

Role of *Cannomys badius* as a Natural Animal Host of *Penicillium marneffei* in India

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Infection by *Penicillium marneffei* in human immunodeficiency virus-positive patients in India has recently been described; the aim of our study was to survey wild rodents and their associated environment in order to identify the natural populations of this fungus. Surveys recovered *P. marneffei* from the internal organs of 10 (9.1%) of 110 bamboo rats (*Cannomys badius*) examined from Manipur state, India, an area endemic for penicilliosis marneffei. Identification of the isolates was based on a detailed study of their morphological characteristics, in vitro conversion to fission yeast form, and exoantigen tests. Multilocus microsatellite typing (MLMT) of the isolates revealed five genotypes. No genotypes were shared between sample sites, and all bamboo rats were infected with a single genotype within sample sites, demonstrating spatial genetic heterogeneity. One MLMT genotype was identical to that seen in a human isolate, suggesting that either coinfection from a common source or host-to-host transmission had occurred. This demonstrates the utility of an MLMT-based approach to elucidating the epidemiology of *P. marneffei*.

Penicillium marneffei is the only dimorphic species of the genus *Penicillium* and is the etiological agent of penicilliosis marneffei. This opportunistic fungal infection occurs among human immunodeficiency virus (HIV)-infected and other immunocompromised patients in several regions of southeast Asia. Areas where *P. marneffei* infection is known to be endemic include Thailand, southern China, Taiwan, Hong Kong, Malaysia, Indonesia, Viet Nam, Myanmar (Burma), and Manipur state in India (10, 11, 23, 27, 28). A single case of the disease in an African from Ghana, who had no history of travel to Asia, has also been described (21). Several cases of penicilliosis marneffei have been reported from Europe, the United States, Australia, and Singapore in patients who had prior history of visits to areas where the infection is endemic (17, 28).

Initial isolation of *P. marneffei* was from a captive bamboo rat (*Rhizomys sinensis*) used for laboratory experiments in South Vietnam (3). The native bamboo rat had been experimentally inoculated with the scrub typhus bacterium *Rickettsia orientalis* (now designated *Rickettsia tsutsugamushi*). At autopsy, the rodent was found to have an enlarged liver and spleen, viscous ascitic fluid, and epiploic nodules. Cultures from all the organs yielded a *Penicillium* species, which proved pathogenic to hamsters (*Mesocricetus auratus*). The fungus was subsequently described as a new species by Segretain (25), who named the fungus *Penicillium marneffei* in honor of Hubert Marneffei, then-director of the Pasteur Institute in French Indochina. In later decades, several workers investigated the prevalence of *P. marneffei* in bamboo rats in different geo-

graphic areas (1, 5, 9, 20, 29, 33). Four species of bamboo rats, *R. sinensis*, *Rhizomys pruinosus*, *Rhizomys sumatrensis*, and *Cannomys badius*, were identified as natural hosts of *P. marneffei* (1, 5, 9). The aim of the present study was to investigate whether *P. marneffei* occurred in bamboo rats in Manipur state, an area where several human cases of penicilliosis marneffei have recently been described (23, 27).

MATERIALS AND METHODS

Description of the study area. The sites where bamboo rats were trapped are located in the Senapati district, about 62 km north of the state capital of Imphal, and the Tamenglong district, approximately 150 km northwest of Imphal in Manipur state (Fig. 1). Manipur is situated from 23.80°N to 25.68°N and from 93.03°E to 94.78°E; it is predominantly a hill state in the northeast of India, with an elevation ranging from 800 to 3,000 m above mean sea level. Geologically, the region is a part of the Manipur-Nagaland orogenic belt, which in turn forms the northern part of the Indo-Burmese range. The region has a subtropical climate, with temperatures ranging between a mean maximum of 32°C and a mean minimum of 0°C. The rainy season lasts from May to October, with an average rainfall of 1,980 mm. The Senapati district is located from 93.29°E to 94.15°E and 24.37°N. The climate is humid and subtropical, with an annual rainfall ranging from 671 to 1,454 mm. The temperature ranges from a minimum of 3.36°C to a maximum of 34.14°C. The Tamenglong district is situated from 93.30°E to 24.59°N. It is entirely composed of hills, ranges, and narrow valleys (altitude, 1,260 m above mean sea level). The temperature ranges from a minimum of 4°C to a maximum of 31°C. The climate is humid and subtropical with heavy rainfall, with an annual mean rainfall of 3,135 mm. Tropical evergreen forest, subtropical forest, and virgin forest are represented in this district. The soil in both districts is clay loam with patches of clay and loam soil. The predominant species of bamboo trees in these places are *Bambusa arundinacea* and *Melocanna bambusoides*. The bushy bamboo species commonest in the Senapati and Tamenglong districts are *Cephalostachyum capitatum* and *Bambusa tulda*, respectively.

Investigation of rodents and soil samples. Professional trappers were contracted to capture the bamboo rats. One hundred and ten bamboo rats of the species *C. badius*, the so-called bay bamboo rat, were trapped on bamboo plantations in Manipur state and subsequently examined. Thirty-five of the animals were trapped on two different bamboo plantations in the Senapati district. The remaining 75 rats were trapped on four different bamboo plantations in the

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FIG. 1. Map of Manipur state, showing the locations of the Senapati and Tamenglong districts where bamboo rats were trapped. Bamboo rats were trapped on four separate bamboo plantations within the Tamenglong district.

Tamenglong district. All the captured rats were brought to the laboratory, euthanized within 1 to 3 days with ether, and aseptically dissected. Portions of lungs, liver, spleen, and pancreas of the animals were removed and minced. These were then inoculated on multiple slopes of Sabouraud dextrose agar (SDA) and brain heart infusion (BHI) agar (HIMedia Laboratories, Mumbai, India), both supplemented with chloramphenicol (0.05 mg ml^{-1}). In 30 of the animals, portions of kidneys and intestines were also similarly processed for culture. Small portions of the internal organs of 25 of the animals were preserved in 10% formalin for histology. Cultures were incubated at 25 to 28°C for 3 to 4 weeks and periodically examined for growths suggestive of *P. marneffei* and other potentially pathogenic fungi. Pure cultures were obtained by subculturing onto SDA slants.

Seventy-two rodents of five other species, *Bandicota bengalensis*, *Rattus norvegicus*, *Rattus rattus*, *Rattus nitidus*, and *Mus musculus*, were trapped on bamboo plantations in Guwahati (Assam), Bara Pani, Umeam, near Shillong (Meghalaya), Imphal (Manipur), and Tandong (Sikkim), India. These species were all investigated for infection by *P. marneffei* by the methods described above.

Twenty-five soil samples collected from the burrows of *C. badius* were also examined for occurrence of *P. marneffei* by dilution plating and the mouse passage technique. Triplicate plates of SDA containing chloramphenicol (0.05 mg ml^{-1}), mold inhibitory agar, yeast extract phosphate agar, and Dichloran Rose Bengal agar (HIMedia Laboratories) supplemented with chloramphenicol (0.05 mg ml^{-1}) were streaked with 0.2-ml quantities of a 1:10 dilution of the suspension of each soil sample. The mouse passage technique was essentially as that described by Beneke and Rogers (2). Three male, 4-week-old Swiss mice were injected intraperitoneally with 0.5-ml quantities of a suspension from each environmental sample. The mice were sacrificed after a period of 4 to 5 weeks, and portions of their internal organs (lungs, liver, and spleen) were cultured on multiple slopes of SDA and BHI agar containing chloramphenicol. Ten samples each of bamboo leaves and shoots were collected from the sites where bamboo rats were trapped and were similarly processed. All the inoculated plates and/or slopes were incubated at 28°C for a period of 3 weeks. Fungal colonies growing in the cultures were purified by subculture and subsequently identified. An additional 120 soil samples were collected and screened from the burrows of other rodent species and from non-rodent-associated sites in bamboo plantations.

Identification of isolates. Isolates were identified as *P. marneffei* by a detailed examination of their gross and microscopic features. Identification was con-

TABLE 1. Prevalence of *P. marneffei* in bamboo rats (*C. badius*) in Manipur state, India, from June 2002 to April 2003

Mo and yr	Locality	No. of rats investigated	No. of rats (% testing positive)
June 2002	Senapati district	8	0
November to December 2002	Senapati district	15	2 (13.3)
January 2003	Senapati district	12	0
February 2003	Tamenglong district	14	0
March 2003	Tamenglong district	48	7 (14.6)
April 2003	Tamenglong district	13	1 (7.7)
Total		110	10 (9.1%)

firmed by (i) in vitro conversion to the fission yeast form on BHI agar (HIMedia) at 37°C and (ii) the exoantigen test. For the latter test, the antigenic extract from 1-week-old SDA slant cultures of each isolate was tested by immunodiffusion against rabbit anti-*P. marneffei* serum with the appropriate controls (26). Proteinase activity of mycelial and yeast forms of all the isolates was tested with 0.4% albumin as a substrate, by using the method of Ruchel et al. (24) with minor modifications recommended by Chakrabarti et al. (4). Mycelial form and yeast form cultures for testing for proteinase were incubated for 10 days at 28 and 37°C, respectively. Both mycelial and yeast forms of all the isolates were also tested for urea hydrolysis with Christensen's urea agar (8). Gelatin and casein hydrolysis was tested by standard procedures (2). Three isolates of *P. marneffei* from cases in Thailand that were obtained from A. Chindamporn and N. Vanittanakom were included in each test series.

Multilocus microsatellite typing (MLMT) of isolates. DNA was extracted from 7-day-old cultures of each isolate as described previously (32). Multilocus genotypes for each isolate were then generated by scoring polymorphisms at 23 microsatellite-containing loci by the protocol described previously (13). Briefly, 1 μl of a 1/10 dilution of the DNA from each isolate was amplified with the QIAGEN Multiplex PCR kit with a working primer concentration of 0.2 μM . Cycling conditions were as follows: 95°C for 15 min; followed by 35 cycles each consisting of 94°C for 30 s, 57°C for 90 s, and 72°C for 60 s; followed by a final extension step of 60°C for 30 min. Subsequently, the PCR products were electrophoresed through a capillary sequencer with a POP6 gel and a ROX-500 internal size standard (Applied Biosystems). Alleles were scored using Genotyper software (Applied Biosystems), and unique genotypes were then assigned a specific microsatellite type (MT) identifier according to the *P. marneffei* MLMT scheme (13). These multilocus genotypes are held in an SQL Server relational database at <http://pmarneffei.multilocus.net/>. Two clinical isolates, CBS 101038 and MT30, were also typed with the MLMT scheme. CBS 101038 was obtained from the Centraalbureau voor Schimmelcultures (CBS) and was recovered by A. Chakrabarti in 1998 from one of the first four autochthonous cases of penicilliosis marneffei detected in Manipur state (27). Isolate MT30 was isolated in 2000 from an HIV-positive patient in Chiang Mai, Thailand, by N. Vanittanakom.

RESULTS

Prevalence of *P. marneffei* in bamboo rats (*C. badius*). Ten (9.1%) of 110 *C. badius* rats examined over a period of 10 months (from June 2002 to April 2003) were positive for *P. marneffei* (Table 1). All the *P. marneffei*-positive bamboo rats appeared healthy. At autopsy, no gross lesions were observed on any of the internal organs of the rats. Two of the isolates were recovered from rats trapped in the Senapati district, and the remaining eight isolates were from rats trapped in the Tamenglong district in Manipur state. In 2 of the 10 positive rats, *P. marneffei* was cultured from the liver and spleen. Cultures of the liver, spleen, and pancreas from three rats yielded *P. marneffei*; cultures of the fungus from lungs, liver, and spleen of another two animals also produced the fungus. For the remaining three *P. marneffei*-positive animals, only spleen tissue cultures yielded the fungus. Histopathological examination

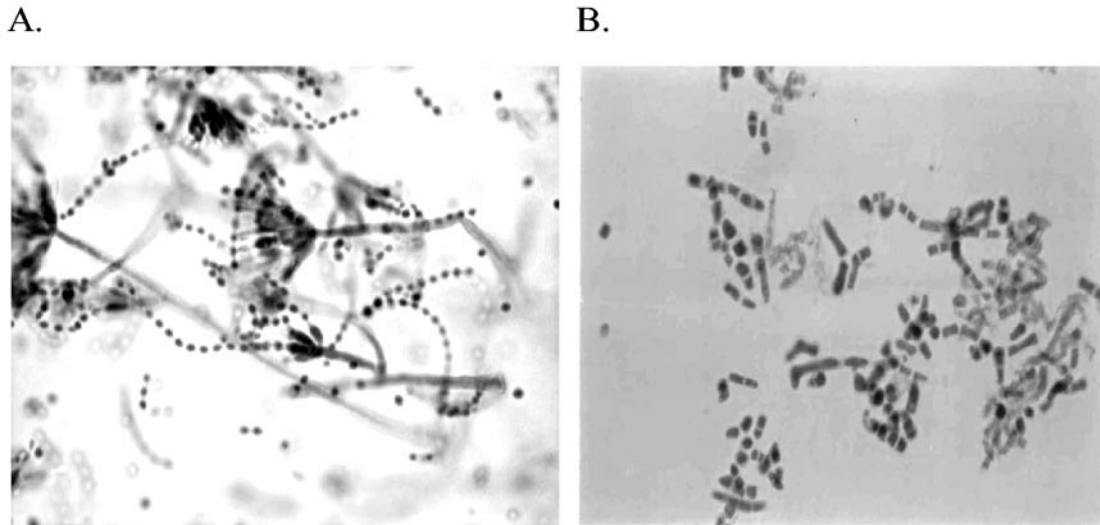


FIG. 2. (A) Lactophenol blue mount of 7 days' growth of *P. marneffeii* (isolate VPCI 214) showing characteristic biverticillate and monverticillate penicilli. Magnification, $\times 425$. (B) Lactophenol blue mount of 3 days' growth of *P. marneffeii* isolate (VPCI 214), showing fission yeast form. Magnification, $\times 425$.

of lungs, liver, and spleen of 15 *C. badius* rats (including 5 of the *P. marneffeii*-positive animals) did not reveal any fungal elements. Unfortunately, we were unable to perform histological analyses of the internal organs of the remaining 10 animals.

P. marneffeii was not recovered from any of the 25 samples of soil from the burrows of *C. badius* or from 10 samples (each) of bamboo shoots and leaves from the surrounding areas. Further sampling of soil samples from the immediate areas surrounding the burrows of bamboo rats and from the burrows of other rodent species (120 soil samples in total) were also negative for *P. marneffeii*.

None of the 72 rodents of the other five species (*B. bengalensis*, *R. norvegicus*, *R. rattus*, *R. niditus*, and *M. musculus*) trapped on bamboo plantations from other areas of northeast India were found to harbor *P. marneffeii*.

Characteristics of the isolates. The characteristics of the isolates were compatible with the standard description of *P. marneffeii* (1, 8, 10, 22). The diffusible red pigment could be easily observed on the reverse of the colonies in young cultures on SDA, later turning the entire medium wine red. Colonies of the isolates on corn meal agar (HIMedia) were relatively slow growing with reduced red pigmentation. Regarding the microscopic features, all the 10 isolates had both symmetrical biverticillate and monoverticillate penicilli (Fig. 2A) with the former generally predominant; occasionally, both types of penicilli were observed in the same branch in some of the isolates. Microscopic examination of growth on BHI at 37°C revealed cylindrical (arthroconidia-like) or ellipsoidal yeast cells, interspersed with a few hyphal fragments (Fig. 2B). The exoantigen extracts of all the isolates gave positive results, as evidenced by multiple precipitation lines of identity with rabbit anti-*P. marneffeii* antiserum and a reference antigen. The results were negative against antigenic extracts of *Aspergillus fumigatus*, *Aspergillus flavus*, and non-*marneffeii* *Penicillium* species. All the tested isolates of *P. marneffeii* demonstrated marked proteolytic activity in both mycelial and yeast form growth, as evidenced by

the zone of clearing of substrate (bovine serum albumin) around the growth. There was no significant difference in the proteinase activity of bamboo rat and human isolates. Results with the urease test were also positive in both mold and yeast forms of the isolates. However, the tests for the hydrolysis of casein and 10% gelatin were negative.

Genotypes of the isolate. MLMT analyses of the 10 isolates recovered five distinct multilocus MTs (Fig. 3). A single isolate, VPCI 230, was found to have a unique genotype. Otherwise, genotypes were shared by two or more isolates. Where multiple isolates were recovered from a single geographic area, all isolates were found to have identical multilocus genotypes (Fig. 3). No MT was recovered from more than a single trapping site, showing that genotypes are geographically patchy. However, one bamboo rat isolate, VPCI 233, had identical alleles at all 23 loci to the human clinical isolate from Manipur state, CBS 101038 (27).

DISCUSSION

The present report establishes the bamboo rat *C. badius* as a natural host of *P. marneffeii* in India. Earlier studies have shown that *P. marneffeii* naturally infects *C. badius* in Thailand. However, the prevalence of infection observed in the present study (9.1%) is much lower than that reported in surveys of *C. badius* in Thailand (1, 5, 29). *C. badius* rats are divided into two groups, primarily on the basis of the color of their fur, which is greyish black or reddish brown. A study by Chariyalertsak et al. (5) found that all 51 greyish-black *C. badius* specimens studied were negative for *P. marneffeii*, while 3 of the 10 rats (30%) in the reddish-brown group were positive for *P. marneffeii*. All *C. badius* rats investigated in the present study were reddish brown and are given a subspecies designation in the Indian zoological literature. In the present study, *P. marneffeii* was recovered more frequently from the spleen than from other organs. This contrasts with previous surveys of *C. badius* in

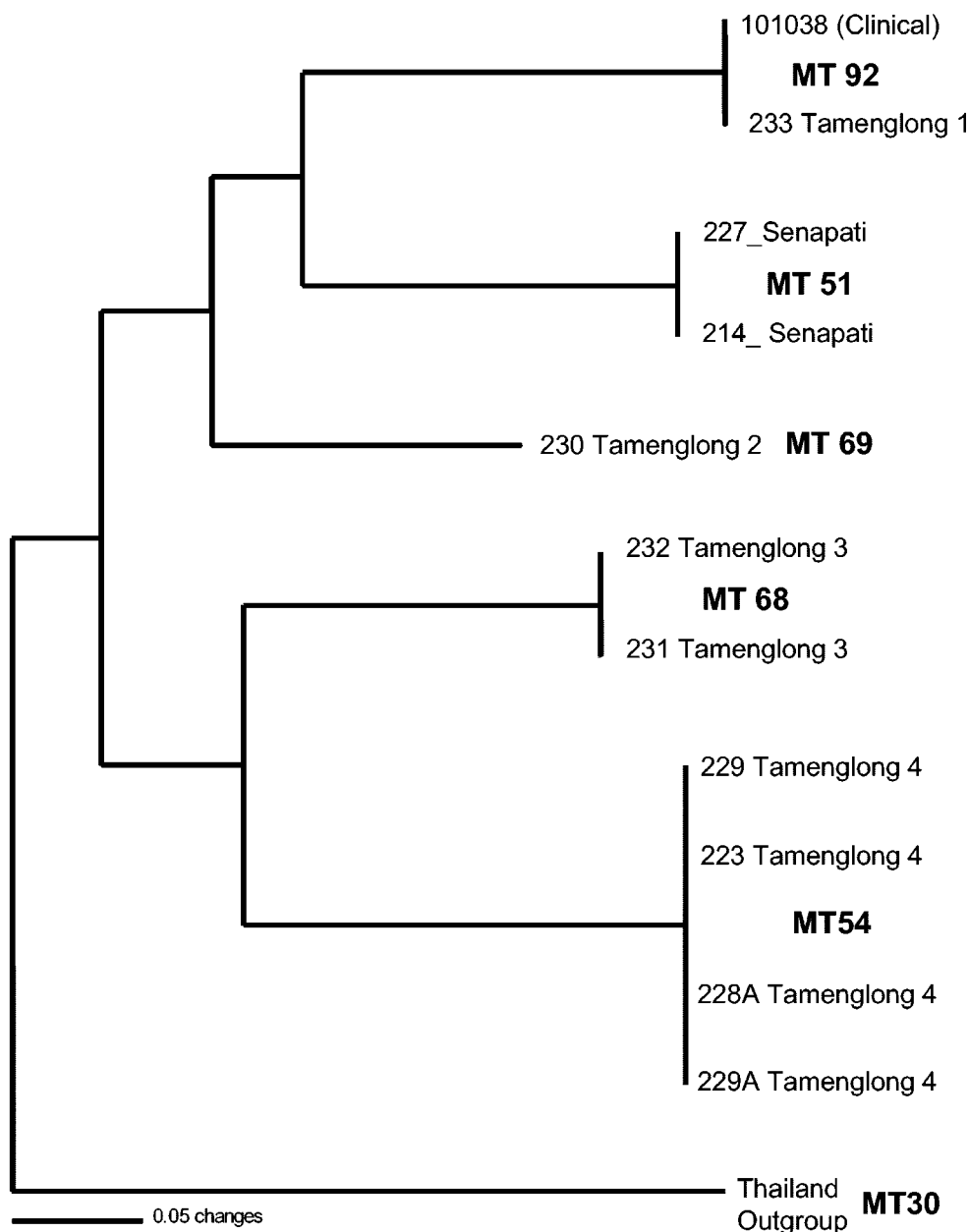


FIG. 3. Neighbor-joining tree showing the relationships between the 10 recovered isolates and the CBS 101038 clinical isolate. Numbers following the districts refer to the bamboo plantations where the bamboo rats were trapped.

Thailand, where the rate of isolation of the fungus was highest from lungs (1, 29).

The prevalence of infection in several bamboo rat species varies widely across their ranges, reviewed here in Table 2. *C. badius* is found in the states of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, and Nagaland, India, and also in Nepal, Bhutan, Bangladesh, Myanmar, Laos, Thailand, and northern Vietnam (7, 12, 19, 27). *R. sinensis* occurs from southern China to northern Myanmar through Thailand to Malaysia (19). The distribution of *R. pruinus* and *R. sumatrensis* ranges from southern China through Thailand to Malaysia, extending to Indonesia in the case of *R. sumatrensis* (19). *R. pruinus* is found in the Indian states of Assam, Manipur, Meghalaya,

Mizoram, and Nagaland, while *R. sumatrensis* is not found in India (7, 12). Geographical and pathological variations in the prevalence of infection in these species suggests that either there are regional variations in the endemicity of infection or there are geographic variations in the predisposition to infection within different species and subspecies of bamboo rats. It would be worthwhile to carry out comprehensive studies of the prevalence of *P. marneffe* in *C. badius* and *R. pruinus* in the northeastern and eastern states of India to further test these hypotheses. However, that we found no sign of infection by *P. marneffe* in the tissues of 72 rodents of five other rodent species is strongly supportive of the hypothesis that there are species-specific variations in susceptibility to *P. marneffe*.

TABLE 2. Prevalence of *P. marneffei* in bamboo rats in Southeast Asia

Country (province, state)	Species of rat (no. of rats examined)	No. (%) of rats positive	Reference
China (Guangxi, Churag)	<i>R. pruinus</i> (19)	18 (94.7)	9
China (Guangxi, Churag)	<i>R. pruinus</i> (179)	14 (63.7)	32
China (Guangxi, Churag)	<i>R. pruinus</i> (16)	15 (93.8)	19
Thailand (Kanchanaburi)	<i>C. badius</i> (31)	6 (19.4)	1
Thailand (Lopburi, Prachuap Khri Khan)	<i>R. pruinus</i> (8)	6 (75.0)	1
Thailand (Chiang Mai)	<i>C. badius</i> (10)	3 (33.3)	5
	<i>R. sumatrensis</i> (14)	13 (92.8)	5
Thailand (Chiang Mai)	<i>C. badius</i> (27)	6 (22.2)	28
	<i>R. pruinus</i> (8)	6 (75.0)	28
India (Manipur)	<i>C. badius</i> (110)	10 (9.1)	This study

Whether this observed variation in susceptibility to infection is driven by ecological, behavioral, or genetic factors awaits further investigation.

Several investigators have reported the use of various molecular techniques in the differentiation of *P. marneffei* strains. Vanittanakom et al. (32) employed restriction endonuclease with HaeII to differentiate *P. marneffei* isolates from northern Thailand. The 22 human isolates in that study were classified into two DNA types: type I comprised 16 (72.7%) isolates, and type II comprised 6 (27.3%) isolates. Of the 23 bamboo rat isolates, 20 isolates from *R. sumatrensis* were type I, and 3 isolates from *C. badius* were type II; the solitary soil isolate investigated was type II. In a study of 20 *P. marneffei* isolates from Taiwanese patients, Hsueh et al. (14) found eight distinct patterns by randomly amplified polymorphic DNA analysis, and two types (types I and II) based on chromosomal DNA restriction fragment-length polymorphism. Imwidthaya et al. (15) carried out fingerprinting of 30 *P. marneffei* isolates recovered from Chiang Mai in northern Thailand and Bangkok in central Thailand by single-nucleotide pattern primers [(GACA)₄] and the phage M13 core sequence. Four types were distinguishable, based on differences in the major bands: type A (6.7% prevalence in Chiang Mai), type B (6.7% prevalence in Chiang Mai), type C (3.3% prevalence in Bangkok), and type D (83.3% prevalence in both Chiang Mai and Bangkok). A separate study analyzing genomic DNA of 64 *P. marneffei* isolates from different regions of Thailand by pulsed-field gel electrophoresis with restriction enzyme NotI (30) revealed two macrorestriction patterns (MPI and MPII) that could be grouped into nine subprofiles (MPIa to MPIf and MPIIa to MPIIc). No correlation between macrorestriction patterns of *P. marneffei* isolates and geographic region or specimen source was observed in these studies. Recently, a study by Lasker and Ran (18) using three microsatellite-containing loci suggested that there was geographic isolation between Chinese and Thai populations of *P. marneffei*.

In the present study, MLMT of the 10 isolates of *P. marneffei* from *C. badius* revealed five genotypes, thus showing that genetically diverse isolates have infected these animals. Due to the large number of microsatellite loci typed ($n = 23$) and the multiallelic nature of the microsatellites (mean number of alleles locus⁻¹ = 5), the chances of finding identical genotypes by chance alone within a recombining population are vanishingly small ($\sim 5^{23}$) (13). However, that we see isolates with identical genotypes within this data set suggests that there is extensive clonal reproduction within the local fungal popu-

lations. The *Talaromyces* sexual stage (teleomorph) that characterizes other biverticilliate *Penicillium* species has never been observed for *P. marneffei*, and this suggests that there will be a strong asexual component to the population structure of *P. marneffei*. Our data are in concordance with this observation. Within our study populations, isolates with identical MT types invariably came from bamboo rats that were trapped at the same site. These data show that *P. marneffei* clones are spatially localized and that the fungal populations are not homogenized over the geographic distances studied here. This finding suggests that long-distance dispersal of *P. marneffei* spores is a relatively rare event; however, larger sample sizes are necessary to corroborate the generality of this finding.

The detection of a genotype that occurs in one of the *C. badius* isolates (VPCI 233) and one of the four human isolates (CBS 101038) of penicilliosis marneffei reported in Manipur state (27) suggests that either coinfection from a common source or host-to-host transmission has occurred. This is the first conclusive genetic evidence that bamboo rats and HIV-positive patients share genetically similar strains of *P. marneffei*. Hypotheses as to how the infection event occurred are at best speculative until the natural reservoir of *P. marneffei* is better understood. So far, it has not been established whether bamboo rats are important natural reservoirs for the transmission of *P. marneffei* to humans in areas of endemicity or whether the fungus resides in soil, and bamboo rats and/or humans are only incidental hosts. However, the evolution to a pathogenic lifestyle may have occurred if death of the host results in the production of large quantities of spores, thus increasing the lifetime reproductive success of the fungus. If this is the case, then bamboo rats may prove an important amplifying host for human *P. marneffei* infections.

The ecology of *P. marneffei* remains enigmatic. Deng et al. (10) isolated *P. marneffei* from three soil samples collected from the burrows of bamboo rats (*R. pruinus*). Chariyalertsak et al. (5) were able to recover *P. marneffei* from 1 (3.6%) of 28 soil samples collected from the burrows of the bamboo rat, *R. sumatrensis*. A case-control study performed in northern Thailand by Chariyalertsak et al. (6) did not indicate that bamboo rats were a reservoir of infection for humans. Rather, age (16 to 30 years) and an occupation involving exposure to plants or animals were found to be factors that were independently associated with an increased risk to infection by *P. marneffei*. These investigators postulated that soil exposure, especially during the rainy season, is a critical risk factor associated with penicilliosis marneffei. In the present study, *P.*

marneffeii could not be isolated from any of the 25 soil samples collected from the burrows of the bamboo rats (*C. badius*), nor could it be recovered from any of the 120 soil samples collected from burrows of other species of rats or from sites other than rodent burrows in bamboo plantations in different parts of northeast India. Vanittanakom et al. (31) demonstrated 80 to 85.1% recovery of CFU after 3 days of incubation from sterilized soil seeded with *P. marneffeii*; however, recovery from nonsterile soil seeded with the fungus was only 6%. A recent study has demonstrated that *P. marneffeii* can survive in sterile soil for several weeks but can survive in nonsterile soil for only a few days (16). Thus, definite evidence of the natural occurrence of *P. marneffeii* in soil is still lacking. Naturally infected bamboo rats may seed the soil with *P. marneffeii*, thus possibly creating new foci of the organism. Further investigations are required to determine the role of bamboo rats in the epidemiology of human infections due to *P. marneffeii*.

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