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Development of a Novel Species-specific DNA Marker for Rapid Detection of the Entomopathogenic Fungus, Nomuraea rileyi, in Infected Insects 【Research report】

快速偵測受感染蟲體內綠殭菌之專一性脫氧核糖核酸標記之開發【研究報告】

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Abstract

A species-specific DNA marker for detection of the entomopathogenic fungus, Nomuraea rileyi, was developed from sequence-characterized amplified regions (SCARs) derived from a random amplification of polymorphic DNA (RAPD) analysis. A common 1.4 kb DNA fragment was amplified in closely related N. rileyi isolates from Taiwan but not in Metarhizium anisopliae or Beauveria bassiana. This fragment was used for designing a pair of 20-mer oligonucleotide primers which were amplified to be a single band of the 284 bp DNA fragment. The predicted size of the DNA fragment was also amplified from 17 domestic N. rileyi isolates and from two other isolates from the United States using a NS1/NS2 primers pair, but they were not from B. bassiana or M. anisopliae. In the sensitivity and interference assays, a predicted amplicon DNA could be detected using a DNA template as low as 0.1 ng, without disturbing it with DNA from the host insect, Spodoptera litura. A 284 bp amplicon DNA was detected from live and mummified S. litura larvae infected with N. rileyi isolates but was not detected with B. bassiana and M. anisopliae isolates using this specific primers pair. These results indicate that detection of N. rileyi in infected larvae is possible one day after inoculation using a NS1/NS2 primer pair. Therefore, this method has the potential for rapidly detecting N. rileyi in infected insects, and is also useful for surveying the distribution of this fungus in the field.

摘要

藉由隨機增幅多型性技術 (RAPD) 分析而產生的特徵性序列增幅區域 (SCAR)，從中發展具種專一性之脫氧核糖核酸標記 (DNA marker)，以偵測蟲生真菌綠殭菌 (Nomuraea rileyi)。在台灣地區親緣關係近似的綠殭菌分離株中，增幅出一條共同的 1.4 kb 長度的DNA序列，但在白殭菌 (Beauveria bassiana) 與黑殭菌 (Metarhizium anisopliae) 並無此一增幅片段。此DNA片段序列被應用於設計一組長度在 20 個核苷酸的脫氧核糖核酸引子對，即NS1/NS2，可增幅出一條長度在 284 個核甘酸的DNA片段。利用NS1/NS2引子對可成功的在本地的綠殭菌 17 株分離株及 2 株美國的綠殭菌品系中增幅出預期長度的DNA片段，但在白殭菌與黑殭菌品系則無法增福出任何片段。在靈敏度與干擾試驗，僅 0.1 ng 的DNA板模即可增幅出預期長度的DNA片段，而且不受宿主昆蟲斜紋夜蛾 (Spodoptera litura) 的DNA干擾，能維持相同高靈敏度。同時也可在受綠殭菌感染的斜紋夜蛾之活幼蟲及殭蟲中，增幅出 284 個核苷酸長度的DNA片段，但受白殭菌及黑殭菌感染的殭蟲則無法增幅出此一DNA片段。另外，本結果亦顯示受綠殭菌感染一天後的幼蟲也能利用此NS1/NS2引子偵測出蟲體有綠殭菌存在。因此，本方法具有快速偵測蟲體受綠殭菌感染之潛力，同時亦可應用於調查綠殭菌在田間之分佈。

Key words: entomopathogenic fungi, Nomuraea rileyi, RADP-PCR, SCAR

關鍵詞: 蟲生真菌、綠殭菌、機增幅多型性技術、特徵性序列增幅區域。

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ABSTRACT

A species-specific DNA marker for detection of the entomopathogenic fungus, *Nomuraea rileyi*, was developed from sequence-characterized amplified regions (SCARs) derived from a random amplification of polymorphic DNA (RAPD) analysis. A common 1.4 kb DNA fragment was amplified in closely related *N. rileyi* isolates from Taiwan but not in *Metarhizium anisopliae* or *Beauveria bassiana*. This fragment was used for designing a pair of 20-mer oligonucleotide primers which were amplified to be a single band of the 284 bp DNA fragment. The predicted size of the DNA fragment was also amplified from 17 domestic *N. rileyi* isolates and from two other isolates from the United States using a NS1/NS2 primers pair, but they were not from *B. bassiana* or *M. anisopliae*. In the sensitivity and interference assays, a predicted amplicon DNA could be detected using a DNA template as low as 0.1 ng, without disturbing it with DNA from the host insect, *Spodoptera litura*. A 284 bp amplicon DNA was detected from live and mummified *S. litura* larvae infected with *N. rileyi* isolates but was not detected with *B. bassiana* and *M. anisopliae* isolates using this specific primers pair. These results indicate that detection of *N. rileyi* in infected larvae is possible one day after inoculation using a NS1/NS2 primer pair. Therefore, this method has the potential for rapidly detecting *N. rileyi* in infected insects, and is also useful for surveying the distribution of this fungus in the field.

Key words: entomopathogenic fungi, *Nomuraea rileyi*, RADP-PCR, SCAR

Introduction

The entomopathogenic fungus, *Nomuraea rileyi*, is distributed worldwide and is pathogenic to lepidopteran larvae (McCoy *et al.*, 1988). It has been found to be epizootic in *Anticarsia gemmatalis* populations as a well-known insecticidal

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agent (Ignoffo, 1981). The isolates of *N. rileyi* collected from different hosts and geographic regions vary in their virulence and specificity (Boucias *et al.*, 1982; Ignoffo and Garcia, 1985). The isolates of *N. rileyi* from Taiwan were examined for use as biocontrol agents for various larval stages of the corn earworm, *Helicoverpa armigera*, and were assessed in field applications (Tang and Hou, 1998; Tang *et al*., 1999). Several fungi with economic potential as mycoinsecticides, e.g., *N. rileyi*, *Beauveria bassiana*, and *Metarhizium anisopliae*, would benefit from advances in biotechnology, especially the development of highly specific and sensitive fungal detection systems. In addition, the need for strict quality control, environmental monitoring, and the persistence of mycoinsecticides require a precise detective system as well (Leathers *et al*., 1993). The detection and classification of these entomopathogenic fungi were previously dependent on biochemical and morphological characteristics, including reproductive spores and colonial morphologies (Samson *et al*., 1988), isoenzymes (Poprawski *et al*., 1988; St. Leger *et al*., 1992), hydrolytic enzymes (Mugnai *et al*., 1989), and immunological cross-reactions (Shimizu and Aizawa, 1988; Tan and Ekramoddoullah, 1991). However, these detection methods include complicated procedures and are time-consuming. Therefore, the development of a rapid and simple diagnostic method is important in the differentiation of *N. rileyi* from other fungi.

In recent years, taxonomy, identification, genotypic properties, and genetic variations in entomopathogenic fungi were studied using molecular genetic techniques (Khachatourians, 1996; Boucias *et al*., 2000; Suwannakut *et al*., 2005). Molecular markers have been utilized to assess genetic variations among isolates of *N. rileyi* and other entomopathogenic fungi, and so provide a method to identify strains of interest, determine the origin of the isolate, and study the population structure or family relationship. For example, polymerase chain reaction-based random amplified polymorphic DNA (RAPD-PCR) has been utilized to differentiate isolates or strains of *N. rileyi*, *B. bassiana*, and *M. anisopliae* (Fegan *et al*., 1993; Bidochka *et al*., 1994; Maurer *et al*., 1997; Castrillo *et al*., 1999; Tigano and Aljanabi, 2000; Kao *et al.*, 2002). The short primers of arbitrary sequence were used and annealed to multiple target sequences, which may produce multiple DNA fragments for diagnostic patterns (Williams *et al*., 1990; Black., 1993). Since the RAPD analytical technique does not require a DNA sequence of the target region, it can be easily applied to detect various entomopathogenic fungi, including poorly studied genomes. However, the RAPD technique requires stringent procedure standardization to ensure reproducibility (Black, 1993).

To utilize the RAPD analysis, some unique PCR products or amplicons were generated in filamentous fungal species or strains of interest. In addition, these DNA amplicons were then converted into species- or strain-specific sequence-characterized amplified region (SCAR) markers (Schilling *et al*., 1996; Abbasi *et al*., 1999; Li *et al*., 1999; Lecomte *et al*., 2000; Castrillo *et al*., 2003; Felici *et al*., 2008; Aveskamp *et al*., 2009; Lim *et al*., 2009). In particular, SCAR primers were designed based on known DNA sequences of the fungi under study which were different from the RAPD primers. This difference permits the development of sensitive and diagnostic methods to amplify specific target fungal DNA because SCAR primers anneal specifically to fungal target DNA sequences.

Currently, reliable assays are required that can differentiate *N. rileyi* isolates and are sufficiently sensitive to serve as monitoring systems. In a previous study, a unique 1.4 kb DNA amplicon sequences of ten isolates of *N. rileyi* had high homology

while four isolates of *B. bassiana and M. anisopliae* were not detected with the same signal (Chang, 2000). Therefore, this unique 1.4 kb DNA fragment was suitable for the development of SCAR primers to distinguish *N. rileyi* from other entomopathogenic fungi. In the present study, we utilized this unique RAPD amplicon DNA which was converted into SCAR markers in order to develop a method that was sensitive enough to specifically detect *N. rileyi* in infected insects. In addition, SCAR assays with diluted DNA templates were used to estimate the sensitivity of the SCAR primers designed in this study.

Materials and Methods

Collection and culture of entomopathogenic fungi

The completely mummified insects, covered by a dense white mycelial mat with a pile of pale green conidia, were collected from various localities in Taiwan. The isolates of *N. rileyi* used in this study were obtained from these collected mummified insects, which were fragmented and washed with 1.5% NaClO and sterile distilled water, twice. The sterilized body fragments were cultured on Sabouraud maltose agar fortified with 1% yeast extract (SMAY) in an incubator at 25°C. After sporulation, a concentration of ca. 1×10^8 conidia/mL was suspended in a 0.1% Tween 80 sterile aqueous solution. This was then added to PDB+Y liquid medium (20% potato, 2% sucrose, and 0.2% yeast extract) to make 1 mL of conidial suspension, and was incubated shakily at 25°C for 5 to 7 days. After condensing the cultured medium, the mycelia were collected on filter papers (Whatman No. 1) by filtration and washed with sterile distilled water twice. The mycelia were harvested by sucking off the excess water, then dried in a freezing drier, and then stored at -20°C.

DNA extraction

Dried mycelia or mummified larvae were ground in liquid nitrogen. The resulting powder was mixed with 3 mL of 50 mM Tris (pH 8.0), 100 mM NaCl, 100 mM EDTA (pH 8.0), and 0.5% SDS, and was then incubated for 30 min at 60°C. Following incubation, the mixture was then gently mixed with 3 mL of sodium acetate (pH 5.2) and incubated for 10 min at -70°C. After centrifugation at 7,000 rpm for 10 min at 4°C, the supernatants were collected and mixed in equal volumes of 100% ethanol for 3 min and centrifuged again at 11,000 rpm for 15 min at 4°C. The resulting sediments were treated sequentially with 20 μL of RNase (Sigma, 10 mg/mL) at 37°C for 5 min. The suspension was extracted twice with equal volumes of phenol: chloroform (1:1). In the aqueous phase, 0.1 volume of 3 M sodium acetate and double the volume of pre-cold 100% ethanol was added and incubated at -70°C for 10 min. The solution was then centrifuged at 11,000 rpm at 4°C for 15 min, and its supernatants were retained. The DNA precipitates were washed twice with 70% ethanol, dissolved in 100 μL of sterile distilled water, and stored at -20°C.

Design and sensitivity of SCAR primers

The 1.4 kb nucleotide sequence of the cloned RAPD fragment was used to design a pair of SCAR primers (Chang, 2000). The NS1 primer (5'CCAAGCCACCAGT CAATTTC3') was designed forward from nucleotide positions 820 to 842, and the NS2 primer (5'TATCACCAGCCTCGATCA CC3') was designed backward from the positions 1,140 to 1,120. A DNA fragment was generated with this pair of NS1/NS2 primers by PCR.

The specificity of the NS1/NS2 primer pair was tested against *N. rileyi*, *B. bassiana* or *M. anisopliae* isolates, using PCR assays. The PCR profile was obtained through initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for

Table 1. Isolates of the entomopathogenic fungi investigated in this study

Isolates	Host insects	Collection locality
Nomuraea rileyi		
BC1	*Helicoverpa armigera*	Yunlin, Taiwan
BC2	*Helicoverpa armigera*	Yunlin, Taiwan
DA1	*Spodoptera exigua*	Taichung, Taiwan
DA2	*Spodoptera exigua*	Taichung, Taiwan
DL	*Spodoptera litura*	Taichung, Taiwan
GL	*Helicoverpa armigera*	Pingtung, Taiwan
HF	*Helicoverpa armigera*	Pingtung, Taiwan
MUL	*Helicoverpa armigera*	Tainan, Taiwan
PZ	*Helicoverpa armigera*	Chiayi, Taiwan
SH1	*Spodoptera exigua*	Changhua, Taiwan
SH2	*Spodoptera exigua*	Changhua, Taiwan
SL	*Spodoptera litura*	Yunlin, Taiwan
TC	*Brithus crini*	Taichung, Taiwan
TP	*Trichoplusia ni*	Taipei, Taiwan
WF1	*Artogeia rapae crucivova*	Nantou, Taiwan
WF2	*Trichoplusia ni*	Nantou, Taiwan
33508	*Spodoptera litura*	Yilan, Taiwan
33732	Unknown	Ivory Coast, ATCC
33733	Unknown	Ivory Coast, ATCC
Beauveria bassiana		
B	*Bombyx mori* L.	Taichung, Taiwan
C	*Cerambycidae*	Nantou, Taiwan
Metarhizium anisopliae		
MSH1	unknown	Pingtung, Taiwan
M-26	unknown	Pingtung, Taiwan

1 min, annealing at 60°C for 20 sec; elongation at 72°C for 30 sec, and a final elongation at 72°C for 7 min. The amplified products were electrophoresed in 1% TAE agarose gel and visualized with ethidium bromide staining. Sensitivity of the NS1/NS2 primer pair was determined by PCR assays with 8 samples containing a serial dilution of DNA templates from *N. rileyi*. Specificity of the NS1/NS2 primer pair was determined by PCR assays with 8 samples containing a serial mixture of DNA templates from *N. rileyi* and *S. litura* larvae.

Infection of *Spodoptera litura* larvae with *Nomuraea rileyi*

A conidial suspension of *N. rileyi* containing 1×10^8 conidia/mL with 0.1% Tween 80 was placed in a 9 cm dia. Petri dish. Ten 5th-instar larvae of *S. litura* were placed in a Petri dish with conidial suspension. They were incubated for 1 min so as to be fully contacted with conidia on the body surface. The inoculated larvae were raised in 30-hole plastic plates at 25°C, 12L:12D photoperiod, and were fed an artificial diet after 8 h. The artificial diet consisted of 5.2% kidney bean, 5.2% soybean powder, 10.4% wheat germ, 4.8% yeast powder, 0.5% ascorbic acid, 0.05% α-cysteine, 0.03% sorbic acid, 0.24% methyl-p-hydroxybenzoate, 1.7% agar and 71.7% distilled water.

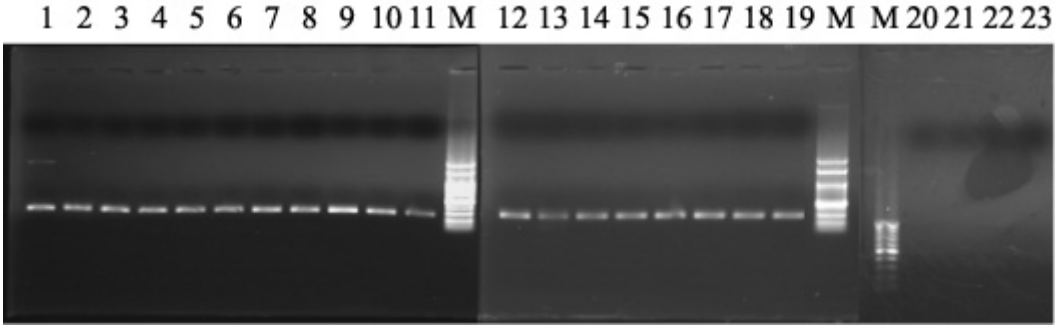

Fig. 1. PCR assays of *N. rileyi, B. bassiana,* and *M. anisopliae* isolates with a NS1/NS2 primer pair, NS1/NS2. (Lane 1: BC; Lane 2: BC2; Lane 3: DA1; Lane 4: DA2; Lane 5: DL; Lane 6: GL; Lane 7: HF; Lane 8: MUL; Lane 9: PZ; Lane 10: SH1; Lane 11: SH2; Lane 12: SL; Lane 13: TC; Lane 14: TP; Lane 15: WF1; Lane 16: WF2; Lane17: 35508; Lane 18: 33732; Lane 19: 33733; Lane20: B; Lane 21: C; Lane 22: M-26; and Lane 23: MSH1(HS); M: 123 bp ladder marker).

Species specificity and detection of infected insects

The mummified larvae of *S. litura* infected with *N. rileyi*, *B. bassiana* or *M. anisopliae* and the live larvae infected with *N. rileyi* were cut into fragments, and were then ground in liquid nitrogen using a pestle. Genomic DNA of mummified and live larvae was extracted using the procedures described above. Species specificity and detection of mummified insects were conducted using PCR with the same NS1/NS2 primer pair.

Results

Development and evaluation of SCAR primers

Since the presence of a unique 1.4 kb DNA fragment from ten isolates of *N. rileyi* was already used to further increase the specificity of DNA markers by generating specific primers able to recognize only *N. rileyi,* one pair of SCAR primers, named NS1 and NS2, were designed for a predicted ORF region,. To test whether the designed NS1/NS2 primers could differentiate *N. rileyi* from other entomopathgenic fungi, the genomic DNAs of nineteen *N. rileyi* isolates and four isolates of *B. bassiana and M. anisopliae* were amplified using a NS1/NS2 primer pair. A 284 bp DNA fragment was generated from nineteen *N. rileyi* isolates while no fragment was produced from four isolates of *B. bassiana and M. anisopliae* (Fig. 1), indicating that the NS1/NS2 primer pair can potentially be applied for the detection of *N. rileyi* genomic DNA.

Sensitivity and species specificity of SCAR primers

To increase the usefulness of the designed NS1/NS2 primer pair, the least amount of genomic DNA template of *N. rileyi* used in PCR was determined. Then a serial dilution of genomic DNA of *N. rileyi* isolate BC1 and a serial mixture of *N. rileyi* isolate BC1 and *S. litura* larvae were carried out in PCR amplification. A 284 bp DNA fragment was amplified in 10 pg of *N. rileyi* genomic DNA (Fig. 2 and 3), indicating that the NS1/NS2 primer pair was highly sensitive in detecting *N. rileyi* genomic DNA.

In addition, the 284 bp DNA fragment was amplified only with the genomic DNA of the mummified larvae infected with *N. rileyi* isolates HF and GL, and no band was formed in the mummified larvae inoculated with *M. anisopliae* and *B.*

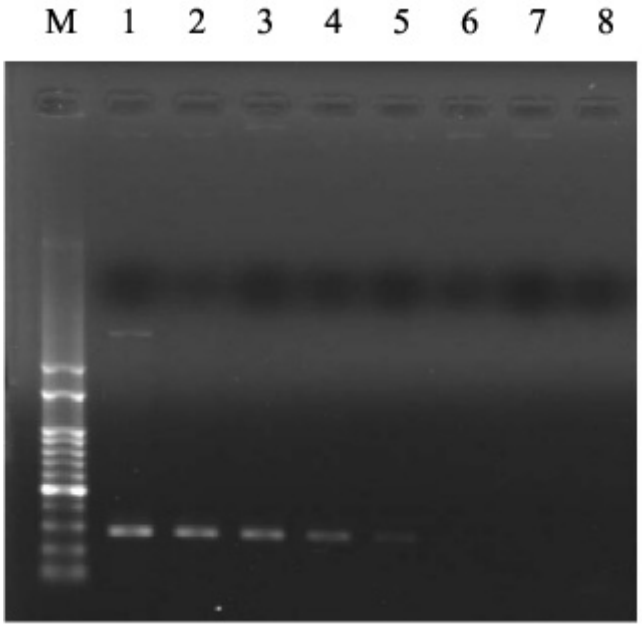

Fig. 2. Sensitivity assay of the NS1/NS2 primer pair in detecting *Nomuraea rileyi* isolate BC1. The template concentrations of genomic DNA were lane 1: 50 ng; lane 2: 10 ng; lane 3: 1 ng; lane 4: 0.1 ng; lane 5: 10 pg; lane 6: 1 pg; lane 7: 0.1 pg; and lane 8: 10 fg.

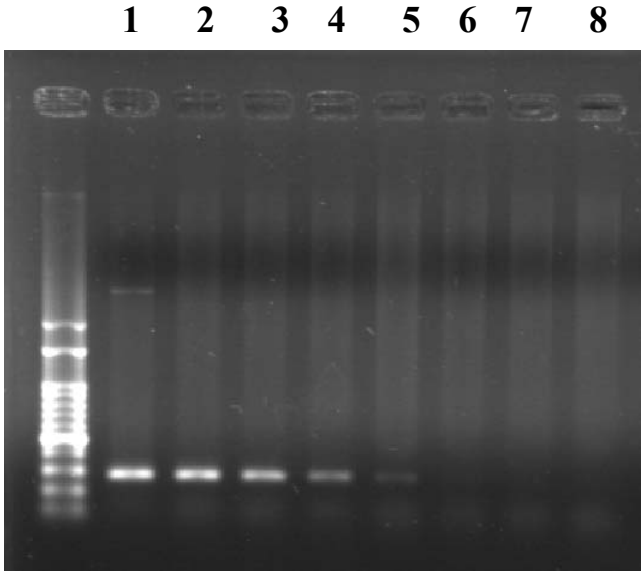

Fig. 3. Detection of *Nomuraea rileyi* isolate BC1 using a NS1/NS2 primer pair. The template concentration of genomic DNA was 100 ng. The DNA ratios of *N. rileyi* BC1 and *Spodoptera litura* were Lane 1: 1/1; Lane 2: 1/10; Lane 3: $1/10^2$; Lane 4: $1/10^3$; Lane 5: $1/10^4$; Lane 6: $1/10^5$; Lane 7: $1/10^6$; and Lane 8: $1/10^7$.

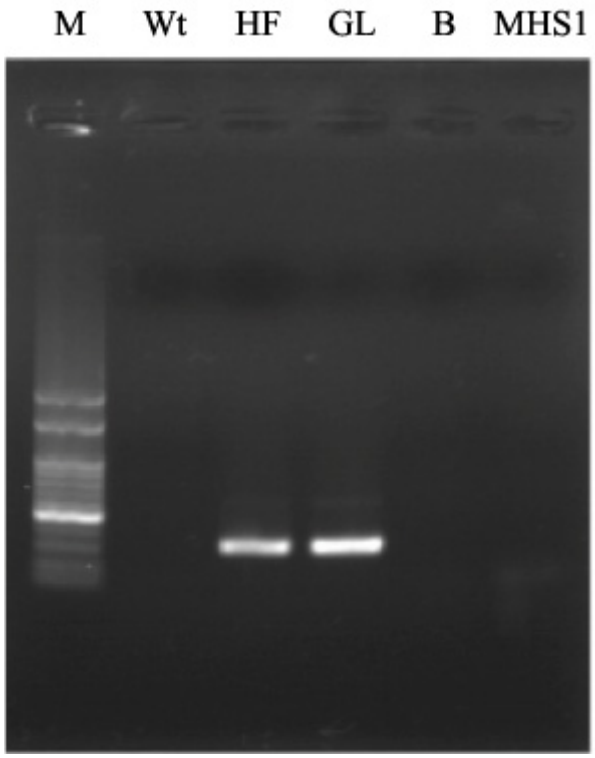

Fig. 4. Detection of mummified *S. litura* larvae infected with different entomopathogenic fungi using a NS1/NS2 primer pair. Wt: healthy larvae; HF: larvae infected with *N. rileyi* isolate HF; GL: larvae infected with *N. rileyi* isolate GL; B: larvae infected with *B. bassiana*; MHS1: larvae infected with *M. anisopliae*.

bassiana (Fig. 4). This proved that the primer pair was highly species-specific to *N. rileyi* in the fungus-infected larvae.

Detection of *Spodoptera litura* live larvae infected with *Nomuraea rileyi* using SCAR primers

A 284 bp DNA fragment was amplified in live larvae infected with HF isolate from 6 to 9 days after incubation. Figure 5 shows that only those larvae infected with *N. rileyi* exhibited reactive bands, whereas larvae infected with either *B. bassiana* or *M. anisopliae* did not form a band. In particular, an intensive signal in the dead larvae at 8 days after inoculation was amplified. The larvae infected with isolate DA1 as well as the fungus by itself were able to successfully amplify a 284 bp DNA fragment from 1 to 6 days after incubation (Fig. 6).

Discussion

A universal DNA fragment from the RAPD analysis was utilized to identify the species or strains of various filamentous fungi and was developed to design species- and strain-specific SCAR markers (Schilling *et al.*, 1996; Abbasi *et al.*, 1999; Li *et al.*, 1999; Lecomate *et al.*, 2000; Castrillo *et al.*, 2003; Felici *et al.*, 2008; Aveskamp *et al.*, 2009; Lim *et al.*, 2009). A SCAR primer pair designed from unknown genetic sequences varies between SCAR and RAPD markers. The SCAR primers, annealing closely to the DNA sequence of fungi in the PCR amplification developed a highly sensitive method for diagnosing fungi and can be applied to detecting a

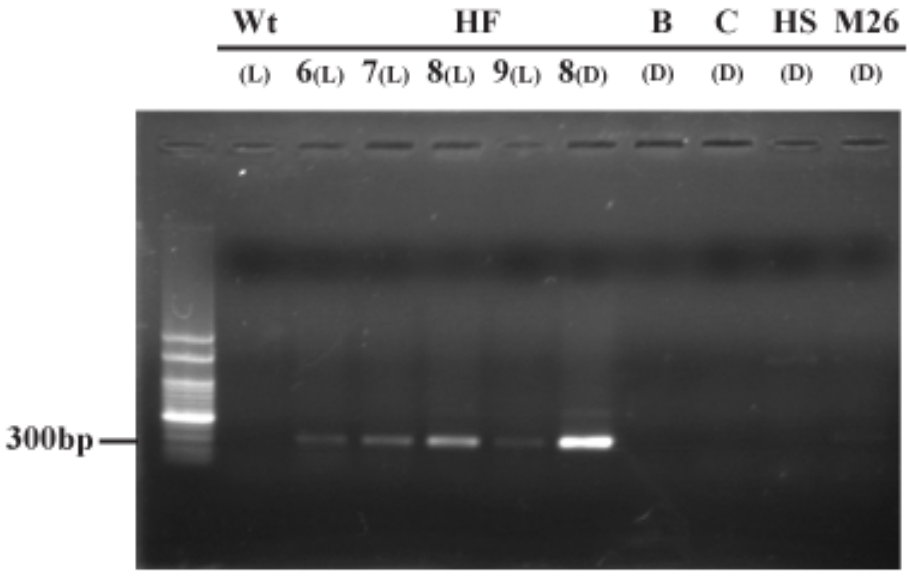

Fig. 5. Detection of live and mummified *Spodoptera litura* larvae infected with different entomopathogenic fungi using a NS1/NS2 primer pair. Numbers indicate the number of days after inoculation; (L) live larvae; (D) dead larvae; Wt: larvae without infection; HF: larvae infected with *N. rileyi* isolate HF; B and C: larvae infected with *B. bassiana*; and HS and M26: larvae infected with *M. anisopliae*.

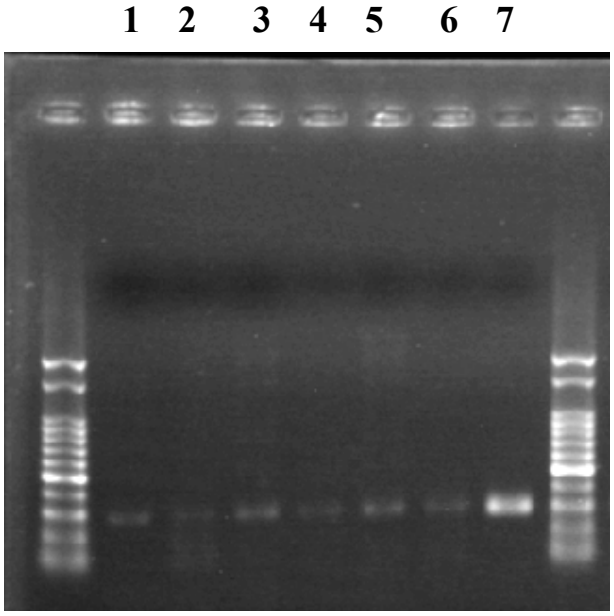

Fig. 6. Detection of *Spodoptera litura* larvae infected with *Nomuraea rileyi* using a NS1/NS2 primer pair. Lane 1-6：One to six days after *S. litura* larvae were inoculated with conidia of *N. rileyi* isolate DA1; and Lane 7, *N. rileyi* isolate DA1 only.

specific fungi in field samples that may be mixed with DNA of other species or strains (Abbasi *et al*., 1999; Castrillo *et al*., 2003; Rubio *et al.*, 2005). In contrast, single species or strain of fungi could be identified using the RAPD technique (Tigano and Aljanabi, 2000; Kao *et al*., 2002). The species-specific NS1/NS2 primer pair in our experiments exhibited high specificity and was reproducible to detect domestic *N. rileyi* isolates when the primer was primer annealed at 60°C.

The main objective of the present study was to find RAPD markers for designing specific SCAR primers that are only detect the target species. Screening with 18 random primers showed one RAPD marker to be reactive with *N. rileyi* but not with *M. anisopliae* and *B. bassiana* (Chang, 2000). However, it is worth noting that SCAR primers seem to be more reproducible than those from the RAPD technique for the identification of entomopathogenic fungi. Although the RAPD technique requires neither cloning nor DNA sequences (Williams *et al.*, 1990), its assay is unstable in terms of reaction conditions, and even some of its banding patterns could be DNA polymerase dependent (Black, 1993; Wolf *et al.*, 1993; Xu and Makalinsky, 1996). These problems can be partly solved by converting RAPD markers into SCAR ones (Paran and Michelmore, 1993). In addition, the usefulness of arbitrary primers for detecting a fungus like *N. rileyi* in infected larvae is ambiguous because the amplification of a mixed sample may lead to the production of DNA fragments which may be different from those generated by the individuals in the mixture (Sweeney and Danneberg, 1994). SCAR markers are also advantageous over RAPD ones because they can be identified based simply on a specific single band instead of on a complex banding pattern.

A 1.4 kb DNA amplicon was found to be specific to *N. rileyi* (Chang, 2000). Since the same size of DNA fragment amplified using random primers from different isolates has little variation in sequences (Castrillio *et al*., 2003), the level of sequence similarity between the unique 1.4 kb DNA fragments amplified by RAPD from ten isolates of *N. rileyi* needs to be determined. The sequence analysis of this amplicon DNA revealed that its fragment did not contain a particular ORF and tandem repeats, and was thus not a complete gene sequence. Due to being highly specific to *N. rileyi*, the amplicon sequence seems to be a part of a specific gene present in *N. rileyi.* However, this needs to be further investigated.

It was found that the multiple characteristics amplified DNA fragment needs at least 200 ng of DNA template when using the RAPD (Davin-Regli *et al*., 1995; Chang, 2000). On the other hand, it requires only 0.1 ng of DNA template to detect fungi, when using a NS1/NS2 primer pair. In addition, the sensitivity and specificity in detecting *N. rileyi* are not disturbed by the presence of larval DNA. Specific SCAR primers were also reported to be highly sensitive in detecting *B. bassiana* (Castrillo *et al.*, 2003). Using 0.1 ng of DNA template for a SCAR primer pair was equally sensitive and specific in detecting fungal samples from soils or leaves in the field. Therefore, the NS1/NS2 primer pair is applicable for detecting the presence of *N. rileyi* in soils, leaves or field-collected larvae, and is also useful for surveying the fungal distribution in the field.

Based on the facts that the content of genomic DNA in *Neurospora crassa* is 4.40 $\times 10^{-2}$ pg/cell (Schulte *et al.*, 2002; Dolezel *et al.*, 2005), and that the NS1/NS2 primer pair designed in this study could amplify a specific fragment in 10 pg genomic DNA of *N. rileyi,* it is estimated that approx. 440 cells are required for the PCR reaction in order to amplify a specific fragment using this primer pair. Furthermore, the sensitivity and species specificity of the NS1/NS2 primer pair is retained when

detecting *N. rileyi* in a mixture of larvae and *N. rileyi* genomic DNA. This makes the NS1/NS2 primer pair useful for the rapid diagnosis of larvae infected with *N. rileyi* and also for monitoring the DNA replication of *N. rileyi* in the infected larvae.

In the past, confirming that larvae were infected with fungi was usually based on the appearance of mycelia in the hemocoel of the larvae, or on the emergence of conidia on the surface of mummified larvae. This made it difficult to diagnose the initial stage of a fungal infection. The development of the NS1/NS2 primer pair makes it possible to successfully and precisely detect a *N. rileyi* infection on the first day of infection and throughout the course of its pathogenesis. Similarly, a strain-specific primer was developed for detecting *B. bassiana* (Castrillo *et al.*, 2003). Our results suggest that the NS1/NS2 primer pair is potentially useful for the rapid detection of *N. rileyi* within the infected larvae as well as in mummified larvae collected from the field.

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快速偵測受感染蟲體內綠殭菌之專一性脫氧核糖核酸標記之開發

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摘　要

藉由隨機增幅多型性技術 (RAPD) 分析而產生的特徵性序列增幅區域 (SCAR)，從中發展具種專一性之脫氧核糖核酸標記 (DNA marker)，以偵測蟲生真菌綠殭菌 (*Nomuraea rileyi*)。在台灣地區親緣關係近似的綠殭菌分離株中，增幅出一條共同的 1.4 kb 長度的 DNA 序列，但在白殭菌 (*Beauveria bassiana*) 與黑殭菌 (*Metarhizium anisopliae*) 並無此一增幅片段。此 DNA 片段序列被應用於設計一組長度在 20 個核苷酸的脫氧核糖核酸引子對，即 NS1/NS2，可增幅出一條長度在 284 個核甘酸的 DNA 片段。利用 NS1/NS2 引子對可成功的在本地的綠殭菌 17 株分離株及 2 株美國的綠殭菌品系中增幅出預期長度的 DNA 片段，但在白殭菌與黑殭菌品系則無法增福出任何片段。在靈敏度與干擾試驗，僅 0.1 ng 的 DNA 板模即可增幅出預期長度的 DNA 片段，而且不受宿主昆蟲斜紋夜蛾 (*Spodoptera litura*) 的 DNA 干擾，能維持相同高靈敏度。同時也可在受綠殭菌感染的斜紋夜蛾之活幼蟲及殭蟲中，增幅出 284 個核苷酸長度的 DNA 片段，但受白殭菌及黑殭菌感染的殭蟲則無法增幅出此一 DNA 片段。另外，本結果亦顯示受綠殭菌感染一天後的幼蟲也能利用此 NS1/NS2 引子偵測出蟲體有綠殭菌存在。因此，本方法具有快速偵測蟲體受綠殭菌感染之潛力，同時亦可應用於調查綠殭菌在田間之分佈。

關鍵詞：蟲生真菌、綠殭菌、機增幅多型性技術、特徵性序列增幅區域。

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