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Genetic diversity and population structure among strains of the entomopathogenic fungus, *Beauveria bassiana*, as revealed by inter-simple sequence repeats (ISSR)

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Inter-simple sequence repeat (ISSR) markers were used to investigate genetic diversity among 39 isolates of *Beauveria* spp. (36 *B. bassiana*, one *B. brongniartii*, one *B. amorpha*, and one *B. velata*) isolated from different insect hosts and geographical origins. Eighteen among 33 primers that contained different simple sequence repeats (SSR) were chosen for their reproducibility and high polymorphism. Considerable intraspecific and interspecific variability among the isolates of *Beauveria* spp. was revealed. One hundred sixty-eight highly reproducible fragments were amplified in all 39 isolates with an average of 9.3 markers per primer; among these, 161 (95.8%) were polymorphic. For 36 *B. bassiana* isolates, 8.9 (1–13) markers per primer were scored, and a total of 123 fragments were amplified, in which 102 (82.9%) were polymorphic. Among the 168 polymorphic bands, 7 bands were considered to be specific for *B. brongniartii* isolate Bbr06, 14 bands for *B. amorpha* isolate Ba08, and 18 bands for *B. velata* isolate Bv01. Within 36 *B. bassiana* isolates, genetic similarity ranged from 0.651 to 0.972. However, the genetic similarity values among different *Beauveria* species ranged from 0.411 to 0.720, suggesting that ISSR technique was successful in differentiating the three closely related species from *B. bassiana*. The results also indicated that there was a certain association between *B. bassiana* isolates and their geographical origins, but no clear correlation between those isolates and their insect hosts. The present study suggested that ISSR markers can be used as robust molecular markers for the population genetics, epidemiological and ecological studies of entomopathogenic fungi.

INTRODUCTION

Beauveria bassiana is a cosmopolitan, haploid, mitosporic and soil-borne entomopathogenic fungus with a very wide and diverse host spectrum reaching to 750 insect species (Inglis *et al*. 2001), and has become one of the seven fungal insecticides registered now on the market (Butt, Jackson & Magan 2001). It has been mass produced in tonnes, and great success has been achieved in large scale application against the Masson's pine caterpillar, *Dendrolimus punctatus*, for over the past 40 yr in China. The recombination through parasexual or sexual cycle in *B. bassiana* and *B. brongniartii* has been revealed (Shimazu, Mitsuhashi & Hashimoto 1988, Li *et al*. 2001, Padmavathi *et al*. 2003). However, this species is widely distributed as an anamorph in nature, and has been reported as a heterogeneous assemblage of strains (Mugnai, Bridge & Evans 1989). Considerable natural genetic variability and heterogeneity within this species complex have been demonstrated (St Leger *et al*. 1992a, Maurer *et al*. 1997, Padmavathi *et al*. 2003). The knowledge of the genetic diversity and intraspecies relationship is of great importance for better understanding population structure, gene flow, isolate typing, ecological and potential impact when used as an insect biological control agents. Furthermore, accurate identification of isolates is a prerequisite for registration and patenting of any biopesticide product, and also for better monitoring the persistence and behavior of an isolate following release into the environment (Pipe *et al*. 1995).

Molecular markers successfully developed during the last two decades have been widely used to study the genetic variability, characterization and relatedness of

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B. bassiana. Initially, isoenzyme analyses (Poprawski *et al.* 1988, St Leger *et al.* 1992a, Bidochka, Menzies & Kamp 2002), telomere fingerprinting (Viaud *et al.* 1996, Padmavathi *et al.* 2003), and RFLPs (Maurer *et al.* 1997, Coates, Hellmich & Lewis 2002a) were used to study phylogenetic relationships within *B. bassiana*. Moreover, similar studies have been performed using several types of markers based on PCR techniques such as RAPDs (Maurer *et al.* 1997, Glare & Inwood 1998), minisatellites (Coates, Hellmich & Lewis 2002b), AFLPs (Aquino de Muro, Mehta & Moore 2003), sequence-characterized amplified region (SCAR) (Castrillo, Vendenberg & Wraight 2003) and simple sequence repeats (SSR, also called microsatellites) (Enkerli *et al.* 2001, Coates *et al.* 2002b, Wang *et al.* 2003a). In all the studies mentioned above, considerable genetic variability within this species was demonstrated. But the conflicting and ambiguous results have been provided in regard to host specialization and geographic location of genetic variability of the fungi (Maurer *et al.* 1997, Bidochka *et al.* 2002, Aquino de Muro, Mehta & Moore 2003, Wang *et al.* 2003a). Therefore, more polymorphic, sensitive, reliable and robust markers are required for reaching a precise strain genotyping and a robust inference into population structure.

The inter-simple sequence repeat (ISSR) is a new kind of molecular marker that amplifies inter-microsatellite sequences at multiple loci throughout the genome by a single primer 16–18 bp long composed of a repeated sequence anchored at the 3′ or 5′ end by 2–4 arbitrary nucleotides (Zietkiewicz, Rafalski & Labuda 1994). Each amplified band corresponds to a unique DNA sequence delimited by two inverted microsatellites, leading to multilocus and highly polymorphic patterns in which fragments are often polymorphic between different individuals (Nagaoka & Ogihara 1997). ISSRs are dominant inheritance markers and can generate large numbers of highly informative and reproducible alleles. Up to now, no reports are available for using ISSR markers to study *Beauveria* spp.

The objectives of the present study are: (1) to test the efficiency of ISSR markers with the goal of using these polymorphisms as genetic markers; (2) to investigate the genotypic variability within *B. bassiana* by analyzing ISSRs of 39 *Beauveria* strains isolated from different insect hosts and geographical origins; and (3) to calculate and to compare genetic diversities, genetic similarities and genetic distances within and among populations, and to reassess the possible correlation between the insect host and the geographic origin.

MATERIALS AND METHODS

Fungal isolates and cultural conditions

36 *Beauveria bassiana* isolates and one isolate of each of *B. brongniartii*, *B. amorpha*, and *B. velata* were obtained from the RCEF (Research Center of Entomopathogenic Fungi, Anhui Agricultural University, Hefei, China) collection. Most of them were originally obtained from different Chinese nature reserves. Isolate Bb61 was obtained from USA, and isolates Bb202 and Bbr06 from Japan, isolates Bb366 and Bb367 from Vietnam, and isolate Bb59 from Yugoslavia were also selected. The fungal strains and their origins are described in Table 1. All strains were propagated on 9 cm diam Sabouraud dextrose agar yeast (SDAY) plates covered with cellophane paper at 25 ± 1 °C. The strains were cultured for 4 d prior to harvesting and freeze-drying.

DNA extraction

DNA extractions were performed using benzyl chloride for chemical cell wall disintegration according to the method of Zhu, Qu & Zhu (1993) with some modifications. Lyophilized mycelium was carefully crushed by spatula in the 1.5 ml microcentrifuge tube before adding the extraction buffer (100 mM Tris-Hcl, pH 9.0; 40 mM EDTA, pH 8.0; 2% SDS) and 30% benzyl chloride. The air-dried DNA pellet after the process of lysis was resuspended in 200 μl sterile distilled water, 2 μl RNase (10 mg ml^{-1}) was added, and the DNA solution was incubated at 37 ° for 1 h. The sample was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and twice with an equal volume of chloroform/ isoamyl alcohol (24:1, v/v), and 1/10 volume of sodium acetate (3 M, pH 5.2) was added before addition of isopropanol and the Eppendorf vial was kept standing at −20 ° for 30 min. The mixture was centrifuged at 12 000 ***g*** at 4 ° for 10 min. The air-dried pellet was resuspended in 100 μl of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) after being washed twice with 70% ethanol and kept at −20 ° The concentration and quality of the purified DNA was evaluated both by 1% agarose gel electrophoresis and by spectrophotometry.

ISSR amplification

33 ISSR markers anchored 3′ end were selected for amplifications (Table 2). Amplification reactions were carried out in 15 μl reaction mixture containing 1.5 μl of 10×PCR buffer (200 mM Tris-HCL pH 8.4, 2.5 mM $MgCl_2$, 500 mM KCL), 0.25 mM each of deoxyribonucleotide triphosphates (dNTP), 0.4 μM of each primer, and 1 U of *Taq* DNA polymerase (TaKaRa Inc.) and 10 ng template DNA. The optimum annealing temperature was determined for each primer. PCR reactions were performed on a Flexigene thermal cycler (Flexigene TECHNE) with following amplification conditions: initial denaturation at 94 ° for 2 min, followed by 30 cycles of denaturation at 94 ° for 45 s, annealing at the specific annealing temperature for 45 s, and extension at 72 ° for 1.5 min. An additional cycle comprising a 5-min extension at 72 ° was included as a

Table 1. *Beauveria* isolates studied here listed by geographical origin, species, insect hosts, and year of collection.

Origin/Species	Isolate	Accession no.[a]	Host	Year of collection
China: Anhui Province, Guniujiang Nature Reserve (GNJ)				
B. bassiana	Bb77	RCEF0060	*Homoptera*, *Membracidae*, *Leptobelus gazelle*	1996
B. bassiana	Bb80	RCEF0063	*Coleoptera*, *Cerambycidae*	1996
B. bassiana	Bb87	RCEF0070	*Hymenoptera*, *Formicidae*	1996
B. bassiana	Bb88	RCEF0071	*Coleoptera*, *Curculionidae*, *Sympiezomias velatus*	1996
B. bassiana	Bb89	RCEF0072	*Homoptera*, *Delphacidae*	1996
B. bassiana	Bb91	RCEF0074	*Hemiptera*, *Pentatomidae*, *Erthesina fullo*	1996
B. bassiana	Bb93	RCEF0076	*Hemiptera*, *Pentatomidae*, *Eurygaster testudinarius*	1996
B. bassiana	Bb370	RCEF0860	*Lepidoptera*, *Lasiocampidae*	2001
China: Anhui Province, Magushan Forest Farm (MGS)				
B. bassiana	Bb143	RCEF0324	*Orthoptera*, *Locustidae*, *Acrida chinensis*	1998
B. bassiana	Bb162	RCEF0343	*Hemiptera*, *Coreidae*, *Riptortus pedestris*	1998
B. bassiana	Bb163	RCEF0344	*Coleoptera*, *Curculionidae*, *Brachyderes incanus*	1998
B. bassiana	Bb164	RCEF0345	*Hemiptera*, *Pentatomidae*, *Halyomorpha picus*	1998
B. bassiana	Bb165	RCEF0346	*Homoptera*, *Cercopidae*, *Aphrophora flavipes*	1998
B. bassiana	Bb168	RCEF0349	*Hymenoptera*, *Ichneumqnidae*	1998
B. bassiana	Bb248	RCEF0477	*Lepidoptera*, *Lasiocampidae*, *Dendrolimus punctatus*	1999
China: Anhui Province, Yaoluoping Nature Reserve (YLP)				
B. bassiana	Bb234	RCEF0420	*Coleoptera*, *Scarabaeidae*	1998
B. bassiana	Bb235	RCEF0421	*Coleoptera*, *Curculionidae*	1998
B. bassiana	Bb241	RCEF0427	*Homoptera*, *Tenthredinidae*	1998
B. bassiana	Bb276	RCEF0583	*Hymenoptera*, *Formicidae*	1999
B. bassiana	Bb278	RCEF0585	*Homoptera*, *Cercopidae*, *Aphrophora flavipes*	1999
B. bassiana	Bb285	RCEF0592	*Orthoptera*, *Locustidae*, *Acrida cinerea*	1999
B. bassiana	Bb287	RCEF0594	*Hymenoptera*, *Iichneumonidae*	1999
B. bassiana	Bb292	RCEF0599	*Orthoptera*, *Pyrgomorphidae*, *Atractomorpha sinensis*	1999
China: Beijing				
B. amorpha	Ba08	RCEF0869	*Coleoptera*, *Tenebrionidae*	2002
China: Guizhou Province				
B. velata	Bv01	RCEF0051 (CGAC86)	Unknown	1996
China: Guizhou Province, Kuankuoshui Nature Reserve (KKS)				
B. bassiana	Bb19	RCEF0224	*Hemiptera*, *Cecidomyiidae*	1996
B. bassiana	Bb54	RCEF0237	*Lepidoptera*, *Lasiocamidae*	1996
B. bassiana	Bb55	RCEF0238	*Lepidoptera*, *Tortricidae*	1996
China: Yunnan Province, Daweishan Nature Reserve (DWS)				
B. bassiana	Bb343	RCEF0812	*Coleoptera*, *Coccinellidae*	2000
B. bassiana	Bb344	RCEF0813	*Coleoptera*, *Elateridae*, *Pleonomus canaliculatus*	2000
B. bassiana	Bb345	RCEF0814	*Lepidoptera*, *Pyralidae*, *Hellula undalis*	2000
B. bassiana	Bb346	RCEF0815	*Lepidoptera*, *Lasiocampidae*	2000
B. bassiana	Bb347	RCEF0816	*Homoptera*, *Membracidae*	2000
Japan: Osaka				
B. bassiana	Bb202	RCEF0383 (F-263)	*Coleoptera*, *Cerambycidae*, *Monochamus alternatus*	2000
B. brongniartii	Bbr06	RCEF0385 (NBL-851)	*Coleoptera*, *Cerambycidae*, *Anoplophora malasiaca*	1978
Serbia: Zabari				
B. bassiana	Bb59	RCEF0043 (=ARSEF 503)	*Lepidoptera*, *Olethreutidae*, *Laspeyresia pomonella*	1980
USA: Massachusetts, Dennis				
B. bassiana	Bb61	RCEF0045 (=ARSEF 1)	*Lepidoptera*, *Lasiocampidae*, *Malacosoma Americana*	1972
Vietnam: Hanoi, Bavi Mountain				
B. bassiana	Bb366	RCEF0835	*Homoptera*, *Aleyrodidae*, *Trialeurodes vaporariorum*	2001
B. bassiana	Bb367	RCEF0836	*Coleoptera*, *Curculionidae*	2001

[a] All cultures are deposited in Research Center of Entomopathogenic Fungi (RCEF), Anhui Agricultural University, Hefei, Anhui Province, China; accession numbers for isolates obtained from other laboratories are given in parentheses.

final step. All ISSR assays were repeated twice, and only the reproducible bands were scored. PCR products were electrophoresed at 100 V in a 2.5% agarose gel for 4 h in 1× TAE buffer (40 mM Tris-Acetic acid, pH 8.0, 1 mM EDTA), and then stained with ethidium bromide. The gel image was recorded by using a Gel Documentation System (Tanon Gis-2008).

Data analysis

The ISSR data were converted into a binary data set (0, absence of band; 1, presence of a band). Percentage polymorphic loci, Nei's genetic diversity, Shannon's diversity index, Nei's (1979) genetic similarity values and genetic distances within pairs and isolates were

Table 2. Inter-simple sequence repeat (ISSR) primers and their properties.

Primer name	Core sequence (5′–3′)	Attached bases	Tm (°C)	Annealing temperature (°C)	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphism (%)	H[e]	PIC[f]
BIS01	$(CA)_6$	AC	47.1	50	–[a]	–	–		
BIS02	$(GT)_6$	CC	50	54	7 (6)[d]	7 (4)	100 (66.7)	0.14	0.25
BIS03	$(CA)_6$	GT	47.1	50	–	–	–		
BIS04	$(CT)_8$	TG	55	54	*[b]	*	*		
BIS05	$(AG)_8$	TA	52.7	54	*	*	*		
BIS06	$(GA)_6$	GG	50	54	12 (9)	12 (9)	100 (100)	0.26	0.41
BIS07	$(TG)_8$	GT	50	54	11 (8)	11 (7)	100 (87.5)	0.23	0.39
BIS08	$(CT)_8$	GC	57.3	54*	*	*			
BIS09	$(AG)_8$	TC	55	54	12 (10)	12 (10)	100 (100)	0.42	0.68
BIS10	$(CT)_8$	AC	55	54	*	*	*		
BIS11	$(GA)_8$	T	49.8	54	3 (1)	3 (1)	100 (100)	0.26	0.42
BIS12	$(AC)_8$	T	49.8	52	5 (3)	5 (3)	100 (100)	0.33	0.55
BIS13	$(AG)_8$	S[c]	49.8	54	13 (9)	13 (9)	100 (100)	0.25	0.40
BIS14	$(AG)_8$	YT	50.3	52	12 (6)	11 (5)	91.7 (83.3)	0.21	0.34
BIS15	$(AG)_8$	YC	52.6	52	11 (9)	7 (4)	63.6 (44.4)	0.10	0.17
BIS16	$(AC)_8$	YC	52.6	52	6 (6)	6 (6)	100 (100)	0.34	0.57
BIS17	$(AC)_8$	YT	50.3	50	7 (4)	6 (1)	85.7 (25.0)	0.06	0.13
BIS18	$(TG)_8$	RC	52.6	54	*	*	*		
BIS19	$(CT)_8$	RT	50.3	54	2 (1)	2 (1)	100 (100)	0.07	0.15
BIS20	$(AT)_8$	ST	32.1	35	–	–	–		
BIS21	$(GTC)_6$		60	54	11 (7)	11 (6)	100 (85.7)	0.22	0.35
BIS22	$(GTG)_6$		61.9	54	19 (13)	19 (12)	100 (92.3)	0.24	0.39
BIS23	$(AAT)_6$		34.5	37	–	–	–		
BIS24	$(CAA)_6$		48.2	50	–	–	–		
BIS25	$(CAC)_4$	GC	55.9	54	–	–	–		
BIS26	$(GAG)_4$	GC	55.9	54	7 (6)	7 (3)	100 (50)	0.13	0.21
BIS27	$(CTC)_4$	GC	55.9	54	10 (9)	10 (9)	100 (100)	0.27	0.43
BIS28	$(GTA)_6$		45.8	48	*	*	*		
BIS29	$(AAG)_6$		45.8	48	11 (8)	11 (8)	100 (100)	0.25	0.42
BIS30	$(GACA)_4$		51.6	54	9 (8)	8 (4)	88.9 (50.0)	0.12	0.19
BIS31	$(GATA)_8$		38.9	42	–	–	–		
BIS32	$(GAGT)_8$		49.2	52	–	–	–		
BIS33	$(GCGT)_4$		59.4	60	*	*	*		
Total					168 (123)	161 (102)	95.8 (82.9)		

[a] Not amplified band.

[b] Weak or ambiguous amplification bands.

[c] Type of degenerate nucleotide: Y, pyrimidine (C, T); R, purine (A, G); S, (C, G).

[d] Left value for all 39 isolates, value in bracket for the 36 *B. bassiana* isolates.

[e] Nei's (1973) gene diversity in the 36 *B. bassiana* isolates.

[f] Polymorphic information content (PIC) value (Botstein *et al.* 1980) in the 36 *B. bassiana* isolates.

computed with POPGENE, version 1.32 (Yeh *et al.* 1997). Dendrograms were also constructed by cluster analysis based upon the unweighted pair group method with arithmetical averages (UPGMA) modified from the NEIGHBOR procedure of PHYLIP, version 3.5. The bootstrap method (Felsenstein 1985) was employed to evaluate the reliability of tree topology. Confidence limits of the dendrograms were estimated using the program WINBOOT software package (Yap & Nelson 1996) using PHYLIP format (Felsenstein 1985) in which 2000 replications were carried out. The polymorphic information content (*PIC*) value (Botstein *et al.* 1980) and Nei's measure of the average gene diversity (*H*) (Nei's 1979) were calculated as follows:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2, \; H_i = \frac{1}{n} \sum_{j=1}^{n} [1 - P_{ij}^2 - (1 - P_{ij}^2)^2]$$

Where P_{ij} is the frequency of the jth allele for marker i and the summation extends over n alleles. The calculation was based on the number of bands/primer in the case of ISSR. An ANOVA and multi-comparison (LSD) were applied to compare genetic diversity indices and genetic similarity values of ISSR markers associated with different geographical locations and insect host populations, which was performed using SPSS 10.0 for Windows.

RESULTS

Evaluation of ISSR-PCR Markers

According to the analysis of the prescreening data using ten *Beauveria bassiana* strains and 33 ISSR primers including 20 dinucleotide, nine trinucleotide, and four tetranucleotide motifs, eight primers that failed to generate any amplification product, seven generated

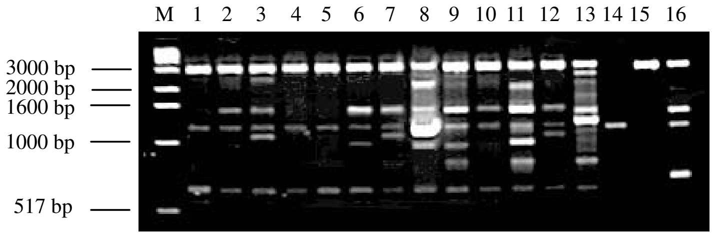

Fig.1. A PCR amplification pattern generated by using the inter-simple sequence repeat primer BIS30 $(GACA)_4$. Lanes 2–16 represent the following strains: 1, Bb276; 2, Bb278; 3, Bb241; 4, Bb345; 5, Bb344; 6, Bb343; 7, Bb347; 8, Bb346; 9, Bb61; 10, Bb202; 11, Bb367; 12, Bb366; 13, Bb59; 14, Bbr06; 15, Ba08; 16, Bv01; M, 1kb DNA ladder.

weak or ambiguous amplification products were discarded and 18 generated bright, polymorphic and reproducible amplification products were selected for further analysis. An example of a PCR fingerprint profile generated using the ISSR primer BIS30 $(GACA)_4$, is shown in Fig. 1. The 18 selected primers amplified consistently in all of the isolates examined (Table 2), and produced a total of 168 reliable fragments for 39 isolates at an average of 9.3 markers per primer, of which 161 (95.8 %) were polymorphic. For 36 *B. bassiana* isolates, 8.9 (1–13) markers per selected ISSR primer were scored, and a total of 123 fragments were amplified, with 102 (82.9 %) polymorphic (Table 2). The highest number of markers was obtained with the primers $(GTG)_6$, whereas the primer $(CT)_8RT$ resulted in the lowest number of markers. From the 168 polymorphic bands, seven bands were considered specific for *B. brongniartii* isolate Bbr06, 14 for *B. amorpha* isolate Ba08 and 18 for *B. velata* isolate Bv01.

The degree of polymorphism detected by each primer, expressed as polymorphic/amplified bands ratio, was calculated. There appeared to be no correlation between the number of bands amplified and the degree of polymorphism. For example, the primers $(AG)_8YC$ and $(TG)_8GT$ generated nine and eight bands, respectively, of which 44.4 % and 87.5 % were polymorphic; on the other hand, 92.3 % of the 13 bands amplified by $(GTG)_6$ primer were polymorphic. Among the 18 primers, nine, $(GA)_6GG$, $(AG)_8TC$, $(GA)_8T$, $(AC)_8T$, $(AG)_8S$, $(AC)_8YC$, $(CT)_8RT$, $(CTC)_4GC$ and $(AAG)_6$, generated 100 % polymorphisms in all the *B. bassiana* isolates.

Genetic diversity was calculated for each of the ISSR primers in the 36 *B. bassiana* isolates. Diversity index permits to evaluate the discriminating ability of each marker. The eight primers $(CTC)_4GC$, $(AG)_8TC$, $(GA)_6GG$, $(GA)_8T$, $(AG)_8S$, $(AC)_8T$, $(AAG)_6$ and $(AC)_8YC$ gave the highest polymorphism (100 %), and the higher diversity indices of more than 0.25 for Nei's (1979) gene diversity index and 0.40 for polymorphic information content (PIC) value. However, primer $(CT)_8RT$ gave a low diversity index, due to the occurrence of only one band. The difference in diversity indices of the other nine primers was as striking as the degree of polymorphism. For example, the primers $(GTG)_6$ and $(AC)_8YT$ recorded the highest and lowest polymorphisms, respectively, and also recorded the highest and lowest diversity values respectively.

Analysis of genetic relationships

The genetic similarity values based on ISSR analysis were obtained by multivariate analysis using Nei's coefficient (data not shown). For 36 *Beauveria bassiana* isolates, the genetic similarity matrix showed that the genetic similarity value ranged from 0.972 for two pairs of strains, Bb143 and Bb162 (both from Magushan Forest Farm), and Bb80 and Bb88 (both from Guniujiang Nature Reserve) to a minimum of 0.651 for accessions Bb344 (Daiweishan Nature Reserve) and Bb87 (Guniujiang Nature Reserve). The genetic similarity values among different *Beauveria* species ranged from 0.411 (between Ba08 and Bb87) to 0.720 (between Bbr06 and Bb367). The considerable intraspecific and interspecific variability among isolates of *Beauveria* spp. were revealed by 18 selected ISSR markers.

The dendrogram showing the genetic relationship among the *Beauveria* strains based on the total number of amplified ISSR fragments is presented as Fig. 2. Each isolate represented a unique haplotype. All strains of *B. bassiana* clustered together to form a specific clade, while the morphological related *B. brongniartii* (Bbr06), *B. amorpha* (Ba08) and *B. velata* (Bv01) formed independent groups, respectively. Cluster analysis demonstrated that there was a genetically distinct variation among *Beauveria* spp. All the non-Chinese strains were genetically separated from Chinese strains. All the strains originated from MGS formed one cluster with the high genetic similarity values, ranged from 0.869 to 0.972 (0.927 ± 0.024), the strains from KKS Nature Reserve also grouped together. However, the *B. bassiana* isolates with the same insect host did not group together and distributed throughout the clusters. For example, the two *B. bassiana* isolates Bb89 and Bb345 share high genetic similarity value (0.925) but they are isolated from Homoptera and Lepidoptera, respectively. Genetic diversity indices and genetic similarity values for different geographical locations and insect host populations are presented in Table 3. The genetic diversity index and genetic similarity value

Table 3. Genetic diversity indices and genetic similarity values of ISSR markers associated with different geographical locations and insect hosts.

Geographical population	Genetic diversity index (DI[a])	Genetic similarity	Insect order	Genetic diversity index (DI)	Genetic similarity
DWS	0.41 ± 0.04	0.85 ± 0.05	*Hemiptera*	0.35 ± 0.05	0.89 ± 0.03
KKS	0.34 ± 0.03	0.89 ± 0.04	*Homoptera*	0.44 ± 0.08	0.84 ± 0.06
GNJ	0.38 ± 0.02	0.89 ± 0.03	*Hymenoptera*	0.39 ± 0.03	0.86 ± 0.05
YLP	0.45 ± 0.07	0.83 ± 0.08	*Coleoptera*	0.47 ± 0.01	0.81 ± 0.02
MGS	0.26 ± 0.02	0.94 ± 0.02	*Lepidoptera*	0.52 ± 0.03	0.78 ± 0.06
			Orthoptera	0.43 ± 0.02	0.83 ± 0.05
Average	0.37 ± 0.08	0.88 ± 0.05		0.43 ± 0.06	0.84 ± 0.04

[a] Shannon's Information index.

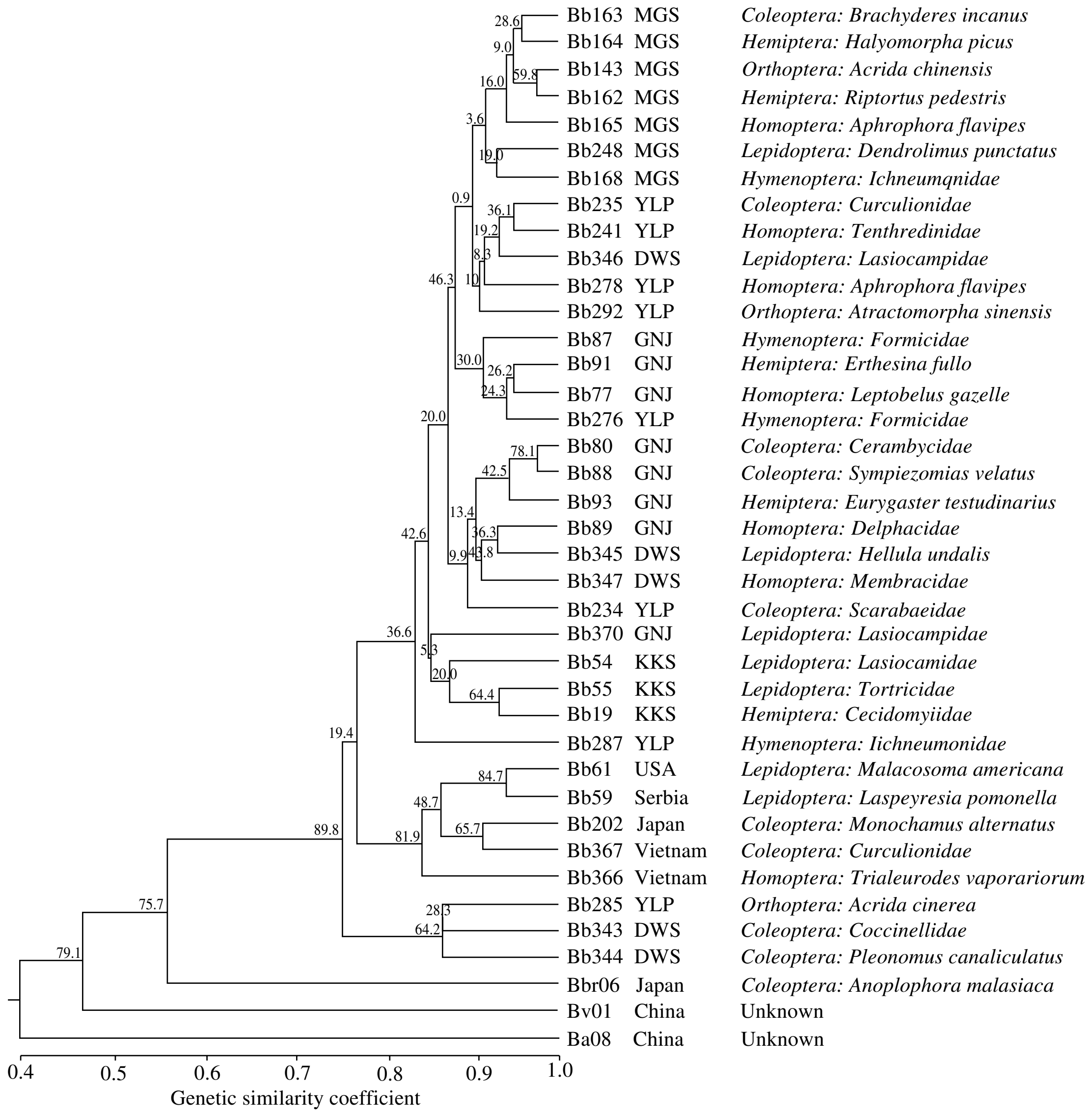

Fig. 2. Dendrograms derived from an unweighted pair group method analysis (UPGMA) cluster based on ISSR markers for 39 *Beauveria* isolates. Numbers on the nodes indicate bootstrap values (%) generated after 2000 replications.

of the MGS population were significantly different ($P < 0.05$) from those of all the other nature reserve populations, suggesting that there was high genetic diversity among *B. bassiana* populations of the nature reserves. The average genetic diversity index with geographical origins was smaller than that associated with insect hosts, but no significant difference was found in the genetic diversity index and genetic similarity value between the geographical locations and insect host populations.

DISCUSSION

In *Beauveria*, at least six species have been distinguished based on morphological and biochemical characteristics (Mugnai, Bridge & Evans 1989), of which three species (*B. bassiana*, *B. brongniartii*, and *B. amorpha*) have been reported in China (Wang *et al.* 2003b). In this study, the considerable variability among *Beauveria* isolates from different hosts and geographical locations analyzed by ISSR technique reflected a high level polymorphism at DNA level. Clustering analysis by UPGMA demonstrated that all isolates of the same taxa *B. bassiana* could be clustered together to form a specific clade. There was genetically distinct variation among the four morphologically similar species; these ISSR markers were successfully used to differentiate the three morphologically similar species (*B. brongniartii*, *B. amorpha*, and *B. velata*) to *B. bassiana*, indicating that ISSR markers are a more suitable technique for interspecific identification. In contrast to other PCR-based techniques, such as RAPD's and AFLP's, ISSR has the advantage of longer primers, allowing for more stringent annealing temperatures (Wolfe & Liston 1998). These higher annealing temperatures apparently provide a higher stability and reproducibility. Furthermore, AFLP is labor intensive, and has high operation and development costs. Microsatellites are specific and highly polymorphous (Coates *et al.* 2002b), but they require knowledge of the genomic sequence to design specific primers and are limited primarily to economically important species.

Entomopathogenic fungi, which are mostly facultative parasites, are subjected to many environmental factors. Environmental habitat selection determines the fungal population structure (St Leger *et al.* 1992b, Bidochka *et al.* 2002). The population structure and gene diversity of isolates can arise either due to diversification of a single genotype into several forms through accumulations of changes in the genome or due to recombination between genetically different individuals either by sexual or parasexual means (Padmavathi *et al.* 2003). Glare & Inwood (1998) demonstrated the importance of geographical origin within the context of observed haplotypes and population structure of *B. bassiana* analyzed by ITS and PCR-RFLP. Wang *et al.* (2003a) suggested that the genetic relatedness of *B. bassiana* strains was more associated with geographical location than with insect host species.

In the present study, all the non-Chinese strains were genetically distinct from Chinese strains. All the strains originating from MGS and from KKS formed independent clusters, respectively. The genetic diversity index of the MGS population was significantly lower than those of all the other populations, indicating that there was high genetic diversity amongst the *B. bassiana* populations of the nature reserves. This might be because the nature reserves had multiple and complex ecosystems at different altitudes and in different seasons, with the temperature, relative humidity, vegetation, and insect host spectrum different and changing in variant habitats. Consequently, the high level of genetic variability among the isolates derived from the nature reserves may be attributed to their diverse habitats. However, the *B. bassiana* isolates with the same host did not group together and were distributed throughout the clusters. At the same time, the average genetic diversity index with geographical origins was lower than that associated with insect hosts. Generally, the above results suggest a certain association between *B. bassiana* isolates and their geographical origins, but no clear correlation between those clusters and their common insect hosts. A similar conclusion was also drawn by Valderrama, Cristancho & Chaves (2000) based on RAPD analyses. Our results suggest that the selection of the strains is of great importance, and only the isolates from similar ecological habitats can be used as effective accessions to reveal potential genetic relationships between the isolates and host and geographical origins. In earlier studies, the strains examined defined as geographical populations were derived from a country or from very disparate areas, and three different classes of conclusions have been drawn in regard to host specialization and geographic distribution of genetic variation of *B. bassiana*: (1) the molecular variation among *Beauveria* isolates was related to insect host range (Viaud *et al.* 1996, Maurer *et al.* 1997, Neuvéglise, Brygoo & Riba 1997); (2) the genetic relatedness of *B. bassiana* strains was associated with geographical origin (St Leger *et al.* 1992a, Glare & Inwood 1998, Bidochka *et al.* 2002, Wang *et al.* 2003a); and (3) no obvious correlation between isolates, hosts and geographical origins was observed (Rivera, Bridge & Bustillo 1997, Berretta *et al.* 1998, Gaitan *et al.* 2002, Aquino de Muro, Mehta & Moore 2003).

In conclusion, ISSR markers detected a very high level of polymorphism between and among *Beauveria* strains. These informative primers could be easily developed for population genetic analysis, epidemiological, and ecological studies of *B. bassiana*. Additionally, ISSR analysis can be utilized to generate unique PCR products or amplicons in different *Beauveria* species or strains of interest, and then may be converted into species- or strain-specific sequence-characterized amplified region (SCAR) markers. SCAR markers can be used to track the fate of experimentally released strains, and to investigate the dynamics of clonal populations. Further studies are needed to investigate the relationship among different *B. bassiana* ecotypes.

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