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

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ABSTRACT

Penicillium marneffei 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. marneffei* - endemic region of Thailand and confirmed by quantitative PCR and sequencing that *P. marneffei* DNA can be detected, a finding that we replicated over three sampling seasons. *P. marneffei*-positive and -negative sampling locations can be viewed using a dynamic browser located at www.spatialepidemiology.net/pmarneffei. We subsequently examined the hypothesis that *P. marneffei* 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. marneffei* 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. marneffei* persisted and increased in biomass when inoculated into sterile soil. *P. marneffei* 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. marneffei*. We conclude that *P. marneffei* 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. marneffei* in soils in natural environments.

Introduction

Penicillium marneffei is a mitosporic (asexual) pathogenic fungus of the Trichocomaceae family, and is endemic to southeast Asia (Ajello *et al.* 1995). *P. marneffei* 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. marneffei* 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

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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; Kjøller & 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 southeast 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 (Iat. 17.885°, Ion. 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 2400*g* 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