Genetic diversity of the fungal pathogen *Metarhizium* spp., causing epizootics in Chinese burrower bugs in the Jingting Mountains, eastern China

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**Abstract** Based on the internal transcribed spacer and inter-simple sequence repeats (ISSR), the phylogenetic relationship and genetic diversity of *Metarhizium* spp., pathogens found in Chinese burrower bugs, *Schiodtella formosana*, were analyzed. The results showed that the causative agents of the epizootic green muscardine disease in populations of *S. formosana* were actually composed of *M. anisopliae* and its sister species, *M. robertsii*. The genetic structure of *Metarhizium* spp. populations were assessed using ten ISSR. A 3D principal component analysis of 51 isolates sampled on different occasions revealed that the *Metarhizium* spp. populations were temporally heterogeneous. They differentiated into two main clades including over 71% of all strains causing epizootics, with a similarity of 83%. The population differentiation was relatively low (G_ST, 0.2080), reflecting a large proportion of gene differentiation (79.2%) within the populations. Further knowledge of the complex species and heterogeneous populations of *Metarhizium* spp. may be necessary for sustainable control methods of *S. formosana*.

**Keywords** *Metarhizium* spp. · *Schiodtella formosana* · Epizootic · Genetic diversity

**Introduction**

*Schiodtella formosana* (Takano and Yanagihara) belongs to the Cydnidae (Hemiptera) [22, 55] and its representatives are usually known under the common name of burrower bugs (or burrowing bugs) due to their specific lifestyle (many of them live in the soil and feed on roots) [28]. The nymphs and adults of *S. formosana* harm wheat, maize, sorghum and Poaceae plants by piercing and sucking their roots, which leads to slowed growth and eventual death [26, 55]. Their biology is very difficult to investigate because most species are small in size and live in the soil [26]. Due to the special characteristics in habits of *S. formosana*, effective integrated control methods include using crop rotation patterns which can change survival conditions and chemical control such as isofenphos-methyl.

*Metarhizium* is a genus of fungi in the family of Clavicipitaceae (class: Pyrenomycetes sphaerales, phylum: Ascomycotina), and is widespread throughout the world [41]. Three *Metarhizium* species, *M. anisopliae*, *M. flavoviride* and *M. acridum*, are among the most abundant and best-studied entomopathogens [34, 41]. On the basis of their morphological features, the first revision of the genus *Metarhizium* was conducted by Tulloch [49], while a phylogenetic analysis also revealed that *M. anisopliae* embody a cryptic phylogenetic species complex [2–4]. Based on the results of the phylogenetic analyses and morphological data, four varieties of *M. anisopliae*, including *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum*, *M. anisopliae* var. *majus* and *M. anisopliae* var. *lepidiotum* were upgraded to species rank. *M. bruneum*, *M. pingshaense*, *M. guizhouense*, *M. robertsii* and *M. globosum* were also recognized as phylogenetic species [4]. Since 1878, when Metschnikoff used *M. anisopliae* to control *Anisoplia austriaca* and *Ceonus*...
punativentris, Metarhizium species and varieties have been widely applied as fungal biocontrol agents for insect control. They have been found and isolated from a wide variety of insects from different orders, and even from mites and nematodes [39]. As important fungal entomopathogens, Metarhizium spp. have great potential against underground pests and possess many advantages, such as effective pathogenicity against target organisms, but no harm to humans or non-target animals. During the last 25 years, several mycopesticides based on M. anisopliae have been commercialized and registered in various countries. Mycopesticides based on M. anisopliae have been recommended for pest control, and their application in some area even exceeded the use of Beauveria. The largest single country of use is Brazil, where commercial biopesticides based on M. anisopliae are used against spittlebugs on around 750,000 ha of sugarcane and 250,000 ha of grassland annually [9, 27]. In addition, species from the genus Metarhizium are used as biological control agents to manage and prevent infestations of various species of pest insects, including locusts [11, 30, 31], grasshoppers [23, 31], malaria mosquitos [6, 44], ambrosia beetles [7], and horn flies [35]. Nevertheless, the major research about Metarhizium is still at an experimental stage and far from large-scale application.

Recently, molecular markers have been used for the identification, taxonomy, evolution and other studies of Metarhizium, and different modern biological techniques have made great contributions, such as enzymology [12, 40, 44], RAPD (random amplified polymorphic DNA) [1, 13, 16, 17, 24, 54], RFLP (restriction fragment length polymorphism) [5, 14, 25, 33, 38], rDNA sequencing [10, 13, 29] and microsatellite markers (SSRs) [15]. With the development of these new techniques, many ecological and taxonomic problems could be resolved. Moreover, molecular tools can provide substantial support for ecological research on Metarhizium and offer insight into this so far inaccessible system.

Although many strains of Metarhizium were isolated from cadavers of insects, the principal habitat of some Metarhizium species may not be insects, but the root rhizosphere [19, 45]. From an epidemiological point, spores residing in soil are the initial source of inocula that causes diseases on insects, but large numbers of cadavers sampled outdoors might be more advantageous to elucidate the epizootic mechanisms of Metarhizium in insects. In the fall of 2009, a dramatic increase of epizootics caused by Metarhizium in burrower bug populations were detected in a limited area of the Jingting Mountain area in southeast Anhui province, China. The average cadaver density reached as many as five individuals per 1 m², although many live individuals were also detected, displaying epizootic symptoms.

Therefore, we conducted an analysis of the phylogenetic diversity of Metarhizium spp. pathogens from Chinese burrower bugs, S. formosana. ISSR markers were also applied to elucidate the population genetic structure of Metarhizium spp. isolates underlying these epizootics. This study provides the first features of epizootic isolates of Metarhizium using rDNA sequencing and ISSR marker information.

Materials and methods

Epizootic sites and fungal isolates

A total of 48 isolates of Metarhizium spp. was obtained from cadavers of burrower bugs and were used for characterization by ISSR-PCR, as shown in Table 1.

Extraction of mycelial genomic DNA

Previous protocols for DNA extraction were used in this study, including the benzyl chloride method [56] with a modification described by Wang et al. [50]. Based on this description, air-dried pellets were resuspended in 500 μl of Tris–EDTA (TE) buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0), to which 2 μl RNase (10 mg ml⁻¹) was added, and incubated at 37 °C for 1 h. The concentration and quality of the purified DNA was evaluated both by 0.8 % agarose gel electrophoresis and by spectrophotometry.

DNA amplification and sequencing

Using aliquots of extracted DNA, we amplified the region of the ribosomal repeat from the 3’ end of 18S rDNA to the 5’ end of the 28S rDNA with partial sequence, spanning internal transcribed spacer (ITS) 1, 5.8S rDNA and ITS2. Primer sequences used were 5’-CTTCGGCTATTGATATGC-3’ (ITS4) and 5’-GGAGTAAAAGTC-GTAACAGG-3’ (ITS5) [51]. All primers were custom synthesized by Shanghai Sangon Biologic Engineering & Technology and Service Co., Ltd, China. PCR reaction were performed in 25 μl reaction volumes containing 20 ng genomic DNA, 1× reaction buffer, 0.5 mM dNTPs, 2 mM MgCl₂, 0.4 μM of each primer and 0.625 U Taq DNA polymerase (Dream Taq™, Fermentas Life Science). The conditions of amplification were 2 min at 94 °C, followed by 35 cycles for 30 s at 94 °C, 30 s at 52 °C, 1 min at 72 °C and a final step for 10 min 72 °C. PCR products were detected by electrophoresis in 0.8 % TAE agarose gels containing 10 μg ml⁻¹ ethidium bromide. Nucleotide sequencing of PCR products was performed by Shanghai Invitrogen Biotechnology Co., Ltd.

ISSR amplification

A total of ten ISSR primers were evaluated by their capacity to produce polymorphic, easy-to-score and
reproducible banding patterns for Metarhizium spp. isolates (Table 1). The ISSR procedure was performed essentially as described by Luan et al. [32] with some modifications. Each PCR reaction contained 1× PCR buffer, 2.0 mM MgCl2, dNTPs at 100 μM each, primers at 0.4 μM, 0.75 U DNA Taq polymerase (Dream Taq™, Fermentas Life Science) and 20 ng template DNA. The PCR reaction mix was adjusted to a final volume of 25 μl with DEPC water (Sangon®, Sangon Biotech). The PCR reactions were performed as follows (with the annealing temperature optimized for each primer): 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 48–54 °C for 1.5 min, 72 °C for 2 min, and a final extension of 10 min at 72 °C. PCR was performed in triplicates using a TPersonal Thermocycler (Biometra®, Germany) and the gel images were recorded by a Gel Documentation System (Tanon Gis-2008).

Data analysis

The ITS1-5.8S-ITS2 gene sequences were aligned with the Clustal X program package [47] at default settings. Selected sequences available from Genbank (accession nos.: M. anisopliae var. anisopliae AF135210, M. majus AF137061, M. anisopliae var. acridum AF137062, M. anisopliae var. lepidiotum AF137065, M. album AF137067, M. flavoviride var. flavoviride AF138270, M. flavoviride var. minus AF138272, AF139850, M. flavoviride var. novazealandicum AF139853 and M. anisopliae ARSEF 2575, 727) were also included in the analysis. Beauveria bassiana JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-parameter distances were computed with JN038193 was used as an outgroup in the phylogenetic analysis. Kimura 2-paramet

Results

Identification of fungal entomopathogens

The 21 ITS sequences were grouped into different sequence lineages (Fig. 1), and our sequences of Metarhizium spp. formed multiphyletic groups with the closely related organisms M. anisopliae var. anisopliae and M. robertsii, respectively. Here, M. robertsii, formerly known as M. anisopliae var. anisopliae, which has a wide insect host range, was described as a new species [4]. Thus, the causative agents of the green muscardine disease in the populations of S. formosana were composed of M. anisopliae and M. robertsii, and Metarhizium species were employed for the nomenclature of fungal entomopathogens in this study.

Polymorphism of ISSR-PCR amplified products

Ten primers generated a total of 93 reliable bands. The different primer pairs amplified between eight and 12 products, 9.3 on average, which ranged in length from 300 bp to 1.7 kb. Of the 93 bands, 80 (86.02 %) were polymorphic (Table 2). As shown in Table 2, the percentage of polymorphic loci (P) ranged from 70 to 100 %. The ISSR marker of P11 in Metarhizium spp. showed the highest Nei’s genetic diversity (h, 0.2534) while P19 had the lowest h (h, 0.0413). Shannon’s information index of the ISSR markers ranged from 0.0977 to 0.4062 (Table 2), suggesting that the eight primers were powerful enough to distinguish diverse isolates of Metarhizium spp. A high level of genetic variation was observed.

Principal coordinate analysis

Based on pair-wise genetic similarity of the isolates, the individual-wise dendrogram constructed by UPGMA cluster analysis displayed a highly heterogeneous relationship, with genetic similarity varying from 0.58 to 1.0. Based on the primitive matrix of ISSR data by NTSYSpc-2.10e software, a principal component analysis (PCA) was used to generate a three-dimensional plot (Fig. 2), with contribution values explained by Dim 1, Dim 2 and Dim 3 at 28.2, 13.2, and 7.5 %, respectively. The complicated relationship between isolates was illustrated by the results of PCA analysis. Of the 51 isolates in three populations, five isolates from the

Table 1 Host, collecting site and time of 51 Metarhizium spp. isolates used in this study.

<table>
<thead>
<tr>
<th>Population ID</th>
<th>Collecting site</th>
<th>Collecting time</th>
<th>Host identity</th>
<th>Isolate number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Jingting Mountain</td>
<td>11-09-2009</td>
<td>Hemiptera: Cydnidae</td>
<td>1–20</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>27-09-2009</td>
<td>Hemiptera: Cydnidae</td>
<td>24–51</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>14-08-2009</td>
<td>Hemiptera: Pentatomidae</td>
<td>21, 22</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>01-08-2009</td>
<td>Hemiptera: Pentatomidae</td>
<td>23</td>
</tr>
</tbody>
</table>
burrowing bugs of populations 1 and 2 were scattered outside the main clades A and B. Among the isolates from the burrowing bugs, 15 isolates from population 1 collected on 11-Sep-2009, and four isolates collected on 27-Sep-2009 clustered into clade A, while 20 isolates from population 2 collected on 27-Sep-2009, four isolates collected on 11-Sep-2009 and three isolates from pentatomid insects clustered into clade B (Fig. 3).

The phylogenetic relationship between the three heterogeneous populations was analyzed after they were homologized through further categorization into homogenous subunits. The homogenous main body of population 1 scattered in clade A was categorized as subpopulation 1-1, while the four isolates in clade B were identified as subpopulation 1-2. The main body of population 2 scattered in clade B was identified as subpopulation 2-1 and the four isolates in clade A were identified as subpopulation 2-2. The five isolates scattering outside the Clade A and B were categorized as subpopulations 1-3 and 2-3 to 2-6, according to the isolate number, respectively (Table 3).

Genetic diversity of populations and subpopulations

**Within population diversity**

The percentage of polymorphic loci ($P$) ranged from 13.11 (population 3) to 76.74 % (population 1), with an overall value of 93.55 %. Assuming Hardy–Weinberg equilibrium, Nei’s gene diversity ($h$) ranged from 0.058 (population 3)
to 0.162 (population 1), with a total value of 0.186. Shannon’s information index \((I_i)\) estimates were slightly higher (0.084 and 0.263, respectively; Table 3), with a total value of 0.312.

### Within subpopulation diversity

Assuming Hardy–Weinberg equilibrium, Nei’s gene diversity \((h)\) ranged from 0.058 (population 3) to 0.127 (sub-population 2-2). Shannon’s information index \((I_i)\) estimates were slightly higher (0.084 and 0.186, respectively).

### Genetic identity and genetic distance of subpopulations

To further evaluate the relationships among the subpopulations, individual ISSR haplotypes were used to estimate subpopulation differentiation. The results shown in Table 4 and Fig. 2 revealed that the largest genetic distance appeared between subpopulations 1-2 and 1-3 (0.6589), while the least distance occurred between subpopulation 2-1 and population 3 (0.0222). The largest genetic identity was observed between subpopulation 2-1 and population 3 (0.9780), while the least identity appeared between subpopulations 1-2 and 1-3 (0.5174).

### Gene flow and genetic differentiation between populations and subpopulations

Gene differentiation and gene flow between subpopulations were calculated with POPGENE (Table 5). The evidence of significant population genetic structure allowed us to calculate ISSR-site frequencies separately for subpopulations and to generate matrices of the coefficients of gene differentiation and gene flow. The maximum \(G_{ST}\) of 0.9217 appeared between the subpopulations 1-2 and 1-3, while the minimum \(G_{ST}\) of 0.1776 occurred between the subpopulations 1-1 and 2-2. The resulting \(G_{ST}\) observed between the subpopulations 1-1 and 2-1 was 0.3730.

Based on POPGENE analysis, the total gene diversity \((H_t)\) of the 51 isolates consisting of three populations was at 0.1286, and gene diversity within the group \((H_s)\) at 0.1019. The distribution of gene diversity revealed a large proportion of gene differentiations (79.2 %) within populations, whereas only 20.8 % was found among populations (Table 6).

### Cluster analysis based on subpopulations

We conducted a UPGMA cluster analysis of the ten subpopulations by using the polymorphic loci based on Nei-Li genetic distance, which fits well to the genetic similarity matrix. The dendrogram comprised two main groups, one including the subpopulations 1-1 and 2-2, and the other group containing the subpopulations 1-2, 2-2 and population 3. The finding that the two major subpopulations 1-1...
and 2-1 are apparently polyphyletic suggests that temporal heterogeneity appeared between these two subpopulations.

**Discussion**

Huang et al. derived their phylogenetic inferences exclusively from ITS, which performs poorly in resolving lineages within *Metarhizium* [20, 21]. Nishi et al. analyzed the phylogenetic relationship of *Metarhizium* spp. isolated from soil in Japan. The results of ITS sequence analysis divided all isolates into three clades, while EF-1α sequence analysis could yet differentiate *M. anisopliae* and *M. robertsii* [37]. Due to relatively slow evolution of the the nuclear sequence (ITS-like), it could provide an important reference for identification of the genus *Metarhizium*. In this study, the ITS sequence data of nine isolates were grouped into the lineage of *M. anisopliae* var. *anisopliae* and *M. robertsii* (Fig. 1). Based on taxa of the species rank *M. anisopliae* [4], we employed the name of *Metarhizium* spp., containing *M. anisopliae* and *M. robertsii*, to the entomopathogenic fungi found in the epizootics. Thus, naturally occurring *M. anisopliae* complex species causing epizootics in the populations of Chinese burrower bugs were observed with molecular techniques.

Tigano-Milani et al. [48] investigated *Paecilomyces fumosoroseus* from epizootics observed in India and Pakistan with arbitrarily primed PCR and tRNA-PCR. They suggested that different genotypes exhibit only 70% similarity [48], which indicated that genetically diverse isolates might be required for the development of an epizootic disease. The results were similar to those observed by Castrillo et al. in a study to assess genetic variation of *Beauveria bassiana* associated with the darkling beetle by using RAPD markers [8]. In our study, phylogenetic analysis of ten ISSR primers revealed polymorphism within isolates that had been phylogenetically

**Table 3** Genetic diversity of population and subpopulation of *Metarhizium* spp.

<table>
<thead>
<tr>
<th>Population &amp; subpopulation</th>
<th>Main Clade</th>
<th>Isolate amount</th>
<th>$N_a$</th>
<th>$N_e$</th>
<th>$h$</th>
<th>$I_s$</th>
<th>$P$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A, B</td>
<td>20</td>
<td>1.692 ± 0.465</td>
<td>1.248 ± 0.294</td>
<td>0.162 ± 0.162</td>
<td>0.263 ± 0.233</td>
<td>69.23</td>
</tr>
<tr>
<td>2</td>
<td>A, B</td>
<td>28</td>
<td>1.767 ± 0.425</td>
<td>1.209 ± 0.262</td>
<td>0.142 ± 0.144</td>
<td>0.241 ± 0.206</td>
<td>76.74</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>3</td>
<td>1.131 ± 0.340</td>
<td>1.105 ± 0.272</td>
<td>0.058 ± 0.151</td>
<td>0.084 ± 0.217</td>
<td>11.13</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>51</td>
<td>1.936 ± 0.247</td>
<td>1.277 ± 0.281</td>
<td>0.186 ± 0.141</td>
<td>0.312 ± 0.188</td>
<td>93.55</td>
</tr>
<tr>
<td>1-1</td>
<td>A</td>
<td>15</td>
<td>1.344 ± 0.479</td>
<td>1.176 ± 0.296</td>
<td>0.108 ± 0.169</td>
<td>0.166 ± 0.249</td>
<td>34.43</td>
</tr>
<tr>
<td>1-2</td>
<td>B</td>
<td>4</td>
<td>1.143 ± 0.353</td>
<td>1.105 ± 0.269</td>
<td>0.060 ± 0.149</td>
<td>0.087 ± 0.215</td>
<td>14.29</td>
</tr>
<tr>
<td>2-1</td>
<td>B</td>
<td>20</td>
<td>1.344 ± 0.479</td>
<td>1.117 ± 0.230</td>
<td>0.079 ± 0.132</td>
<td>0.130 ± 0.202</td>
<td>34.38</td>
</tr>
<tr>
<td>2-2</td>
<td>A</td>
<td>4</td>
<td>1.309 ± 0.466</td>
<td>1.222 ± 0.350</td>
<td>0.127 ± 0.195</td>
<td>0.186 ± 0.282</td>
<td>30.91</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>3</td>
<td>1.131 ± 0.340</td>
<td>1.105 ± 0.272</td>
<td>0.058 ± 0.151</td>
<td>0.084 ± 0.217</td>
<td>11.11</td>
</tr>
</tbody>
</table>

**Table 4** Genetic identity $I$ and genetic distance $D$ of different subpopulations of *Metarhizium* spp.

<table>
<thead>
<tr>
<th>Subpop ID</th>
<th>1-1</th>
<th>1-2</th>
<th>2-1</th>
<th>2-2</th>
<th>3</th>
<th>1-3</th>
<th>2-3</th>
<th>2-4</th>
<th>2-5</th>
<th>2-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>****</td>
<td>0.8631</td>
<td>0.9226</td>
<td>0.9729</td>
<td>0.9009</td>
<td>0.6523</td>
<td>0.7380</td>
<td>0.7946</td>
<td>0.6680</td>
<td>0.8415</td>
</tr>
<tr>
<td>1-2</td>
<td>0.1472</td>
<td>****</td>
<td>0.9597</td>
<td>0.8622</td>
<td>0.9655</td>
<td>0.5174</td>
<td>0.6055</td>
<td>0.7486</td>
<td>0.6881</td>
<td>0.8587</td>
</tr>
<tr>
<td>2-1</td>
<td>0.0805</td>
<td>0.0411</td>
<td>****</td>
<td>0.9157</td>
<td>0.9780</td>
<td>0.5648</td>
<td>0.6511</td>
<td>0.7507</td>
<td>0.7175</td>
<td>0.8845</td>
</tr>
<tr>
<td>2-2</td>
<td>0.0275</td>
<td>0.1482</td>
<td>0.0881</td>
<td>****</td>
<td>0.9116</td>
<td>0.6523</td>
<td>0.7255</td>
<td>0.7761</td>
<td>0.6748</td>
<td>0.8154</td>
</tr>
<tr>
<td>3</td>
<td>0.1044</td>
<td>0.0351</td>
<td>0.0222</td>
<td>0.0926</td>
<td>****</td>
<td>0.5577</td>
<td>0.6348</td>
<td>0.7375</td>
<td>0.7302</td>
<td>0.8807</td>
</tr>
<tr>
<td>1-3</td>
<td>0.4272</td>
<td>0.6589</td>
<td>0.5713</td>
<td>0.4272</td>
<td>0.5838</td>
<td>****</td>
<td>0.5806</td>
<td>0.5376</td>
<td>0.5591</td>
<td>0.5914</td>
</tr>
<tr>
<td>2-3</td>
<td>0.3038</td>
<td>0.5017</td>
<td>0.4291</td>
<td>0.3210</td>
<td>0.4544</td>
<td>0.5436</td>
<td>****</td>
<td>0.6559</td>
<td>0.5269</td>
<td>0.6882</td>
</tr>
<tr>
<td>2-4</td>
<td>0.2299</td>
<td>0.2895</td>
<td>0.2868</td>
<td>0.2535</td>
<td>0.3044</td>
<td>0.6206</td>
<td>0.4217</td>
<td>****</td>
<td>0.6129</td>
<td>0.7742</td>
</tr>
<tr>
<td>2-5</td>
<td>0.4035</td>
<td>0.3738</td>
<td>0.3320</td>
<td>0.3933</td>
<td>0.3144</td>
<td>0.5814</td>
<td>0.6408</td>
<td>0.4895</td>
<td>****</td>
<td>0.6882</td>
</tr>
<tr>
<td>2-6</td>
<td>0.1726</td>
<td>0.1523</td>
<td>0.1227</td>
<td>0.2040</td>
<td>0.1271</td>
<td>0.5253</td>
<td>0.3737</td>
<td>0.2559</td>
<td>0.3737</td>
<td>****</td>
</tr>
</tbody>
</table>

Note: Nei’s genetic identity (above diagonal) and genetic distance (below diagonal).
and morphologically classified as *Metarhizium* spp. In conclusion, based on our phylogenetic analysis we found that the isolates causing the same epizootic did not form a monophyletic cluster. Among populations, two main groups sharing more than 85% similarity were observed among 43 (43/48, 89.6%) strains associated with Chinese burrower bugs.

Due to its versatile life history pattern, a broad host range is required by *M. robertsii* to accommodate for diverse lifestyles and allow host switching [18, 45]. In this study, three *Metarhizium* isolates from Pentatomidae insects clustered in clade B, and showed a close relationship to samples collected on 27-Sep-2009. Gene differentiation and gene flow between population 3 and subpopulation 2-1 were 0.2157 and 0.9091, respectively. These findings suggest that the pathogens found in the population of bugs on 27-Sep-2009 might have originated from other insect hosts.

This study further demonstrates that ISSRs are a reliable genotyping technique for estimating inter-/intraspecific variation of *Metarhizium* spp. The genetic diversity of *Metarhizium* populations and subpopulations in the present study ranged from 0.058 to 0.162, and Shannon’s information index ranged from 0.084 to 0.263 (Table 3). The total gene diversity (*Ht*) of the *Metarhizium* spp. population was 0.1286, and gene diversity within the group (*Hs*) was 0.1019. However, the coefficient of gene differentiation of the two main subpopulations (subpopulations 1-1 and 2-1) sampled on different time points was 0.3730 (Table 5). According to qualitative guidelines by [52], *F*<sub>ST</sub> (or *G*<sub>ST</sub> in this case) values above 0.25 indicated higher genetic heterogeneity between these two subpopulations. Apart from these two predominant subpopulations sharing 85% similarity, there several minor subpopulations and scattered strains were found. These enhanced the population heterogeneity and increased the environmental adaptability and naturally occurring complexity of *Metarhizium* spp. populations. The predominant subpopulations result in a rapid increase of incidences, thus forming an epizootic outbreak, while the minor subpopulations or scattered strains have an enzootic impact on their host population, which benefits the long-term ecological balance. The heterogenous populations of entomopathogenic fungi are characteristic for the coexistence of epizootic and enzootic strains. Heterogenous populations might facilitate the pathogen’s occurrence, persistence and future outbreaks.

In summary, the most significant finding of this study is that pathogens causing the green muscardine disease in populations of *S. formosana* are actually composed of *M. anisopliae* and its sister species *M. robertsii*. The *Metarhizium* spp. isolates showed high genetic diversity, which is consistent with their temporal heterogeneity during the different epizootic outbreaks. Therefore, these data illustrate the importance of successive sampling to determine the influence, extent and strength of fungal entomopathogens associated with epizootics. Further investigations of fungal entomopathogens are needed to ascertain their ecology and coevolution with their hosts in a specific environment. Understanding the metapopulation ecology of fungal entomopathogens will provide information for developing mycofungicides and for planning appropriate control strategies.

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**References**

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