Morphological and phylogenetic analysis of Fusarium species associated with vertical system of Orobanche spp.

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Abstract: Broomrapes (Orobanche spp.) are parasitic weeds and considered as a major limiting factor for the cultivation of various crops in many parts of the world. Due to the special biology of broomrape, including seed production, dispersal and longevity, the control of these species are often extremely difficult. Broomrape poses a serious threat to Iran’s agriculture; therefore exploring potential biological agents for these species are necessary. In this study, samples of infected broomrape plants (brown rot on vertical systems) collected from ten provinces of Iran, over the summer period (2014-2015). Fusarium isolates were identified according to their cultural and morphological characteristics. For phylogenetic analysis, a part of the tef1-a gene was amplified and examined. Based on morphological characters, fourteen species of Fusarium, including F. andiyazi, F. equiseti, F. flocciferum, F. foetens, F. hostae, F. lactatarum, F. oxysporum, F. proliferatum, F. redolens, F. sambucinum, F. solani s. l., F. thapsinum, F. torulosum and F. verticilloides, were identified. F. solani s. l., with 25% frequency, was the most common species among species. Eight species namely F. andiyazi, F. hostae, F. flocciferum, F. foetens, F. lactatarum, F. redolens, F. thapsinum and F. torulosum on broomrape are being reported for the first time on global-scale and F. lactatarumis being reported for the first time in Iran.

Key words: Fusarium lactatarum, biological agent, new species, tef1-a gene.

INTRODUCTION

Broomrapes (Orobanche spp.) are one of the most important weeds around the world (Ghotbi et al., 2011). Broomrapes are distributed in more than 80 countries and invaded almost 16 million hectares of agricultural lands around the world. Depending on rate and the amount of infection, broomrape can reduce yield quality and quantity between 30 to 100 percent. Broomrapes only germinate in response to specific chemicals released by the host plants (Perez-de-Luque et al. 2010). After germination, the seedlings attach to the host roots by the production of specialized feeding structures, described as haustoria to develop and accumulate nutrient resources from host plant (Joel et al. 2007). Therefore, broomrape is a damaging and destructive weed to crops and is difficult to control.

Although broomrapes are efficient in mechanisms such as seed production, dispersal and longevity and host roots attachment ability (reaching and entering the vascular tissue and underground development), the control of these species are extremely difficult (Montazeri, 2011; Amsellem et al. 2001b; Mazaheri & Ershad, 1995). Despite the various control practices against broomrapes, such as cultural and mechanical methods, soil fumigation, soil solarization, trap crops and resistant cultivars (Jacobsohn et al., 2001), these available control techniques have not yet proven to be as effective, economical and applicable as expected (Alejandro et al. 2010; Goldwasser & Kleifeld 2004).

Phytopathogenic fungi such as Fusarium species (F. oxysporum Schelcht, F. oxysporum f. sp. orthoceras, F. solani Mart., F. arthrosporoides Sherb., F. nygamai Burgess & Trimbol and F. semiteectum Berk. & Ravenel, especially F. semiteectum var. majus) were reported to be associated with Orobanche spp. These Fusarium species have shown significant pathogenicity against Orobanche spp. when tested under controlled or field conditions (Amsellem et al. 2001b; Bedi & Donchev, 1991; Cohen et al. 2002; Muller-Stover et al. 2002). These fungal pathogens demonstrate their potential ability to be used as bio-herbicides.

TEF is a protein that is translated into an essential part of the encoding high phylogenetic region (Geiser et al. 2004). This gene first was used as a marker for identification and phylogenetic relationship of the species belongs to noctuid moths subfamily Heliothinae in Lepidoptera Order (Cho et al. 1995).
Since the issue of broomrape has caused serious problems to Iran’s agriculture; this study introduce a proper method to overcome the problems associated with Orobanche spp. The aim of this study was to identify Fusarium species associated with Orobanche species that potentially used as biological control agents of broomrape using indigenous antagonistic Fusarium species.

MATERIALS AND METHODS

Sampling
Infected broomrape plants (Orobanche spp.) with vertically brown rot symptoms were randomly collected from tomato farms in ten provinces of Iran, including Alborz, Tehran, Kermanshah, Kurdistan, Hamadan, Zanjan, East Azerbaijan, Razavi Khorasan, Fars and Markazi provinces, during summertime in 2014-2015. Samples were picked up from their roots using trowel and transferred to the laboratory in paper packets.

Fungal isolation and preservation
Isolation of fungi was carried out according to Nash and Snyder (1962) medium. The roots were separated and rinsed in tap water for 20 minutes to wash away soil particles. The Root part of each samples were then cut into 2 cm pieces and the sterilization steps took place. The pieces were soaked at 1% sodium hypochlorite for 2 minutes and rinsed in sterilized distilled water and air dried on sterile filter paper. The disinfected pieces were cut into 2 cm pieces, placed on Peptone PCNB Agar (PPA) medium and incubated at 25°C in the dark for 3 days. The isolates were sub-cultured into Water Agar (WA) medium and a tip of the hyphae was picked up and transferred to PDA (potato dextrose agar) medium. The purified isolates were then stored on sterile filter papers at -20°C. Fungal isolates were deposited in fungal culture collections of University of Tehran (UTFC).

Morphological characterization
Isolates of Fusarium were identified according to their cultural and morphological characteristics as described by (Gerlach & Nirenberg, 1982; O’Donnell et al., 2004; Leslie & Summerell, 2006; Saremi, 2005). The isolates were grown on PDA medium to determine their growth rate and colony pigmentation, so the cultures were incubated at 26°C and 30°C for 7–10 days in the dark. Colony diameter was measured and Colony color recorded with naked eyes. Isolates were also placed on CLA and SNA, then incubated for 14 days under fluorescent and near-ultraviolet lights conditions to investigate the presence and shape of the macroconidia, microconidia and chlamydospores.

Phylogenetic analysis
DNA extraction: Liquid cultures were initiated by adding 2 pieces of 5 days old fungal cultures to 250-mL Erlenmeyer flasks containing 100 mL PDB medium (potato dextrose broth plus 2 g yeast extract per liter). Flasks were incubated at room temperature approximately 25°C on a rotary shaker for 6–8 days. Mycelium was collected by filtration through the sterile filter paper with a vacuum funnel. Mycelia were harvested, frozen and stored at −20°C. DNA was extracted using a modified hexadechltrimethyl-ammonium bromide (CTAB) procedure (Doyle and Doyle 1987). The DNA was visualized on a 1% agarose gel (wt/v) (Boehringer Mannheim) stained with ethidium bromide and viewed under ultra-violet light. DNA concentrations were estimated by comparing the intensity of ethidium bromide fluorescence of the DNA sample to a known concentration of lambda DNA marker (marker III, Roche Diagnostics). Extracted DNA (50–90 ng) was used as the template for the PCR reaction.

Molecular characterization
A part of the tef1-a gene was amplified by PCR using the primers Ef1 F (5-ATGGGTAAGGAGCAAGAC-3) and Ef2 R(5GGGAATCAGTGTACATGGTTG-3) (O’Donnell et al. 1998) in a final volume of 25 μL containing 50-60 ng of DNA, 0.1 μM of each primer, 150 μM dNTP, 3 U Taq DNA polymerase and PCR reaction buffer. Amplifications were conducted in a Master-cycler (Eppendorf) with an initial denaturation of 5 min at 95°C followed by 35 cycles of 60 s denaturation at 95°C, 75 s annealing at 56°C, 60 s extension at 72°C and a final extension of 7 min at 72°C. The presence of PCR products was confirmed by gel electrophoresis. The tef1-a amplicons were sequenced by Macrogen Co. (South Korea) using the two PCR primers as sequencing primers. Sequence identities were determined using Blast analysis from NCBI available online and most identical sequences from each species were recorded together with their information to use in phylogenetic analysis (table 1).

Sequence analysis
Sequences were aligned and compared by Kimura’s two parameters distance model and the neighbor-joining (NJ) and Maximum Likelihood (ML) methods with Tamura-Nei distance model using the program MEGA ver. 6.0 software (Gouy et al. 2010). The topology of the resulting tree was tested by bootstrapping with 1000 re-samplings of the data.

RESULTS AND DISCUSSION
A total of 203 isolates from 385 collected samples were identified as the genus of Fusarium. Based on morphological characters, only fourteen Fusarium species, including F. andiyazi, F. equiseti, F. fuscum, F. foetens, F. hostae, F. laceratum, F. oxysporum, F. proliferatum, F. redolens, F. sambucinum, F. solani s. l., F. thapsinum, F. torulosum and F. verticilloides, have been identified. Fusarium solani, F. oxysporum and F. redolense with 25%, 20% and 15% frequency are common among all the species, respectively (table 2).
Fungi associated with Orobanch spp. including Alternaria spp., Bipolaris australis, F. equiseti, F. oxysporum, F. semitectum, F. solani, Rhizoctonia solani, Ulocladium atrum and Verticillium albo-atrum, were isolated from O. aegyptiaca (Mohammadi et al. 2014). Fusarium species are well distributed across many geographical regions and substrates, and also widely distributed in different soils, plants and air (Booth 1971; Burgess et al. 1994; Nelson et al. 1994; Summerell et al. 2003). So, in this research different species of genus Fusarium associated with Orobanch spp. from different geographical regions of Iran were identified.

The morphological identification of the Fusarium species was confirmed by the sequencing of tef1-α gene. So, the Standard Nucleotide BLAST search for similarities showed the similarity percentage of the strains ranged from 98 to 99 percent. The tef1-α sequence of Fusarium strains were searched for homology in GeneBank database. Then, the result of tef1-α gene sequencing demonstrates that all tested isolates belong to the genus Fusarium. Also, the similarities of tef1-α sequence between our isolates and the reference sequences from the GeneBank, were supported by bootstrap values of more than 50 percent. All the analyzed sequences data were deposited in the GeneBank database. To our knowledge, this is the first report of eight species including F. redolens, F. torulosum, F. hostae, F. foetans, F. andiyazi, F. flocciferum, F. lacertarum and F. thapsinum on
Table 2. Information of fifteen species of Fusarium isolated from infected broomrapes.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Sampling region</th>
<th>GB Accession no.</th>
<th>Collection Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH1</td>
<td><em>F. flocciferum</em></td>
<td>Alborz province</td>
<td>MF588955</td>
<td>ABRII 10265</td>
</tr>
<tr>
<td>FSE3-1</td>
<td><em>F. foetens</em></td>
<td>Fars province</td>
<td>MF588956</td>
<td>ABRII 10257</td>
</tr>
<tr>
<td>HT1-2</td>
<td><em>F. solani</em></td>
<td>Alborz province</td>
<td>MF588957</td>
<td>ABRII 10258</td>
</tr>
<tr>
<td>KG1</td>
<td><em>F. torulosum</em></td>
<td>Kermanshah province</td>
<td>MF611744</td>
<td>ABRII 10259</td>
</tr>
<tr>
<td>KG7-1</td>
<td><em>F. hostae</em></td>
<td>Kermanshah province</td>
<td>MF611745</td>
<td>ABRII 10260</td>
</tr>
<tr>
<td>KK23-2</td>
<td><em>F. verticillioides</em></td>
<td>Kurdistan province</td>
<td>MF611746</td>
<td>ABRII 10261</td>
</tr>
<tr>
<td>KK27-2</td>
<td><em>F. andiyazi</em></td>
<td>Kurdistan province</td>
<td>MF611747</td>
<td>ABRII 10262</td>
</tr>
<tr>
<td>KK32-1</td>
<td><em>F. sambucinum</em></td>
<td>Kurdistan province</td>
<td>MF611748</td>
<td>ABRII 10263</td>
</tr>
<tr>
<td>KK32-2</td>
<td><em>F. verticillioides</em></td>
<td>Kurdistan province</td>
<td>MF611749</td>
<td>ABRII 10264</td>
</tr>
<tr>
<td>KM4</td>
<td><em>F. lacertarum</em></td>
<td>Kermanshah province</td>
<td>MF611751</td>
<td>ABRII 10266</td>
</tr>
<tr>
<td>NA2-1</td>
<td><em>F. equiseti</em></td>
<td>Hamedan province</td>
<td>MF611752</td>
<td>ABRII 10267</td>
</tr>
<tr>
<td>NA9</td>
<td><em>F. redolans</em></td>
<td>Hamedan province</td>
<td>MF611753</td>
<td>ABRII 10268</td>
</tr>
<tr>
<td>ZCH2-2</td>
<td><em>F. oxysporum</em></td>
<td>Zanjan province</td>
<td>MF611755</td>
<td>ABRII 10269</td>
</tr>
<tr>
<td>ZCH6</td>
<td><em>F. proliferatum</em></td>
<td>Zanjan province</td>
<td>MF611754</td>
<td>ABRII 10270</td>
</tr>
</tbody>
</table>

*Orobance* spp. in global scale. Identification of *F. lacertarum* from *Orobanch* spp. is recorded for the first time for the mycobiota of Iran.


Colonies on PDA were reached to 38-44 mm average growth after three days at 26°C. Mycelia were white and the bottom of the colony showed brown-orange pigmentation after seven days. On CLA, the aerial mycelium produced solitary chlamydospores with 7.7-12.3µm diameters, and no microconidia was formed. Macroconidia originated from abundant sporodochia with strong orange color, in short monophialids, usually with not more elongated apical cells, rarely curved and the basal cell of macroconidia presented a foot form. Apical cell form in hook form, smoothly curved, 5 septa, 42-58×3.5-4.5 µm (Fig. 1). Morphological characters of *F. lacertarum* approved by sequencing results of *tef1* - α part gene.


In this study, based on molecular data and morphological characterization, *F. lacertarum* is reported for the first time in Iran and also on broomrape across the world. Morphology of examined specimens agrees with the description provided by Poletto et al. (2015).

![Fig. 1. Morphological characters of F. lacertarum. a. A single spore of Macroconidia. b. A clump of clamydospores. c. Front and d. back views of PDA cultured colony. Scale bar: 10 µm.](image-url)
Phylogenetic analysis

A phylogenetic tree with 3 clades including clades A, B and C was drawn during phylogenetic analysis of tested taxa based on NJ method (Fig. 2). Clade A was containing Gibberella fujikuroi species complex and divided into four subclades. Sublade A1 showed strain of F. andiyazi that were supported by bootstrap value of 99%, sublade A2 consisted of isolates F. verticilliodes and sublade A3 including strains of F. foetans, F. oxysporum and F. thapsinum. An isolate which was morphologically identified as F. verticilliodes (kk 23-2), according to no observation of globose microconidia, was placed within the F. thapsinum isolates from NCBI and sublade A4 contained strains of F. proliferatum species.

Clade C is a group with members of Gibbosum complex of Fusarium species and divided into four subclades names C1, C2, C3 and C4. Subclade C1 was supported by the strong bootstrap value of 94% and included F. laceratum strains. Subclade C2 included strains of F. scirpi and was located as sister group of subclade C3 including, strains of F. equiseti and some Fusarium sp. isolates.

Clade B includes other species and divided into four subclades (B1, B2, B3 and B4). Subclade B1 included the strain HT 1-2 and demonstrated high similarities of tef1-a gene sequence to the referred isolates of F. solani, subclade B2 contained two species of F. hostae and F. redolens by strong bootstrap value of 98%, sublade B3 consists of F. torulosum and F. flociferatum strains, sublade B4 consisted of strain KK32-1 which gave high similarity of tef1-a gene sequence to the referred F. sambucinum from NCBI and was supported by the strongest bootstrap value of 100%.

Maximum likelihood (ML) analysis of the tef1-a gene sequence alignment recovered a tree with significant similarities to the phylogenetic tree in NJ method (Fig. 3). However, there are some differences between the two trees. For example, subclade B1 in NJ analysis which includes F. solani strains is located as a separate clade in maximum likelihood method that
was named as clade D. Subclade B4 consisted of strain KK32-1 and strains of *F. sambucinum* was placed in clade C in maximum likelihood analysis by the strongest bootstrap value of 100%. The location of isolate KM4 (*F. lacertarum*) in ML tree is the same as NJ tree.

In this study, in addition to the morphological identification, the sequence data analysis of the *tef1*-α region was employed. The molecular data and phylogenetic relationships allow reliable differentiation between the major *Fusarium* species. For this purpose, sequence of *tef1*-α gene was used to assess 15 identified species of genus *Fusarium* isolated from broomrape samples. Use of translation elongation factor 1α (*tef1*-α) and β-tubulin genes can lead to more clear fungal identification (Vitate et al. 2011; Wang et al. 2011). Overall, Molecular analysis had harmony with morphological grouping, except for isolate kk23-2 in clade A and subclade A3, where *F. verticillioides* isolate (kk23-2) was located between the *F. thapsinum* isolates. Some strains of *F. thapsinum* that do not produce diagnostic yellow pigment, is morphologically identical to *F. verticillioides* therefore in similar situations molecular characterization can be helpful in identifying these isolates (Leslie & Summerell, 2006). This sort of discrepancy between morphological and molecular data in fungal studies has been seen frequently (Darvishnia 2013; Watanabe 2013).

Although the morphological species concept does not completely reflect the phylogenetic tree of the genus *Fusarium* (O'Donnell et al., 2000), this does not imply that morphological characteristics are not useful for identification and taxonomy. To identify unknown species, morphological characteristics can be widely applied to any species, not only the genus *Fusarium* but also to other fungi (Taylor et al. 2000). *Fusarium* isolates can be initially classified on the basis of morphological similarity, with the awareness that sections are in fact a means of artificial grouping, but the morphological approach fails to detect many biological factors but phylogenetic approach can be useful in detection of this factors (Liddell, 2003)

**Fig. 3.** Phylogenetic tree showing the relationship of 34 *Fusarium* species strains based on *tef1*-α gene sequence using the Maximum likelihood (ML) method. The percentage values of replicate trees in which the linked taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The bootstraps values > 50% were shown next to the branches. *Microdochium majus* was used as an out-group.
Due to the deficiencies and problems within morphological identification knowledge and also due to the large number of fungal species and inadequate available morphological information, the use of molecular information can be helpful (Davari et al. 2013). So, we need to construct more reliable taxonomic-mic system in combination with the morphological, phylogenetic, toxicological, biological, and other recognition methods. The use of another gene in molecular identification can be useful in better identifying and better phylogenetic analyses.

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REFERENCES


شناسایی ریخت شناختی و فیلوژنیک گونه های علف هرز گل جالیز

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چکیده: گل جالیز (Orobanche spp.) از علف های هرز انگلی به عنوان یکی از عوامل مهم محدود کننده در کشت محصولات مختلف در بسیاری از نقاط جهان مورد توجه می‌باشد. با توجه به ویژگی‌های موتور این علف هرز از جمله: تولید بذر، پراکندگی و طول عمر زیاد، کنترل آن بسیار دشوار است. گل جالیز یک مشکل جدی در کشاورزی ایران می‌باشد. لذا شناسایی عوامل زنده با قابلیت کنترل این علف هرز حائز اهمیت می‌باشد. در این مطالعه در طول نیمه سال 93 و 94 از نمونه‌های علف هرز گل جالیز از مزارع گوجه‌فرنگی در استان زنجان جمع‌آوری گردیدند. هر گونه ریخت شناختی با ویژگی‌های خاصی در دسترس داشتند. از جمله چهارده گونه از جنس Fusarium، جایگاه گونه Fusarium solani s. l. با فراوانی ۲۵٪، بهترین گونه در دست انداخته. برای تکثیر و تجزیه و تحلیل فیلوژنیک مورد بررسی قرار گرفت. بر اساس ویژگی‌های ریخت شناختی، چهارده گونه از جنس Fusarium، Fusarium thapsinum، Fusarium solani s. l، Fusarium sambucinum، Fusarium redolens، Fusarium proliferatum، Fusarium oxysporum، Fusarium lacertarum، Fusarium hostae، Fusarium equiseti، Fusarium andiyazi، Fusarium flocciferum، Fusarium andiyazi، Fusarium foetens و Fusarium verticillioides اطلاعاتی در مورد گونه جدید، Fusarium lacertarum، ثبت شد. برای گونه جدید، نام جدیدی به نام Fusarium lacertarum نامگذاری گردید. برای اولین بار در مقياس جهانی و برای اولین بار در فلور قارچی ایران، گزارش می‌شود.

کلمات کلیدی: Fusarium lacertarum، عوامل بیولوژیک، گونه جدید، زن

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