This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>

available at www.sciencedirect.com

journal homepage: www.elsevier.com/locate/funeco

Environmental detection of *Penicillium marneffe* and growth in soil microcosms in competition with *Talaromyces stipitatus*

Elizabeth PRYCE-MILLER¹, David AANENSEN^a, Nongnuch VANITTANAKOM^b,
Matthew C. FISHER^{a,*}

^aDepartment of Infectious Disease Epidemiology, Imperial College Faculty of Medicine, London, United Kingdom

^bDepartment of Microbiology, Microbiology Unit, Central laboratory, Faculty of Medicine, Chiang Mai University, Thailand

ARTICLE INFO

Article history:

Received 5 December 2007

Accepted 14 February 2008

Published online 18 April 2008

This manuscript is dedicated to the memory of Dr Pryce-Miller, 7th November 1976 to 11th September 2007.

Keywords:

Endemic

Environmental

Fungus

Mycosis

Penicillium

Quantitative environmental PCR

ABSTRACT

Penicillium marneffe is an endemic mycosis of humans in southeast Asia. Epidemiological data have shown that exposure to soil and season increase the risk of infection, and it is assumed that the main environmental reservoir is in soil. We sampled soils from a *P. marneffe* - endemic region of Thailand and confirmed by quantitative PCR and sequencing that *P. marneffe* DNA can be detected, a finding that we replicated over three sampling seasons. *P. marneffe*-positive and -negative sampling locations can be viewed using a dynamic browser located at www.spatial-epidemiology.net/pmarneffe. We subsequently examined the hypothesis that *P. marneffe* isolates representing the two major phylogeographic clades of this species can grow in: (i) soil and (ii) competition against the closely related species, *Talaromyces stipitatus*, in a model soil environment. *P. marneffe* was not detected in non-sterile soil microcosms 14 d post inoculation, showing that the pathogen is unable to compete against complete soil fauna under our laboratory conditions. However, both isolates of *P. marneffe* persisted and increased in biomass when inoculated into sterile soil. *P. marneffe* stably co-existed with *T. stipitatus*, and that the main competitive interaction was the inhibition of *T. stipitatus* growth at low spore application by the 'Eastern' isolate of *P. marneffe*. We conclude that *P. marneffe* is present in soils within endemic regions, and is able to grow in soil under certain conditions. More research is required to ascertain the specific conditions that regulate the growth of *P. marneffe* in soils in natural environments.

© 2008 Elsevier Ltd and The British Mycological Society. All rights reserved.

Introduction

Penicillium marneffe is a mitosporic (asexual) pathogenic fungus of the Trichocomaceae family, and is endemic to southeast Asia (Ajello et al. 1995). *P. marneffe* has emerged as a significant human mycosis since the 1980's, paralleling the increasing incidence of the Human Immunodeficiency Virus

(HIV) within this region. The genus *Penicillium* contains over 200 species, many of which have a world-wide distribution in soil or decaying vegetation (Pitt 1988). *P. marneffe* is, however, unusual for several reasons: (i) the fungus is highly endemic, only being found across a narrow band of tropical southeast Asia (Supparatpinyo et al. 1994); (ii) it is the only member of the genus that exhibits temperature-dependent

* Corresponding author. Department of Infectious Disease Epidemiology, Imperial College Faculty of Medicine, St. Mary's Campus, Norfolk Place, London W2 1PG, United Kingdom. Tel.: +44 207 594 3801; fax: +44 207 262 8140.

E-mail address: matthew.fisher@imperial.ac.uk (M.C. Fisher).

URL: <http://www1.imperial.ac.uk/medicine/people/matthew.fisher>

¹ Deceased author.

1754-5048/\$ – see front matter © 2008 Elsevier Ltd and The British Mycological Society. All rights reserved.

doi:10.1016/j.funeco.2008.02.002

dimorphic growth as an intracellular fission yeast; and (iii) it is the only member of the genus that can behave as a primary animal pathogen.

Whilst many studies have focused upon defining the ecotypes where certain *Penicillium* species dominate (Grishkan et al. 2003; Johansson 2001; Kjoller & Struwe 1982; Paul & Clarke 1970), similar studies have not been carried out for *P. marneffei*. It has been shown that a history of exposure to soil, especially in the rainy season, increases the risk of infection with *P. marneffei* (Chariyalertsak et al. 1997). It has only been cultivated once from a soil sample (Chariyalertsak et al. 1996), it is slow-growing, and this low rate of recovery may be due to the lack of a specific, selective, medium. The fungus has been found to naturally infect a high proportion of burrowing bamboo rats, an animal common to the region (Capponi et al. 1956; Chariyalertsak et al. 1996; Deng et al. 1986). However, it is not yet clear whether these bamboo rat infections represent an obligate stage in the *P. marneffei* life cycle. *P. marneffei* isolates with identical multilocus genotypes are shared between humans and bamboo rat species in Thailand and India (Fisher et al. 2005), showing bamboo rats are a possible zoonotic source for human infections (Fisher et al. 2004b). On the other hand, it is equally possible that bamboo rats and humans have become infected from a common environmental source. Determining the ability of this fungus to survive and compete in the natural environment is central to elucidating whether *P. marneffei* is a saprotrophic fungus, and is the central hypothesis that we test here.

Surveys have shown that *P. marneffei* is endemic to India, Thailand, the Guangxi region of China, Vietnam, Taiwan and Hong Kong (Singh et al. 1999). Analysis of clinical isolates from these regions using MultiLocus Microsatellite Typing (MLMT) (Fisher et al. 2004a), has determined the population genetic structure of this fungus (Fisher et al. 2004b). Extensive phylogeographic structure was identified showing two deeply divided clades, corresponding to Eastern and Western south-east Asia. Within the Western clade, genetic differentiation was observed between spatially separated populations (Fisher et al. 2005). A consequence of this finding is that clinical isolates can be readily assigned to a geographic source-population. As these different genotypes may represent niche-adapted lineages, we included a representative isolate from both the 'East' and 'West' clades in our experiments, to compare their growth responses.

P. marneffei is closely related to other *Penicillium* species within the subgenus *Biverticillium* (Pitt 1979), and the genus *Talaromyces* has been identified as the closest relative to *P. marneffei*, which can exhibit sexual biverticilliate states (LoBuglio & Taylor 1995). Of these, *Talaromyces stipitatus* is readily recovered from Thailand soils (Fisher, unpub. obs.). If niche-adaptation is important in generating the observed patterns of spatial genetic variation in *P. marneffei*, then it is likely that isolates from two different geographical regions, and representing both 'Western' and 'Eastern' clades, will show differing competitive abilities in soil. Further, if *P. marneffei* is a niche-specialist rather than an ubiquitous soil pathogen, then we can predict that it will perform poorly in competition with closely related fungal taxa.

This paper describes the detection of the pathogenic fungus *P. marneffei* from soils in Thailand. We then investigated

the growth potential of the two *P. marneffei* genotypes in soil from northern Thailand, and measured the ability of these genotypes to compete directly with *T. stipitatus* in soil microcosms. Relative growth of the fungi in our microcosms was measured using a rapid and reliable quantitative TaqMan probe-based quantitative PCR (qPCR) reaction (Haughland et al. 2004; Lotrario et al. 1995) following environmental DNA extraction. These experiments address an urgent need to better understand *P. marneffei*'s survival and ecology in soil as infection is an important cause of morbidity and mortality in HIV patients living or travelling in Southeast Asia.

Materials and methods

DNA sampling and extraction from soil

Soil samples were collected over 3 years from locations where cases of penicilliosis had occurred and from a range of ecotypes in Northern Thailand within a circle of radius 60 km, centered on Chiang Mai (lat. 17.885°, lon. 98.986°). Environments sampled included the environs of eight patients houses who had fallen ill with penicilliosis marneffei, different forest and grassland environments, and sites associated with animals, such as bat-caves and an elephant camp. From each site, 20 g of soil was taken from the 'A' subsurface soil horizon to a maximum depth of 5 cm. Total DNA was extracted from the soil samples using a modified flotation method (Larsh et al. 1953; Vanittanakom et al. 1995). Briefly, 30 ml of phosphate buffered saline (PBS) solution was added to 15 g soil in a 50 ml tube (Nunc GmbH & Co. KG, Wiesbaden, Germany). The tubes were agitated by vortexing for 10 min. The soil solution was then left to settle for 20 min at room temperature. As much supernatant as possible (approx 25 ml) was transferred with care to a sterile 50 ml tube and topped up to 30 ml with PBS solution. These soil extracts were centrifuged at 2400g at 4 °C for 10 min and the supernatant was discarded. The pellet was suspended in 250 µl of sterile, distilled water and introduced into a Bead Solution tube (UltraClean™ Soil DNA Isolation Kit, Mo Bio Laboratories, Inc., Carlsbad, USA). Samples were bead-beaten (MiniBeadBeater-8, Biospec Products, Bartlesville, USA) for 2 min in the presence of 60 µl of solution 1 and 200 µl of IRS solution (Biospec). The manufacturer's guidelines were then followed with the resulting DNA resuspended in 100 µl sterile, distilled water. These soil DNA extracts were subsequently stored at -20 °C. This protocol was followed for both environmental samples and soil microcosms.

Quantitative environmental real-time PCR

The qPCR assays were designed to target and amplify a 90 bp portion of the β -tubulin locus, and were designed by alignment of β -tubulin sequences from *Penicillium* subgenus *Biverticillium*. DNA sequences for this region for 50 *Penicillium* and *Talaromyces* species were provided by the Centraalbureau voor Schimmelcultures (CBS). The sequences were aligned using ClustalW in MEGA version 3.0 (Kumar et al. 2004) and Primer Express 2.0 (Applied Biosystems) was used to design genera specific primers and species specific probes for *P. marneffei* and *Talaromyces stipitatus*. Real-time quantitative PCR

utilised an internal probe which hybridised to a specific target sequence. The probe was labelled with two dyes, a fluorescent reporter at the 5' end, a nonfluorescent quencher and minor groove binder at the 3' end. The probes were designed adhering to the recommendations by Applied Biosystems so that there was less than 1 °C difference between the two probe melting temperatures; each probe was positioned as closely as possible to the 3' end of the TsF forward primer and the polymorphic site was placed in the central third of both probes.

We designed genera specific primer sequences as follows: TsF 5'CAAATCGGTGCTGCTTTCTG, TsR 5'CAGAGATGATTTGCCTGAAATAGTCA. The TaqMan probes that were used to discriminate the two species were: *P. marneffei* probe 5' VIC-C TCTCAACAAACACATCAC-MGB 3' and *T. stipitatus* probe 5' 6-FAM-TGCTTGGGTAACACTCA-MGB 3'.

The specificity of the MGB probes were tested by attempting to amplify the DNA extracted from several *Penicillium* and *Talaromyces* species: *T. stipitatus* (CBS375.48, USA); *T. derxii* (CBS412.89, Japan); *P. verruculosum* (CBS312.59, Japan); *P. purpurpogenum* (CBS286.36, Japan); *P. funiculosum* (CBS433.89, India) and *P. rubrum* (CBS184.27, USA). Amplifications were conducted in 96-well polypropylene plates on an ABI Prism 7300 (Applied Biosystems, Warrington, UK). Each 25 µl reaction contained 12.5 µl of Taqman Universal PCR Mastermix (Applied Biosystems), 900 mM of each primer (Eurogentec Ltd., Southampton, UK), 200 mM of each minor groove binding (MGB) probe (Applied Biosystems), 5 µl of a 1 in 10 dilution of the soil extract and 2.0 µl of de-ionised water. The amplification conditions were 50 °C for 2 min, 95 °C for 10 min and then 50 cycles of 95 °C for 15 sec and 1 min at 60 °C. Each soil sample was tested in duplicate, only being considered positive if the cycle threshold (Ct) value was under 45 cycles (Brancart et al. 2005). Standard curves based on Ct for a 10-fold dilution series of *P. marneffei* genomic DNA ($n = 3$) were developed. The standard curves were obtained by plotting the Ct threshold versus the logarithm of the concentration of each 10-fold dilution series. The dilution series ran over 4 orders of magnitude.

Isolates used in microcosm competition experiments

The following isolates were used: Western clade *P. marneffei* (CM; MMC47P11-5, Chiang Mai), Microsatellite Type (MT) = 10; Eastern clade *P. marneffei* (TW; MY3370, Taiwan), MT = 146 (<http://pmarneffei.multilocus.net/>); *Talaromyces stipitatus* (strain CBS375.48; Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). All isolates were grown on 2 % malt extract agar (Smith 1994) at 28 °C for 1 week. Spore dilutions were produced by scraping spores into 10 ml of sterile distilled water. Subsequently, 20 µl was added to a Neubauer chamber (Weber Scientific International Ltd, Teddington, England) and the number of spores counted under a microscope (Laborlex 12, Ernest Leitz Wetzlar, Germany). The average total number of *P. marneffei* spores ml⁻¹ for each isolate was calculated. The concentrated spore solution was then diluted appropriately, with sterile distilled water, to produce a dilution series of 10⁸, 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10², 10 and 0 spores for each *P. marneffei* isolate and 10⁶, 10⁵, 10⁴, 10³, 10², 10 and 0 spores for *T. stipitatus*.

Competition experiment design

Soil was collected from a single site outside the Maharaj Nakorn Chiang Mai Hospital, Chiang Mai (lat. 18.7899°, lon. 98.975°). The soil was sieved and processed by serial autoclaving. Sterilisation was at 121 °C for 1 h, followed by cooling at room temperature for 24 h, and then sterilised as before. This was repeated three times to ensure that any spores/enzymes stimulated by the first round of sterilisation would be destroyed in the second or third round (Bogan et al. 1996; Warcup, 2003).

Each microcosm comprised 15 g of sterile soil added to a Petri dish (Sterilin, Billingshurst, UK) and was inoculated with: 1 ml of the appropriate *P. marneffei* spore dilution (focal species), 1 ml of the appropriate *T. stipitatus* spore dilution (associate species) and the volume made up to 5 ml of liquid with the addition of sterile distilled water. The spore numbers used for both strains were 0, 10³, 10⁴ and 10⁵, co-inoculated with *T. stipitatus* at 0, 1, 10², 10³, 10⁴ and 10⁵ in all combinations, plus a control of no added spores of either species (Table 1). The competition experiments were performed in duplicate for each spore inoculum combination. The microcosms were all incubated at 28 °C in the dark for 14 d. As a non-sterile control, the same numbers of *P. marneffei* described above were inoculated in duplicate into air-dried, but non-sterile, soil to determine the ability of *P. marneffei* to survive 'naturally' in a soil microcosm that is complete with its normal complement of microbes.

Statistical analyses

We determined whether the increasing addition of *T. stipitatus* spores had either a negative or positive impact upon: (1) *P. marneffei* survival; and (2) final DNA concentration at the end of the experiment. To aid data interpretation the cycle threshold (DNA detection) values were transformed into spore equivalents. As observation that the amplification reactions in this study sometimes did not exhibit perfect exponential amplification due to the presence of inhibitors from the soil extracts, detection rates (Ct value, Table 1) from a spore dilution series amplified in the presence of soil ($t = 0$ d) were used to construct a standard curve plot in order to estimate fungal biomass increase as 'spore equivalents'.

Statistical tests were carried out using ANOVA in S-PLUS (Lucent Technologies 2003). Due to non-normal distribution of the experimental residuals, an inverse of the cube transformation was used to normalise the distribution of data.

Table 1 – Spore equivalent values for the two isolates of *P. marneffei* (CM, TW) after 0 and 14 d calculated from an extrapolated standard curve Fig 2

Spores inoculated (0 d)	<i>P. marneffei</i> CM, spore equivalents (14 d)	<i>P. marneffei</i> TW, spore equivalents (14 d)
10 ³	$9.8 \times 10^4 \pm 18\,000$	$9.7 \times 10^4 \pm 11\,000$
10 ⁴	$5.7 \times 10^5 \pm 1000$	$2.7 \times 10^5 \pm 27\,000$
10 ⁵	$8 \times 10^5 \pm 6600$	$3.8 \times 10^5 \pm 28\,000$

However, actual results using transformed and non-transformed data did not differ substantially and, in particular, there was little difference in the final levels of significance.

Results

Real-time primer and TaqMan® probe design and specificity

Generic primers TsF/R, were chosen from the alignment of β -tubulin sequences from biverticillate and terverticillate *Penicillium* and *Talaromyces* species. Using this primer pair to amplify genomic DNA from 9 isolates of *Penicillium* and *Talaromyces*, resulted in PCR products that were in all cases c. 90 base pairs consistent with the *in silico* predictions of 90 base pairs for *P. marneffei* and 91 base pairs for *T. stipitatus*. This size PCR product allowed species specific probes to be developed – *P. marneffei* (Pm_Btub) and *T. stipitatus* (Ts_Btub); there was no detection of the seven other *Penicillium* and *Talaromyces* species. A threshold cycle, Ct, value of zero was determined for the 5 isolates ($n = 9$ per isolate). Only *P. marneffei* and *T. stipitatus* were amplified with Ct values of 18.9 and 20.3, respectively.

Extraction, then amplification of DNA from soil samples that had been spiked with *P. marneffei* spores showed that DNA was successfully detected down to 10 spores ml^{-1} (Fig 1).

Environmental detection of *P. marneffei*

Of 137 sites tested for *P. marneffei*, seven were positive (5.1%). Of the positive sites, one of eight patient's homes – a sample from the base of a tree – was associated with a positive qPCR test. Correct detection of *P. marneffei* was confirmed in some cases by sequencing the amplification products for the intragenic (ITS) spacer region. In all cases, there was 100% homology with published *P. marneffei* ITS sequences (data not shown). The positive soil samples were from: (1) inside a cave populated by bats; (2) an elephant camp; (3) outside a patient's house; and (4) near a Buddhist Temple. Positive samples were recovered from Elephant-camp soils for each

Fig 1 – Detection of *P. marneffei* from a dilution series of soils spiked with 10^8 – 1 spores.

of the three sampling years (Fig 2; www.spatial-epidemiology.net/pmarneffei).

P. marneffei survival in sterile and non-sterile soil

Neither *P. marneffei* isolate could be detected, after 14 d, by real-time PCR ($n = 8$, for each isolate) when spores were inoculated into non-sterile soil, irrespective of the initial spore concentration (Fig 3). However, both *P. marneffei* isolates, CM (Chiang Mai) and TW (Taiwan), were detected in the sterile soil microcosms (Fig 3) after the same time. The lowest spore inoculum, 10^3 spores, was detected for both CM and TW isolates with similar Ct values ($\text{Ct}38.99 \pm 1.82$ for CM and $\text{Ct}39.80 \pm 1.14$ for TW) following 14 d incubation (Table 1).

Growth of *P. marneffei* CM and TW isolates in sterile soil with no added *T. stipitatus*

Increasing spore inoculum resulted in increased DNA detection (Fig 1). After 14 d of incubation, 9.8×10^4 (± 18000) spore equivalents were detected where 10^3 spores of CM had been inoculated, increasing to 8.2×10^5 (± 6600) spore equivalents for 10^5 spores of CM inoculated (Tables 2 and 3). Similarly, after 14 d, 9.7×10^4 (± 11000) spore equivalents were detected in microcosms inoculated with 10^3 *P. marneffei* TW spores. Microcosms inoculated with 10^5 spores of TW showed increased biomass equal to the detection of 3.8×10^5 (± 28000) spore equivalents. There was no significant difference ($P > 0.05$) in the biomass produced by *P. marneffei* CM and TW at equivalent spore inoculum concentrations (Fig 4A).

P. marneffei competitive response to increasing *T. stipitatus* spore concentrations

Real-time PCR detected both *P. marneffei* strains in all of the DNA extracts from the sterile microcosms to which they were added. The initial *P. marneffei* spore inoculum

Fig 2 – Map of the locations of sample sites around Chiang Mai, Thailand. ★ Site positive for *P. marneffei* by qPCR. ● Site negative for *P. marneffei* by qPCR. For dynamic colour map see: <http://www.spatial-epidemiology.net/pmarneffei/env/>.

Fig 3 – Detection of the *P. marneffei* isolates (CM – from Chiang Mai and TW – from Taiwan) 14 d after inoculation into sterile and non-sterile soils.

significantly effected the level of *P. marneffei* detection (CM $F_{1,112} = 37.7$, $P = 1.27 \times 10^{-8}$; TW $F_{1,77} = 1.3$, $P = 1.8 \times 10^{-5}$). The impact of *T. stipitatus* on the growth of the two *P. marneffei* isolates in soil, as estimated using ANOVA, was non-significant (CM $F_{1,112} = 37.7$, $P = 0.075$; TW $F_{1,77} = 1.3$, $P = 0.26$).

Growth of *P. marneffei* TW was reduced by 11 %, in the treatment with 10^5 *T. stipitatus* spores added compared to microcosms with no added spores, but this was not significant ($P > 0.05$). Both *P. marneffei* isolates persisted and increased in biomass in all microcosms.

T. stipitatus* response to increasing spore concentrations of *P. marneffei

ANOVA showed that initial spore concentration was the most significant factor influencing Ct end point biomass (CM, $F_{1,74} = 79.3$, $P = 2.53 \times 10^{-13}$; TW, $F_{1,81} = 29.6$, $P = 5.4 \times 10^{-7}$). When co-inoculated with *P. marneffei* CM, *T. stipitatus* was not detected in six of the treatments (Fig 4B). Fewer than 10 to 100 spore equivalents were present after 14 d of competition with *P. marneffei* CM.

Table 3 – Mean cycle threshold value for *T. stipitatus* at the five spore concentrations co-inoculated with the two isolates of *P. marneffei*

Number of <i>T. stipitatus</i> spores inoculated	<i>P. marneffei</i> isolate (Chiang Mai, CM)	<i>P. marneffei</i> isolate (Taiwan, TW)
10^1	0	39.4 ± 1.04
10^2	0	39.9 ± 1.46
10^3	40 ± 0.76	38.1 ± 1.42
10^4	36.67 ± 1.28	33.27 ± 1.42
10^5	32.70 ± 1.22	32.3 ± 0.90

T. stipitatus was however detected in all of the microcosms into which it was inoculated with *P. marneffei* TW (Fig 3). There was a significant ($P \leq 0.05$) decrease in the Ct value when over 10^6 spores were added to the microcosms. Detection of *T. stipitatus* occurred earlier in the microcosms which were inoculated with *P. marneffei* TW isolate (Table 2; $F_{1,155} = 4.6$, $P = 0.03$).

Discussion

Our results have shown that *P. marneffei* is clearly present in soils within Thailand, but as DNA was only detected in seven of 137 sampled environments, it was not possible to analyse specific associations between occurrence and environmental variables, or whether there was a non-random distribution of *P. marneffei* in the environment. However, it is worth noting that three of the seven sites are heavily used by animals (a bat cave, an elephant camp and a human habitation with a known history of *P. marneffei* infection), and in one site (the elephant camp) *P. marneffei* DNA was detected over several years. As *P. marneffei* is known to be associated with Bamboo rats, our observations are circumstantial evidence that the organism may be associated with a wider range of species than is currently known.

The molecular qPCR test that we have developed is shown here to be highly sensitive, detecting 10 *P. marneffei* spores ml^{-1} in spiked soil samples. However, in our environmental survey for the pathogen, we were not able to distinguish between live, viable *P. marneffei* conidia and inviable conidia. We attempted to culture the organism from PCR-positive sites, however, were not successful. The use of mRNA in reverse transcriptase PCRs is today enabling both the symptomatic and presymptomatic detection of viable

Table 2 – Cycle threshold (Ct) values and number of spores for the two isolates of *P. marneffei* (CM, TW) after 0 and 14 d in soil microcosms

Number of spores inoculated	<i>P. marneffei</i> at 0 d cycle threshold value	<i>P. marneffei</i> at 14 d		<i>P. marneffei</i> at 14 d	
		Ct value	Spores	Ct value	Spores
10^3	a	38.99 ± 1.82	$9.8 \times 10^4 \pm 18000$	39.80 ± 1.14	$9.7 \times 10^4 \pm 11000$
10^4	a	35.70 ± 1.04	$5.7 \times 10^5 \pm 10000$	37.63 ± 2.67	$2.7 \times 10^5 \pm 27000$
10^5	38.46 ± 0.86	34.06 ± 0.66	$8.2 \times 10^5 \pm 6600$	36.83 ± 2.84	$3.8 \times 10^5 \pm 28000$
10^6	32.9 ± 0.22	b	–	b	–
10^7	28.34 ± 0.51	b	–	b	–

a Not detected.

b Spore concentration not used.

Fig 4 – Quantification of A. *Penicillium marneffeii* and B. *Talaromyces stipitatus* when grown in direct competition in sterile soils after 14 d. (A). *P. marneffeii* grown in competition with 100 000 *T. stipitatus* spores. (B). *T. stipitatus* grown in competition with 100 000 *P. marneffeii* spores. CM = Chiang Mai *P. marneffeii* and TW = Taiwan *P. marneffeii* isolate.

fungal pathogens in plants (Gough & Lee 1985; McMaugh & Lyon 2003) and our future experiments aim to investigate cell viability by using *P. marneffeii*-specific probes to the mRNA of a constitutively-expressed gene such as beta tubulin (Yan & Liou 2006).

P. marneffeii is not very competitive with soil microflora and microfauna in non-sterile microcosms and DNA was below the limits of detection by 14 d after inoculation. The only other studies to date examining the efficacy of detecting *P. marneffeii* in seeded non-sterile and sterile soil used traditional culturing methods (Vanittanakom et al. 1995; Joshi et al., 2003). In Vanittanakom et al.'s study the recovery rate from 100 *P. marneffeii* spores in non-sterile soil was 6 % compared to 84.3 % from sterile soil, mirroring our finding that *P. marneffeii* does not flourish naturally in soil. As yet, no other published study has attempted to monitor *P. marneffeii* in soil using molecular means. However, a 2-fold reduction in fungal detection efficiency using real-time PCR was recorded when directly quantifying *Glomus intraradices* from non-sterile soil (Filion et al. 2003). Our study suggests that not only does *P. marneffeii* survival in non-sterile soil suffer, but so does qPCR efficiency. There are several reasons for the differences between sterile and non-sterile soil. Autoclaving alters the chemical composition of soil (Salonius et al. 1967), removes competition from soil organisms, makes available nutrient resources and

slightly raises the pH (Lotrario et al. 1995). In natural soils mycostatic and mycolytic factors commonly occur and are known to adversely affect the development of some human pathogenic fungi (McDonough 1963). Such factors may be negatively affected by the sterilization process. Further, in non-sterile soil microcosms, intact non-target soil micro-flora present may negatively influence real-time PCR amplification and in turn the levels of fungi detected (Filion et al. 2003).

In the present study, growth of *P. marneffeii* was readily detected in the sterile microcosms. Since the detectable level of inoculated soil at 0 d was 10⁵ spores, but after 14 d detection was possible in the microcosms to which 10³ spores were added, DNA extraction has increased 100-fold. This presumably reflects an increase in fungal biomass over the 14 d. *P. marneffeii* has also been isolated from inoculated sterile Thai soil in two other studies (Vanittanakom et al. 1995).

The model microcosm experiments demonstrated that, when in direct competition with a closely related Trichocomaceae species, *P. marneffeii* establishment and growth was not significantly affected. *T. stipitatus*, when added to the study system, did not elicit a negative competitive response from *P. marneffeii* CM, which was not competitively excluded from any of the microcosms. Although not statistically significant, it was noted that *P. marneffeii* TW did not increase in biomass as consistently when in the presence of a high concentration

of *T. stipitatus* spores. This is preliminary evidence that *P. marneffei* CM exhibited higher relative fitness in the Chiang Mai soil microcosms when compared to *P. marneffei* TW, although the effect was not statistically significant under our current levels of experimental replication. The data suggest that *T. stipitatus* was able to grow more effectively when in competition with *P. marneffei* TW than with *P. marneffei* CM.

However, it should be noted that the many studies on DNA extraction methods have highlighted the difficulty of consistent extraction efficiency and PCR processing, especially in relation to extractions from soil (Filion *et al.* 2003; Martin-Laurent *et al.* 2001; Miller *et al.* 1999; Yeates *et al.* 1999). Extraction efficiency varies due to factors such as adsorption to soil colloids (Frostegard *et al.* 1999) and the co-extraction of humic substances (Zhou *et al.* 1996). Whilst further optimisation of our experimental setup is required, such methodological variables should not impact on the findings of our study as all fungal microcosms were processed in an identical manner.

As both fungal species used in this study are closely related, it is likely that they share similar life strategies. Typically, *Penicillium* and *Talaromyces* include *r*-selected characteristics required for successful primary resource capture, namely good dispersal, rapid spore germination, rapid mycelial extension, and the ability to utilise organic substances available in previously uncolonised resources (Cooke & Rayner 1984). While, there was some negative interaction at low *T. stipitatus* spore concentrations, there were no dramatic competitive responses by either fungus. This may be explained by either: (1) the two fungi occupying different niches; or (2) sharing similar functional capabilities and initial resources important for colony establishment, but with their functional capabilities diversifying and separating with colony maturation.

P. marneffei exhibits highly structured populations (Fisher *et al.* 2005), and one mechanism by which this structure could have evolved is local adaptation to heterogeneous environments. This apparently, clonal asexual fungus co-colonised the soil microcosms successfully and flourished in the presence of a closely related competitor. We observed a significant effect of altering soil type on the growth of *P. marneffei*, however we were not able to do the reciprocal experiment using a 'typical' Taiwanese soil. Until this experiment is repeated with greater numbers of isolates from a larger number of regions, it is not possible to say whether our results have a general significance. However, our data raise interesting questions, and potential answers, as to why there are such deep divisions in the population genetic structure of *P. marneffei*.

Our finding that *P. marneffei* is unable to grow within an unsterilised environment suggests that its niche in nature may be more specific than that which we used here. The dispersal and growth of fungi strongly depends on environmental factors; among these soil type, rainfall, humidity are all expected to control germination and growth (Frostegard *et al.* 1999). Our finding that *P. marneffei* cannot survive in non-sterile microcosms is not a refutation of the soil microenvironment as a reservoir of the fungus. Rather, these data show that we need to consider wider ranges of environmental variables and spatial scales when investigating the metapopulation structure of these pathogenic fungi in nature.

In conclusion, this study has shown that *P. marneffei* is present in soil in endemic regions in Thailand, and that soil microcosms provide a tool by which the dynamics of its establishment, growth and resource partition can be monitored by quantitative molecular methods. Further studies on the ability of *P. marneffei* to function in more complex fungal communities, and in different environmental backgrounds, will provide a better understanding of the interplay between fitness, function and the population genetics on the metapopulation dynamics of pathogenic fungi.

Acknowledgements

The Taiwanese isolate of *P. marneffei* was supplied by PR Hsueh, β -tubulin alignments were provided by S de Hoog, Centraal Bureau voor Schimmelcultures, the Netherlands, and the research was funded by a Wellcome Trust Biodiversity grant to MC Fisher.

REFERENCES

- Ajello LP, Sukroongreung S, Nilakul CH, Tantimavanich S, 1995. Occurrence of *Penicillium marneffei* infections among wild bamboo rats in Thailand. *Mycopathologia* **131**: 1–8.
- Bogan B, Schoenike B, Lamar R, Cullen D, 1996. Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **62**: 2381–2386.
- Brancart F, Rodriguez-Villalobos H, Fonteyne P-A, Peres-Bota D, Liesnard C, 2005. Quantitative TaqMan PCR for detection of *Pneumocystis jirovecii*. *Journal of Microbiological Methods* **61**: 381–387.
- Capponi M, Sureau P, Segretain G, 1956. Penicilliose de *Rhizomys sinensis*. *Bulletin of the Exotic Pathology Society* **49**: 418–421.
- Chariyalertsak S, Vanittanakom P, Nelson KE, Sirisanthana T, Vanittanakom N, 1996. *Rhizomys sumatrensis* and *Cannomys badius*, new natural animal hosts of *Penicillium marneffei*. *Journal of Medical and Veterinary Mycology* **34**: 105–110.
- Chariyalertsak S, Sirisanthana T, Supparatpinyo K, Praparattanapan J, Nelson KE, 1997. Case-control study of risk factors for *Penicillium marneffei* infection in human immunodeficiency virus-infected patients in northern Thailand. *Clinical Infectious Diseases* **24**: 1080–1086.
- Cooke RC, Rayner ADM, 1984. *The Ecology of Saprotrophic Fungi*. Longman, London.
- Deng ZL, Yun M, Ajello L, 1986. Human *Penicilliosis marneffei* and its relation to the bamboo rat (*Rhizomys pruinosus*). *Journal of Medical and Veterinary Mycology* **24**: 383–389.
- Filion M, St-Arnaud M, Jabaji-Hare SH, 2003. Direct quantification of fungal DNA from soil substrate using real-time PCR. *Journal of Microbiological Methods* **53**: 67–76.
- Fisher MC, Aanensen D, de Hoog S, Vanittanakom N, 2004a. Multilocus microsatellite typing system for *Penicillium marneffei* reveals spatially structured populations. *Journal of Clinical Microbiology* **42**: 5065–5069.
- Fisher MC, de Hoog GS, Vanittanakom N, 2004b. A highly discriminatory Multilocus Microsatellite Typing System (MLMT) for *Penicillium marneffei*. *Molecular Ecology Notes* **5**: 231–234.
- Fisher MC, Hanage WP, de Hoog S, Johnson E, Smith MD, White NJ, Vanittanakom N, 2005. Low effective dispersal of

- asexual genotypes in heterogeneous landscapes by the endemic pathogen *Penicillium marneffeii*. *PLoS Pathogens* **1**: 159–165.
- Frostegard A, Courtois S, Ramisse V, Clerc S, Bernillon D, Le Gall F, Jeannin P, Nesme X, Simonet P, 1999. Quantification of bias related to the extraction of DNA directly from soils. *Applied and Environmental Microbiology* **65**: 5409–5420.
- Gough FJ, Lee TS, 1985. Moisture effects on the discharge and survival of conidia of *Septoria tritici*. *Phytopathology* **75**: 180–182.
- Grishkan I, Nevo E, Wasser SP, Beharav A, 2003. Adaptive spatiotemporal distribution of soil microfungi in 'Evolution Canyon' II, Lower Nahal Keziv, western Upper Galilee, Israel. *Biological Journal of the Linnean Society* **78**: 527–539.
- Haugland RA, Varma M, Wymer LJ, Vesper SJ, 2004. Quantitative PCR analysis of selected *Aspergillus*, *Penicillium* and *Paecilomyces* species. *Systematic and Applied Microbiology* **27**: 198–210.
- Johansson M, 2001. Composition of the saprotrophic fungi in Calluna heathland soil and the influence of ammonium nitrate application. *Water, Air and Soil Pollution: Focus* **1**: 231–239.
- Joshi A, Guhnani HC, Vijayan VK, 2003. Survival of *Penicillium marneffeii* in sterile and unsterile soil. *Journal of Medical Mycology* **13**: 211–212.
- Kjøller A, Struwe S, 1982. Microfungi in ecosystems: fungal occurrence and activity in litter and soil. *Oikos* **39**: 391–422.
- Kumar S, Tamura K, Nei M, 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150–163.
- Larsh H, Hinton A, Furcolow M, 1953. Laboratory studies of *Histoplasma capsulatum* III. Efficiency of the flotation method in isolation of *Histoplasma capsulatum* from soil. *The Journal of Laboratory and Clinical Medicine* **41**: 478–485.
- LoBuglio K, Taylor J, 1995. Phylogeny and PCR identification of the human pathogenic fungus *Penicillium marneffeii*. *Journal of Clinical Microbiology* **33**: 85–89.
- Lotrario JB, Stuart BJ, Lam T, Arands RR, O'Connor OA, Kosson DS, 1995. Effects of sterilization methods on the physical characteristics of soil: implications for sorption isotherm analyses. *Bulletin of Environmental Contamination and Toxicology* **54**: 668–675.
- Lucent Technologies. I. S plus. 2003.
- Martin-Laurent F, Philippot L, Hallet S, Chaussod R, Germon JC, Soulas G, Catroux G, 2001. DNA Extraction from soils: old bias for new microbial diversity analysis methods. *Applied and Environmental Microbiology* **67**: 2354–2359.
- McDonough E, 1963. Effects of natural soils on *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Allescheria boydii*. *American Journal of Hygiene* **77**: 66–72.
- McMaugh SJ, Lyon BR, 2003. Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. *Biotechniques* **34**: 982–986.
- Miller DN, Bryant JE, Madsen EL, Ghiorse WC, 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Applied and Environmental Microbiology* **65**: 4715–4724.
- Paul EA, Clarke FE, 1970. The microflora of grassland. *Advances in Agronomy* **22**: 375–435.
- Pitt JI, 1979. *The Genus Penicillium and its Teleomorphic States Eupenicillium and Talaromyces*. Academic Press, Inc., New York.
- Pitt JI, 1988. *Laboratory Guide to Common Penicillium Species*. Academic Press, London, New York, Toronto, Sydney, San Francisco.
- Salonius PO, Robinson JB, Chase FE, 1967. A comparison of autoclaved and gamma-irradiated soils as media for microbial colonisation experiments. *Plant and Soil* **27**: 239–248.
- Singh PN, Ranjana K, Singh YI, Singh KP, Sharma SS, Kulachandra M, Nabakumar Y, Chakrabarti A, Padhye AA, Kaufman L, Ajello L, 1999. Indigenous disseminated *Penicillium marneffeii* infection in the state of Manipur, India: report of four autochthonous cases. *Journal of Clinical Microbiology* **37**: 2699–2702.
- Smith DO, 1994. *The Preservation and Maintenance of Living Fungi*. In: *IMI Technical Handbook*, second ed, vol 2. CAB International, Wallingford.
- Supparatpinyo KC, Khamwan V, Baosoung KE, Nelson KE, Sirisanthana T, 1994. Disseminated *Penicillium marneffeii* infection in southeast Asia. *Lancet* **344**: 110–113.
- Vanittanakom N, Mekaprateep M, Sriburee P, Vanittanakom P, Khanjanasthiti P, 1995. Efficiency of the flotation method in the isolation of *Penicillium marneffeii* from seeded soil. *Journal of Medical and Veterinary Mycology* **33**: 271–273.
- Warcup JH, 2003. Soil steaming: a selective method for the isolation of ascomycetes from soil. *Transactions of the British Mycological Society* **34**: 515–518.
- Yan HZ, Liou RF, 2006. Selection of internal control genes for real-time quantitative RT-PCR assays in the oomycete plant pathogen *Phytophthora parasitica*. *Fungal Genetics and Biology* **43**: 430–438.
- Yeates CG, Davison AD, Altavilla N, Veal DA, 1999. Methods for microbial DNA extraction from soil for PCR amplification. *Biological Procedures Online* **1**: 40–47.
- Zhou J, Bruns M, Tiedje J, 1996. DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* **62**: 316–322.