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Use of Random Amplified Polymorphic DNA to Characterize Entomopathogenic Fungi, Nomuraea spp., Beauveria spp., and Metarhizium anisopliae var. anisopliae, from Taiwan and China【Research report】

利用 RAPD 方法分析比較蟲生病原真菌Nomuraea spp.、 Beauveria spp.和Metarhizium anisopliae var. anisopliae 分離株【研究報告】

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Abstract

Levels of virulence of entomopathogenic fungi among the genera Beauveria, Metarhizium, and Nomuraea are widely diverse. Random amplified polymorphic DNA (RAPD) was used to differentiate a total of 38 strains of entomopathogenic fungi isolated from 20 geographic regions of Taiwan and mainland China. Fungal isolates were obtained from 15 insect species. Banding patterns were generated from 3 selected primers (OPM 12, 18, and 20). Isolates were grouped into 10 clusters according to similarity, following cluster analysis using Jeffrey' s coefficients. Three distinct genotypes were observed among the 38 isolates tested. On the basis of RAPD patterns, 2 genera and 1 species were recognized, namely Beauveria, Metarhizium anisopliae var. anisopliae, and Nomuraea. Nomuraea exhibited a more
conservative banding pattern than each of the other genera. RAPD markers may be useful as identification biomarkers of specific biocontrol strains in a limited geographical area.

摘要

利用 RAPD (random amplified polymorphic DNA)分析方法進行來自灣本地(36 株)和大陸(2 株) Beauveria 、Metarhizium 和 Nomuraea 三個屬五個種(Beauveria amorpha (Ba), Beauveria bassiana (Bb), Beauveria brongniartii(Bbr), Nomuraea rileyi (Nr), Nomuraea viridulus (Nv) 和Metarhizium anisopliae (Ma)共38 株蟲生真菌的鑑別分析研究，以三引子OPM 12、18、20 進行反應的結果分析，Beauveria、Metarhizium 和 Nomuraea 三個屬可明顯被區隔開來，38 株菌可被區分成10 個clusters，但部份M. anisopliae 菌株(Ma5,6,7)和N. rileyi 的親緣性反較N. rileyi 與N. viridulus 之間為高。

Key words: random amplified polymorphic DNA (RAPD), Beauveria, Metarhizium, Nomuraea.

關鍵詞: random amplified polymorphic DNA (RAPD), Beauveria, Metarhizium, Nomuraea.

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Use of Random Amplified Polymorphic DNA to Characterize Entomopathogenic Fungi, *Nomuraea* spp., *Beauveria* spp., and *Metarhizium anisopliae* var. *anisopliae*, from Taiwan and China

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ABSTRACT

Levels of virulence of entomopathogenic fungi among the genera *Beauveria*, *Metarhizium*, and *Nomuraea* are widely diverse. Random amplified polymorphic DNA (RAPD) was used to differentiate a total of 38 strains of entomopathogenic fungi isolated from 20 geographic regions of Taiwan and mainland China. Fungal isolates were obtained from 15 insect species. Banding patterns were generated from 3 selected primers (OPM 12, 18, and 20). Isolates were grouped into 10 clusters according to similarity, following cluster analysis using Jeffrey's coefficients. Three distinct genotypes were observed among the 38 isolates tested. On the basis of RAPD patterns, 2 genera and 1 species were recognized, namely *Beauveria*, *Metarhizium anisopliae* var. *anisopliae*, and *Nomuraea*. *Nomuraea* exhibited a more conservative banding pattern than each of the other genera. RAPD markers may be useful as identification biomarkers of specific biocontrol strains in a limited geographical area.

Key words: Random amplified polymorphic DNA (RAPD), *Beauveria*, *Metarhizium*, *Nomuraea*.

Introduction

Entomopathogenic fungi of the genera *Beauveria*, *Metarhizium*, and *Nomuraea* are widely dispersed among different hosts and geographical regions. Growth of *Beauveria bassiana* in industrial mass cultures for biological control of insects has been reviewed (Desgranges *et al.,* 1993). In addition, several commercial products incorporating entomopathogenic fungi, including *Metarhizium anisopliae* var. *anisopliae* (Khachatourians, 1991) and *Nomuraea rileyi* (Bartlett *et al.,* 1988) are available. These 3 species display quite distinct biological characteristics and infectivities.

Diversity within the entomo-

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pathogenic fungi has been analyzed using morphological features, biochemical characteristics (Rombach *et al.,* 1987；Mugnai *et al.,* 1989) and isozyme analysis (deConti *et al.,* 1980；St Leger *et al.,* 1992). Entomopathogenic fungi are used as biological control agents for many insect pests, and their demonstrable differences in infectivity makes it important to identify isolates accurately (Yip *et al.,* 1992).

Polymerase chain reaction (PCR) is a powerful technique for amplifying specific regions of DNA. Random amplified polymorphic DNA (RAPD) fragments, DNA sequences separated by gel electrophoresis after PCR, are useful genetic markers for a variety of eukaryotic organisms, including humans, fungi, and plants (Welsh, *et al.,* 1991；Williams, *et al.,* 1991).

Our goal was to use RAPD to examine differences among entomo-pathogenic fungal genera and isolates from Taiwan and mainland China. The results of this study imply that considerable genetic diversity exists. This diversity may be associated with geographical location and original insect host. Furthermore, an assigned genetic basis permits the prediction of patterns of different genera and isolates.

Materials and Methods

Thirty-eight isolates of *M. anisopliae* var. *anisopliae*, *Beauveria* spp., and *Nomuraea* spp. were obtained from infected insects. Thirty-six samples were collected from Taiwan and 2 (*B. bassiana,* Bb-9 and *B. brogniartii,* Bbr-1) from mainland China. The locations and original host species are presented in Table 1. Single-spore cultures were isolated and cultured in V8 medium (Difco Laboratories, Detroit, MI, USA) for *Beauveria* spp., in Sabouraud's maltose agar plus 1% yeast extract (SMAY medium) for *Nomuraea* spp., and in PDA medium for *M. anisopliae* var. *anisopliae,* at 25℃ for 14 days.

Genomic DNA was extracted from lyophilized mycelia and conidia according to the method described by Pfeifer and Khachatourians (1993) with minor modifications. Mycelia were recovered from media by filtration, and 80 mg of mycelia or conidia was extracted in 0.6 ml of lysis buffer (0.1 M Tris (pH 8.0), 1% SDS, 0.1 M EDTA, 0.1 M NaCl, 0.1 mg/ml proteinase K). The suspension was extracted twice with Tris-saturated phenol (pH 8.0)/ chloroform (1:1, v/v) using 10-min centrifugations at 10,000 x*g* for phase separations. DNA in the aqueous phase was precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.4) and 2.5 volumes of 95% ethanol. The sample was then centrifuged at 10,000 x*g* for 20 min. The pellet was washed twice with 70% (v/v) ethanol and dried under a vacuum. DNA was resuspended in 0.05 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0) containing 2 mg/ml RNase A (Sigma Chemical, St. Louis, MO).

RAPD products are frequently amplified introns or internal transcribed spacer regions. A total of twenty 10-mer oligonucleotide primers was utilized for RAPD analysis (Table 2). These primers were purchased from Operon Technologies (Alameda, CA). A typical mixture consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 200 µM each of dNTP (Pharmacia), 0.05 mM primer, 100 ng DNA, 0.5 unit Taq DNA polymerase (DyNAzyme) in a 50-µl volume, overlaid with mineral oil. The temperature cycle was as follows: initial denaturation for 5 min at 95℃, followed by 45 cycles at 95 ℃ for 1 min, 33℃ for 1.5 min, and 72℃ for 2.5 min, with a final for extension at 72 ℃ 10 min. Amplification was performed using a Perkin-Elmer Cetus DNA thermal cycler model 480. Ten microliters of amplification products was

Table 1. Original host, location, and year isolation of *Beauveria bassiana* (Bb), *Beauveria brongniartii* (Bbr), *Nomuraea rilieyei* (Nr), *Nomuraea viridulus* (Nv), and *Metarhizium anisopliae* (Ma).

Isolate	Original host species	Location (Geographic region)	Year of isolation	Cluster
Bb-1	*Echinocnemus squameus*	Taoyuan (2)	1991	II
Bb-2	*Plutella xylostella*	Peitou, Changhua (9)	1992	I
Bb-3	*Cylas formicarius*	Mt. Seven Star (1)	1990	II
Bb-4	*Brontispa longissima*	Taichung (5)	1992	II
Bb-9	*Carposina nipponensis*	mainland China	1995	X
Bb-13	*Plutella xylostella*	Jenai, Nantou (8)	1995	II
Bb-14	*Coccinella septempunactata*	Shanping, Kaohsiung (17)	1995	III
Bb-15	*Echinocmemus* sp.	Shanping, Kaohsiung (17)	1995	III
Bb-16	Lepidoptera (unidentified)	Shanping, Kaohsiung (17)	1995	II
Bb-17	*Plutella xylostella*	Wufeng, Taichung (6)	1995	II
Bbr-1a	*Lachnosterna horishana*	Mainland China	1995	IV
Ba-1b	Arachnida (unidentified)	Shanping, Kaohsiung (17)	1995	II
Ma-1	*Plutella xylostella*	Jenai, Nantou (8)	1995	VIII a
Ma-2	Coleoptera	Tungshi, Taichung (4)	1995	VIII a
Ma-3	Coleoptera	Ilan (3)	1995	VIII b
Ma-4	*Brontispa longissima*	Pingtung (19)	1995	IX
Ma-5	Lepidoptera (unidentified)	Tapu, Chiayi (16)	1996	VII
Ma-6	House fly	Tapu, Chiayi (16)	1996	VII
Ma-7	Lepidoptera (unidentified)	Fanlu, Chiayi (16)	1996	VII
Nv-1	Hymenoptera (Cicada)	Wufeng, Taichung (6)	1995	V
Nr-2	*Spodoptera litura*	Sanhsing, Ilan (3)	1995	VI a
Nr-3	*Spodoptera exigua*	Tuku, Yunlin (12)	1995	VI b
Nr-4	*Spodoptera exigua*	Paochung, Yunlin (11)	1995	VI b
Nr-5	*Spodoptera exigua*	Tuku, Yunlin (12)	1995	VI b
Nr-6	*Helicoverpa armigera*	Tacheng, Changhua (7)	1995	VI b
Nr-9	*Helicoverpa armigera*	Peikong, Yunlin (13)	1995	VI b
Nr-10	*Helicoverpa armigera*	Yuanchang, Yunlin (13)	1995	VI b
Nr-11	*Helicoverpa armigera*	Tuku, Yunlin (12)	1995	VI b
Nr-12	*Spodoptera litura*	Hsilo, Yunlin (10)	1995	VI b
Nr-13	Lepidoptera (unidentified)	Shanping, Kaohsiung (17)	1995	VI b
Nr-14	*Helicoverpa armigera*	Hsikiu, Chiayi (14)	1995	VI a
Nr-15	*Helicoverpa armigera*	Chiayi (14)	1995	VI a
Nr-16	*Spodoptera exigua*	Luchu, Kaohsiung (18)	1995	VI a
Nr-17	Lepidoptera (unidentified)	Tungshi, Taichung (4)	1996	VI c
Nr-18	Lepidoptera (unidentified)	Tapu, Chiayi (16)	1996	VI c
Nr-19	Lepidoptera (unidentified)	Jenai, Nantou (8)	1996	VI a
Nr-20	Lepidoptera (unidentified)	Luku, Nantou (8)	1996	VI a
Nr-21	Lepidoptera (unidentified)	Jenai, Nantou (8)	1996	VI c

The code numbers for geographic regions correspond to the locations indicated in Fig. 3. Cluster no. is defined in Fig. 2.

Table 2. Primers used in this study and the numbers of informative RAPD bands generated.

Code	Sequence 5' to 3'	No. of informative bands		
		Beauveria spp.	*Nomuraea* spp.	*Metarhizium* spp.
OPM-01	GTTGGTGGCT	0	4	7
OPM-02	ACAACGCCTC	2	3	4-7
OPM-03	GGGGGATGAG	0	0-1	2-3
OPM-04	GGCGGTTGTC	3	2-3	0
OPM-05	GGGAACGTGT	2	4-5	3-4
OPM-06	CTGGGCAACT	0	5	3-6
OPM-07	CCGTGACTCA	3	3-5	3-5
OPM-08	TCTGTTCCCC	0	1-2	2-4
OPM-09	GTCTTGCGGA	0	1-3	3-7
OPM-10	TCTGGCGCAC	0	3-5	6
OPM-11	GTCCACTGTG	0	2-3	2-3
OPM-12	GGGACGTTGG	1-2	3-4	8
OPM-13	GGTGGTCAAG	0-1	4	6
OPM-14	AGGGTCGTTC	0	7	4-6
OPM-15	GACCTACCAC	0-1	4	8
OPM-16	GTAACCAGCC	0-1	7	6
OPM-17	TCAGTCCGGG	0-3	3-7	5
OPM-18	CACCATCCGT	3	1-3	4
OPM-19	CCTTCAGGCA	2-7	3	3
OPM-20	AGGTCTTGGG	7	5	6

loaded onto a 1.5% (w/v) agarose gel containing 0.5 µg/ml (w/v) ethidium bromide and electrophoresed at 100 V for 0.5 h. Amplified DNA fragments on the gel were visualized using a UV transilluminator.

Polymorphisms between isolates were scored from gel photographs (Fig. 1). Scored amplified fragments ranged in size from approximately 300 to 2000 bp. The proportion of shared RAPD products between isolates (F) was calculated using the formula proposed by Nei and Li (1979): $F = 2 mxy/(mx + my)$; where mx and my are the number of amplification products produced by each isolate, and mxy is the number of products shared by the isolates. In addition, pooled data (*F* values) from 20 primers were analyzed using an unweighted pair group option with the Gel Compar software package (Applied Maths BVBA, Kortrijk, Belgium) and the arithmetic mean (UPGMA) procedure. A dendrogram was produced from the data (Fig. 2).

Results and Discussion

The regions of Taiwan from which isolates were obtained are shown in Fig. 3. These isolates were collected from regions located as far as apart as 293 km. Isolates Bb-9 and Bbr-1 were collected from mainland China.

The RAPD marker patterns of DNA extracted from 38 fungal isolates were assessed with 20 primers (Kit M). We analyzed 10 isolates of *B. bassiana*, 1 isolate of *B. brongniartii* (Bbr-1a), 1 isolate of *Beauveria amorph* (Ba), 7 isolates of *M. anisopliae* var. *anisopliae* (Ma), 18 isolates of *N. rileyi* (Nr), and 1 isolate of *N. viridulus* (Nv). The primers used in this investigation and the number of informative bands generated are listed in Table 2. Not all primers performed with the same efficiency. In some cases, no amplified bands were

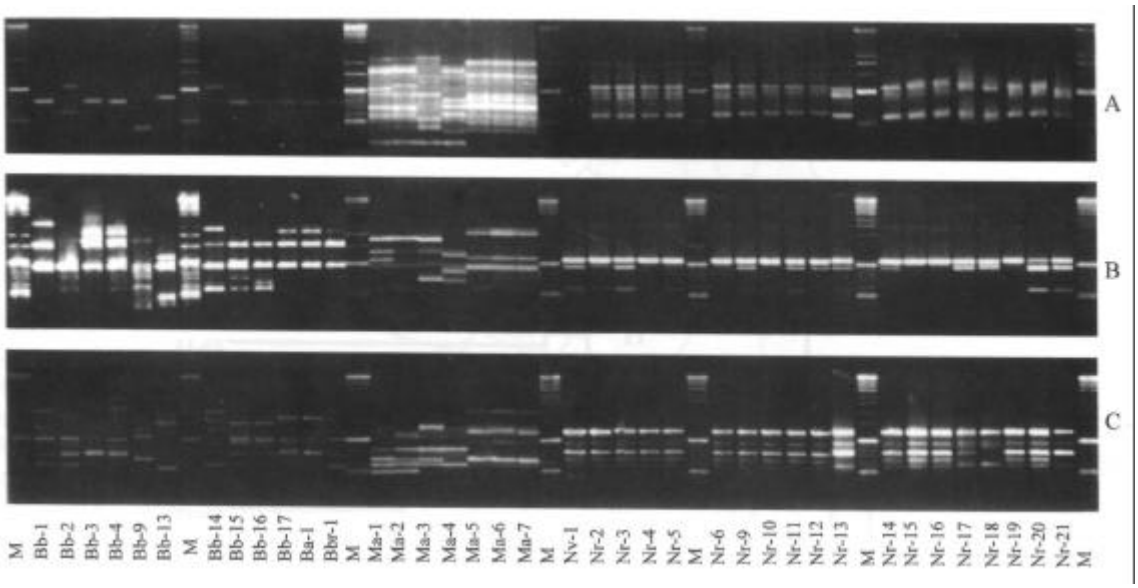

Fig. 1. Ethidium-bromide stained agarose gels of PCR products from DNA samples of 38 isolates of entomopathogenic fungi, *Beauveria bassiana*, *Nomuraea rileyi*, and *Metarhizium anisopliae* generated using primers OPM-12, OPM-18, and OPM-20. The code numbers above each lane indicate isolate identification numbers as listed in Table 1. The lanes marked with the letter M are Pharmacia Kilobase DNA markers and 100-bp mass standards. For all RAPD assays, duplicate tests were conducted for each sample. A, OPM-12 primer tested; B, OPM-18 primer tested; C, OPM-20 primer tested.

obtained, presumably due to the lack of suitable priming sites in the genomic DNA of *Beauveria* spp. (OPM-1, 3, 6, 8, 9, 10, and 14) and *Metarhizium* spp.(OPM-4). The greatest number of bands was found with primer OPM-20. This primer produced 7 different bands for *Beauveria* spp., 5 for *Nomuraea* spp. and 6 for *M. anisopliae* var. *anisopliae* isolates (Table 2, Fig. 1).

A dendrogram was constructed for all isolates using data pooled from primers OPM-12, OPM-18, and OPM-20. On the basis of similarity coefficients, this comprised 3 distinguishable groups of isolates: group A (isolates of *Beauveria* spp.), group B (isolates of *Nomuraea* spp., and *M. anisopliae* var. *anisopliae*), and group C (part of the isolates of *M. anisopliae* var. *anisopliae*); which separated at branch points of a 55% and a 67% similarity coefficient, respectively. The main coefficients of similarity obtained from pairwise comparisons were 76% to 97.5% for isolates derived from *Nomuraea*, 58% to 89.5% for isolates derived from *Beauveria,* and 67% to 98% for isolates derived from *M. anisopliae* var. *anisopliae*. In general, the clusters of isolates defined by RAPD analysis correlated with the respective genera of the entomopathogenic fungi tested, except for Bb-9, which was isolated from mainland China.

Clusters of *Beauveria* spp. and *M. anisopliae* var. *anisopliae* isolates defined by RAPD analysis did not correlate strictly with host species or with the region from which they were obtained. Cluster VIII consisted of 3 isolates from

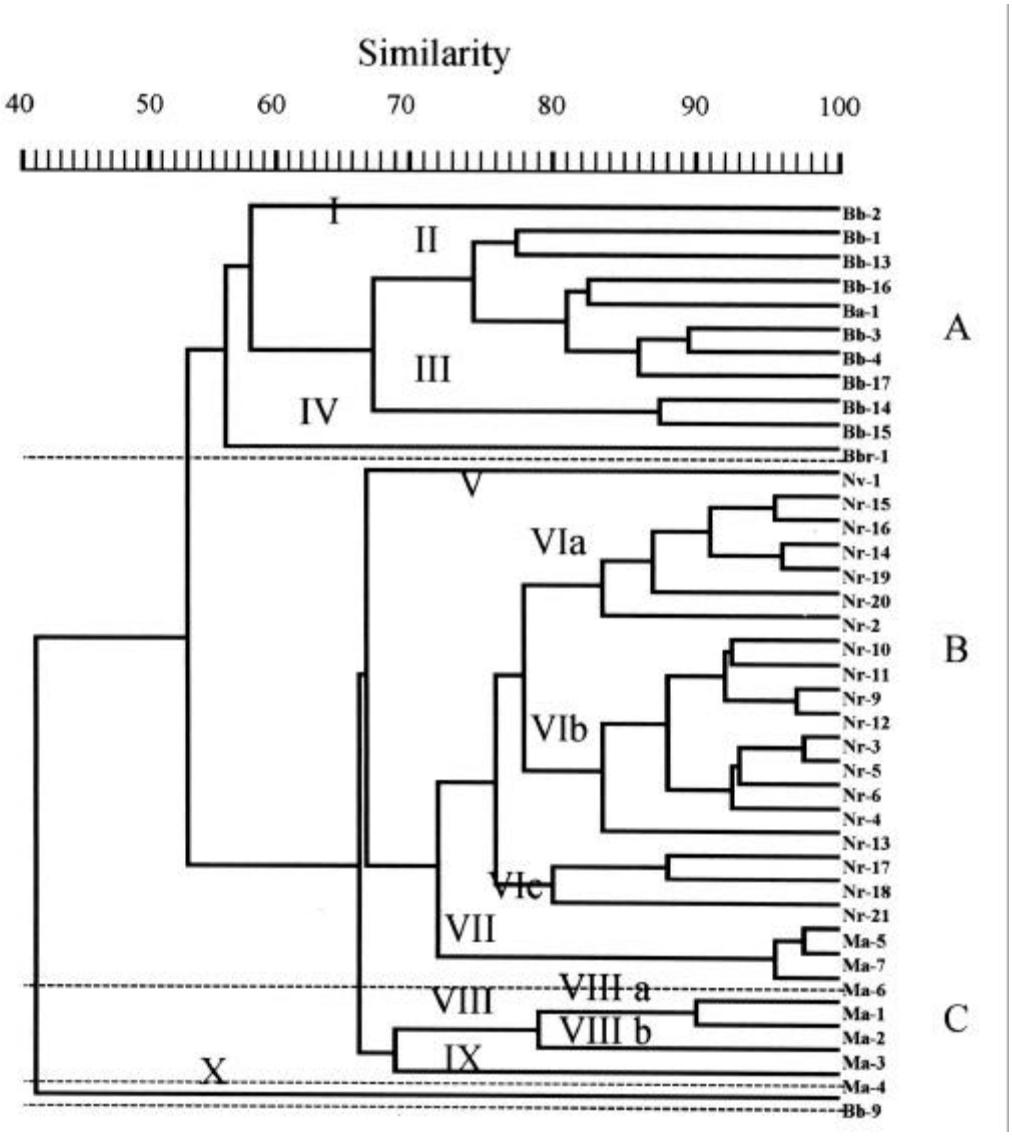

Fig. 2. Dendrogram of 38 isolates of entomopathogenic fungi based on RAPD data. All isolates could be separated into 3 distinguishable groups (A, B, and C) and 14 clusters by similarity coefficient analysis.

the 3 different regions and 2 different insect hosts. Bb-2, Bb-13, and Bb-17, which originated from the same host, *Plutella xylostella,* showed high diversity. Isolates Bb-14, Bb-15, and Bb-16 all originated from geographic region 18, and were classified into 2 separate clusters. Isolates Ma-5, Ma-6, and Ma-7 of cluster VII originated from Chiayi, Taiwan, but exhibited closer similarity to *N. rileyi* than to other *M. anisopliae* var. *anisopliae* isolates. But, *N. rileyi* isolates exhibited a high level of similarity, as this species' hosts all belonged to the

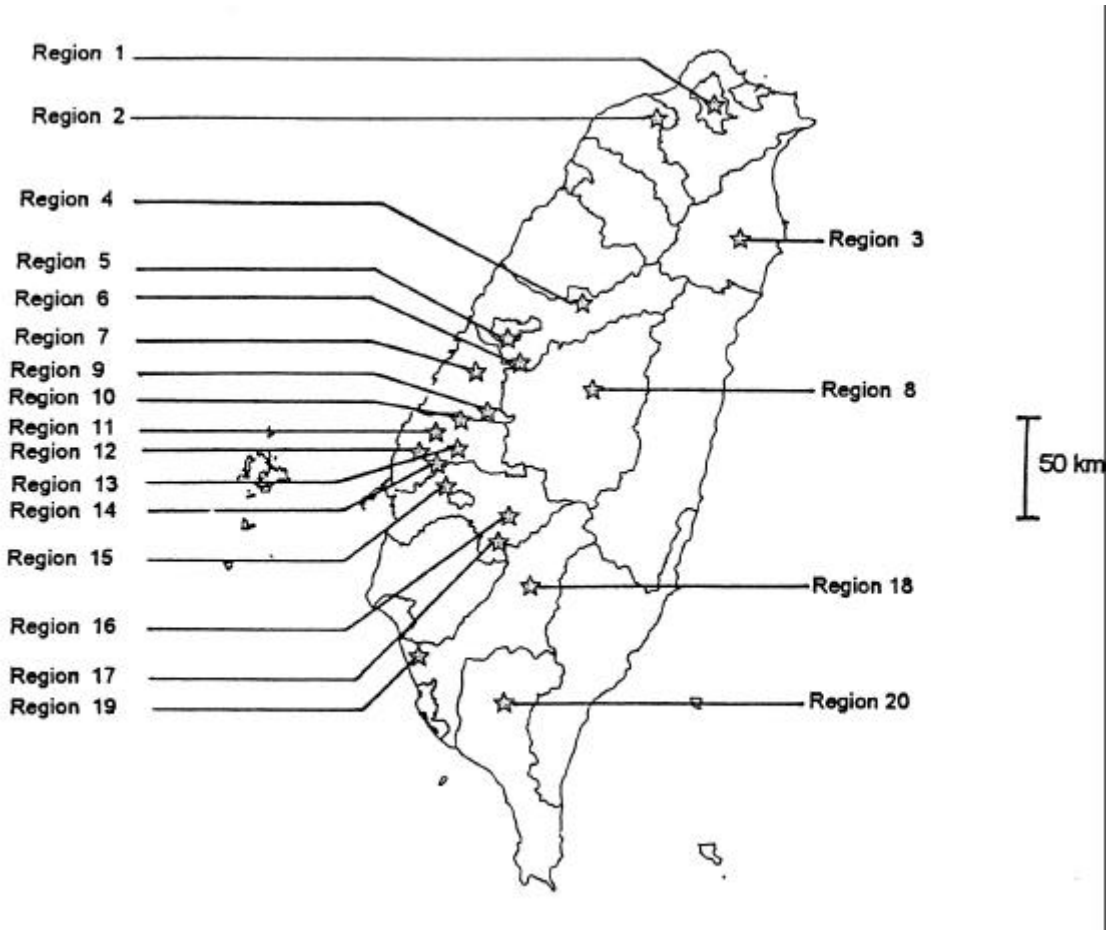

Fig. 3. Regions of Taiwan from which isolates were obtained.

Lepidoptera. These isolates were all obtained from peanut fields and asparagus fields of 3 centrally located regions of Yunlin, Taiwan, and demonstrated stablilities similar to those of *N. rileyi* in central Taiwan.

The findings presented herein, using RAPD to describe the great genetic diversity in entomopathogenic fungi, were consistent with those of RFLP and the Southern blot technique (Kosir *et al.,* 1991 ; Bridge *et al.,* 1993), as well as with other biochemical markers. Allozyme analysis of *M. anisopliae* also showed the diversity of this species (Riba *et al.,* 1985 ; St. Leger *et al*., 1992) with calculated genetic differences ranging from 0 to 0.8 based on allelic frequencies at 8 biochemical loci (St. Leger *et al*., 1992). The accuracy of RAPD markers for predicting genetic relationships has been demonstrated by previous studies. Groupings of individuals within several species, determined by RAPD, coincide with taxonomic systems based on morphological and genetic differences, biotypes, and conidia types (Fegan *et al.,* 1993; Johnson *et al.,* 1997). More recently, fungal universal primers ITS 1 and ITS 4 have been used to amplify ITS regions and 5.8 S rDNA from various fungal genomic DNA (White *et al.,* 1990 ;

Johnson *et al.,* 1997), as well as in internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA to help clarify interspecific relationships (Shih *et al.,* 1995). The nature and extent of genetic variation among fungal entomopathogens are largely unknown and require prior knowledge of the genome to be analyzed. The RAPD technique is adequate because the method relies on the presence of priming sites for a single primer, 1 of which has an inverted orientation and is close enough to permit PCR amplification. RAPD markers represent a convenient means of scanning and comparing the genomes of individuals which is consistent with established phenotypic schemes (Kazan *et al.,* 1993).

The most important finding of the current investigation is that isolates of entomopathogenic fungi can be classified into different genera. Thus, the identity of specific isolates can be determined by the RAPD procedure to avoid error judgements by direct sequencing among RAPD products in the future.

The dendrogram produced from the RAPD data in the present study splits the 38 isolates into 3 major groups. The results presented herein show the genetic variability among the entomopathogenic fungus genera, *Nomuraea* and *Beauveria,* and isolates of *M. anisolpliae* var. *anisolpliae.* The relationship between RAPD profiles of entomopathogenic fungal isolates and pathogenicity against insects should be further studied using biocontrol tests.

Acknowledgments

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利用 RAPD 方法分析比較蟲生病原真菌 *Nomuraea* spp.、*Beauveria* spp.和 *Metarhizium anisopliae* var. *anisopliae* 分離株

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摘 要

利用 RAPD (random amplified polymorphic DNA)分析方法進行來自灣本地(36 株)和大陸(2 株) *Beauveria*、*Metarhizium* 和 *Nomuraea* 三個屬五個種(*Beauveria amorpha* (Ba), *Beauveria bassiana* (Bb), *Beauveria brongniartii* (Bbr), *Nomuraea rileyi* (Nr), *Nomuraea viridulus* (Nv) 和 *Metarhizium anisopliae* (Ma)共 38 株蟲生真菌的鑑別分析研究，以三引子 OPM 12、18、20 進行反應的結果分析，*Beauveria*、*Metarhizium* 和 *Nomuraea* 三個屬可明顯被區隔開來，38 株菌可被區分成 10 個 clusters，但部份 *M. anisopliae* 菌株(Ma5,6,7)和 *N. rileyi* 的親緣性反較 *N. rileyi* 與 *N. viridulus* 之間為高。

關鍵詞： random amplified polymorphic DNA (RAPD), *Beauveria*, *Metarhizium*, *Nomuraea.*