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Original paper

True truffle diversity in Iran

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Abstract
Inside commercial batches of *T. aestivum* imported from Iran to Italy the ascomata of six *Tuber* spp. (*Tuber borchii*, *Tuber brumale*, *Tuber macrosporum*, *Tuber rufum* f. *lucidum*, *Tuber excavatum* and *Tuber fulgens*) were found and described. Sixteen specimens were analyzed; most of them were completely immature probably because truffles in Iran are harvested by digging all the soil forest without using trained dogs. The morphology of the Iranian ascomata and their spore dimensions are similar to those of the European ascomata and phylogenetic analyses based on their ITS sequences placed them inside the European clades of *Tuber*. In Iran, truffles are found in the northern part of the country with the highest production in the Hyrcanian region (between Guilan and Golestan provinces). To date, Iran seems to be the most Eastern location where European species of *Tuber* are found.

Keywords
Iran, true truffles, *Tuber borchii*, *Tuber brumale*, *Tuber macrosporum*, *Tuber rufum*, *Tuber excavatum*, *Tuber fulgens*

Introduction
Truffles are generally considered to be all those fungi that form subterranean fruiting bodies and live in a symbiotic relationship with the roots of one or more host plants. They are a large polyphyletic group with representatives in the Glomeromycota, Ascomycota, and Basidiomycota (Læssøe et al., 2007). However, strictly speaking "true truffles" are limited to those ascomycetes belonging to the pan-global genus *Tuber* (Zambonelli et al., 2016) which comprises around 200 species worldwide distributed (Bonito et al., 2010). There are 35 species of *Tuber* in Europe (Leonardi et al., 2021) but only a few of them are highly appreciated because of their special taste and smell. In Italy, for example, only seven species of *Tuber* are harvested and commercialized after the national law L.N. 752/1985: *Tuber magnatum* Picco (the Italian white truffle), *Tuber melanosporum* Vittad. (the Perigord black truffle), *Tuber aestivum* Vittad. (the summer truffle), *Tuber borchii* Vittad. (the bianchetto truffle), *Tuber brumale* Vittad. (the winter truffle), *Tuber macrosporum* Vittad. (the smooth black truffle) and *Tuber mesentericum* Vittad. (the ordinary truffle). Although no species in the genus are considered poisonous some are considered inedible either because they have an unpleasant aromas, like *Tuber rufum* Vittad. and *Tuber maculatum* Vittad. or have a hard flesh, like *Tuber excavatum* Vittad.

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Overexploitation of truffle resources, climate warming, a decrease of land surface devoted to truffles and natural habitat deterioration due to the abandoned management of the old truffle orchards have been considered the major causes of natural truffle production decline in Europe (Hall et al., 2007; Büntgen et al., 2012; Le Tacon et al., 2014).

The cultivation of truffle species (*T. melanosporum*, *T. aestivum* and *T. borchii*) has been successfully achieved in Europe and other continents, like Australia, New Zealand, the USA, Chile and South Africa. Nevertheless, the availability of these truffles is still insufficient to satisfy the increasing market demand. For this reason, truffle companies are constantly searching for new supplies. Starting around 1994 more than 20 tons of Chinese black truffles (especially *Tuber indicum* Cooke & Massee) have been imported into Europe annually (Riousset et al., 2001). In the same years, European truffles were re-discovered in the Balkan Peninsula, especially in Hungary, after the harvesting restrictions of the communist era were relaxed (Gógán Csorbai et al., 2007). Consequently, *T. magnatum, T. aestivum* and *T. brumale* began to be intensively harvested and exported to other European countries, especially Italy.

More recently, *T. aestivum*, as well as other *Tuber* species, were also found in the Caucasus and surrounding countries such as Iran (Badalyan et al., 2005; Bagi and Fekete, 2010; Jamaly, 2017; Ammarellou and Alvarado, 2018; http://www.mnp.am/red_book_fauna/eng/p520.html) but the knowledge on the diversity of truffles from this area is limited particularly in Iran truffles. In recent years Puliga and colleagues (2020) have reported that tons of *T. aestivum* ascomata have been imported into Italy from Iran and while inspecting some commercial batches they found and described a new species *Tuber iranicum*. Regarding the presence of *T. brumale* in Iran it has been reported by the sequences of its mycorrhizas (Bahram et al., 2012), but the ascomata were not described. To the best of our knowledge, no other species of *Tuber* have been reported from Iran.

In the work, 16 ascomata from Iran morphologically similar to European *Tuber* species were DNA barcoded by sequencing their ITS rDNA regions and phylogenetically compare them with morphologically similar European species belonging to the same *Tuber* clade (Bonito et al., 2010; 2013). We also make morphological descriptions of their spores and report the distribution and ecological features of the truffle areas of Iran where they are collected.

Materials and methods

The *Tuber* spp. ascomata analyzed in this study were found in commercial batches of *T. aestivum* that had been imported from Iran to Italy in 2018 (29th August, 15th November, 7th December, 19th December) and 2019 (16th March). A part of each ascoma was either dried and preserved in the CMI-Unibo Herbarium of Bologna University, another portion was fixed in FAA (90% ethanol 70%, 5% acetic acid and 5% formaldehyde) for later micro-morphological analyses and the remaining portion was freeze-dried for 48 h in a Virtis Benchtop 2 K lyophilizer (SP Industries) and then preserved at −20 °C pending molecular analysis.

Each ascoma was tentatively assigned to a *Tuber* species on the basis of their macro-microscopic characters (Pegler et al., 1993; Montecchi and Sarasini, 2000; Zambonelli et al., 2000; Ceruti et al., 2003). Microscopic observations were made on gleba fragments mounted in Hoyer's medium (Glime and Wagner 2017; Ita Paz personal communication) using an Eclipse TE 2000-E microscope (Nikon). Spore measurements were made by Nis-Elements AR (v 3.10) software (Zeiss) from images captured with a DXM1200F digital camera (Nikon).

At least 30 measurements for 3-4 spore asci, depending on the specimens, were taken (Gross, 1987; Zambonelli et al., 2000) and reported as (minimum), mean ± standard deviation (maximum). The average spore length/width ratio (Qm) was also calculated. The degree of maturity was calculated

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on the basis of the percentage of asci having formed spores: immature 0%, partially immature 1-25%, slightly immature 25-50%, mature 50-100%.

For molecular analyses, DNA was extracted from 20 mg of two lyophilized ascomata using NucleoSpin® Plant II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. ITS region of the rDNA was amplified using primer pairs ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993). PCRs were carried out in 50-µl reaction volumes using 2x BiomixTM (Bioline) and amplifications were run in a SimpliAmp thermal cycler (ThermoFisher) as follows: 95 °C for 6 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s. PCR products were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and sequencing was performed with both ITS forward and reverse Sequences were aligned and edited manually with BioEdit 7.0.5.3 (Hall, 1999) and compared to those deposited in GenBank (www.ncbi.nlm.nih.gov).

Phylogenies of Iranian truffles were constructed using Maximum Likelihood (ML) and Bayesian Inference (BI) in raxmlGUI 1.5b2 (Silvestro and Michalak 2012) and MrBayes 3.2.2 (Ronquist et al., 2012), respectively. Sequences generated in this study were analyzed separately for each *Tuber* clade (Bonito et al., 2010) using GenBank ITS sequences obtained from ascomata collected in Europe. *Tuber brumale* accession MT495426 was used as an outgroup for all phylogenetic reconstructions except for the Melanosporum clade where it was substituted by *T. melanosporum* sequences (KM659870, KF591751, KM659874). Multiple sequence alignments were performed with CLUSTALW (Thompson et al., 1994). ML analyses were performed with 1,000 throughout bootstrap replicates (100 runs) and applying the GTRGAMMA model of nucleotide substitution for phylogenetic reconstructions in Puberulum, Melanosporum, Excavatum and Macrosporum clades and GTRCATI for Rufum clade. BI analyses were conducted using the best fit-models of nucleotide substitution selected by JModelTest v2.1.4 (Darriba et al., 2012) under the Akaike information Criterion (Puberulum and Melanosporum: HKY+G; Excavatum: TrNef+G; Macrosporum: TIM2+G; Rufum: TIM2+I). To ensure convergence, two independent MCMC runs with six chains each were performed in parallel for 5,000,000 generations, sampling every 100 generations. A majority-rule consensus tree was generated after the exclusion of the first 250 trees and the posterior probabilities of each node were computed. ML and BI trees were edited using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

The distribution of truffles in Iran was sourced from Ali Baghdadi and Ali akbar Mozafari by interviewing local harvesters and traders. They also provided the climate characteristics of the areas.

Results and discussion

Morphological analyses

On the basis of the descriptions available in the literature (Pegler et al., 1993; Montecchi and Sarasini, 2000; Zambonelli et al., 2000; Ceruti et al., 2003) the 16 representative ascomata of truffles purporting to be *Tuber aestivum* were found to be *T. borchii, T. brumale, T. rufum* f. *lucidum*, *T. macrosporum, T. excavatum* and *Tuber fulgens* Quél. (Figs 1 and 2). The main difference between the imported *T. brumale* and Italian specimens was that two out of four ascomata had a basal cavity which is rarely found in Italian specimens (Montecchi and Sarasini, 2000; Zambonelli et al., 2000; Ceruti et al., 2001) and more akin to French specimens (Riousset et al., 2001).

The micro-morphological characteristics of the analyzed truffles are reported in table 1 and Figures 1 and 2. Seven ascomata were immature and the asci empty of spores probably because truffle harvesting in Iran is carried out by excavating the ground as if digging for potatoes without using trained dogs (Puliga et al., 2020). As a consequence, to confirm the macro morphological identification, we had to resort to DNA barcoding (Supplementary Figs 1-5).

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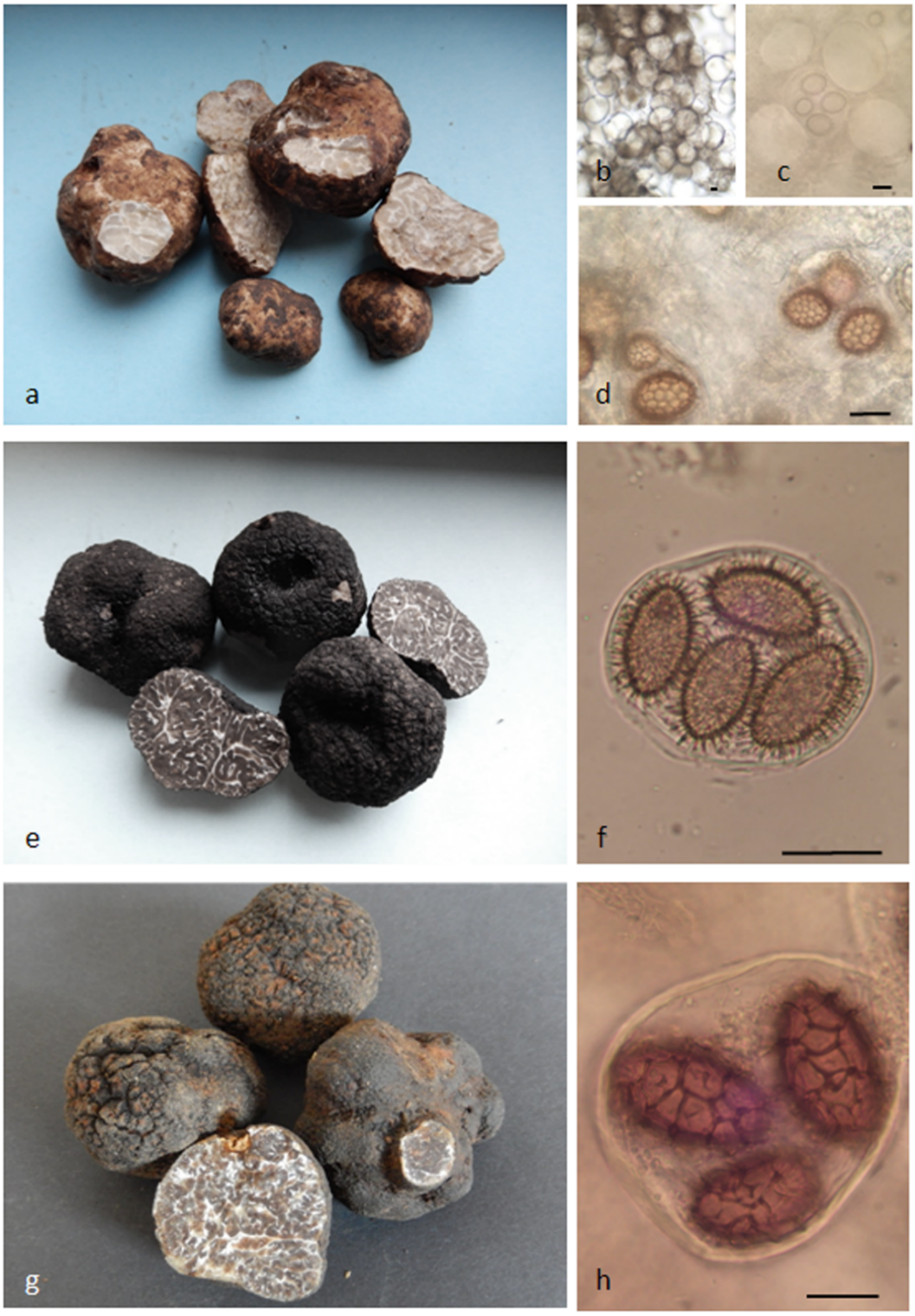

Fig. 1 – Ascomata (a) and spores of immature (b) and partially mature (c and d) *T. borchii* n. 5005, 5006, 5007 and 5008. Ascomata (e) and spores (f) of *T. brumale* n. 5000 and 5001. Ascomata (g) and spores (h) of *T. macrosporum* n. 4937 and 4038. Bars = 20 µm.

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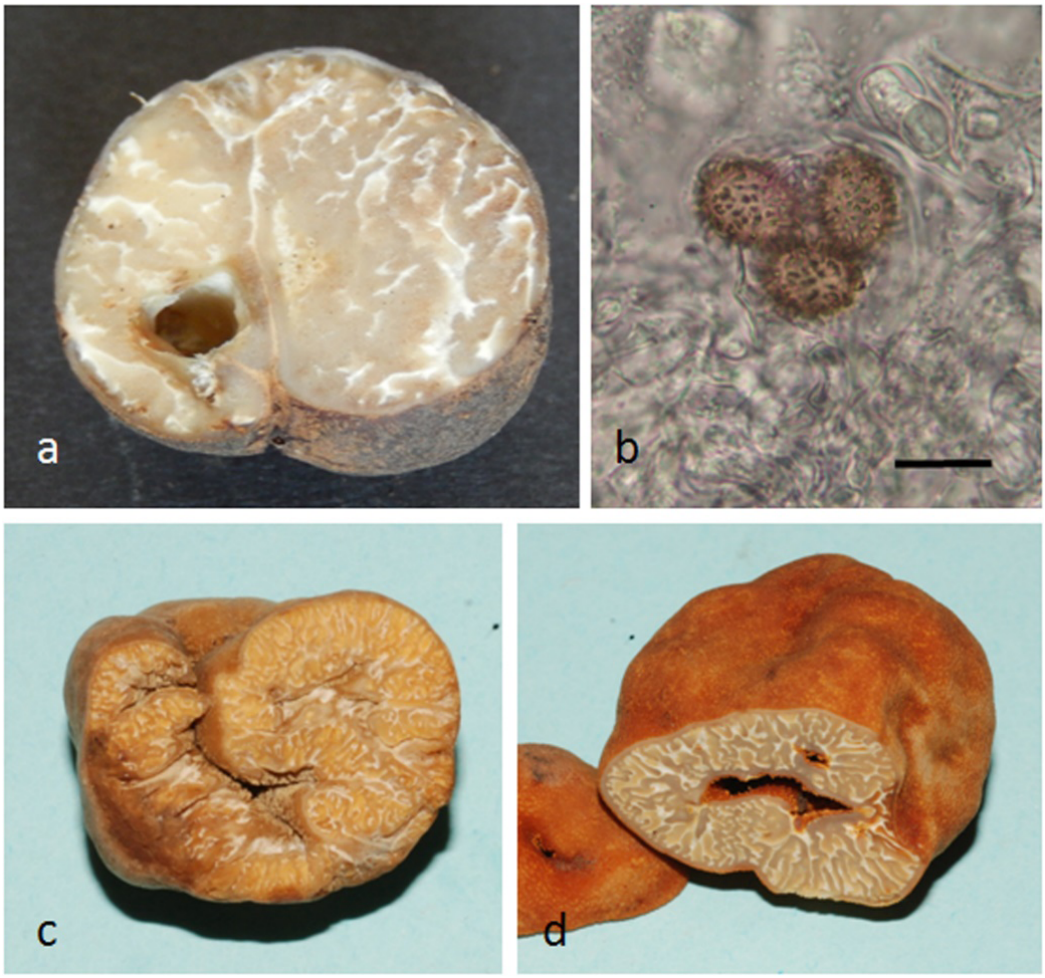

Fig. 2 – Ascoma (a) and spores (b) of *Tuber rufum* f. *lucidum* n. 4944. Immature ascomata of *Tuber excavatum* (c) n. 5036 and *Tuber fulgens* (d) n. 5034. Bar = 20 µm.

It was not always possible to make spore measurements in 4-spored asci in the mature specimens because *T. borchii* 5007 and the two *T. macrospoum* ascomata had only a maximum of 3-spored asci. The spore dimensions of all *Tuber* species were similar to those previously reported by Zambonelli et al. (2000). Only *T. macrosporum* spores appeared slightly smaller than those measured by Zambonelli et al. (2000) and Ceruti et al. (2003) but similar to those of Montecchi and Sarasini (2000). Although the spores of *T. brumale* 4967 in 4-spored asci were similar or slightly smaller than those of the other Iranian *T. brumale* specimens and congruent with the measurements reported in the literature (Montecchi and Sarasini, 2000; Zambonelli et al., 2000), this ascoma had spores up to 55 µm long in 1-spored asci (data not shown). It is known that spore dimensions are inversely proportional to the number of spores per ascus (Gross, 1987) and the anomalous abundance of one-spored asci may have favored the formation of exceptionally large spores. The unusual number of spores per ascus is not surprising due to its high variability even in the ascomata of a single species (Weden et al., 2005).

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Table 1 – Morphological characteristics and GenBank accession numbers of the *Tuber* ascomata found inside commercial batches of *T. aestivum* from Iran.

CMI-Unibo n.	species	Ascoma characteristics	arrival date	spore measures µm[1]	spore/ascus	QM	Orn. µm	Accession number
4980	*Tuber borchii*	immature	15/11/2018	n.a.	n.a.	n.a.	n.a.	MW829424
5005	*Tuber borchii*	immature	07/12/2018	n.a.	n.a.	n.a.	n.a.	MW829427
5006	*Tuber borchii*	immature	07/12/2018	n.a.	n.a.	n.a.	n.a.	MW829425
5007	*Tuber borchii*	partially immature	19/12/2018	(24) 28- 39 (45) x (18) 22-28 (34)[2]	1-**3**	1.3	2-5	MW829423
5008	*Tuber borchii*	slightly immature	19/12/2018	(24) 27- 33 (36) x (20) 21-25 (27)	1-4 (5)	1.3	2-4	MW829426
5047	*Tuber borchii*	mature	16/03/2019	(22) 27- 34 (39) x (21) 24-28 (30)	1-3 (4)	1.2	3-6	MW829422
4946	*Tuber brumale*	immature	28/08/2018	n.a.	n.a.	n.a.	n.a.	MW829418
4967	*Tuber brumale*	mature	15/11/2018	(20) 24- 30 (33) x (13) 15-18 (19)	1-4 (5)	1.6	3-6	MW829421
5000	*Tuber brumale*	Mature, with a basal cavity	07/12/2018	(22) 27- 32 (34) x (14) 16-18 (20)	(2-3) 4-6	1.7	3-5	MW829420
5001	*Tuber brumale*	Mature, with a basal cavity	07/12/2018	(22) 26- 34 (36) x (13) 16-18 (20)	(2-3) 4-6	1.8	3-10	MW829419
4937	*Tuber macrosporum*	mature	29/08/2018	(32) 41- 51 (55) x (21) 24-29 (32) [2]	1-3	1.7	2-6	MW884553
4938	*Tuber macrosporum*	mature	29/08/2018	(39) 42- 49 (54) x (22) 24-28 (29) [2]	1-3	1.7	3-7	MW884554
4944	*Tuber rufum* f. *lucidum*	slightly immature	29/08/2018	(19) 21- 26 (28) x 16-19 (22)	1-4	1.3	2-5	MW829417
5036	*Tuber excavatum*	immature	16/03/2019	n.a.	n.a.	n.a.	n.a.	MW884550
5034	*Tuber fulgens*	immature	16/03/2019	n.a.	n.a.	n.a.	n.a.	MW884551
5035	*Tuber fulgens*	immature	16/03/2019	n.a.	n.a.	n.a.	n.a.	MW884552

[1] The spore measures were taken in 4-spore-asci with the exception of the ascomata n. 5007, 4937 and 4938. n.a.= not applicable

[2] Spore measures taken in 3-spore-asci

Molecular analyses

Molecular analysis confirmed morphological identifications of the examined specimens. Phylogenetic analyses clustered all *Tuber* specimens from Iran in clades formed by European species (Supplementary Figs 1–5).

The Iranian sequences of the ascomata 4980, 5005, 5006, 5007, 5008 and 5047 belong to *T. borchii* and all clustered with those of the haplotype I identified by Bonuso et al. (2010) (Supplementary Fig. 1). The specimen 4944 classified as *T. rufum* f. *lucidum* for the dark color of the peridium (Montecchi and Sarasini, 2000) falls within the "*Tuber lucidum* complex" clade defined by Healy et al. (2016) (Supplementary Fig. 2).

Although morphological differences were found between the four Iranian *T. brumale* ascomata examined, genetic analyses showed the absence of genetic variability in their ITS regions (Supplementary Fig. 3). All the specimens (4946, 4967, 5000, 5001) morphologically classified as *T. brumale* clustered together with the sequences of Haplogroup II of *T. brumale* after Merényi et al. (2014; 2016). This haplogroup comprises ascomata from Eastern Europe as well as other 2 accessions from Iran (FR852065 and FR852067).

No nucleotide polymorphisms were found in ITS sequences of the specimens 4937 and 4938, which did not show evident morphological differences with respect to European *T. macrosporum*. They seem to belong to the clade I in *T. macrosporum* identified by Benucci et al. (2016) althought their sequences form a separated subclade poorly supported by both bootstrap value and posterior probability. Deeper molecular investigations will be needed to define the phylogenetic relationships between European and Iranian *T. macrosporum* (Supplementary Fig. 4).

Molecular analysis confirmed also the morphological identifications of ascomata attributed to the

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Excavatum clade. The specimen 5036 belongs to the cryptic species of *T. excavatum* identified as clade III after Puliga et al. (2020) while the ascomata 5034 and 5035 fall among *T. fulgens* specimens. It is surprising the diversity of Iranian *Tuber* species inside the Excavatum clade. Until now, three European species have been already described (*T. fulgens*, *T. excavatum* and *T. iranicum*) and other cryptic species could be found with a more in-depth study of Iranian truffles (Supplementary Fig. 5).

Distribution and ecology

Iran seems to be the most Eastern location of European native *Tuber* species. Although it was not possible to know the exact collection localities of the Iranian truffles analysed in this work, they should have be harvested in the same localities where *T. aestivum* is found. *Tuber aestivum* were first found in the eastern part of Iran in the Tangrah region but the highest truffle production is concentrated in the Hyrcanian region (from Guilan to Golestan provinces) (Hamza, 1994) (Fig. 3). In these areas, *T. aestivum* is mostly found in sandy high pH soils with a high $CaCO_3$ content, and containing a low amount of organic matter (Jamali, 2017).

Fig. 3. Distribution and productivity of truffles in the Northern parts of Iran (a). Typical truffle producing forests (b and c).

It grows especially in broad leaved forests populated by *Quercus infectoria* Oliv. and also other oak species (*Quercus brantii* Lindl., *Quercus castaneifolia* C. A. Mey, *Quercus macranthera* Fisch. & C.A. Mey) Persian ironwood (*Parrotia persica* (DC.) C.A. Mey), medlar (*Mespilus germanica* L.), whitethorn (*Crataegus oxycantha* L.), hawthorn (*Crataegus pontica* K. Koch), and hazelnut (*Corylus avellana* L.).

The highest truffle presence (Fig. 3) is concentrated in the central areas of Golestan province at 37°N and 55°E, at 150-500 m above sea level. This region consists of the mountain and plain area. Regarding data from meteorological stations of the region over the period 2010–2020 (IRIMO, 2021), the climate is humid temperate, the highest, lowest and average annual temperatures are +40.4 °C, -9.2 °C and +17.58 °C, respectively. Furthermore, the average annual rainfall is about 676 mm. The maximum relative humidity is 85.8%, minimum 23.8% and the annual average is 68.2%. The

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maximum daily rainfall is 90 mm and the number of rainy days is 59 days/y. The average of number of frost days is 24 days/y and the average of daily sunny hours is 5.49 h (Supplementary Fig. 6a).

Good truffle productions have been also reported in southern and western regions of Golestan province at 36°N and 54°E, at 140 m above sea level. The climate in these areas is humid temperate and the maximum daily rainfall is 65.2 mm and also the average annual rainfall is 350 mm. The highest, lowest and average annual temperatures are +44 °C, -12 °C and +17.98 °C, respectively. In addition, the number of frost days is 12.7 days/y and the average of daily sunny hours is 7.38 h. The maximum relative humidity is 99.6%, the minimum 11.6% and the annual average is 59.26% (Supplementary Fig. 6b).

In the central and eastern part of Mazandaran province at 36°N and 53°E, at 50-120 m above sea level, truffle production is low; the maximum relative humidity is 98.6%, minimum 41.6% and the annual average is 74.56%. The average annual rainfall is up 423 mm and the maximum daily rainfall is 73 mm. The highest, lowest and average annual temperatures are +39.6 °C, -8.4 °C and +17.67 °C, respectively. The average number of frost days is 16.6 days/y and the average of daily sunny hours is 4.14 h (Supplementary Fig. 6c).

Conclusions

This work provides new insights into the truffle diversity of Iran near the previously reported *T. aestivum* site (Jamaly, 2017; Ammarellou and Alvarado, 2018), and *T. iranicum* (Puliga et al., 2020), another six European *Tuber* species from Iran are described. Three of them, *T. borchii, T. macrosporum* and *T. brumale* are economically important for their gastronomic qualities; that opens the possibility of developing the commercial harvesting and trade of also these truffle species in Iran.

However, in order to preserve this important resource, it is important that proper truffle harvesting rules will be introduced in order to avoid overexploitation that is a common problem in the countries where truffle resources have only were recently been discovered (Chen et al., 2018). The introduction of trained dogs is mandatory because they detect only mature truffles with the typical aroma. The use of spades, hoes or other inappropriate tools to collect truffles represents a waste of valuable resources as well as a serious threat to conservation of natural habitats. It should be avoided the same was done in China in the 1980s for the matsutake areas where inexperienced Chinese collectors used rakes or small adzes to remove the litter layer, dig through the topsoil and find the priced grade 1 matsutake [*Tricholoma matsutake* (S. Ito et Imai) Sing]. This process caused irreparable damage not only to the shiros but also to the understory vegetation and soil structure (Ian R. Hall, personal communication). The sustainable utilization of natural resources, such as the edible ectomycorrhizal mushrooms, should be imperative in the modern society to protect our forests and environment.

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