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New *Entoloma* species from Iran

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Abstract: Three specimens of *Entoloma* genus were collected from Kermanshah province, west of Iran. Macro and micro-morphological features of the fungal specimens were examined. The ITS1 and ITS4 Primer pair was used to amplify ITS-rDNA. Phylogenetic analyses of ITS-rDNA sequences were carried out using Maximum Likelihood method with 1000 bootstrap repetitions. Based on the results obtained from morphological examinations along with data obtained from ITS- rDNA sequences, three species including *Entoloma phaeocyathum*, *E. sinuatum*, *E. undulatosporum* were identified. *E. sinuatum* has previously been reported from Iran, therefore this study represents the first record of *E. phaeocyathum* and *E. undulatosporum* from Iran.

Key words: Agaric, *Entoloma phaeocyathum*, *Entoloma undulatosporum*, Kermanshah, Qalajeh.

INTRODUCTION

The agaric family *Entolomataceae* is one of the most species-rich families within the *Agaricales* with more than 2,200 species described worldwide (Kirk 2019). According to molecular analyzes, the species in the *Entolomataceae* are a monophyletic group (Moncalvo et al 2002; Matheny et al 2006). The family is characterized by pink spore prints and distinctive spores. Traditionally, *Entolomataceae* consists three genera viz. *Entoloma* (Fr.) P. Kumm. *Rhodocybe* Maire and *Clitopilus* (Fr. ex Rabenh.) P. Kumm. which are morphologically separated from each other based on spore characteristics (Co-David et al 2009). The genera all share ornamented spores as a unique character. The spore ornamentation is formed by local thickenings in the spore wall, which is called the epicorium (Clémençon et al 2004).

The members of the family *Entolomataceae* which have spores with irregular bumps and ridges are classified in the genus *Rhodocybe*, those with longitudinally ridged spores are classified in the genus *Clitopilus* and the members with angular spores in all views are classified in the genus *Entoloma* (Co-David et al 2009).

The genus *Entoloma* which comprises about 1700 species is the largest genus in this family (Kirk 2019). *Entoloma* is globally distributed from the Arctic to the tropical habitats (Noordeloos 1984; Morgado et al 2013). Morphological features commonly used to identify different species of this genus include the habit and color of the basidiocarp, the pileus hygrophaneity, the shape and size of the spore, and the type of structure and pigmentation of the pileipellis (Knudsen & Vesterholt 2008).

According to the literatures, till now, 11 species of *Entoloma* including *E. clypeatum. E. mammosum, E. rhodopolium, E. sericellum* (Saber 1993), *E. incanum* (Zokaei 2002) *E.cinnabarina* (Saber & Pegler 2000), *E. hirtipes, E. majaloides, E. sinuatum* (Ershad 1995), *E. griseoluridum* (Asef 2007) and *E. griseorubellum* (Fadavi et al 2013) have been reported from Iran.

Here, we present descriptions and illustrations of two *Entoloma* species reported for the first time from Iran.

MATERIALS AND METHODS

Sampling and Morphological Examinations Through field surveys of agaric fungi conducted from 2014-2017, three specimens belonging to the genus *Entoloma* were collected from the Qalajeh area, Kermanshah, western Iran. For each specimen collected, habit, habitat and GPS coordinates were recorded at the collecting site. To save and illustrate diagnostic details of fresh basidiocarp, habit and habitat, the specimens were photographed before collection. Macroscopic characteristics including shape and dimensions of pileus and stipe, gill attachment, the spore print color, etc., were examined on freshly collected specimens. Microscopic features of each specimen including basidia, basidiospores and

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cystidia were also observed, measured and illustrated using the OLYMPUS BX51 microscope.

After preliminary examination, the specimens were dried using a drying chamber. The specimens were identified using available literatures (Moser 1978; Hansen & Knudsen 1992; Knudsen & Vesterholt 2008). A specimen from each species was kept in the fungal herbarium of the Iranian Research Institute of Plant Protection, Tehran, Iran.

Extraction and amplification of genomic DNA

Fungal genomic DNA was extracted from internal tissue of dried basidiocarp using fungal DNA extraction kits manufactured by Denzaist Asia Company, following the instructions provided by the manufacturer. PCR amplifications of the internal transcribed spacers and 5.8S ribosomal DNA (ITS-rDNA) was performed using primer pairs ITS1 and ITS4 (White et al 1990). To prepare the PCR reaction mixture, about 50 ng of DNA template, 10 µM of each primer and *25 µl* of the 2x *master mix* (Sinagen Company, Iran) were mixed in a final volume of 50 µl.

The PCR amplification protocol included: 2 min pre-denaturation at 90 °C followed by 35 cycles of 40 sec denaturation at 95 °C, 40 sec primer annealing at 57 °C, 50 sec primer extension at 72 °C and 10 min final primer extension at 72 °C. The PCR products were separated using a 1% agarose gel. To confirm DNA amplification, the gel visualized by staining with Red Gel and photographed under UV light. The amplification products were purified and sequenced by Macrogen, Inc., Seoul, South Korea.

Phylogenetic analyses

The target sequences were compared with related sequences to find the most similar ones using the BLAST searches against GenBank database. The sequences derived from GenBank and the sequences generated in this study are listed in Table 1. Multi-sequence alignments was carried out using Clustal W (Thompson et al. 1997), checked and manually edited. The maximum likelihood (ML) analyses were performed using MEGA software ver. 6.0. To assess branch supports, The bootstrap values were performed with 1000 replications (Tamura et al 2013).

Table 1. *Entoloma* specimens used in the phylogenetic analyses. Iranian specimens are shown in bold.

Species	Locality	Herbarium Code	Accession Number
Clitopilus hirneolus	Netherlands	MEN 199956	KC710132
E. araneosum	United Kingdom	K(M):190503	MF977956
E. araneosum	Netherlands	MEN 200314	KC710056
E. excentricum	Canada	-	KY706186
E. excentricum	Italy	6103	JF907996
E. graphitipes	Spain	AC1559	KJ001446
E. graphitipes	Spain	JVG 1121026-1	KJ001442
E. phaeocyathum	Spain	SFC 11020301-01	KJ001419
E. phaeocyathum	Spain	JC-20060204.2 (Ex-1859)	KJ001422
E. phaeocyathum	**Iran**	**16979**	**MH453501**
E. rusticoides	Spain	SFC 1107171	KJ001437
E. sinuatum	Netherlands	J.Wisman 2003-09-19	KC710109
E. sinuatum	Netherlands	J.Vauras 8181F	KC710116
E. sinuatum	Finland	H:6003960	GU373512
E. sinuatum	USA	MLS007	GQ397994
E. sinuatum	**Iran**	**16980**	**MH447332**
E. sinuatum	USA	BHS2009-07	GU289652
E. subsinuatum	Canada	UBC F-23966	MF955124
E. subsinuatum	Canada	ANT274-QFB28797	MN992385
E. undulatosporum	Spain	SFC 11021902	KJ001408
E. undulatosporum	**Iran**	**16981**	**MH453494**
E. versatile	United Kingdom	24168	MF977945
E. versatile	United Kingdom	K(M):142307	MF977969
Lyophyllum leucophaeatum	France	FR2014002	KP192581
Rhodocybe matesina	Italy	MCVE:29262	KY629961

RESULTS

Morphological examinations and comparison of generated ITS sequences against databases of GenBank led to the identification of three species, namely, *E. phaeocyathum*, *E. sinuatum* and *E. undulatosporum*. According to the literatures, *E. phaeocyathum* and *E. undulatosporum* are new records for the mycobiota of Iran. Morphological descriptions and illustrations of these two species are as follows:

Entoloma phaeocyathum Noordel., Persoonia 12(4): 461 (1985)

Macroscopical description: Pileus 10-20 mm in diam, convex, umbilicate to funnel-shaped, Surface, at first, tomentose, and then becoming finely scaly. Blackish brown, dark red brown or grayish brown, paler towards the margin and weakly hygrophanous, translucently striate at margin (fig.1-a). Lamellae decurrent, distant, thickish, arcuate, at first dark grey brown and then brown pink (Fig.1- b). *Stipe* 5-20 × 1-2 mm, paler than pileus, yellow brown to very dark brown, hollow, cylindrical with small bulb at base.

Microscopical description: Spores 7.9-8.9 × 9-10.3 µm, isodiametrical; 5-9 angled in side—view, very thin-walled (Fig.1-d),.*Basidia*, 4—spored, clavate, clampless (Fig.1- c). Cystidia, cylindrical to clavate.

Habit and Habitat, Found in small groups in relatively dry forest of *Quercus* on calcareous soil.

Specimens examined: IRAN, Kermanshah province, Gilan-e Gharb, Dar Badam, 1710 m, E46º 25′ 39″, N34º 01′08″, 12 May 2016, leg. E. Seidmohammadi, (IRAN 16979F).

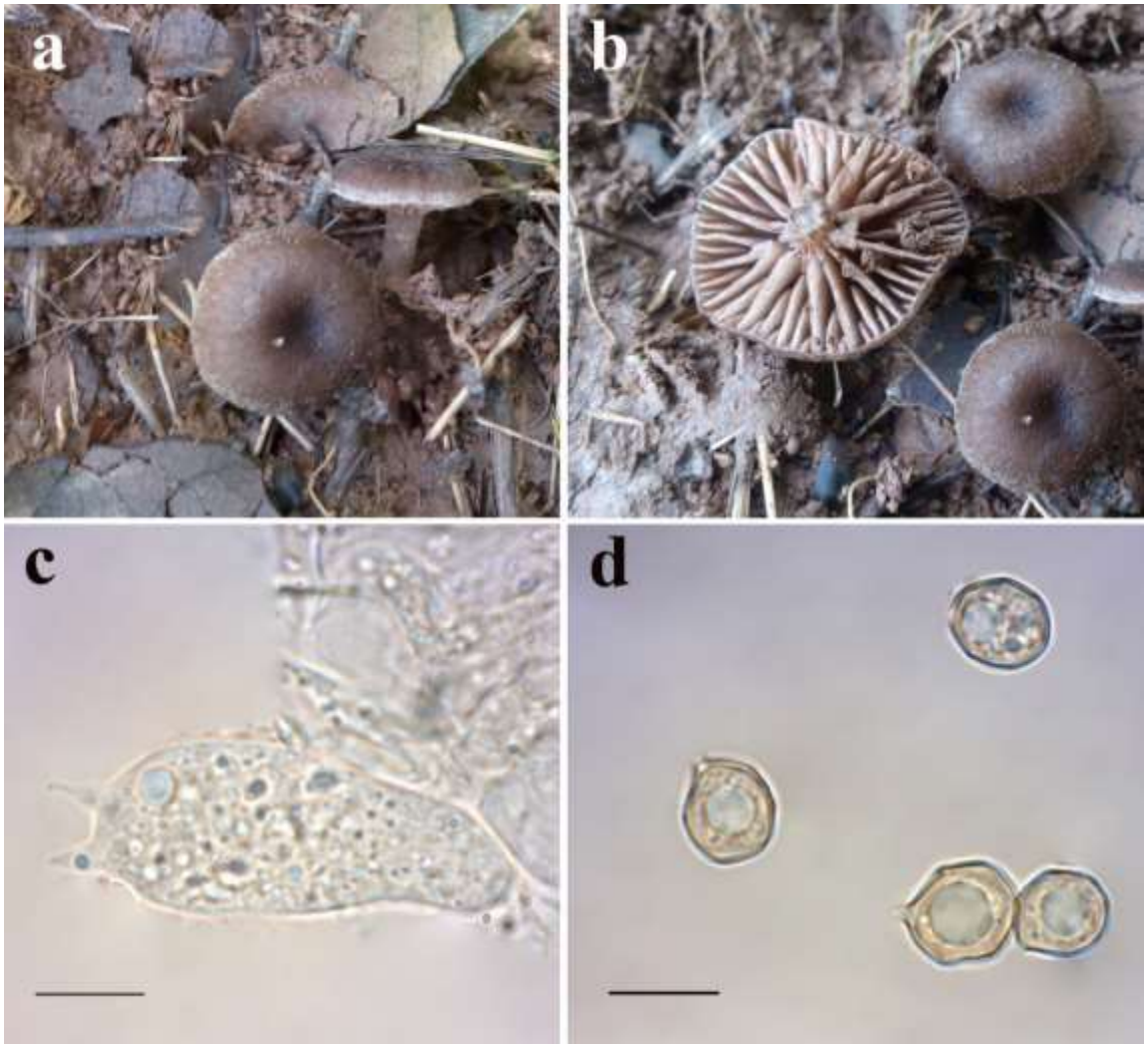

Fig. 1. *Entoloma phaeocyathum*: a, Pileus; b: Lamellae; c: Basidia; d: Basidispore. Scale bar: 10 µm.

Entoloma undulatosporum Arnolds & Noordel., Persoonia 10(2): 295 (1979)

Macroscopical description: Pileus 20-30 mm in diam, convex to funnel—shaped, hygrophanous, when moist, shining blackish brown, paler at margin (Fig. 2-a*). Lamellae* medium spaced, adnate to deccurret, pale grey-brown, usually with a pink tinge when mature and with sligthly paler edge. *Stipe* 15-20 × 2-4 mm, cylindrical, brown to dark brown, paler than pileus, at base usually with a white tomentum (Fig. 2-b). Spore deposit pink with a brownish shade.

Microscopical description: Spores 7.1-8 × 9.5-10.8 µm, very thin-walled and irregularly many-angled (Fig. 2-a). Basidia,4-spored, clavate and clamped (Fig. 2-c). Cystidia, cylindrical to clavate (Fig. 2-d, e). Clamps abundant in hymenium, Habit and Habitat, Found in small groups in grassland.

Specimens examined: IRAN, Kermanshah province, Mele Nai, 1206 m, E46º 03´ 44″, N34º 05´51″, 12 February 2016, leg. E. Seidmohammadi, (*IRAN* 16981F).

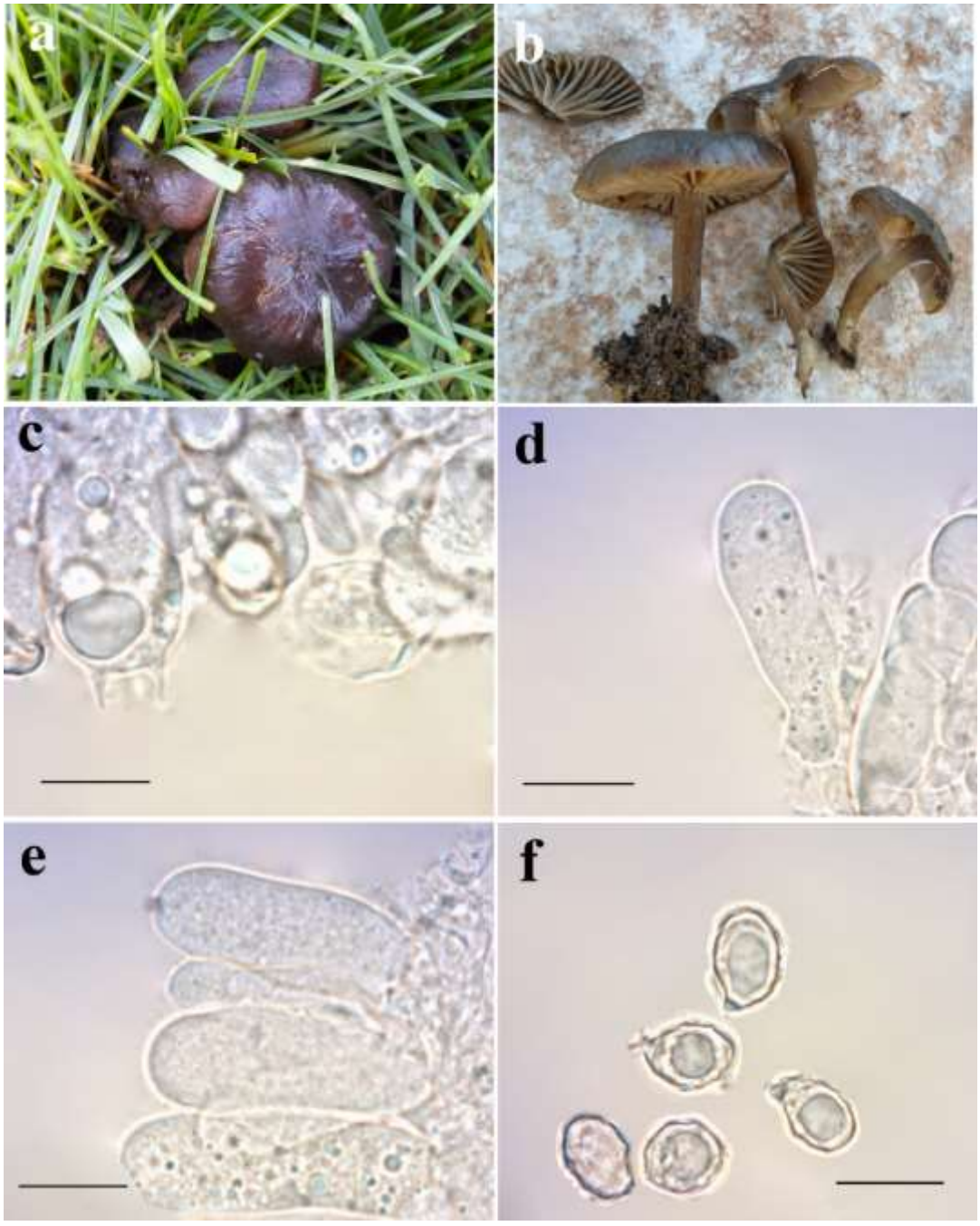

Fig. 2. *Entoloma undulatosporum*: a-b, Basidiocarp; c: Basidia; d and e: Cystidia: f: Basidiospores. Scale bar: 10 µm.

The phylogenetic trees were constructed from the ITS sequence data of *Entoloma* species using three approaches: Maximum parsimony (MP), Neighbor-Joining (NJ) and Maximum Likelihood (ML). The resulting phylogenetic trees showed the same topologies. The phylogenetic tree inferred from the ITS dataset of *Entoloma* species based on the ML analyses is presented in Fig. 3. As shown, Iranian specimens of *E. phaeocyathum* and *E. undulatosporum* were placed in separate well-supported clades. The Iranian specimen of *E. sinuatum* strain MH447332 clustered with two *E. sinuatum* specimens from USA (GQ397994) and Finland (GU373512) within a fully supported clade, however, the specimens belonged to *E. sinuatum* were divided into two completely supported subclade.

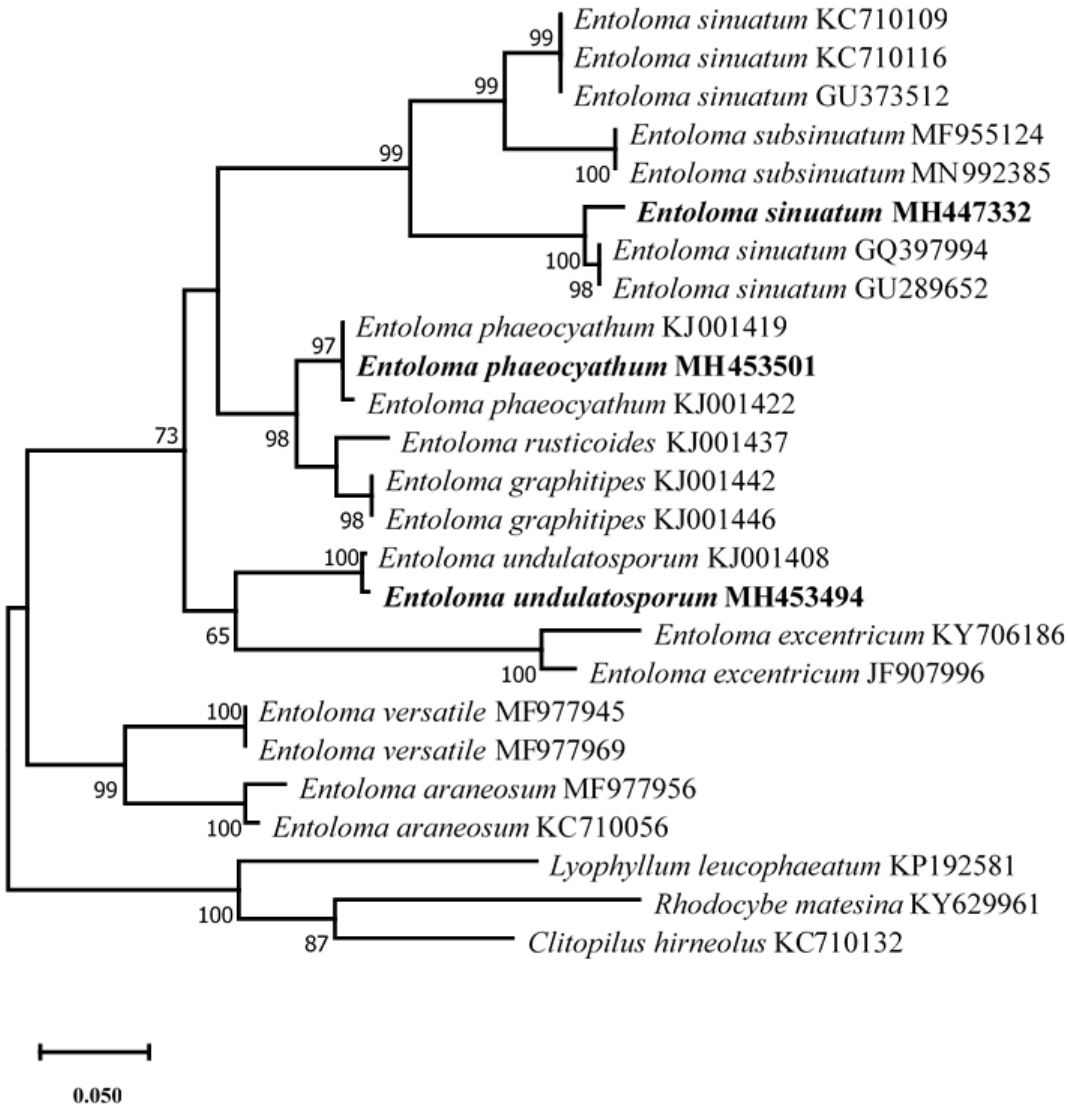

Fig. 3. Phylogenetic tree reconstructed from the ITS sequence alignment of *Entoloma* species based on the maximum likelihood method. The Iranian specimens shown in bold. The bootstrap value were performed with 1000 replications.

DISCUSSION

Our results in the present study revealed the presence of two rare species of *Entoloma viz E. phaeocyathum* and *E. undulatosporum* in the west of Iran. As well as we identified *E. sinuatum* that previously not recorded from Kermanshah Province. Clear morphological characteristics along with the phylogenetic data confirmed the accurate identification of the specimens. Nevertheless, in the case of *E. sinuatum*, as shown in Fig. 2, the specimens used to construct phylogenetic tree were divided into two subclade. This may be attributed to the presence of cryptic species within *E. sinuatum*. However, analysis of more genes seems necessary to address the question.

To our knowledge, *E. phaeocyathum* and *E. undulatosporum* have been reported only from Europe (Knudsen & Vesterholt 2008; Vila et al 2014). Our findings in this study indicated that the distribution range of the above-mentioned species extend to western part of Iran, at least.

Entoloma is a species- rich genus with approximately 1,700 species (Kirk 2019) and is considered to be cosmopolitan in distribution (Noordeloos 1984). According to the literatures, prior to this study, only 11 species of *Entoloma viz* 11 species of *Entoloma* including *E. clypeatum. E. mammosum*, *E. rhodopolium*, *E. sericellum* (Saber 1993), *E. incanum* (Zokaei 2002) *E.cinnabarina* (Saber & Pegler 2000), *E. hirtipes*, *E. majaloides*, *E. sinuatum* (Ershad 1995), *E. griseoluridum* (Asef 2007) and *E. griseorubellum* (Fadavi et al 2013) have been reported from Iran. The small number of *Entoloma* species reported from Iran may represent insufficient studies for identification of macrofungi in Iran. Over the past years, several researchers have identified agaric fungi in Iran (Saber 1991; Saber 1993; Zokaei 2002; Asef 2007; Hosseini et al 2010; Asef 2014; Fadavi et al 2015; Mahdizadeh et al 2016; Seidmohammadi et al 2018). However, most of these studies have been conducted in the north of the country and other areas such as Kermanshah province have been less studied.

Kermanshah province is in the middle of the western side of the country, a mountainous region with an average annual rainfall of about 480 mm. The region is located between the Iranian plateau and the Mesopotamian plain. Decreasing elevation from east to west has caused significant climatic diversity in the province. Therefore, Kermanshah province is considered a four-season region. The climate of the highlands of the province is temperate during summer and cold in winter with heavy snowfall, while the climate of the western parts is temperate during winter and hot and dry in summer (Borjian 1396). Due to the different climatic conditions and plantation in the region, many fungal species are likely to be found in Kermanshah province. Therefore, it is necessary to pay more attention to the identification of macrofungi in this region and other less explored areas in the country.

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گونه‌های جدید *Entoloma* در ایران

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چکیده: سه نمونه از جنس *Entoloma* از استان کرمانشاه در غرب ایران جمع‌آوری شد. ویژگی‌های ریخت‌شناختی ماکروسکوپی و میکروسکوپی نمونه‌ها مورد بررسی قرار گرفت. جفت آغازگر ITS1/ITS4 برای تکثیر و تعیین توالی ITS-rDNA استفاده شد. واکاوی تبارشناختی داده‌های توالی ITS-rDNA با استفاده از رویکرد حداکثر احتمال با ۱۰۰۰ تکرار بوت استرپ با استفاده از نرم افزار MEGA6 انجام شد. بر اساس نتایج به‌دست‌آمده از بررسی‌های ریخت‌شناختی و داده‌های به‌دست‌آمده از توالی‌های ITS-rDNA، سه گونهٔ *Entoloma phaeocyathum*، *E. sinuatem* و *E. undulatosporum* شناسایی شدند. گونه *E. sinuatem* قبلاً از ایران گزارش شده است، لذا این مطالعه، اولین گزارش گونه‌های *E. phaeocyathum* و *E. undulatosporum* از ایران است.

کلمات کلیدی: آگاریک، *Entoloma phaeocyathum*، *Entoloma undulatosporum*، کرمانشاه، قلاجه

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