

# Evidence of *Zelkova carpinifolia* as a potential host of *Tuber uncinatum* from the Quaternary of Hyrcanian temperate forests

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## Research Article

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# Abstract

*Tuber uncinatum* (Tuberaceae, Pezizales) is a commercially important ectomycorrhizal (EcM) fungal species associated with many deciduous EcM host species belonging to the Fagales lineage. Because of its host multiplicity among deciduous species, we hypothesized that it might associate with other plant lineages of Rosids as the most recent common ancestral of the ectomycorrhizae (MRCA-EcM) of Ulmaceae radiated preceding Fagaceae. The samples (host plant specimens, fungal ascomata, and soil) were from homogeneous Caucasian elm populations of the Hyrcanian temperate forests of Iran. Diagnosing the host plant was performed by using available botanical sources. The EcM symbiosis evidence and EcMF identity were illustrated by surveying gleba, spore shape, and EcM root anatomy using SEM, light, and stereo-microscopy, followed by molecular analysis. The micrographs obtained from root examination demonstrated the Hartig net penetration among cortical and epidermal cells and the presence of mantle as the EcM sheath. This study supports the EcM symbiosis of *T. uncinatum* with *Zelkova carpinifolia* from the Quaternary period in the Hyrcanian temperate forests.

## Introduction

Mycorrhizae is a prevalent symbiosis in terrestrial ecosystems (Trappe 1962; Smith & Read 1997, 2008; Wang and Qiu 2006; Brundrett 2009), which is formed between mycorrhizal fungi and most higher plants (Brundrett and Tedersoo 2020). Ectomycorrhizae (EcM), one of the most important mycorrhizal types distinguished based on the phylogenetic lineage of the host plant (Tedersoo & Brundrett 2017) and root morphological feature (Brundrett 2004), including the presence of the mantle and the Hartig net and intercellular hyphae that have not penetrated the cells (Smith & Read 2008). There are about 7750 known species of ectomycorrhizal fungi (EcMF) worldwide (Rinaldi et al. 2008), including 250 genera belonging to 80 independent lineages (Tedersoo & Smith 2013; Geml 2017), mainly belonging to Basidiomycota and Ascomycota (Tedersoo et al. 2010).

According to a recent phylogenetic study, lineages of EcM host plants are related to ancestral relationships (Tedersoo & Brundrett 2017). The Angiosperms species forming EcM involve 33 families (Wang & Qiu 2006). The EcM habit status in the Fagales lineage seems to be ancestral, given that it involves more than 80% of its genera (Larson-Johnson 2015).

The most diverse and major lineages forming ECM are located in rosids (Wang & Qiu 2006). The rosids are the earliest clade of angiosperms forming ECM inferred from molecular clock analyses at about 100-109 million years ago (Mya). The most recent common ancestral of the ectomycorrhizae (MRCA-EcM) forming the rosids clade provides a logical range of EcM participation that does not overestimate the origin time of EcM in angiosperms hosts (Wikström et al. 2001). Also, the occurrence of the MRCA-EcM of Ulmaceae in the Early Cretaceous (110 Mya) preceded the MRCA-EcM of Fagaceae which radiated in the Late Cretaceous (89 Mya) (Wang & Qiu 2006; Wikström et al. 2001) (Fig. 1).

The MRCA of *Zelkova* occurred in East Asia in the early Paleocene and scattered to Europe from the mid-Paleocene to the early Eocene. After then, it diverged into East Asian and European biogeographic clades (Zhang et al. 2021; Ma et al. 2016). The MRCA of *Zelkova carpinifolia* in 12Mya (Zhang et al 2021) radiated preceding *Quercus castaneifolia* (5.5 Mya) (Hipp et al. 2019) (Fig. 1). *Zelkova carpinifolia* (Pall.) K. Koch. (Caucasian elm, its common name) is distributed in the Transcaucasian region (Kozłowski and Gratzfeld 2013), extended from Georgia and Azerbaijan to northern and northeastern Iran (Euxino-Hyrcanian province) and a few spots in eastern Turkey (Zieliński 1979; Browicz & Zieliński 1982; Khatamsaz 1991; Güner

and Zieliński 1998). The western Colchic region and the eastern Hyrcanian region have been reported as its two major populations relying on a substantial genetic variation (Maharramova et al. 2015). *Z. carpinifolia* is located biogeographically in the temperate Ulmaceae clade belonging to the European clade (Zhang et al. 2021). It is an Arcto-Tertiary relict specie (Leroy and Roiron, 1996; Ramezani et al., 2008; Zohary, 1973), which survived in the Hyrcanian temperate forests (HTFs) (Akhani et al., 2010), but severely under extinction in other regions (Bétrisey et al. 2018). Despite the uncertainty about the expanding realm of the HTFs preceding the Last Glacial Maximum (LGM) (Leroy et al., 2013), it is a contiguous region along with the Colchic forest of Georgia in the European Pliocene and Early Pleistocene vegetation era (Knapp, 2005).

*Tuber* Mich. ex F.H. Wigg. (Micheli 1780) is an EcM genus of the *Tuberaceae* family belonging to the order Pezizales (Bonito et al. 2009). *T. aestivum* includes two varieties, var. *aestivum* Vittad. (Vittadini 1831) and var. *uncinatum* Chatin. (Chatin 1887). The mean origin time of the *Tuberaceae* has been estimated in the late Jurassic at 156 Mya. *Tuber* is estimated to have diverged from other taxa in the early Cretaceous. The majority of its lineages from extant subgenera emerged in the late Cretaceous era at 65 Mya, although their primary radiations occurred during the Paleogene. The mean ages of the MRCA for the *aestivum* clade are estimated at 101 Mya (Fig. 1).

Although *T. aestivum* has several hosts of deciduous trees (Hilszczanska et al. 2008), a wide extent of temperate forests is considered natural habitats for its anonymous host species, as reported by recent studies (Stobbe et al. 2012; Benucci et al. 2012). In this study, we sampled *T. uncinatum* populations and associate hosts to examine (i) the other likely deciduous host species of *T. uncinatum* and (ii) its spatiotemporal distribution pattern along the alluvial chronosequence in Hyrcanian temperate forests (ACHTFs).

## Materials & Methods

### *Sampling and field description*

*T. uncinatum* ascomata, *Z. carpinifolia* mycorrhizal root, and the adjacent soil were sampled from *Z. carpinifolia* homogeneous populations to avoid misdiagnosis of mycorrhizal root species caused by potential sampling error in mixed forests. Ecological surveys were conducted along the HTFs with a wide range of elevation differences (from 230 to 1100 m A.S.L.) and homologous climate. The specimens were collected from all the natural habitats of *T. uncinatum* in the northern region of Iran during spring and autumn. The ACHTFs surrounded by the mountains along the southern coasts of the Caspian Sea are one of the very rare temperate deciduous forests in the semi-arid region of the Middle East. The studied regions of the HTFs considered humid temperate zone accompanied by high relative humidity based on bio-climatic classification (Khatibi & Saberi 2020).

### *Pedological examination and geological interpretation*

The collected soil samples from harvested points of *T. uncinatum* ascomata symbiont with *Z. carpinifolia* were dried using an oven at 120°C. The soil texture and granulation tests were accomplished using sieving series (5, 10, 35, 70, 230, and <230 meshes) by the USDA method (Ditzler et al. 2017); then the soil type was recognized by the soil texture chart. Based on data bring from the maps of soil, soil potentially, and geology that were all taken from the European Soil Data Centre (ESDAC), the sampling sites contain Lithosol-Brown forest soil and Rendzina ([https://esdac.jrc.ec.europa.eu/images/Eudasm/Asia/images/maps/download/IR2000\\_1SO.jpg](https://esdac.jrc.ec.europa.eu/images/Eudasm/Asia/images/maps/download/IR2000_1SO.jpg), Dewan et al.

1961) with soil limitations due to dissected relief and/or shallow depth (available on [https://esdac.jrc.ec.europa.eu/ESDB\\_Archive/EuDASM/Asia/images/maps/download/IR2000\\_3SO.jpg](https://esdac.jrc.ec.europa.eu/ESDB_Archive/EuDASM/Asia/images/maps/download/IR2000_3SO.jpg), Dewan & Famouri 1963) along the ACHTFs ([https://esdac.jrc.ec.europa.eu/ESDB\\_Archive/EuDASM/Asia/images/maps/download/IR2000\\_2SO.jpg](https://esdac.jrc.ec.europa.eu/ESDB_Archive/EuDASM/Asia/images/maps/download/IR2000_2SO.jpg), NIOC 1957, The Geological Staff of the Iran Oil Company). All the maps overlaid on Google Earth available on <https://github.com/kuboosoft/google-earth-installer.git> can be run on fedora 37 OS. ArcGIS Ver. 10.8.2 was employed for the distribution reconstruction of *Z. carpinifolia* and *T. uncinatum* on the geological map (Fig. 7).

### ***Macroscopic and microscopic examination***

The collected specimens were identified using the diagnostic key for *Zelkova* from present **resources** of flora Iranica (Zieliński 1979), the flora of Iran (Khatamsaz 1991), and the flora of Turkey (Davis 1972). The genus *Zelkova* belongs to the family of Ulmaceae (Denk and Grimm 2005), related to the order Rosales.

Specimens identity of collected samples of truffles was accomplished according to the characteristics mentioned by Chevalier and Frochot (1997). Ascomata were identified according to the diagnostic keys for hypogeous fungi (Szemere 1965; Pegler et al. 1993; Zambonelli et al. 2000; Montecchi & Sarasini 2000; Ceruti et al. 2003).

Preliminary examinations were performed on collected samples of truffles under a Nikon stereomicroscope based on the color and size of gleba and peridium of ascomata. The examination processes were performed on young mycorrhizal roots collected from several stands according to the flowchart of Brundrett & Tedersoo in 2018. The evidence from the survey of EcM root samples was then compared with that of *Corylus avellana* accessed in DEEMY (An Information System for Characterization and DEtermination of EctoMYcorrhizae) online key available in [www.deemy.de](http://www.deemy.de) documented by Agerer (Agerer 1987–2012; Agerer & Rambold 2004–2020). Sectioning of the truffle fruiting body and mycorrhizal roots for examination under light microscopy (LM) was implemented manually using a titanium razor blade under the stereomicroscope.

For the histological study of the root cortex, the preparation of samples and proceeding was performed according to the Johansen method (Johansen 1940). Initially, the root samples were fixed using a common fixative solution (formaldehyde/acetic acid/water by volume ratio of 1:1:1) at 5 °C. Dehydration was then performed on the samples using an ethanolic series graded by 60%, 70%, 80%, 90%, and 96% v/v. Polychromatic staining was accomplished on longitudinal-sectioned root samples using 0.05% O-Toluidine Blue solution (O'Brien et al. 1964). The sectioned samples from the gleba of the fruiting body were stained using Melzer's reagent (Baral 1987). The histologic characteristics of mycorrhizal roots and *Tuber* specimen spores were examined under Olympus BH-2 LM (×100). The micrograph was prepared of mycorrhizal roots and spores and surrounded ascus using a digital camera.

Scanning Electron Microscopy (SEM) was applied for getting a micrograph of the mycorrhizal root surface. In this order, cross-sectioning of the mycorrhizal root was prepared by hand using a titanium razor blade. A modified method was used for specimen preparation. The sample fixed with 1% Glutaraldehyde and 2.5% Formaldehyde was then dehydrated through an ethanolic series graded by 50% to 96% (v/v) and immediately dried with HMDS (hexamethyldisilazane) within the 30s for preparation of fungal soft tissue to examination under SEM (Gibbons & Geetha-Loganathan 2019), a suitable alternative to Critical Point Drying (CPD) (Rumph & Turner 1998). The

specimen attached to the stub was gold coated under vacuum within 2 min by Vactech HVT-16-3HD-LD magnetron sputtering coater and then observed in Hitachi SU3500 15.0KVX120 SEM.

### ***PCR, sequencing, and phylogenetic analysis***

The Internal Transcript Spacer region of nuclear DNA (nrDNA ITS) was used to identify the *Tuber* specimen. ITS molecular analysis and sequencing performed by the Institute of Ecology and Earth Sciences, University of Tartu. About 20 mg of gleba was used for DNA extraction. The following PCR program was used: initial denaturation at 94 °C for 5 min, which was followed up by 35 cycles of denaturation at 94 °C within 30 s, 52 °C for 30 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 min.

The collected specimen sequence derived from ITS1 and ITS2 were analyzed; then the obtained ITS sequences comparison in FASTA format was performed in the UNITE database (<https://unite.ut.ee/> Kõljalg et al. 2005) using BLASTn, and the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) database using MegaBLAST tool (Altschul et al. 1990). The most similar ITS sequences with the most reliable resources were downloaded for each morphotype of some selected *Tuber* species in the NCBI database to analyze the ML phylogenetic tree of ITS regions. *Choiromyces alveolatus* (Harkn.) a species of Tuberales was selected as the outgroup. Alignment was accomplished with the MAFFT V. 7.475-1 program (Katoh et al. 2002) using the FFT-NS-2 alignment algorithm. Maximum likelihood (ML) analyses were carried out by the ITS data set to estimate phylogenetic relationships with Randomized Accelerated Maximum Likelihood (RAxML V. 8.1.17; Stamatakis 2014) to obtain the highest likelihood in a heuristic search (Lu & Zhang 2013) based on the GTRGAMMA model for nucleotide with 10,000 rapid bootstrap trees on starting. The ML tree visualized in FigTree V. 1.4.

## **Results**

### ***Soil type, bedrock characteristics and geological era***

The results obtained from the soil granulation test indicated that *T. uncinatum* is mainly located on limestones. Based on soil, soil potentiality, and geological data (Fig. 7) taken from ESDAC maps, the ascomata have been solely harvested from Chomerli forest park and Daland forest park that contain Lithosol and Rendzina along the Quaternary alluvial chronosequence in the HTFs (Dewan et al. 1961).

### ***Mycobiont diagnosis***

The fruit bodies are overall similar to those of *T. aestivum* but had a more intense odor and spicy taste. The average weight of the collected samples varied between 6 and 18 g. The average diameter of the samples was measured as 1.4 - 6.2 cm. The specimen ascocarps are sub-globose or irregularly globose, 2–5 cm in diameter, and black or grayish black peridium (Fig. 2a). The specimens were distinguishable as *T. uncinatum* by a darker gleba, smaller peridium warts, and non-striated warts than *T. aestivum* under a stereomicroscope with 3x magnification (Figs. 2b & 2c). It mainly diagnosed micromorphologically by globose asci, about 62.5 µm, with 2–4 spores irregularly clustered inside (Fig. 2d). Spores are broadly ellipsoid or sub-globose, yellow to brown,

ornamented by 5 µm height of mesh reticulum (alveolate septa) that have hooked thorns in spore reticulum (Figs. 2e & 2f).

### ***Molecular identification of mycobiont***

The collected *Tuber* specimens located inside the branch of the *aestivum* clade beside the other genotype of *T. uncinatum* were taken from NCBI, which can be seen on the ML phylogenetic tree (Fig. 6).

### ***EcM symbiosis evidence***

The presence of EcM mantle was demonstrated by the micrographs obtained from the examination of the exterior side of the root by SEM microscopy (Fig. 3c) and root longitudinal section under LM (Figs. 4a & b). Also, the penetration of *T. uncinatum* Hartig net around the cortical, epidermal, and hypodermal cells and among the vascular elements indicated in the longitudinal section of *Z. carpinifolia* root examined under LM (Fig. 4).

### ***Host diagnosis***

Specimens were identified by surveying mature organs. It was identified as *Z. carpinifolia* (Fig. 5). It is a large deciduous tree, that stands 20 to 35 meters tall; but sometimes the height solely reaches half a meter in dwarf creeping bushes (Figs. 5b, d & e). It is typically a stout, upright tree with a short, robust trunk that the numerous nearly straight branches made the canopy cover, which is distinguished by a very unusual vase-like form. Its alternate leaves are papery, rough, elliptic or oval, serrate, and have comb veins, by a deep green color that changes to orange-brown in the fall. *Z. carpinifolia* has dimorphous leaves including coarse leaves on sterile shoots and fine leaves on fertile bushes (Kozłowski & Gratzfeld 2013; Jasińska et al. 2021 & 2015). Its size is varied from 4 to 10 cm in length, 2.5 to 6 cm in width, recognizable by 7 to 12 indents on either side of a sharply serrated edge. Its unblooming flowers are small, greenish, without petals, and pollinated by the wind. Its tiny green drupe fruit, 5 to 6 mm in diameter, is directly attached to the side of the leaf (Zieliński 1979; Khatamsaz 1991).

## **Discussion**

### ***Habitat evidence***

Since it is possible that being mixed with other EcM hosts facilitates the desired host in contamination to fungus, the examined specimens in the present study were collected from homogeneous Caucasian elm communities to avoid misdiagnosis of specimens. In mixed communities, *Z. carpinifolia* species solely along with *Quercus castaneifolia* vegetation elements constitute Quercetum associations. According to field observations, the reported EcM association observed in the natural habitats where *Pinus* species was a constant but not dominant element of the plant association.

Our finding (Fig. 5b) is consistent with the empirical observation of Martegoute and Courdeau (2002), who introduces the *brûlé* condition (Martegoute and Courdeau 2002; González-Armada et al. 2010).

The lithosol, rendzina, and alluvial soil types recognized on the ESDAC map (Dewan et al. 1961) are the same as the soil type of *T. aestivum* which in turn reported for *Corylus avellana*, *Carpinus betulus*, and *Betula pendula* (Stobbe et al. 2013; Gryndler et al. 2013; Marjanović et al. 2010).

### ***Microscopy evidence of EcM***

The micrograph obtained from root examination under LM demonstrates the Hartig net has surrounded cortical and epidermal cells as the same hyphal coils were observed among vascular bundles that all are considered the most reliable evidence for establishing the EcM associations (Fig. 4). The presence of mantle as the EcM sheath is visible in the examination of root longitudinal sectioning under LM (Figs. 4a & 4b) and the exterior formations of root under SEM indicated in the form of a cobwebs-like structure (Fig. 3c). The root deformation of *Z. carpinifolia* especially dichotomous branches on lateral roots, as well the abnormal forms such as tipstaff and/or Scorpius tail are the obvious features for typical EcM root characteristics which observed under the stereomicroscope and SEM (Fig. 3).

### ***Historical evidence***

The recorded geographic locations in the present study and geological era taken from the ESDAC maps and overlaying them on Google Earth indicated that the discussed EcM association was noted only in the habitats have belonged to the Loess (Caspian area) Quaternary period; Whereas, *Z. carpinifolia* is distributed widely in different geological periods throughout the ACHTFs (Fig. 7; Brovicz & Zielinski 1982). According to some palaeobotanical studies, *Z. carpinifolia* indicated an extant widespread along with the Transcaucasia in 6-5.5 Kya (Leroy and Roiron, 1996; Ramezani et al., 2008; Zohary, 1973). A report studying the population genetics of *Z. carpinifolia* in the western Colchic region of Georgia and the eastern Hyrcanian region of Iran indicated that gene flow has mediated by wind pollination but not by seed dispersal (Maharramova et al. 2015). Although, the distribution map of *Z. carpinifolia* (Browicz and Zielinski 1982) indicated that its distribution pattern as the interrupted patches in the HTFs refugia (Fig. 7) along with the Transcaucasia seems to be affected by climatic changes in the LGM (27 Kya) and then by the dry period in the Early-Holocene (11.7-8.2 Kya) (Leroy et al. 2013) (Fig. 1). So, the discussed ECM association seems to have formed during the Mid and Late-Holocene epoch synchronized with the extant widespread of *Z. carpinifolia* after the LGM and drought period. The field observations and outcomes from GIS on the geological map indicated the EcM association of *T. uncinatum* with *Q. castaneifolia* in the areas related to the Neogene, Paleogene, and even the Late Cretaceous periods.

## **Conclusion**

The identified symbiotic associations could be the first EcM-hosting report of *Tuberaceae* from the Pezizales clade in Ulmaceae (Rosids clade). Also, *Z. carpinifolia* seems to be as the posterior EcM host of *T. uncinatum* in deciduous species of the HTFs during the Neogene era, who itself might have engaged from the other deciduous species, or basal eudicots, and Pinales that have already radiated there in the Paleogene era. According to a recent IUCN report, *Z. carpinifolia* has been reported as a vulnerable species under threat (Bétrisey et al. 2018); So in-situ species conservation is a necessity.

## Declarations

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**Author contribution** S.M.A. and H.R. planned the study. The fieldwork, laboratory examinations and diagnosis, molecular and phylogenetic analyses, and writing the manuscript were performed by S.M.A. H.R. revised the manuscript.

**Competing interests** The authors declare no competing interests.

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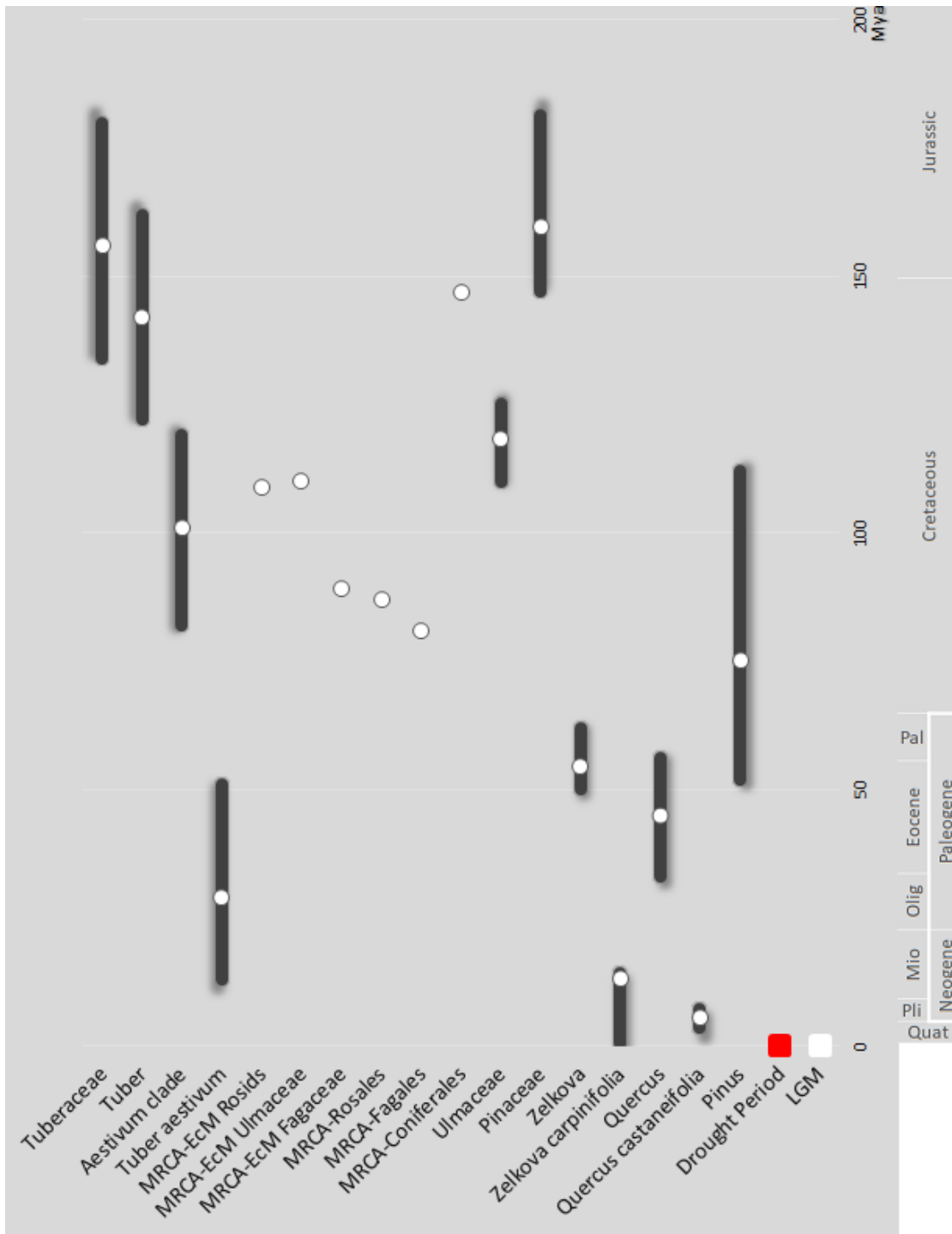
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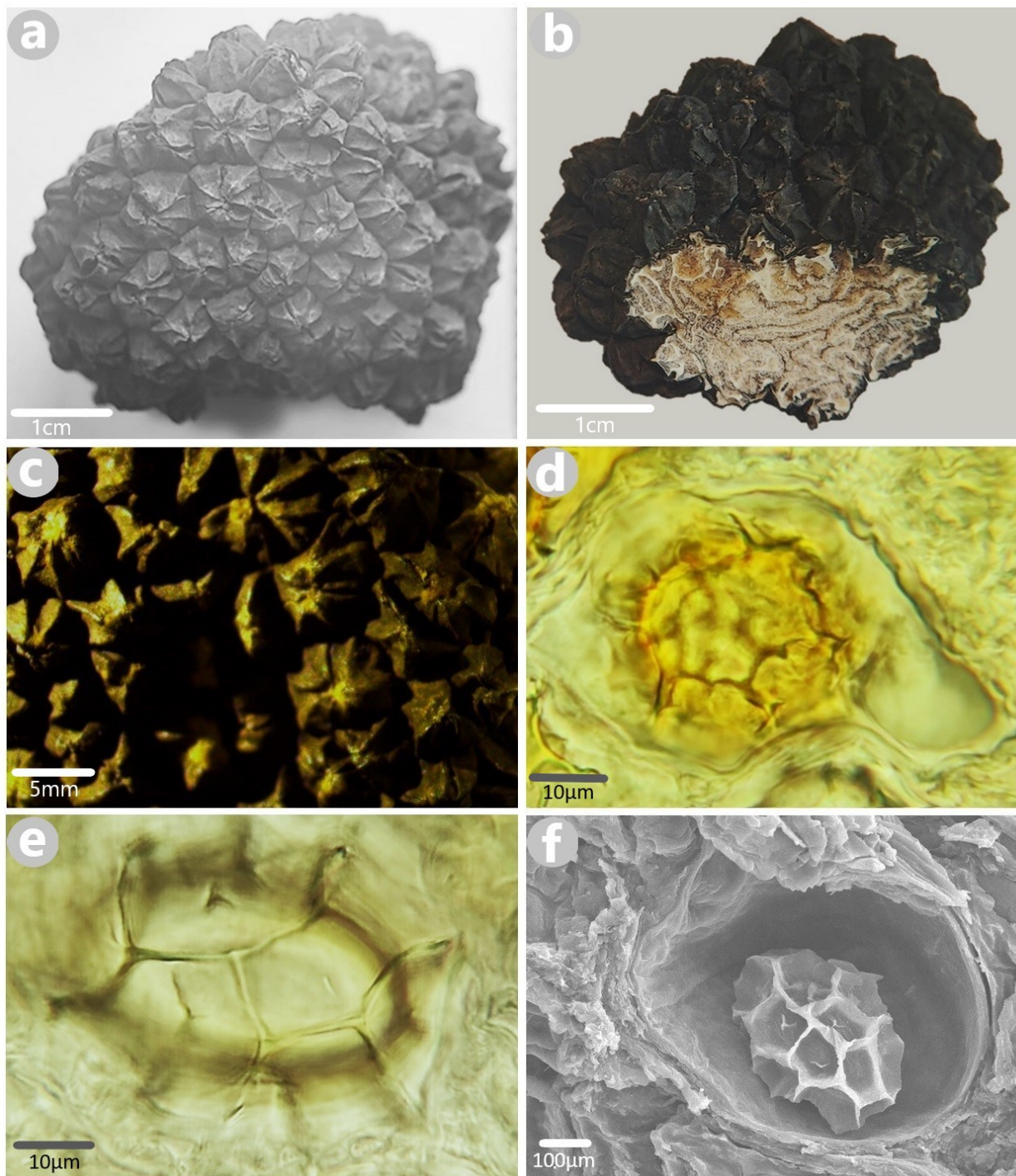
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## Figures



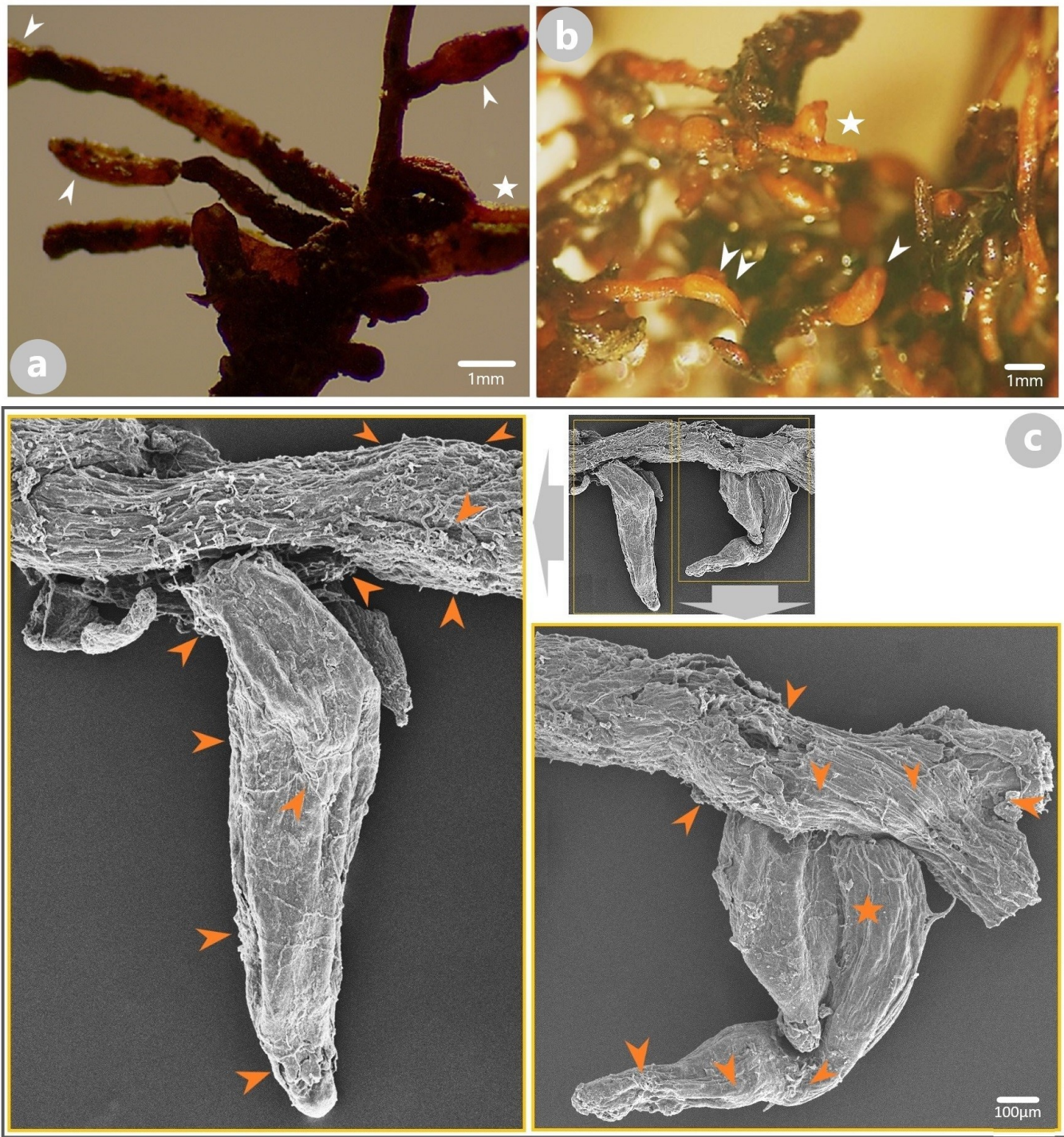
**Figure 1**

Radiation range and nodes (MRCA/EcM-MRCA) of *Tuber* clade and some selected containing EcM-forming clades from Ulmaceae, Fagaceae, and Pinaceae during the geological era based on Bonito et al. 2013; Bruns and Shefferson 2004; Clark et al. 2011; Hipp et al. 2019; Wang and Qiu 2006; Xiang et al. 2014; Zhang et al. 2021 & 2022



**Figure 2**

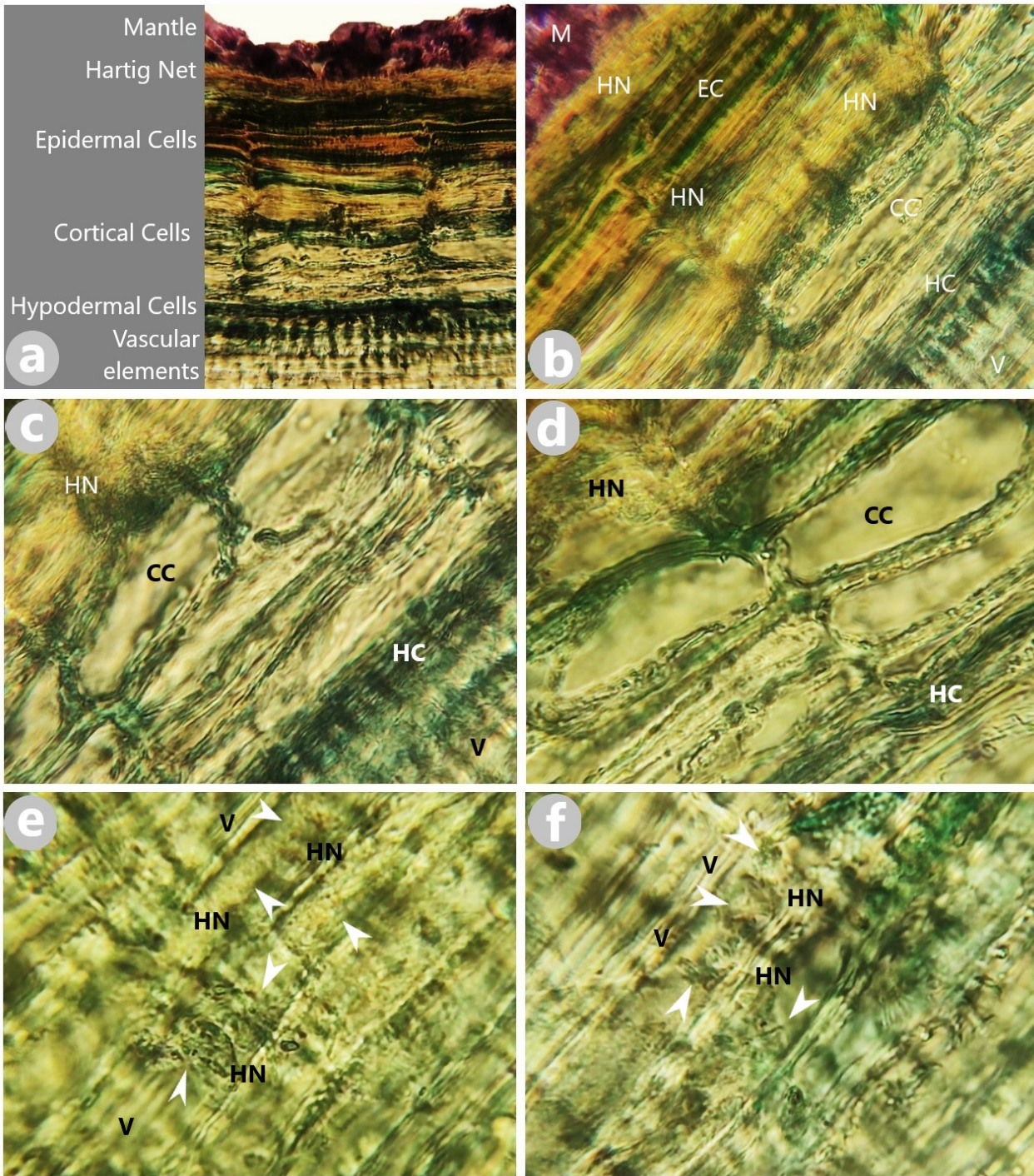
*T. uncinatum* fruiting-body 1x (a), gleba 1x (b), and non-striated peridium warts 3x (c) under stereomicroscope; ascus sac (d) and mature spore with some formations including surrounded by mesh reticulum and hooked thorns appendices on the surface under 100x LM (e) & 500x SEM (f)



**Figure 3**

The EcM roots of *Z. carpinifolia* deformed as dichotomous branches of lateral root (by the asterisk), tipstaff (by single pointed arrow), and/or Scorpius tail (double pointed arrows) under the stereomicroscope (a & b); The dichotomous branches of lateral root (by the asterisk) with the exterior formations of the EcMF sheath (mantle) of *T. uncinatum* indicated in the form of a cobwebs-like structure (by pointed arrows) under SEM (c)





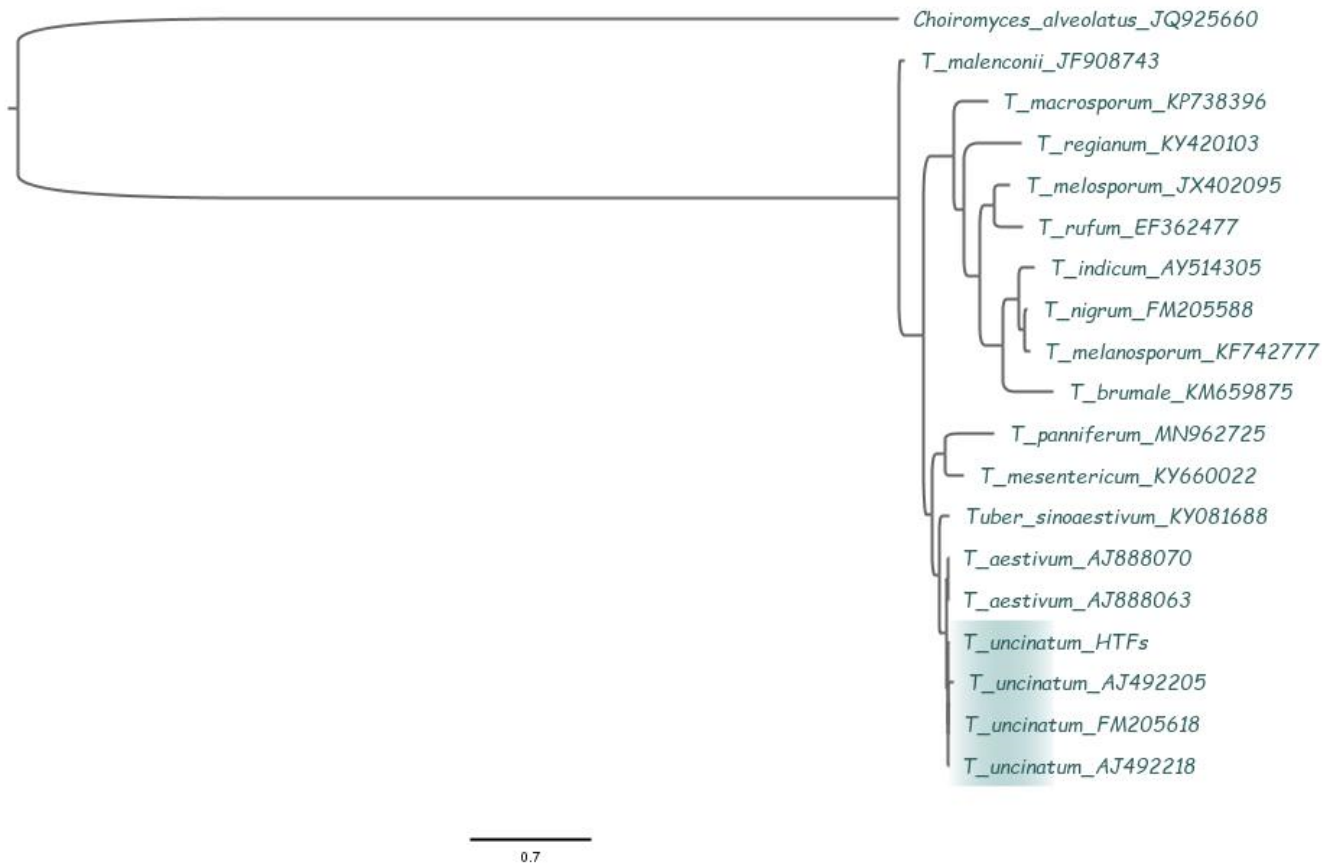
**Figure 4**

Evidence of EcM symbiosis indicated by the mantle formation and Hartig net penetration of *T. uncinatum* around the cortical and epidermal cells of *Z. carpinifolia* obtained from longitudinal sectioning micrograph under LM; (M) as Mantel, (HN) as Hartig Net, (EC) as Epidermal cell, (CC) as Cortical cell, (HC) as Hypodermal cell, and (V) as Vascular elements



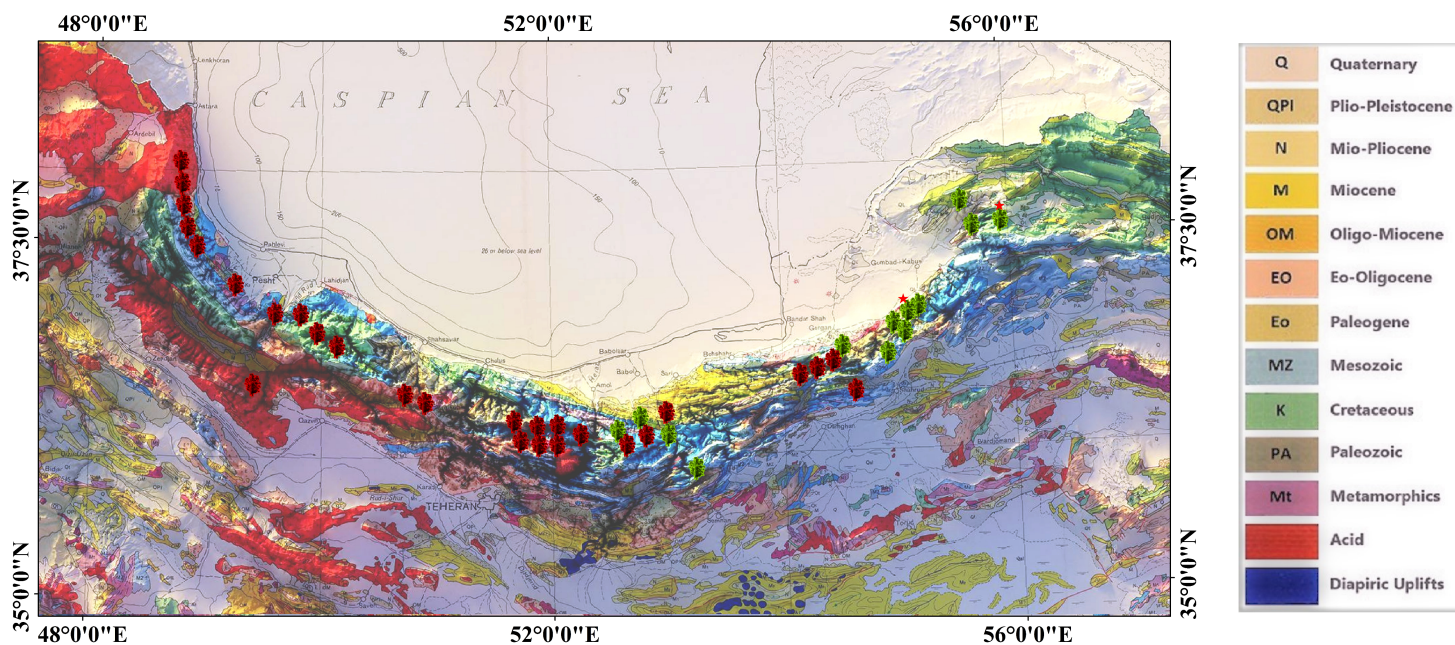
**Figure 5**

*Z. carpinifolia*; standing mass (a), formation of the *brûlé* condition (by arrows) around the dwarf creeping bushes at the interval of tall trees in a homogeneous association of Caucasian elm (b), branch comprising coarse leaves in the rear side (c), dwarf creeping bushes on the forest floor containing fine leaves (d), summer truffle (by pointed arrow) under the dwarf bush (e)



**Figure 6**

ML phylogenetic tree generated using RAxML from the alignment of ITS1, 5.8S sub-unit, and ITS2 regions sequences of 18 datasets of *Tuber* species (including their GenBank accession numbers) using GTR+G model with 10000 replication bootstrapping; the studied specimen is labeled as *T. uncinatum*HTFs; *Choiromyces alveolatus* selected as the outgroup



## Figure 7

Distribution reconstruction of *Z. carpinifolia* based on Brovicz & Zielinski 1982 in the geological map of Iran (NIOC, 1957); Green trees illustrate the attendance of *T. uncinatum*; But its symbiotic regions with *Z. carpinifolia* have been illustrated by red asterisks observed at only the regions relevant to the Quaternary period

## Supplementary Files

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