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Molecular identification of several wild species of oyster mushroom (*Pleurotus sp.*) Isolates from Iran

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ABSTRACT: Cultivation of edible mushrooms as a way to use renewable resources in the production of protein-rich foods is considered one of the ways to ensure food security in the world. Among the edible mushrooms, oyster mushroom (Pleurotus sp.) has the second rank in the world production of edible mushrooms. One of the important steps in creating new3 breeds and producing hybrids with high productivity in this edible mushroom is collecting isolates from nature and identifying them at the species level. Through the identification of species, we will be able to identify suitable parents based on desired agronomic traits in crosses and be used in targeted breeding programs. With the expansion of molecular techniques and the use of ITS sequences in different species, it has been possible to identify the investigated species more accurately. 18 isolates of oyster mushrooms were collected from 6 different geographical regions in Razavi, North and South Khorasan provinces. These isolates were identified morphologically and genetically. For genetic study, genomic DNA was extracted from the selected breeds. ITS1 and ITS4 primers were used to amplify genes encoding ITS1, ITS2 and 5.8S rDNA. As a result, out of 18 investigated isolates, 3 isolates that had a significant difference in growth with other isolates were selected. After examining the amplified sequences and their affinity in the databases with the existing identified sequences, it was determined that isolates A2 and B3 belonged to Pleurotus floridanus species and E2 isolate belonged to Pleurotus cystidiosus species.

Key words: edible mushroom, genetic distance of species, taxonomy, ITS sequences

INTRODUCTION

Cultivation of edible mushrooms is one of the biological solutions for using lignocellulosic wastes in the agricultural sector. Biotransformation of lignocellulosic compounds is done by edible fungi, and the use of renewable resources in the production of protein-rich foods provides food security for developed countries (Mandeel et al., 2005). Edible oyster mushrooms have unique enzyme systems that give them the ability to use organic compounds. Millions of tons of unused agricultural waste are used as a substrate for the cultivation of edible mushrooms every year. The used substrates of edible mushrooms are also used as organic fertilizers (Maher, 1991) or animal feed (Grabbe, 1990) and poultry. The unique feature of edible fungi in the extensive use of waste and lignocellulosic biomass enables them to produce a product with food and medicinal quality (Baysal and Peker, 2001).

Edible oyster mushrooms (Pleurotus spp.) occupy the second place in the world production of edible mushrooms, and its annual production is increasing all over the world (Yildiz and Yesil, 2006). Oyster

mushroom cultivation has increased in the last decade due to the simplicity of cultivation, variety of different substrate combinations, no need for complex technologies and high yield potential. Edible oyster mushrooms can use a wide range of agricultural waste and adapt themselves to different environmental conditions. Edible mushrooms can be cultivated in small spaces by smallholder farmers and provide good sources of protein for these growers (Gregori et al., 2007).

In fact, the cultivation of edible mushrooms is the process of protein recovery from lignocellulosic compounds. The protein content of edible mushrooms is known as the main characteristic of these mushrooms. In the sources, an average value of 19 to 35% of the dry weight of mushrooms has been reported compared to 13.2% protein in wheat and 25.2% in milk. Edible mushrooms are also a good source of various vitamins, carbohydrates and fiber (Waser and Weis, 1999).

Generally, morphological and molecular identification methods provide the same results at the genus level. All three investigated isolates belonged to the subfamily Basidiomycota and the genus Pleurotus. However, molecular identification methods enable the identification of other taxonomic units such as species. In the past, oyster mushrooms were placed in the Agaricaceae family based on morphological identification methods until they were established in the Pleurotaceae family by molecular identification methods. This difference among the mentioned methods is due to the difference of taxonomic systems and also the accuracy of molecular methods compared to morphological methods. Morphological classifications are based on the classification of Ainsworth and Webster (Sharma, 1989), while molecular classification systems are based on the comparison of gene sequences of major fungal races, especially rRNA coding genes.

By having reliable molecular identification methods, edible mushroom species can be identified in the shortest time. In breeding programs for performing hybridization or gene transfer, the identification of species of oyster mushrooms will be of great help in the results.

More than 5000 species of edible mushrooms are used as food and medicine all over the world. In the scientific classification of fungi announced by Ainsworth and Webster, most edible fungi are members of the Basidiomycotina and Ascomycotina subfamilies (Sharma, 1989). One of the important steps in creating new races in this edible mushroom is collecting isolates from nature and identifying them at the species level (Agrawal, et al., 2010). By identifying the species and examining the similarities, we can choose suitable parents for crossbreeding and gene transfer. Identification of species in the past years has been done by morphological characteristics. Currently, with the expansion of molecular techniques and the identification of ITS sequences in different species, it has been possible to identify the studied species more accurately. Phylogenetic studies based on the sequence of ITS genes in rDNA show that the asexual species of Neotyphodium are derived from the sexual species of Epichloe. These findings are also confirmed by molecular information obtained from isozyme markers, microsatellites (Brem and Leuchtmann, 2003). Dehghanpour et al. (2006) used ITS sequences and 5.8S rDNA gene to determine the taxonomy of Neotyphodium endophytic fungi.

Edible oyster mushrooms (Pleurotus spp.) have acceptable popularity all over the world, but the type of texture, shape, taste, color and even the place and location of its cultivation are very important in attracting consumers and using these mushrooms (David et al. ., 2007). Several commercial breeds with desirable characteristics have been improved and introduced through their collection and cultivation, but the existence of a collection of wild breeds with suitable traits for exploitation in breeding programs seems essential. Hence, the aim of this research is to conduct genetic studies on wild isolates collected from oyster mushrooms in order to molecularly identify these species using ITS sequences.

Materials and methods

Collection of samples and isolates of oyster mushrooms

Wild species of oyster mushrooms were collected from nature surveys in Razavi, North and South Khorasan provinces. Fresh, healthy and undamaged samples were kept for tests and molecular studies. The mushrooms were washed 2-3 times using running water and for surface disinfection, the collected mushrooms were immersed in 70% ethanol for 5 minutes. Then, under the laminar hood, small pieces of the central part of the mushroom stem and cap were separated and kept in PDA and PGA cultures at 25±2°C for the growth of mushroom mycelium. To obtain pure cultures, subcultures were prepared several times from the samples and then the isolated cultures were kept at 4˚C.

Preparation of seeds from wild breeds of oyster mushroom (spawn)

In order to grow the mycelium of oyster mushrooms collected in the culture medium and obtain mushroom fruit, seeds (spawn) of these breeds were prepared. For this purpose, wheat seeds were used as intermediate culture medium as mushroom seeds. Wheat seeds were boiled and autoclaved in nylon bags with high temperature tolerance. Then, under a sterile hood, these seeds were inoculated with a piece of culture medium containing the mycelium of wild oyster mushroom isolates. Then the spawn bags were kept at 25˚C for 8 days.

Test the ability to grow and colonize the culture medium

To investigate the growth rate and status of oyster mushroom mycelium, the vessel medium was prepared according to the method described by Rajendran et al. (1991). The ingredients in this culture medium were: wheat stubble extract (1%), peptone (0.1%) and agar 1.5%. After cultivation, the mushrooms were kept at a temperature of 25±2°C for 6 days, and after this period, the growth rate was recorded daily.

To investigate the growth characteristics in the culture media, crushed wheat stubble was used as the main media. Wheat stubble was pasteurized using steam at 60°C for 60 minutes. Next, pasteurized cultures were inoculated in nylon bags with dimensions of 40 x 20 cm along with 3% of freshly prepared seeds (spawn) of isolated breeds. These bags were placed at 23-25 ˚C and in the dark for mycelium growth (Mandeel et al., 2005).

Molecular identification of isolates using ITS sequence

The obtained isolates were examined in terms of morphological characteristics in conventional scientific classifications and identification keys (Dung, 2003). For genetic characteristics, from all selected isolates, genomic DNA was extracted using the method of Liu et al. (2000) with some modifications. became Polymerase chain reaction was performed using Bio-Rad CFX96 PCR machine. The materials and concentrations of the compounds used in the PCR reaction are according to Table 1. The sequence of ITS1/ITS4 primers used in this experiment is as follows (White et al., 1990):

ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'

ITS4: 5'-TCCTCCGCTTATTGATATGC-3'

Thermal program of polymerase chain reaction using Taq polymerase enzyme as follows: first cycle: 3 minutes at 94˚C, 35 next cycle: 94˚C for 45 seconds, 60˚C for 50 seconds and 72˚C for 2 minutes and Final cycle: 72˚C for 5 minutes. The PCR reaction products were stored at 4°C and in the refrigerator. To observe and examine the amplified products, 1% agarose gel electrophoresis and TAE buffer with a constant voltage of 7 v/cm were used, and the gel was stained with ethidium bromide.

Amplified fragments were sequenced at Biotech ® in South Korea and blasted with the information available on the NCBI website at http://blast.ncbi.nlm.nih.gov/Blast.cgi to identify edible oyster mushroom species.

Results and discussion

18 isolates were collected from six different geographical regions (Table 2). The isolates obtained from North Khorasan and Razavi provinces had white mycelium, while the mycelium network of mushrooms collected from South Khorasan province were white and gray in color. The appearance of mycelium growth is shown in Figure 2. The results of growth measurements of wild isolates as well as growth characteristics along with their names are given in Table 2.

Most of the strains grew well in the selected culture medium, but this result by itself cannot indicate the proper growth of these isolates in the usual oyster mushroom cultivation media. However, it can be concluded that the species that actively grow in this culture environment can have a good spread in the culture medium. The results of statistical analysis showed a significant difference ($p<0.05$) in the growth rate of this isolate. As a result, three isolates were selected in terms of growth type, which had significant differences with each other, and the next studies were carried out on these three isolates.

Examining the morphological characteristics of mushroom isolates

All three selected isolates had septa and clamp junctions in their mycelia (Figure 1). According to the observations of Sharma (1989), clamp connections have been seen in the cell wall of the secondary hyphae of fungi in most of the genera of the Basidiomycete family. With the naked eye, the hyphae of these isolates were seen as white, but under the light microscope, they were transparent and colorless. Two isolates A2 and B3 were similar and had white mycelium, while isolate E2 had gray hyphae (Figure 2).

Oyster mushrooms are saprophytic fungi and most of its species are edible (Jedinak and Sliva, 2008). In the life cycle of this fungus, its specific sexual structure is the fruiting organ or sporocarp, which consists of a large accumulation of intertwined mycelia. Except for the difference in mycelium color of these isolates, their sporocarps had many common characteristics. Their fruits were fleshy, juicy and with the ability to increase in size during ripening. There were many gills on the underside of the cap of the fruiting body. On the outer layer of these gills, which is called hymenium, spores are produced, which are released after development. On the hymonium layer, there are many basidia with short stalks and four basidiospores. Each basidiospore develops and is released into the air when mature (Dung, 2007). The spores of the examined isolates were colorless, transparent and bean-shaped. At the time of collection, the spore print prepared from them was observed in white color. Based on the morphological classification system of fungi (Sharma, 1989 and Dung, 2003), it was determined that the three isolates A2, B3 and E2 belong to the subphylum Basidiomycota, order Hymenomycetes, order Agaricales, family Pleurotaceae and genus Pleurotus.

Molecular studies in selected isolates

In the extracted DNA samples, the absorption ratio of A260/A280 was in the range between 1.8 and 1.9 and the DNA concentration in the samples was 700-800 ng/µl. These results showed that the extracted DNAs are qualified for the next stages of molecular studies. PCR products using ITS1/ITS4 primer pair had a clear single band between 590-670bp. Figure 3 shows a diagram of the amplified region with ITS1/ITS4 primers. In order to remove waste materials left from PCR and also unused primers, the amplified fragment was isolated from agarose gel using commercial gel purification kit (K-3035-1) of Bioneer company. Repeating the electrophoresis on the sample purified from the gel confirmed the quality and quantity of the isolated fragment for the sequencing process (Figure 3).

The amplified DNA sequence in the identification region of the ITS1/ITS4 primer pair in isolates A2, B3 and E2 with the number of nucleotides 635, 597 and 661, respectively, is as follows:

A_2

01 AGAATTACTA TGGAGTTGTT GCTGGCCTCT AGGGGCATGT GCACGCTTCA
51 CTAGTCTTTC AACCACCTGT GAACTTTTGA TAGATCTGTG AAGTCGTCTC
101 TCAAGTCGTC AGACTTGGTT GCTGGGATTT AAACGTCTCG GTGTGACTAC
151 GCAGTCTATTTACTTACACA CCCCAAATGT ATGTCTACGA ATGTCATTTA
201 ATGGGCCTTG TGCCTTTAAA CCATAATACA ACTTTCAACA ACGGATCTCT
251 TGGCTCTCGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
301 TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACCT TGCGCCCCTT
351 GGTATTCCGA GGGGCATGCCTGTTTGAGTG TCATTAAATT CTCAAACTCA
401 CTTTGGTTTC TTTCCAATTG TGATGTTTGGATTGTTGGGG GCTGCTGGCC
451 TTGACAGGTC GGCTCCTCTT AAATGCATTA GCAGGACTTC TCATTGCCTC
501 TGCGCATGAT GTGATAATTA TCACTCATCAATAGCACGCA TGAATAGAGT
551 CCAGCTCTCT AATCGTCCGC AAGGACAATTTGACAATTTG ACCTCAAATC
601 AGTAGGACTA CCCGCTGAAC TTAAGCATAT GACCT

B_3

01 AGTCTTCCCA ACCACCTGTG AACTTTTGATAGACAGTGAA GTCGTCTCTC
51 AAGTCGTCAG ACTTGGTTGC TGGGATTTAA ACGTCTCGGT GTGACTACGC
101 AGTCTATTTA CTTACACACC CCAAATGTAT GTCTACGAAT GTCATTTAAT
151 GGGCCTTGTG CCTTTAAACC ATAATACAAC TTTCAACAAC GGATCTCTTG
201 GCTCTCGCAT CGATGAAGAA CGCAGCGAAA TGCGATAAGT AATGTGAATT
251 GCAGAATTCA GTGAATCATC GAATCTTTGA ACGCACCTTG CGCCCCTTGG
301 TATTCCGAGG GGCATGCCTG TTTGAGTGTC ATTAAATTCT CAAACTCACT
351 TTGGTTTCTT TCCAATTGTG ATGTTTGGAT TGTTGGGGGC TGCTGGCCTT
401 GACAGGTCGG CTCCTCTTAA ATGCATTAGC AGGACTTCTCATTGCCTCTG
451 CGCATGATGT GATAATTATC ACTCATCAAT AGCACGCATG AATAGAGTCC
501 AGCTCTCTAA TCGTCCGCAA GGACAATTTG ACAATTTGAC CTCAAATCAG
551 GTAGGACTAC CCGCTGAACT TAAGCATATC AATAGACGGA GGAAGGA

E_2

01 ATACATTCAA CCACTTGTGC ACTTTTGATA GATTCGCAGA GTTGCCCTCT
51 CAGGTCAGTA AATGACTTGG TTGGTCGGGATTGTCACAGT CCTGGCTTTG
101 ACTTTGTGGG TCTATTATCT TATACACACT TGTATGTCCA TGAATGTTAT
151 TTTCTTGGGC CATGTGCCTA TAAAACCTAA TACAACTTTC AACAACGGAT
201 CTCTTGGCTC TCGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG
251 TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC ACCTTGCGCC
301 CCTTGGTATT CCGAGGGGCA TGCCTGTTTG AGTGTCATTA AATTCTCAAA
351 TCTATAGAGC TTTTTTGTGA TATAGATTTG GATTGTTGGG GGCTGCTGGC
401 TTTTTACCAA GTTGGCTCCT CTTAAATGCA TTAGCGGGAC TTTATTGCCT
451 CTGCGCACAG TGTGATAATT ATCTACGCTG GCCGACATGC AATGACTTTA
501 CAAGTCCAGC TTTCTAACTG TCTTTCAAGA CAATGACTTG ACAATTTGAC
551 CTCAAATCAG GTAGGACTAC CCGCTGAACT TAAGCATATC AATAAGCGGA
601 GGAAAGATCA TTAATGAATT ACTCATGAAG CTGATGCTGG TCTCTCGGGA
651 CATGTGCACG C

The obtained sequences were compared with the information available in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Comparison of the amplified fragment in A2 and B3 isolates with

the identification region of the ITS1/ITS4 primer pair (which includes ITS1 gene sequence, 5.8S rDNA gene, ITS2 and part of the 28S rDNA gene sequence) with Pleurotus floridanus shows a great similarity in It was the mentioned region, so that the two isolates A2 and B3 in the amplified region had a similarity of 99% with the sequences registered under accession numbers GU721058 and FJ810170.1. Therefore, two isolates belonging to Pleurotus floridanus species were introduced.

Fungi (branch), Basidiomycota (suborder), Agaricomycotina (order), Agaricomycetes (suborder), Agaricomycetidae (order), Agaricales (suborder), Pleurotaceae (family), Pleurotus (genus), Pleurotus floridanus (species)

In isolate E2, 99% homology with the identification region of the ITS1/ITS4 primer pair was observed with Pleurotus cystidiosus with accession number DQ978222.1, and this oyster mushroom isolate was placed in the following grouping.

Fungi (branch), Basidiomycota (suborder), Agaricomycotina (order), Agaricomycetes (suborder), Agaricomycetidae (order), Agaricales (suborder), Pleurotaceae (family), Pleurotus (genus), Pleurotus cystidiosus (species).

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