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Morphological and molecular characterization of Arbuscular Mycorrhizal fungus, *Acaulospora punctata* in Iran

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Abstract: A new species of *Acaulospora* was found in the mountainous region of Iran (Kerman province, Raviz), at 2118 m asl around the roots of *Prunus cerasus*. The new species was named *A. punctata* by Ohel, 2013, because of its ornamentation on the outer spore wall with point-like. The spores are yellow-white, globose to subglobose, 105–139 µm in diameter, and have three walls that each of which consists of some layers. Phylogenetic analyses of sequences obtained from the ITS and partial LSU & SSU of the ribosomal genes confirm the new species in a separate clade within the Acaulosporaceae. According to the phylogenetic analysis, the fungus as named b-pooneh placed in a monophyletic group within the genus *Acaulospora* next to *Acaulospora covernata*, and *A. ignota*.

Key words: Arbuscular mycorrhizal fungi, Glomeromycetes, mountainous region, Kerman

INTRODUCTION

Arbuscular mycorrhizal fungi are the obligate symbiont of many terrestrial plants. These fungi are of great economic and ecological importance. Arbuscular mycorrhizal fungi (AMF) help preserve the productivity and diversity of plant species. They can improve plant nutrition, make water available to plants, improve soil structure and increase plant tolerance to pathogens and environmental stresses. According to our knowledge, these species as asexually reproductive organisms; we cannot define them as biological species (Senés-Guerrero & Schüßler 2016). The number of species described of arbuscular mycorrhizal fungi (AMF) compared with other fewer fungi is a scare species (Ohsowski et al. 2014; Hall 1984). The history of the identification of these fungi is through their spore morphology. This method alone can cause errors in the identification of these fungal species because some species have several forms of spores. On the other hand, a group of these fungi has a period of resting spores, and they cannot be considered active mycorrhizal populations in this period (Oehl et al. 2011a; Hempel et al. 2007). For all the reasons mentioned, molecular identification is a reliable way to identify this group of fungi.

Recently, new species of *Acaulospora* have been described using morphological and molecular phylogenetic analysis with SSU, ITS, and LSU rRNA sequences. (Palenzuela et al. 2014; Oehl et al. 2011b; Furrazola et al. 2013). Acaulosporoid spores of the board majority of known *Acaulospora* spp. consist of three walls: a colorless or colored outer wall forming the spore surface (spore wall 1), and two colorless inner walls (spore walls2 and 3), each of these walls usually comprises two or three layers; none of them is in contact with the others (Błaszkowski 1995; Oehl et al. 2006; Stürmer & Morton 1999). The upper surface of spore wall1 is bi-layered and be even or decorated, and layer1 sometimes balloons in polyvinyl alcohol / lactic acid/glycerol (PVLG). Spore wall2 is always even, and spore wall3 maybe even or decorated with granular processes. In many species of *Acaulospora*, the spore wall3 stains or does not color at all in the Melzer's reagent and does not alter the shape in PVLG or becomes formless (Błaszkowski et al. 2015; Palenzuela et al. 2013). The decoration of the surface of the spores was a valuable character for comparison between species. An ornamented *Acaulospora* species was discovered during a survey of AM fungi in clay soil from Rafsanjan, Kerman province, in Iran. Until now, scattered research has been done on mycorrhizal fungi in this area (Aminizadeh et al. 2018; Aminian et al. 2021; Yazdanpanah et al. 2017). Only one study has been done on the mycorrhizae of the vegetation of this region, and these fungi have been isolated and identified from the plants of wild rye, sunflower, onion, and almond (Aminian et al. 2021). So far, mycorrhizal species symbiosis with the roots of these

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plants has been identified from stone fruit trees (Calvet et al. 2004; Summuna et al. 2019). Zangeneh (2021) reported *A. punctata* from wheat field soil in Sistan and Balochestan, Iran. Therefore, this study aims to describe the new species, combining the morphological and molecular phylogenetic analysis of partial SSU, ITS, and partial LSU rRNA sequences.

MATERIALS AND METHODS

Locality and host plant

Raviz region, which was sampled for this study, is located in Rafsanjan city, Kerman province, Iran. In this site, weather is cold and mountainous climate with an average annual temperature of about 16°C and an average annual rainfall of 1100 mm. The fungus hereafter described was found at the mountainous site where woody and herbaceous plants grow.

Plant and Soil Sampling

Soil samples were collected in Raviz (Kerman province, Iran) in clay soil from the rhizospheres of sour cherry (*Prunus cerasus*). Three plants and the soil around the roots were collected at the sites in February 2020. Three samples of the rhizospheric soil of each plant (1-2 kg) were taken from a depth of 5–30 cm and blended to provide a compound soil sample per individual plant. These samples were used to separate AM spores (Palenzuela et al. 2013). AM fungal trap cultures: To cultivate AM fungi from samples, trap cultures with *Sorghum vulgare* and *Zea mays* were established in cylindrical 1000 mL pots (12 cm diam) filled with the soil around the root samples the field. The pots were irrigated three times per week. The cultures have been maintained in the greenhouse under natural light and temperature conditions for more than two years. Despite several tries since 2020, trap culturing of the new fungi has not been a positive outcome.

Morphological analyses

AM spores were isolated from soil samples by a wet sieving method (Sieverding et al. 1991). The morphological characteristics of the spores and their structures were described from samples prepared in PVLG and Melzer reagent combination (Brundrett 1994). Spore structure terminology follows (Oehl et al. 2011b) for species with acalosporoid spore formation. Photos were taken with a Nikon DSFi1 camera on a compound microscope (Nikon Eclipse 80i).

Molecular analyses

DNA extraction: Spores were washed in pure water and sonicated. The extracts were obtained from three individual spores. The spores were put on a slide in a drop (5–10 µl) of pure water, then were crushed with a sterile needle in 20µl of a solution including 10µl of 5X GoTaq PCR buffer and 10µl of sterile distilled water and then placed at 60 to 65 ° C for 15 minutes. After 10 minutes of centrifugation at 13000 g, the supernatant was used as the template DNA in PCR. PCR reactions were performed according to Goto et al. (2012). The DNA extract was used as a template for a nested PCR using the primers SSUmAf) TGGGTAATCTTDTGAAACTTY (and LSUmAr) TGCTGWHACTCAAWYCTATCRAW (. The second round of PCR was performed with specific primers of arbuscular mycorrhizal fungi, SSUmCf (TATYGYTCTTNAACGAGGAATC) and LSUmBr (DAACACTCGCAYAYATGYTAGA), which were able to amplify fragments of 1500 bp of ribosomal DNA (Krüger et al. 2009).

The first amplification reaction was performed with a volume of 25µl and including 5.5µl of deionized distilled water, 12.5µl of Amplicon *Taq* DNA Polymerase 2x, 1µl of each primer, and 5µl of DNA extracted from single spores. In the second amplification reaction, instead of the extracted DNA, 5µl of the first PCR product was used as the template DNA. At the first stage, cycling parameters were 3 min at 95°C (1 cycle), the 30s at 95°C, 1 min at 55°C, 90s at 72°C (40 cycles), and a final elongation of 10 min at 72°C followed the last cycle and a second stage, 3 min at 95°C (1 cycle), 30s at 95°C, 1 min at 60°C, 90s at 72°C (30 cycles), and a final elongation of 10 min at 72°C followed the last cycle. We analyzed PCR products by electrophoresis in a 1.5% agarose gel dyed with Gel Red™ and viewed by UV illumination. The expected amplicons were sent to Topaz Gene Company for sequencing in both directions by the Sanger method.

Phylogenetic analyses

The nucleotides with low score quality at 3′ and 5′ end have been removed using seqtK (Shen et al. 2016). Then forward and reverse have been assembled using CAP3 (Huang & Madan, 1999). The sequence has been edited manually concerning the available reference genomes in Mesquite (Maddison 2005). The edited sequence has been analyzed using BLASTn(https://blast.ncbi.nlm.nih.gov/Blast.cgi).
The AM fungal sequence acquired was aligned with other AMF sequences from GenBank. The new sequence was deposited in the EMBL database under the accession number OK184927. We selected 40 AMF sequences that include b-pooneh after editing, all available *Acaulospora* sequences with known species and families. The sequence was aligned using Linux version of MAFFTv.7 with 'Auto' settings; afterward, the sequence alignment was checked using Mesquite. The alignment has been displayed with "NCBI multiple sequence alignment viewer" (Rangwala et al. 2021) and "Weblogo3" (Crooks et al. 2004).

To create the phylogenetic tree, the completed sequence has been subjected to IQtree (Linux version) (Nguyen et al. 2015) and MrBayes 3.2.7 (Linux version) (Ronquist et al. 2012), which are an efficient maximum-likelihood-based (ML) and Bayesian interface-based (BI) phylogenetic tree-building tools. Ultrafast bootstrap (UFBoot) was used

to validate phylogenetic trees for 1000 replications. Bayesian interface analysis using MrBayes 3.2.7, has been carried out by following settings: four MCMC, two runs, 1 × 107 generations, the sampling frequency of 10,000, and burn-in proportion of 0.25.

RESULTS

Acaulospora punctata Oehl, Palenz., I.C.Sánchez, G.A.Silva, C.Castillo & Sieverd. **sp. nov.** MycoBank MB 561543

Etymology: punctata (Latin), referring to the small, round pits that are regularly formed at a short distance to each other on the outer spore wall. The spores are yellow-white, globose to subglobose,105–139 µm in diameter, rarely ovoid to ellipsoid, (105–)110 133(148) × (96–)105–120 µm in diameter. The outer spore wall includes three layers (OWL1–OWL3) with 3.2–6.2 µm width. The outer layer (OWL1) is evanescent with 0.5–1.0 µm thick. The second layer (OWL2) is yellow-white, laminated, .02–4.1 µm thick, containing the ornamentation with arranged and circular pits. The inner layer of the outer wall (OWL3) is 0.5 µm thick and hard to see. The middle wall is hyaline and bi-layered; 1.3–1.2 µm thick in total. These two layers (MWL1 and MWL2) are semi-flexible, firmly adherent to each other, and thus, appear as one wall layer. The inner wall is hyaline with three layers (IWL1–IWL 3) 2.5–2.3 µm thick. The IWL1 is about 0.7–1.2 µm thick. IWL2 is 0.8–1.5 µm thick. IWL3 is very fine and hard to see because of its close sticking to IWL2 (Fig. 1.).

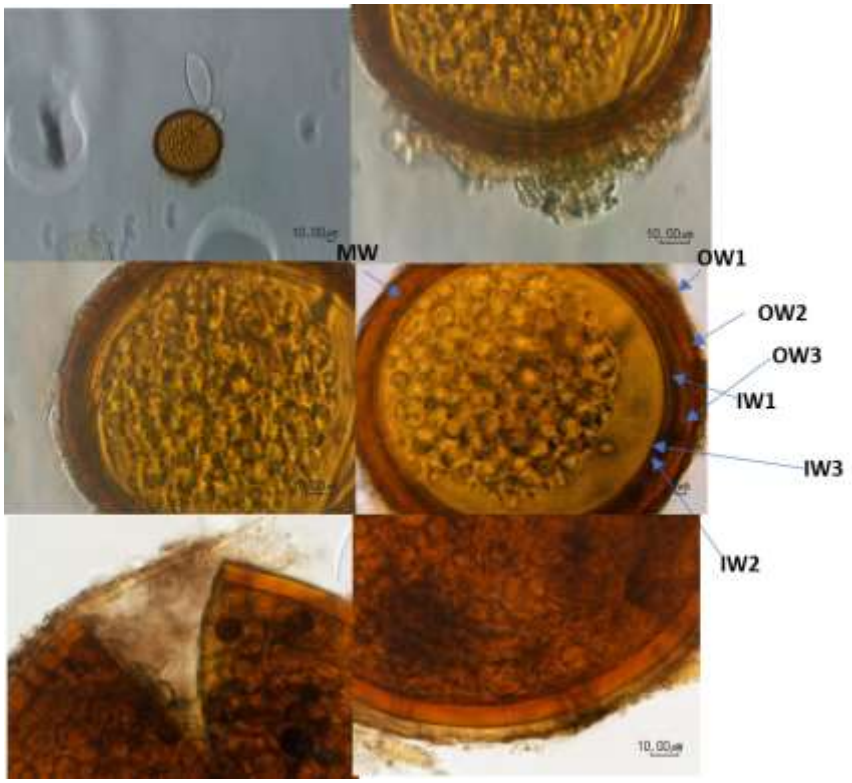

Fig. 1. *Acaulospora punctata*, Spores with point-like (punctuate) pitted ornamentation, spores with triple layered outer (OWL1-3), bi-layered middle (MWL1-2) and triple layered inner wall (IWL1-3).

Molecular analyses

The alignment with SSU–ITS–LSU sequences of *A. punctata* had a length of 1924 characters, of which 824 were informative. The reconstructed tree of AMF isolates, containing 40 terminals, rooted by a *Paraglomus brasilianum* isolate is described in Fig. 2. The best-fitting model selected by IQtree for the created tree based on BIC criteria was GTR [42] +F + I + G4. Both BI and ML phylogenetic analyses placed *A. punctata* in a separate clade. Phylogenetic analyses on sequences, comprising the ITS1, the 5.8S rDNA subunit, and ITS2 regions of the ribosomal gene (about 1500 bp), firmly placed the sequence of *A. punctata* into the genus *Acaulospora* adjacent to *A. covernata* Błaszk. (Błaszkowski 1989). According to the phylogenetic analysis, the fungus as named b-pooneh placed in a monophyletic group within the genus *Acaulospora* next to *Acaulospora covernata*, and this group, i.e. *Acaulospora punctata* with bootstrap = 100 is a monophyletic group that b-pooneh with bootstrap = 97 placed in this clade. All members of this clade are isolated from *Diversispora* with bootstrap = 100.

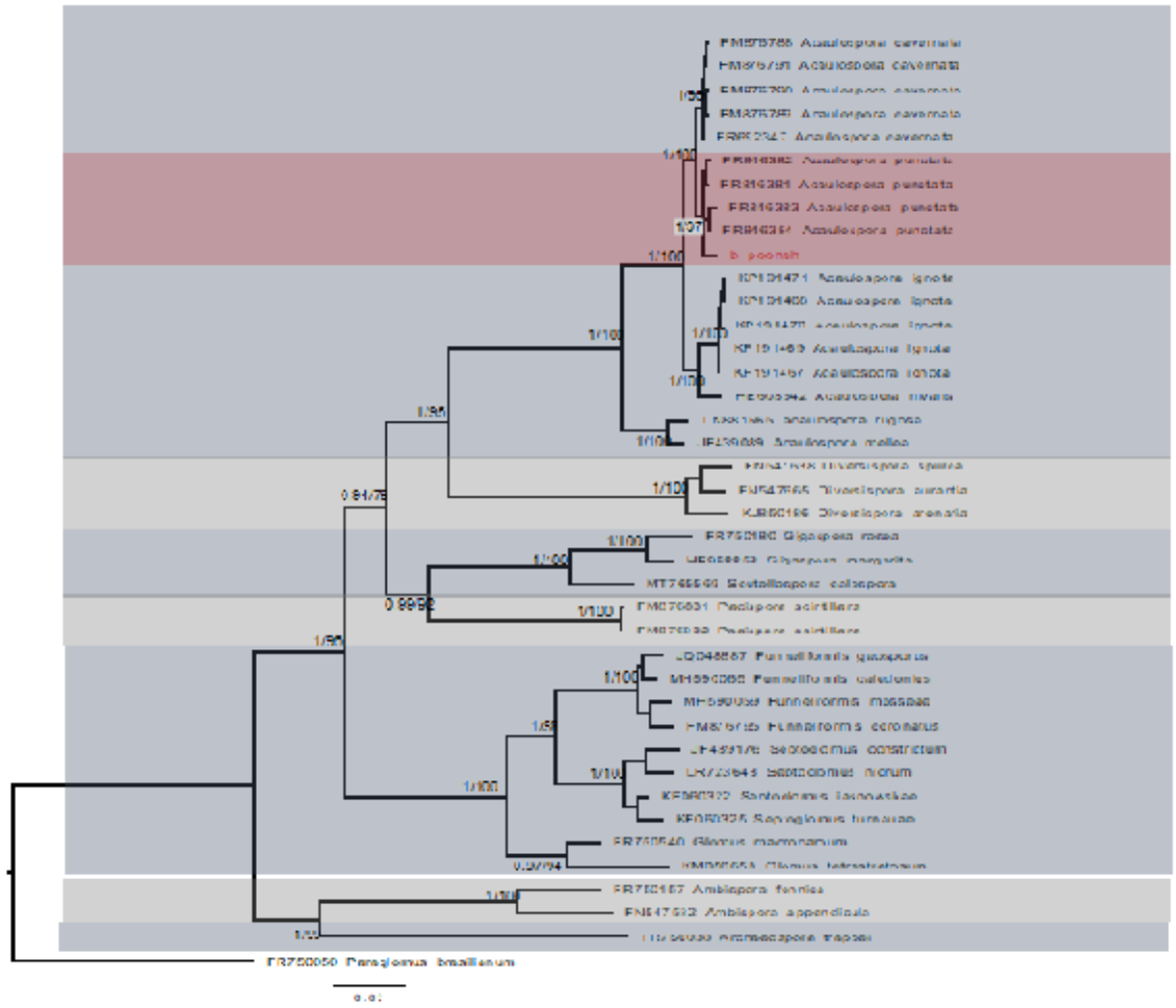

Fig. 2. The BI phylogenetic tree of 40 terminals reconstructed using MrBayes, rooted by a *Paraglomus brasilianum* isolate. The estimated posterior probability /bootstrap are indicated at the main nodes.

To establish the results of the tree, we plotted a network in which the fungus is separated from the others, as shown in figure 3. As shown in the two figures displayed using "NCBI multiple sequence alignment viewer" (Fig. 4) and "Weblogo 3" (Fig. 5), a conservation pattern alignment is shown between the sequences examined. And as you can see, the conserved and variable regions have almost the same pattern among the same species.

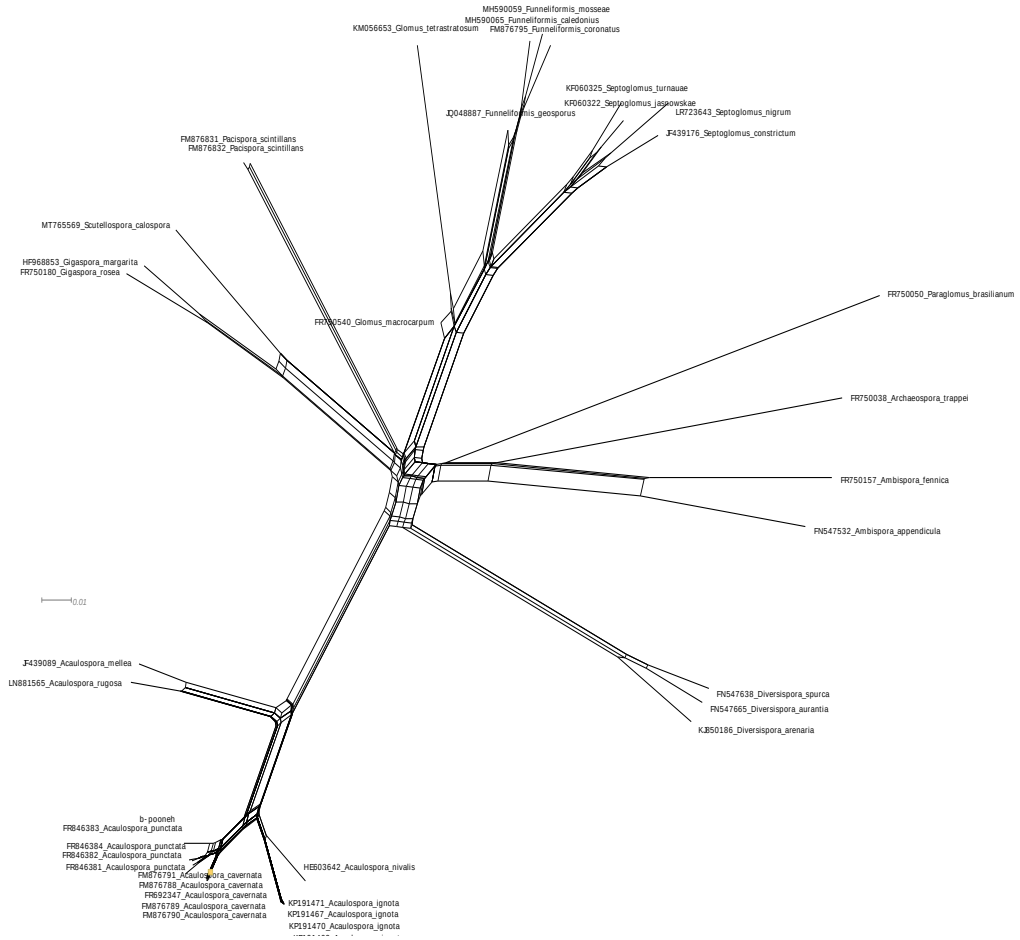

Fig. 3. The reconstructed NJ network based on 40 terminals (*Acaulospora punctate* are highlighted in red)

Fig. 4. Illustration of multiple sequence alignment, In the first 100 records resulted from BLASTn, we could find 18 *Acaulospora* with known 6 species. In this picture the multiple sequence alignment including b-pooneh and other available known species related to *Acaulospora* has been summarized. Red areas show variable regions.

Distribution and habitat

Spores of *Acaulospora punctata* were found in the sites in Raviz-Kerman, Iran (3o°28′16″N, 55°27′25″E) during December 2020. In the field samples, it is possible to find it alone or associated with the other described species, including *Funneliformis caledonium*, *Funneliformis mosseae*, and *Funneliformis coronatum*.

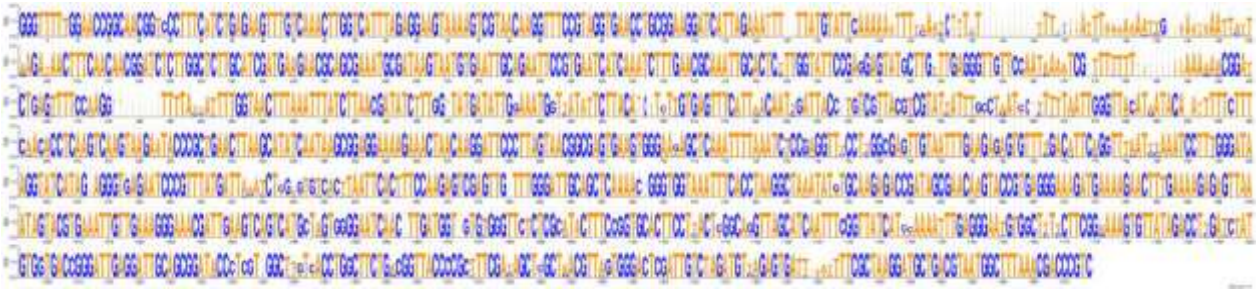

Fig. 5. Weblogo of the multiple sequence alignment. Lowercase and overlapped letters indicate variable and uppercase letters indicate conserved areas

DISCUSSION

The spores of the genus *Acaulospora* are shaped laterally on the neck of sporiferous saccules. The saccule is created at the end of the hyphae. It usually degenerates after the spore ball is constituted and separated from adult spores in soil samples (Oehl et al. 2006). In this study, saccule was decomposed in soil samples and was not observed. *Acaulospora* spores have several characteristics. Except for a few species, all species have three walls: outer wall (OW), middle wall (MW), and inner wall (IW). The outer wall is usually three-layer, the middle wall is two-layer, and the inner wall is two or three layers. Each species of this genus has a unique feature that discerns it from other species. For example, *A. viridis* can easily be distinguished from all other *Acaulospora* species, by the regularly greenish brown appearance of the spores above all in water and PVLG (Palenzuela et al. 2014) *A. pustulata* has minuscule spores among Acaulosporaceae (Palenzuela et al. 2013) *A. herrerae* can be distinguished from other species through its raised and dense reticulum and the shape of its rounded, elliptical to elongated pits (Furrazola et al. 2013), or the morphological character that most distinguish *A. ignota* is the ornamentation of the upper surface of spore wall1 layer 2, which consists of pale warts or leveled elevations (Błaszkowski et al. 2015). *Acaulospora punctata* is one of several species of *Acaulospora* that have been thoroughly analyzed using morphological and molecular identification tools (Oehl et al. 2011b, 2006). The new fungus can be discerned from all species of *Acaulospora* by its characteristic hollow ornaments on the outer surface of the spores. Morphologically, *A. punctata* spores are similar to *A. scrobiculata* and *A. cavernata* spores (Oehl et al. 2011b). However, *A. scrobiculata* has more irregular pits in more irregular distance to each other (Trappe 1977; Oehl et al. 2006), and *A. cavernata* has more giant pits (Błaszkowski 1989; Oehl et al. 2006).

Phylogenetically, *A. punctata* is similar to four *Acaulospora* species having each a distinct characteristic ornamentation on the spore surface (*A. cavernata*, *A. sieverdingii*, *A. paulinae*, and *A. denticulata*).

The other ornamented *Acaulospora* species analyzed on the ITS-rDNA region of the ribosomal gene i.e., *Acaulospora scrobiculata* Trappe (Trappe 1977), *A. alpina* (Oehl et al. 2006), and *A. lacunosa* J. B. Morton (Morton 1986), are phylogenetically clearly more distant to *Acaulospora punctata*. Species of *Acaulospora* forming soft, colored spores of a wall structure similar to that of *A. pumctata* are. *A. pumctata* was found in mountainous areas in Iran and before that in soil of wheat field in Sistan and Balochestan province of Iran (Zangeneh 2021). It was found in orchard stands dominated by *Prunus cerasus*, *Prunus avium*, and *Prunus armeniaca*. For the first time, it was found in the Swiss Alps, and the Chilean in pioneer stands dominated by *Epilobium fleischeri*, *Saxifraga oppositifolia*, *Poa alpina*, and *Notofagus alpine*. The ecosystem studied in this research is mountainous, and in Switzerland, its occurrence was restricted to mountainous to subalpine (Oehl et al. 2011b). Species of *Acaulospora* forming smooth, colored spores of a wall structure similar to that of *A. punctata*. *A. capsicula*, *A.longula* Spain & N. C. Schenck, *A. mellea* Spain & N. C.Schenck, *A.*

morrowiae Spain & N. C. Schenck, and *A. fhomii* Blaszak.

Since the morphological results cannot be completely accurate, molecular identification was performed, confirming the morphological results. And according to our finding, this species was similar to *Acaulospora*, with a high percentage of 98 of identity in the BLASTn analysis.

Previous researches have shown that if the similarity of ITS, LSU, and SSU regions is above 97% identity, they are known as the same species, and in this study, this similarity was even higher, then according to these results and according to the results from the morphology, it is confirmed that this species belongs to *A. punctata* and formed a sister group with the other *Acaulospora* used in the phylogenetic tree, are all in the same clade with Bootstrap = 100. To confirm the phylogenetic relationships of the tree, it was observed that in the network, all *A. punctata* are in a separate clade with other *Acaulospora*. In different *Acaulospora* species, the pattern of conservation was examined. As can be seen in the figures, some regions are variable, and some of them are conserved; these variable and conserved points correspond to the pattern of other studies.

The development of molecular methods for identifying rDNA has reinforced the research of AMF communities. The small subunit (SSU) and large subunit (LSU) rRNA genes and the internal transcribed spacer (ITS) region are applicated markers for the identification of AMF species (Senés-Guerrero & Schüßler 2016). In this study, three rRNA regions are also used as molecular markers: the partial small subunit (SSU) rRNA gene, the internal transcribed spacers (ITS1, 5.8S, and ITS2), and the partial large subunit (LSU) rRNA gene. The choice of rRNA region is vital because rRNA regions vary in their ability to distinguish AMF species. The AMF SSU sequences have relatively low variability compared to the ITS region. The ITS rRNA region shows large sequence variability within the Glomeromycota and a high power down to the species level (Stockinger et al. 2010). In the fungal kingdom, the level of intraspecific nucleotide variation within the nuclear ribosomal region is highest in the ITS, followed by the LSU and SSU (Schoch et al. 2012), and the same pattern was found in this study as seen in figures 2 and 3.

There are regions within the nuclear ribosomal operon of high and low nucleotide conservation. In eukaryotes, the SSU and LSU are relatively conserved compared to the ITS, due to their biological role in encoding ribosomal RNA (Abeyrathne & Nazar 2005). ITS regions, which are under lower selective pressure, are expected to show the highest variation that can be seen in our pattern.

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خصوصیات ریخت شناسی و مولکولی گونه‌ی قارچ میکوریز آربسکولار *Acaulospora punctata* در ایران

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چکیده: گونه‌ای قارچ میکوریز آربسکولار از جنس *Acaulospora* در رشته کوه‌های ایران (استان کرمان ، راویز) ، در ارتفاع ۲۱۱۸ متری در اطراف ریشه های *Prunus cerasus* جداسازی و شناسایی شد. این گونه توسط Ohel ، در سال ۲۰۱۳ به دلیل تزئینات روی دیواره خارجی اسپور *Acaulosora punctata* نامگذاری شده است. اسپورهای این گونه زرد مایل به سفید، کروی تا نیمه کروی به ضخامت ۱۳۹-۱۰۵ میکرومتر بوده که سه دیواره داشته و هرکدام از دیواره‌ها شامل تعدادی لایه می‌شوند. بررسی فیلوژنتیکی توالی‌های به دست آمده از ITS و بخشی ازLSU و SSU ژن‌های ریبوزومی، قرار گرفتن این گونه را در Acaulosporaceaeتأیید می‌کند. طبق این بررسی‌ها، این گونه قارچی که b-pooneh در این پژوهش نام گرفت، در گروهی تک نیایی یعنی جنس *Acaulospora* و در مجاورت *A. cavernata* و *A. ignota* قرار می‌گیرد.

کلمات کلیدی: قارچ‌های میکوریزا آربسکولار، Glomeromycetes ، منطقه کوهستانی

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