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A Molecular Marker Specific to *Metarhizium anisopliae* var. *majus*

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Abstract More innovative molecular markers were investigated for rapid and consistent differentiation of *Metarhizium anisopliae* var. *majus* from *M. anisopliae* var. *anisopliae.* A total of 28 isolates were obtained from various countries and hosts: 13 isolates of *M. anisopliae* var. *anisopliae*, 12 isolates of *M. anisopliae* var. *majus*, and 3 isolates of *M. anisopliae* collected in Korea. This study involved restriction enzyme digestions of a PCR product amplified from nuclear internally transcribed spacer (ITS) and a portion of the 28S rDNA regions. Among 11 different restriction enzymes used in this study, *Mbo*I digestion particularly produced a restriction pattern that had characteristics of *M. anisopliae* var. *majus.* This restriction pattern was consistent in all isolates of *M. anisopliae* var. *majus* regardless of their geographic origins and insect hosts. Mapping experiments revealed that *Mbo*I sites of *M. anisopliae* var. *majus* are identical to those of *M. anisopliae* var. *anisopliae* with an exception for the presence of an additional site in the PCR product. Results from this study provide an additional method for identification and differentiation of isolates of these two varieties of *M. anisopliae* for use in the field and laboratory experiments.

Key words: *Metarhizium anisopliae* var. *anisopliae*, PCR-RFLPs, molecular marker

Entomopathogenic fungi have drawn great attention for the control of agricultural pests because they provide an environmentally benign alternative to chemical pesticides [3]. In particular, *Metarhizium anisopliae* (Metschn.) Sorokin is one of the most promising candidates as a mycoinsecticide due to its broad host range, ease of mass production, and the persistence of its conidia in the environment [1, 2].

Within *M. anisopliae*, two varieties are recognized based on the size of the conidia: var. *anisopliae* (3.5–9×3.5 μm) and var. *majus* (Johnst.) Tulloch (9–18×1.8–4.5 μm) [10]. However, a single character such as the size of the conidia may be insufficient in correctly identifying isolates because of the variation of conidial length on different culture media [4]. Therefore, stable and objective criteria for delimitation of these two varieties would be useful for researchers who must be able to identify the correct variety of *M. anisopliae* for laboratory and field experiments and for commercial purposes.

Several studies have examined taxonomic and phylogenetic relationships of *Metarhizium* species, including these two varieties, employing molecular tools such as random amplified polymorphic DNA (RAPD) markers [5], restriction fragment length polymorphisms (RFLPs) of rDNA [7], and various rDNA sequences [2, 8]. However, these studies did not emphasize the development of molecular markers specific to the two varieties. In particular, Pipe *et al.* [7] were able to distinguish European and S. American isolates of *M. anisopliae* var. *anisopliae* from all the *M. anisopliae* var. *majus* isolates by polymerase chain reaction (PCR)-RFLPs of the rDNA repeat unit. Unfortunately, their study failed to differentiate *M. anisopliae* var. *anisopliae* from the Philippine Islands, New Zealand, and U.S.A. from the *M. anisopliae* var. *majus.*

The present study was designed to investigate new and additional objective molecular markers for differentiation of the two varieties of *M. anisopliae.* For this purpose, 28 isolates from various geographic origins and insect hosts were examined for RFLP markers of the region amplified from the 5' end of the nuclear internal transcribed spacer (ITS) to approximately 600 bp downstream from the 5' end of 28S rDNA. In addition, restriction sites were mapped out for the enzyme, *Mbo*I, which generated variety-specific restriction patterns.

MATERIALS AND METHODS

Fungal Isolates and Preparation of DNA

The various host insects of the strains and their geographic location are shown in Table 1. For DNA isolation, 100 ml

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Table 1. Variety, hosts, and geographic locations of *Metarhizium anisopliae* isolates used.

Isolate	Host	Geographic location
M. anisopliae		
C 396[a]	Phasmida	Korea
C 835[a]	Phasmida	Korea
C 642[a]	Phasmida	Korea
M. anisopliae var. *anisopliae*		
ARSEF 538[b]	*Oryctes rhinoceros* (Coleoptera)	Thailand
ARSEF 1448[b]	*Scaptores castanea* (Hemiptera)	Brazil
ARSEF 1968[b]	*Strigoderma arboricola* (Coleoptera)	USA
ARSEF 2080[b]	*Nilaparvata lugens* (Homoptera)	Indonesia
ARSEF 2106[b]	*Nephotettix virescens* (Homoptera)	Indonesia
ARSEF 2140[b]	Larva (Lepidoptera)	Canada
ARSEF 2156[b]	*Oryctes* sp. (Coleoptera)	New Guinea
ARSEF 2231[b]	*Zygogramma bicolorata* (Coleoptera)	India
ARSEF 2469[b]	Larva (Coleoptera)	Mexico
ARSEF 2974[b]	*Aedes crinifer* (Diptera)	Argentina
ARSEF 3148[b]	*Haphochelus marginalis* (Coleoptera)	France
ARSEF 3187[b]	*Tenebrio molitor* (Coleoptera)	Philippines
ARSEF 3329[b]	*Popillia japonica* (Coleoptera)	Portugal
M. anisopliae var. *majus*		
ARSEF 297[b]	*Xyloryctes jamaicensis* (Coleoptera)	Western Samoa
ARSEF 298[b]	*Xyloryctes jamaicensis* (Coleoptera)	Western Samoa
ARSEF 978[b]	*Oryctes rhinoceros* (Coleoptera)	France
ARSEF 988[b]	*Bombyx mori* (Lepidoptera)	Japan
ARSEF 1015[b]	*Bombyx mori* (Lepidoptera)	Japan
ARSEF 1092[b]	Soil	Japan
ARSEF 1858[b]	Coleoptera	Poland
ARSEF 1859[b]	Coleoptera	Poland
ARSEF 1914[b]	*Oryctes* sp. (Coleoptera)	Philippines
ARSEF 1946[b]	*Oryctes rhinoceros* (Coleoptera)	Philippines
ARSEF 2151[b]	*Oryctes rhinoceros* (Coleoptera)	Indonesia
ARSEF 3145[b]	*Oryctes rhinoceros* (Coleoptera)	France

[a]Obtained from Department of Agricultural Biology, Kangweon National University, Korea.
[b]Obtained from Dr. R. A. Humber, ARSEF, Plant Protection Research Unit, U.S. Plant, Soil & Nutrition Laboratory, Ithaca, U.S.A.

of Sabouraud's dextrose broth with 1% yeast extract in a 250 ml flask was inoculated with 1 ml of conidial suspension (10^7/ml), and incubated in an orbital shaker (100 rpm) at 25°C for four days. The mycelium was harvested by filtration, blotted on paper towels, and stored at -20°C. DNA isolation was performed as described previously [13].

PCR Amplification

PCR reactions were performed for amplification of the ribosomal repeat spanning from the 5' end of the nuclear ITS1-5.8S-ITS2 to approximately 600 bp downstream from the 5' end of 28S rDNA (Fig. 1) in an automated thermocycler device (Perkin-Elmer-Cetus, Foster City, U.S.A.). Primer sequences of ITS1 and LR3 used in the study are listed in White *et al.* [12] and Vilgalys and Hester [11], respectively. The PCR mixture consisted of 100 μM of each dNTP, 1.5 mM $MgCl_2$, 2 units of *Taq* DNA polymerase (Promega), 10 μl of 10x buffer (Promega), and 25 ng of template DNA. Amplification was performed in a Perkin

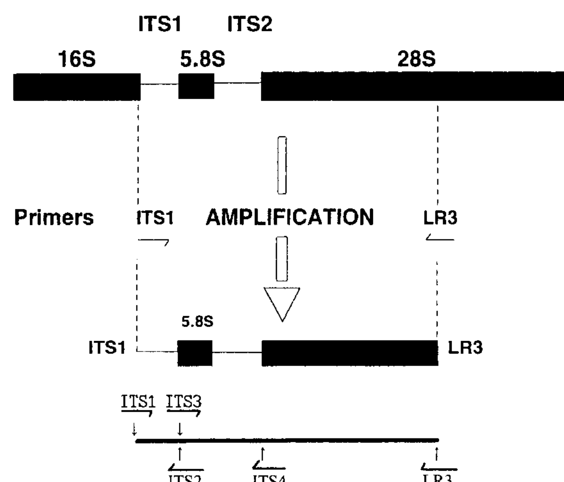

Fig. 1. The region of ribosomal repeat used in PCR amplification extending from the 5' end of nuclear ITS to approximately 600 bp downstream from the 5' end of 28S rDNA. Additional primer sites used for mapping are shown at the bottom.

Elmer 9600 thermocycler as follows: 5 min at 94°C for an initial cycle; 1 min at 94°C, 1 min at 50°C, 2 min at 72°C for 35 cycles; and 4 min at 72°C for a final extension cycle.

Restriction Enzyme Digestions

Amplified PCR products were digested with each of 11 restriction endonucleases (*Hae*III, *Alu*I, *Hpa*II, *Cfo*I, *Hsp*92II, *Mbo*I, *Rsa*I, *Taq*I, *Tru*9I, *Eco*RI, and *Eco*RV) according to the manufacturer's protocols (Promega, Madison, U.S.A.). Digested DNA fragments were resolved by electrophoresis (7 V/cm) for 3 h in 3% agarose gels (2.25% FMC NuSieve GTG agarose and 0.75% Seakem GTG agarose in 2 M Tris-HCl, pH 8.0, 5.7% glacial acetic acid, 0.05 M EDTA, pH 8.0), using the 100 bp DNA ladder (Promega) as a molecular size standard. After EtBr-staining, the fragments were visualized and photographed on a UV transilluminator (310 nm) in order to record the results.

Mapping of Restriction Sites

To map out restriction sites for the enzyme, *Mbo*I, which had generated variety-specific markers, the procedure of Vilgalys and Hester [11] using the primer pairs ITS1/ITS2, ITS3/ITS4, ITS1/ITS4, and ITS3/LR3 was used. Primer sequences of ITS2, ITS3, and ITS4 are listed in White *et al.* [12]. In addition, restriction sites not resolved by these procedures were mapped out by the additivity of pairs of restriction fragments generated by full and partial digestions with *Mbo*I. For partial digestions, approximately one unit of *Mbo*I was added in 100 μl of reaction mixtures containing each of PCR products. The reaction mixtures were then incubated at 37°C, and resolved by loading 20 μl of each sample in an agarose gel each hour starting at 1 h after the incubation until partial-digested fragments were observed. The three-hour-incubation exhibited the most partially-digested fragments.

RESULTS

PCR Products and RFLPs

Amplification of DNA from 28 isolates of *M. anisopliae* with primers ITS1 and LR3 produced a common PCR product of approximately 1,177 bp (Fig. 2A). Length variation was not observed among the isolates from different geographic origins and hosts, or between isolates of *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus*.

With the exception of *Eco*RV, all restriction enzymes digested the PCR products amplified from DNA of the 28 isolates. The total sum of the size of visible restriction fragments generated by each of the 10 enzymes was comparable to the size of the PCR product. Due to the limited resolution of the 3% agarose gels, the size of restriction fragments less than 50 bp was estimated by subtracting the sum of the size of scorable restriction fragments from the size of the PCR product. Three enzymes, *Alu*I, *Cfo*I, and *Mbo*I, generated polymorphic restriction patterns. Only one Korean isolate (C396) showed

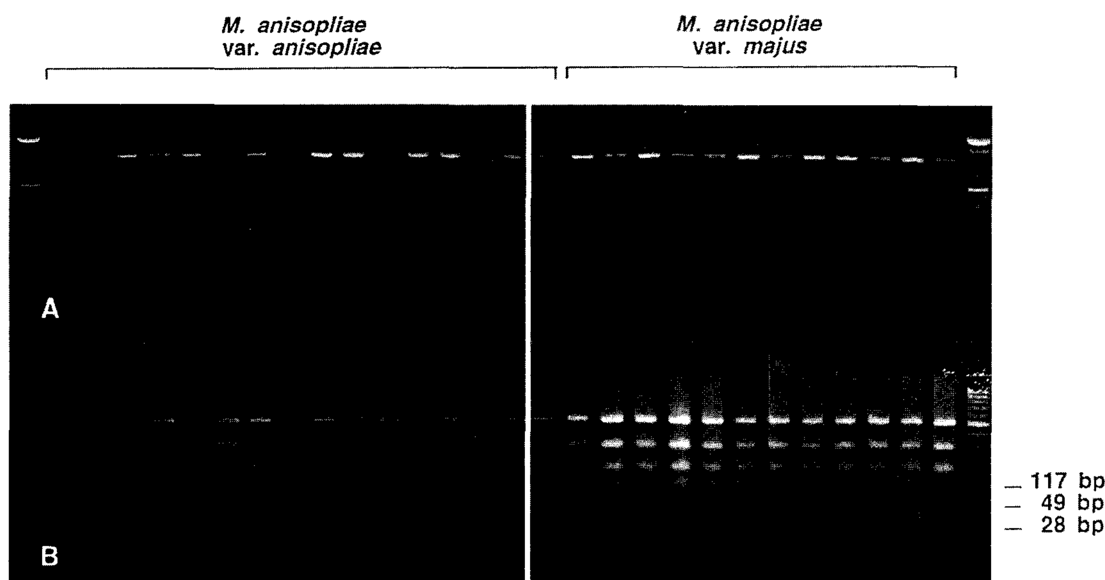

Fig. 2. PCR products and RFLPs of rDNAs from 28 isolates of *M. anisopliae*.
A, Primers ITS1 and LR3 were used to amplify the region corresponding to the 5' end of ITS approximately 600 bp downstream from the 5' end of 28S rDNA. The size of the PCR product is approximately 1177 bp. Size variation was not observed in all isolates. B, PCR products from the 28 isolates were digested with *Mbo*I. The restriction pattern of *M. anisopliae* var. *majus* isolates is distinguished from those of *M. anisopliae* var. *anisopliae* isolates. The bars indicate the 117, 49, and 28 bp fragments specific in isolates of *M. anisopliae* var. *majus*. The 49 and 28 bp fragments in *M. anisopliae* var. *anisopliae* are not clearly resolved due to limited resolution of a 3% agarose gel. Most of the left and right lanes contain the 100 bp DNA ladder.

a different restriction pattern from other isolates in the *Alu*I-digest. Six isolates of *M. anisopliae* var. *anisopliae* (ARSEF2080, 2140, 2231, 2469, 3187, 3329) revealed an identical pattern distinct from other isolates in *Cfo*I-digest (data not shown). The *Mbo*I-digestion clearly differentiated isolates of *M. anisopliae* collected in Korea and *M. anisopliae* var. *anisopliae* from *M. anisopliae* var. *majus*. As shown in Fig. 2B, digestion of isolates of *M. anisopliae* collected in Korea and *M. anisopliae* var. *anisopliae* resulted in five restriction fragments (28, 166, 175, 298, 510 bp), whereas those of *M. anisopliae* var. *majus* revealed six fragments (28, 49, 117, 175, 298, 510 bp). The 175, 298, and 510 bp fragments were shared by both varieties but the 166 bp fragment was not present in *M. anisopliae* var. *majus*. Instead, the 49 and 117 bp fragments were observed. These results indicated that the 49 and 117 bp fragments were produced due to an additional *Mbo*I site present within the 166 bp fragment shown in *M. anisopliae* var. *anisopliae*. Such restriction patterns were consistently observed in all isolates of *M. anisopliae* var. *majus* regardless of their geographic origin and host insect.

The *Mbo*I-restriction pattern of the three Korean isolates of *M. anisopliae* (C396, 835, 642) was identical to that of *M. anisopliae* var. *anisopliae* isolates, indicating their close genetic affinity with *M. anisopliae* var. *anisopliae*. Conidial sizes of the Korean isolates were measured, and estimated to be within the range of the conidial size of *M. anisopliae* var. *anisopliae*. Their conidial length and width were 4.0-4.5 μm and 2.1-2.2 μm, respectively These results indicate that *Mbo*I-restriction patterns of the ITS1-LR3 region are specific for varieties of *M. anisopliae*.

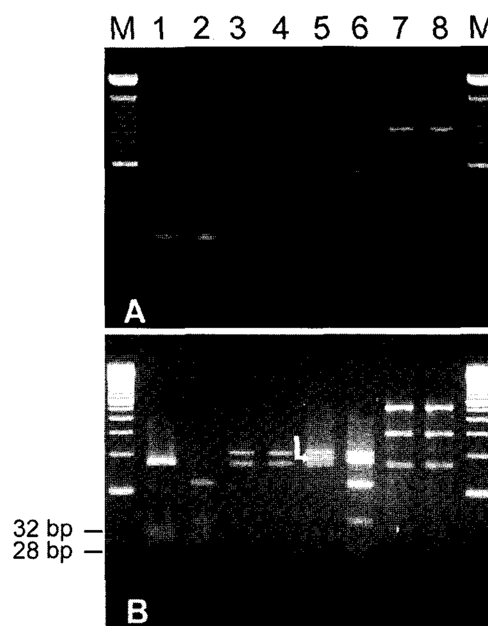

Fig. 3. Sizes and *Mbo*I-digestions of incremental PCR products from *M. anisopliae* var. *anisopliae* (Lanes 1, 3, 5, 7) and *M. anisopliae* var. *majus* (Lanes 2, 4, 6, 8).
A, PCR products amplified from each of four incremental fragments were loaded in a 2% agarose gel (8 V/cm for 1 h 20 min); M: 100 bp ladder; Lanes 1, 2: ITS1-ITS2 (216 bp); Lanes 3, 4: ITS3-ITS4 (358 bp); Lanes 5, 6: ITS1-ITS4 (548 bp); Lanes 7, 8: ITS3-LR3 (972 bp). B, *Mbo*I-restriction fragments from each PCR product were loaded in a 4% agarose gel (16 V/cm for 40 min). The 175 bp fragment is indicated by the white arrow.

Mapping of *Mbo*I-restriction Sites

To determine the positions of the *Mbo*I-restriction fragments shown in Fig. 2B, four incremental PCR fragments were amplified from one isolate of each variety, using the primer pairs ITS1/ITS2, ITS3/ITS4, ITS1/ITS4, ITS3/LR3 (Figs. 1, 3A), and digested with *Mbo*I (Fig. 3B). The resulting restriction fragments were run adjacently on a 4% agarose gel, and the 175 bp fragment of both varieties could be mapped out. As shown in restriction fragments of *M. anisopliae* var. *anisopliae* (Fig. 3B), the 28 and 166 bp fragments were present in restriction fragments of the ITS1-ITS2 and ITS1-ITS4 regions but not in the ITS3-ITS4 and ITS3-LR3 regions. In addition, the 175 bp fragment was observed only in those of the ITS1-ITS4 region. These results indicated that the position of the 175 bp fragment is next to the 28 and 166 bp fragments. However, this result did not elucidate the order of the 28 and 166 bp fragments shown in *M. anisopliae* var. *anisopliae*, the 49 and 117 bp fragments in *M. anisopliae* var. *majus*, and the 298 and 510 bp fragments in both varieties.

In order to locate the 49 and 117 bp fragments specific isolates of *M. anisopliae* var. *majus*, and the 28 bp fragment observed in both varieties, the PCR products amplified from both varieties with primers ITS1/ITS2 were subjected to full and partial digestions with *Mbo*I (Figs. 3B, 4A). As shown in Fig. 3B, full digestion resulted in three restriction fragments (28, 32, and 166 bp) in *M. anisopliae* var. *anisopliae*, and four restriction fragments (28, 32, 49, and 117 bp) in *M. anisopliae* var. *majus*. This indicated that the 32 bp fragment is located to the 3' end of the ITS1-ITS2 region. Among the partially-digested fragments of the ITS1-ITS2 region, the 81 bp fragment was observed in *M. anisopliae* var. *majus* (Fig. 4A). In a situation where the 49 bp fragment of *M. anisopliae* var. *majus* shown in Fig. 2B is located immediately before or after the 28 bp fragment, the 77 bp fragment that accounted for the sum of 28 and 49 bp should be observed among partially-digested fragments. However, this fragment was not revealed. Instead, the 81 bp fragment resulted from the undigested site located between the 49 and 32 bp fragments. This result clearly indicated that the 28 bp fragment is present at the 5' end of the ITS1-ITS2 region, and followed by the 117, 49, and 32 bp fragments in order.

In a similar fashion, the 298 and 510 bp fragments in Fig. 2B could be located by referring to a full- and partially-digested fragments of the ITS3-LR3 region. In a full digestion (Fig. 3B), three fragments (164, 298, and 510 bp)

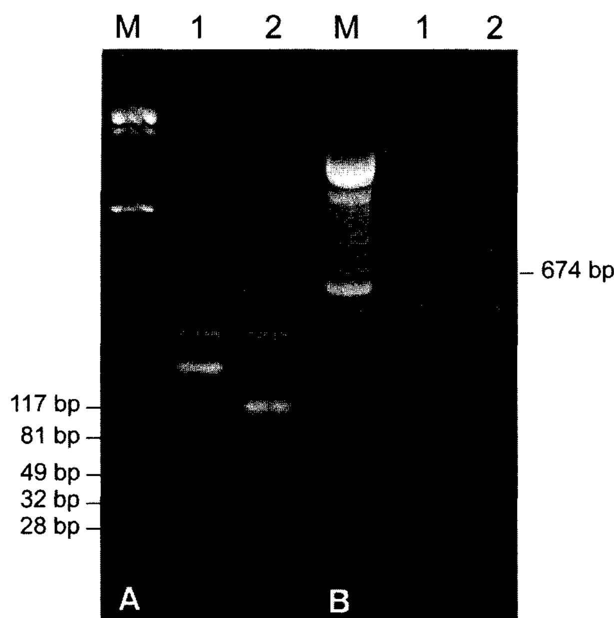

Fig. 4. *Mbo*I-partial digestion of PCR products amplified from *M. anisopliae* var. *anisopliae* (Lane 1) and *M. anisopliae* var. *majus* (Lane 2); M: 100 bp ladder.

A, Partial-digested fragments of the ITS1-ITS2 region were loaded in a 4% agarose gel (16 V/cm for 40 min). Resulting 28, 32, 49, 81, and 117 bp fragments are indicated by the bars. B, Partial-digested fragments of the ITS3-LR3 region were loaded in a 2% agarose gel (8 V/cm for 1 h 20 min). Resulting 674 bp fragment is indicated by the bar.

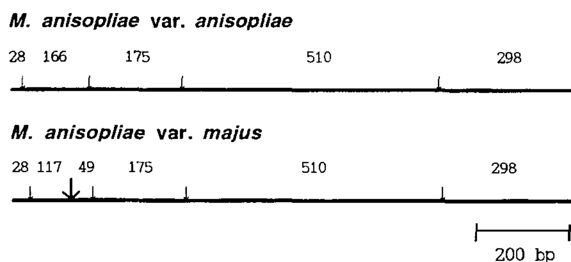

Fig. 5. *Mbo*I-restriction maps of the ITS1/LR3-amplified product from *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus*.

The numbers on the maps indicate approximate sizes of restriction fragments. Arrows indicate *Mbo*I restriction sites. Additional *Mbo*I site on the map of *M. a.* var. *majus* is indicated as the bold arrow.

were revealed in both varieties, showing that the 164 bp fragment is located to the 5' end of the ITS3-LR3 region. An approximately 674 bp fragment was observed among partially-digested fragments of the ITS3-LR3 region (Fig. 4B). If the 510 bp fragment is present to the 3' end of the PCR product, the 462 bp fragment, corresponding to the sum of the 164 and 298 bp fragments, must be shown among the partially-digested fragments. But since this fragment was not revealed, it indicated that the 298 bp fragment is present to the 3' end of the ITS3-LR3 region. The resulting *Mbo*I restriction sites within the ITS1-LR3 region identified in this study are shown in Fig. 5 for the two varieties.

Discussion

Differentiation of *M. anisopliae* var. *majus* and *M. anisopliae* var. *anisopliae* has been largely dependent on the size of the conidia [9, 10]. Recently, several studies were made to characterize the varieties of *M. anisopliae* with the use of molecular approaches such as analyses of RAPD [5], RFLPs [7], and various rDNA sequences [2, 8]. However, these studies focused largely on grouping isolates of different taxa on the basis of their overall genotypic differences. For a rapid and easy identification of strains isolated in the field for commercial purposes, more innovative criteria are needed. The present study employed PCR-RFLPs of a nuclear ITS region and a portion of 28S rDNA to identify molecular markers specific to either of the two varieties of *M. anisopliae*. With 11 restriction enzymes, the authors were able to find molecular markers characteristic to *M. anisopliae* var. *majus* in the *Mbo*I digest.

In the *Mbo*I digest, five restriction fragments (28, 166, 175, 298, 510 bp) were observed in the isolates of *M. anisopliae* collected in Korea and *M. anisopliae* var. *anisopliae,* whereas six fragments (28, 49, 117, 175, 298, 510 bp) were exhibited in those of *M. anisopliae* var. *majus*. Such restriction patterns were consistently observed in all isolates regardless of different geographic origins and host insects. Mapping experiments revealed that the restriction fragments (49 and 117 bp) observed only in *M. anisopliae* var. *majus* are attributed to an additional *Mbo*I site within the 166 bp fragment of *M. anisopliae* var. *anisopliae*. These results are consistent with the previous study [2].

Curran *et al.* [2] examined phylogenetic relationships of *Metarhizium* species including the two varieties of *M. anisopliae* with a sequence analysis of nuclear ITS1-5.8S-ITS2. However, restriction site differences between the two varieties were ignored. When their sequence data was re-examined, the same *Mbo*I-restriction sites were found as shown in our maps. There was little difference in size between the corresponding restriction fragments shown in our restriction maps and their sequence data. Such size differences that were noted resulted from the fact that size estimation of restriction fragments was dependent on the molecular size marker. Nevertheless, both the number and order of restriction fragments shown in Fig. 5 are consistent with those in their study.

In addition, an observation was made in their sequence data which suggests that a guanine within an additional *Mbo*I-recognition site (GATC) of *M. anisopliae* var. *majus* was replaced by thymine at the corresponding site of *M. anisopliae* var. *anisopliae*. Such a single transversional substitution may have led to the loss of the *Mbo*I site originally present within the 161 bp fragment of *M. anisopliae* var. *anisopliae*. This hypothesis can be drawn from the theory [6] that the loss of the restriction site is more likely to occur than an addition. However, more

thorough investigation in conjunction with other approaches is needed to clarify the evolutionary history of these organisms. Based on the evidence of the other study [2], the 117 and 49 bp fragments could be used as molecular markers of *M. anisopliae* var. *majus*. On the other hand, because of the limited resolution of the 49 bp fragment in agarose, the 117 bp fragment would represent a more useful marker for identification of *M. anisopliae* var. *majus*.

In conclusion, this study, which involved PCR-RFLPs of nuclear ITS and a portion of 28S rDNA, provides an additional criterion to differentiate *M. anisopliae* var. *majus* from *M. anisopliae* var. *anisopliae*. The results indicate that the *Mbo*I-restriction patterns observed are associated with types of *M. anisopliae*. Since the 117 bp fragment generated from *Mbo*I digestion is consistent in all isolates of *M. anisopliae* var. *majus*, it should be a useful molecular marker to differentiate two varieties of *M. anisopliae*.

Acknowledgments

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