Molecular Epidemiology Reveals Genetic Diversity amongst Isolates of the Cryptococcus neoformans/C. gattii Species Complex in Thailand

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Abstract

To gain a more detailed picture of cryptococcosis in Thailand, a retrospective study of 498 C. neoformans and C. gattii isolates has been conducted. Among these, 386, 83 and 29 strains were from clinical, environmental and veterinary sources, respectively. A total of 485 C. neoformans and 13 C. gattii strains were studied. The majority of the strains (68.9%) were isolated from males (mean age of 37.97 years), 88.5% of C. neoformans and only 37.5% of C. gattii strains were from HIV patients. URA5-RFLP and/or M13 PCR-fingerprinting analysis revealed that the majority of the isolates were C. neoformans molecular type VNI regardless of their sources (94.8%; 94.6% of the clinical, 98.8% of the environmental and 86.2% of the veterinary isolates). In addition, the molecular types VNI (2.4%; 66.7% of the clinical and 33.3% of the veterinary isolates), VNIV (0.2%; 100% environmental isolate), VGI (0.2%; 100% clinical isolate) and VGII (2.4%; 100% clinical isolates) were found less frequently. Multilocus Sequence Type (MLST) analysis using the ISHAM consensus MLST scheme for the C. neoformans/ C. gattii species complex identified a total of 20 sequence types (ST) in Thailand combining current and previous data. The Thai isolates are an integrated part of the global cryptococcal population genetic structure, with ST30 for C. gattii and ST82, ST83, ST137, ST172 and ST173 for C. neoformans being unique to Thailand. Most of the C. gattii isolates were ST7 = VGIIb, which is identical to the less virulent minor Vancouver island outbreak genotype, indicating Thailand as a stepping stone in the global spread of this outbreak strain. The current study revealed a greater genetic diversity and a wider range of major molecular types being present amongst Thai cryptococcal isolates than previously reported.

Citation: Kaocharoen S, Ngamskulrungroj P, Firacative C, Trillo L, Piyabongkarn D, et al. (2013) Molecular Epidemiology Reveals Genetic Diversity amongst Isolates of the Cryptococcus neoformans/C. gattii Species Complex in Thailand. PLoS Negl Trop Dis 7(7): e2297. doi:10.1371/journal.pntd.0002297

Editor: Bodo Wanke, Fundação Oswaldo Cruz, Brazil, United States of America

Received January 15, 2013; Accepted May 23, 2013; Published July 4, 2013

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Funding: S. Kaocharoen was supported by Chulalongkorn University Graduate Scholarship to commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej. This work was also supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) #2/2551 to A. Chindamporn and S. Kaocharoen, a New Researcher Scholarship of CSTS, NSTDA Ministry of Science and Technology, to P. Ngamskulrungroj and an NH&MRC project grants #352303 and APP1031943 to W. Meyer. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The members of the Cryptococcus neoformans/C. gattii species complex are the causative agent of cryptoccocosis, which is a systemic mycosis, in a wide range of animals and humans [1–4]. Inhalation of infectious propagules (basidiospores or blastoconidia) are proposed to be the source of the infection [1]. C. neoformans comprises two varieties and three serotypes: C. neoformans var. grubii (serotype A), C. neoformans var. neoformans (serotype D) and a hybrid (serotype AD), whereas C. gattii comprises two serotypes, B and C [1,3].

Extensive surveys of the yeast have shown that the ecological niches of both species are different. C. neoformans has been associated worldwide with soil enriched with pigeon excreta and decaying wood [1,5,6]. On the contrary, C. gattii was until recently thought to be geographically restricted to tropical and subtropical regions and thought to be related mainly to eucalyptus trees [7–9]. Further environmental studies in South America and Asia pointed out several species of tropical trees as the natural habitat of C. gattii such as oiti (Licaria tomentosa), almond trees (Terminalia cathappa), cassia (Cassia grandis), potter tree (Ficus microcarpa) and Syzygium cumini [10–12]. A recent ongoing outbreak of C. gattii on Vancouver Island, Canada, a temperate area, indicated an environmental shift of this species [13]. Moreover, in contrast to previous known environmental sources of this species, C. gattii has been found in association with a number of native tree species (Douglas fir, alder, maple and Garry oak) on Vancouver Island rather than with eucalypt trees [13].

A number of molecular typing techniques have been used to study the molecular epidemiology of the C. neoformans/C. gattii species complex.
Author Summary

The most common fungal pathogen of the central nervous system in humans is Cryptococcus neoformans. The species complex, var. gattii, causes opportunistic infection in humans with normal or compromised immune systems. Cryptococcus neoformans is found in the environment and has been isolated from pet birds, a common cause of cryptococcosis in immunocompromised individuals. The molecular epidemiology of the species complex has been studied using various typing methods, such as random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and multi-locus sequence typing (MLST). These methods have been used to identify the genetic diversity of Cryptococcus neoformans var. gattii strains in Thailand, which are mainly the cause of cryptococcosis in immunocompromised individuals.

Methods

Strains and media

The 498 cryptococcal isolates were recovered from the culture collections of the Molecular Mycology and Mycobacteriology Laboratory, Faculty of Medicine, Siriraj Hospital, Mahidol University, the Mycology Unit Laboratory, King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, the Mycology Laboratory of the National Institute of Health, Nonthaburi, Thailand and the Molecular Mycology Research Laboratory, Westmead Hospital, Westmead, NSW, Australia (see Table S1). All strains were maintained in glycerol at −80°C.

Environmental sampling

The environmental strains were collected from pigeon droppings in Bangkok during the years 2002–2005 using a method previously described [26]. Briefly, the pigeon droppings were collected into sterile zip-lock bags from 21 districts in Bangkok and transferred to the Mycology Unit Laboratory, King Chulalongkorn Memorial Hospital for isolation and identification. The pigeon droppings were dissolved in 0.85% normal saline, vigorously vortexed, and centrifuged. The supernatant was diluted, then spread onto Sabouraud Dextrose Agar plates supplemented with 40 μg/ml chloramphenicol and incubated at 37°C and observed for growth of yeast colonies every day. The yeast colonies were transferred to new media and identified as C. neoformans var. gattii species complex and included into the study. Two additional environmental strains isolated from pigeon droppings were kindly provided from the mycology laboratory, Chiang Mai University. The strain information is listed in Table S1.

URAS5-RFLP

Genomic DNA was isolated as described previously [19]. The major molecular types were determined by URAS5-RFLP analysis as previously described [19]. Briefly, the URAS5 gene was amplified with the following primers URAS5 (5′ATGGTCCTCG-CAAGCCCTCGACTCCG3′) and SJ01 (5′TTAAGACC-TCTGAACACCGTACTC3′). The obtained amplification products were digested with the restriction enzymes HindIII and SmaI. The digested PCR products were visualized and compared to the reference strains on a 3% agarose gel after staining with ethidium bromide.

PCR-fingerprinting

PCR-fingerprinting was carried out as described previously [18] using the microsatellite specific primer M13 (5′-GAGGTTGCGGCGTTC3′). The PCR-fingerprinting profiles were visualized and compared on 1.4% agarose gels containing ethidium bromide using the 1D gel analysis module BioGalaxy in the software package BioMICS ver. 7.5.30 (BioAware, Hannut, Belgium). Strains with identical M13 PCR-fingerprints were grouped in the same M13 type (see Table S1).
MLST

VNI strains representative of the different M13 types identified by PCR-fingerprinting and all VNII, VNIV and VGII strains were chosen for MLST analysis. Using the ISHAM consensus MLST typing scheme, seven unlinked genetic loci, including conserved and variable regions of CAP59, GPD1, LAC1, PLB1, SOD1, URA5 and the IGS1 region, were amplified using the primers and amplification parameters described by the ISHAM Cryptococcal Working Group [21], sequenced and analyzed as reported previously [35,36]. To put the newly identified molecular patterns into context with previous Thai studies, sequences of additional strains of the C. neoformans/C. gattii species complex were retrieved from those studies [39,37] (see Tables S2 and S3). The previously published Thai sequence types [33] were downloaded from the mlst.mycologylab.org webpage. The generated sequences were manually edited using the software Sequencher 4.9 (Gene Codes Corporation, MI, USA) and aligned using Clustal W [38], part of the program Bioedit 7.0.9.0 [39]. The concatenated alignments were then imported to the program MEGA 5.09 [40] and analyzed using the neighbor-joining method with p-distance [41]. Bootstrap analysis [42] with 1000 replicates was used to estimate support for clades of the concatenate dataset. The genetic network analysis was performed using the software Network 4.5.1.6 (Fluxus Technologies Ltd., Suffolk, UK). All allele types and subsequently the combined sequence types were assigned using the ISHAM consensus database at mlst.mycologylab.org, as described previously [35]. All MLST sequences are deposited at mlst.mycologylab.org webpage. The sequences of the herein determined alleles of the seven MLST loci are deposited in GenBank (Table S4).

Reference strains

The following set of laboratory standard reference strains representing each of the eight major molecular types of the Cryptococcus neoformans/C. gattii species complex were used: WM 148 (serotype A, VNI/AFLP1), WM 626 (serotype A, VNII/AFLP1A), WM 628 (serotype AD, VNIII/AFLP2), WM629 (serotype D, VNIV/AFLP3), WM 179 (serotype B, VGI/AFLP4), WM 178 (serotype B, VGII/AFLP5), WM 779 (serotype C, VGIII/AFLP6), R265 (VGII/AFLP3), R272 (VGIIb) [19,35].

Patient data

Demographic and clinical data for each isolate, including isolation site and date, patient’s residence, age, gender and HIV status, were retrieved from the clinical records if they were available and are listed in Table S1. These isolates are anonymous and the data cannot be used to trace back to individuals.

Table 1. Distribution of the major molecular types among strains of Cryptococcus neoformans and C. gattii from different sources in Thailand.

| Source     | Molecular type | VNI | VNII | VNIV | VGI | VGII | Total |
|------------|----------------|-----|------|------|-----|------|-------|
| Human      | 365 (94.6%)    | 8 (2.1%) | 0 | 1 (0.3%) | 12 (3.1%) | 386 |
| Environment| 82 (98.8%)     | 0 | 1 (1.2%) | 0 | 0 | 83 |
| Animal     | 25 (86.2%)     | 4 (13.8%) | 0 | 0 | 0 | 29 |
| Total      | 472 (94.8%)    | 12 (2.4%) | 1 (0.2%) | 1 (0.2%) | 12 (2.4%) | 498 |

doi:10.1371/journal.pntd.0002297.t001

Table 2. Distribution of the M13 types among Thai VNI isolates.

| M13 type | Frequency |
|----------|-----------|
| A        | 426 (90.3%) |
| B        | 1 (0.2%) |
| C        | 3 (0.6%) |
| D        | 9 (1.9%) |
| E        | 1 (0.2) |
| F        | 32 (6.8%) |
| Total    | 472 |

doi:10.1371/journal.pntd.0002297.t002

Data analysis

Statistical analysis was performed using the SPSS software package ver. 18.0.0 (IBM, Armonk, New York). Unknown data were regarded as missing data and excluded from the calculations.

Results

Demographic data

Among the 498 strains collected, 386, 83 and 29 strains were from clinical, environmental and veterinary sources, respectively. Of the clinical strains, 68.9% were from male and 31.1% from female patients, with an average age of 37.97 years. A total of 405 C. neoformans and 13 C. gattii strains were studied. Most of the C. neoformans clinical strains were from HIV positive patients (88.5%). In comparison, only 37.5% of the C. gattii strains were from HIV positive patients. The clinical strains were collected from all areas of Thailand with most strains originating from Bangkok (47.3%) and the central part of Thailand (27.0%). From the clinical strains, 80% were recovered from CSF, 17.5% from blood and 2.5% from other sites. All environmental isolates were obtained from pigeon droppings. The most common site of cryptococcal isolation in veterinary cases was the nasal cavity of cats (72.4%). For further information, see Table S1.

Major molecular types

To examine the genetic diversity of Thai cryptococcal strains, the major molecular types were determined by M13 PCR-fingerprinting and/or URA5-RFLP analysis [19]. As seen globally [43], VNI was the most common molecular type among Thai human (94.6%), environmental (98.8%) and animal (86.2%) isolates, though less frequent, VNII, VNIV, VGI and VGII were also found (Table 1).

M13 PCR-fingerprinting of VNI Thai isolates showed six different subtypes

As the majority of VNI Thai isolates were reported previously to be clonal [33], M13 PCR fingerprinting analysis, which has shown to differentiate cryptococcal molecular subtypes in several previous studies [13,18,37], was performed to check for clonality amongst the collected Thai VNI isolates. The obtained PCR fingerprinting patterns were assigned with a M13 type. Six M13 types (A, B, C, D, E, and F) were identified amongst all studied isolates, with type A being the most common type identified (90.3%) (Table 2). The genetic diversity identified by M13 PCR-fingerprinting analysis suggested that the Thai isolates are more genetically diversified than previously reported [33].
Table 3. Allele types and sequence types of selected Thai cryptococcal isolates.

| Strain name | Mol. type | Source | M13 type | CAP59 | GPD1 | IGS1 | LAC1 | PLB1 | SOD1 | URA5 | ST |
|-------------|-----------|--------|----------|-------|------|------|------|------|------|------|----|
| 47-2158     | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| 47-4995     | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| 47-5055     | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| 47-5061     | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| DMST20763   | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| DMST20764   | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| DMST20765   | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| DMST20766   | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| DMST20767   | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| DMST20768   | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| MC-S-265    | VGII      | Human  | ND       | 2     | 6    | 10   | 4    | 2    | 15   | 2    | 7  |
| MC-S-115    | VGII      | Human  | ND       | 2     | 6    | 32   | 4    | 2    | 15   | 2    | 7  |
| A13         | VNI       | Animal  | A        | 1     | 1    | 1    | 4    | 2    | 1    | 5    | 4  |
| B1          | VNI       | Human   | A        | 1     | 1    | 1    | 4    | 2    | 1    | 5    | 4  |
| C110        | VNI       | Human   | A        | 1     | 1    | 1    | 4    | 2    | 1    | 5    | 4  |
| C121        | VNI       | Human   | A        | 1     | 1    | 1    | 4    | 2    | 1    | 5    | 4  |
| E1          | VNI       | Environment | A     | 1     | 1    | 1    | 4    | 2    | 1    | 5    | 4  |
| P17         | VNI       | Human   | A        | 1     | 1    | 1    | 4    | 2    | 1    | 5    | 4  |
| P4          | VNI       | Human   | A        | 1     | 1    | 1    | 4    | 2    | 1    | 5    | 4  |
| A25         | VNI       | Animal  | A        | 1     | 1    | 1    | 3    | 2    | 1    | 5    | 6  |
| B59         | VNI       | Human   | A        | 1     | 1    | 1    | 3    | 2    | 1    | 5    | 6  |
| CM1         | VNI       | Human   | A        | 1     | 1    | 1    | 3    | 2    | 1    | 5    | 6  |
| CM19        | VNI       | Environment | A    | 1     | 1    | 1    | 3    | 2    | 1    | 5    | 6  |
| S24         | VNI       | Human   | A        | 1     | 1    | 1    | 3    | 2    | 1    | 5    | 6  |
| T28         | VNI       | Environment | A     | 1     | 1    | 1    | 3    | 2    | 1    | 5    | 6  |
| C146        | VNI       | Human   | A        | 1     | 1    | 1    | 3    | 2    | 1    | 5    | 137 |
| A19         | VNI       | Animal  | B        | 1     | 1    | 10   | 3    | 2    | 1    | 1    | 31 |
| A17         | VNI       | Animal  | C        | 1     | 1    | 10   | 3    | 2    | 1    | 1    | 31 |
| A18         | VNI       | Animal  | C        | 1     | 1    | 10   | 3    | 2    | 1    | 1    | 31 |
| S87         | VNI       | Human   | D        | 1     | 1    | 1    | 3    | 4    | 1    | 1    | 3  |
| E12         | VNI       | Environment | D    | 1     | 1    | 25   | 3    | 2    | 1    | 1    | 77 |
| B31         | VNI       | Human   | E        | 1     | 1    | 1    | 4    | 2    | 1    | 5    | 4  |
| B2          | VNI       | Human   | F        | 1     | 3    | 1    | 5    | 2    | 1    | 1    | 5  |
| CM17        | VNI       | Human   | F        | 1     | 3    | 1    | 5    | 2    | 1    | 1    | 5  |
| E38         | VNI       | Environment | F    | 1     | 3    | 1    | 5    | 2    | 1    | 1    | 5  |
| C140        | VNI       | Human   | F        | 1     | 1    | 1    | 5    | 2    | 1    | 1    | 81 |
| 47-1104     | VNIi      | Human   | ND       | 2     | 9    | 14   | 8    | 11   | 12   | 4    | 40 |
| 47-7559     | VNIi      | Human   | ND       | 2     | 9    | 14   | 8    | 11   | 12   | 4    | 40 |
| 48-1350     | VNIi      | Human   | ND       | 2     | 9    | 14   | 8    | 11   | 12   | 4    | 40 |
| 48-1398     | VNIi      | Human   | ND       | 2     | 9    | 14   | 8    | 11   | 12   | 4    | 40 |
| 48-1643     | VNIi      | Human   | ND       | 2     | 9    | 14   | 8    | 11   | 12   | 4    | 40 |
| 48-1663     | VNIi      | Human   | ND       | 2     | 9    | 14   | 8    | 11   | 12   | 4    | 40 |
| 46-2852     | VNIi      | Human   | ND       | 8     | 10   | 15   | 8    | 12   | 3    | 11   | 42 |
| 48-2323     | VNIi      | Human   | ND       | 2     | 9    | 14   | 8    | 11   | 11   | 4    | 43 |
| A10         | VNIi      | Animal  | ND       | 2     | 9    | 14   | 8    | 11   | 11   | 4    | 43 |
| A11         | VNIi      | Animal  | ND       | 2     | 9    | 14   | 8    | 11   | 11   | 4    | 43 |
| A5          | VNIi      | Animal  | ND       | 2     | 9    | 14   | 11   | 11   | 16   | 15   | 172 |
| A6          | VNIi      | Animal  | ND       | 2     | 9    | 14   | 11   | 11   | 16   | 15   | 172 |
| CBS7816     | VNIv      | Environment | ND  | 17    | 21   | 28   | 19   | 14   | 1    | 20   | 126 |

ND = not done, ST = sequence type, Mol. = molecular, bold = new ST types of Thai isolates identified in this study.
doi:10.1371/journal.pntd.0002297.t003
Figure 1. Phylogram of the Thai C. neoformans isolates. Phylogram depicting the genetic relationships between the Thai C. neoformans isolates based on neighbor joining analysis of the concatenated seven ISHAM consensus MLST loci using the program MEGA 5.03. Bold numbers on the branches indicate bootstrap support above 75%. Underlined strain numbers indicate STs identified in a previous study [33]. C = clinical, E = environmental, V = veterinary.
doi:10.1371/journal.pntd.0002297.g001

* = unique ST of Thailand
underlined = identified in the previous study
Thai C. neoformans isolates are genetically diversified

To verify the obtained diversity and to enable comparison with previous studies, MLST analysis, which has a superior discriminatory power and reproducibility over M13 PCR-fingerprinting [37], was performed. MLST analysis was performed on representative strains of each M13 type from the VNI isolates (14 strains of M13 type A, 1 of M13 type B, 2 of M13 type C, 2 of M13 type D, 1 of M13 type E, and 4 of M13 F) and for all VNII and VNI isolates. Eight additional sequence types (STs), with ST3, ST31, ST77, and ST137 for VNI and ST40, ST42, ST43 and ST172 for VNII (Table 3, Table S1 and Figure 1), were identified amongst the studied C. neoformans isolates when compared with a previous report, which had identified the following STs: ST4, ST5, ST6, ST81, ST82, ST83, ST95 and ST141 for VNI; and ST173 for VNII [33]. Network analysis showed that the Thai C. neoformans isolates are an integral part of the global population structure of this species, with nine ST’s being unique to Thailand, but closely related to other global isolates (Figure 2).

Almost all clinical VGII isolates belong to the same ST as the low virulent Vancouver Island outbreak strain

As VGII isolates are the causative agent of the ongoing outbreaks on Vancouver Island, Canada and Pacific Northwest region of USA [35,44], MLST analysis was performed to determine the relationship of the Thai VGII isolates to the outbreak strains. Surprisingly, 11 out of the 12 VGII isolates were identical to the genotype of the low virulent Vancouver Island outbreak strains, VGIIb/ST7 (Figure 3). One isolate had a ST, which was unique to Thailand (ST30) (see Tables 3 and S1 and Figure 3).

Discussion

The obtained data concerning the demographics and the HIV status of the patients were in line with previous reports of cryptococcosis from Thailand [25,31]. Besides some missing demographic data it is clear from the available data that C. neoformans was the most common species identified among HIV positive patients, while C. gattii was mainly a primary pathogen in immunocompetent patients, which is in accordance with a previous global study [18]. The fact that most isolates were recovered from male HIV positive patients with an average age of infection of 37.97 years represents the HIV demography in Thailand, with 60% of the HIV infected patients being male with an average age of 30–34 years [45].

C. neoformans has been found worldwide, with VNI being the most common molecular type, including recent reports from...
Thailand [33]. The molecular typing in the current study confirmed this paradigm, where VNI is predominant regardless of the isolate source. Moreover, the rare molecular type VNII, for which only one isolate had been reported previously from Thailand [33], has now been identified from several strains from both humans and animals. As the natural reservoir of VNII has never been reported, the herein presented data allow to suggest: that a close relationship between animal and human VNII isolates may exist, as strains from humans and animals share the same genotype, ST43 (Figure 1 and Table 3). However, further studies are needed to draw a definite conclusion as the numbers of the studied VNII strains are very small and other human VNII strains showed no relationships with animal strains. On the other hand, a strong relationship between VNI clinical and environmental strains is evident, as they share the same STs (Figure 1 and Table 3).

The correlation between C. neoformans and HIV in Thailand is supported by the low prevalence of the genotype VNIc/M5, corresponding to M13 type F in the current study, which is known to be associated with non-HIV patients in China [46], Korea [37] and Japan [36]. In fact, only 6% (23 out of 386 isolates) of the clinical cases were of M13 type F (VNIc/M5) (Figure S1) and only one of them (P21) had been isolated from a HIV positive patient. All other cryptococcal isolates form HIV positive patients had either the M13 type A or D (Table S1).

The herein obtained MLST data when combined with data previously reported [33] showed clearly, that the STs present in Thailand are an integral part of the global population genetic structure, and are not as unique as previously reported [33]. For the C. neoformans molecular type VNI, seven of the STs are shared with global strains and six are unique for Thailand (Figure 2). All of the isolates form a close network with a number of Thai specific STs and are directly linked to other globally present STs (Figure 2).

The current study describes for the first time molecular typing of C. gattii isolates from Thailand, taken into account the literature since the 1990’s [30,31]. The high percentage of the VGII molecular type (92.3%) amongst the studied C. gattii isolates is in contrast to a report from a neighboring country, Malaysia, where 76.5% of the C. gattii isolates belonged to the molecular type VGI [47]. No VGIII or VGIV isolates have been found in the current study and the fact that they have never been reported from this region may suggest that those molecular types are not endemic in this area. The geographically closest related place from which VGIII and VGIV isolates have been reported is India [43,48].

Before the AIDS epidemic, a predominance of C. gattii as the causative agent of cryptococcosis was found in Thailand, which was possibly related to non-HIV immunocompromised conditions [25,26]. A recent study on non-HIV cryptococcosis cases suggested that the disease was not such a rare event in HIV negative patients and is also associated with high mortality rates [49,50], a fact also seen with the cases of C. gattii infection investigated in the current study.

Moreover, the predominance of the VGII molecular type in this tropical region revealed in the current study is of special interest, as a similar situation was only described from the northern part of Brazil [51], which is in contrast to most of the described isolations.
which are associated mainly with areas of temperate climate or in the high mountain regions of Colombia [24,32,33]. In addition, the fact that several strains (the DMST strains) were isolated more than 10 years ago (Table S1), [30,34] suggests that this molecular type is prevalent in Thailand, as it is in South America [19], but unlike Australia [55] or Europe [56] where the molecular type VGI is predominant.

The fact that 11 out of the 12 C. gattii strains studied showed an identical ST type to the one of the Vancouver Island outbreak strain, VGIIb = ST7 is remarkable. It reveals the high clonality that this VGIIb C. gattii population has in Thailand, which is similar to the situation described in Australia [35]. It confirms the point previously made, that this low virulent outbreak strain is globally present, with Australia and Thailand being important stepping-stones in the global spread of this outbreak strain, linking South America, via Australia with North America and Europe.

In summary, as in other worldwide studies, the same distribution of cryptococcal genotypes has been found in Thailand, with a predominance of C. neoformans var. grubii, molecular type VNI, isolated from HIV positive patients. Our study suggests a greater genetic diversity among Thai cryptococcal isolates especially amongst VNI strains with 13 different STs than reported previously [33]. The majority of Thai C. gattii isolates are clonal and identical to the Vancouver Island outbreak strain with VGIIb = ST7, identifying Thailand as a stepping-stone in its global spread. In addition, a strong linkage between environmental and clinical strains was found for the VNI isolates. A connection between other rare molecular types, such as VNII for C. neoformans or VGI and VGII for C. gattii and the environment in Thailand could still not be found and needs further investigation. Extensive environmental and veterinary sampling would be of great help to fill this gap. Moreover, despite an advanced development of HIV treatment, cryptococcosis is still a major problem as an opportunistic infection in Thailand, making further studies, concerning the epidemiology and virulence of the Cryptococcus neoformans/C. gattii species complex mandatory for a proper management of the disease in the future.

Supporting Information

Figure S1 Phylogram correlating the newly identified Thai C. neoformans sequence types with previously reported types. Phylogram depicting the genetic relationships between the Thai VNI isolates studied herein in combination with previously published data representing the following M13 PCR-fingerprinting patterns VNIa, VNIb, VNIc/M5 and VNIc (32) based on neighbor joining analysis of the concatenated seven ISHAM consensus MLST loci. Bold numbers on the branches indicate bootstrap support above 75%. Letters in brackets indicate the M13 type. WM140 = VNI standard, WM626 = VNI standard.

Table S1 Strains used in this study and associated demographic and molecular data.

Table S2 Correlation between old and new allele and sequence type numbering from the Simwami et al. 2011 (28) publication and the new C. gattii MLST database at mlst.mycologylab.com for the MLST data used in the current study.

Table S3 MLST data for the additional published C. neoformans strains used in this study.

Table S4 GenBank accession numbers for all strains of the MLST alleles obtained from Thai C. neoformans and C. gattii isolates used in this study.

Acknowledgments

The authors would like to thank the staff and students of the Molecular Mycology and Mycobacteriology Laboratory, Faculty of Medicine Siriraj Hospital, Mahidol University and the Mycology Unit Laboratory, Faculty of Medicine, King Chulalongkorn Memorial Hospital for the preparation of the strains and providing the patient demographic data for this study and the Mycology Laboratory, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand for kindly providing the two environmental strains.

Author Contributions

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