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Original Research

Morphological and molecular characterization of coprinoid fungi newly recorded for the mycobiota of Iran

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Abstract: Twenty-five specimens of coprinoid fungi were collected during an ongoing survey of agaric fungi in Kermanshah Province, western Iran. The specimens were identified based on morphological characteristics and molecular analysis of internal transcribed spacer sequences. Five species of *Coprinellus viz C. disseminates, C. flocculosus, C. micaceus, C. radians and C. xanthothrix*, three species of *Coprinopsis viz C. atramentaria, C. insignis* and *C. semitalis* and two species of *Coprinus viz C. pinetorum* and *C. sterquilinus* were identified. Among the species identified in this research, three unreported species from Iran namely *C. insignis, C. semitalis* and *C. pinetorum* are reported. Detailed morphological descriptions and illustrations of this three newly-recorded species were provided here and their evolutionary relationships were presented by the constructing of a phylogenetic tree.

Key words: *Coprinopsis*; *Coprinellus*; *Coprinus*; Kermanshah.

Introduction

The coprinoid or inky cap fungi have long been classified under the family name Coprinaceae and the genus *Coprinus* Pers. (1, 2). The genus was characterized by a unique set of characters including the deliquescent nature of the lamellae, sequential development of basidia, the dark pigment of the basidiospores and presence of pseudoparaphyses in the hymenium (3). However, molecular phylogenetic studies revealed that the coprinoid fungi are a highly diverse and polyphyletic group. In fact, *Coprinus comatus* along with one close ally, C. *sterquilinus* appeared that are more related to the family Agaricaceae (4). Consequently, *Coprinus sensu lato* subdivided into two families and four genera. *Coprinus sensu stricto* assigned to the Agaricaceae and the rest species split into three newly defined genera including *Coprinellus*, *Coprinopsis* and *Parasola* in the family Psathyrellaceae (5).

Coprinoid fungi are saprotrophic and found solitary, gregarious or in big clusters on a variety of substrates including: humus, dead wood, on vegetal debris, dung, etc. (3).

In the genus *Coprinellus*, immature lamellae are not pinkish, the veil may be present or absent; pileus ranging from deliquescent to non-deliquescent during sporulation; pileipellis consists of a hymeniderm or cystoderm of globose or piriform cells and ozonium may present or absent. The genus *Coprinopsis* has a cutis-like pileipellis, universal veil remnants appear as shaggy scales or broad membranous patches on the surface of the pileus; lamellae in this genus are always deliquescent. *Coprinus* is characterized from other coprinoid fungi by the appearance of veil remnants as floccose scales firmly attached to tramal tissues, cottony annulus and pseudovolva, the presence of a central suspended strand in the hollow stipe and the absence of pleurocystidia (5).

According to the literature, two species of *Coprinus*, 10 species of *Coprinellus*, 16 species of *Coprinopsis* and six species of *Parasola* have been reported from Iran (Table 1).

Here, we provide macro and micro-morphological descriptions and illustrations of three coprinoid species previously not-reported from Iran.

Materials and Methods

Sampling and morphological Identification of Fungi

Coprinoid specimens were collected from Kermanshah Province, western Iran, during frequent field surveys from 2014 to 2017. The fresh specimens were photographed before collection to save distinctive characters. Moreover, GPS coordinates were recorded for each collecting site. The specimens were examined based on macro and micro-morphological characteristics. Morphological examinations were performed using fresh specimens. Hand sections of the gills and the surface of the pileus and stipe were mounted in water and micromorphological features including basidiospores, basidia, cystidia and pileipellis were observed, measured and photographed using a light microscope (OLYMPUS BX51). The dimensions of spores, basidia and cystidia were calculated from measurements of 25- 30 randomly selected structures. The specimens were identified using

Table 1. List of Coprinoid fungi reported from Iran.

Species	Locality	References
Coprinus comatus	Arasbaran, Nur, Orumieh, Shandiz, Tehran	(6-10)
C. sterquilinus	-	(11)
Coprinellus truncorum	Arasbaran	(12)
C. micaceus	Ghamsar, Karaj, Orumieh, Shandiz	(10, 13-17)
C. angulatus	Evin	(9)
C. disseminatus	Tehran, Zabol	(8, 18, 19)
C. domesticus	-	(18)
C. impatiens	-	(18)
C. flocculosus	Tehran	(8, 18)
C. subimpatiens	Karaj	(9)
C. tardus	-	(18)
C. xanthothrix	-	(18)
Coprinopsis atramentaria	Shandiz, Ghamsar, Chalus, Karaj	(10, 19, 20)
C. picacea	Arasbaran, Karaj, Khorramabad, Shandiz	(7, 9, 10, 18, 19)
C. nivea	Arasbaran, Karaj	(9, 18)
C. cinerea	Karaj, Shahriar	(9)
C. friesii	-	(18)
C. gonophylla	-	(18)
C. lagopus	Babolsar, Karaj	(8, 18)
C. brunneofibrillosa	Nur, Tehran	(8, 9)
C. ephemeroides	Karaj	(9)
C. lagopoides	Tehran	(9)
C. macrocephalus	Tehran	(9)
C. martinii	Kashan	(19)
C. cordispora	Tehran	(9)
C. scobicola	Evin	(21)
C. urticicola	Amol	(22)
C. spelaiophila	Gorgan	(23)
Parasola leiocephala	Arasbaran	
P. miser	Ghamsar	(13, 18)
P. auricoma	-	(18)
P. hemerobia	-	(18)
P. kuehneri	-	(18)
P. plicatilis	Mashhad, Nur, Tehran	(8, 10, 21)

available literature (2, 3, 24, 25). A specimen from each identified species collected from the different geographical region was deposited in the herbarium of fungi, Iranian Research Institute of Plant Protection, Tehran, Iran.

DNA extraction and PCR amplification

Genomic DNA of selected specimens was extracted using the Fungi DNA extraction kit (Denazizst Asia, Iran). The ITS-rDNA region was amplified using the primer pairs ITS1/ ITS4. The PCR mixture was prepared by mixing about 50 ng of template DNA, 10 µM of each primer and 25 µL of a ready master mix (Sinagen Company, Iran) in a final volume of 50 µL. Cycling conditions consisted of an initial denaturation at 90 °C for 2 min, 35 PCR cycles of denaturation at 95 °C for 40 s, annealing at 57 °C for 40 s and extension at 72 °C for 50 s. These were followed by a final extension at 72 °C for 10 min using a Biometra thermocycler (Tpersonal, Germany). The amplification products were separated on a 1% agarose gel. The gel was stained with Red Gel and visualized under UV to confirm DNA amplification. The PCR products were purified and sequenced by Macrogen, Inc. (South Korea).

The sequences were manually edited using the Bioedit software (26). Edited sequences were submitted to the GenBank (http: //www.ncbi.nlm. nih.gov/genbank) (Table 2).

Phylogenetic analysis

The sequences were compared with the sequences available in the GenBank database to find the most similar ones using Basic Local Alignment Search Tool (BLAST) algorithm. Multiple sequence alignments of the newly generated sequences and sequences of the valid species, derived from the GenBank (Table 2) were performed with Clustal X software version 2.0.11 (27), checked and improved manually where necessary. The maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses were performed

Table 2. The list of the species and specimens of coprinoids were used for phylogenetic analyses based on ITS sequence in this study.

Species	Locality	Herbarium Code	Accession Number
Coprinellus disseminatus	**Iran**	**16895**	**MH178667**
C. disseminatus	Hungary	-	LT549082
C. disseminatus	Hungary	-	JN159561
C. xanthothrix	**Iran**	**16901**	**MH259846**
C. xanthothrix	Hungary	-	HQ847044
C. xanthothrix	Hungary	-	JN943112
C. xanthothrix	Hungary	-	JN159578
C. flocculosus	**Iran**	**16892**	**MH174090**
C. flocculosus	**Iran**	**16900**	**MH179321**
C. flocculosus	Hungary	-	FN396138
C. flocculosus	Hungary	-	JN159576
C. flocculosus	USA	MICH:232861	KM403380
C. radians	**Iran**	**16976**	**MH259844**
C. radians	**Iran**	**-**	**MH715394**
C. radians	USA	SDR-MM5680	MG748587
C. radians	China	-	HQ380760
C. radians	China	-	KT192203
C. micaceus	**Iran**	**16893**	**MH715395**
C. micaceus	**Iran**	**-**	**MH259302**
C. micaceus	**Iran**	**-**	**MH259838**
C. micaceus	**Iran**	**16899**	**MH259839**
C. micaceus	USA	DBG:24506	KR338834
C. micaceus	Hungary	-	GU227721
C. micaceus	Hungary	-	JN943115
Coprinopsis insignis	Iran	16905	MH259868
C.insignis	Tunisia	-	KU973838
C. insignis	Hungary	-	FN396124
C.insignis	Hungary	SZMC-NL-1510	JX118738
C.semitalis	Iran	16906	MH304260
C.laanii	Netherlands	CBS476.70	GQ249276
C. sclerotiger	USA	-	KR869759
C. sclerotiger	**Iran**	**-**	**MF161091**
C. atramentaria	**Iran**	**16907**	**MH259868**
C.atramentaria	China	SX2014092607	KR733589
C. atramentaria	United Kingdom	-	KF897018
C. atramentaria	Hungary	-	FN396110
Agrocybe praecox	USA	MSC 378486	AY194531
Agaricus bisporus	China	-	FJ223230
Coprinus sterquilinus	**Iran**	**16902**	**MH715356**
C.sterquilinus	Korea	-	AF345821
C.sterquilinus	Italy	15595	JF907843
C. pinetorum	**Iran**	**16903**	**MG372061**
C. pinetorum	Spain	AH:45815	KU686927
C. pinetorum	Spain	AH:45798	KU686926
C. pinetorum	Spain	AH:45797	KU686925

Newly generated sequences are in bold.

using the MEGA5 software. The bootstrap values with 1000 replicates were performed to determine branch support. (28).

Results and Discussion

During the field surveys, a total of 25 specimens of coprinoid fungi were collected. PCR amplifications of the ITS–rDNA region with primers ITS1 and ITS4 generated DNA fragments about 550–670 bp. (Fig. 1). The fragments were successfully sequenced for selected specimens. Based on the morphological examinations and rDNA-ITS sequences data, five *Coprinellus*, three *Coprinopsis*, and two *Coprinus* species were identified.

The five *Coprinellus* species included *C. disseminates, C. flocculosus, C. micaceus, C. radians* and *C. xanthothrix*. The *Coprinopsis* species included *C. atramentaria, C. insignis* and *C. semitalis*. The *Coprinus* species included *C. pinetorum* and *C. sterquilinus*.

Among the species identified in this study, *C. insignis, C. semitalis* and *C. pinetorum* are new records to the mycobiota of Iran. Morphological descriptions and illustration of this three newly-recorded species in this study were given in alphabetical order as follows:

***Coprinopsis insignis* (Peck) Redhead, Vilgalys & Moncalvo 2001**

Pileus 4-8 × 3-4 cm when still closed, ovoid or ellipsoid, then, expanding to conical to campanulate, at first white with a silvery delicate fibrillose veil, then, becoming greyish pale-brown to gray toward margin and pale brown toward the centre (Fig 2, a). Veil remnants are evanescent. Gills, free, crowded, deliquescent, first white, then greyish-brown to black. The spore print is dark brown. Stipe 8-10×1-1.5 cm, white in colour, hollow with the clavate base (Fig 2, c). Spores 11-14.7 × 6.7-8.3 µm, amygdaloid, warty, rounded or slightly conical at the base, papillate at apex, very dark red-brown; with central narrow germ pore (Fig 2, e). Basidia, clavate 23.8-30.9 × 10.3-11.5 µm, four-spored. Pleurocystidia, utriform to subcylindrical (Fig 2, b). Veil consisted of cylindrical hyphoid elements (Fig 2, d). This species is easily characterized from closely related species by its amygdaloid, warty spores and its velar hyphoid elements.

Specimens examined. IRAN, Kermanshah province, Gilan-e Gharb, Qalajeh, found in a cluster on soil near *Querqus* tree, 19 May 2015, P. Seidmohammadi, IRAN 16904F; Kermanshah province, Islamabad-e Gharb, Beigrezaei, found in a cluster on soil near *Querqus* tree, 15 May 2015, P. Seidmohammadi, IRAN 16905F.

***Coprinopsis semitalis* (P.D. Orton) Redhead, Vilgalys &Moncalvo 2001**

Pileus 3-2×0.6-1 cm in diameter, when still closed, subglobose, ovoid to oblong, grey to grey-brown, covered with a powdery floccose veil (Fig 3, a). Gills, free, crowded, deliquescent, first white, and finally black. Stipe, 2-4× 0.2-0.4, whitish; cylindrical and hollow. Basidia 4-spored, Pleurocystidia and cheilocystidia not observed because of deliquescent gills. Spores 10.3-10.8×7.5-8.1 µm, ellipsoid or ovoid, dark red-brown, have a loosening perispore that makes the spores winged (Fig 3, b), germ pore central. Veil consisted of globose, verruculose cells (Fig 3, d).

Specimen examined. IRAN, Kermanshah province, Islamabad-e Gharb, Beigrezaei, found in a cluster on animal dung, 31 March 2016, E. Seidmohammadi, IRAN 16906F.

***Coprinus pinetorum* G. Moreno, Carlavilla, Heykoop & Manjón, in Crous et al., Persoonia 36: 427 (2016)**

Pileus 4-5 × 3–4 cm when still closed and young, ellipsoid to subcylindrical (Fig 4, a), then, the margin begins to flare out and split. Meanwhile, it appears to melt into an inky, black liquid. The *Pileus* is white at first with fibrillose to the flocculose surface, then, covered with whitish to light brownish scales (Fig 4, a). Gills

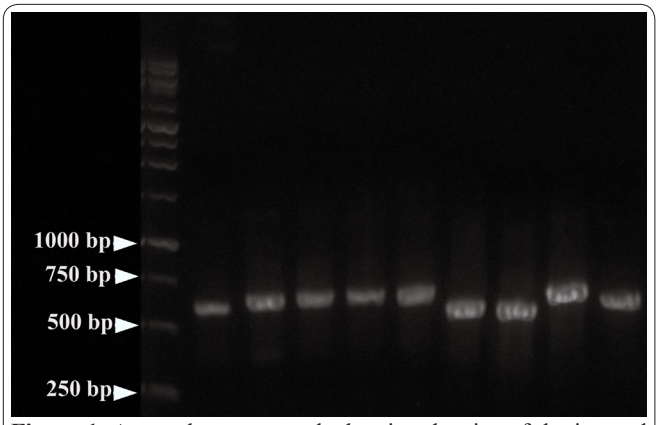

Figure 1. A sample agarose gel, showing the size of the internal transcribed spacer (ITS) region of some coprinoid fungi measured by gel electrophoresis of PCR products, amplified by primers ITS1 and ITS4.

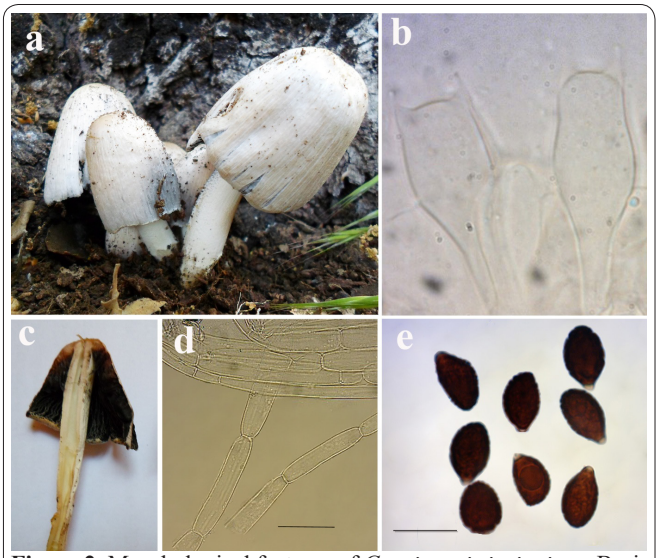

Figure 2. Morphological features of *Coprinopsis insignis*; **a.** Basidiocarp; **b.** Basidia; **c.** Vertical section of basidiocarp showing hollow stipe; **d.**Hyphoid elements of veil; **e.** Spores. Scale bar: 10 µm.

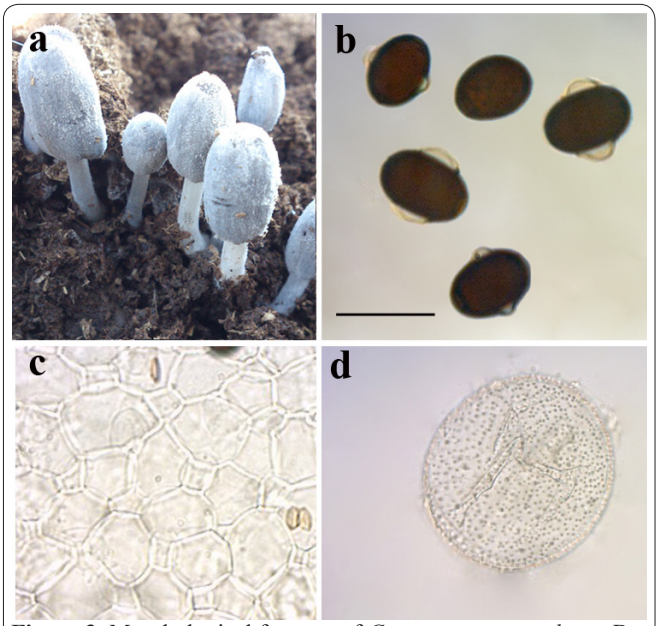

Figure 3. Morphological features of *Coprinopsis semitalis*; a. Basidiocarps; b. Winged spores; c. Pileipellis; d. Verruculose element of the veil. Scale bar: 10 µm.

are free, crowded and white when young, then become pinkish and finally black. They are strongly deliquescent when mature. The spore print is black. Stem, 5–7 ×

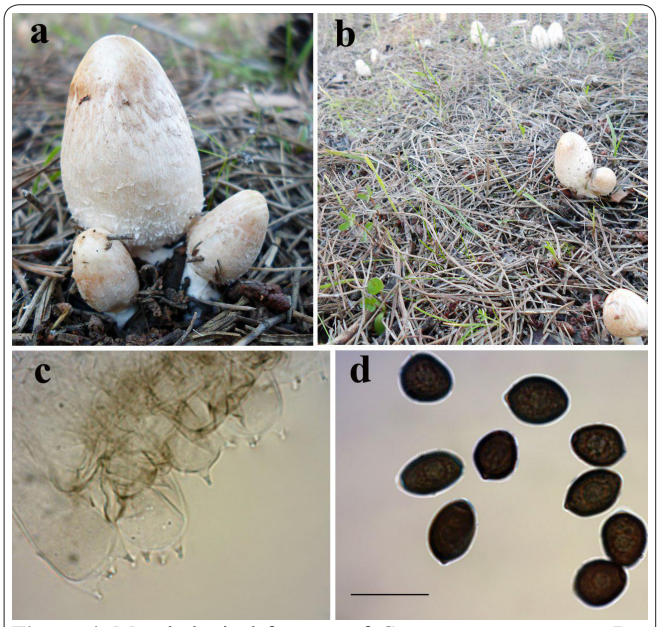

Figure 4. Morphological features of *Coprinus pinetorum*; a. Basidiocarps before expansion; b. Fairy ring; c. Basidia; d. Spores. Scale bar: 10 µm.

1–1.5 cm, slender, with a movable ring on the lower half part, white in colour, hollow, with central strand mycelium and slightly bulbous at the base. Spores 8-9.6 × 5.9-7 µm, ellipsoid, sometimes slightly broadened at base, smooth, dark brown, with central germ pore (Fig 4, d). Basidia, clavate, 18–36 × 9.9–11.7 µm, four-spored(Fig 4, c); Pleurocystidia and Cheilocystidia not observed. Veil consisted of cylindrical hyphoid elements.

C. pinetorum comparing with *C. comatus,* have smaller spores; this species grows among needles of *Pinus halepensis*, whereas *C. comatus* appears on lawns and other nitrate-rich sites. *Coprinus sterquilinus* have also larger spores than *C. pinetorum* and grows on animal dung (3).

Specimen examined. IRAN, Kermanshah province, Islamabad-e Gharb, Badrei, found in a fairy ring on humus alongside pine trees (*Pinus halepensis*), 25 December 2015, E. Seidmohammadi, IRAN 16903F.

Phylogenetic analyses of ITS-rDNA sequence data of coprinoids using MP, NJ and ML approaches yielded in phylogenetic trees with the same topologies. The phylogenetic tree inferred from ITS sequence data based on the ML analyses is presented in figure 5. As shown, the genera *Coprinus, Coprinipsis* and *Coprinellus* were clustered independently. Except for *C. radians,* the species were also clustered within separate well-supported clades, confirming the morphological identification. Two *Coprinellus* specimens which morphologically identified as *C. radians* were clustered on two separate clades both together with the specimens of the same species published in the GenBank. This may be attributed to the polyphyletic nature of *C. radians.* More detailed morphological characterization of *C. radians* and analysis of more genes seems necessary to address the exact position of this specimens.

Our results in the present study revealed the presence of three rare coprinoid fungi *viz C. insignis, C. semitalis* and *C. pinetorum* in western Iran. To our knowledge, *C. semitalis* and *C. pinetorum* have been reported only from Europe. *Coprinopsis insignis* has also been reported from Europe and America. So, the results of this study indicated that the distribution range of above-mentioned species extends to Iran, at least.

During the past years, several studies have been conducted on the identification of agaric fungi in Iran. However, most of these studies have been dedicated to the identification of macrofungi in the north of the country and other regions including Kermanshah Province have poorly studied.

Kermanshah Province situated in western Iran. The province is a mountainous region between the Iranian plateau and the Mesopotamian plain. From the east of the province towards the west, elevation drops progressively, until the vast plains of Iraq fill the horizon. The climate of the highlands is mild in summer and cold in winter, with heavy snowfall, whereas, the weather in the west area of the Province is mild in winter but hot and dry during the summer. The average annual rainfall of the Province is 480 mm (29). Regarding the various climatic conditions and plantation in Kermanshah Province, many fungal species could be found in the region. So, greater attention to identifying macrofungi in this region and other poorly investigated areas in the country is needed.

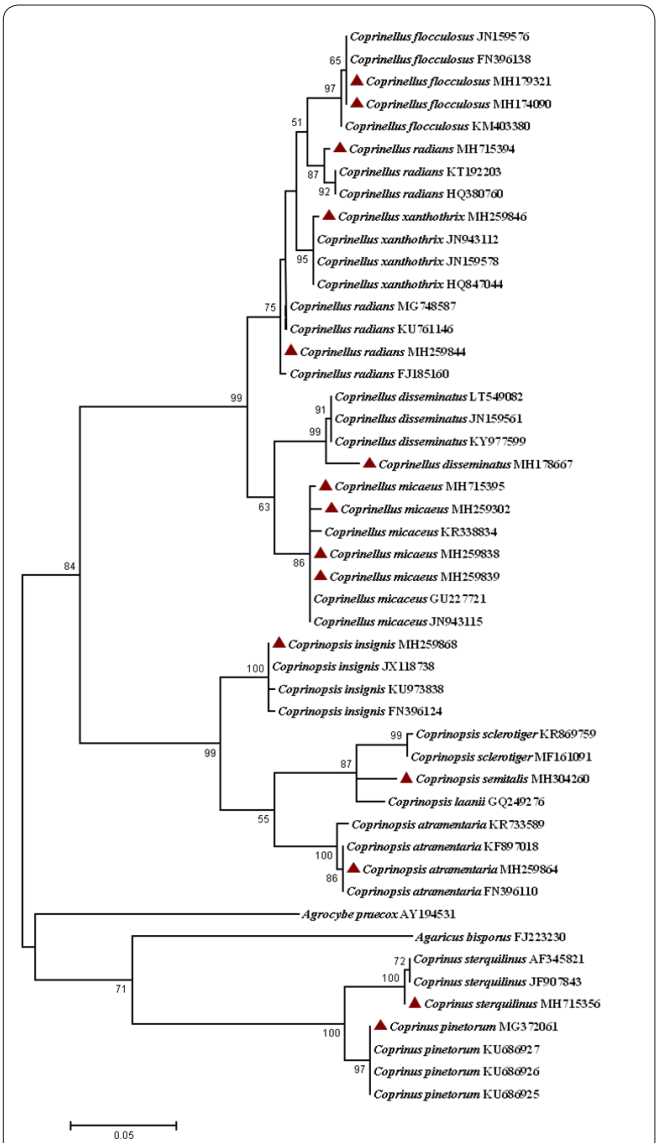

Figure 5. Phylogenetic tree constructed from the ITS sequence alignment of *Coprinus, Coprinipsis* and *Coprinellus* Species based on the maximum likelihood (ML) approach, with 1000 bootstrap replicates. The Iranian specimens are shown with triangle labels.

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