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# MOLECULAR IDENTIFICATION OF *MARASMIUS OREADES* - AN EDIBLE MUSHROOM FROM PAKISTAN BASED ON ITS-rDNA SEQUENCE DATA

**A. Razaq[*], S. Ilyas and A.N. Khalid**

**Department of Botany, University of the Punjab, Lahore, Pakistan**
**[*]Corresponding author's e-mail: ectomycorrhiza@gmail.com**

*Marasmius oreades* is common edible species which has been collected from Himalayan moist forests of Pakistan. This species has been characterized on morphological and molecular basis. This study is first imitative to identify this species using morpho-taxonomical characteristics and sequencing of internal transcribed spacers (ITS). Taxonomically, this species was previously not described from Himalayan part of Pakistan. Earlier studies remained restricted to few macroscopic studies. Macrosopic characters like size, shape and colour of pileus, stipe and spore are compared in literature for its identification. The gene sequence of this sample thus obtained where BLAST and checked for homology searches using NCBI. The results depicted 99% homology of these samples with same species reported from other parts of the world. Maximum likelihood method of phylogenetic analysis also helps to separate it from closely related species.
**Keywords:** ITS-rDNA, edible mushroom, Himalayan moist forests, Marasmiaceae

## INTRODUCTION

*Marasmius* Fr. (Agaricales, Marasmiaceae) has approximately 600 species worldwide (Wannathes *et al.*, 2009). This euagaric (gilled mushrooms) genus has small to medium basidiocarps with smooth pileus, adnexed to free, spaced lamellae, white, apiculate basidiospores. Moncalvo *et al.* (2002) determined the polyphyletic nature of this genus using molecular tools which changed the monophyletic idea about this group (Antonín and Noordeloos, 1993). This is the reason why mycologists now have switched to molecular taxonomy for identification of fungi especially based on ITS-rDNA marker.

The internal transcribed spacer (ITS) region contains two polymorphic/ variable non-coding regions that are nested within the rDNA repeat between the highly conserved small subunit (18S), 5.8S and large subunit (28S). This region has interspecific variable but intraspecific conserved sequences, high copy number, shorter size (650-900bp), conserved priming sites and a huge amount of sequence data (38,089 accessions in the database) in GenBank for comparison, so these properties make this region more powerful tool for identification of mushrooms (Gardes and Bruns, 1993; Bruns and Shefferson, 2002, 2004). This marker is used for fungal barcoding and phylogenetic studies of macrofungi (Razaq *et al.*, 2012a, Razaq *et al.*, 2013). *Marasmius* is distributed in North America, Europe and Asia (Wannathes *et al.*, 2009).

From Southeast Asia a number of renowned publications on *Marasmius* using molecular markers, (Desjardin *et al.*, 2000) in Indonesia, (Tan *et al.*, 2007) in Malaysia, and (Desjardin *et al.*, 2004) and (Wannathes *et al.*, 2004, 2007 and 2009) in Thailand are available. But from south Asia recently mycologists have started to describe species on phylogenetic species concept for identification of euagrics (Razaq *et al.*, 2012b) in Pakistan, (Aravindakshan *et al.*, 2012) in India.

We are describing molecular identification of *Marasmius oreades* collected from Himalayan part of Pakistan on the basis of ITS-rDNA marker to update mycological flora of this region. Pakistani people of hilly areas commonly used it as food and thinking it medicinal herb for stomach. The presence of trehalose sugar makes this mushroom sweet in taste and only the caps are used for edible purpose while the stems are unappetizing. The fairy rings of *M. oreades* are infamous as growth repressor for tree in their vicinity (Blenis *et al.*, 1997). The genetic variation of ITS region and morphological characters confirm the identification of Pakistani collection as *M. oreades*.

## MATERIALS AND METHODS

***Morphological characterization*:** Basidiocarps of the species were carefully dugout with the help of a sharp knife and photographed on the spot. Morphological characters were noted in the field. Sections of lamellae were prepared stained with Congo Red and Melzer's reagent. Size dimensions were determined for 25 basidiospores, 20 basidia, 20 cystidia and 20 pileal element from each basidiomata under the light microscope equipped with camera lucida.

***DNA Isolation and amplification of ITS*:** Dried tissue (approx. 100mg) was macerated by pestle mortar using liquid nitrogen. The macerated tissue was gently shifted in to 1.5ml centrifuge tube and 1ml of 2% CATB buffer was added; it was mixed gently. All tubes were carefully incubated at 65°C for one hour in water bath but after each

twenty minutes the tubes were inverted and mixed gently. For removal of cellular junk or proteins, 600 µl of Isoamly alcohol: Chloroform (1:24) was added in each tube and gently vortex to mix all in the tubes. The mixture was clarified by centrifugation for 10min at maximum speed. The supernatant was taken in another tube by careful pipetting without touching the pellet between two layers. The DNA was precipitated by adding 2/3 volume of ice-cold Isopropanol or ½ volume 95% ethanol and placed at room temperature for a few minutes or in freezer for overnight. DNA was collected by centrifugation at 15,000rpm and pellet was washed with 70% ethanol or wash buffer. The final pellet was dried at room temperature or at 370C in an oven and dissolved in 50 µl TE buffer or distilled H2O. ITS region of rDNA was amplified using fungal primer pair ITS1F and ITS4 (White *et al.,* 1990). PCR was performed in 20-µl reaction volume following the protocol given by Gardes and Bruns (1993). The size of PCR product was determined on 1.5% agrose gel in Gel documentation system (UVtec, Avebury House, Cambridge CB4 1QB UK) using default setting. PCR product was directly sequenced in both directions using the same pair of amplification primers (Macrogen, Korea).

***Sequence analysis and phylogeny*:** The generated sequence was edited manually using BioEdit sequence alignment editor version 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, California). For initial comparison and alignment of the sequence, BLAST (Basic Local Alignment Search Tool) analysis was performed using the National Center for Biotechnology Information (NCBI), USA database. For further phylogenetic analysis and alignment of sequence, closely related 28 sequences were retrieved from GenBank. The newly generated sequence and those which retrieved from the GneBank were aligned by using Cluatal W program of Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura *et al.,* 2011). Phylogenetic analysis was determined following Razaq *et al.,* (2012b). Consensus nucleotide sequence of *M. oreades* was submitted to European Molecular Biology Laboratory (EMBL) database under accession number HF546217.1.

## RESULTS

**Morphology and taxonomy:**

***Marasmius oreades (Bolt.: Fries) Fries Epicr. Myc. 375. 1838*:** Pileus, 3.0–5.5 cm wide, pale brownish to pale ochraceous, centrally ochre-brown fading towards margins, darker when young, convex-conical with broad obtuse umbo, then plano-convex to applanate with undulate to crenulated margins, glaborous, hygrophorus, context, fragile


**Figure 1.** ***Marasmius oreades.*** **1, 2 Basidiomata with pileus and lamellae. 3 Basidiospores. 4 Hymenium with basidia. 5 Pileal elements. 6 Cauilocystidia. Bars = 1–2= 1.2 cm, 3= 3 µm, 4, 6= 13.5 µm, 5= 7 µm.**

to thin, soft, unchanging when bruised or cut (Fig.1). Lamellae whitish to pale cream-yellow, free to adnate, emarginated, subdistant to distant, broad, 33mm thick, intervenose when old, edges entire to blunt, concolour with pileus; lamellulae absent. Stipe 6.0 × 0.38 cm, cylindrical to slightly broaden at base, pale brown when young, darker with age, light brown to dark brown towards the apex, smooth; context pale brown, uniform, not bulbous toward base, without volval scales or zones, without ring; universal veil absent; Odor and taste: not recorded.

Basidiospores 7.5–9.5 × 3.6–5.0 µm, elliptical to ellipsoid, inequalateral, apiculate (pointed at one end, smooth, hyaline, non-amyloid. Basidia 35–48 × 7.0–9.5 µm, 4-spored, clavate. Basidioles 33.0–41.5 × 6.0–9.5 µm, cylindrical, clavate or subfusoid. Pleurocystidia and cheilocystidia absent. Pileipellis is hymeniderm composed of cells 18.0–31.5 × 12.5–20.0 µm, clavate or pyriform, regular to irregular or with scattered projections, thin to slightly thick-walled, smooth, hyaline to pale yellowish in 5% KOH. Caulocystidia 39.5–86.5 × 9.0–12.5µm, variable in shape, clavate, fusoid, subcylindrical, mostly irregular, lobate, branched to coralloid, thin-walled.

***Material examined*:** Pakistan: Khyber Pakhtunkhwa, Abbotabad, Ayubia-Khanspur, on the ground humified soil under *Pinus wallichina* vegetation, small groups, Abdul Razaq, KP-03, 18-08-2009, submitted to herbarium, Department of Botany, University of the Punjab, Lahore. LAH. 18080903.

***Molecular characterization*:** *Marasmius oreades* species collected from Pakistan is described on molecular basis using base sequence of rDNA gene. The two non-coding (ITS1 &ITS2) species specific region of Internal transcribed spacers (ITS) of rDNA along with 5.8S region are amplified and sequenced. The sequence was analyzed by comparing with the data in the GenBank using BLAST (Basic local alignment search tool) searches and phylogenetic analysis of sequence as well.

***Sequencing of ITS-rDNA*:** The target region comprising of internal transcribed spacers (ITS1 & ITS2) and 5.8S of rDNA generated fragments of approximately 800bp on amplification in polymerase chain reaction (PCR) using fungal universal primers pairs (ITS1&ITS4). Initial BLAST analysis of nucleotide sequences revealed that Pakistani collection matches maximum with *Marasmius oreades* (GenBank accession # FJ481042.1) 99%, Query coverage 100 %, E-value 0. All the sequences in Blast analysis of Pakistani sequence belong to the same genus or different genus of the same family. The percentage base similarity of local collection is noted maximum with *M. oreades* (FJ481042) from China. Other closely related sequences in the Blast below the closest sequence showed 90%, 89%, 88% and 87% with *M. maximus* Hongo (FJ904977.1, Republic of Korea), *M. nivicola* Har.Takah. (FJ904973.1, Republic of Korea), *M. wynneae* Berk. & Broome (FJ904979.1, Republic of Korea) and *M. graminum* (Lib.) Berk. (GenBank accession # JN943595.1, Denmark) respectively.

***Phylogenetic analysis*:** The phylogenetic analysis of *Marasmius oreades* collected from Pakistan was carried out using maximum likelihood method. The sequences included in this analysis had 1031 characters, from which 749 characters were used in final analysis after trimming the alignment from both 5' and 3' sides. In phylogenetic analysis gaps are treated as data. A total 28 sequences of *Marasmius* species were included in phylogenetic analysis out of which two have been collected from Pakistan. To clarify the phylogenetic position of each Pakistani collection, sequences of different sections of the genus were retrieved from the GenBank database. There are two major clades in the phylogenetic tree (Fig. 2) which have been labeled as clade I and clade II. All the Pakistani species lie in clade II of the tree and none clustered in clade I. The Clade II contains some closely related species like *Marasmius albimyceliosus* (EU935544.1), *M. brunneospermus* (FJ904966.1, FJ904967.1, FJ904968.1, FJ904969.1), *M. oreades* (FJ481042.1) and *M. maximus* Hongo (FJ904974.1, FJ904975.1, FJ904976.1, FJ904977.1). Pakistani collection of *M. oreades* (HF546217) clustered with the same Chinese collection (FJ481042) under a significant bootstrap value (97%).

## DISCUSSION

*Marasmius oreades* is characterized by glabrous, cream to light-colored, umbonate pileus, adnate to nearly free, well spaced lamellae, tough, and white spores, cystidia absent. It forms distinctive circular fruitings in grassy areas for it is called its name. It was described first time by Fries (1838). It is a common edible euagric mushroom which is distributed worldwide through all the continents except Antarctica (Thiers, 1982) Europe to North America (Antonín and Noordeloos, 2010, Desjardin, 1987) but rarely described from south Asia especially from Himalayan range.

Desjardin (1987) described the 7–8.5 × 4–5.5 µm, hyaline, smooth spore for *M. oreades* and Thiers (1982) given this range as 6.6–9.0 × 3.9–6.0 µm for *M. oreades*. But spore size of Pakistani collections (7.5–9.5× 3.6–5.0 µm) is almost same as given by these authors. This spore size and shape indicated that Pakistani population of *M. oreades* is similar with North American and European individuals. It is also evident that this edible species is found not only in European continent but also in Asian earth indicating its cosmopolitan habitat. Sometimes the only spore size is not the good indicator of species differentiation, for example, Antonín *et al.* (2010) also had given spore size for *M. maximus* Hongo (7.0–9.5 × 4.5–6.0 µm) which is closely related to *M. oreades*. The other important character, in this case, for species delimitation is the presence of cheilocystida in

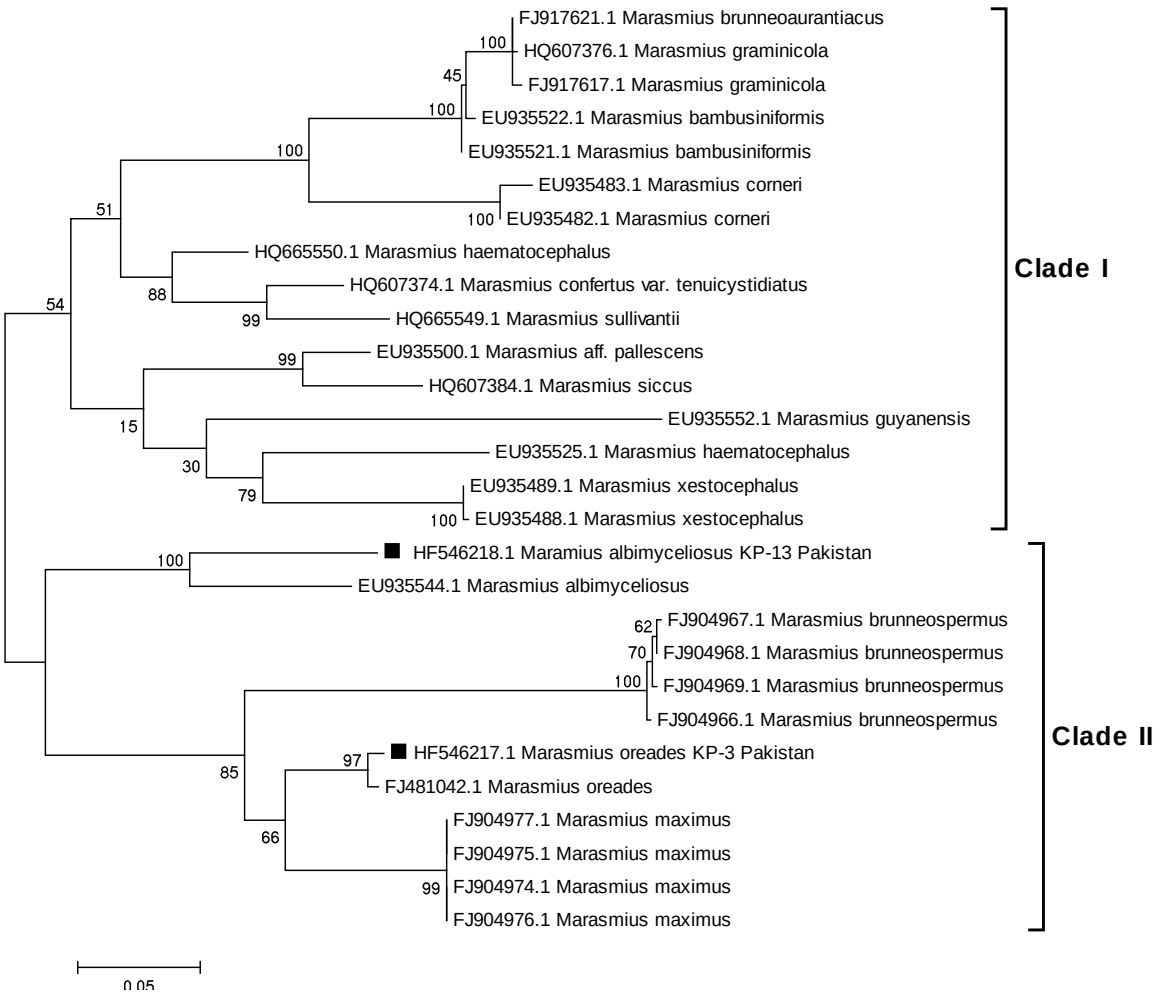


**Figure 2. Phylogenetic analysis of *Marasmius oreades* collected from Pakistan based on nrITS-rDNA regions. This tree is based on maximum likelihood method using Jukes-Cantor model. The values given above branches represent bootstrap values while values given below represent branch lengths.**

former which are lacking in latter. The similar morphology, same spore size and easily overlooking of cheilocystidia in *M. maximus* may lead to misidentification of this species. Therefore this situation is confusing but the molecular approach has solved this problem of misidentification because this morphological plasticity remains transparent on DNA nucleotide bases level.

In the molecular analysis, ITS-rDNA sequence of Pakistani *M. oreades* (HF546217.1) matched 99% with Chinese sequence of same species (FJ481042.1) indicating that the genetic makeup of both individuals are almost same though both populations belong to two different regions. Generally, the sequence similarity of two individuals above 97% shows that both individuals are conspecific (Vellinga, 2003; Razaq *et al.,* 2012a, 2013). The remaining 1% dissimilarity is also due to bio-geographical variation of populations. Similarly, the same co-relation is also determined by maximum likelihood, phylogenetic method, based on ITS-rDNA sequences.

In the phylogenetic analysis, all *Marasmius* sequences formed two clades on the basis of sequence similarity. Pakistani sequences lies in clade II of Fig. 2, *M. oreades* from Himalayan region clustered with Chinese sequence very clearly showing conspecific relationship between the both. The higher bootstrap value (97%) and similar branch lengths of both nodes of this species also indicate that these two are different individuals of the same species. Morphologically, *Marasmius maximus* is very closely related species with *M. oreades* which has very different genetic makeup. In the phylogenetic analysis (Fig. 2, Clade II), both *M. oreades* and *M. maximus* form very distinct sub-clades in clade II of Fig. 2 confirming that the presence of cheilocystidia is a good taxonomic character for species delimitation. These findings are in accordance with Wannathes *et al.* (2009) and Antonín *et al.* (2010). In both

these articles *M. oreades* always separated from *M. maximus* in the same section very clearly. All the above discussion can be concluded that Pakistani *M. oreades* is a different individuals of the same population which is distributed in Europe, North America and China. Although, the geographic distribution of this population is different yet the basic morphology and molecular similarity is same among all populations.

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