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Morphological and molecular identification of ectomycorrhizal fungi associated with Persian oak (*Quercus brantii* Lindl.) tree

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Abstract: Identification of ectomycorrhizal (ECM) fungi in different ecosystems has major significance. In this research, to identity ECM fungi, we used two methods including the morphological method and the molecular method that is more precise. Basidiocarp collection of fungi associated with oak tree (*Quercus brantii* Lindl.) roots was carried out in the spring season 2016 and was identified by morphological and molecular methods. We also checked macroscopic and microscopic features and measured each structure using BioloMICS Measures software. To verify the morphological identification, the internal transcribed spacer (ITS) region was amplified by PCR using the primer pair ITS1/ITS4, and the sequences were analyzed. According to the morphological observations, the identified species were *Amanita crocea*, *Boletus comptus*, *Tricholoma giganteum*, and *Inocybe rimosa*. Besides, based on molecular techniques by comparing sequences, we identified five species out of the eight ones as *A. crocea* and other species as *T. giganteum*, *I. rimosa* and *B. comptus*. Both morphological and molecular methods are necessary for identifying ECM fungi associated with tree roots in the Zagros zone in the west of Iran.

Keywords: mycorrhizal symbiosis; forest ecosystem; *Amanita*; *Tricholoma*; *Inocybe*; *Boletus*

In recent decades, oak tree decline has been a widespread problem in the Zagros zone in the west of Iran. Different conditions lead to the decline, including drought, soil nutrient depletion, attack of pests and diseases, and dust storm (Sagheb-Talebi et al. 2014). Mycorrhizal symbiosis is especially ectomycorrhizal by increasing drought resistance and enhancing the ability of plants to uptake nutrient elements, decreasing tree decline and mortality (Brady, Weil 2008).

In the forest, there is an obligatory symbiotic relationship between tree roots and a certain group of fungi called ectomycorrhiza in the soil, which brings benefits for them (Brundrett 2009; Itoo, Reshi 2013; Wurzburger et al. 2017). Numerous studies have indicated that the mycorrhiza can promote plant growth and survival by increasing their tolerance to biotic and abiotic stresses (Sylvia, Williams 1992; Wu 2017).

ECM are commonly found in temperate forests or in taiga, where other fungi such as truffles, boletes, amanitas, and chanterelles exist. They form a symbiosis with tree roots including oak, pine, birch, eucalyptus, and European spruce (Querejeta et al. 2014;

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Korhonen et al. 2019). ECM have an essential role in the forest growth and health by improving the uptake of nutrients such as phosphorus, nitrogen, and micronutrient elements from the soil (Smith, Read 2008). Mirzaei and Heydari (2014) studied the relationship between environmental factors, colonization and abundance of arbuscular mycorrhizal fungi associated with *Amygdalus scoparia* in Zagros forests. They identified 7 species of AMF including *Glomus fasciculatum*, *G. intraradices*, *G. mosseae*, *G. claroideum*, *G. drummondi*, *G. caledonium* and *Gigaspora gigantea* through morphological characteristics in the region.

Molecular techniques and the DNA sequencing analysis can be used as faster and more accurate methods for the identification of ECM with high morphological diversity. In many studies, molecular techniques have been used to identify ECM (e.g. Larsson et al. 2009; Jairus et al. 2011; Bahram et al. 2013; Sebastiana et al. 2013; Lothamer et al. 2014; Pushpa et al. 2014; Vizzini et al. 2014; Latha, Manimohan 2016). Many researchers have used the markers to identify the genus and species of ECM (White et al. 1990; Pushpa et al. 2014). In Iran, the surface area of forest land (% of land area) was 6.6% in 2016. So, it is essential to identify ectomycorrhizal symbiosis fungi in these ecosystems. Therefore, the aim of this research is to provide a morphological and molecular characterization of ECM that increase drought resistance via a symbiotic relationship between oak tree roots and ECM.

MATERIAL AND METHODS

Site description. The geographical location of the studied area is between longitude 46° 41' 23.76'' E and latitude 33° 19' 28.95'' N, with a total area of about 4 417 hectares. The elevation range varies from 1 350 m to 1 756 m a.s,l. with slopes between 10 and 15% and north-south facing aspect. The major vegetation cover is composed of Persian oak (*Quercus brantii* Lindl.) trees which are accompanied by wild pistachio (*Pistacia* spp.) trees and hawthorn (*Crataegus pontica* C. Koch) trees (Sagheb-Talebi et al. 2014). The average age of forest trees is over 150 years. According to the De Martonne climatic classification, the climate is temperate semi-humid with mean annual precipitation of 592.5 mm and mean annual temperature of 19.4 °C. The soils in the study area are classified as Inceptisols with silty clay loam texture and soil pH ranges from 7 to 7.8. Fungal samples were collected during the growing season in May 2016. Fruit bodies were collected within a distance of 2–3 m from the tree trunk on the soil surface.

Morphological identification. We investigated morphological features including the shape and dimensions of the pileus and stipe, the presence or absence of the volva, gill attachment, the spore print colour, clamp connection, shape and size of basidiospores, shape, size and position of cystidia, shape and number of stigmata. Specimens were identified based on available taxonomic keys and publications (Moser, Kibby 1983; Knudsen, Vesterholt 2008). After the preliminary investigation, the specimens were dried using a dehydrator.

Molecular identification. Doyle and Doyle (1990) method with slight modification was used to extract the total DNA from 5 mg of fresh basidiocarps. The fungal tissue (fruit body) and the extracted DNA were transferred to Eppendorf tubes. The samples were then placed in a mortar containing liquid nitrogen and powdered using a porcelain pestle. The fungal tissue was then suspended in 700 ml extraction buffer consisting of 100 mM Tris-HCl (pH 9.0), 0.5 M EDTA (pH 8.0), 1.4 M NaCl, 2% hexadecyltrimethylammonium bromide (CTAB), 0.2% mercaptoethanol and centrifuged for 30 min at 65°C. The tubes containing the samples were inverted every 10 min, and 700 µl chloroform/isoamyl alcohol (24:1, v/v) was added to the tubes. The tubes were centrifuged for 20 min at 14 000 g for the phases to be separated. Then, the supernatant was removed and 2 µl of RNase A was added to the aqueous phase which was incubated at 37 °C for 30 min. Seventy µl of sodium acetate 2.5 M (or potassium acetate) and 400 µl isopropanol which had been stored for 30 min–24 h at –20 °C was added to each tube. The solution was centrifuged at 14 000g for 10 min. for developing the DNA pellet. The upper phase was discarded and washed away with 70% (v/v) ethanol, and centrifuged for 60 seconds min at 12 000g. The pellet containing DNA was solubilized in 100 µl sterile deionized water and kept at 4 °C for 24 h. Finally, the tubes were stored at –20 °C until further use. The quality of the extracted DNA was determined by electrophoresis on 0.8% agarose gel, followed by staining using ethidium bromide. In the last analysis, the gel was exposed to UV radiation.

Amplification of ITS by PCR. Amplification of the internal transcribed region of nuclear ribosom-

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al DNA (rDNA) was done using universal primers of ITSl and ITS4 with the respective sequences of 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' (White et al. 1990). The mixture used for PCR included 12.5 µl of Master Mix (2X), 1 µl from each of the primers mentioned above (100 pmol/µl), 4 µl of diluted DNA sample (100 pmol/µl) and 6.5 µl of sterile deionized water for the total 25µl reaction volume. The PCR program used for amplification consisted of 3 min. of initial denaturation at 95°C, denaturation and annealing for 35 cycles at 94 °C and 60 °C and for 60 seconds, respectively and 72 °C of extension for 90 seconds. The final extension stage had duration of 5 min. at 72 °C. To test whether there are any contaminations by reagents and reaction buffers, every amplification set contained DNA-free control samples. The quality of the extracted DNA was determined by electrophoresis on 0.8% agarose gel followed by staining using ethidium bromide (Sambrook et al. 1982) and photographed under UV light.

Sequencing of amplified ITS regions. The amplified segment was purified using a GeneJET PCR Purification Kit (Fermentas, UK). The sequencing was done using the primers ITS1 or ITS4, and the nucleotide sequence comparisons were performed using Blast Multiple Alignment Tool (BLASTn) network sequences against the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA) database. All DNA sequences of the ITS regions from fruit bodies were submitted to the GenBank (NCBI).

Statistical and phylogenetic analyses. Sequences of the ITS regions were used to study phylogenetic relationships of the studied taxa. Multiple alignments were performed with CLUSTALW (Thompson et al. 1994) and BIOEDIT v.7.0.9 (Hall 1999) tools were used for multiple alignments and manually optimized, respectively. Distance methods were used for phylogenetic analysis. The neighbour-joining (NJ) method proposed by Saitou and Nei (1987) was applied in constructing tree topology. Bootstrap resampling was computed 1 000 times for assessing branching confidence (Felsenstein 1985).

RESULTS

In this research, we tried to recognize fungi growing around trees as ectomycorrhiza and symbiotic fungi with oak trees by their collection and identification. In 2016, the specimens were retrieved from Bivereh oak forest in Malekshahi region, Ilam province (west of Iran).

Morphological identification

In the present study, four species of ectomycorrhizal fungi (ECM) associated with oak trees were identified which are described as follows:

***Amanita crocea* (Quel.) Singer**

A. crocea has a cap of 4–10 cm in size, convex, saffron orange colour in the centre and brown in the margin. The gills are free and cream in mass. Stipe 90–120 × 15–25 mm, cream to pale orange, without annulus, almost equal and has a volva. Lamellae cream-colored and free. Spores hyaline, smooth, globose to subglobose and 8–12 × 7–9.5 µm. Spore print white (Figure 1; A, B).

***Boletus comptus* Simonini 1993**

In this fungus, the cap is convex, 8–10 cm, pinkish or greyish pink. The tubes are yellow, pores small, orange-brown, turn black when pressed. The stipe is thick, clavate or club-shaped, 7–9 × 3–4 cm, yellow to brown and without annulus. The stem base is bulbous, rooted in the base and immersed in the earth. Spores 10–15 × 6–7 µm and spores print are brown. Reaction with Melzer's reagent was positive (Figure 1; C, D).

***Tricholoma giganteum* Massee, Bull. Misc. Ihf. Kew 1912**

Tricholoma giganteum has a cap of 10–30 cm in size, the colour is cream, white to greyish-white, initially convex, then flat and glabrous. Gills greyish-white, sinuate (notched), crowded of several lengths and 5–11 cm. The stipe is central, white, glabrous, solid, 5–10 × 3–5 cm and without annulus. Spores hyaline, smooth, obviate and 8–10 × 6–7 µm. Spore print white. Cystidia not seen. Hyphae with clamps (Figure 1; E, F).

***Inocybe rimosa* (Bull.)**

This fungus has a small cap, 2–8 cm; conical with a bump in the centre and yellow to brown. Gills attached to the stem in immature state and away from it in mature state and grey to brown. Stipe 50–90 × 7–9 mm, equal, without annulus and whitish to pale yellowish. Spores smooth, ellipsoidal and 9–12 × 4.5–7 µm. Spore print is brown. Cheilocystidia cylindrical and 30–65 x 10–22 µm (Figure 1; G, H).

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Figure 1. *Amanita crocea* (A) and its spores (B); *Boletus comptus* (C) and its spores (D); *Tricholoma giganteum* (E) and its spores (F); *Inocybe rimosa* (G) and its spore (H)

Molecular Identification

Comparing the reported sequences (Table 1) suggested that out of the eight ECM species gathered from various regions in the study area, five were identified as *Amanita crocea*. Other species were identified as *Inocybe rimosa*, *Tricholoma giganteum* and *Boletus comptus*. As well as, the e-value column shows that there is a very high similarity between our sequences and the sequences in NCBI. The internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal gene of all specimens were amplified using ITS4 and ITS1 universal primers, and a single approximately 600–625 bp band was obtained via agarose gel analysis for

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Table 1. The results of DNA sequence analysis of the ITS region based on GenBank BLAST searches

Code	EMF taxon name	ID Accession	BLAST best match to vouchered specimen	Maximum ident. (%)	Base pairs used (number)	Query coverage (%)	*E*-value
AmCr1	Amanitaceae	MF278764	*Amanita crocea*	99	600	98%	0.0
AmCr2	Amanitaceae	MF278765	*Amanita crocea*	98	600	97%	0.0
TrGi1	Tricholomataceae	MG867660	*Tricholoma giganteum*	99	600	99%	0.0
AmCr3	Amanitaceae	MF278766	*Amanita crocea*	98	600	98%	0.0
InRi1	Inocybaceae	MF278770	*Inocybe rimosa*	99	625	99%	0.0
AmCr4	Amanitaceae	MF278767	*Amanita crocea*	99	600	99%	0.0
AmCr5	Amanitaceae	MF278768	*Amanita crocea*	99	600	97%	0.0
BoCo1	Boletaceae	MF278769	*Boletus comptus*	99	600	98%	0.0

BLAST – program that finds similar protein or nucleotide sequences to your target sequence, *E*-value – probability due to chance

each species. The ITS sequences of five specimens of *Amanita* (Accession no.: MF278764 to MF278768), one specimen of *Tricholoma* (Accession no.: MG867660), one specimen of *Boletus* (Accession no.: MF278769) and one specimen of *Inocybe* (Accession no.: MF278770) from Iran showed 98–99% homology with *A. crocea*, 99% homology with *T. giganteum*, 99% homology with *B. comptus* and 99% homology with *I. rimosa* deposited in GenBank. Using the cladistic method, the phylogenetic tree length was 734 with *CI* = 0.87; *RI* = 0.94; *RCI* = 0.82 for all sites and parsimony-informative sites were *iCI* = 0.87 and *iRI* = 0.94. According to phylogenetic analysis, the studied isolates were clustered in a distinct monophyletic group related to *A. crocea*, *T. giganteum*, *B. comptus*, and *I. rimosa* from other authors (Figure 2).

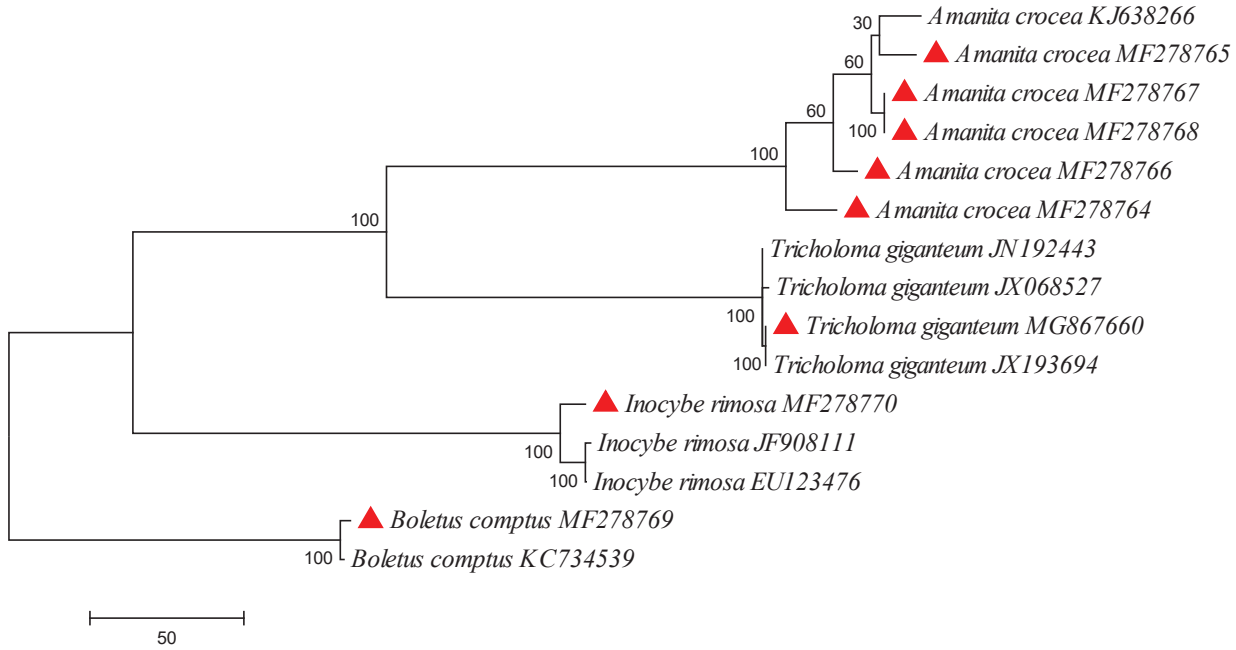

Figure 2. Rooted 50% majority rule consensus tree resulting from 1 000 bootstrap replications of the parsimony analysis of the *ITS* rDNA sequences (Consistency index: *CI* = 0.87; Retention index: *RI* = 0.94; Rescaled consistency index: *RCI* = 0.82). The analysis was conducted using the heuristic search algorithm. Numbers on the branches are the bootstrap values (%). The red triangles refer to our specimens in Iran

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DISCUSSION

In this paper, we recognized four agaric fungi associated with oak trees in Zagros forest according to morphological and molecular characteristics. The correct identification of the fungi, especially agaric fungi, is not always easy (Diez et al. 2002). Identification of ectomycorrhizal (ECM) fungi is traditionally carried out with the help of morphological characteristics (Amicucci et al. 1998) or by biochemical, olfactory, biophysical, and immunological techniques (Papa et al. 1987; Zambonelli et al. 1993; Gandeboeuf et al. 1994; Neuner-Plattner et al. 1999). According to the studies mentioned before, very similar morphological features and the requirement of biological material using accurately identified mycorrhizal fungi is limited (Jamali, Banihashemi 2013).

In recent decades, one useful method for precise identification, taxonomic and phylogenetic studies of ECM has been the amplification of the internal transcribed spacer (ITS) and the intergenic spacer (IGS) (Henrion et al. 1994; Paolocci et al. 1999). In our molecular study, each specimen was amplified using the primer pair ITS1 and ITS4, and an amplicon of about 650–660 bp was obtained for most specimens which was in accordance with others (Diez et al. 2002; Ferdman et al. 2005). Based on the ITS region sequences, these specimens showed 98–99% homology with authentic specimens deposited in GenBank. The accuracy of species identification was confirmed by phylogenetic analysis. The genus *Amanita* (Figure 1; E) has an ectomycorrhizal symbiosis with a variety of host plants, especially oak, beech, and conifers. It is widely found in North America and Europe (Pande et al. 2004). This fungus was previously identified in Iran and the authors reported the morphological characteristics of *A. crocea* and several other species of this genus and achieved similar results (Bahram et al. 2006).

The results of molecular identification for one of the samples led to the identification of *T. giganteum*. In a similar study with molecular sequencing, the identification of this genus and species was carried out using basidiocarps and pure culture of fungi and the authors obtained similar results (Pushpa et al. 2014). The individuals of the genus *Tricholoma* (Figure 1; H) are usually ECM that have a symbiotic relationship with different species of broadleaved and coniferous trees. *Tricholoma giganteum* is edible. In a study regarding the morphological characteristics of this fungus, Kim et al. (1998) gathered similar results. The genus *Inocybe* and the species *I. rimosa* (Figure 1; F) are commonly found in rich soils, and often on limey soils. These fungi have an ectomycorrhizal symbiosis with a wide range of host trees, especially conifers and oak, alder, etc. (Jacobsson 2008; Kirk et al. 2008). The genus *Inocybe* is not usually suitable for eating. The results of morphological identification are in line with the findings of other researchers (Cripps 1997).

Temperate broadleaved forests are the habitat of *Boletus comptus* (Figure 1; G) which has an ectomycorrhizal symbiosis with oak trees (*Quercus*) (Estadès et al. 2004). This fungus found in Europe and in the Mediterranean region such as Italy, Montenegro and Spain is not edible either. The results of morphological identification are consistent with some previous studies (Simonini 1992; Simonini 1998).

In summary, we applied morphotyping and molecular methods to characterize ECM fungi associated with oak trees and four ECM including *I. rimosa*, *B. comptus*, *A. crocea* and *T. giganteum* were identified which are suitable for mycorrhizal symbiosis with the ecological characteristics of Iranian oak trees in the Bivareh habitat. However, with more than 1.8 million hectares of forest coverage with different tree species in the Zagros zone, there is many more ECM that remained to characterize. Moreover, according to the results, the molecular technique was more precise than the morphological method because it characterized other four species. Therefore, the identification of ECM fungi by both methods in these ecosystems is crucial and it should be noted that in poor soils and when there is water shortage due to the low rainfall, the presence of ECM increases soil fertility and water availability.

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