Int. J. Indust. Entomol. Vol. 8, No. 1, 2004, pp. 77~80

International Journal of
Industrial Entomology

Identification of Entomopathogenic Fungus, *Beauveria* spp. F-101 Isolated from *Thecodiplosis japonensis* Using Internal Transcribed Spacer Sequence

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(Received 17 December 2003; Accepted 30 January 2004)

For the development of the alternative control system against the major forest pests, *Beauveria* spp. F-101, isolated from a dead larva of *Thecodiplosis japonensis*, was selected because this isolate showed high pathogenicities against *T. japonensis* and *Acantholyda parki*. *Beauveria* spp. F-101 had irregular clustered conidiophores and conidia borne on a distinctive apical zigzag extension, and it showed typical characteristic of the genus, *Beauveria* in morphology. For molecular based-identification, the ribosomal ITS region of *Beauveria* spp. F-101 was amplified with ITS1 and ITS4 primers, and cloned into pGEM-T Easy vector. The amplified PCR product was 569 bp in size and completely sequenced. The similarities of the cloned ITS sequence were 99% and 97% to those of *B. bassiana* and *B. brongniartii*, respectively. In comparison to other species among the genus *Beauveria*, the ITS region of *Beauveria* spp. F-101 showed a similarity of 95% to *B. amorpha*, 95% to *B. tenella*, 89% to *B. vermiconia* and 69% to *B. alba*, respectively. In addition, in comparison to different genus, it had 95% similarities to *Cordyceps militaris* and 91% to *Paecilomyces tenuipes*. Accordingly, the current result suggests that *Beauveria* spp. F-101 was a variant of *B. bassiana* and it seems to be a new isolate considering sequence variation in ITS region.

Key words: *Beauveria bassiana* F-101, Pathogenicity, *Thecodiplosis japonensis*, *Acantholyda parki*, Internal transcribed spacer, Forest pest control

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Introduction

Thecodiplosis japonensis (the pine needle gall midge) and *Acantholyda parki* (the black-tipped sawfly) are two major pests on pine trees such as *Pinus densiflora* and *P. koraiensis*, etc. and also the notorious forest pests in Korea (Chung and Shin, 1994; Shin *et al.*, 1998). To control these two pests, the chemical insecticides were mainly used through tree trunk injection, soil treatment and air dispersal. Although chemical control has been efficacious, the drawbacks such as impact on the non-target organisms including human, environmental pollution and high cost have necessitated the more selective control methods compatible with the environment.

Entomopathogenic fungi are capable of causing high levels of mortality in insect population and widely distributed in the taxa of Ascomycotina and Zygomycotina. Since sericulturists in Asia firstly reported *Beauveria bassiana* infections in silkworm (Steinhaus, 1956), several strains of *B. bassiana* have been formulated and registered as commercial products against a wide range of insect pests (Feng *et al.*, 1994). *B. bassiana* strains control insects by causing disease: spores of the fungus land upon the cuticle of susceptible insects, germinate and force a germ-tube through the cuticle by a combination of enzymatic action and physical pressure. Growth of the fungus inside the insect causes death by attrition and by disruption of physiological processes of the insect (Samson *et al.*, 1988).

Beauveria comprises two main insect pathogenic species, *B. bassiana* and *B. brongniartii*, which are mainly parasitic on Lepidoptera and Coleoptera. *Beauveria* species are classified by the shape of their conidia and the placement of conidia on the conidiogenous apparatus (Glare and Inwood, 1998). Up to now, molecular based approaches such as restriction fragment length polymor-

phism (RFLP; Maurer *et al.*, 1997), random amplification of polymorphic DNA (RAPD; Urtz and Rice, 1997) and rDNA sequence comparison (Shih *et al.*, 1995) are popularly used. Among them, the sequence analysis of internal transcribed spacer (ITS) and 28S rDNA D3 region has been used for assumption of phylogenetic relationship or identification (Bruns *et al.*, 1991; Driver *et al.*, 2000).
In our previous report, eight *Beauveria* strains, which had over 60% mortality to larvae of *T. japonensis*, were selected (Shin *et al.*, 1998). Among them, *Beauveria* spp. F-101, isolated from the dead larvae, showed 67.2% mortality at 2.4×10^7 spores/ml against *T. japonensis* and also had high pathogenicity against *A. parki* (90% mortality at 3×10^7 spores/ml). In this study, we cloned and analysed ITS region for identification of *Beauveria* spp. F-101 which had high pathogenicities against *T. japonensis* and *A. parki*.

Materials and Methods

Fungal isolate and media

Beauveria spp. F-101 was isolated from a dead larva of *T. japonensis* collected in Korean forest. The isolate was maintained on a Sabouraud dextrose agar (4% dextrose, 1% bacto-peptone, 1.5% agar powder) plus 2% yeast extract (SDA+Y) medium at 25 and a photoperiod of 15:9 (L : D) hrs (Vandenberg, 1996).

Electron microscopy

The scanning electron microscopy of *Beauveria* spp. F-101 was performed according to Cryo-SEM method (Strivastava *et al.*, 1997). The isolate, *B. bassiana* F-101 was cultured on a SDA + Y medium for 14 days and then 5×5 mm size-cultured piece was dipped in liquid nitrogen bath for fixation. The specimen was dried, coated with carbon, stained with gold and observed by scanning electron microscope (JEOL, JSM-5410 LV, Japan).

Genomic DNA isolation

The isolation of genomic DNA was performed according to method of Lee and Taylor (1990), with slight modification. Viable spores were collected from potato dextrose broth agar (Vandenberg, 1996) and used as inoculum. The fungus was cultured 50 ml potato dextrose broth in 250 ml flask for 7 days at 25°C in darkness with vigorous agitation. The cultured beer was harvested and the collected sample was ground well in a mortar in the presence of liquid nitrogen. The ground sample was then resuspended with DNA extraction solution [3% SDS, 50 mM EDTA, 50 mM Tris-HCl (pH 7.2), 1% 2-mercaptoethanol] at a ratio, 400 µl per 100 mg and incubated for 1 hr at 65°C. Thereafter, phenol/chloroform extraction and isopropanol precipitation were performed. The dried DNA pellet was dissolved with TE buffer (pH 8.0) containing RNase A (10 mg/ml) and used as template DNA for PCR amplification.

Oligonucleotides and PCR

For the amplification of internal transcribed spacer (ITS) region of rDNA from *Beauveria* spp. F-101, oligonucleotides, ITS1 and ITS4 were designed (White *et al.*, 1990) and their sequences and binding sites were shown in Fig. 2. For amplification of ITS region, a Polymerase chain reaction (PCR) was performed with Ex-Taq™ DNA polymerase (Takara Co., Japan) using a DNA Thermal Cycler (Perkin Elmer Co., USA), based on a 30-cycle program, with each cycle consisting of denaturation at 94°C for 1 min, annealing at 65°C for 1.5 min, and extension at 72°C for 1 min. Following amplification, the PCR products was purified using Qiaquick PCR purification kit (Qiagen Co., Germany) according to manufacturers protocol and analyzed by 0.8% agarose gel electrophoresis.

Cloning and nucleotide sequence analysis

For the DNA sequence analysis of the amplified ITS region, the PCR product was cloned in pGEM-T Easy vector (Promega Co, USA). The DNA sequence of ITS region was determined on an ABI sequencer Model 377 (ABI system, USA). The obtained sequence was compared with the known ITS regions of several *Beauveria* species and other entomopathogenic fungi using BLAST search.

Results and Discussion

We isolated *Beauveria* spp. F-101 from a dead larva of *T. japonensis* for the development of the alternative control system against the major forest pests. This isolate particularly had high pathogenicities against *A. parki* as well as *T. japonensis* Furthermore, *Beauveria* spp. F-101 strain exhibited rapid germination at relatively low temperatures (4 days at 15°C and 6 days at 10°C) in comparison with the mean germination time of other *Beauveria* spp. (5 days at 15°C and 13 days at 10°C) and it was even capable of germinating at 5°C after 18 days (data not shown). The germination at low temperature may be a powerful merit for *Beauveria* spp. F-101, because this ability can efficiently kill the larvae of *T. japonensis* regardless of temperature in Korea (Li *et al.*, 2000).

For the morphological identification, the conidia of *Beauveria* spp. F-101 were observed by scanning electron microscopy (Fig. 1). In the genus *Beauveria*, the conid-

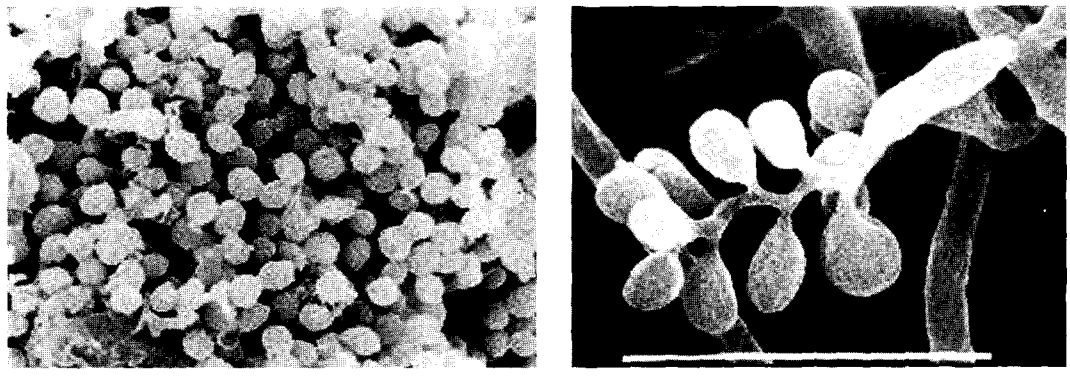

Fig. 1. Scanning electron microscopy of *Beauveria* spp. F-101 isolated from a dead larvae of *T. japonensis*. Size bar indicates 10 μm.

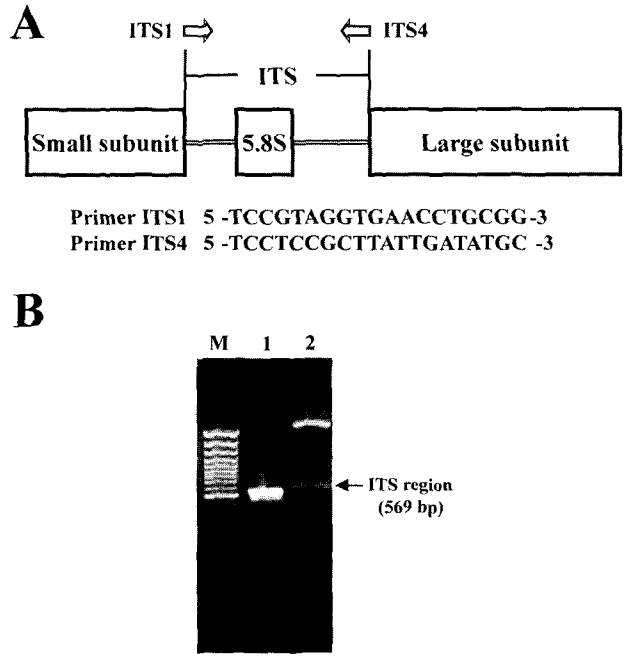

Fig. 2. Primer design for the amplification of internal transcribed spacer (ITS) of rDNA (A) and, PCR product and clone (B) of *Beauveria* spp. F-101 (M, 100 bp ladder; lane 1, PCR product; 2, ITS region cloned in pGEM-T Easy vector).

iophore is characterized by bearing hyaline conidia singly on zigzag rachis or sterigmata (Tanada and Kaya, 1993). *Beauveria* spp. F-101 strain also had irregular clustered conidiophores and conidia borne on a distinctive apical zigzag extension and this shape is a typical feature of *Beauveria* species in morphology.

To further identify *Beauveria* spp. F-101, the ribosomal ITS region flanking the 5.8S subunit was amplified with ITS1 and ITS4 primers and cloned into pGEM-T Easy vector (Fig. 2). The amplified PCR product was 569 bp in size and ITS region was completely sequenced (Fig. 3).

The sequenced ITS region of *Beauveria* spp. F-101 was analysed with ITS regions of other fungi using Blast program (Table 1). The similarities of cloned ITS sequence were 99% and 97% to those of *B. bassiana* (GenBank Acc. No. AB027382) and *B. brongniartii* (GenBank Acc. No AB027381), respectively (Nikoh and Fukatsu, 2000). In comparison to other species among the genus *Beauveria*, *Beauveria* spp. F-101 had 95, 95, 89 and 69% sim-

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1   TCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTG
61  AACCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGGACCAGCGG
121 CCCGCCGGGGACCTCAAACTCTTGTATTCCAGCATCTTCTGAATACGCCGCAAGGCAAAA
181 CAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAG
241 CGAAATGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCA
301 CATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCGAC
361 CTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGCCGGCCCTGAAATGGAGT
421 GGCGGCCCGTCCGCGGCGACCTTTGCGTAGTAATACAGCTCGCACCGGAACCCCGACGCG
481 GCCACGCCGTAAAACACCCAACTTCTGAACGTTGACCTCGAATCAGGTAGGACTACCCGC
541 TGAACTTAAGCATATCAATAAGCGGAGGA
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Fig. 3. Nucleotide sequence of the ITS region in *Beauveria* spp. F-101. Primer binding sites are underlined.

Table 1. The rDNA sequence similarity based on ITS region of *Beauveria* spp. F-101

Species	Nucleotide sequence similarity (%)	GenBank Acc. No.
B. bassiana	99	AB027382
B. brongniartii	97	AB027381
B. amorpha	95	U18960
B. tenella	95	U35287
B. vermiconia	89	U18959
B. alba	69	U18961
Cordyceps militaris	95	AJ243774
Paecilomyces tenuipes	91	AB027380

ilarities with *B. amorpha* (GenBank Acc. No. U18960), *B. tenella* (GenBank Acc. No. U35287), *B. vermiconia* (GenBank Acc. No. U18959) and *B. alba* (GenBank Acc. No. U18961), respectively. In addition, in comparison to different genus, this isolate had 95% and 91% similarities to *Cordyceps militaris* (GenBank Acc. No. AJ243774) and to *Paecilomyces tenuipes* (GenBank Acc. No. AB027380; Nikoh and Fukatsu, 2000).

In conclusion, *Beauveria* spp. F-101 in this study was identified as a variant of *B. bassiana* but this strain seemed to be a new isolate according to the current result. In view of forest pest control system, *B. bassiana* F-101 strain could be extensively used because of high pathogenicities against *T. japonensis* and *A. parki* and germination property at low temperature. Further studies will be required in order to clarify whether there are pathogenicities of *B. bassiana* F-101 against other major forest pests such as the alder leaf beetle (*Agelastica coerulea*) and the black pine bast scale (*Matsucoccus thunbergianae*), etc.

Acknowledgements

This work was supported by the Brain Korea 21 project and a grant funded by Ministry of Science and Technology of Korean Government to Korea Forest Research Institute.

References

Bruns, T. D., T. J. White and J. W. Taylor (1991) Fungal molecular systematics. *Annu. Rev. Ecol. Syst.* **22**, 525-564.

Chung, S. B. and S. C. Shin (1994) Studies on the effects of black-tipped sawfly, *Acantholyda posticalis posticalis* Matsumura on the growth of the Korean white pine, *Pinus koraiensis* S. *J. Korean Forestry Soc.* **83**, 450-459.

Driver, F., R. J. Milner and J. W. H. Trueman (2000) A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rRNA sequence data. *Mycol. Res.* **104**, 134-150.

Feng, M. G., T. J. Poprawski and G. G. Khachatourians (1994) Production of formulation and application of the entomopathogenic fungus *Beauveria bassiana* for the insect control: current status. *Biocontrol Sci. Technol.* **4**, 3-34.

Glare, T. R. and A. J. Inwood (1998) Morphological and genetic characterization of *Beauveria* spp. from New Zealand. *Mycol. Res.* **102**, 250-256.

Lee, S. B. and J. W. Taylor (1990) Isolation of DNA from fungal mycelia and single spores; in *PCR protocols, A guide to methods and applications*. Innis, M. A., D. H. Gelfand, J. J. Sninsky and T. J. White (eds.), pp. 282-287, Academic Press, New York.

Li, Y., H. Gong and H. Park (2000) Biochemistry and physiology of overwintering in the mature larva of the pine needle gall midge, *Thecodiplosis japonensis* (Diptera: Cecidomyiidae) in Korea. *Cryo Lett.* **21**, 149-156.

Maurer, P., Y. Couteaudier, P. A. Girard, P. D. Bridge and G. Riba (1997) Genetic diversity of *Beauveria bassiana* and relatedness to host insect range. *Mycol. Res.* **101**, 159-164.

Nikoh, N. and T. Fukatsu (2000) Interkingdom host jumping underground: phylogenetic analysis of entomoparasitic fungi of the genus *Cordyceps*. *Mol. Biol. Evol.* **17**, 629-638.

Samson, R. A., H. C. Evans and J. P. Latge (1988) Atlas of entomopathogenic fungi. Springer-Verlag. Utrecht.

Shih, H. L., C. P. Lin, R. F. Liou and S. S. Tzean (1995) Complete nucleotide sequence of *Beauveria bassiana* 5.8S rRNA coding gene and flanking internal transcribed spacers. *DNA Seq.* **5**, 381-383.

Shin, S. C., S. J. Park, S. M. Lee, K. S. Choi, S. G. Lee and C. Y. Lee (1998) Pathogenicity of *Beauveria* spp. isolated from Korea against the pine needle gall midge (*Thecodiplosis japonensis* Uchide et Inouye) and their physiological characterization. *KFRI. J. For. Sci.* **59**, 57-63.

Srivastava, A., A. R. Menon and J. R. Bellare (1997) Electron microscopy of modified aluminum alkoxide microstructures on freeze-drying. *J. Colloid Interface Sci.* **1991**, 521-524.

Steinhaus, E. A. (1956) Microbial control the emergence of an idea. *Hilgardia* **26**, 107-160.

Tanada, Y. and H. K. Kaya (1993) Fungal infection; in *Insect Pathology*. Tanada, Y. and H. K. Kaya (eds.), pp. 318-387, Academic Press, San Diego.

Urtz, B. E. and W. C. Rice (1997) RAPD-PCR characterization of *Beauveria bassiana* isolates from the rice water weevil *Lissorhoptrus opyzpphillus*. *Lett. Appl. Microbiol.* **25**, 405-409.

Vandenberg, J. D. (1996) Standardized bioassay and screening of *Beauveria bassiana* and *Paecilomyces fumosoroseus* against the Russian wheat aphid (Homoptera: Aphididae). *J. Econ. Entomol.* **89**, 1418-1423.

White, J. J., J. Bruns, S. B. Lee and J. Taylor (1990) Amplification and direct sequencing of fungus ribosomal RNA genes for phylogenetics; in *PCR protocols, A guide to methods and applications*. Innis, M. A., D. H. Gelfand, J. J. Sninsky and T. J. White (eds.), pp. 315-322, Academic Press, New York.