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Detection of local isolates of the entomopathogenic fungus *Metarhizium anisopliae* using specific primers

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Abstract

The entomopathogenic fungus *Metarhizium anisopliae* is a promising biological control agent of several insect pests in agriculture. Molecular approaches (PCR and DNA sequence analysis) were used as tools for the identification of different local isolates (Ma88, Ma19 and Ma29) and imported isolate (Ma-h). Primer sequences used were 5'-TCCTCCGCTTATTGATATGC-3' (ITS4) and 5'-GGAAGTAAAAGTCGTAACAAGG-3' (ITS5). The DNA sequences of the amplified regions showed that the high homology (90%) was between the isolates Ma88 and Ma29, while it was 88% between Ma88 and Ma19 isolates and 89% between the Ma19 and Ma29 isolates. there were more heterogeneous between the three local isolates (Ma88, Ma19 and Ma29) and the imported isolate (Ma-h). The homogeneity among local isolates and their heterogeneity with imported isolate is due to many reasons, including geographical isolation and differences in environmental conditions such as high or low temperature and humidity.

Keywords: Local isolates, entomopathogenic fungus *Metarhizium anisopliae*, specific primers

Introduction

Entomopathogenic fungi are biological agents that can be used to control pest insects (Golshan *et al.*, 2013) [14]. The fungal species of *Beauveria bassiana* and *Metarhizium anisopliae* have been reported to very efficiently affect some insect pests, especially Lepidoptera, Hemiptera, Homoptera and Coleoptera (Herdatiarni *et al.*, 2014) [15]. The use of entomopathogenic fungi as biological agents has shown some advantages such as increased disease control efficiency; reduce the insecticides applied, minimize environmental contamination hazards, and decrease pest resistance (Ambethgar, 2009) [1].

The genus *Metarhizium* of ascomycete fungi contains entomopathogenic species. Some species of this genus cause Green muscardine disease in insects and arthropods, as first described by microbiologist Elie Metchnikoff in 1880. The host-range of the genus covers insect pests important in commercial agriculture including locusts, grasshoppers, termites, crickets, and hemipterans (Zimmermann, 2007) [19]. However, some species might be more host-specific, one example is *Metarhizium acridum*, former *M. anisopliae* var. *acridum*, a species which infects only locusts and grasshoppers. This species has been used as a biological control agent commercialized under the trade name Green Muscle or Green Guard (Becker underwood 2014; Esser *et al.*, 2002) [2, 11]. Other virulent species such as *M. anisopliae* are also frequently studied with hopes of developing active agents against ticks, mites and malaria carrying mosquitoes (Ren *et al.*, 2014; Mugisho *et al.*, 2014; Thomas & Read, 2007).

Polymerase Chain Reaction (PCR) and DNA sequencing are extensively used as standard detection, diagnostic and phylogenetic evolutionary tools for many fungal species (Bindslev *et al.*, 2002) [5]. rDNA sequences are used to study the taxonomic relationships and genetic variations in fungi that encode the construction of ribosome RNA (Hibbert, 1992) [16]. rRNA sequences are available at both the nucleus and the mitochondria, which include a gene under large units 28S rRNA and 5.8S rRNA) and the gene under small subunit (18S rRNA) (White *et al.*, 1990). The rRNA genes occur in tandem frequencies and hundreds of copies along the genome (Bruns *et al.*, 1992) [6]. The regions between the genes of the large and small units are called the Internal Transcribed Spacers (ITS), which are characterized as relatively large heterogeneous compared to the sequence of large and small cell genes and

has relatively short sequences with base pairs of 400-800 bp. Therefore, ITS regions are used in molecular diagnostic studies and heterogeneity of fungi (Buscot *et al.*, 1996) [7]. Destéfano *et al.* (2004) [10] designed specific primers to diagnose *M. anisopliae*, *M. album* and *M. flavoviride* within infected larvae of *Diatraea saccharalis*, after completion of PCR and electrophoresis steps, a single band was obtained at 540 bp, they performed a DNA sequencing of the regions ITS1, 5.8S, and ITS2, the study found that *M. anisopliae* has an evolutionary line that differs from *M. album* and *M. flavoviride* depending on the specialized primers, the three fungi were diagnosed simply and safely, in spite of isolated them from *Diatraea saccharalis* infected larvae.

In order to diagnose and differentiate the differences between the fungal isolates of the same species and some of the local isolates of *M. anisopliae*, they must be studied genetically by the Polymerase Chain Reaction (PCR) technique (Sun *et al.*, 2016) [18] and DNA sequencing (Destéfano *et al.*, 2004) [10]. Therefore, this study aimed at estimating the molecular heterogeneity of *M. anisopliae* isolates using PCR and DNA sequence.

Fungal culture

Four *M. anisopliae* isolates (Ma88, Ma29, Ma19 and Ma-h) were taken from the IPM Center of Agricultural Research Department \ Ministry of Science and Technology \ Iraq, three local isolates (Ma88, Ma29 and Ma19) from different regions and the fourth isolate (Ma-h) imported from India. *M. anisopliae* were cultured on potato dextrose agar medium (PDA) autoclaved at 121°C (15 Psi) for 15-20 minutes and poured into sterilized Petri plates. The Petri plates containing PDA medium were incubated at 27 ± 1°C, 80 ± 5% relative humidity and photoperiod of 12 hours.

A fungal genomic DNA extraction

Genomic DNA of *M. anisopliae* isolates was extracted from 5-7 days fungal cultures as described by Lee and Taylor (1990) [17] with some modification as follows: the fungal mass from the fungal cultures plate was scraped and placed in the Eppendorf tube 500 µl of CTAB extraction solution and 2 µl of Mercaptoethanol was added and mixed with a vortex. After that, the tubes were placed in the water bath at 65 °C for 90 minutes and then 300µl of the chloroform solution was added with manual shaking. The samples were centrifuged at 5000 cycles/min for 5 minutes, then 300 µl of the upper aqueous layer was taken in a new Eppendorf tube, add to it 50 µl of sodium acetate and 450 µl of isopropanol alcohol and mixed well with stirring gently to precipitate the DNA that kept at -20 °C for 30 minutes. The whole content was centrifuged at 12,000 rpm for 10 min to pellet down the DNA. The DNA pellet was washed with 70% ethanol and centrifuged at 12,000 rpm for 5 min. After removing the alcohol, 50 µl of de-ionized water were added and then centrifuged at 13,500 rpm for 20 seconds. DNA concentration and purity were measured by Nanodrop.

PCR amplification and nucleotide sequencing:

Sun *et al.* (2016) [18] designed the primers pairs to a diagnosis of *M. anisopliae*, Primer sequences used were 5'-TCCTCCGCTTATTGATATGC-3' (ITS4) and 5'-GGAAGTAAAAGTCGTAACAAGG-3' (ITS5), it was obtained by Bioneer, The polymerase chain reaction was performed using the AccuPower® PCR PreMix kit reaction mixture and the PCR thermal protocol consisted of an initial

denaturation step at 95 °C for 5 min, 35 amplification cycles of denaturation step 95 °C for 45 sec, primer annealing step at 53 °C for 45 sec, primer extension step at 72 °C for 45 sec, and a final extension step of 72 °C for 10 min. After the electrophoresis we cut the band that appeared on agarose gel for each isolate and sent it with their primers to the Bioneer to sequence the base pairs of the required area (ITS) in the Eppendorf tube, through a program Bioedit, the sequences were compared with two isolates of *M. anisopliae* (FJ545326.1 and JF792884.1) from the gene bank in the National Center for Biotechnology Information (NCBI).

Results and discussion

The results showed that DNA purity was between 1.68-1.88. The DNA concentrations of all tested isolates ranged between 110-420 ng / µl. The DNA analysis of *M. anisopliae* isolates (Figure 1) showed that the molecular weight of the bands produced by the electrophoresis of the amplification of the ITS region is approximately 620 base pairs. This is consistent with a study conducted by Sun *et al.* (2016) [18], in which they investigated some of the isolates of *M. anisopliae* and their virulent for *Rhynchophorus ferrugineus*, where the molecular weight had a length of ITS- rDNA was 685 base pairs, As well as with Curran *et al.* (1994) in the study of the analysis of rDNA sequence of the *Metarhizium* sp. The molecular weight of single packs and all isolates was 601 base pairs, within the normal range of ITS-rDNA (bp 800-400) (Buscot *et al.*, 1996) [7].

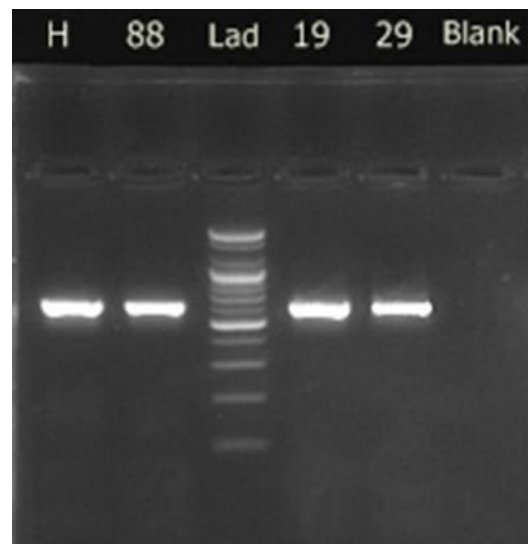


Fig 1: The product of amplification of the ITS region *M. anisopliae* isolates passed on 1% agarose gel for 90 min after dyeing with ethidium.

The alignments of the nucleotide sequences of the ITS region of the strains investigated by us with GenBank ITS sequences for the same region and species of fungi are shown in Figure 2. The nucleotide sequences of four isolates of *M. anisopliae* were done to the ITS region of nucleus and mitochondria DNA, the study revealed changes in the level of the nucleotides of the studied area (Point mutations). In order to compare the results of the sequencing of studied fungal isolates and they are similar with the same species isolates, sequences were taken from Gene Bank for published studies on the NCBI website through the link <http://www.ncbi.nlm.gov>. A comparison was also made among the local isolates sequences and with the imported

isolate by using a ready computer program called Bioedit. Sometimes the results of the nucleotide sequences at both ends of the studied genome are not sufficiently clear, the unclear part is left in order to obtain more accurate results, so it was taking 532 base pairs out of 620 base pairs.

The sequences of local and imported isolates were compared with each other, and their compatibility with global isolates at the Gene Bank. As shown in (Figure 2) there was found that the isolates, Ma88 and Ma19 had 88% homology. The variation was represented by point mutations of the type of deletion and substitution at different sites of the bases. The most frequent substitution in different ranges was 1-37, 148-78, 376-495, the deletion mutation in Ma88 isolate was at sites 109, 119, 449, 464, 491, 492, 516, 517 and 521. The deletion mutation of Ma19 was at locations 339, 340, 341, 369, 380, 381, 382, 449, 464, 465, 516, 517, 521. Comparing Ma88 and Ma29 isolates, showed the homology ratio was 90%. The substitution was found in the most frequent ranges at 5-61, 78-123, 376-393, and 440-512. The deletion mutation in Ma29 isolate was at locations 60, 363, 380, 381, 382, 449, 464, 470, 516, 517 and 521. The compatibility ratio between the Ma19 and Ma29 isolates was 89% and the most frequent of the substitution mutation was at ranges 1-60, 80-133 and 400-512. Sequencing of three local isolates (Ma88, Ma19, and Ma29) the imported isolate (Ma-h) showed more heterogeneous. The homogeneity between Ma88 and Ma-h was 80% by increasing of substitution sites and decreasing the number of deletion mutations relative to the number of them in local isolates, their number in an imported isolate (Ma-h) were four mutations (414, 449, 492, 501). The homogeneity of an imported isolate with the Ma19 was 81% while it was 80% with Ma29. Sequences of studied local isolates were high homology (82-83%) with the sequencing of the same fungus isolates recorded in the World Gene Bank (FJ545326.1 and JF792884). greater homology ratio appeared between the sequence of FJ545326.1 and JF792884.1 with the imported isolate (Ma-h), the ratio was 89%.

The homogeneity among local isolates and their heterogeneity with imported isolate is due to many reasons, including geographical isolation and differences in environmental conditions such as high or low temperature and humidity. This is consistent with a study conducted by Fernandes *et al.* (2009) ^[12] In Characterization of *Metarhizium* sp. That there is a strong correlation between the tolerance capacity of high and low temperatures with the classification of species and strains of fungi.

In another study, Bidochka *et al.* (2005) ^[4] in his study in Canada revealed that all isolates of *M. anisopliae* were similar when using molecular tests regardless of geographic origin. On the other hand, there was a significant genetic variation between isolates, including the United States of America isolates but they were all within the *M. anisopliae* group. The difference between the isolates of the same species is due to the genetic variation between the isolates. This difference was recorded between the isolates of *B. bassiana* and *M. anisopliae* in many studies (De la Rosa *et al.*, 2002; Garcia *et al.*, 1984) ^[9, 13].

Bidochka *et al.* (2001) ^[3] reported that fungal genotypes are associated with the environmental region rather than with the host insects, Heat tolerant isolates can be more applicable to pest control in open agricultural environments, while non-tolerant isolates can be more applicable to pests in cold forest environments.

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