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Molecular Identification of Glomus mosseae from Wheat Rhizosphere in Damghan Region, Iran

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Abstract

Arbuscular mycorrhizas (AM) have mutualistic symbiosis with plants and thus efforts have been placed on application of these symbiotic relationships to agricultural and environmental fields. In this study, soil and root samples were collected from 40 sites of wheat rhizospheres in Damghan region located in central parts of Iran. Fungal structures as well as colonization were observed by root staining and clearing. Also, trap cultures established using maize in greenhouse condition. AM fungal spores were extracted and identified using morphological method. Using morphological characters, 12 species of what were identified. The most abundant species was *G. mosseae*. DNA was extracted from different plant and fungal structures including colonized and non-colonized roots, sporocarps, extraradical mycelia as well as single spores. Partial regions on 18S rDNA as well as 28S rDNA were amplified using PCR with AM fungal specific primers (AML1/NS31) and G. mosseae specific primers (NDL22/5.21).

Keywords: Arbuscular Mycorrhizal Fungi, *Glomus mosseae*, PCR, Identification, Diversity

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi have mutualistic symbiosis with most vascular plants and provide many benefits to the plants including increased uptake of inorganic nutrients, enhanced tolerance to many environmental stress and protection from pathogens [1-2]. Also, species diversity of AM fungi influences plant diversity and ecosystem productivity [3]. Thus efforts have been placed on application of these symbiotic relationships to agricultural and environmental fields. In spite of these advantages, application of these fungi has been limited because their relationships with plants have not been clearly understood. It would be mainly due to the inability to culture these fungi because they are obligate symbionts which can only be cultured under the presence of their hosts. Identification of AM fungi have been relied on microscopic observations of spores collected from soil [4-5]. About 200 different AM fungal taxa in order Glomales of Zygomycota have been described on the basis of morphological characteristics of their asexual spores [6]. However, these fungal organs have a limited number of morphological features. In recent years, molecular techniques have been used to study phylogenetic relationships and genetic variations of AM fungi. Several attempts have made to use ITS region of rDNA as a tool for identification of AM fungi [7] and phylogenetic studies with DNA extractions and sequencing from AM fungal spores [8-9]. This fungal group has recently elevated to the status of a new monophyletic phylum Glomeromycota [10]. This is based on the analysis of small subunit rDNA sequences. Spores are formed in soil during certain period of their life cycle and related to their host plants and environmental stress. Also, some species of AM fungi may not produce their spores. The contrary to spores in soil, hyphae colonizing the root are an active part of the fungus involved in interconnections between plant and soil environment. However, hyphae in roots can not be identified to species level with morphological methods. Thus, it is important to develop techniques identifying AM fungi colonizing roots for further studies and applications of these fungi. The molecular technique allows identifying hyphae within roots with AM specific PCR primers targeted rDNA regions. The specificity of primers is an important factor for molecular identification of AM fungi within roots. Recently, AM specific primer set AML1/AML2 has been developed. These primers amplified most of the fungi belonging to Glomeromycota and excluded DNA of other organisms such as plant, bacteria and other fungi inhabiting roots, suggesting high specificity of the primers. Also, because molecular identification of AM fungal hyphae colonizing roots depends on molecular information of spores, it is important to obtain morphological and molecular data of spore for molecular identification of the fungi in roots. About 65 species of AM fungi have been reported in Iran and their identification was based on morphological characteristics of mostly field collected spores. There is no information on nucleotide sequences of AM fungal spores found in Iran. In this study, soil samples were collected from different wheat rhizospheres in Damghan region, central parts of Iran and identified using morphological characteristics. Also, partial 18S rDNA as well as 18S rDNA genes were amplified for specific identification of *Glomus mosseae.*

MATERIALS AND METHODS

Soil collection and culture of AM fungal spores

During the years 2008-2009, soil and fine root samples were collected from Damghan region in central parts of Iran from about 40 sites of wheat rhizospheres (Fig 1). Root samples were stained with 0.05% trypan blue [11] and colonization of AM fungi were observed. Then, collected soils were mixed with sterilized sand at the rate of 1:1 (w/w) and were used for trap cultures. Pots (15 cm diameter.) for trap culture were maintained with maize as host plants under greenhouse condition for four months. The pots were watered as needed and fertilized every 2 weeks with 100 ml of low P (1/20 phosphate) Hoagland Nutrient Solution (2.8g H_3BO_3, 3.4g $MnSO_4 \cdot H_2O$, 0.1g $CuSO_4 \cdot 5H_2O$, 16.22g $ZnSO_4 \cdot 7H_2O$, 0.1g $(NH_4)_6MO_7O_2 \cdot 4H_2O$, 5ml H_2SO_4, 6.72g Na_2EDTA, 5.58g $FeSO_4$, 0.94g $Ca(NO_3)_2 \cdot 4H_2O$, 0.52g $MgSO_4 \cdot 7H_2O$, 0.66g KNO_3, 0.06g $HN_4H_2PO_4$). After 4 months of growth, above ground parts of plants were removed and soils in the pots were stored at 4°C until used.

Morphological identification of AM fungal spores

Spore of AM fungi were extracted from 35g of soil using wet-sieving and sucrose density gradient centrifugation methods [12]. The extracted spore was observed under a light microscope and identified morphologically based on spore color, shape, surface ornamentation, spore contents and wall structures [4-5].

Molecular Identification of AM fungal spores

Different plant and fungal structures including colonized and non-colonized roots, sporocarps, extraradical mycelia as well as single spores were separated and used for molecular identification. DNA from roots as well as mycelia was extracted using CTAB method [13]. Also, Spores and sporocarps were sonicated and washed with distilled water three times; crushed to extract DNA in a 0.2ml PCR tube and sterilized water 1µl added to a 0.2ml PCR tube. The concentration of DNA was estimated by absorbance at 260nm. DNA were amplified (25µl) including 2.5µl PCR buffer (10X), 0.7µl Taq DNA polymerase, 2.5µl dNTP (2mM), 1.25µl of each primer (0.1µg/ml) and 0.3µl BSA (Bovine Serum Albumin) with 18S rDNA primers including general fungal primer AM1 (GTTTCCCGTAAGGCGCCGAA) designed to exclude plant DNA [14] and universal eukaryotic primer NS31 (TTGGAGGGCAAGTCTGGTGCC) [15]. The PCR was performed for 35 cycles (at 94°C for 1min, 58°C for 1min, 72°C for 1min, 72°C for 10min). The PCR products were separated by electrophoresis on 1.5% agarose gel and stained with Ethidium Bromide. PCR products were used as template for the second amplification (30µl) including 3µl PCR buffer (10X), 1µl Taq DNA polymerase, 2µl dNTP (2mM), 1.5µl of each primer (0.1µg/ml) with 28S rDNA primers specific for *G. mosseae* including NDL22 (TGGTCCGTGTTTCAAGACG) as well as 5.21 (CCTTTTGAGCTCGGTCTCGTG) [16]. Amplification was done for 40 cycles (at 94°C for 1min, 60°C for 1min, 72°C for 1min, 72°C for 10min). Products were separated on 1.5% agarose gel.

RESULTS AND DISCUSSION

Study showed that arbuscular mycorrhizal fungal spores could be observed in all of collected soil samples especially in samples with more fine roots. Different fungal structures including vesicles, extraradical mycelia, arbuscules and intraradical spores could be observed after root staining and clearing (Fig 2). The most abundant structures were vesicles and extraradical mycelia which confirmed other findings [17]. Spores isolated from soils were separated and identified using morphological characters including spore size, color, wall structures and reactions with Melzer's reagent. Totally, 12 morpho-species from *Glomus* (11 species) and *Scutellospora* (1 species) genera of Glomerales and Diversisporales orders and 2 families Glomeraceae and Scutellosporaceae were identified (Fig 3). The most abundant species was *G. mosseae* (78%). Host plant species has been suggested a possible factor for affecting AM fungal sporulation and community composition [18-19]. However, AM fungal composition in soils tested might be influenced by other factors as well as host plant species [20]. Changes in fungal spore populations in agricultural soils have been observed following different cropping histories [21] and some management practices, e.g. the use of fungicides or soil disinfection, can have negative effects on the symbiotic fungal population. It could be a reason for relatively low number of

Fig 1. Site of soil and root sampling in Damghan region, central part of Iran

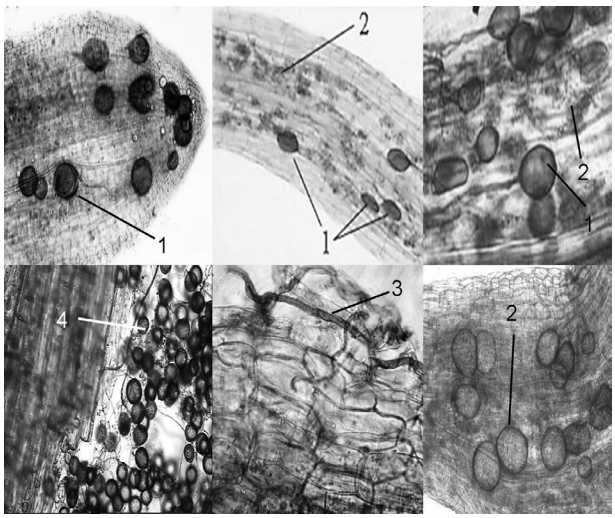

Fig 2. Different fungal structures after root staining and clearing. 1: Vesicles; 2: Arbuscules; 3: Extraradical mycelia; 4: Spores.

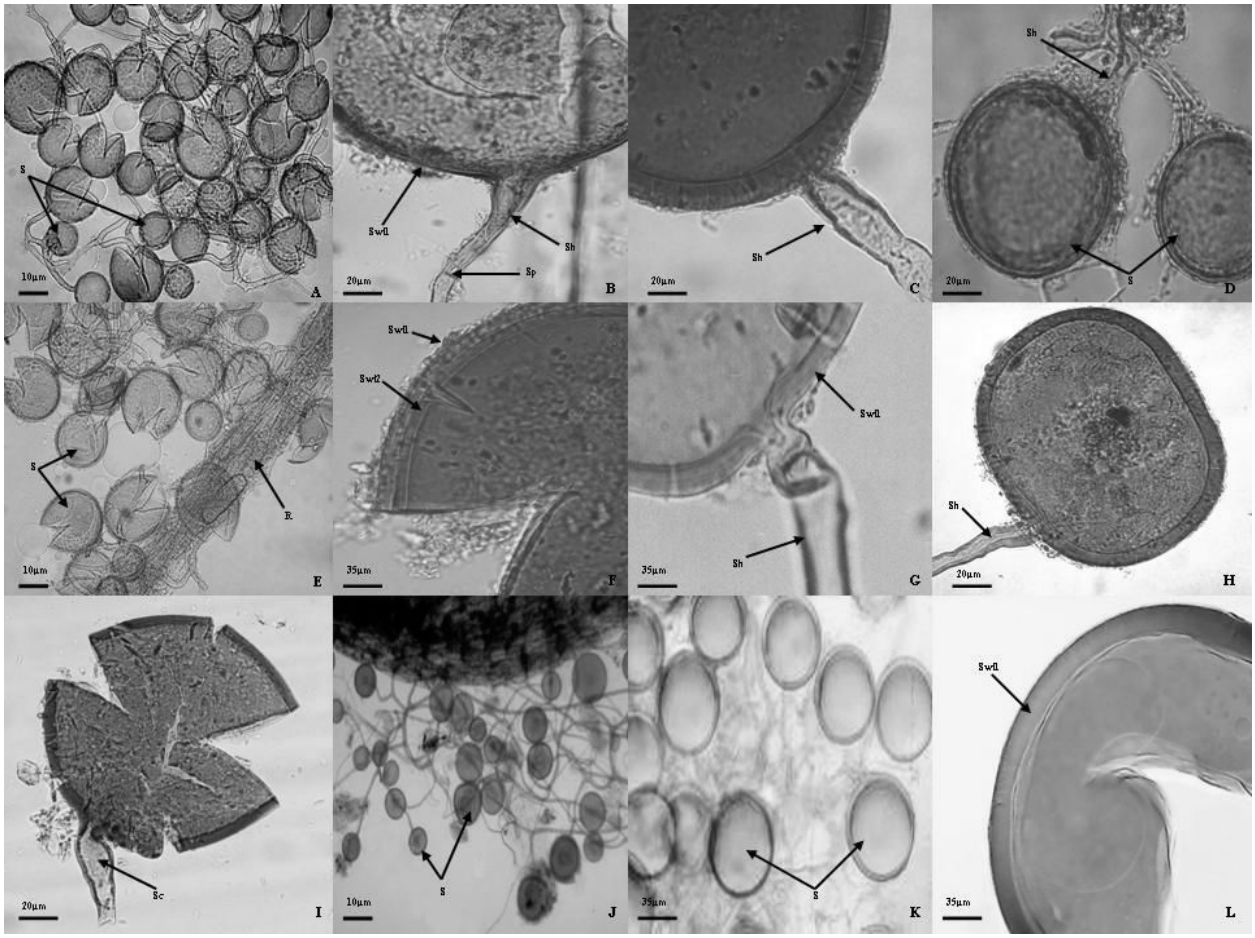

Fig 3. Different fungal species identified. A: *G. fasciculatum*; B: *G. mosseae*; C: *G. macrocarpum*; D: *G. geosporum*; E: *G. intraradices*; F: *G. caledonium*; G: *G. constrictum*; H: *G. etunicatum*; I: *Scutellospora callospora*; J: *G. aggregatum*; K: *G. glomerulatum*; L: *G. versiforme*.

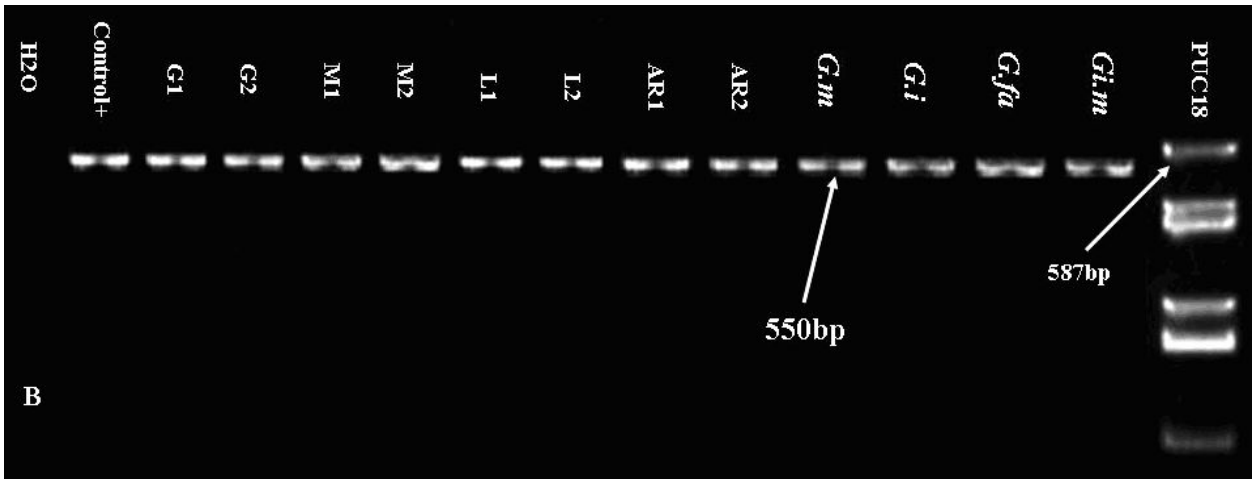

Fig 4. Fragment (550bp) amplified from extracted DNA using AM1/NS31 primers on 1.5% agarose gel. H_2O: Negative control; Control+: Colonized root; G_1-AR_2: DNA extracted from sporocarps, spores, extraradical mycelia and roots; *G.m:* DNA from pure *G. mosseae* spores*; G.i:* DNA from pure *G. intraradices* spores; *G.fa:* DNA from pure *G. fasciculatum* spores; *Gi.m:* DNA from pure *Gigaspora margarita* spores; PUC18: Size marker.

species found in roots of both crop species. Helgason et al. [14] found significantly lower diversity of AM fungal communities in roots from arable fields than those from forest sites. Also, they found that the most dominant species was *G. mosseae* in arable study sites and not found in woodland. Mycorrhizal root colonization rate was about 70%. Significant differences in mycorhizal colonization rate and mycorrhizal structures between the sites were not detected under microscopes. However, there was much evidence that the spore counts or microscopic observations of roots do not reflect composition or abundance of AM fungi [22]. In the past decades, many progresses have been made in agricultural and ecological studies.

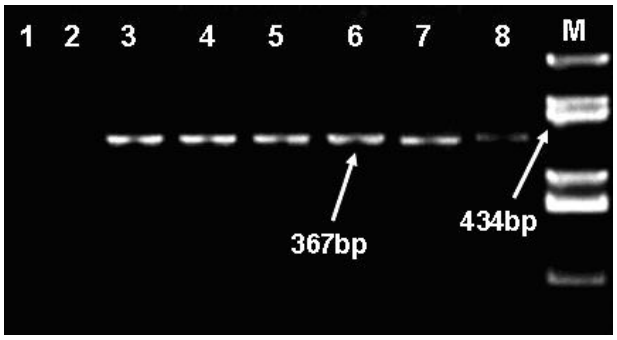

Fig 5. Fragment (367bp) amplified from extracted DNA using NDL22/5.21 primers specific for *G. mosseae* on 1.5% agarose gel. 1: Negative control (H_2O); 2: DNA from non-colonized root; 3: DNA from pure *G. mosseae* BEG12 spores; 4: DNA from colonized root by pure *G. mosseae* BEG12; 5: DNA from sporocarps; 6: DNA from spores; 7: DNA from extraradical mycelia; 8: DNA from roots; M: PUC18 size marker.

Host plants benefit from AM fungi in increased growth through phosphorus uptake, and also mycorrhizal associations is related to environmental aspects. However, in practice, AM fungal colonization in roots was able to observe under microscopes due to the morphological and histological differences from other pathogenic fungi. However, it was not possible to differentiate AM fungi in colonized roots at the below level of genus. Spore communities in soil do not reflect AM fungal populations in active symbionts. Recent progresses of molecular techniques allow being able to resolve these problems. Partial SSU of rDNA fragment of AM fungi from roots and spores were amplified using AM1 and NS31 primers and the length of PCR products was approximately 550bp (Fig 4).

NS31-AM1 primers used in this study would be one of the useful primers to study AM fungal community in roots because these primers were designed to exclude plant DNA from DNA extracts from plant roots [14]. However, as previously published specific primers, including AM1 primer used in this study for AM fungi have shown several mismatches, it was a particular interest to establish new primers which match to all the AM fungal species and exclude plant and other fungal DNA. One of problems of AM1-NS31 primers was that they could not amplify a certain group of AM fungi including genus *Archaeospora* and *Paraglomus* and it should be solved for further ecological and physiological studies of AM fungi. In second amplification using NDL22/5.21 primers, the length of PCR products was about 367 bp (Fig 5). These bands were more clear in DNA amplified of fungal structures such as sporocarps, spores and mycelia but was a little faint with samples of colonized roots. Also, there are no bands on control (DNA of non-colonized roots), which shows the specificity of primers. Combination of these primers designed from LSU rDNA could be identified *G. mosseae* in different samples. Our findings were compatible with others [16,23].

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