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Occurrence and diversity of insect-associated fungi in natural soils in China

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ABSTRACT

In the present study, the occurrence and species diversity of insect-associated fungi in soil collected mainly from forest habitats in different regions of China were compared by using the '*Galleria* biat method'. Insect-associated fungi were defined to include known insect pathogenic fungi, opportunistic pathogens and secondary colonizers isolated from the *Galleria mellonella* bait insect exposed to the soil samples in question. Insect-associated fungi were detected in 55.5% of the 425 soil samples. A total of 377 fungi belonging to 46 species and 27 genera were isolated and identified. Among them, 6 species were known insect pathogenic fungi, 21 were opportunistic pathogens and 19 were secondary colonizers. Insect pathogenic fungi were most prevalent and *Paecilomyces farinosus*, *Beauveria bassiana* and *Metarhizium anisopliae* var. *anisopliae* (Hyphomycetes) were the most common species, comprising 19.6%, 14.1% and 10.6% of the total number of isolates, respectively. Opportunistic pathogens also had high occurrences in the soil with the percentage frequency added up to 36.9%. Among the opportunistic fungi, *Fusarium oxysporum*, *F. solani*, *Geomyces pannorum*, *Clonostachys rosea* f. *catenulata* and an unidentified *Fusarium* sp. resulted in the highest *G. mellonella* mortality in the preliminary pathogenicity test. Using principal component analysis, two components accounted for 76.5% of the total variance were extracted. Component 1 was positively correlated with species richness and species diversity and negatively correlated with the average altitude of the sampling region. Component 2 was negatively correlated with species evenness and positively correlated to the level of insect pathogenic fungi. The two-axis ordination of communities showed clear separation of the fungal community in South Central China, indicating higher occurrence of insect-associated fungi in the soil of subtropical humid region than the other regions.

1. Introduction

The soil habitat is considered as excellent habitat for insect pathogenic fungi and other microorganisms since it is protected from UV radiation and buffered against extreme biotic and abiotic influences (Keller and Zimmerman, 1989). Insect pathogenic fungi in the genera *Beauveria*, *Conidiobolus*, *Metarhizium* and *Paecilomyces* are all commonly found in the

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soil (Domsch et al., 1980). Many other fungal species have also been reported on diseased soil-inhabiting insects in various regions of the world and fungal epizootics in soil insect populations are also well documented (Samson et al., 1988; Keller and Zimmerman, 1989; Klingen and Haukeland, 2006).

To detect insect pathogenic fungi in soil, various selective media have been used (Veen and Ferron, 1966; Doberski and Tribe, 1980; Chase et al., 1986) which approximated the density of fungal propagules in soil. However, these selective media were developed only to several known species such as *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* var. *anisopliae* (Metschnikov) Sorokin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith. The '*Galleria* bait method' was first introduced by Zimmermann (1986) as a sensitive method to detect a broad spectrum of insect pathogenic fungi in soil samples. By comparing the two methods, Enkerli et al. (2004) found that *Beauveria brongniartii* (Sacc.) Petch isolates could be retrieved by '*Galleria* bait method' even when the fungus was at the level that could not be detected by using selective medium method. For those advantages, the '*Galleria* bait method' has been widely used in detection of insect pathogenic fungi in soil. The occurrence and distribution of insect pathogenic fungi in agricultural field soils have been extensively investigated in previous studies (Chandler et al., 1997; Bidochka et al., 1998; Ali-Shtayeh et al., 2002; Klingen et al., 2002; Keller et al., 2003; Meyling and Eilenberg, 2006). Some studies have also been conducted on the occurrence in adjacent hedgerows that are less affected by human cultivation activities (Klingen et al., 2002; Meyling and Eilenberg, 2006). These show a higher occurrence of insect pathogenic fungi in hedgerow and woodland soil. Higher density of insect pathogenic fungi was also founded in no-tilled soybean field soils than that under conventional tillage (Sosa-Gómez et al., 2001).

Other fungal species have also been found on insects in soil. During the study of the fungi from cadavers of the cave cricket *Troglophilus neglectus* Krauss (Rhaphidophoridae, Orthoptera), Gunde-Cimerman et al. (1998) found that *Mucor* spp., which have been considered as opportunistic pathogenic fungi, were isolated with the highest frequencies from the larval cadavers of *T. neglectus*. Species of the genera *Fusarium* and *Penicillium* have also been found in soil by using the '*Galleria* bait method' (Mietkiewski et al., 1991). Ali-Shtayeh et al. (2002) suggested that an essential weakly pathogenic fungus might at times become associated as the causal agent of epizootics in predisposed insects. Their preliminary pathogenicity test proved that isolates of the genera *Absidia*, *Aspergillus*, *Fusarium* and *Mucor* could kill the larvae of *Galleria mellonella* L. (Lepidoptera, Pyralidae). Actually, host insects in the soil are subject to challenge from many pathogen species. Avirulent pathogens have been reported to play significant role in insect-pathogen dynamics (Thomas et al., 2003). Therefore, we choose to include opportunistic fungi and secondary colonizers in our study of the naturally occurring fungi associated with insects in China. In using the '*Galleria* bait method', opportunistic pathogens can infect the weakened or wounded larvae during the baiting of soil samples and the secondary colonizers are those colonize the insect cadavers after the death of *G. mellonella*.

Gao et al. (1995) obtained *Beauveria* spp. and *Metarhizium* spp. from 78.7% of 47 soil samples collected in Northern China. This indicated high occurrence of insect pathogenic fungi in the soil in China. No detailed studies have been conducted on the occurrence of soil dwelling insect pathogenic fungi in China till this time. Further, the occurrence and diversity of insect pathogenic fungi in natural soils were not well documented. In this study, soil samples were collected in forestry and mountain habitats in different regions of China, the occurrence and distribution of insect-associated fungi were investigated by using the '*Galleria* bait method' (Zimmermann, 1986). To distinguish the relationship between the fungi and the insect, all fungi except the well-known insect pathogenic species were tested for their pathogenicity to *Galleria* larvae.

2. Materials and methods

2.1. Collection of soil samples

Soil samples were collected from different parts of China from the year 2003 to 2005. The samples were taken by cylindrical soil core borer (Φ = 20 mm) to a depth of 20 cm. Five columns of soil from an area about 5 m^2 were mixed as one sample. The samples were placed into plastic bags and stored at 4 °C. The samples were baited within 2 months.

A total of 425 soil samples originating from forests or mountains of 10 provinces in China were collected (Table 1). The sampling sites were grouped into six regions according to the climatic zone which they belonged to: Northern China included Dongling mountain and Wulingshan nature reserve; Western China included Linzhi, Mengda nature reserve and Beishan forestry center; Northeastern China included Liangshui national reserve and Changbai mountain; Middle-southern China included Tianmu mountain, Zhangjiajie national forest park and Jinggang mountain. Hainan Island included Wuzhi mountain, Jianfengling nature reserve and Diaoluo mountain; Himalayas, where soil samples were collected in Qomolangma Mountain above snow line.

2.2. Isolation and identification of fungi

Insect-associated fungi were isolated from soil samples by using the '*Galleria* bait method' (Zimmermann, 1986). The soil sample was passed through 2-mm pore sieve to remove plant tissues and molding gravels or blocks, and then mixed thoroughly and placed in a plastic bag. If the soil samples were too dry, they were moistened with sterile distilled water. Each of three autoclaved 50 mL centrifuge tubes was filled with soil of each sample and remained 1 cm of free air at the top of tube.

The wax moth, *Galleria mellonella* L. was reared continuously in constant darkness at 28 °C. The third or fourth instar larvae (approximately 30 days after hatching) were used as baits. Five larvae were placed on the soil surface in each tube and covered with lid, and then the samples were kept at the temperature of 20–25 °C for two weeks. During the first 4 days the tubes were upended twice a day to keep the larvae moving in the soil. The larvae were examined on 7th and 14th days after inoculation, respectively. Dead larvae were surface sterilized with 3% sodium hypochlorite for 3 min and then

Table 1 – Characteristics of sampling sites and regions

Region	Site[a]	Sampling time	Geographical location (lat. N, long. E)	Altitude (m)	Climatic zone
Northeastern China	LS	Sep/2004	47°10′N, 128°53′E	700	Mid-temperate humid zone
	CBM	Sep/2004	42°30′N, 127°53′E	1000	
Northern China	DLM1	Sep/2003	39°55′N, 115°25′E	2200	Warm-temperate subhumid zone
	WLS	May/2004	40°33′N, 117°25′E	2100	
Western China	BS	Aug/2004	36°30′N, 102°45′E	3600	Plateau climate zone
	LZ	Jul/2004	29°30′N, 94°43′E	3000	
	MD	Aug/2004	35°50′N, 102°40′E	3100	
South central China	JGM	Oct/2005	26°34′N, 114°10′E	1000	North subtropical humid zone
	TMM	Apr/2005	30°21′N, 119°23′E	1200	
	ZJJ	Sep/2005	29°20′N, 110°24′E	1200	
Hainan Island	DLM2	Dec/2003	18°48′N, 109°48′E	900	North tropic humid zone
	JFL	Dec/2003	18°44′N, 109°02′E	800	
	WZM	Dec/2003	18°54′N, 109°39′E	1200	
Himalayas	QMLM	Apr/2005	27°54′N, 86°54′E	>5200	Plateau climate zone, Snow-covered area

[a] LS, Liangshui national reserve, Heilongjiang province; CBM, Changbai mountain, Jilin province; DLM1, Dongling mountain, Beijing; WLS, Wulingshan nature reserve, Hebei province; BS, Beishan forestry centre, Qinghai province; LZ, Linzhi, Tibet; MD, Mengda nature reserve, Qinghai province; JGM, Jinggang mountain, Jiangxi province; TMM, Tianmu mountain, Zhejiang province; ZJJ, Zhangjiajie national forest park, Hunan province; DLM2, Diaoluo mountain; JFL, Jianfengling mountain; WZM, Wuzhi mountain, Hainan province; QMLM, Qomulangma mountain, Tibet.

rinsed twice with sterile distilled water. After removing free water of the larvae surface, they were placed onto potato dextrose agar plates containing 0.1 g/L streptomycin and 0.05 g/L tetracycline. Same fungal species was only recorded once for each tube, regardless isolate numbers from different larvae.

The fungi were identified mainly based on the morphological characteristics of reproductive structures with the aid of several taxonomic keys (Domsch et al., 1980; Nelson et al., 1983; Samson et al., 1988). For those poorly sporulating isolates, the internal transcribed spacer (ITS) of ribosomal DNA were amplified and sequenced following the procedure of White et al. (1990). Similar taxon retrieved by Basic Local Alignment Search Tool (BLAST) in GenBank/NCBI was used as reference for further morphological examination and identification.

2.3. *Preliminary pathogenicity test (Koch's postulates)*

Infections of isolates with no knowledge about their pathogenicity to the *G. mellonella* larvae were bioassayed. The tested fungus was grown on PDA plate for 10–12 days and its spores were washed with 1 mL sterile water into 1.5 mL tubes. The final instar larvae of *G. mellonella* were immersed into the spore suspension with forceps for about 3–5 s, and then transferred into 9 cm diameter Petri dishes with moistened filter paper. Petri dishes were sealed with parafilm to maintain the humidity, and were incubated at the temperature of 20–25 °C in darkness. Infected larvae were inspected daily until dead or pupation. Fungal structures growing out of the dead larvae were identified to evaluate whether the fungus was the same as inoculated. Five larvae were treated for a fungus and the experiment was carried out three times to evaluate the pathogenicity of the fungus.

2.4. *Data analysis*

Species richness was measured by using the formula $Ma = (S - 1)/\ln N$ (Magurran, 2004), in which S is the number of species and N is the total number of isolates. The species diversity was measured using the Shannon–Wiener index (H') and the formula was $H' = -\sum_{i=1}^{n} P_i \ln P_i$ (Pielou, 1975), where P_i is the proportion of the ith species and n is the number of species at the site. The evenness (J) of fungal communities was represented by $J = H'/H'_{max}$ (Pielou, 1975).

The software SPSS 15.0 was used for the statistical data processing. Analyses were made of frequencies of occurrence of the different species of insect-associated fungi in soil of different regions by standard χ^2 tests. Multiple pairwise comparisons were performed using the Table procedure of SPSS software and the Bonferroni method was used to adjust P-values. One way analysis of variance (ANOVA) was used to test differences of the occurrences of the common species. Parameters including occurrence, species diversity of fungal community and the altitude of the six studied regions were subjected to principal component analysis (PCA) to elucidate the major variation patterns. The altitude value were logarithm transformed (base = 10) prior to analysis. A component loading was considered significant when it was >0.7. The analysis was performed using the Data Reduction procedure of SPSS software.

3. Results

3.1. *Occurrence and pathogenicity of insect-associated fungi*

Insect-associated fungi were found in 55.5% (236 of 425) of the soil samples examined and 377 fungal isolates belonging to 46

species and 27 genera were obtained (Table 2). Insect-associated fungi had high frequency of occurrence in soils from South Central China (SCC) and could be obtained (positive sample) from 80.0% (40 out of 50) of the samples collected there. Regions belonging to the temperate zone (Northern China, Northeastern China) also had higher levels of positive samples (56.3% and 53.9%, respectively). The regions belong to plateau zone (Western China, Himalayas) had lower levels of positive samples (49.5% and 45.7%, respectively). The occurrence of insect-associated fungi were not significantly different between Northeastern China, Northern China, Western China, Hainan Island and Himalayas but were significantly different between South Central China and the other five regions ($\chi^2 = 15.39$; d.f. = 5; $P = 0.009$).

3.1.1. *Pathogenicity tests*

Other than insect pathogenic fungi, all isolates (174) were tested for their pathogenicity to *Galleria* larvae (Table 3). The genera *Aspergillus*, *Cladosporium*, *Fusarium*, *Geomyces*, *Clonostachys*, *Gloeotinia*, *Lecythophora*, *Mariannaea*, *Mortierella*, *Mucor*, *Penicillium* and *Pestalotiopsis* did all killed the *Galleria* larvae. *Fusarium* spp., especially *Fusarium oxysporum Schlecht. emend. Sny. & Hans.*, *Fusarium solani* (Mart.) Sacc. and an unidentified species *Fusarium* sp., showed mortalities of up to 93.3%, 86.7% and 93.3%, respectively. The levels of mortality of *F. oxysporum* and *F. solani* were not significantly different between regions (ANOVA, for *F. oxysporum*, $P = 0.091$; for *F. solani*, $P = 0.411$). Fungal species other than well-known insect pathogens but can infect insects, whether reported by other authors or showed in the present study are grouped into opportunistic pathogens.

There were also nineteen species showed no pathogenicity to *Galleria* larvae. Species that had not been reported on insects by other authors and resulted in no *Galleria* larvae mortality in the pathogenicity test are considered secondary colonizers in present study. Thus, the insect-associated fungi were grouped into three groups: (1) fungi already reported to be insect pathogens; (2) opportunistic pathogens and (3) secondary colonizers (Table 2).

3.1.2. *Insect pathogenic fungi*

Insect pathogenic fungi were the most abundant fungal species and the frequency of occurrence was 53.8% (203 out of the 377 isolates). The most commonly isolated fungal species were *Paecilomyces farinosus* (Holm: Fries) A.H.S. Brown & G. Smith (19.6%), *Beauveria bassiana* (14.1%) and *Metarhizium anisopliae* var. *anisopliae* (10.6%) (Table 2). There was no significant difference in the occurrences of these three fungal species ($F = 1.305$; $P = 0.300$). *Paecilomyces farinosus* was distributed in soils in all six regions, and the frequencies of occurrence were significantly different between South Central China and the other five regions ($\chi^2 = 12.19$; d.f. = 5; $P = 0.032$). There were significantly more *P. farinosus* isolates obtained in soils in South Central China. *Beauveria bassiana* was not obtained in Himalayas while abundant in soils in Northern China, and its frequency was significantly different between Northern China and the other four regions ($\chi^2 = 47.20$; d.f. = 4; $P < 0.001$). *Metarhizium anisopliae* var. *anisopliae* was not isolated in soils of Himalayas region, and its frequency was significantly higher in Hainnan Island and South Central China and lower in Northern China, Northeaster China and Wester China ($\chi^2 = 16.61$; d.f. = 4; $P = 0.002$). *Paecilomyces fumosoroseus* occurred in regions except that of Hainan Island and Himalayas, and significantly more in South Central China than the other three regions ($\chi^2 = 12.11$; d.f. = 3; $P = 0.007$). *Lecanicillium lecanii* (Zimm.) Viegas and *Tolypocladium inflatum* Gams occurred with low frequencies in the soil of natural habitats in China. When insect pathogenic species were pooled, the occurrence of these fungi were significantly higher in Northern China and South Central China and lower in the other four regions ($\chi^2 = 64.86$; d.f. = 5; $P < 0.001$).

3.1.3. *Opportunistic fungi*

Aspergillus, *Fusarium*, *Clonostachys*, *Mortierella*, *Mucor* and *Penicillium*, were opportunistic pathogens, and the frequency of occurrence of this group of fungi was 36.9% (139 out of 377 isolates) (Table 2). The most commonly isolated species of this group were *Fusarium oxysporum* (9.3%), *Fusarium solani* (4.8%), *Aspergillus flavus* Link (3.5%) and *Mortierella* spp. (3.2%). The occurrence of *F. oxysporum* was significantly higher in South Central China and lower in Northern China, Northeastern China, Western China and Hainan Island ($\chi^2 = 19.49$; d.f. = 4; $P = 0.001$). *Fusarium solani* showed an even distribution between the six sampling regions ($\chi^2 = 7.37$; d.f. = 5; $P = 0.098$). Other opportunistic pathogens isolated, but only at low frequencies, belong to the following genera: *Cladosporium*, *Geomyces*, *Gloeotinia*, *Lecythophora*, *Mariannaea* and *Pestalotiopsis*.

3.1.4. *Secondary colonizers*

Secondary colonizers were isolated only at low levels, and the total frequency of occurrence in this group of fungi was 9.3% (35 out of 377 isolates). Most of these species have not been reported to be isolated from insects before (Table 2).

3.2. *Regional variation of insect-associated fungi community*

The insect-associated fungi obtained from soil samples of the same region can be treated as a fungal community. To elucidate the major variation patterns, parameters of the fungal community of studied regions including species richness, species diversity index, evenness, levels of positive samples, levels of pathogenic species and the average altitude of the sampling regions were subjected to the principal components analysis (PCA). Two principal components (Eigenvalue > 1) were extracted and they explained 47.8% and 28.7% of the total variance, respectively (Table 4).

The first component (PC1) was positively correlated with species richness and species diversity and negatively correlated with the average altitude of the sampling region. PC1 representing the altitude of the sampling regions had strong negative influence on both the species richness and the species diversity of insect-associated fungi. The second component (PC2) was negatively correlated with species evenness and positively correlated to the level of insect pathogenic fungi.

Based on the scores on the two principal components, the fungal communities were arranged in the scatter graph which showed the ordination of communities (Fig. 1). The results

Table 2 – Distribution and frequency of insect-associated fungi in soils of different regions

Fungal species[a]		Frequency of occurrence[b]						%F[c]	Reported on insects by other authors
		SCC (*N* = 50)	NC (*N* = 96)	NEC (*N* = 76)	WC (*N* = 111)	HI (*N* = 57)	HM (*N* = 35)		
All species		80.0	56.3	53.9	49.5	52.6	45.7		
Known insect pathogen	*Beauveria bassiana*	6.0	34.4	5.3	8.1	7.0	–	14.1	Yes, several
	Metarhizium anisopliae var. *anisopliae*	20.0	12.5	2.6	5.4	17.5	–	10.6	Yes, several
	Lecanicillium lecanii	2.0	1.0	3.9	2.7	1.8	–	2.4	Yes, several
	Paecilomyces farinosus	34.0	16.7	14.5	17.1	10.5	14.3	19.6	Yes, several
	Paecilomyces fumosoroseus	18.0	3.1	6.6	5.4	–	–	6.1	Yes, several
	Tolypocladium inflatum	2.0	2.1	1.3	–	–	–	1.1	Yes, several
Opportunistic pathogen	*Aspergillus flavus*	6.0	–	9.2	0.9	1.8	2.9	3.5	Yes, Domsch et al., 1980; Gunde-Cimerman et al., 1998
	Aspergillus sydowii	–	–	1.3	–	–	–	0.3	No, only present study
	Cladosporium cladosporioides	–	1.0	–	–	–	–	0.3	Yes, Gunde-Cimerman et al., 1998
	Clonostachys rosea f. *catenulatum*	2.0	–	–	–	–	–	0.3	No, only present study
	Clonostachys rosea f. *rosea*	2.0	3.1	–	1.8	–	2.9	1.9	Yes, Toledo et al., 2006
	Fusarium avenaceum	–	1.0	–	1.8	–	22.9	2.9	Yes, Li, 1988
	Fusarium oxysporum	24.0	8.3	7.9	2.7	10.5	–	9.3	Yes, Ali-Shtayeh et al., 2002
	Fusarium redolens	–	–	1.3	–	–	–	0.3	No, only present study
	Fusarium solani	4.0	4.2	3.9	1.8	10.5	2.9	4.8	Yes, Ali-Shtayeh et al., 2002
	Fusarium sp.	8.0	–	7.9	0.9	–	–	2.9	No, only present study
	Geomyces pannorum	–	3.1	–	–	–	–	0.8	Yes, Gunde-Cimerman et al., 1998
	Gloeotinia temulenta[d]	–	1.0	–	–	–	–	0.3	No, only present study
	Lecythophora sp.	2.0	–	–	–	–	–	0.3	No, only present study
	Mariannaea elegans	2.0	–	1.3	–	–	–	0.5	No, only present study
	Mortierella spp.	6.0	–	3.9	3.6	3.5	–	3.2	Yes, Gunde-Cimerman et al., 1998
	Mucor spp.	8.0	2.1	2.6	–	–	–	2.1	Yes, Gunde-Cimerman et al., 1998; Ali-Shtayeh et al., 2002
	Penicillium brasilianum	–	–	1.3	–	–	–	0.3	No, only present study
	Penicillium chrysogenum	4.0	1.0	–	0.9	5.3	5.7	2.4	No, only present study
	Penicillium corprophlium	–	–	–	–	1.8	–	0.3	No, only present study
	Penicillium thomii	–	–	1.3	–	–	–	0.3	No, only present study
	Pestalotiopsis theae	–	1.0	–	–	–	–	0.3	No, only present study
Secondary colonizer	*Absidia glauca*	–	–	1.3	–	–	–	0.3	No, only present study
	Chaetomium globosum	–	–	–	2.7	–	2.9	1.1	No, only present study
	Clonostachys sp.	–	–	1.3	–	–	–	0.3	No, only present study
	Fusarium equiseti	–	1.0	–	–	–	–	0.3	No, only present study
	Fusarium sambucinum	–	–	–	–	–	2.9	0.3	No, only present study
	Fusarium tricinctum	–	–	–	0.9	–	2.9	0.5	No, only present study
	Penicillium simplicissimum	–	–	1.3	2.7	–	–	1.1	No, only present study
	Penicillium italicum	–	–	–	–	1.8	–	0.3	No, only present study
	Periconiella mucunae	4.0	–	–	–	–	–	0.5	No, only present study
	Pseudeurotium zonatum	–	1.0	–	–	–	–	0.3	No, only present study
	Rhizopus oryzae	–	–	1.3	–	–	2.9	0.5	No, only present study

Talaromyces flavus[d]	4.0	–	–	0.9	3.5	–	1.3	No, only present study
Talaromyces trachyspermus[d]	–	–	1.3	–	–	–	0.3	No, only present study
Trichoderma aureoviride	–	–	–	–	1.8	–	0.3	No, only present study
Trichoderma koningii	2.0	–	–	–	–	–	0.3	No, only present study
Trichoderma parceramosum	–	–	–	–	–	5.7	0.5	No, only present study
Trichoderma virens	–	1.0	1.3	0.9	–	–	0.8	No, only present study
Williopsis saturnus (yeast)[d]	–	–	1.3	–	–	–	0.3	No, only present study
Unidentified basidiomycetous yeast	–	–	1.3	–	–	–	0.3	No, only present study

Abbreviations: SCC, South central China; NC, Northern China; NEC, Northeastern China; WC, Western China; HI, Hainan Island; HM, Himalayas.

[a] Total number of species = 46.

[b] Frequency of occurrence = number of samples with a specific species/total number of samples collected in the region.

[c] Percentage frequency (%F) = number of isolates of a species/total number of isolates of all species (377).

[d] rDNA-ITS sequences were amplified and sequenced: *Gloeotinia temulenta*, EU287812; *Talaromyces flavus*, EU287814, EU287815; *Talaromyces trachyspermus*, EU287809; *Williopsis saturnus*, EU287816.

showed distinct separation of the community of South Central China. Other communities were not clearly separated. This indicates higher species richness and species diversity of insect-associated fungi, especially the insect pathogenic species in the soil of subtropical humid region in China.

4. Discussion

This is the first detailed investigation of insect pathogenic fungi in the soil environment in China. The insect pathogenic fungal species detected in China were similar with other parts of the world (Vänninen et al., 1989; Vänninen, 1995; Bidochka et al., 1998; Meyling and Eilenberg, 2006), the wide distributions and high occurrences of *B. bassiana* and *M. anisopliae* var. *anisopliae* in natural soil habitats in China have been showed. *Beauveria bassiana* was most frequently found in colder regions (Northern China) and *M. anisopliae* var. *anisopliae* in warmer regions (South Central China and Hainan Island). This corresponds well with earlier investigations (Vänninen et al., 1989; Bidochka et al., 1998). However, *P. farinosus* was the most frequently found species in this survey and was distributed in the soil of tropical forest in Hainan Island to the frozen soil in Himalayan region. Further, *P. farinosus* was found at significantly higher frequency in soils in South Central China. This is in contradiction to studies in Finland. Vänninen et al. (1989) found that *P. farinosus* was significantly more common in samples originating from the north of Finland. Different distribution pattern of *P. farinosus* in different regions indicated big difference in temperature requirements between isolates. Klingen and Haukeland (2006) indicated that both *B. bassiana* and *P. farinosus* could tolerate a wide range of climatic conditions. The difference in temperature requirement among isolates of a species may because of the large variation of the species. Actually, there are big discussions about whether many of the soil insect pathogenic fungal species are actually species complex rather than specie, and that fungi considered to be different strain groups of the same fungal species are rather different species (Bidochka et al., 2001; Rehner and Buckley, 2005; Devi et al., 2006).

When the fungal species were pooled, Vänninen et al. (1989) found that the distribution of insect pathogenic fungi in Finland was very even between the north and south. However, occurrence of insect pathogenic fungi was significantly different between the studied regions in China. Higher frequency occurred in South Central China and Northern China and lower frequency occurred in the other four regions. This might because of the larger variation of the climatic type in the studied regions than that of in Finland. The principal component analysis showed that insect-associated fungi, especially the insect pathogenic species, were more frequent in soil of the subtropical humid region in China, and relatively less in temperate and tropic regions. According to the previous study, humid tropical forests had a rich and varied insect pathogenic fungal species and the great majority of species belong in the genus *Cordyceps* (Ascomycota; Hypocreales) (Evans, 1982). While Hyphomycetes such as *Beauveria*, *Metarhizium* and *Paecilomyces* were the dominant fungi found on soil insects (Samson et al., 1988; Keller and Zimmerman, 1989). There must be different insect pathogenic mycofloras

Table 3 – Pathogenicity test (Koch's Postulates) results

Fungus species	No. of isolates	Mortality (%)	Percentage of pathogenic isolates (%)
Absidia glauca	1	0	0
Aspergillus flavus	13	0–6.7	30.8
Aspergillus sydowii	1	26.7	–
Chaetomium globosum	4	0	0
Cladosporium cladosporioides	1	6.7	–
Clonostachys rosea f. *catenulatum*	1	73.3	–
Clonostachys rosea f. *rosea*	7	0–6.7	42.9
Clonostachyssp.	1	0	–
Fusarium avenaceum	11	0–26.7	27.3
Fusarium equisetii	1	0	–
Fusarium oxysporum	35	0–93.3	80.0
Fusarium redolens	1	26.7	–
Fusarium sambucinum	1	0	–
Fusarium solani	18	0–86.7	66.7
Fusarium sp1.	11	0–93.3	81.8
Fusarium tricinctum	2	0	0
Geomyces pannorum	3	0–93.3	33.3
Gloeotinia temulenta	1	6.7	–
Lecythophora sp.	1	13.3	–
Mariannaea elegans	2	0–6.7	50.0
Mortierella spp.	12	0–13.3	16.7
Mucor spp.	8	0–13.3	37.5
Penicillium brasilianum	1	6.7	–
Penicillium chrysogenum	9	0–6.7	22.2
Penicillium corprophlium	1	6.7	–
Penicillium italicum	1	0	–
Penicillium simplicissimum	4	0	0
Penicillium thomii	1	26.7	–
Periconiella mucunae	2	0	0
Pestalotiopsis theae	1	6.7	–
Pseudeurotium zonatum	1	0	–
Rhizopus oryzae	2	0	0
Talaromyces flavus	5	0	0
Talaromyces trachyspermus	1	0	–
Trichoderma aureoviride	1	0	–
Trichoderma koningii	1	0	–
Trichoderma parceramosus	2	0	0
Trichoderma virens	3	0	0
Williopsis satumus	1	0	–
Unidentified basidiomycetous yeast	1	0	–

between the soil and the overground environment in tropical forest.

Among the opportunistic fungi found in this study *F. oxysporum*, *F. solani*, and unidentified *Fusarium* sp., *Geomyces pannorum* (Link) Sigler & J.W. Carmich.and *Clonostachys rosea* f. *catenulatum* (J.C. Gilman & E.V. Abbott) Schroers resulted in the highest *G. mellonella* mortality in the preliminary pathogenicity test. The characteristics of the fast growing opportunistic fungi may result in infection of injured or weakened insect. They may also live as non-pathogenic insect associates (Hajek et al., 1993). *Geomyces pannorum* and *Clonostachys rosea* f. *rosea* (Link: Fr.) Schroers, Samuels, Seifert & W. Gams have been

Table 4 – Loading capacity matrix of principle components

Community parameter	Principal component[a]	
	PC1 (EV[b] = 2.870)	PC2 (EV = 1.720)
Level of positive sample	0.584	0.463
Species richness	**0.875**	0.014
Species diversity	**0.955**	−0.111
Species evenness	0.358	**−0.866**
Level of pathogenic species	0.150	**0.862**
Altitude	**−0.838**	−0.006
Cumulative explained variance (%)	47.8	76.5

[a] Bold values indicate community parameters dominating principal components, PC1 and PC2.
[b] Eigenvalue (EV).

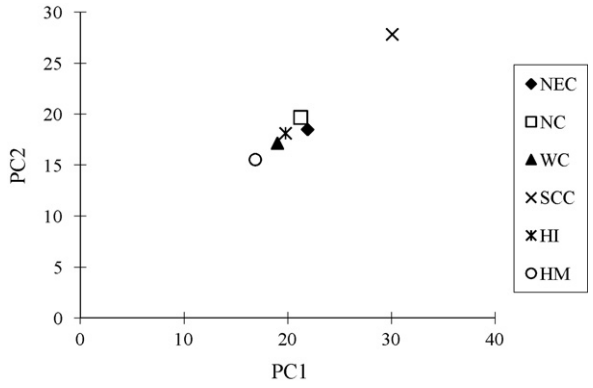

Fig. 1 – Score plot of principal component analysis showing the separation of the fungal community of South Central China; abbreviations: NEC, Northeastern China; NC, Northern China; WC, Western China; SCC, South Central China; HI, Hainan Island; HM, Himalayas.

considered as saprophytic fungi by other authors (Gunde-Cimerman et al., 1998). *Clonostachys rosea* f. *rosea* have, however, also been reported to be pathogenic to *Oncometopia tucumana* and *Sonesimia grossa* (Hemiptera: Cicadellidae) (Toledo et al., 2006). On the other hand, Milner (1992) report that some isolates of the well-known insect pathogenic fungus *M. anisopliae* may result in a very low mortality of insects even when they are rolled in a Petri dish with sporulating *M. anisopliae*. The infection events of fungi in invading insects may be affected by factors including fungal inoculation dosage, propagule quality, host preference, defense, activity as well as environmental conditions such as humidity, temperature, etc. The difference in pathogenicity between isolates is understandable.

As showed in this study, both *B. bassiana* and *M. anisopliae* var. *anisopliae* had high occurrence in the soil of natural habitats and their occurrences were not significantly different. However, the phenomenon that *B. bassiana* preferred natural habitat and *M. anisopliae* var. *anisopliae* preferred cultivated habitats was observed by several authors (Mietkiewski et al., 1991; Rath et al., 1992; Vänninen, 1995; Bidochka et al., 1998). Quesada-Moraga et al. (2007) reported no significant effect of habitat on the occurrence of *B. bassiana*, but strong association between *M. anisopliae* var. *anisopliae* and soils from cultivated habitats. More studies on the effect of habitat type on occurrence of insect-associated fungi are needed. Great effect of altitude on the species diversity of insect-associated fungi had been shown in our study. While in the study of Quesada-Moraga et al. (2007), which most samples were taken between 50 and 1000 m, the interaction between altitude and fungal species was not significant. The reason might be that altitude do not affected the occurrence of insect-associated fungi until the variance was great (e.g. >1000 m).

Only a few fungal species that associated with insects, mostly insect pathogenic species had been recorded in the soil (Keller and Zimmerman, 1989; Klingen and Haukeland, 2006). This may be jointly because: (1) infected insects remain hidden in the soil; (2) research work was restricted to agricultural habitats and nearby non-agricultural habitats and (3) the opportunistic pathogens or avirulent pathogens have largely been overlooked to date. Quite a few opportunistic pathogens had been obtained in the present study (accounted for 36.9% of total isolates) and some of the isolates showed high pathogenicity to insects. The study of Thomas et al. (2003) showed that pre-inoculation of avirulent pathogens produced significantly negative effect of reducing mortality and sporulation of insect pathogens. The opportunistic pathogen even the secondary colonizer may play significant role in mediating the insect-pathogen interactions. Consequently, to understand more about the insect-pathogen dynamics in the soil, studies on the natural occurrence and distribution of insect pathogenic fungi in different soil types and in different geographical regions are necessary. Ecological studies on the occurrence of all insect-associated fungi in the soil are needed too.

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