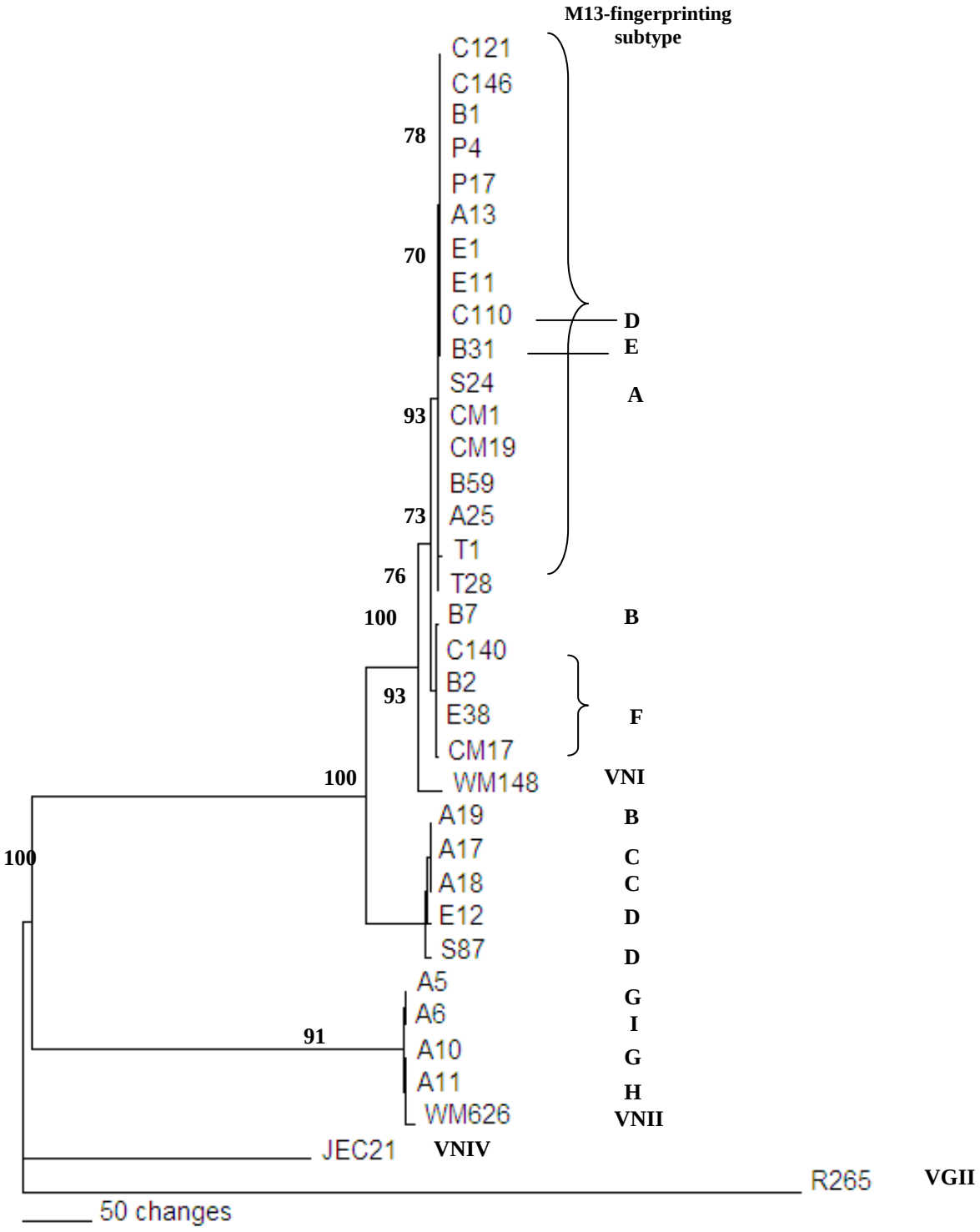


Figure33. The topology of MLST result compare with M13-fingerprinting result.

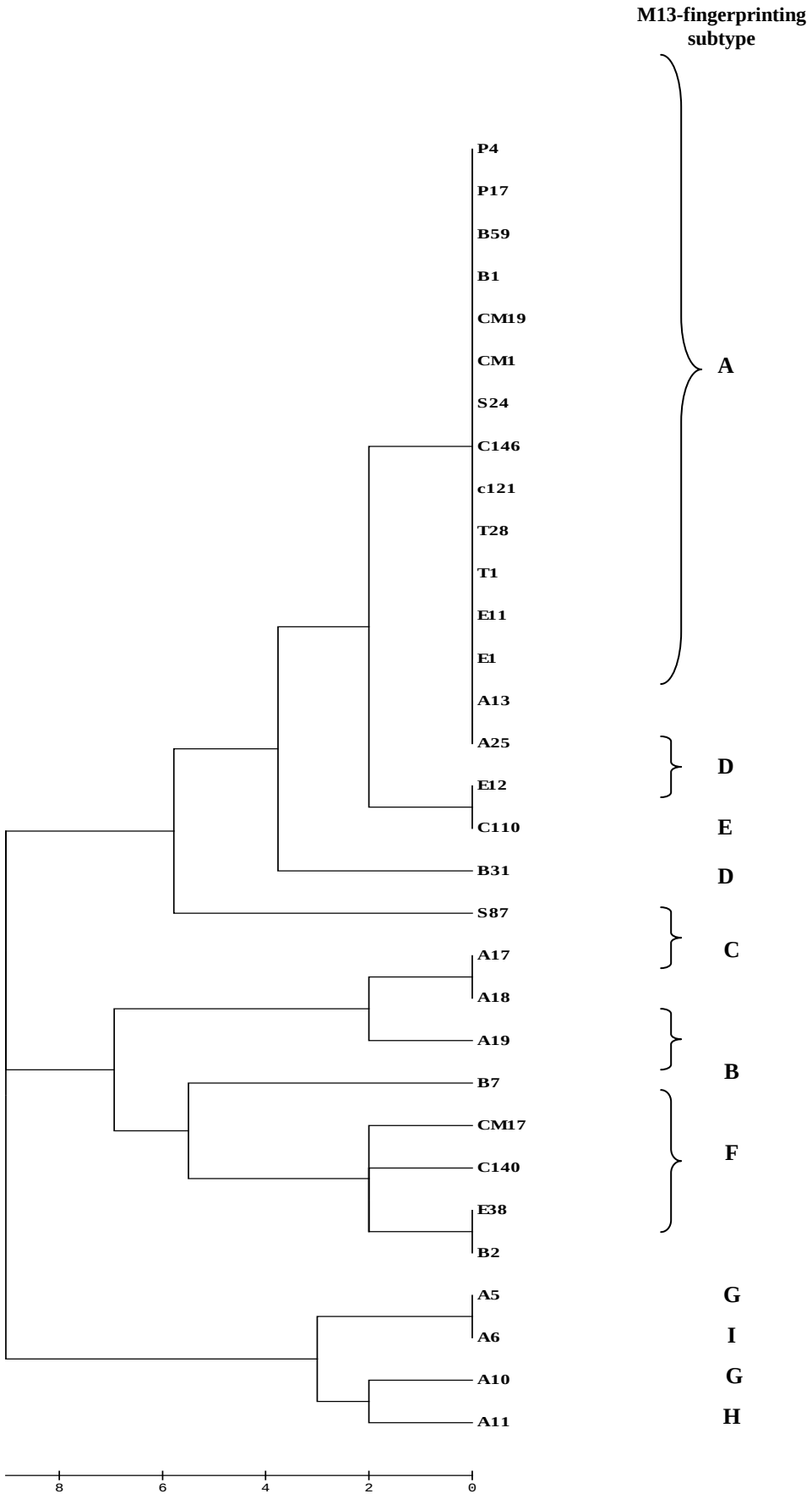


Figure34. The topology of MLMT results compare with M13-fingerprinting subtype.

Chapter V

Discussion

Four hundred eighty-one *C. neoformans* Thai isolates from three sources including clinical, veterinary and environmental origins were subjected to this genotyping study. The variety was selected for preliminary screening the variety of *C. neoformans* species complex. The results showed that all 481 isolates were *C. neoformans* var. *grubii* molecular type VNI and VNII (serotype A). All VNII isolates were the isolated from feline and classified as *C. neoformans* var. *grubii* molecular type VNII. These varieties were investigated using the rapid and reliable *URA5*-RFLP developed by Meyer et al. (14). Eight standard strains of *C. neoformns* species complex showed the unique different *URA5*-pattern; molecular type VNI and VNII classified as *C. neoformans* var. *grubii*, molecular type VNIII classified as *C. neoformans* AD hybrid, VNIV classified as *C. neoformmans* var. *neoformans.* While *C. gattii* showed the *URA5*-pattern as VGI-VGIV, which unable to distinguish the difference between serotype B and C. This result was corresponded to the previous epidemiological studies that *C. neoformans* var. *grubii* is existed as the dominant variety in Thailand and also those are in worldwide (24, 111, 132). As all 7 isolates of *C. neoformans var. grubii* molecular type VNII could isolate only from feline cases among 32 isolates (21.88%) of 2/8 cases (25%) for veterinary source (21.88%). None of *C. neoformans* var. *grubii* molecular type VNII was found in 361 clinical isolates and 80 environmental isolates this phenomenon may indicated that *C. neoformans* var. *grubii* molecular type VNII is a common in feline in Thailand more than in human.

URA5-RFLP typing of *C. neoformans* species complex Thailand has not been reported to classify the variety before, only serotype was reported from these studies as *C. neoformans* serotype A using the latex agglutination test (24). The detection by this technique is absolutely easy to distinct each variety by performinmg together with molecular typing strain. From this experiment, *URA5*-RFLP is recommended as an alternative variety differentiation technique for research or any available laboratory.

Mating type is one of potential virulence factor of *C. neoformans* species complex since there mating type α is a majority mating type in the isolates (8).The mating type determination of 481 *C. neoformans* var. *grubii* isolates resulting mating type **α.** This finding is in accordance with the previous study that mating type α is more frequent than mating type **a** in *C. neoformans* population (134). Incomplete hypothesis was purposed to explain this finding (98). Under the appropriate condition, fungal hyphal could develop from the haploid *C.neoformans* and producing basidiospores which is the association of hyphal phase with the mating type **α** may explain more frequency of this mating type in *C. neoformans* isolates.

M13-PCR fingerprinting was one of typing tools to discriminate the strains variation by detecting microsatellite DNA region using a single specific primer M13 pahge sequence. This technique combines the specificity of hybridization fingerprinting with the rapid simplicity of PCR reaction and suitable for study of genetic diversity within *C. neoformans* species complex (111). In this study, it was shown that M13-fingerprinting is able to discriminate the polymorphic microsatellite sites among 481 Thai isolates into 9 subtypes (subtype A-I) which subtype A is a majority subtype (88.35%). Only few isolates were classified as subtypes E, H and I. These subtypes might be the rarely subtype in Thailand; the large numbers of isolates

is required to confirm this evidence. However, these patterns also can be the real unique fingerprinting patterns since there was a report indicated the substantial genetic diversity, which may be patient specific fingerprinting pattern. (111).

After M13-fingerprinting was analysed, twenty-eight cases of 1 to 2 weeks consecutive sample collection al least twice were revealed. It was found that 25 out of these 28 cases demonstrated the identical M13-fingerprinting profile mostly were subtype A. This finding is in agreement with the previous study that human cryptococcosis is typicaly caused by a single cryptococcal strain (111). The major genetic variation *in vivo* is not common after the infection. Or in the other hands, the microevolution of *C. neoformans* during chronic human infection was found (135), three of serial isolated cases, nine isolates, showed the different M13-fingerpritning patterns. In this previous study, mice were introduced to infect by serial isolates after that the different virulent to infected mice were monitored. This phenomenon call 'microevolution' which may allow a cryptoccocal yeast to change, escape eradication by the immune system and finally cause chronic infections. From this study, three out of eight feline cases showed mix infection with difference M13-fingerprinting profile. All of these diversified yeasts were collected at the same time and the same sits in each case. The difference in inter-variety and intra-variety level was observed.

After M13-fingerpritng was used as the preliminary typing technique of *C. neoformans* var. *grubii* Thai isolates. Thirty-one isolates as a representative from each subtype were subjected to perform the further study; MLST and MLMT, two new approaches typing of *C. neoformans* var. *grubii*. All these M13-fingerprint of 31 selected isolates were confirmed the phygenetic classification by performing the separate M13-fingerprint experiment. These fingerprints were analyzed based on the

same criteria by an expert in the M13-leader Laboratory as blind study (data not shown). The clusters of both phylogenetic trees showed indifference. This indicated that the M13-fingerprint in this study is consistence and these isolates can be representative to the further analysis by MLST study.

MLST is a power DNA-typing method for the evaluation of intra-species genetic relatedness. It relies on DNA sequence polymorphism analysis in certain genes and has also shown a high discriminatory power for bacterial and fugal pathogen (17). The first evident to apply MLST technique in *C. neoformans* species complex was the outbreak study in British Columbia, Vancouver, Canada (121). The result revealed that the parents of the *C. gattii* outbreak strain were from endemic areas, Australia and South America. These parents had the same sex mating alpha. This finding is indicated that MLST is able to reveal the epidemiology and also the origin of the strain. From this MLST study, nine DNA fragments were amplified in the certain region of the eight genes of house keeping and virulence genes from each strain. After analysis of eight gene's sequences, it was demonstrated that the *Actin I* fragment showed the most variable site whereas the CAP59 fragments (*CAP59* primer) was the least variation one. Regarding CAP59 gene, two regions using CAP59 primers and CAPCN59 primers were tried. With the latter primers CAP59CN more variable of allele type (AT) was detected or high discriminatory power was demonstrated.

Hence, the selection of the gene is one of the most significance factors for MLST, affecting the demonstration of the polymorphic site. It was mentioned that the optimal gene-number for MLST study is 5 to 10 genes (18). Among 9 gene fragments in this study, the AT from *CAP59* revealed only 2 types whereas *CAP59*CN showed the highest, 6 allele types. In contrast, the number of AT from the remaining genes,

including *ACT1*, *PLB1*, *LAC1*, *IGS1*, *SOD1*, *TEF1* and *URA5* varied from 3-5 types. After combine all these ATs of these eight genes, the genetic variation in *C. neoformans* var. *grubii* Thai population in this study is shown. Moreover, this is the first MLST technique exploring the genetic character of *C. neoformans* Thai isolate from Thailand.

Each step of MLST technique was based on the common sequencing protocol, thus all the obtained informatics data are able to share with those from other studies in both national level and global level. As the advantage of this technique, the data in this study was subjected to analyze with the MLST global network website data (http:www.mlst.net , which derived from worldwide 93 *C. neoformans* var. *grubii* isolates (122). It is required to mentioned that MLST using the combination of eight genes in this work was finished prior to the publication of the consensus MLST which claimed six genes are qualified (*CAP59*, *IGS1*, *PLB1*, *LAC1*, *TEF1* and *SOD1*). The common genes are *ACT*1 , *CAP59*, *IGS1*, *PLB1*, *LAC1*, *TEF1*, *SOD and URA5*. Te result obtained from the analysis with the same genes showed thirteen new polymorphic alleles of *C. neoformans* var. *grubii*. This indicated that thirty one isolates in this study share certain genetic informations and clonality with the isolates from worldwide (Fig 31). It is of noted that 13 new polymorphic allele of *C. neoformans* var. *grubii* are also detected in this experiment.

Not only MLST method was performed, MLMT technique focused on genetic polymorphism along the regions of microsatellite, spreading through out the genome was also tried. Microsatellite is known as one of the genetic markers. This technique has been used to classify the genetic polymorphism in other fungi, such as *Penicillium marneffei, Candida albicans* (137). In terms of the discrimination of the repeating unit

variation in *C. neoformans* var. *grubii*, MLMT was used to classify the allele distribution and the allele specific to geographic area has been observed. Six isolates from Thailand was also included (23). The common allele types were found in isolates from worldwide excluding from Asia. It is interesting that among isolates from Asia have the specific geographic allele type. Another MLMT study similar ti Hanafy et al (23) was performed By Zhu et al. (136) to show the polymorphism of clinical and environmental *C. neoformans* var. *grubii* isolates. The analysis of MLMT can discriminate the isolates between clinical and environment sources. They also suggested that the possibility of human cryptococcal infection might derive from environmental source (136).

The same representative isolates of MLST were tested the ability to use microsatellite variations as the tool for epidemiology analysis. This study tried the detect the MA variation based on the length of each loci fragment composed of repeating region and the flanking sequences and the numbers of each repetitive microsatellite locus. Regarding the loci fragment size, the certain allele specific to indicate the variety, *C. neoformans* var. grubii –VNI and VNII was shown (Table 10 and Figure 32). However, this result is hardly demonstrate the relationship of the isolated sources and the MA (Figure 32) with the limited numbers of the isolates. The unique MA was revealed in all these loci. It is hardy to conclude that the certain allele specific found in some isolates, B7 and B31 was present only in these isolates. This allele might be the rarely microsatellite allele in *C. neoformans* var. *grubii* Thai isolates. As mentioned above that the number of repeating units has been reported as the criteria for MLMT analysis (24). This study also performed the analysis using this criteria too. Sixteen isolates represented from each MT classified by the other criteria

were subjected to do the sequencing. Only one of the five loci, CAA8, revealed the association of the repeating unit number and the locus size. Whereas another four loci showed the dissociation. This result is in agreement with Hanafy et al's study (24) that the allele size was affected from both different repetitive number and the variation of its flanking regions.

This study was compared three typing techniques, M13-fingerprinting, MLST, and MLMT to demonstrate the genetic variation of Thai *C. neoformans* var. *grubii* isolates. Comparing the cluster genotype grouping between M13-fingerprinting and MLST, the concordance was mostly demonstrated (Figure 33) except three isolates. The isolates C110 and B31was identified as subtype D and E, in order, by M13-fingerprinting whereas these two isolates were placed in the same cluster of MLST which composed of the isolates from M13-subtype A. Another isolate, B7 was classified as subtype B by M13-fingerprinting but was placed in same MLST cluster of subtype F based on RAPD method. In fact, B7 and A19 should be in the same group but this tree, they were separated as shown (Figure 33). Based on the MLMT phylogentic tree, B7 placed separately like MLST tree. On the other hand, all these mentioned isolates were arranged in concordance to the MLMT –tree. Only one isolate, S87, subtype D of RAPD technique was located in the separated cluster analyzed by MLMT (Figure 34). These three isolates, B7, B31, and S87 contained unique AT and/or MA. Even though these three typing techniques were demonstrating the concordance genotypic grouping of *C. neoformans* var. *grubii*, few isolates were classified in difference cluster but in the close neighbor position. These might be due to the distinct of the certain selected genetic markers in each typing technique. These studies were explored the MLST and MLMT techniques comparing the standard M13-

fingerprinting technique to analyze the genotypic variation as one of the tool for epidemiology study in Thai *C. neoformans* var. *grubii*. However, to clarify few puzzles as mentioned, more isolates should be tested.

At this point, each typing technique has its pro and con and depending on the research questions (Table 12). The consideration criteria of the appropriate typing technique should cover the standard isolates, the experience personals, the available equipments, the analytical programs, the reproducibility of the results, the possibility to compare between laboratories, the standard data base and the power of discrimination. Up to the present, MLST has been purposed as the recommended typing method for *C. neoformans* var. *grubii* due to the established consensus network and the MLMT is another new approach to perform the research.

Focus on the environmental population, the isolates collected in the year 2003 and 2005 were included for this three typing methods. No distinct genotype was found. This indicated that the genetic variation in this source is independent to the period. Even though only two isolates were from the Northern part, these were also placed in the same major genotype which contained the Metropolitan isolates.

Beside the genetic polymorphism was performed, the genetic distance by MLMT data was additional explored to view the association between sources whether this yeast is the communicable agent and or the zoonotic agent. The value of genetic distance between two certain origins was calculated by Pairwise Population Matrix of Nei Unbiased Genetic Distance Here, to decrease the bias analysis, the data of molecular type of VNII were excluded. Clinical population was related to the environmental population (0.019) more than to animal population (0.333) and the animal group was far from the environmental one (0.408). It seems that this yeast is

possible to be the communicable infection source than to be the zoonotic source. However, the genotypic variation was found in the animal group than other groups. The strong evidence of the communicable infection agent is still ambiguous. As we know that the infection depends on the association between the causative agent and the host conditions. More patient demography and the higher number of the isolates might be the way to answer this hypothesis.It is still questions that which source is the origin of the infection and how the yeast transfers to the host and cause the pathogenesis.

The ideal genetic variation tool should contain these factors: reproducible profiles, low cost and less consuming the time and labor for processing, unambiguous in interpreting the result, possible to transform the data as e-database, making the network database (16, 18). Among the recently methods, MLST and MLMT are mostly touch these ideal requirements. Anyhow, the selection of both appropriate genes fragments and loci are necessary for this approach. The epidemiology structure of *C. neoformans* var. *neoformans* Thai isolated from clinical , veterinary and environmental sources were demonstrated including; *URA5*-RFLP, mating type analysis, M13-fingerpritning and also demonstrate the genetic variation using MLST and MLMT.

Table12. Advantage and disadvantage of three typing techniques, M13-fingerprinting, MLST and MLMT techniques

Techniques	Advantage	Disadvantage
M13-fingerprininting	1. Simple, laborious technique 2. Not require the specific equipment 3. In expensive 4. Visual /program analysis	6. Conditional variation error 7. In comparable between laboratories due to the method in analysis
MLST	1. Simple technique 2. Not required specific equipment 3. Established standard protocol 4. Reference data base 5. Established the global communication as e-network	1. Selection of the analytical gene (s) 2. Costly
MLMT	1. New approach for analytical study, criteriae, markers 2. Cost depend on the criteria of analysis	1. No consensus yet: difference loci and primers 2. The standard techniques –under established

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APPENDIX

1. Recipes for the buffers, media and solutions used in this thesis

LI-COR loading dye	Stop stock: 49 ml Formamide 20 mg Sodium Hydroxide 1 ml 0.5 M EDTA Dye stock 21 mg Fuchsin in 1 ml stop stock Loading Dye 500 ul dye stock 9.5 ml stop stock
Sabouraud agar (SAB)	10 g peptone 40 g glucose 15 g agar dH2O to make 1 L
10X TBE buffer	107.8 g Tris base 55 g Boric acid 7.4 g EDTA dH2O to make 1 L

2. List of reagents used in this thesis

Acrilamide	Biorad Laboratories, Hercules, CA, USA
Agar	Oxoid Ltd., Hampshire, England
	Difco, Beackton-Dikinson Microbiology system, Sparks, MD, USA
Agarose	LE, Promega Corp., Madison, WI, USA
Amplicilin	CAlbiochem-Novabiochem, San-Diego, CA, USA
Amplitaq DNA polymerase	Perkin elmer, Roche Applied Biosystems Division,Brachburg, NJ, USA
B-mercaptoethanol	Sigma Chemical Co., St. Louis, MO, USA
Boric acid	Ressearch Organics Inc., Cleveland, Ohio, USA
Bovine serum albumin	New England Biolabs, Beverly, MA, USA
Chloroform	Merk pty. Ltd., Kilsyth, VIC, Australia
dNTP	
Dimethyl sulfoxide	Ressearch Organics Inc., Cleveland, Ohio, USA
DNA ladder	1 kb ladder, GIBCO-BRL, Life technologies, Rockville, MD, USA
	100 bp
EDTA	Ressearch Organics Inc., Cleveland, Ohio, USA
Ethanol	Merk pty. Ltd., Kilsyth, VIC, Australia
Ethidium bromide	Sigma Chemical Co., St. Louis, MO, USA
Glycerol	Progen Industrie Ltd., Ipswich, QLD, Australia

IPTG	Promega Corp., Madison, WI, USA
Size standard IRDyeTM700/800	LI-COR inc., Lincoin, NE, USA
Isopropanol	Sigma Chemical Co., St. Louis, MO, USA
Magnesium acetate	Sigma Chemical Co., St. Louis, MO, USA
Mineral oil	Sigma Chemical Co., St. Louis, MO, USA
Phenol	
Primer supplies	Sigma Genosys, Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia
Restriction nuclease	New England Biolabs, Beverly, MA, USA
SDS	Biorad Laboratories, Hercules, CA, USA
Sodium chloride	Merck, Kilsyth, VIC, Australia
Sodium hydroxide	Merck, Kilsyth, VIC, Australia
TEMED	Biorad Laboratories, Hercules, CA, USA
Tris base	Ressearch Organics Inc., Cleveland, Ohio, USA
X-gal	Progen Industrie Ltd., Ipswich, QLD, Australia

3. List of kits used in this thesis

DNA purification kit	WizardTM DNA clean up system, Promega Corp., Madison, WI, USA
Gel extraction kit	GFX extraction kit, Amersham-Pharmacia Biotech Australia Pty. Ltd., Castle-Hill, NSW, Australia

PCR cloning kit	pGEM-T Easy vector systems, Promega Corp., Madison, WI, USA
PCR purification kit	QIAquick PCR purification kit, Oiagen GmbH Hilden, Germany
Plasmid miniprep kit	Qiagen plasmid miniprep kit, Oiagen GmbH Hilden, Germany

4. List of computer software and websites used in this thesis

BioLoMICs	
SAGATM Generation 2 version 2.1	LI-COR Inc., Lincoln NE, USA
BioEdit	
Sequencer	
PAUP version 4.0b 10 for Macintoash	Sinauer Associates, Sunderland, Massachusetts, USA
C. neoformans Sequence type	http;//www.cgrubii/mlst.net

3. List of laboratory supplies and equipments used in this thesis

0.5 and 1.5 ml polypropylene tube	Eppendrof-Netherler-Hinz GmbH, Humburg, Germany
Benchtop centrifuge	Eppendrof-Netherler-Hinz GmbH, Humburg, Germany
Eppendorf Concentratr, model 5301	Eppendrof-Netherler-Hinz GmbH, Humburg, Germany
LI-COR DNA sequencer Long redir 4200	LI-COR Inc., Lincoln, NE, USA
Dry block heater	Selby Scientific Ltd., Mulgrave Nth, VIC , Australia
Electrophoresis chamber	Biorad Laboratories, Hercules, CA, USA
	Jordan Scientific Co. Inc., Bloomington, IN, USA
Miniature pastel	Konte, Vineland, NJ, USA
Pipette	Pipetman, Gilson Medical Electronics, Villers-le-Bel, France
UV transluminator	Uniequip, Martinsreid, Munich, Germay
Thermocyclers	ThermoCycler model 480, Perkin Elmer, Roche, Applied Biosystems Division, Branchburg, NJ, USA
	Hybaid PCR Express, Hybaid Ltd., Middlesex, UK
Vortex	IKA Works, Wilmington, NC, USA

VITAE

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PUBLICATION

2008 — Multilocus microsatellite typing for *Cryptococcus neoformans* var. *grubii* Hanafy, Ahmed; **Kaocharoen, Sirada**; Jover-Botella, Alejandro, Katsu, Masakazu; Iida, Soji; Kogure, Takahisa; Gonoi, Tohru; Mikami, Yuzuru; Meyer, Wielannd , *Medical Mycology* 2008, Volume 46(7) ,685 – 696.

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