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ORIGINAL PAPER

Determination of genetic variability among the isolates of *Metarhizium anisopliae* var. *anisopliae* from different geographical origins

Shoaib Freed · Feng-Liang Jin · Shun-Xiang Ren

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Abstract The entomopathogenic fungus *Metarhizium anisopliae* is currently used as an efficient biological control agent against different insects. The prevalence and genetic variability of the entomopathogenic fungus *Metarhizium anisopliae* var. *anisopliae* in Asian countries (China, Laos, Singapore and South Korea) and a European country (The Netherlands) was examined. The fungus was found to be widespread in agricultural and forest soils throughout China especially in the south and south western regions, with a maximum recovery percentage of 81.6, while in Laos it was found to be abundant in the forest soils only, the soil of Netherlands also seemed to be an excellent source of entomopathogens with a significant recovery of insect pathogenic fungi. Simple sequence repeats (SSR) and ITS-rDNA sequence data showed that the isolates are closely linked to each other. The gene diversity (He) and polymorphic information content values of sixty two isolates showed mean values of 0.37 and 0.63. The majority of the isolates belonged to one of the closely related genotypes and these were found to be dominant in the agricultural as well as forest ecosystem. Genetic distances among the isolates according to locations and different sources showed minimal variation ranging from 0.23 to 0.34%, with maximum genetic distance of 0.34% among the isolates from Laos and The Netherlands. The reason for the limited variation is uncertain, however even the similarity index among the isolates can endow with sufficient knowledge for the implications of the methods to develop this fungus as a microbial control agent.

Keywords *Metarhizium anisopliae* · Genetic diversity · Simple sequence repeats · ITS-rDNA · Biocontrol · Phylogenetic analysis

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Introduction

The mitosporic haploid fungus *Metarhizium anisopliae* var. *anisopliae* commonly known as "green muscardine fungus" is a globally distributed entomopathogenic fungus that has been isolated from more than 200 host species (Butt et al. 2001). These entomopathogens offer an attractive alternate to the use of chemical insecticides in agricultural crop protection (Lacey and Goettel 1995). In order to enhance the efficacy of microbial control programs and selecting an efficient genotype for the control of insect pests it is necessary to understand the phylogeny/population genetics of the particular ecosystem (Inglis et al. 2008).

To study the population genetics of a particular ecosystem for the purpose of screening an efficient genotype has spearheaded an interest in the documentation of molecular differences and the genetic identification of a particular strains (White et al. 1990). The application of molecular techniques in mycology has shed new light on the systematics, biochemistry, and ecology of entomopathogenic fungi (Entz et al. 2005). Different kinds of molecular techniques have been used to study the population genetics, e.g., restriction fragment length polymorphism (RFLP) (Hegedus and Khachatourians 1993; Maurer

et al. 1997), and random amplified polymorphic DNA (RAPD) (Fegan et al. 1993; Piatti et al. 1998; Jensen et al. 2001). In the recent years the use of microsatellite markers, simple sequence repeats (SSR) have gained more attention as molecular markers for genome mapping as well as population genetics in plants, animals and fungi (Goldstein and Schlotterer 1999; Tenzer et al. 1999; Kretzner et al. 2000; Enkerli et al. 2001, 2005), while internal transcribed spacer (ITS-rDNA) sequence analysis have been an effective tool for detecting the genetic diversity in many fungal species (Coates et al. 2002; Enkerli et al. 2005).

Considerable work has been done on the genetic diversity of *Metarhizium* using different molecular and traditionally used classification techniques within a defined geographical region (Enkerli et al. 2005; Bidochka et al. 2001) which reveal significant genetic variability in this biocontrol agent. Inglis et al. (2008) examined the abundance and genetic diversity of *Metarhizium* in two different geographical locations of Canada but the genetic variability was observed minimal due to some uncertain factors. Keeping in view all the previous research works, a study was planned to investigate the genetic diversity and variability of *Metarhizium anisopliae* isolated from diverse soil sources of different countires and for the screening of an efficient isolate to release in the enviornment as an effective biocontrol agent. In the present study we isolated *Metarhizium ansiopliae* from soils of different countries (China, Laos, South Korea, Singapore and The Netherlands), and measured the genetic variability among different *Metarhizium* isolates from different soils sources (e.g., urban, agricultural and forest) by microsatellite markers (SSR) and genotypes were determined by ITS-rDNA and 5.8S rRNA gene sequence from representative countries.

Materials and methods

Sampling locations

The soil samples from a variety of habitats including urban, agricultural and forest ecosystems were collected from China, (China was divided into four main sections for soil sampling i.e, north and north eastern regions, south and south western regions, central and western region) (Fig. 1), Laos, South Korea, Singapore and The Netherlands. A total of 140 sites from 13 different provinces of China were sampled, while for Laos out of 5 provinces (Fig. 2), 25 cites were sampled. Four cities of the Netherland's soil was sampled at five different cites, while for South Korea (Cheju) and Singapore one city was sampled (Fig. 3, Table 1). At each sampling location, the soil was taken from the A-horizon ($\approx$10 cm deep) with a cylindrical soil core borer (2.0 cm diameter, 5–15 cm deep) from three different sites 10 m apart. The soil cores were mixed to form one sample ($\approx$10 g), which were placed in a plastic bag and stored at 4°C before further processing and isolation of *Metarhizium anisopliae*.

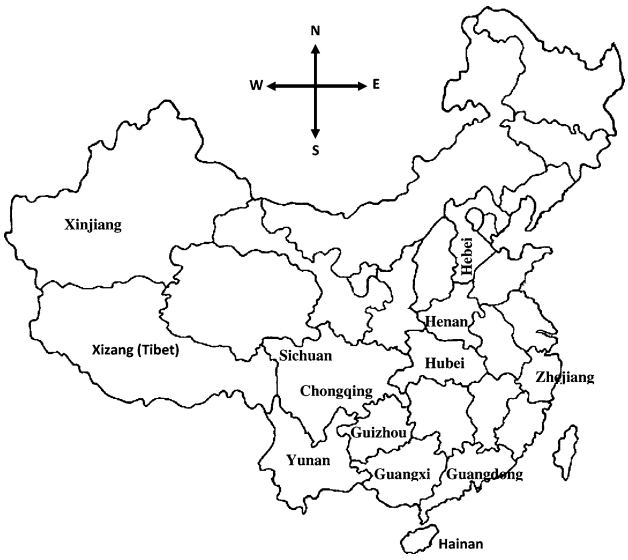

Fig. 1 Map of China showing the provinces for the soil sample collection

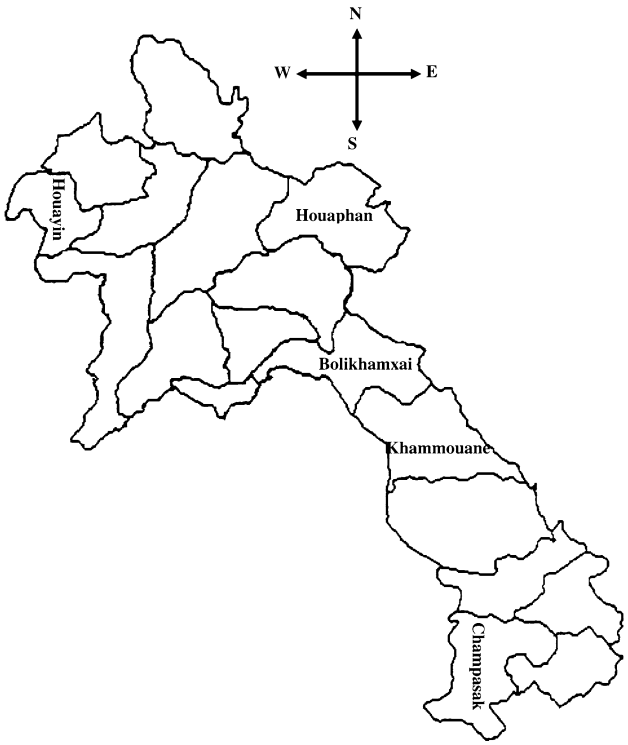

Fig. 2 Map of Laos showing the provinces for the soil sample collection

Fig. 3 Map of The Netherlands showing the locations for the soil sample collection

Isolation of fungi

For soil samples collected from different locations, *Metarhizium anisopliae* was isolated on semi-selective medium described by (Veen and Ferron 1966). The medium consisted of glucose (10 g), peptone (10 g), bile (15 g) (Sigma), rose Bengal (1:15,000), and agar (30 g) in 1 L of deionized water. The medium was amended with 0.002 gL^{-1} dodine (N-dodecylguanidine monoacetate; American Cyanamid), 0.12 gL^{-1} cycloheximide (Sigma) and 0.25 gL^{-1} chloramphenicol (Sigma). Sub-sample of soil (10 g) was placed into 90 ml of sterile water, and the sample was homogenized using a surface-sanitized blender. The homogenate was diluted three times in a ten-fold dilution series in sterile phosphate buffer (100 mM, pH7.0) and from each dilution, 100 µl was spread onto the semi-selective medium, and cultures were maintained at 25°C in dark. Cultures were examined after 4 days and daily thereafter for three additional days for confirmation and growth of fungi. Conidia characteristic of *Metarhizium* were transferred onto PDA, cultures were purified, and examined microscopically for characteristic conidiogenesis.

All isolates were subsequently propagated from a single conidium (Table 1). A suspension (100 µl) containing a low density of conidia in phosphate buffer was spread onto PDA. After 24 h growth at 25°C, a single germinated conidium separated from other conidia was identified by dissecting microscope using 20 times magnification, a piece of the medium encompassing only the target conidium was aseptically removed and transferred to PDA, and culture was maintained at room temperature. After 5 days, morphological characteristics of *Metarhizium* were confirmed microscopically; conidia were collected and stored in sterile 30% glycerol at −80°C until used for further propagation.

Genomic DNA isolation

The genomic DNA was extracted by the method described by Liu et al. (2000) with some modifications. Extracted genomic DNA was identified by 1% TBE–agarose gel (Invitrogen Corp., Burlington, ON) electrophoresis, stained with ethidium bromide and visualized under UV light. DNA was purified by using the E.Z.N.A. Gel Extraction Kit (OMEGA bio-tek, USA) for PCR amplification.

SSR amplification

Sixty two isolates of *Metarhizium anisopliae* were analyzed with ten SSR primers (Ma097, 099, 142, 145, 164, 165, 195, 210, 307 and 325) (Enkerli et al. 2005). The PCR reactions were performed in a Bioer XP Cycler using 2 µl of genomic DNA from each isolate and the PCR solution comprised of total volume of 25 µl containing 1 ×PCR buffer, 0.2 mM of dNTPs, 2.5 mM $MgCl_2$, 200 µM of forward and reverse primers, and 1 U Taq DNA polymerase (Takara Japan). Amplification reaction consisted of an initial step of 4 min at 95°C, followed by 38 cycles of 1 min at 95°C, 1 min at 50°C (44–56°C depending on the primers) and 1 min at 72°C, with a final 4 min extension at 72°C. Products were separated on 6% denaturing polyacrylamide gel in 1 ×TBE buffer at 1,800 V for about 2 h. DNA fragments were stained with silver nitrate and fragment sizes determined based on internal and external size standards.

PCR amplification for ITS-rDNA regions

The primers, SSZ (5′-ATAACAGGTCTGTGATG-3′) and LSU4 (5′-TTGTGCGCTATCGGTCTC-3′) were used to amplify the partial large and small rRNA genes, ITS regions and the 5.8S rRNA gene (Hausner et al. 1993). The PCR mixture consisted of a total volume of 25 µl containing 1 ×reaction buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.5 µM of each primer (Invitrogen, USA), and 1U Taq DNA polymerase (Takara Japan) and 2.0 µl of DNA template. The amplification conditions consisted of one cycle at 95°C for 3 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, finishing up with 1 cycle at 72°C for 10 min. The PCR products were identified and sized by agarose gel electrophoresis in a 1.5% TAE-agarose gel with a 100 bp DNA ladder and

Table 1 *Metarhizium anisopliae* var. *anisopliae* isolates from different geographical origin and source

No.	*M. anisopliae* isolates	Geographical origin	Source	Accession No's
1.	CNCG	Zhongxian, Chongqing, China	Agricultural soil	FJ545275
2.	CNGD1	Zhuhai, Guangdong, China	Agricultural soil	FJ545276
3.	CNGD2	Zhaoqing, Guangdong, China	Orchard soil	FJ545277
4.	CNGD3	Yingde, Guangdong, China	Agricultural soil	FJ545278
5.	CNGD4	Cheping, Guangdong, China	Agricultural soil	FJ545279
6.	CNGD5	Kaiping, Guangdong, China	Orchard soil	FJ545280
7.	CNGD6	Maoming, Guangdong, China	Agricultural soil	FJ545281
8.	CNGD7	Shaoguan, Guangdong, China	Agricultural soil	FJ545282
9.	CNGD8	Huizhou, Guangdong, China	Forest soil	FJ545283
10.	CNGD9	Yangjiang, Guangdong, China	Forest soil	FJ545284
11.	CNGD10	Huizhou, Guangdong, China	Forest soil	FJ545285
12.	CNGD11	Yang Jiang, Guangdong, China	Orchard soil	FJ545286
13.	CNGU1	Leishan, Guizhou, China	Forest soil	FJ545287
14.	CNGU2	Taiba, Guizhou, China	Mountain soil	FJ545288
15.	CNGU3	Leishan, Guizhou, China	Forest soil	FJ545289
16.	CNGX	Dongxing, Guangxi, China	Forest soil	FJ545290
17.	CNHB1	Shennogjia, Hubei, China	Forest soil	FJ545291
18.	CNHB2	Shennogjia, Hubei, China	Forest soil	FJ545292
19.	CNHB3	Shennogjia, Hubei, China	Forest soil	FJ545293
20.	CNHE	Linzhou, Henan, China	Mountain soil	FJ545294
21.	CNHN1	Lingshui, Hainan, China	Forest soil	FJ545295
22.	CNHN2	Baoling, Hainan, China	Forest soil	FJ545296
23.	CNHN3	Lingshui, Hainan, China	Forest soil	FJ545297
24.	CNHN4	Lingshui, Hainan, China	Forest soil	FJ545298
25.	CNSN1	Dujiang Yan, Sichuan, China	Forest soil	FJ545299
26.	CNSN2	Baoxing, Sichuan, China	Cicadas	FJ545300
27.	CNXJ1	Hami, Xinjiang, China	Agricultural soil	FJ545301
28.	CNXJ2	Hami, Xinjiang,China	Agricultural soil	FJ545302
29.	CNXZ	Linzhi, Tibet, China	Agricultural soil	FJ545303
30.	CNYN1	Pingbian, Yunan, China	Forest soil	FJ545304
31.	CNYN2	Yingjiang, Yunan, China	Forest soil	FJ545305
32.	CNYN4	Libo, Yunan, China	Forest soil	FJ545306
33.	CNZH	Hangzhou, Zhejiang, China	Forest soil	FJ545307
34.	CNXJ3	Tekesi, Xinjiang, China	Forest soil	FJ589644
35.	CNXJ4	Wen Xuxian, Xinjiang, China	Agricultural soil	FJ589645
36.	CNXJ5	Ku erle Shi, Xinjiang, China	Peer orchard soil	FJ589646
37.	CNXJ6	Ba Chuxian, Xinjiang, China	Forest soil	FJ589647
38.	CNXJ7	Wen Quanxian,Xinjiang, China	Sunflower field	FJ589648
39.	LSHY	Houayin, Champasak, Laos	Forest soil	FJ545308
40.	LSNA1	Namphao, Bolikhamxai, Laos	Forest soil	FJ545309
41.	LSNA2	Namphao, Bolikhamxai, Laos	Forest soil	FJ545310
42.	LSNM1	Namsanam, Khammouane, Laos	Forest soil	FJ545311
43.	LSNM2	Namsanam, Khammouane, Laos	Forest soil	FJ545312
44.	LSNM3	Namsanam, Khammouane, Laos	Forest soil	FJ545313
45.	LSPK	Khouay NBCA, Vientiane Prefecture, Laos	Forest soil	FJ545314
46.	LSTC1	Tadchampa, Champasak, Laos	Forest soil	FJ545315
47.	LSTC2	Tadchampa, Champasak, Laos	Forest soil	FJ545316
48.	LSTC3	Tadchampa, Champasak, Laos	Forest soil	FJ545317

Table 1 continued

No.	*M. anisopliae* isolates	Geographical origin	Source	Accession No's
49.	LSVT1	Vientiane, Laos	Forest soil	FJ545318
50.	LSVT2	Vientiane, Laos	Forest soil	FJ545319
51.	LSVY	Viengxay, Houaphan, Laos	Forest soil	FJ545320
52.	NLAN	Arnhem, Gelderland, The Netherlands	Potato field soil	FJ545321
53.	NLHN2	Heteren, Gelderland, The Netherlands	Vegetable field soil	FJ545322
54.	NLHN3	Heteren, Gelderland, The Netherlands	Corn field soil	FJ545323
55.	NLUT	Utrecht, The Netherlands	Urban land soil	FJ545324
56.	NLWN	Wageningen, Gelderland, The Netherlands	Grass field soil	FJ545325
57.	SKCJ1	Cheju island, South Korea	Volcano soil	FJ545326
58.	SKCJ2	Cheju island, South Korea	Urban land soil	FJ545327
59.	SKCJ3	Cheju island, South Korea	Urban land soil	FJ545328
60.	SKCJ5	Cheju island, South Korea	Volcano soil	FJ545329
61.	SPCT1	Singapore City, Singapore	Urban soil	FJ589649
62.	SPCT2	Singapore City, Singapore	Urban soil	FJ589650

were purified by E.Z.N.A. Gel Extraction Kit (OMEGA bio-tek, USA) for DNA sequencing.

DNA sequencing and data analysis

DNA sequencing was done by using the primers, SSU3 (5′-GTCGTAACAAGGGTCTCCG-3′), LSU-2 (5′-GATA TGCTTAAGTTCAGCG-3′) and 5.8SB (5′-TGTACACAC CGCCCGTC-3′) with the same concentrations i.e., 0.5 μM. Nucleotide sequences for the isolates were searched for their similarity index by using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ClustalW was used for multiple sequence alignment. Divergence of each pair of sequence was calculated by DAMBE (Xia and Xie 2001). Phylogenetic tree was constructed on the bases of neighbor-joining method using MEGA 4.0 software (Tamura et al. 2007). ITS1 and ITS2 regions and 5.8S rRNA gene of sixty two isolates were sequenced and subsequently submitted to GenBank under the Accession Nos. FJ545275-FJ545329 and FJ589644- FJ589650.

SSR data analysis

PowerMarker program (Liu and Muse 2005) was used to calculate the number of alleles per locus, gene diversity (He) and the polymorphic information content (PIC) for allelic profile based SSR analysis. The phylogenetic tree was visualized by TreeView (Page 2001). The Analysis of Molecular Variance (AMOVA) and fixation index (Fst, θ) was calculated to clarify the molecular patterns of within population variation of the isolates of *Metarhizium anisopliae* collected from different geographical locations. Arlequin 3.01 software was used for all calculations (Excoffier et al. 2005).

Results

Genetic diversity by microsatellite markers analysis

Analysis of sixty two *Metarhizium anisopliae* isolates using microsatellite markers gave minimum level of genetic variability in entomopathogenic fungus. Total twelve primers were used in the study, while two primers (Ma327 and Ma375) didn't amplify any isolate. Total of 54 alleles were detected with a mean number of 5.4 per locus in a range of 3 to 7 (Table 2). In our study the locus Ma307 was the most frequent allele found in each isolate.

The gene diversity (He) values estimate the probabilty that two alleles at any randomly chosen locus are different from each other. In our experiment sixty two isolates showed a mean value of 0.37. The polymorphic information content is a measure of the probability that two randomly sampled genotypes have different allelic profiles, the mean PIC value for *Metarhizium anisopliae* isolates used in this study was 0.63 (Table 2). This indicates that *Metarhizium anisopliae* isolates used in the study have high degree of biodiversity.

The dendogram based on the neighbor joining (NJ) method gave the genetic similarity and separated the *Metarhizium anisopliae* isolates into three clades (Fig. 4), while the dendogram generated by the SSR data was statistically supported by a cophenetic correlation value ($r = 0.85$). The clade I showed maximum genetic linkages as it comprised of the *Metarhizium* isolates from four different countries i.e, (China, Laos, South Korea and The Netherlands) with majority of the isolates belonging to forest ecosystem. The clade II mainly clustered the Chinese isolates from different provinces, 83.3% of the isolates from clade II originated from agricultural field soils and the

Table 2 Allele size range, allele number, genetic variability (He) and Polymorphic information content (PIC) of SSR markers from the isolates of *M. anisopliae* var. *anisopliae*

SSR markers	*M. anisopliae* var. *anisopliae*				
	Primer Sequence (5′-3′)	Size range	Allele number	He	PIC
Ma097	F:AGGAAGTCAAATAGAATACGTACCG R:CCTTTTGTCGCTTGCTTG	150–180	3	0.45	0.54
Ma099	F:CAAGTTTACGCATATTGGTTACGATA R:TCACCGGCCATCTCATTAGAT	150–160	6	0.24	0.65
Ma142	F:GACGGTATATTTATGATCAGCTCG R:TCGGGAACTAGACTTTAAGTATCAC	100–130	6	0.57	0.71
Ma145	F:CCGTACTTGGTACATATTCCTGATG R:GGGATGTCCGCATTCGAA	100–120	5	0.79	0.53
Ma164	F:GCACTCAACCAAGAGCGAGG R:CAACGTCACCTGTGGCACAC	100–120	7	0.83	0.61
Ma165	F:CGACATTTCACCGTTGTACATATG R:GGACTGGGAGTTTGGAGCTC	130–150	5	0.32	0.70
Ma195	F:AATTATAAAACTGAAGAAACAGAAA R:GTGTTCCTAGTGACCTCCTTACT	100–120	5	0.21	0.75
Ma210	F:CCCGAGGCCTGTAGTCTACG R:TTTCCTGGAAAGGCAAGAACTT	90–150	6	0.19	0.48
Ma307	F:CATGCTCCGCCTTATTCCTC R:GGGTGGCGAAGAAGTAGACG	150–200	6	0.09	0.71
Ma325	F:TTTATTGTGGTTGGAGATGCCA R:CATGATAAAAGGTCATGTTTGCC	150–170	5	0.07	0.63
Mean			5.4	0.37	0.631

rest of these belonged to forest ecosystem. In contrast to this clade III clustered the isolates mainly originated from forest ecosystem, with one isolate CNSN2, recovered from an insect (cicada), it also showed less genetic difference with other isolates as compared to CNSN1, isolated from forest soil of the same province i.e Sichuan, China.

The AMOVA results for SSR data (Table 3) indicated that the variance within population accounted for 80.65% of the total genetic variation, while the genetic variance between population reported for 19.35%. The fixation index (Fst = 0.1975) showed that little genetic differentiation had occurred between the isolates of *Metarhizium anisopliae* (Table 3).

Prevalence and diversity of *Metarhizium anisopliae* in China

The Fig. 1, shows the information regarding the sampling sites. Soil samples were taken from two main provinces (Hebei and Henan) of north and north-eastern region of China. Only three isolates of *Metarhizium anisopliae* were isolated from samples collected from twenty sites in Hebei province. While from Henan province, ten different sites of agricultural and forest ecosystem were sampled but only one isolate of *Metarhzium anisopliae* was recovered. In case of south and south western China, we observed that *Metarhzium anisopliae* was widely dispersed in these regions. From six provinces out of forty locations (including agricultural, forest and urban lands) forty nine isolates (twenty one included in this study) were isolated. While from the western region of China, Xinjiang and Xizang (Tibet) were selected for soil sampling. Xinjiang is the largest province according to area and Xizang is the highest place and famous for its extreme temperature, with an average temperature ranging from −18–3.6°C in winter and 7–19°C in summer (http://www.cbw.com/general/gintro/tibet.html). From Xinjiang out of fifty locations only seven isolates were isolated, while from Tibet out of twenty locations, one isolate was recovered. This shows the limited prevalence of *Metarhizium anisopliae* in these regions.

Prevalence and diversity in Laos and other countries

To detect the genetic diversity among the isolates of *Metarhizium anisopliae* in China and the effect of geographical locations on the genetic differentiation, the soil samples were collected from the neighboring countries i.e.,

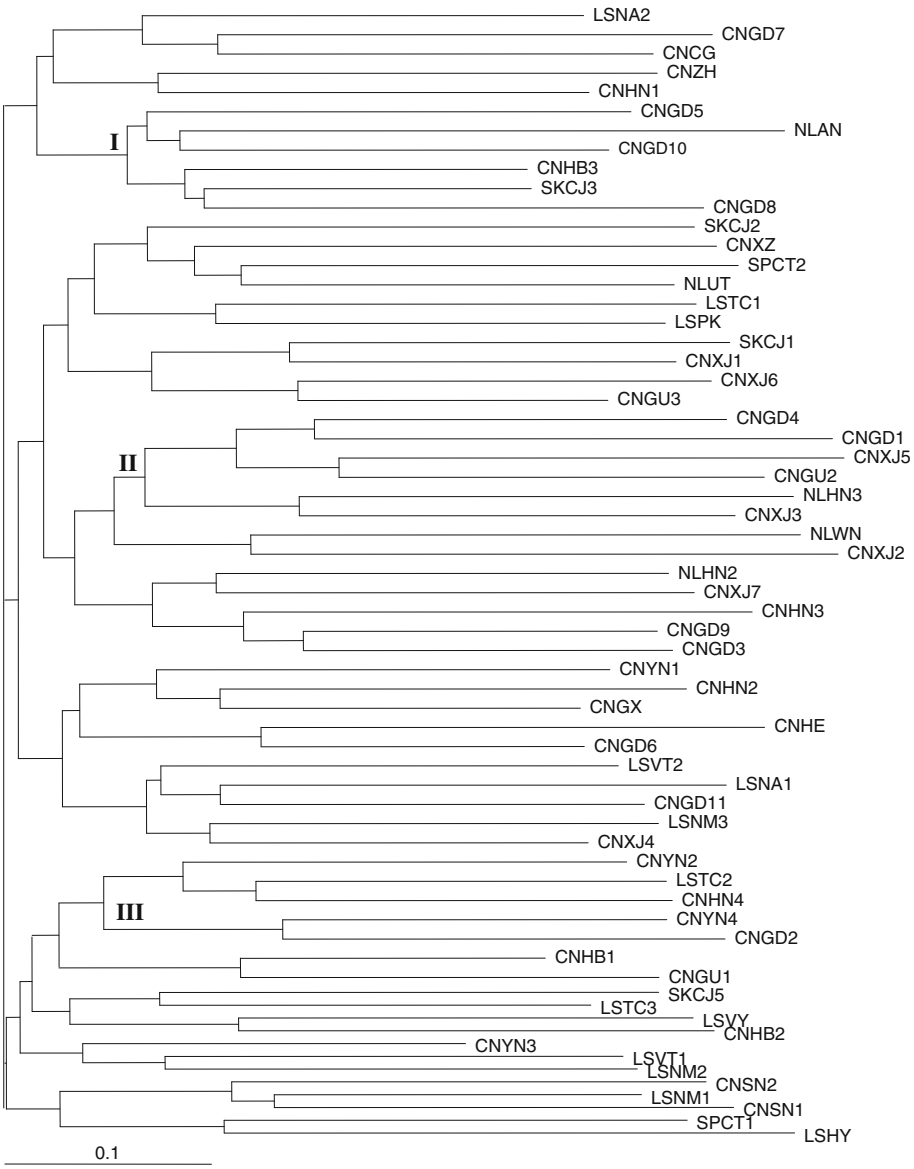

Fig. 4 Dendogram based on simple sequence repeat (SSR) analysis of 62 isolates of *Metarhizium anisopliae* var. *anisopliae* from different geographical origins. (*CN* China, *GD* Guangdong, *CG* Chong Qing, *HB* Hubei, *ZH* Zhejiang, *HN* Hainan, *YN* Yunan, *HE* Henan, *GX* Guangxi, *GU* Guizhou, *XZ* Xizang (Tibet) and *XJ* Xinjiang), (*LS* Laos, *NA* Namphao, *VY* Viengxay, *VT* Vientiane, *NM* Namsanam, *TC* Tadchampa, *PK* Khouay Prefecture and *HY* Houayin), (*SK* South Korea, *CJ* Cheju), (*NL* Netherlands, *HN* Heteren, *WN* Wageningen, *UT* Utrecht and *AN* Arnhem) and (*SP* Singapore, *CT* City)

Table 3 Analysis of molecular variance (AMOVA) results and fixation index (F_{ST} θ) of the simple sequence repeat (SSR) for the isolates of *Metarhizium anisopliae* var. *anisopliae* ($n = 62$, degree of freedom (df) = $(n-1)$

Source of variation	*df*	Sum of squares	Variance components	Percentage variation
SSR				
Among populations	1	48.451	1.9501va	19.35
Within populations	61	641.035	8.6593vb	80.65
Total	62	689.486	10.6094	
Fixation index	F_{ST}	0.1975		

Laos (Fig. 2), South Korea, Singapore and one European country i.e, The Netherlands (Fig. 3). From our results Laos seemed to have a significant distribution of *Metarhizium anisopliae*. Laos was included in the study due to its forest ecosystem. All the samples included in this study were isolated from forest soil and out of twenty five sampling sites from different provinces of Laos (Table 1, Fig. 2), seventeen isolates of *Metarhizium anisopliae* were recovered (Thirteen used in the study), this shows high prevalence of *Metarhizium anisopliae* in this regions.

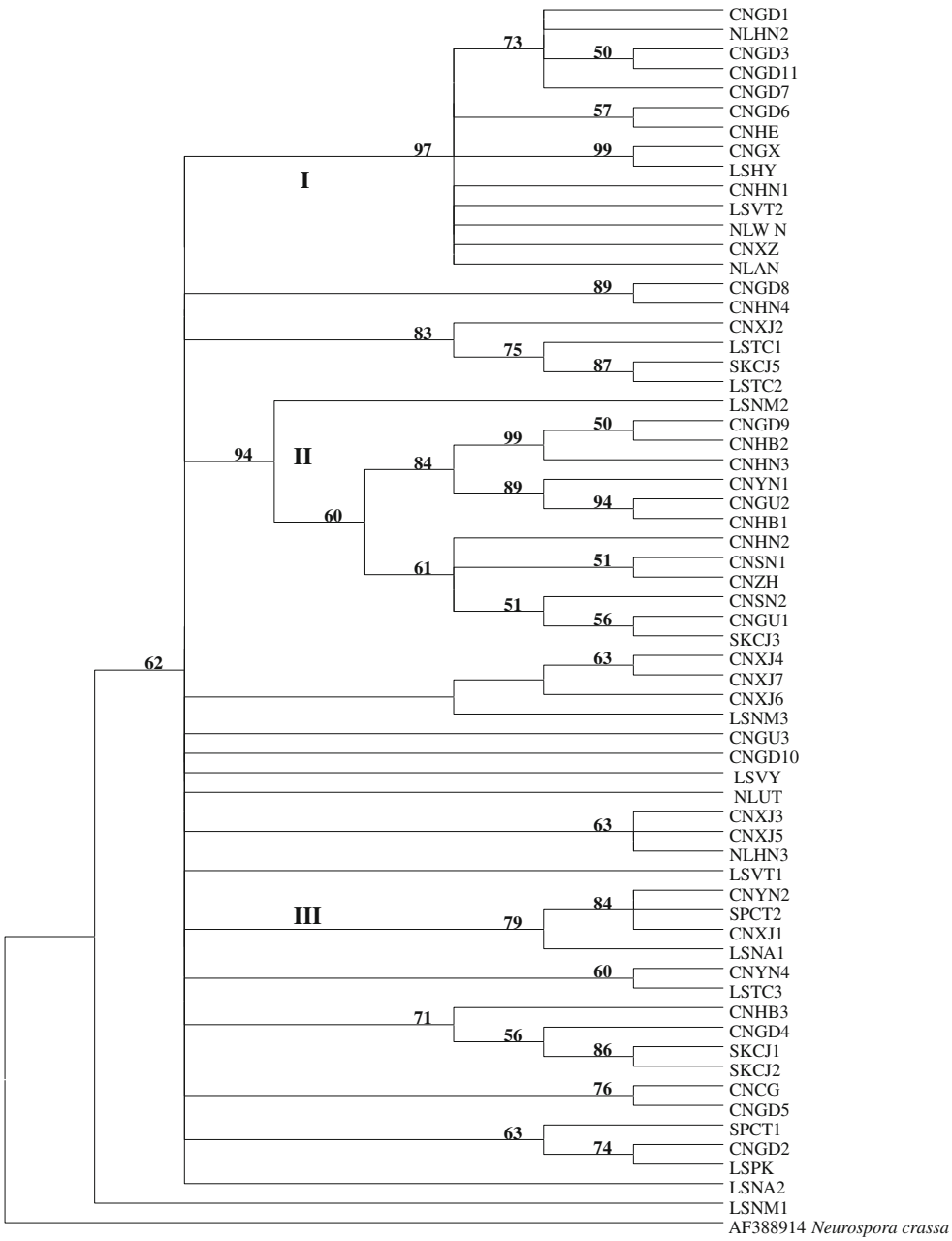

Fig. 5 Dendogram based on Neighbor joining (NJ) method by analysing the internal transcribed spacer(ITS) 1, 2 and 5.8S ribosomal gene of 62 isolates of *Metarhizium anisopliae* var. *anisopliae* from different geographical origins. *Neurospora crassa* was used as outgroup. The numbers at the nodes indicate for the internal branches within the tree obtained by bootstrap analysis (% of 1,000 bootstraps); (*CN* China, *GD* Guangdong, *CG* Chong Qing, *HB* Hubei, *ZH* Zhejiang, *HN* Hainan, *YN* Yunan, *HE* Henan, *GX* Guangxi, *GU* Guizhou, *XZ* Xizang (Tibet) and *XJ* Xinjiang), (*LS* Laos, *NA* Namphao, *VY* Viengxay, *VT* Vientiane, *NM* Namsanam, *TC* Tadchampa, *PK* Khouay Prefecture and *HY* Houayin), (*SK* South Korea, *CJ* Cheju), (*NL* Netherlands, *HN* Heteren, *WN* Wageningen, *UT* Utrecht and *AN* Arnhem) and (*SP* Singapore, *CT* City)

Similar type of results were obtained form the Netherlands soil samples i.e., from five sampling sites, six isolates of *Metarhizium anisopliae* were recovered, in addition to this ten isolates of *Isaria fumosoroseus* were also isolated, that shows the enrichment of Netherland's soil with entomopathogenic fungi. Soil samples were also taken from South Korea and Singapore but incase of South Korean soil, out of twenty sampling sites only four isolates of *Metarhizium* were recovered (Table 1). Soil of Singapore seemed to be a poor source of entompathognes as from eighteen sampling sites only two isolates of *Metarhizium anisopliae* were obtained. This shows that the soils of these regions harbor less entomopathogenic fungi. ITS-1 and ITS-2 region sequencing confirms that all isolates are *Metarhizium anisopliae* var. *anisopliae.*

Overeall genetic diversity by ITS-rDNA sequence data

In total, sixty two isolates of *Metarhizium anisopliae* were isolated from agricultural, forest and urban soils from China, Laos, South Korea, Singpaore and The Netherlands (Table 1). The ITS-1 and ITS-2 regions along with the 5.8S rRNA gene were sequened form all isolates. The dendogram obtained by the NJ method using MEGA 4.1 clustered all isolates into three clades (Fig. 5).

The clade I clustered mainly the isolates of *Metarhizium anisopliae* from three countries i.e, China, Laos and The Netherlands. The south and south western regions of China had wide distribution of *Metarhizium anisopliae*, but minimal genetic variablity was observed in this clade, which may be due to the same soil source in this region. The clade II showed significant diversity and variability among the isolates of *Metarhizium anisopliae* from China, Laos, Netherlands and South Korea.. In clade II one isolate CNSN2 isolated from an insect (cicada) was clustered with the isolate of its neighboring province (Guizhou) and little genetic differecne was observed between them. Some isolates in clade II like CNGU3, CNGD10, LSVY (isolated from forest soils) and NLUT (isolated from urban soil) showed some diversity at genetic level but still these isolates have not much genetic variation to be grouped in separate clade.

Similar results were observed in clade III as it clustered only the isolates from south western China and from other countries. The dendogram (NJ analysis) obtained by clustering sixty two isolates showed one isolate i.e., LSNM1 with maximum variation from the other isolates, as it didn't group with any other isolates.

Genetic distances of *Metarhizium anisopliae* according to locations

The genetic distances among the isolates from different locations were calculated by Kimuras two parameter model (Table 4). Apparently minimal significant distances among the isolates of different countries were observed. The genetic distance among the isolates range from 0.23 to 0.34%, with maximum genetic distance of 0.34% among the isolates of Laos and Netherlands.

Genetic distances of *Metarhizium anisopliae* according to different sources

The results for the genetic distances among isolates from different sources were 0.23–0.32%, which are similar to the values obtained for the isolates of differnet geographical locations (Table 5). Maximum genetic distance of 0.32% was observed between the isolates recovered from mountainous and urban soils.

Table 4 Genetic distances of *Metarhizium anisopliae* var. *anisopliae* according to locations

	China	South Korea	Netherlands	Laos	Singapore
China	N/C				
South Korea	0.031				
Netherlands	**0.023**	0.029			
Laos	0.032	**0.034**	0.028		
Singapore	0.029	0.033	0.027	0.031	N/C

* N/C = not calculated

The bold font represents the range of genetic distance between the isolates of *Metarhizium anisopliae* from different countries

Table 5 Genetic distances of *Metarhizium anisopliae* var. *anisopliae* according to different sources

	Agricultural	Forest	Mountain	Insect	Volcano	Urban
Agricultural	N/C					
Forest	0.029					
Mountain	**0.023**	0.026				
Insect	0.031	0.030	0.027			
Volcano	0.028	0.029	0.027	0.030		
Urban	0.031	**0.032**	0.029	0.030	0.030	N/C

* N/C = not calculated

The bold font represents the range of genetic distance between the isolates of *Metarhizium anisopliae* from different soil sources

Discussion

In this study the prevalence and genetic diversity of the entomopathogenic fungus *Metarhizium anisopliae* in various geographical regions including China, Laos, South Korea, Singapore and The Netherlands were examined. China was divided into four main sections for soil sampling i.e, north and north eastern regions, south and south western regions, central and western region. The samples were taken from thirteen provinces (twenty nine cities) on the bases of different geographical location and source. For Laos the sampling locations (five provinces, seven cities) were assigned on the basis of maximum area coverege to get the best possible results for the variability of entomopathogenic fungus. In addition to this, complementary sampling locations were selected from the neighboring countries like, Singapore, South Korea and from a European country, the Netherlands, to get the maximum variability ratios among the isolates.

Metarhizium anisopliae is an entomopathogenic fungus with worldwide distribution. It has been isolated from a variety of habitats like agricultural, forest and urban soils from different geographical origins (St. Leger et al. 1992). From our finding it is obvious that this fungus prevails in abudance in south-south western regions of China, as the isolates of *Metarhizium anisopliae* were recovered from all of the six provinces including agricultural, forest and urban habitats. This can be due to the climatic conditions of this area as these regions have long summer with an average temperature of 27–33°C and a short winter without snow fall. The average rainfall in these regions is 760–1,000 mm/year (http://www.cma.gov.cn/english/). These

climate conditions may help and are suitable for the growth of *Metarhizium anisopliae*. Under dry conditions, fungus may survive in the hyphal stage, but fail to produce conidia on the outside of the body, but below 10°C and above 35°C no sporulation occurs. The optimal temperature for sporulation is 25–30°C. (http://ec.europa.eu/food/plant/protection/evaluation/existactive/BIPESCO-5_F52.pdf; Sun et al. 2003). In contrast to this, the extreme climate conditions like long winter with snow fall and annual rain fall ranging upto 1,150 mm/year in north-north-eastern regions may results in less prevalence of *Metarhizium anisopliae*. Similar type of results were observed for the western part (Xizang, Tibet) due to extreme temperature conditions in this region, with an average temperature ranging from −18 to 3.6°C in winter and 7–19°C in summer (http://www.cbw.com/general/gintro/tibet.html). Therefore, it can be spaculated that due to harsh weather conditions occurrence of *Metarhizium anisopliae* is scarce. This fungus has been already reported in different parts of China (Sun and Liu 2008). In case of isolates recovered from Laos, an interesting fact related to soils of Laos was observed, as none of the isolate was recovered from the agricultural or urban soil but from the forest ecosystem. This indicates that the forests of Laos are good source of entomopathogens. Studies have proved that the isolates receovered from the forests of Laos are effective against the control of *Pieris rapae* (Freed et al. unpublished data).With regards to the occurrence of insect pathogenic fungi in South Korea and Singapore, more sampling is needed to perceive the presence of entomopathogens in these regions.

To determine the genetic diversity of *Metarhizium anisopliae* in Asian countries and European country, we used microsatellite markers (SSR technique) and ITS-rDNA regions sequencing data including ITS-1, ITS-2 and 5.8S gene regions as previosuly studied for *Metarhizium anisopliae* and other fungi (Entz et al. 2005; Márquez et al. 2006). From the SSR results it was found that a wide distribution of *Metarhizium anisopliae* existed in the dendogram but the low level of polymorphism was observed. It can be spaculated that ten SSR markers used in this study only detected similar genotypes as in case of clade I and II. Since these loci displayed less polymorphism (Tables 2, 3), therefore more SSR markers may be required to find the differentiation between all genotypes. The low differentiation can be due to the presence of large number of common alleles co-evolved by mutation or these were identical by gene flow. The less genetic demarcation among the isolates may be related to the origin of isolates as approximately 95% of the isolates belonged to the soil samples collected from agricultural land (orchards, cereals, fiber crops etc.), forest ecosystem and with one isolate CNSN2 that was isolated from insect (cicadas). Results described herein are in accordance with another study undertaken by Velasquez et al. (2007), in which less polymorphism was observed in the thirty eight isolates collected from Chile.

The results of ITS-rDNA sequence data also confirmed the SSR results as minimal genetic variation was observed in all sixty two isolates, even the isolates were collected from different ecological regions with different climatic zones. It is also very interesting that in clade II the isolates from south and south western part of China were clustered with the isolates of Laos and South Korea (Fig. 5). The results showed that some characters are being shared by these isolates or the isolates from these regions are influencing other isolates. The grouping of Laos isolates in this clade show that all these isolates in the same clade might have some shared alleles or same genetic make up. In contrast to present results, considerable genetic variability for mitosporic fungi including the entomopathogenic fungus *Metarhizium anisopliae* has been reported in previous studies (Driver et al. 2000; Aquino de Muro et al. 2003; Enkerli et al. 2005). The isolate LSNM1 showed maximum variance in case for the ITS-rDNA sequence data as it didn't grouped in any of the clade, in contrast to this for the SSR results it was grouped in the clade III (Figs. 4, 5), with possible reason that the different gene regions can give some what different results with respect to one another, while for the variation in ITS-rDNA regions this can be assumed that its genetic make up is different from other isolates. The predominance of the closely related genotypes across different habitats suggests that sympatric lines are uncommon in these areas as it was observed in case of studies by Inglis et al. (2008). The genetic distances among the isolates from different locations were calculated (Tables 3,4), the results showed that minimum differences exists among the isolates of different locations, host range or different ecosystem. These results confirm the findings of Inglis et al. (2008) that the adjoining areas can have minimal genetic variations. In clade II an isolate CNSN2 recovered from insect was clustered with an isolate CNGU1, isolated from forest soil but minimal distance was observed between these two isolates. (Fig. 5). The minimal difference between these isolates shows a weak association between insect host range and isolate genotype as previously described by (Cobb and Clarkson 1993; Bidochka et al. 1994). The less diversity of *Metarhizium anisopliae* in all the regions studied can be due to their similar geography or the topography of the sampling sites. The biogeography theory suggests that local species richness is affected by past event and the enviornmetnal conditions (Hughes Martiny et al. 2006). It is also suggested that minimal variability observed can be the result of immigration of *Metarhizium anisopliae* to neighboring regions or the isolates have evolve from common ancestor strain.

In conclusion, closely related genotypes of *Metarhizium anisopliae* were found dominant in different geographical ecosystems (agricultural, forest and urban) in south-southwestern regions of China. These results may provide an overview for the development of an effective entomopathogenic biocontrol agent for enviornmental friendly control system. Still more work is needed to check the effectiveness of these isolates against different insect pest complex.

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