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IDENTIFICATION OF POTENTIAL ENTOMOPATHOGENIC FUNGI OF *Tetranychus kanzawai* Kishida (Tetranychidae: Acarina) USING ITS-5.8s rDNA REGION AS MOLECULAR MARKER

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ABSTRACT

Fungi has been tested as one of the potential control agents for insect pests, which raises hopes for developing fungi as good biopesticides. The high variation within fungi species made taxonomic identification procedures more complex, thus molecular identification techniques are needed in addition to traditional morphological characteristics currently used as primary methods to classify fungi species. The objective of this research was to identify the species of the most pathogenic fungi to *Tetranychus kanzawai* Kishida using RAPD-PCR. The internal transcribed spacer of 5.8s rDNA (ITS-5.8s rDNA) sequence of these fungal isolates were amplified using two sets of universal primers for ITS and then analyzed. Molecular identification showed that these isolates had a higher of similarity to *Metarhizium anisopliae* than *Metarhizium flavoviride*.

Keywords: entomopathogenic fungi, ITS, molecular characterization, *Tetranychus kanzawai*

INTRODUCTION

The use of fungi as biopesticide covers a wide range of applications, that includes their use as control agents for insect pests - One of fungi classes recently used for that purpose is Hyphomycetes (Deuteromycetes), which is distinguished from other fungal groups by the morphology of its conidia, conidiogenous cells, and by hosts. However, it is almost impossible to distinguish individual isolates using only morphological characters because of limited distinctive characteristics (Samson, 1974). Moreover, neither standard laboratory bioassays nor interactions with their natural hosts offer sufficient information to identify fungi on the subspecies level (Osborne and Landa 1992). Once selected for development as pest control agent, fungi isolates must be identified at the subspecies level (Jenkins and Grzywacz, 2000). This will place the fungi isolate within a taxonomic rank, preferably at species level. The ensuing problem is that fungi species are widely distributed in the environment worldwide and thus have an enormous number of strains. For instance, more than 700 species of fungi from about 90 genera are known as entomopathogens, composed of various subspecies, pathotypes, strains, and isolates (Charnley, 1989). Individual isolates of a particular entomopathogenic and mycoparasitic fungi may display considerable specialization in host range, and it is becoming increasingly apparent that identification at the species level is no longer adequate (Humber, 1997).

Several methods have been used to describe the variation within a species of entomopathogenic and mycoparasitic fungi. These include morphological characteristics of spores and colonies, extracellular protein profiles, pathogenicity, and growth or nutrient requirements (Samson, 1981). Obviously, taxonomic procedures are becoming more complex, and it is generally accepted that some forms of molecular identification techniques are needed in addition to the traditional morphological characteristics formally used to classify fungi species (Bridge and Arora, 1998). Different molecular techniques are used for various applications and on different entomopathogenic and mycoparasitic fungi, including identification of fungi isolates based on DNA polymorphism using RAPD-PCR technique

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(Joshi and St Leger, 1999). The RAPD (Random Amplified Polymorphic DNA) technique was introduced in 1990 (Samšiňáková *et al*., 1983). This technique could reveal polymorphism within completely unknown samples without the need of probe hybridization or DNA sequencing. Only one short oligonucleotide primer (6–12 bases) is used for the reaction, and primers sequence is fully arbitrary. The product is a spectrum of DNA fragments differing from each other in length and nucleotide sequence. The total number of products and the length of each DNA sequence depend on DNA and primer templates used and are specific for a particular combination. The application of RAPD markers is similar to those of other DNA polymorphism detection methods. It can be used also for characterization of a fungi isolate by constructing a specific finger print or for genetic stability testing of an individual isolate. RAPD has already been used to estimate the diversity of a population, for genotype characterization or constructing the molecular phylogeny of closely related taxons (Tigano-Milani *et al*., 1995). Previous study showed that among the seven entomopathogenic fungi isolates tested against *T. kanzawai*, isolates Ma4, Ma5 and Ma6 were the most pathogenic (Sanjaya *et al*., 2013). The objective of this research was to identify the most pathogenic fungi using RAPD technique.

MATERIALS AND METHODS

Molecular Characterization

DNA extraction. Mycelia and conidia from each of the three most pathogenic isolates previously identified (Sanjaya *et al*., 2013) were inoculated on potato dextrose agar (PDA) and single spore colony were grown on potato dextrose broth (PDB), incubated on shaker (150 rpm) at 20˚C for 5-7 days. DNA extraction was done following the manufacturer's protocol using Animal and Fungi DNA Preparation Kit (Jena Bioscience, Germany).

RAPD-PCR Amplification. Internal transcribe spacer (ITS) region for 5.8s ribosomal DNA (rDNA) used for the classification of the unknown fungi were amplified using three different types of ITS primers: ITS 1, ITS 2 and ITS 4 (Table 1). The primer-pairing were set based on reported ITS region for ITS-5.8s rDNA of the common fungi associated with insect. Amplification reactions were performed using 2x Taq Master Mix (Vivantis Technologies, Malaysia) with a total volume of 50 µl, consisting of 20 µl nuclease free sterile water, 25 µl 2xTaq Master mix, 1 µl of 2.5 mM $MgCl_2$, 1 µl 5 µMol each primer, and 2 µl of DNA template (20-25 ng). Controlled reactions were also run containing all components except genomic DNA. Thermal conditions was as follows: one cycle of initial denaturation at 95°C for five minutes, follow by 35 cycles with denaturation at 94°C for one minute and 30 sec, annealing at 55°C for two minutes, and extension at 72°C for three minutes, and a final extension at 72°C for five minutes.

Table 1. Internal Transcribe Spacer (ITS) primers used in the amplification of the target ITS-5.8s rDNA sequence of *Metarhizium anisopliae* Ma4, Ma5 and Ma6 isolates fungal isolates

ITS Primer Code	Sequence
ITS 1	5'-TCCGTAGGTGAACCTGCGG-3'
ITS 2	5'-GCTGCGTTCTTCATCGATGC-3'
ITS 4	5'-TCCTCCGCTTATTGATATGC-3'

PCR products were separated by electrophoresis in 1% agarose gel, containing 0.01% SYBR safe DNA gel stain (Invitrogen molecular probe), run with TBE (Tris-Boric EDTA) buffer by comparison with 1 kbp DNA Ladder conduct at 3 V cm^{-1}. The gel was viewed and examined under ultra-violet (UV) light for band confirmation.

DNA Sequencing. PCR products of 25 µl amplified DNA sequence of each sample genes were placed in a new and sterilized 1.5 ml micro centrifuge tube and then brought to MACROGEN Inc. in Seoul, South Korea (www.macrogen.com; email: info@macrogen.com) through FedEx® Express (www.fedex.com/ph) for sequencing.

Data Analysis

ITS DNA sequences were analyzed by sequence homologous alignment. Similarities tests were carried out using BLAST Biological Software and multi-aligned with CLUSTALW Biological Software. Sequences were compared with published sequences of known fungi on the NCBI GenBank Database and data of mite mortality were submitted for analysis of variance using F test and the means were compared by

Duncan multiple range test (P<0.05) using the SAS software package.

RESULTS AND DISCUSSION

Molecular Characterization

Polymerase Chain Reaction (PCR). The development of PCR and the design of primers for the amplification of the various rDNA regions have considerably facilitated taxonomic studies of fungi. ITS sequences are generally constant, or show little variation within species but vary between species in a particular genus. The results of gel electrophoresis are shown in Figure 1.

A PCR product of approximately 200 bp was obtained from the three entomopathogenic isolates using the ITS1-ITS2 primer pair. This 200 bp PCR product, however, was not observed in Ma4. All of PCR products sizes can be amplified with the primer pairs ITS1, ITS2, and the 5.8s rDNA gene and also with 50 bp of the 3' end of the 18s rDNA and 50 bp of the 5' end of the 28s rDNA. A PCR product of approximately 560 bp was amplified from all the isolates, Ma4, Ma5, Ma6, using the ITS1-ITS4 primer pair. Fungal universal primers of ITS1 and ITS4 have been commonly used to amplify ITS regions. Three *Metarhizium* isolates can be detected with ITS1 and ITS4 primers.

TheITS-5.8s rDNA sequence of the three fungi isolates were also observed using ClustalW multiple sequence alignment (Figure 2). Differences in the nucleotide sequence imply that Ma4, Ma5 and Ma6 were three different species. The BLAST similarity search sequences were obtained from samples of commonly known as entomopathogenic fungi, namely from *Beauveria, Metarhizium,* and *Paecilomyces* genus, from the GenBank. The identity of Ma4, Ma5 and Ma6 isolates as a species under the genus *Metarhizium* was verified using multiple sequence alignment of the ITS and 5.8s region sequence shown in (Table 2). Further, phylogenetic sequence analysis using different *Metarhizium* species showed a close relationship of Ma4, Ma5 and Ma6 with *Metarhizium anisopliae* (Strain CZ592) (Figure 2).

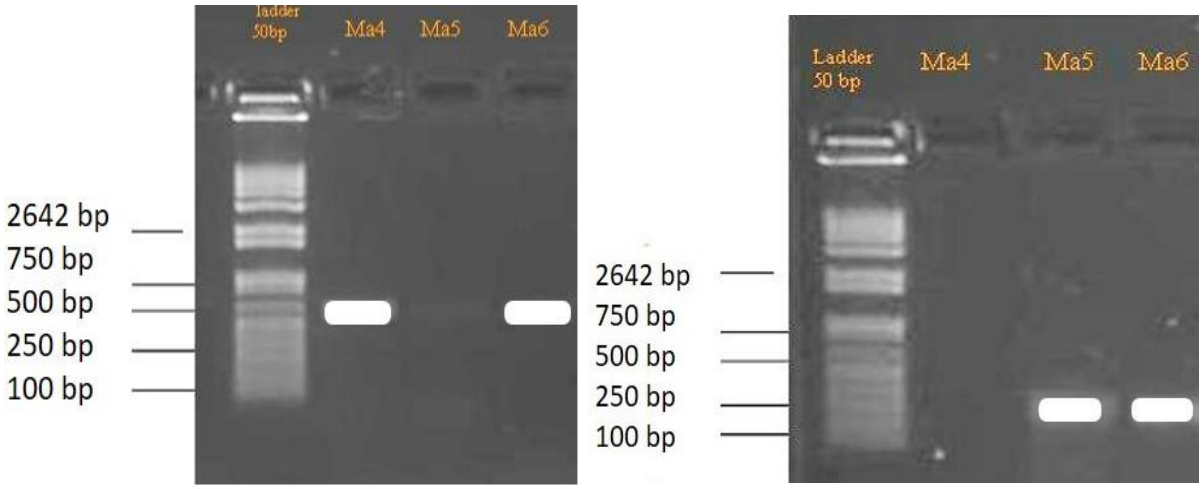

Figure 1. Amplicons from the three fungal isolates, Ma4, Ma5 and Ma6, found to be most pathogenic to *Tetranychus kanzawai* Kishida using ITS1-ITS2 and ITS1-ITS4 primer pairs.

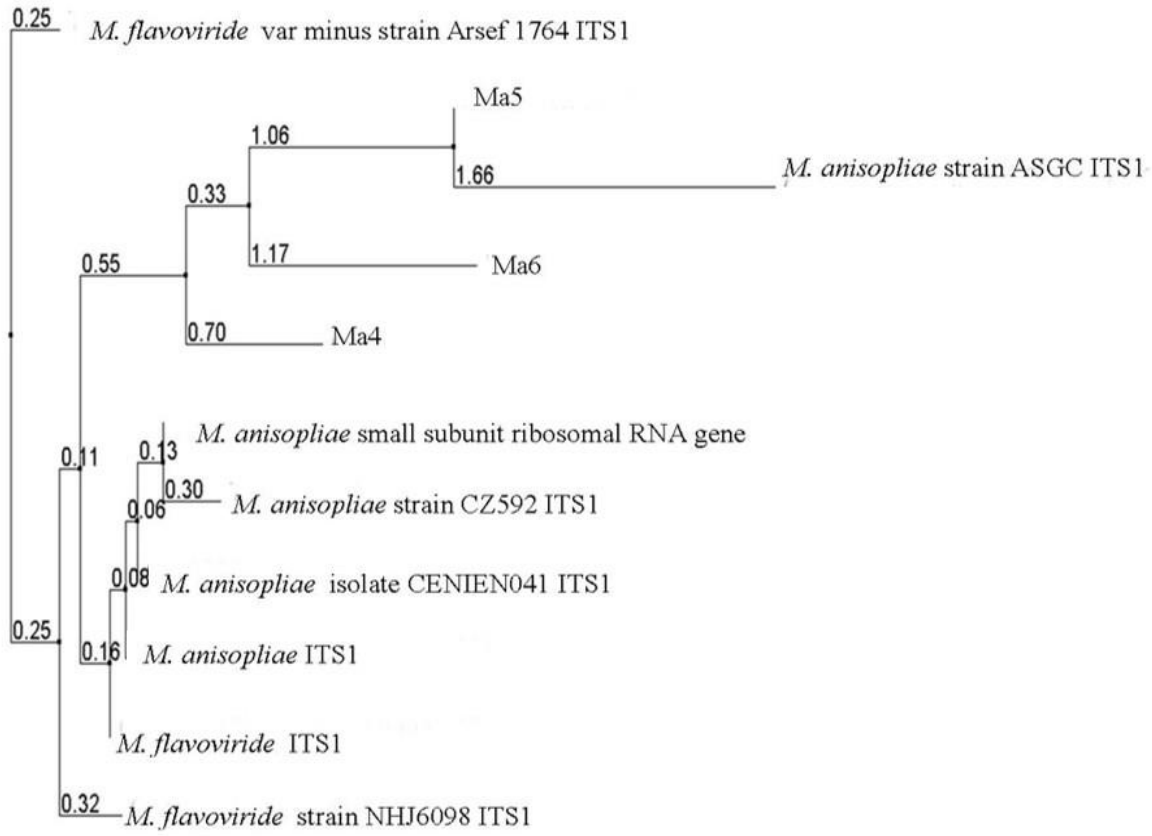

Figure 2. Rooted phylogenetic tree of ITS-5.8s rDNA region of *Metarhizium anisopliae* isolates Ma4, Ma5, and Ma6 compared with *M. flavoviride* and *M. anisopliae*.

Phylogram

The use of ITS 5.8s rDNA sequence has been found to be a powerful tool for *Metarhizium anisopliae*. Observations showed that it can distinguish not only between isolates from different geographical origins, but also between isolates from the same country (Figure 1). The Blast of Ma4, Ma5 and Ma6 with isolates of *Metarhizium* from GenBank was obtained to identify the percent score of similarity (Table 2).

The sequencing showed that Ma4, Ma5 and Ma6 when aligned with some isolates of *M. anisopliae* had a higher percent of similarity than *M. flavoviride* (Table 2). The identity of Ma4, Ma5, and Ma6 isolates as species under the genus *Metarhizium* were verified using multiple sequence alignment of the ITS and 5.8s region sequence (Figure 1). It also showed that three species of *Metarhizium* were *M. anisopliae* when compared to *M. anisopliae* CZ592.

Table 2. ITS-5.8s DNA sequence homology of *Metarhizium anisopliae* Ma4, Ma5 and Ma6 isolates compared with *other M. anisopliae* ITS-5.8s rDNA sequences in the Gen Bank

Fungi Species	Base Pair Length	Description	% Score Similarity		
			Ma4	Ma5	Ma6
Metarhizium anisopliae Small subunit ribosomal RNA gene	1293	18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	65	66	59
Metarhizium anisopliae Strain CZ592	1290	18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	78	88	89
Metarhizium anisopliae Strain CENIEN041	1291	18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	64	68	59
Metarhizium anisopliae Strain ASGC	2080	18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	78	79	75
Metarhizium anisopliae ITS1	487	18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	67	58	57
Metarhizium flavoviride ITS1	397	18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	70	55	57
Metarhizium flavoviride NHJ5733	1129	18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	64	55	55
Metarhizium flavoviride ARSEF17	1129	18S rRNA gene, partial sequence; ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence; and 28S rRNA gene, partial sequence	67	56	55

Molecular Identification and Pathogenicity

Phylogenetic sequence analysis showed a close relationship of Ma4, Ma5 and Ma6 with *Metarhizium anisopliae* (Strain CZ592) (Figure 2). In regards to the performance variability of the different isolates, Sosa-Gómez and Alves (1983) reported a high enzymatic activity in more pathogenic isolates of *M. anisopliae* from several Brazilian regions, and suggested that they are probably associated with the presence of enzymes that influence the penetration process of the fungus (St Leger *et al*., 1988; De La Rosa *et al*., 1997), as well as with toxins such as destruxins and beauvericin, present in *M. anisopliae* and *B. bassiana* respectively, which vary in different isolates (Roberts and St. Leger, 2004). However, unlike insecticides, fungal infection takes 4-6 days after application to kill a mite. During this time the infected mite can cause serious damage to the crops (St. Leger *et al*., 1996).

In the study of Tamai *et al*., (1998) on the mite *T. urticae* using *Beauveria* spp. isolates at a concentration of 5×10^8 conidia ml^{-1}, mortality ranged from 5.5 to 100% in total. Similarly, Oliveira *et al*. (2012), working with *B. bassiana* isolates at 10^8 conidia mL^{-1} and the red mite *Oligonychus yothersi* (McGregor), recorded 77 to 98% mortality. On the other hand isolates of *M. anisopliae*, causes 12.0 to 45.0%, and LT_{50} of 8.6 to 18.4 days.

CONCLUSION

The internal transcribed spacer of 5.8s rDNA (ITS-5.8s rDNA) characteristics confirmed that Ma4, Ma5 and Ma6 were *Metarhizium anisopliae*.

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