Genetics and Molecular Research

Molecular tracing of white muscardine in Asian corn borer using inter-simple sequence repeat (ISSR) analysis

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Genet. Mol. Res. 14 (4): 18720-18730 (2015)
Received August 26, 2015
Accepted October 19, 2015
Published December 28, 2015
DOI http://dx.doi.org/10.4238/2015.December.28.21

ABSTRACT. *Beauveria bassiana* is a soil fungus that parasitizes arthropod species, and is used to control the Asian corn borer in Northeast China. In this study, *B. bassiana* was investigated in Xiaoxian County and Baicheng City, and the results were compared with those of Gongzhuling City, where the fungus was not applied. Using the inter-simple sequence repeat (ISSR) molecular marker technique, 198 isolates were extracted from Asian corn borer and other insect cadavers, and soil and air, and two released strains were analyzed to trace the infection source. In Xiaoxian and Baicheng populations, artificially released *B. bassiana* subpopulations were more abundant than indigenous fungi, and the released strains were the main cause of disease in those areas. Artificial *B. bassiana* displayed positive effect on overwintering of Asian corn borers in corn straw stacks in Xiaoxian County. Indigenous populations in Gongzhuling City showed

higher genetic variation. In summary, we identified a significant correlation between genetic distance and geographic distance ($P < 0.01$).

Key words: Asian corn borer; *Beauveria bassiana*; Molecular tracing; Population genetic structure; Inter-simple sequence repeat; Genetic distance

INTRODUCTION

Beauveria bassiana (*B. bassiana*) is an entomopathogenic fungus with broad specificity, capable of infecting over 750 arthropod species (Bidochka et al., 1994). It plays a significant role in the biological regulation of insect populations and is a source of fungal insecticides. In China, *B. bassiana* has been broadly applied to control diverse pests in agriculture and in forestry, both in greenhouses and in the field (Li et al., 2006). The Asian corn borer (ACB), *Ostrinia furnacalis* Guenée, is a major insect pest that threatens corn production in China. Use of the fungus in the biological control of the ACB has received considerable attention (Pu & Li, 1996). From as early as the 1920 and 1930s, Metalinkov and Toumanoff (1928), and Lefebvre (1931) separately began to apply *B. bassiana* to control the European corn borer (ECB). Carruthers et al. (1985) established a mathematical model for predicting biological relationships between *B. bassiana* and the ECB. Xu et al. (1973, 1986, 1987) confirmed the bio-control capabilities of *B. bassiana* on the ACB, and this has since become the major control approach in Northeast China. A number of different application processes have been tried in China, such as covering corn straw stacks with *B. bassiana* conidial powder. This method increases the number of parasitized larvae in stacks and reduces the number of insects in fields. It is suitable in corn producing regions suffering intermediate levels of crop damage (<50%) and is now largely promoted by agricultural departments (Zhang, 2013). For regions with >50% damage, this approach was combined with releasing *B. bassiana* in the field during the late whorl stage, to give a control effect of 56.98% on second generation ACB in summer corn (Pu and Li, 1996).

B. *bassiana* populations were reported to be genetically diverse (Takatsuka, 2007; Fernandes et al., 2009), and host specialization varied among different isolates (Vestergaard et al., 2003). Molecular ecological approaches provide a precise and credible way to investigate genetic diversity and the population genetic structure of *B*. *bassiana*. These approaches enable in-depth study of artificially induced, or naturally occurring infectious diseases, in order to establish disease characteristics and dynamics, determine sources and spreading routes, and assess bio-control strategies (Zhao et al., 2007). The inter-simple sequence repeat (ISSR) molecular marker technique in the present studyused16-18 artificially synthesized primers that have 2-4 randomly selected nucleotides at the 3' or 5' flanking regions, for PCR amplification of the repeating sequences. In comparison to other DNA molecular marker methods, ISSR was more easily used and reliable for assessing genetic diversity. In addition, ISSR has been used in previous studies of plants, animals, and fungi (Gupta et al., 1994; Zietkiewicz et al., 1994; Kerrigan et al., 2003; Wang et al., 2005; Takatsuka, 2007; Lihme et al., 2009). ISSR analysis of hundreds of isolates from a silkworm-producing region, allowed Li et al. (2010, 2011) to confirm that silkworm diseases are genetically distant from those of pine moths. They found that silkworm diseases always remained indigenous, regardless of whether pine moths surrounding the silkworm nursery were naturally occurring or released populations. These researchers concluded that applying *B*. *bassiana* to control pine moths in silkworm-producing regions does not adversely affect the silkworm industry (Li et al., 2010, 2011). ISSR is therefore an effective technique for monitoring the incidence and spread of *B*. *bassiana*.

A key activity in insect epidemiology research entails the monitoring of the extent and route of disease spread. Long-distance spread of fungal spores has been well documented. Gregory (1968) reported that uredospores of *Puccinia striiformis* were dispersed over distances ranging from a few centimeters to over a thousand kilometers. During the period from 2000 to 2002, Hovmøller et al. (2008) demonstrated that rapid global spread of two aggressive strains of a wheat rust fungus, *P. striiformis* f. sp. *tritici*, over three continents (North America, Australia, and Europe), included areas where the occurrence of yellow rust disease in wheat was previously considered insignificant (eastern USA), or even absent (West Australia). Although there is no similar evidence for *B. bassiana*, Elkinton et al. (1991) estimated that *E. maimaiga* spread over 100 km in one season. Such evidence suggests that the effects of wind critically influence the movement of air-borne fungi such as *B. bassiana*. Large indigenous populations of *B*. *bassiana* occur in forests (Ding et al., 2004; Zhao et al., 2007), which work together with released fungi to control forest insect pests. *B*. *bassiana* can live parasitically in insect hosts, or saprophytically in the environment, when host insects are not present (Studdertand Kaya, 1990). *B*. *bassiana* can possibly become established in corn plantations from naturally occurring or artificially released fungi. To date, the population dynamics of released and indigenous *B*. *bassiana* isolates have not been reported, and the sources of natural epidemics are unknown.

In the present study, ISSR markers were employed to investigate the genetic structure of *B*. *bassiana*. Molecular tracing of the sources of *B*. *bassiana* was performed for Xiaoxian County (Anhui Province) and Baicheng City (Jilin Province), where it was artificially released for control of the ACB.The data for these two areas were compared with those for Gongzhuling City, where *B*. *bassiana* was not applied. The results provide a basis for the rational application of *B*. *bassiana* as a bio-control agent.

MATERIAL AND METHODS

Study locations

Three areas, Xianxian, Baicheng, and Gongzhuling were selected for the study (Table 1).

On May 10, 2010, *B*. *bassiana* conidial powder was sprayed on corn straw stacks in experimental plots in Baicheng, and on July 25, 2010, granular preparations of *B*. *bassiana* were applied during the late whorl stage, to experimental plots in Xiaoxian. *B*. *bassiana* was not applied to the experimental plots in Gongzhuling.

Table 1. Locations in the study.

Location	Province	Country	Longitude and latitude
Xiaoxian	Anhui	China	N34º11', E116º56'
Baicheng	Jilin	China	N45º43', E122º52'
Gongzhuling	Jilin	China	N43º52', E124º45'

Strains tested

Two hundred isolates of *B. bassiana* were characterized by ISSR-PCR in the present study. IsolateNo.1 (A1) was a released strain, collected from ACB cadavers in corn fields of Xiaoxian. Taobei District Agricultural Technology Extension Service Center (China) provided the isolation No. 105 (B1),a released strain, isolated from ACB cadavers in Baicheng. The other 198 isolates were

derived from the cadavers of the insects that were parasitized by the fungus in corn fields, corn straw stacks, soil, and air in the three locations under investigation. Isolations from Xiaoxian (104) were designated as population A,70 isolations from Baicheng as population B, and 26 isolations from Gongzhuling as population C. The detailed serial numbers, substrates, and collection times of each isolate are listed in Table 2.

Cadavers and soil samples were cultured in selective Dodine-crystal oatmeal medium. Air samples were collected after direct exposure of the selective medium plates to air and culture in Sabouraud-dextrose-yeast extract agar (SDAY) medium (Wang et al., 2000). All *B. bassiana* isolates were subjected to microscopic and subsequent molecular identification, by ITS1-5.8S-ITS2 rDNA and Bloc sequencing and preserved in the Anhui Provincial Key Laboratory of Microbial Control (China).

Table 2. Origin of the *B. bassiana* isolates.

Population (Locality)	Subpopulation number	Substrate	Collection time	Isolate number
A (Xiaoxian County, Anhui Province)	A1	Production strain (released isolate)		1
	A2	Asian corn borer cadavers in treated field	16-09-2010	2-30
	A3	Cadavers of other insects in treated field, including 4 *Heliothis armigera*, 5 *Propylea japonoca*, a chrysopid, 2 *Cryptothelea variegata* and a hemipteran insect	16-09-2010	31-43
	A4	Soil in treated field	16-09-2010	74-84
	A5	Air flow above treated field	16-09-2010	96-104
	A6	Asian corn borer cadaver in corn straw stack	26-05-2011	44-73
	A7	Soil beneath corn straw stack	26-05-2011	85-95
B (Baicheng City, Jilin Province)	B1	Production strain (released isolate)		105
	B2	Asian corn borer cadavers in stacks treated with *B. bassiana*	03-06-2010	106-136
	B3	Soil beneath treated corn straw stack	03-06-2010	158-167
	B4	Air flow above treated stacks	03-06-2010	168-174
	B5	Asian corn borer cadavers in field near treated stacks	02-10-2010	137-157
C (Gongzhuling City, Jilin Province)	C1	Asian corn borer cadavers in stack	02-06-2010	175-191
	C2	Asian corn borer cadavers in field	01-10-2010	192-200

Mycelium preparation

Isolates were inoculated separately on SDAY plates covered with glass paper and incubated in an illuminator at 25°C for 4 days, until the petri dishes were fully coved by the of mycelium The thallus was stripped from the glass paper, and mycelia were stored at -20°C following lyophilization.

DNA extractionand screening of ISSR primers

Total DNA from thallus samples was extracted as described by Zhu et al. (1994) and electrophoresed on 0.8% agarose gel to determine quality. DNA samples were quantified using SmartSpec plus (Labsystems, Bio-Rad, USA), diluted to 20 ng/μL, and stored at -20°C. Four *B. bassiana* isolates from each of the three study populations were selected for primer screening, employing the methods of Li *et al*. (2006) and Yu et al. (2003). Nine of the selected isolates showing clear amplification bands and abundant polymorphisms were selected for PCR amplification (Table 3).

Table 3. ISSR primers and their amplification properties.

Primer	Sequence (5'-3')	Annealing temperature (°C)	No. of amplified loci	No. of polymorphic loci	PPL (%)	H_E	I_S
P5	(AAG)6	48	15	15	100	0.3205 ± 0.1851	0.4768 ± 0.2362
P6	(AG)8S	54	14	14	100	0.3299 ± 0.1767	0.4894 ± 0.2285
P10	(AC)8YC	52	11	11	100	0.2193 ± 0.2053	0.3401 ± 0.2706
P11	(AG)8C	54	12	12	100	0.1913 ± 0.1993	0.3040 ± 0.2608
P12	TG(CA)6C	50	13	12	92.31	0.3139 ± 0.1971	0.4637 ± 0.2575
P13	CCA(GTG)4	54	14	13	92.86	0.2133 ± 0.1927	0.3339 ± 0.2583
P19	DD(CCA)5	53.5	20	20	100	0.2936 ± 0.1774	0.4445 ± 0.2320
P26	(GTC)5TC	54	15	15	100	0.2597 ± 0.1954	0.3990 ± 0.2496
P28	(AG)8YC	50	20	20	100	0.2893 ± 0.1645	0.4443 ± 0.2098
Mean			14.9	14.7			
Total			134	132	98.51	0.2743 ± 0.1863	0.4168 ± 0.2428

Y represents C or T; S represents C or G.

ISSR amplification

ISSR-PCR amplification was conducted using a Bio-Rad PCR System (T100™ Thermal Cycler, USA). PCR buffer (including 25 mM $MgCl_2$) and DreamTaq DNA polymerase were purchased from Fermentas (USA), and dNTPs and the GM339 DNA ladder were purchased from Bio Basic (Canada). Primers were synthesized by Jierui Biological Engineering (Shanghai). ISSR-PCR amplifications and analyses were conducted as previously described (Wang et al., 2005) with the following modifications: 1.0 U DNA Taq polymerase was decreased to 0.5 U, and the template DNA was increased to 20 ng. PCR was performed in triplicate. Each PCR reaction contained 1.5 µL 10X PCR buffer, 0.25 Mm dNTPs, 0.6 µM primers, 0.5 U Taq DNA polymerase, and 20 ng template DNA. The PCR reaction mix was adjusted to a final volume of 15µL with diethylpyrocarbonate (DEPC)-treated water (Sangon Biotech, Sangon). PCR products were separated by 1.5% agarose gel electrophoresis, visualized, and recorded by using the AlphaEase FC software and the Alpha Imager TM IS-2200 system(USA).

Data analysis

ISSR electrophoretograms were converted to primitive (0, 1) matrices that were subsequently used to calculate: Nei's genetic diversity index (H_E); Shannon's information index (I_S); percentage of polymorphic loci (*PPL*); genetic diversity within population (H_S); total gene diversity (H_T); coefficient of genetic differentiation ($G_{st}=H_T-H_S/H_T$); and gene flow [$N_M= (1-G_{ST})/(4G_{ST})$]; using POPGENE version32 software (Yeh et al.,1997).

RESULTS

Polymorphism of ISSR amplification products

Nine primers showing polymorphism and high stability in ISSR amplification were selected from the initial 23 primers that were screened (Table 3). These nine primers amplified 134 bands from the 200 isolates, of which 132 (98.51%) were polymorphic. Each primer amplified 11–20 bands, with an average of 14.9. Primers P19 and P28 amplified the highest number (20) of total and polymorphic loci, whereas primer P10 produced the lowest number of amplification products

(11). Primers P5, P6, P10, P11, P19, P26, and P28 all showed the highest polymorphic percentage (100%), and P6 gave the highest H_E and I_S values.

Genetic consistency and genetic distance in *B. bassiana* subpopulations

To determine the genetic relationships between the *B. bassiana* subpopulations in Xiaoxian and Baicheng, POPGENE32 software was employed to survey the genetic similarity (*I*) and genetic distance (*D*). The results showed that *D* was 0.0011-0.1185 for subpopulations in Xiaoxian, with an average of 0.0381, whereas *I* was 0.8883-0.9989, with an average of 0.9629 (Table 4).

Table 4. Genetic similarity (*I*) and genetic distance (*D*) in population A.

Subpopulation	A1	A2	A3	A4	A5	A6	A7
A1	-	0.9899	0.9650	0.9635	0.9964	0.9314	0.8883
A2	0.0101	-	0.9869	0.9797	0.9989	0.9688	0.9343
A3	0.0356	0.0132	-	0.9888	0.9803	0.9697	0.9603
A4	0.0372	0.0206	0.0113	-	0.9775	0.9553	0.9384
A5	0.0036	0.0011	0.0199	0.0228	-	0.9566	0.9249
A6	0.0710	0.0317	0.0307	0.0458	0.0443	-	0.9670
A7	0.1185	0.0680	0.0405	0.0635	0.0781	0.0336	-

Genetic similarity lies above the diagonal and genetic distance lies below the diagonal.

For Baicheng, *D* was higher than that of Xiaoxian, being 0.0143-0.1006, with an average of 0.0478. In contrast, *I* was 0.9043-0.9858, with an average of 0.9537 (Table 5), lower than that of Xiaoxian.

Table 5. Genetic similarity (*I*) and genetic distance (*D*) in population B.

Subpopulation	B1	B2	B3	B4	B5
B1	-	0.9832	0.9043	0.9722	0.9279
B2	0.0169	-	0.9294	0.9858	0.9638
B3	0.1006	0.0732	-	0.9367	0.9549
B4	0.0282	0.0143	0.0654	-	0.9786
B5	0.0748	0.0368	0.0461	0.0216	-

Genetic similarity lies above the diagonal and genetic distance lies below the diagonal.

Comparison of the released strains (A1 and B1) revealed that isolations from corn fields and stacks, which were treated by these strains (A2 and B2), showed higher *I* (0.9899 and 0.9832, respectively) and lower *D* (0.0101 and 0.0169, respectively), which confirmed the insecticidal efficacy of artificially released *B. bassiana*. For both treatments (corn fields and stacks, A2 and B2, respectively), *D* was similar, whereas *I* was highest for subpopulations A5 and B4 from airflow samples, suggesting that treatments A2 and B2 contributed to the increased *B. bassiana* density in airflow isolates. In contrast, *D* was the lowest with subpopulations A7 and B3 from soil for both treatments, suggesting that soil is the primary reservoir of the organism, independent of released strains. This is of great significance to maintaining the genetic diversity of indigenous *B. bassiana*. In addition, ACB cadavers from corn stacks in Xiaoxian (A6) and from the field in Baicheng (B5), gave low *D* values to subpopulations A1 and A2, and B1 and B2 (just below the values for soil subpopulations A7 and B3). These results demonstrate that the cadavers of A6 in corn stacks of Xiaoxian and B5 in Baicheng fields were not caused by artificial release in A1 and B1, but by indigenous strains at the respective locations.

The *D* value between subpopulations of Cadavers of other insects (A3) and ACB cadavers (A2) in treated fields in Xiaoxian was markedly lower (0.0132), suggesting that release of *B. bassiana* has some influence on other host insects.

The genetic distance *D* between different ACB cadaver subpopulations from fields and corn stacks in the three experimental plots are shown in Table 6.

Table 6. Genetic similarity (*I*) and geographic distance (*D*) in subpopulations from Asian corn borer cadaver isolates.

Subpopulation	A2	A6	B2	B5	C1	C2
A2	-	1	1949	1949	1548	1548
A6	0.0317	-	1949	1949	1548	1548
B2	0.3818	0.2527	-	1	406	406
B5	0.3450	0.2231	0.0368	-	406	406
C1	0.3412	0.2377	0.1609	0.1144	-	1
C2	0.3905	0.2663	0.1067	0.0864	0.0716	-

1) Geographic distance lies above the diagonal and genetic similarity lies below the diagonal. 2) the geographic distance between two subpopulations of the same population is assumed to be 1km, while the subpopulations of different populations is assumed to be identical: A-B, 1949 km; B-C, 406 km; A-C, 1548 km.

The *D* values for Xiaoxian (A2-A6) and Baicheng (B2-B5) were 0.0317 and 0.0368, respectively. Both values were considerably lower than that of C1-C2 in Gongzhuling (0.0716), suggesting that artificial release of *B. bassiana* to control the target species also spread to non-target species. However, the genetic distance between C1 and C2 in Gongzhuling was lower than that between C1 and B5 (0.1144), C2 and B5 (0.0864), C1 and B2 (0.1609), and C2 and B2 (0.1067) in Baicheng, as well as between C1 and A2 (0.3412), C2 and A2 (0.3905), C1 and A6 (0.2377), and C2 and A6 (0.2633) in Xiaoxian. These results confirm that the genetic distance between populations was significantly correlated with geographic distance ($r = 0.894$, $P < 0.01$).

Gene flow and genetic differentiation between *B. bassiana* subpopulations

POPGENE software was used for further analysis of gene flow and genetic differentiation among the subpopulations of *B. bassiana*. As shown in Table 7, the gene flow (N_M) of subpopulations in Xiaoxian was 0.2999-10.3983, with an average of 2.1350. The genetic differentiation index (G_{ST}) was 0.0235-0.4546, with an average of 0.1652 (Table 7). These results suggest that gene flow varies greatly between *B. bassiana* in different niches.

Table 7. Gene flow (N_M) and genetic differentiation coefficient (G_{ST}) in Population A.

Subpopulation	A1	A2	A3	A4	A5	A6	A7
A1	-	2.1796	0.6997	0.4589	2.5484	0.7754	0.2999
A2	0.1029	-	3.5161	2.0321	10.3983	2.5887	0.9494
A3	0.2633	0.0664	-	3.0388	1.8229	2.5547	1.4539
A4	0.3527	0.1095	0.0760	-	1.3037	1.5850	0.8276
A5	0.0893	0.0235	0.1206	0.1609	-	1.5411	0.6470
A6	0.2438	0.0881	0.0891	0.1362	0.1396	-	2.6143
A7	0.4546	0.2084	0.1467	0.2320	0.2787	0.0873	-

Gene flow (N_M) lies above the diagonal, and genetic differentiation coefficient is below the diagonal.

ACB cadavers in subpopulation A2 (released strain region) gave the largest N_M but the lowest G_{ST} (0.0235) in comparison to subpopulation A5 in air, which represented a non-genetic differentiation grade of < 0.05 according to the criteria of Wright (1931). Released strain A1 and

subpopulation A7 from soil beneath corn stacks, had the lowest N_M (0.0299) and the largest G_{ST} (0.4546), which represented extensive differentiation of >0.25.The gene flow of subpopulations in Baicheng was between 0.4689 and 3.2384, with an average of 1.5761, and G_{ST} was between 0.0717and 0.3478, with an average of 0.1746 (Table 8).

Table 8. Gene flow (N_M) and genetic differentiation coefficient (G_{ST}) in Population B.

Subpopulation	B1	B2	B3	B4	B5
B1	-	1.5184	0.4689	0.9355	0.5585
B2	0.1414	-	1.0999	3.2384	1.9763
B3	0.3478	0.1852	-	1.2033	1.9347
B4	0.2109	0.0717	0.172	-	2.8272
B5	0.3092	0.1123	0.1144	0.0812	-

Gene flow (N_M) lies above the diagonal, and genetic differentiation coefficient is below the diagonal.

The gene flow in Baicheng was significantly lower than that in Xiaoxian, but the general trends were consistent. ACB cadavers in subpopulation B2 from the corn stacks showed the largest N_M (3.2384) but lowest G_{ST} (0.0717), in comparison to subpopulation B4 in air. The released strain B1 showed the lowest N_M (0.4689) but largest G_{ST} (0.3478), in comparison to subpopulation B3 from soil beneath the corn stacks, reflecting extensive differentiation. These results further confirm that soil is the main reservoir of *B. bassiana* from which new fungal infections can arise and spread.

Released *B. bassiana* strains and in Xiaoxian (A1) and Baicheng (B1), showed high N_M (2.1796 and 1.5184, respectively) and low G_{ST} (0.1029 and 0.1414, respectively) with subpopulations A2 and B2 from ACB cadavers in treated regions. This indicates that there was only moderate genetic differentiation between released strains and subpopulations from ACB cadavers in treated regions. This further confirms the colonization effect of *B. bassiana* release, and suggests that the released strains were the main source of fungus in ACB. The N_M value between ACB cadavers in the treated field in Xiaoxian (A2) and other subpopulations (cadavers of other insect hosts (A3), soil (A4), air (A5), and corn stacks (A6)) were 3.5161, 2.0321, 10.3983, and 2.5887, respectively. These values were all far higher than the threshold value of 1.0 for gene differentiation, indicating that gene introgression and flow was strengthened. In addition, released *B. bassiana* could be dispersed by ACB cadavers, soil, and air to treated regions. Furthermore, the N_M between A2 and A3 was 3.5161, and G_{ST} was only 0.0664, suggesting minimal genetic differentiation between the two subpopulations. This finding further demonstrates that release of *B. bassiana* for bio-control has influenced the other insects.

As shown in Table 9, G_{ST} values of subpopulations A2-A6 and B2-B5 isolated from cadavers in the treated regions of Xiaoxian and Baicheng were 0.0881 and 0.1123, respectively, reflecting moderate differentiation. These G_{ST} values were markedly lower than those of the two highly differentiated subpopulations (C1 and C2) in Gongzhuling (0.2045; relative gene flow of 0.9726), which is consistent with the genetic distance analysis. These findings suggest that gene introgression and flow in areas where fungi was artificially released, was more extensive than that in untreated regions, further confirming the influence of released *B. bassiana* for bio-control purposes.

With the exception of B5-C1 and B5-C2, which gave G_{ST} values of 0.2407 and 0.2190 respectively, G_{ST} values between subpopulations from ACB cadavers were consistently higher than 0.25, representing extensive differentiation. This indicates that gene flow between subpopulations in the three experimental plots was blocked to a certain extent. The genetic and geographic distance in the three experimental plots were clearly correlated, and likewise, the genetic differentiation index was significantly correlated with geographic distance (r = 0.796, P < 0.01). These results

reflect the regional characteristics of *B*. *bassiana* widely prevalent in the population of ACB in southern and northern China.

Table 9. Geographic distance and genetic differentiation coefficient (G_{ST}) between the subpopulations from Asian corn borer cadaver isolates.

Subpopulation	A2	A6	B2	B5	C1	C2
A2	-	1	1949	1949	1548	1548
A6	0.0881	-	1949	1949	1548	1548
B2	0.5686	0.3683	-	1	406	406
B5	0.4924	0.3072	0.1123	-	406	406
C1	0.5105	0.3329	0.3433	0.2407	-	1
C2	0.5730	0.3797	0.2938	0.2190	0.2045	-

Geographic distance (km) lies above the diagonal, genetic differentiation coefficient is below the diagonal.

DISCUSSION

In the present study, the application of granule preparations of *B*. *bassiana* to corn fields (during the late whorl stage) in Xiaoxian County, and powder preparations of *B*. *bassiana* to corn stacks in Baicheng City, both resulted in a certain degree of efficacy. The induced epidemics in corn fields and stacks killed a large number of ACBs, but also infected other host insects, soil, and airflow in treated regions. The released strains also infected ACBs in corn stacks in Xiaoxian and corn fields in Baicheng that did not receive artificial *B*. *Bassiana* treatment. This suggests that released strains also contribute to the development of enzootics. Disease in untreated areas was likely caused by non-dominant indigenous strains, although dominant strains did contribute to the incidence of endemic disease. This resulted in robust heterogeneous subpopulations within different ecological niches of the two regions, which is favorable for strengthening genetic diversity.

In Gongzhuling, where *B*. *bassiana* was not applied, the populations consisted entirely of indigenous organisms that exhibited greater genetic variation (higher genetic distance and genetic differentiation) than isolates from Xiaoxian and Baicheng, both of which had clearly been affected by artificial inoculation. Indigenous fungus causeda low rate of endemic disease in ACB populations, whereas artificial inoculation reduced the number of insects by further enhancing disease. In the present study, we have shown that this limitation may be overcome by artificially releasing *B*. *bassiana* on corn plants during the late whorl stage, and/or by treating corn stacks.

Analysis of the phylogenetic relationships between *B*. *Bassiana* from different geographical sources and insect hosts has yielded disparate results. Viaud et al. (1996) and Maurer et al. (1997) reported that the host, rather than the geographic source, determines the phylogeny of *B*. *bassiana*, whereas Berretta et al. (1998) and Gaitan et al. (2002) found no correlation between host and source. However, more recent studies do suggest that geographical source determines phylogeny (Bidochka et al.,1997; Wang et al., 2003; Aquino de Muro et al., 2005; Wang et al., 2005). The sample sizes in the aforementioned studies were limited; therefore, He et al. (2012) and Hu et al. (2013) separately investigated *B*. *bassiana* populations in 622 isolates from 13 provinces, and 568 isolates from 9provinces, in northern and southern China respectively, using the ISSR technique. Both studies reported that regional differences in phylogeny could only be detected in large sample populations (almost the entire northern or southern regions of China). Phylogeny across subpopulations within small populations indicates a random arrangement and combination, regardless of geographic distance, neighboring relationship, or phylogenetic relationship of host insects. In the present study, we found clear regional characteristics in phylogeny using only 200

isolates, and identified a significant correlation between genetic distance and geographic distance ($P < 0.01$). This could be because our study involved only one host insect (ACB) in the three experimental plots. Given an increased number of experimental plots and/or insect hosts, the phylogeny may become scrambled in a larger dataset. This hypothesis remains to be tested.

In addition, ACBs have a different voltinism in Jilin Province and Anhui Province. Populations in Baicheng and Gongzhuling exhibited digoneutism, whereas in Xiaoxian, trigoneutism was common. The genetic relationships between the subpopulations of cadavers of ACBs in Baicheng (B2 and B5) and the subpopulations in Gongzhuling (C1 and C2), were found to be closer than those between the subpopulations in Baicheng and Gongzhuling (B2, B5, C1, and C2) and the subpopulations in Xiaoxian (A2 and A6). Whether this was related to their differences in voltinism requires further investigation.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by a grant from the Natural Science Foundation of China (#30972368). We are grateful to Xiangyang Wang and Yunfeng Tan for their help during field investigations and Shengli Zhang for his technical assistance with laboratory work.

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