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Molecular identification of entomopathogenic *Fusarium* species associated with *Tribolium* species in stored grains

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ABSTRACT

Fusarium species are common pathogens of plants, animals and insects worldwide, including Iran. The occurrence of entomopathogenic *Fusarium* species isolated from *Tribolium* species as one of the most important insect pests of stored grains were sampled from various provinces in western Iran. In total, 15 *Tribolium* species belonging to *T. castaneum* (Herbst) and *T. confusum* (Du Val) (Col: Tenebrionidae) were detected and 8 isolates from *Fusarium* spp. were collected from them. Based on morphological features, the *Fusarium* isolates were classified into *F. keratoplasticum* and *F. proliferatum*. The phylogenetic trees based on *tef1* dataset clearly separated all morphological taxa. DNA sequences of ITS regions and β-tubulin gene were also confirmed morphological taxa. All of the *Fusarium* isolates were evaluated for their pathogenicity on *T. confusum*. Maximum mortality rate was observed for *F. keratoplasticum* (isolate FSSCker2) and this isolate may be considered as a good candidate for biological control in the ecosystem of stored grains. This is the first report on molecular identification of *Fusarium* species isolated from insects in Iran and *F. keratoplasticum* and *F. proliferatum* were isolated for the first time from *Tribolium* species as two entomopathogenic fungi.

1. Introduction

Stored grain commodities are usually infested by various insects. It is generally considered that more than 15% grain losses in different stored grain commodities occur as a result of insect pest infestation, which reduces the quality of grains and as a consequence their commercial value (e.g., Togola et al., 2013). Flour beetles of the genus *Tribolium* are major and cosmopolitan pests of stored products and owing to their high reproductive rate, sometimes annual damage from this pest is up to 80 percent (Modares Najafabadi, 2002). *Tribolium* species are also considered as the vectors of aflatoxigenic fungi (Payne, 1998). Fumigation plays a very important role in insect pest elimination of stored products, however the choice of desired pesticides is very limited because of the strict requirements imposed for the safe use of synthetic insecticides on or near foods (Suchita et al., 1989). Therefore, successful biological control of stored grain pests with entomopathogenic fungi could be useful for integrated pest management programs (IPM; Golshan et al., 2014; NouriAiin et al., 2014).

Successful control of insect pests with entomopathogenic fungi such as *Metarhizium anisopliae* (Michalaki et al., 2006), *Beauveria bassiana* (Lord, 2007), *Purpureocillium lilaci* (Barra et al., 2013), *Verticillium lecanii* (Bosly and El-Banna, 2015), and *Fusarium* spp. (Mendel et al., 2012; Kasson et al., 2013) have been previously reported. *Fusarium* species have been known as one of the major groups of causal agent of various infections of human, domesticated animals and insects (Teetor-Barsch and Roberts, 1983; Claydon and Grove, 1984; Nucci and Anaissie, 2007). They are being developed worldwide to control some weeds of agricultural importance and some are already available commercially to limit various species of fungi (Benhamou et al., 2002; Boari and Vurro, 2004). Although, many *Fusarium* species are able to perform as weak entomopathogenic fungi and some may invade dead insects' body as saprophytes (Booth, 1971), although it has been discerned that some species kill the host insects by inserting hyphae into their body and injecting toxins (Gupta et al., 1991). In this regard, relationship between members of *F. solani* species complex (FSSC) and ambrosia beetles, *Euwallacea* is one of the well-studied entomopathogenicity (Mendel et al., 2012; Kasson et al., 2013). Moreover, pathogenicity relationships were reported between *F. verticillioides* and *T. confusum*, and *F. circinatum* and *T. piniperda*, as well (Gupta et al., 1991). There are reports of entomopathogenicity of *F. avenaceum* and *F. verticillioides* against rice weevil (*Sitophilus oryzae*) and grasshopper (*Tropidacris collaris*), respectively (Batta, 2012; Pelizza et al., 2011). Therefore, *Fusarium* such as *Metarhizium*, *Beauveria*, *Purpureocillium*, and *Verticillium*

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genera have great potential to be applied as biological control agents against insects and could be exploited for the development of novel microbial products which would protect stored grains against pest (Hajek et al., 1997). The taxonomic status of these fungi is being studied, but no attempt has been made to identify those isolated from insects in Iran. Therefore, the objective of this study was to identify pathogenic fungi isolated from stored grain insect pests from various provinces in western Iran based on the morphological and molecular characteristics.

2. Materials and methods

2.1. Isolation and identification of entomopathogenic fungi

Died *Tribolium* species were collected from different stored grain markets and warehouses in western Iran. All *Tribolium* species were identified based on morphological characteristics as *T. confusum* (9) and *T. castaneum* (6). All samples were immediately transferred to the mycology laboratory and surface sterilized by ethanol (70%) and air-dried at room temperature for 1 min. All fungal species were isolated from insects using peptone pentachloronitrobenzene agar plates supplemented with neomycin and streptomycin. Plates were incubated at 25 ± 2 °C for 48 h and emerging colonies were transferred to potato dextrose agar (PDA) plates. All isolates were purified by the single-spore isolation technique. After 24 h of incubation, single germinated conidia were transferred onto PDA and carnation leaf-piece agar (CLA) plates to study their morphological characteristics.

2.2. DNA extraction

DNA was extracted from isolates using the method previously described (Chehri, 2014). Briefly, all *Fusarium* isolates were grown on PDA with sterile dialysis membranes (Lui et al., 2000). All plates were placed in a incubator with controlled conditions (25 ± 2 °C, with 12:12 h light:dark cycle). Mycelia were harvested and DNA was extracted using a DNeasy® Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol.

2.3. Amplification and sequencing of tef1, β-tubulin, and ITS

Amplification of the *tef1* and β-tubulin genes and ITS regions was conducted utilizing the primers *ef1* (5′-ATGGGTAAGGAGGACAAGAC-3′) and *ef2* (5′-GGAAGTACCAGTGATCATGTT-3′) (O'Donnell et al., 1998), T1 (5′-AACATGCGTGAGATTGTAAGT-3′) and T2 (5′-TAGTGACCCTTGGCCCAGTTG-3′) (O'Donnell and Cigelnik, 1997) (O'Donnell and Cigelnik, 1997), and ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990), respectively. PCR products were purified using Qiagen columns according to the manufacturer's protocol and stored at −20 °C. Sequences were used to query two databases, GenBank and Fusarium ID using BLAST to determine sequence similarity of all tested isolates. A phylogenetic tree inferred from *tef1* gene sequences was generated using maximum parsimony (MP) analysis with the MEGA4.0 program (Tamura et al., 2007).

2.4. Lethal time and percentage of mortality

The lethal time (LT_{50}; the numbers of days until 50% of insects were dead) was determined as reported previously (Barra et al., 2013) with minor modifications. Briefly, all *Fusarium* isolates were cultured on PDA plates (75 ml) including 50 g sterilized maize grains. After 5 days two sterilized *T. confusum* adults (one month old) were placed on PDA plates containing *Fusarium* isolates. The plates were incubated under 12 h alternating light (black/white) at 25 to 27 °C and 70–75% relative humidity for 21 days. Mortality was analyzed during 3 weeks and compared with the untreated control samples. The inoculated fungi were re-isolated from the dead insects to prove the Koch's postulates and to confirm that the inoculated fungus was the causal agent of the death of insects. The experiments were arranged in a completely randomized design with 5 replications.

2.5. Statistical analysis

Statistical analysis (LT50 and mortality percentage) was performed using SPSS 16 software. All data were analyzed by one way analysis of variance, followed by *post hoc* Duncan's multiple range test at significant level of $p < 0.05$.

3. Results

3.1. Isolation and identification of entomopathogenic fungi

Fifteen dead insects from *Tribolium* species were collected from different stored grain markets and warehouses in western Iran. All *Tribolium* species were identified based on morphological characteristics as *T. confusum* (9) and *T. castaneum* (6). Eight isolates belonged to two *Fusarium* spp. were obtained from both identified *Tribolium* species in western Iran. All the isolates belonged to *F. keratoplasticum* (5) and *F. proliferatum* (3) through morphological characters (Short et al., 2013; Leslie and Summerell, 2006). *F. keratoplasticum* produces yellowish white mycelium. Aerial conidiophores, either unbranched or branched, up to 100 µm long, were formed abundantly on CLA. The macroconidia were small length, dorsiventral, with 3-4-septate, with notched basal cell and blunt apical cell (Fig. 1). Microconidia were oval and sometimes clavate (Fig. 1). Chlamydospores formed were relatively abundant in mycelium, mostly globose, subglobose, intercalary or terminal, and smooth to rough-walled, 8–15 µm diam. Chlamydospores were formed singly, and in cluster, or in chains (Fig. 1). Features showed, 5 isolates belonged to *F. keratoplasticum*. *F. proliferatum* produced purple-violet mycelium. The abundant aerial conidiophores formed on CLA. The macroconidia were slender, almost straight, with 3-5-septate, with poorly developed basal cell and curved apical cell (Fig. 2). Club shaped microconidia form in chains and false heads. Conidiogenous cells were polyphialides and monophialides (Fig. 2). Features showed, 3 isolates belonged to *F. proliferatum*.

3.2. Molecular analysis

DNA sequences of the *tef1* gene for all *Fusarium* isolates, β-tubulin gene for *F. proliferatum* isolates and ITS regions for *F. keratoplasticum* isolates were used to confirm the species identity. A single band of DNA fragments 700-bp, 500-bp, and 550-bp was amplified for the *tef1*, β-tubulin genes and ITS regions, respectively, from all *Fusarium* isolates. From similarities searched at NCBI (O'Donnell et al., 2012) and FUSARIUM-ID (Geiser et al., 2004) database, identification of all isolates was confirmed with statistical significance. A list of species names and GenBank accession numbers of the strains used in this study are provided in Table 1.

All *Fusarium* isolates were included in the phylogenetic analysis, constructed based on *tef1* dataset (Fig. 3). The analyzed dataset consisted of 1000 sites and 46 taxa (including the outgroup). Maximum parsimony of 46 taxa of the *Fusarium* inferred from *tef1* gene sequences revealed isolates FFSCPor1, FFSCPor2, and FFSCPor3 formed a monophyletic group with *F. proliferatum* (NRRL 22944 and NRRL 31071) with strong phylogenetic affinity (95% MP