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RESEARCH PAPER

The first report of *Choiromyces venosus* and a new report of *Tuber aestivum* from north Hyrcanian forest of Iran

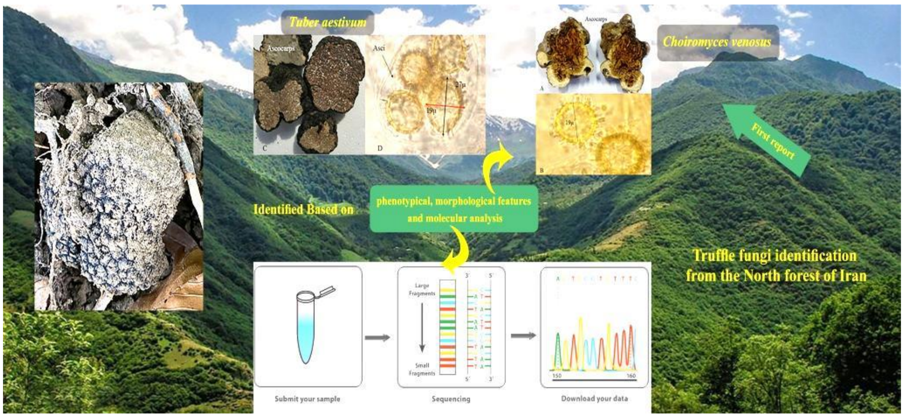
Delnia Sepahvand [1]*, Anoshirvan Shirvany [1], Vahid Etemad [1], Mohammad Javan-Nikkhah [2], Thomas Henry DeLuca [3], Abbas Atashi Khalilabad [2]

[1] Department of Forestry and Forest Economy, Faculty of Natural Resources, University of Tehran, Karaj, Iran
[2] Department of Plant Protection, Faculty of Agricultural Science and Engineering, College of Agriculture and Nature Resources, University of Tehran, Karaj
[3] Department of Ecosystems and Society, Oregon State University, Corvallis, USA

Highlights

- Identification of truffle fungi in Iran is as important as in the world because there are the rich diversity forests in Iran but the introducing and investigation on truffles are limited.
- The first report of *Choiromyces venosus* and fourth report of *Tuber aestivum* the edible truffle mushroom in Iran.

Graphical Abstract



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Abstract

Luxurious truffles have always been among the most popular symbiotic microorganisms, but there have been limited efforts to identify specific truffle species in forest systems around the world. Recent developments in molecular techniques have rapidly improved our ability to identify microorganisms responsible for the formation of truffles with different tree species. In the present study, an attempt was made to identify and describe two species of truffles occurring in deciduous forests of Golestan and Mazandaran provinces of Iran. *Choiromyces venosus* and *Tuber aestivum* were identified by a combination of morphological characterization, cytological methods and molecular analysis. Molecular characterization was performed by amplification and sequencing of the internal transcribed spacer regions (ITS) of nuclear rDNA. Based on the ITS sequences, Iranian specimens were placed in the same branch in a clade with *T. aestivum* and *C. venosus*. All sequences of *C. venosus* and *T. aestivum*, including the Iranian specimens, had an average similarity of 99.5% (the range was from 99 to 100%). Moreover, the diverse forests of Iran are a rich habitat for the ectomycorrhiza of truffle fungi. Due to the wide range of climatic conditions and forest communities in Iran, further studies are needed on the identification, diversity, ecology, phylogeny and economic value of Iranian truffles.

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* Corresponding author: t.sepahvand@ut.ac.ir (D. Sepahvand)

1. Introduction

True truffles represent a group of ectomycorrhizal fungi that form subterranean fruiting bodies that are exceptionally popular in culinary circles worldwide; however, there remains only limited understanding of the occurrence of truffles in forest ecosystems around the world. Truffles belong to the genus *Tuber* in order Pezizales of Ascomycota. These hypogeous fungi colonize the roots of dominant tree species such as oak, pine, poplar, birch, or aspen creating a mutual symbiosis that has existed for approximately 120 million years (Brundrett, 2002). The ECM fungi provide the host tree with improved access to minerals and water and in response, the plant can convey up to one third of the photosynthetic ingredients to the fungus (Nehls et al., 2007). The sharing of metabolites is vital for the perdurability of both tree and fungus, mainly in poor-conditioned soils and the ECMs offer an important strategy to dissolve the lack of nutrient and carbohydrate encountered by trees and fungi in forest (Kottke and Oberwinkler, 1986). Truffles create edible hypogenous fruiting bodies in which some of *Tuber* species such as *T. magnetum*, *T. melanospurom*, and *T. aestivum* have had worldwide mercantile importance. One of the controversial species in terms of being edible or being toxic is *Choiromyces venosus*, an ectomycorrhizal ascomycete, probably associated with oak and other broadleaved trees (Pegler et al., 1993; Hall, 1999; Mello et al., 2002).

Truffle varieties have different popularity for consumption in different regions. The ascomycete truffle *Choiromyces venosus* (Fr.) Th. Fr. (synonym: *Choiromyces meandriformis* Vittad.), the so-called pig truffle, is an ectomycorrhizal species belonging to the *Tuberaceae* family in the Pezizomycetes. *C. venosus* forms hypogeous, tuberculate fruiting bodies (ascomata) with a strong, distinctive odour. Gastronomic value of this whitish truffle is considered differently through Europe This symbiotic species is associated with deciduous and coniferous trees, and prefer clayey soils (Murat et al., 2018). In some parts of Sweden, with *C. venosus* people make delicious food and it is one of the most popular spices, and in other countries it is sold as an expensive Italian white truffle *T. magnatum* Picco due to its phenotypic and local nominal similarity. Given the old Swedish vernacular name 'vit tryffel' (white truffle), it is understandable how it is commonly mixed up with 'Tartufo bianco' in Italian. Many changes have occurred in the naming and placing of truffles in a fixed phylogenetic position because of its impressive size, exceptional morphology and unique aroma. Depending on the genetic study, it is phylogenetically more related to truffles of the genus *Tuber*, than to the desert truffles *Terfezia* (Percudani et al., 1999). To date, seven species have been accepted into the genus *Choiromyces*. *C. alveolatus* (Harkn) Trappe, *C. cookei* Gilkey, *C. ellipsosporus* Gilkey, *C. meandriformis* Vittad., *C. tetrasporus* Velen., C. *venosus* (Fr.) Th. Fr.and *Choiromyces helanshanensis* Juan Chen & P.G. Liu. *Choiromyces venosus* occurs in Europe (Pegler et al., 1993). In Germany it is considered a favorite mushroom also in central Spain and Sweden, while in France and Italy it is considered toxic. There are only three sites have known from the North American (California, Oregon and West Virginia), where it has been reported in coniferous forests (Castellano et al., 2003; Csorbainé et al., 2009; Christina et al., 2009).

There are an estimated 200 species of *Tuber* that have been reported from around the Northern hemisphere (Hall and Haslam, 2012; Leonardi et al., 2021). A few numbers of the *Tuber* genus are highly economically valued due to their unique organoleptic aroma. One of these species is the Burgundy truffle (*Tuber aestivum* Vittad.), which has an excellent flavor (Mello et al., 2006). *Tuber aestivum* is widely distributed throughout Europe from southern Europe to Ireland, Great Britain and Sweden (Gotland Isle) and East to the Caucasus Mountains, North Maghreb (rare), near and north middle Asia to Turkmenistan/ Iran borders. Under broadleaved and coniferous trees and shrubs; its ascomata develop a bit throughout the year, commonly from summer to late autumn (Molinier et al., 2016; Leonardi et al., 2021) and found in a variety of forest types (Gryndler et al., 2011), to date it has been reported from Israel (Turgeman et al., 2012), France, Italy, New Zealand, North Africa, Spain, Turkey, Algeria, and Hungary (Stielow and Menzel, 2010), Czech Republic (Gryndler et al., 2011). *Tuber aestivum* grows in an ectomycorrhizal association with many different tree species, such as *Corylus avellana*, *Quercus spp*, (Turgeman et al., 2012), *Tilia cordata*, *Carpinus betulus* (Gryndler et al., 2011). According to Brundret (2002), *C. avellana*, *Q. pubescens*, *Carpinus betulus*, and *Ostrya carpinifolia* are the usual hosts of *Tuber* species in nature and truffle orchards (Brundret, 2002). This species prefers moderately alkaline soils with a pH of 8, a $CaCo_3$ content greater than 10%, a good aeration, and permeability. Ascocarps of *Tuber* species are generally distinguished by their phenotypic characteristics, mainly based on morphological features of the asci and spores. Due to limited morphological characters, these fungi are difficult to identify at species level. Molecular phylogenetic studies have recently demonstrated that morphological characters of hypogeous Ascomycetes can be unreliable (Ferdman et al., 2005). According to phylogenetic studies based on the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA), the genus *Tuber* is composed of nine distinct species groups (Clade) : Aestivum, Excavatum, Gennadii, Macrosporum, Maculatum, Melanosporum, Puberulum, Regianum, Rufum (Leonardi et al., 2021). Studies in Iran on truffles coexisting with trees have been reported in only some limited case studies of the fungi with the host trees. Jamali (2014) reported that *T. aestivum* from the oak forests of Mazandaran and strategies were presented to prevent improper harvesting and destruction of its natural habitat (Jamali, 2014). *Tuber aestivum* var. *uncinatum* was recorded by Ammarellou and Alvarado (2018) with molecular identification from Mazandaran forests (Ammarellou and Alvarado, 2018). In the other study, a third report from an oak forest in Zagros region was reported (Jamali, 2017). In the other research, recorded the ascomata of six *Tuber* spp. (*Tuber borchii*, *Tuber brumale*, *Tuber macrosporum*, *Tuber rufum* f. *lucidum*, *Tuber excavatum* and *Tuber fulgens*) inside commercial batches of *T. aestivum* imported from Iran to Italy (Puliga et al., 2021). The following research was conducted in an attempt to identify and characterize two truffle specimens from Iran obtained from Hyrcanian forest by using both morphological examination and molecular analyses.

2. Materials and Methods

2.1. Truffle sampling

In this study, two specimens of Truffles associated with roots of oak (*Quercus castaneifolia*) were collected by using a trained dog from two different regions of Hyrcanian forests (deciduous forest) in northern Iran. One truffle of creamy white appearance was obtained from Loveh forest in the Golestan province in the eastern part of the Hyrcanian forests which is a young protected forest covered with an oak stand approximately 40-60 years in age. The other truffle specimen was black in color and found in a young even-aged oak stand that was about 25-30 years in age in Mazandaran province in Pol Sfid (in the middle part of Hyrcanian Forests) in the north of Iran. The exact location is not reported here to protect the truffles from illegal harvesting. The hypogenous fruit bodies collected were kept on dry ice until transferred to a laboratory where samples were stored at -20°C in laboratory of forest biology, university of Teheran until processing for analysis.

2.2. Morphological characterization

Morphological analysis was performed on fresh fruit bodies. Thin sections of the fruiting bodies were mounted on glass slides using distilled water as mounting media for microscopic analysis. Fruit-body characterization was based on a number of parameters such as peridium color, gleba texture and color, shape, color of asci, number of ascospores per ascus, sizes, color and ornamentation of the ascospores. Specimens were identified based on available taxonomic keys (Hawker 1954; Pegler et al., 1993).

2.3. Phylogeny

DNA extraction was performed using the method described (Zhong and Steffenson, 2001). The complete internal transcribed spacer (ITS1-5.8S-ITS2) region of rDNA were amplified using the ITS4 and ITS5 primers (White et al., 1990). The PCR amplification was carried out in a thermocycler with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation step at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, and terminated with a final extension step at 72 °C for 10 min. The PCR product was analyzed in 1.5% agarose gel by electrophoresis with 1x Tris-Boric acid-EDTA buffer (TBE) and sent to Cardiogenetic Research Center (IRAN) for sequencing. After sequencing (Kumar et al., 2004), sequence was manually edited with Chromas 2.6.6 software (Technelysium, Australia) and the edited sequence was saved in FASTA format. The resulting sequence (500-550 bp) was subjected to BLAST search to find the most similar sequences in the National Centre of Biological Information (NCBI) (Altschul et al., 1990). Seventeen reference ITS sequences of *Choiromyces*, *Tuber*, as well as our isolates and *Globisporangium ultimum* as the out group, were selected for phylogenetic analyses. Then, the sequences were aligned with Clustal W (Thompson et al., 1994). Maximum likelihood (ML) analysis (Felsenstein, 1973) was performed by heuristic search with Mega X (Kumar et al., 2018). Bootstrap analysis (Felsenstein, 1985) of the ML tree was performed with 1000 replicates. Newly obtained sequence in this study were deposited in GenBank (*Tuber aestivum* with Accession No. MW682332 and *Choiromyces venosus* with Accession No. MW682330).

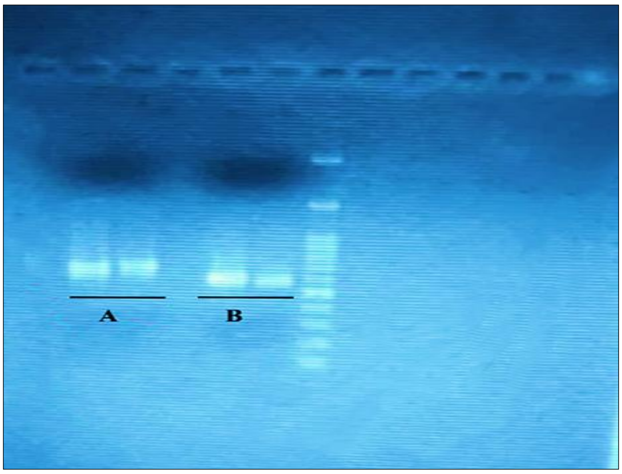

Figure 1. Gel electrophoresis of ITS-PCR products *Tuber aestivum* (A), and *Choiromyces venosus* (B), specimens using primer pair ITS1/ITS4 on 1% agarose gel, 550 bp.

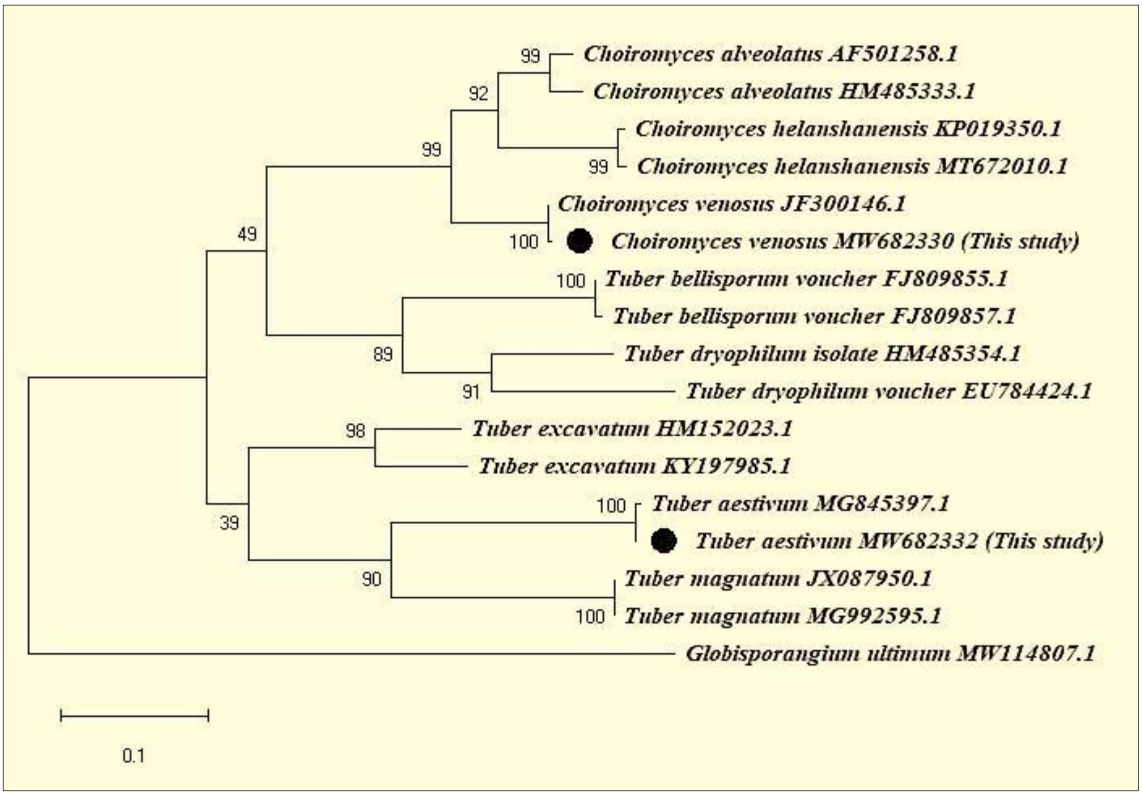

Figure 2. Phylogram Maximum Likelihood (ML) tree based on aligned sequences of ITS region of 17 isolates of *Tuber*, *Choiromyces* and *Globisporangium ultimum* as out-group generated in MEGA X. Bootstrap values (1000 replicates) indicated at the nodes. The scale bar indicates 0.1 expected changes per site. The black circles refer to Iranian *Choiromyces venosus* and *Tuber aestivum*.

3. Results and Discussion

The white truffle has the following characteristics: Ascoma globose to subglobose, irregularly lobulated, 9×11cm in diameter. (Fig. 3). The peridium was whitish to creamy-white or brownish with a small dark spots. The gleba was whitish near the edge to yellow-brownish 'due to an onset of decaying'the center with sinuous veins similar to those of the genus *Tuber* (Fig. 3A). It has a high pungent almost pleasant aroma. Pseudoparenchimatic peridium. The asci were claviform, pedunculated, and contained 6-8 spores per ascus. Spores globose 17-21 μm in diameter, yellowish, with isolated curvy spines with a truncated tip. According to morphological and molecular examination the fruit-body is *Choiromyces venosus* (Fr.) Th. Fr. It is the first report of this species from Iran.

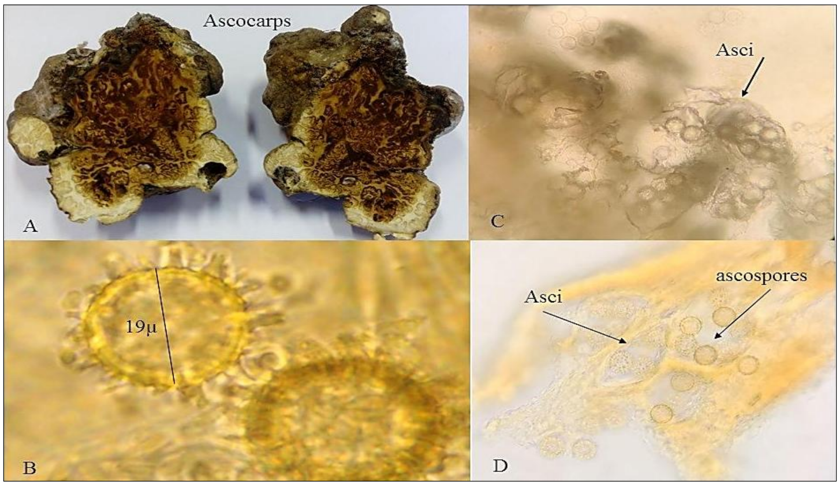

Figure 3. Morphological characteristic of *Choiromyces venosus* specimens collected in northern Iran; ascocarps (A), spore size (×100) (B), the asci and ascospores (×40) (C, D).

Specimen of black Truffle has following features: Ascomata were subglobose, heavily lobed and irregular, 8-10 cm in diameter (Fig. 4). The peridium was warty and black in color and pseudoparenchimous in texture. The gleba was firm, solid, brown to black, and with brown and white veins sinuous similar to those of the genus *Tuber* (Fig. 4.C). The truffle possessed a strong aroma, perfectly pleasant. Asci were globose to subglobose in form and contained 3-6 ascospores per ascus. Spores globose to broadly ellipsoid (24×18) 24-29x18-27 μm in diameter, yellow, translucent, ornamented with a coarse irregular reticulum (Paolocci et al., 2000). According to morphological resembling and molecular result, the fruit-body is *Tuber aestivum* Vittad. This represents the fifth documented report of this species of truffle from Iran.

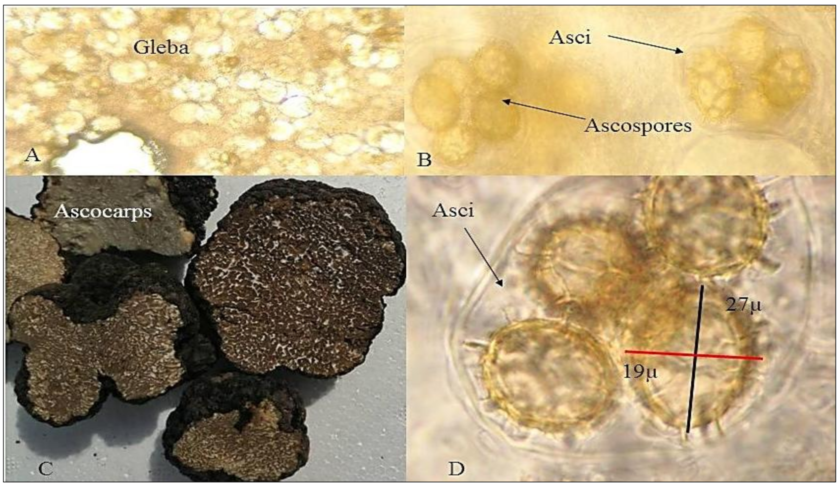

Figure 4. Morphological characteristic of a black truffle, *Tuber aestivum* collected from an oak forest in northern Iran. The panels present the gleba (×10) (A), the asci and ascospores (×40) (B), ascocarps (C), spore size and ascus (×100) (D).

Table 1. Morphological description of *Choiromyces venosus* and *Tuber aestivum* specimens collected in Northern Iran.

Characteristics	*Choiromyces venosus*	*Tuber aestivum*
Location	Golestan province	Mazandaran province
Date of hunting	November 2020	June 2020
Ascomata	Globose 9–11cm in diameter	Subglobose to much lobed and irregular, 8 cm in diameter
Peridium color	Whitish to creamy- white / brownish with a smal darker spots	Warty and pale Black to jet-black
Gleba color	whitish near the edge to Yellow-brownish in the center	Solid, brown to black, and with brown veins
Aroma	Very pungent, almost pleasant	Strong and perfectly pleasant
Peridium texture	Pseudoparenchimous	Pseudoparenchimous
Asci form	Claviform, pedunculated, containing 6-9 spores	Globose to subglobose containing 3-6 spores
Spores size	globose 17–21 μm diam roundish	Globose to broadly ellipsoid,24-29x 18-27 μm
Spores color	yellowish, with isolated curvy spines with truncated tip	yellow, translucent, ornamented with a coarse irregular reticulum

From oak stand in the Hyrcanian forest in northern Iran, two truffle species, *Choiromyces venosus* and *Tuber aestivum*, with significant commercial interest were identified on the basis of morphological features and molecular analysis (Iotti et al., 2010). For instance, a considerable level of effort has been invested into molecular differentiation of *T. aestivum* and *T. aestivum* forma *uncinatum* (Paolocci et al., 2004; Ammarellou and Alvarado, 2018). Using PCR with the primers ITS4 and ITS5, a fragment of 550 bp was amplified for *T. aestivum* and *Choiromyces venosus* specimens separately. Based on ITS gene sequences, these sequences showed 100% homology with the corresponding sequences of *T. aestivum* and *C. venosus* from Gen Bank (O'Donnell and Cigelnik, 1997). Subsequently, both phenotypical and molecular data confirmed the identification of the both isolates as *T. aestivum* and *C. venosus*. Based on ITS sequences, our specimens were clustered in a distinct monophyletic group related to *T. aestivum* and *C. venosus* reported from others. In agreeing with literatures, in Kermanshah province, *T. aestivum* was found to be associated with *Quercus brantii* in Zagros forest due west of Iran (Jamali, 2017). Also the previous studies are in alignment with a current investigation announcing the identification of *T. aestivum* from the Alborz forest in the north of Iran. However, our report of *C. venosus* is the first of such from Iran, this identification shows that forested territory in Iran has a significant potential to grow various natural truffles (Pacioni et al., 2007).

In this respect, recently six *Tuber* spp. including *Tuber borchii*, *Tuber brumale*, *Tuber macrosporum*, *Tuber rufum* f. *lucidum*, *Tuber excavatum* and *Tuber fulgens* have been identified and recorded through. Imported bunch of truffes from Iran to Italy that seems Iran to be the most Eastern location where European species of *Tuber* are found. (Puliga et al., 2021). Similar to other ECM Pezizomycetes (i.e., *Tuber* spp. And *Choiromyces*), confirming that the genomes of ECM species are generally larger than those of saprotrophic species (Payen et al., 2014). The ascomycete truffle *C. venosus* is still largely unknown with respect to its biology, ecology and physiology. Preliminary results of cytotoxicity data indicate that the amounts of *C. venosus* extract required to achieve cell death in the cytotoxicity assay is similar to that of other commonly consumed fungi including *Agaricus bisporus* and *T. aestivum* (Hansen and Pfister, 2006). Also in the Golestan province of Iran it consumed without any food poisonous reports. Most reports on the consumption or biology of this species have been in Sweden where it establishes mycorrhizae mainly with *Quercus*, *Tilia*, *Betula* and *Corylus*. Its typical habitats are light deciduous woodlands, wooded pastures and parks, on somewhat loamy, neutral to slightly basic, calcareous soils. Wild boars (*Sus scrofa* L.) are attracted by *Choiromyces* but it is argued also the common magpie (*Pica pica*) as a potential dispersal vector. *Choiromyces* is considered an edible species in Sweden despite its somewhat rubberlike smell (Massei, and Genov, 1995). The *C. venosus* has been found mainly in July and August in throughout Europe, to a large extent in gardens or along roadsides (Hawksworth et al., 1996). It is possible that the frequent appearance in July and August while it completes its life cycle in October to November in Iran because of lower latitude geographically (Giovannetti and Fontana, 1982; Jeandroz et al., 2008). Although Truffles are most often reported in association with forest trees, there are numerous examples of the hypogeous fungus *C. venosus* being present in cultivated gardens or with broad-leaved fruit and ornamental shrubs and trees in the Czech Republic (Kottke and Oberwinkler, 1986). These observations require more investigation to determine presence, diversity, ecology, phylogeny, and economics value of Iranian truffles.

4. Conclusion

The Hyrcanian forests in the north of Iran are home to a large number of ectomycorrhizal fungi. The aim of this study was to identify and introduce two species of edible ectomycorrhizal fungi. From oak stand in the Hyrcanian forest two truffle species, *Choiromyces venosus* and *Tuber aestivum*, with significant commercial interest were identified on the basis of morphological features and molecular analysis. Identification of symbiotic fungi with trees provides a comprehensive perspective on the diversity of existing fungi and the increasing knowledge of the ecosystem biology and also the path of future studies in Iran.

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