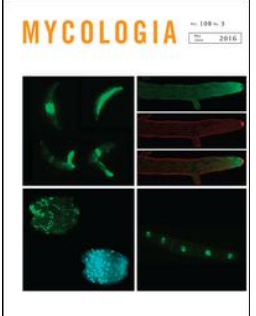


Mycologia

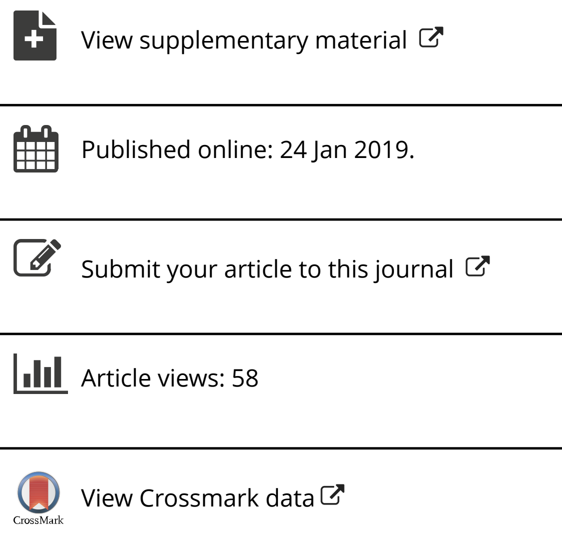
ISSN: 0027-5514 (Print) 1557-2536 (Online) Journal homepage: https://www.tandfonline.com/loi/umyc20

Ribosomal DNA polymorphisms reveal genetic structure and a phylogeographic pattern in the Burgundy truffle *Tuber aestivum* Vittad.

Claudia Riccioni, Andrea Rubini, Aziz Türkoğlu, Beatrice Belfiori & Francesco Paolocci

To cite this article: Claudia Riccioni, Andrea Rubini, Aziz Türkoğlu, Beatrice Belfiori & Francesco Paolocci (2019): Ribosomal DNA polymorphisms reveal genetic structure and a phylogeographic pattern in the Burgundy truffle *Tuber aestivum* Vittad., Mycologia, DOI: 10.1080/00275514.2018.1543508

To link to this article: https://doi.org/10.1080/00275514.2018.1543508



View supplementary material

Published online: 24 Jan 2019.

Submit your article to this journal

Article views: 58

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=umyc20

MYCOLOGIA
https://doi.org/10.1080/00275514.2018.1543508

Ribosomal DNA polymorphisms reveal genetic structure and a phylogeographic pattern in the Burgundy truffle *Tuber aestivum* Vittad.

Claudia Riccioni [a], Andrea Rubini [a], Aziz Türkoğlu [b], Beatrice Belfiori [a], and Francesco Paolocci [a]

[a]Institute of Biosciences and Bioresources Perugia Division, National Research Council, Via Madonna Alta n. 130, 06128 Perugia, Italy; [b]School of Environmental and Forest Sciences, Box 352100 University of Washington, Seattle, Washington 98195-2100

ABSTRACT

Ectomycorrhizal ascomycetes belonging to the genus *Tuber* produce edible fruiting bodies known as truffles. *Tuber aestivum*, in particular, is a fungus appreciated worldwide and has a natural distribution throughout Europe. Most of the molecular studies conducted on this species have been focused on the question as to whether or not *T. aestivum* and the morphologically similar *T. uncinatum* are conspecific. Conversely, only a handful of studies have assessed the level and distribution of genetic diversity and occurrence of phylogeographic patterns in this species. Here, we analyzed the genetic diversity of *T. aestivum* over a wide geographic range, performing an extensive sampling of specimens from Turkey, which is novel, to the best of our knowledge. We compared the internal transcribed spacer (ITS) profiles of 45 samples collected in different Turkish areas with those of 144 samples from all over Europe. We identified 63 haplotypes, 32 of which were exclusively present in Turkey. The majority of these haplotyes were also population specific. Haplotype network analysis and statistical tests highlighted the presence of a genetic structure and phylogeographic pattern, with three spatially distinct genetic clusters (northeastern Europe, southern Europe, and Turkey), with Turkey representing a diversity hotspot. Based on these results, we hypothesize the presence of glacial refugia for *T. aestivum* in Turkey, whereas European populations likely experienced a population bottleneck. The possible occurrence of cryptic species among Turkish *T. aestivum* samples also emerged. Our results are of practical relevance for the marketing of *T. aestivum* truffles and mycorrhizal seedlings and the preservation of the biodiversity of this species.

ARTICLE HISTORY
Received 4 October 2017
Accepted 19 October 2018

KEYWORDS
Ectomycorrhizal fungi; genetic pattern; internal transcribed spacer; phylogeography; truffles; Turkey

INTRODUCTION

Macrofungi are fungi producing visible fruiting bodies and are characterized by a saprotrophic, parasitic, or symbiotic lifestyle. Plant-fungus symbioses play key ecological roles in agroforest ecosystems (Smith and Read 2010). Some fungi, belonging to the Ascomycota and Basidiomycota phyla, establish symbiotic associations with the lateral roots of many tree and shrub species by developing structures known as ectomycorrhizae. The ectomycorrhizal associations are beneficial to both partners: the fungi use the carbon compounds photosynthesized by the host plants while providing their hosts with nutrients, water, and protection against biotic and abiotic stresses. Besides their ecological role, ectomycorrhizal ascomycetes belonging to the order Pezizales genus *Tuber* produce hypogeous fruiting bodies, which are the true truffles. These are edible macrofungi that in some *Tuber* species hold distinctive aromatic properties, which make them appreciated and marketed worldwide as food delicacies. Truffles live in native forests throughout the Northern Hemisphere (Bonito et al. 2013), with patterns of geographic distribution depending on the species. The most valuable white and black truffle species, *T. magnatum* and *T. melanosporum*, respectively, have very limited geographic ranges: *T. magnatum* is only harvested in Italy and in some Balkan areas, although records from a few other countries have been reported (Tabouret 2011; Riccioni et al. 2016); *T. melanosporum* grows spontaneously in Italy, France, and Spain (Riccioni et al. 2008) but has been introduced in several countries around the world (Chen et al. 2016). Conversely, other truffle species display a wider distributional range and among them is *T. aestivum*, one of the most economically important truffles, better known as the Burgundy truffle. This species is widespread in nearly all European countries, as well as in North Africa (Chevalier et al. 1979) and in Turkey (Türkoğlu et al. 2015). In sharp

CONTACT Francesco Paolocci francesco.paolocci@ibbr.cnr.it

Claudia Riccioni and Andrea Rubini contributed equally to this work.
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/umyc.
Supplemental data for this article can be accessed on the publisher's Web site.

© 2019 The Mycological Society of America

Published online 24 Jan 2019

contrast to other *Tuber* species, *T. aestivum* is successfully cultivated in diverse environments and countries, both within and outside its natural geographic range (Chevalier 2008; Chevalier and Sourzat 2012; Stobbe et al. 2013; Morcillo et al. 2015), due to its adaptation to a large spectrum of climatic and pedological conditions.

Due to the presence of truffles having different morphological characteristics (i.e., spore ornaments), bouquet, ripening period, and ecological requirements, in the late 19th century Chatin (1887) split *T. aestivum* into two distinct species: *T. aestivum* and *T. uncinatum*, the former representing truffles that ripen in summer, the latter in the fall. However, in the last few years, phylogenetic studies carried out by different research groups have all come to the conclusion that the phenotypic differences between *T. uncinatum* and *T. aestivum* are not enough to justify the separation into two distinct species and recommend the use of *T. aestivum* Vittad. as the scientific name (Paolocci et al. 2004; Wedén et al. 2004, 2005; Chevalier and Sourzat 2012; Molinier et al. 2013).

Assessing the extent and distribution of genetic diversity of fungal species, on both large scale and small scale, is crucial to understanding their biology and demographic history and to guide biodiversity conservation programs (Douhan et al. 2011). Concerning truffles, large-scale genetic diversity studies have been carried out on *T. magnatum* (Rubini et al. 2004, 2005) and *T. melanosporum* (Bertault et al. 1998; Murat et al. 2004, 2011; Riccioni et al. 2008; García-Cunchillos et al. 2014). Geographically structured populations and phylogeographic signals were identified in both species. Moreover, these studies suggested that both *T. magnatum* and *T. melanosporum* experienced a population bottleneck during the last ice age and that their refugia were located in the southernmost areas of their distributional ranges. Using different markers, such as random amplification of polymorphic DNA (RAPD), internal transcribed spacers (ITS) of rDNA, amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSRs), several authors also reported the existence of genetic diversity among populations of *T. aestivum* (Gandeboeuf et al. 1997; Mello et al. 2002; Wedén et al. 2004; Splivallo et al. 2012b; Molinier et al. 2016b). However, these studies were centered only on the European populations.

Interestingly, in accordance with its wider habitat range, *T. aestivum* displays a higher rate of genetic and morphological diversity with respect to other *Tuber* species of economic relevance (Rubini et al. 2005 and references therein; Molinier et al. 2016b). More in depth, in a global survey conducted on *Tuber* species, *T. aestivum* was shown to be the most polymorphic, since its ITS variation was up to 3.7% (Bonito et al. 2010). More recently, four genetic groups have been recognized in the European populations of *T. aestivum*, by using SSRs, and the presence of an ecotype, potentially adapted to the climate of southern Europe, has been envisaged (Molinier et al. 2016b). On a smaller spatial scale, some studies have focused on the spatial and temporal genetic structure of *T. aestivum* in both natural and man-made truffle orchards, as well as on the potential link between the genetic diversity of the fruiting body and the aromatic profiles (Splivallo et al. 2012b; Molinier et al. 2015, 2016a). Here, we extended the sampling to include the natural populations of *T. aestivum* of Turkey, with the aim of evaluating the extent and distribution of genetic diversity and of disclosing the phylogeographic patterns in this species on a larger spatial scale compared with previous studies. To this end, we characterized the ITS profiles of the 45 Turkish specimens from 11 populations and compared their haplotypes with those of 144 previously characterized specimens from all over Europe.

MATERIALS AND METHODS

Sample source, DNA isolation, PCR, and sequencing. —A total of 45 *T. aestivum* ascocarps were collected in the years 2010–2014 from 11 natural areas in Turkey (TABLE 1; FIG. 1; SUPPLEMENTARY TABLE 1). The Aegean region (populations 1–7 and 11) was the most sampled, followed by the Marmara (population 9), Mediterranean (population 8), and Black Sea (population 10) regions. Dried herbarium specimens as well as fresh specimens were used. Soon after collection, the peridium was removed from the fresh ascocarps and the inner part of the gleba was cut into slices, frozen in liquid nitrogen, and stored at −70 C until DNA extraction.

All ascocarps were morphologically checked according to Chevalier et al. (1979). Spore examination was performed to exclude the presence of similar species such as *Tuber mesentericum*. Genomic DNA was isolated from 0.3 g of gleba. The internal transcribed spacer (ITS) of the rDNA region was amplified by polymerase chain reaction (PCR), as previously described by Paolocci et al. (2004)(). Dimethyl sulfoxide (DMSO) 10% (*v*/*v*) was added to the PCR reaction mixtures to amplify most of the DNA, due to the presence of a G/C-rich region in the *T. aestivum* ITS (Paolocci et al. 2004). In addition, DNA amplification from certain herbarium specimens was obtained only after adding bovine serum albumin (BSA; 7 mg/ml;

Table 1. Geographic information and diversity of the *T. aestivum* populations.

Population	Origin	Country	Latitude	Longitude	N	nH	nHG
1	Bozkurt	Turkey	37.82	29.61	11	9	0.82
2	Acipayam	Turkey	37.42	29.35	3	1	0.33
3	Honaz	Turkey	37.76	29.27	9	9	1.00
4	Cal	Turkey	38.08	29.4	9	4	0.44
5	Fethiye	Turkey	36.58	29.25	1	1	1.00
6	Burdur	Turkey	37.42	30.67	1	1	1.00
7	Izmir	Turkey	38.2	26.84	1	1	1.00
8	Hatay	Turkey	36.2	36.16	1	1	1.00
9	Çatalca	Turkey	41.14	28.46	1	1	1.00
10	Ordu	Turkey	41.02	37.5	1	1	1.00
11	Muğla	Turkey	37.21	28.36	7	4	0.57
	Turkey				**45**	**32**	**0.71**
12	Emilia Romagna	Italy	44.6	11.22	3	2	0.67
13	Umbria	Italy	42.94	12.62	9	5	0.56
14	Abruzzo	Italy	42.34	13.45	9	6	0.67
15	Molise	Italy	41.56	14.66	2	2	1.00
16	Liguria	Italy	44.32	8.4	3	3	1.00
17	Lombardia	Italy	45.48	9.84	9	2	0.22
18	Piemonte	Italy	44.8	9.29	11	6	0.55
19	Marche	Italy	43.63	12.76	2	1	0.50
20	Italy	Italy	41.87	12.57	2	2	1.00
21	Teruel	Spain	40.34	-1.11	1	1	1.00
22	Castilla	Spain	41.75	-4.79	2	2	1.00
23	Dordogne	France	45.1	0.75	2	1	0.50
24	Bouches du Rhone-Drome	France	44.36	4.91	5	4	0.80
	Southern Europe				**60**	**24**	**0.4**
25	Yonne-Haute Marne	France	48.8	6.09	8	4	0.50
26	Oxford	England	51.75	-1.26	1	1	1.00
27	Gotland	Sweden	57.65	18.71	35	4	0.11
28	Oland	Sweden	56.66	16.67	2	1	0.50
29	Aarhus	Denmark	56.16	10.2	1	1	1.00
30	NPR Karlstejn	Czech Republic	49.93	14.18	9	3	0.33
31	Damborice-Klinek	Czech Republic	49.03	16.91	1	1	1.00
32	Teplice	Czech Republic	50.64	13.83	3	1	0.33
33	Chodec	Czech Republic	50.41	14.51	1	1	1.00
34	Pest-Baranya	Hungary	47.16	20.18	3	3	1.00
35	Tribeč	Slovakia	48.68	18.39	12	4	0.33
36	Povazsky Inovec	Slovakia	48.77	18.03	8	3	0.38
	Northern Europe				**84**	**13**	**0.15**
		Total			189	63	0.33

Note. N = number of samples; nH = number of haplotypes; nHG = ratio nH/N.

Sigma-Aldrich, Steinheim, Germany) to the reaction mixture (Paolocci et al. 1999). The amplified fragments were purified using the EuroGold Cycle-Pure kit (Euroclone, Milan, Italy).

ITS sequencing was carried out using the primers ITS1, ITS4, 5.8sf, and 5.8sb (Rubini et al. 1998) and the BigDye Terminator Cycle Sequencing Kit (version 3.1; Applied Biosystems, Foster City, CA, USA), according to the supplier's instruction manual. DMSO 10% (*v*/*v*) was also added to the sequencing reaction mixtures. An ABI 3130 Genetic Analyzer (Applied Biosystems) was used for capillary electrophoresis. Electropherograms were analyzed with FinchTV 1.3.1 (Geospiza, Seattle, Washington; http://www.geospiza.com). A BLASTn analysis (Altschul et al. 1990) was carried out for each sequence to confirm the species. The ITS sequences were deposited in GenBank under the following accession numbers: KU664080 to KU664124 (SUPPLEMENTARY TABLE 1).

Sequence mining from GenBank.—In order to obtain a *T. aestivum* sampling representative of the entire species distributional range, 144 ITS sequences from specimens collected throughout Europe, and referenced in previous papers (Mello et al. 2002; Paolocci et al. 2004; Wedén et al. 2005; Gryndler et al. 2011), were downloaded from GenBank (SUPPLEMENTARY TABLE 1). Thus, we obtained a data set consisting of 189 *T. aestivum* specimens, by combining the 45 ex novo sequenced samples from Turkey with the GenBank samples. These were grouped in 36 populations according to their place of origin (TABLE 1; SUPPLEMENTARY TABLE 1). Assembly, editing, and alignment of the ITS sequences were performed by the software Geneious 4.8.5 (Biomatters, Auckland, New Zealand; http://www.geneious.com).

Data analyses.—The number of ITS haplotypes and their frequency in the entire collection and at the population/area level were calculated using the software Arlequin 3.5.1.2 (Excoffier and Lischer 2010). To evaluate the representativeness of our sampling, a rarefaction analysis was performed by plotting the

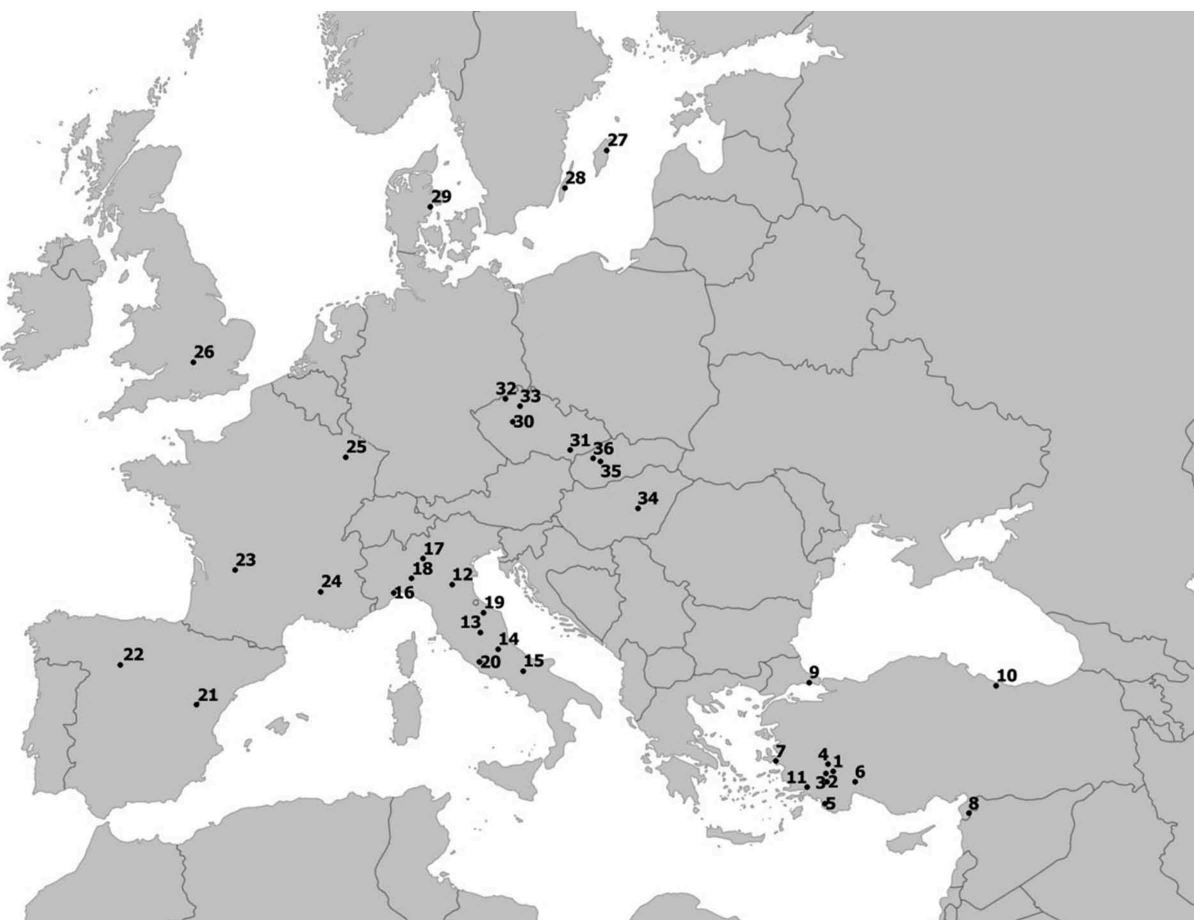


Figure 1. Geographic map showing the locations of the populations of *T. aestivum* under study.

number of haplotypes as a function of sample size using the software EstimateS 9.1. A phylogenetic tree was inferred with the software MEGA6 (Tamura et al. 2013) by using the neighbor joining method and the composite maximum likelihood distance. The alignment gaps were considered using the pairwise deletion option. Bootstrap analysis was performed with 100 replicates. The alignments were submitted to TreeBASE (S22696). The analysis of the haplotype network was performed with the software Haploview (http://www.cibiv.at/~%20greg/haploviewer) using the maximum parsimony (MP) method implemented in the DNAPARS module of the PHYLIP package 3.696 (Felsenstein 1989).

The clustering of samples was examined by the software BAPS (Bayesian Analysis of Population Structure) 4.14 (Corander et al. 2003, 2008). The genetic divergence between the groups identified by BAPS was estimated using MEGA6 (Tamura et al. 2013) as an average proportion (*p*) of nucleotide sites at which two sequences being compared are different (p-distance).

To check for deviations from neutrality, i.e., to see whether DNA sequences evolve in manners inconsistent with the neutral theory of molecular evolution, we performed Tajima's D and Fu and Li's D* and F* neutrality tests by using DnaSP 5.1 (Librado and Rozas 2009). A significant deviation from genetic neutrality could be interpreted as a result of a recent population expansion or bottleneck (Tajima 1989). The mismatch distribution analysis, i.e., the distribution of pairwise differences in our set of sequences, was performed as an additional test for demographic expansion using Arlequin 3.5.1.2. The graph resulting from this analysis is usually multimodal in samples drawn from populations at equilibrium, whereas it is unimodal in populations that have been through a recent demographic expansion.

The hierarchical analysis of molecular variance (AMOVA) was performed using Arlequin 3.5.1.2. The spatial structure of populations was inferred using the software Spatial Analysis of Molecular Variance (SAMOVA) 2 (Dupanloup et al. 2002; http://cmpg.unibe.ch/software/samova2/). SAMOVA defines groups of populations that are geographically homogeneous and maximally differentiated from each other on the basis of Fct statistics. Fct indicates the proportion of

Table 2. Relative numbers of haplotypes at different latitudinal ranges.

Latitudinal range (°N)	N	nH	nH/N
58–51 (populations 26–29)	39	6	0.15
50–47 (populations 25, 30–36)	45	10	0.22
45–42 (populations 12–14, 16–19, 23, 24)	53	20	0.38
41–36 (populations 1–11, 15, 20–22)	52	39	0.75

Note. N = number of samples; nH = number of haplotypes; nHG = ratio nH/N.

molecular variance among groups of populations. The pairwise nucleotide difference was used as a measure of distance, and the significance of the Fct index was evaluated through a permutation test (1000 permutations). The haploid genetic distance among populations was calculated after conversion of the DNA sequence data into numeric codes using GenAlEx 6.501 (Peakall and Smouse 2012). To evaluate the presence of a pattern of isolation by distance (Rousset 1997), we performed the Mantel correlation test between genetic distance and the natural logarithm of geographic distances (km) among populations using the software GenAlEx and 1000 random permutations.

RESULTS

Successful PCR amplification and sequencing of the ITS region was obtained for all 45 samples from Turkey. The nucleotide alignment between these sequences and those downloaded from GenBank was 542 bp long and contained a total of 108 variable sites (single-nucleotide polymorphisms [SNPs] and indels).

By considering these polymorphisms, 63 haplotypes, namely, H1 to H63, were detected within the 189 *T. aestivum* samples under study (SUPPLEMENTARY TABLE 1; SUPPLEMENTARY FIG. 1). An even higher number of haplotypes should be expected by increasing the sample size, as is shown by the rarefaction curve (SUPPLEMENTARY FIG. 2).

Out of the 63 haplotypes identified, 32 exclusively belonged to the 11 Turkish collection sites (SUPPLEMENTARY TABLES 1 and 2). In addition, with the only exception of H9, which was shared between two nearby populations (Honaz and Bozkurt), all the Turkish haplotypes were also specific to single populations (SUPPLEMENTARY TABLE 2).

As shown in TABLE 1, the ratio between number of haplotypes and sample size (nHG) in Turkey was about 2- and 5-fold higher than that of southern and northern Europe, respectively. This ratio increased from north to south if the entire population set was subdivided into four groups according to latitude (TABLE 2).

The phylogenetic tree of the haplotypes highlighted the presence of three main clusters (FIG. 2). Cluster I grouped most of the European haplotypes (25), since only 5 were the Turkish haplotypes, whereas most (25) of the Turkish haplotypes belonged to cluster II, which only included 6 haplotypes from Europe. A third, small cluster (III) only included 2 haplotypes from Turkey.

We performed a network analysis that also included geographic information and haplotype frequencies in order to infer relationships among the haplotypes (FIG. 3). The existence of three main clusters of haplotypes, corresponding to three major geographic areas, emerged from this analysis: southern Europe (cluster A), northeastern Europe (cluster B), and Turkey (cluster C). Only a few haplotypes, all belonging to the Turkish samples, were not included in these clusters. The three most frequent haplotypes (H33, H36, and H57) were largely shared between European populations, with H36 mainly found in Italy (cluster A). Conversely, H33 and H57 were mainly found in northern and eastern Europe, respectively (cluster B), and were very similar to each other, only differing by one SNP. Many rare haplotypes, which only differed by one to three polymorphisms compared with the most common haplotypes, were also present (FIG. 3).

In contrast to what emerged in the European populations, where some haplotypes dominated whole regions, Turkey presented a high diversity and was characterized by many exclusive haplotypes. These haplotypes were very different not only from the European ones but also between each other, both at intra- and interpopulation levels (FIG. 3; SUPPLEMENTARY FIG. 1). The most frequent Turkish haplotype, although only shared by four individuals from Cal, was H19 (FIG. 3; SUPPLEMENTARY TABLE 2). A few haplotypes (4), which only differed from H19 by one to two polymorphisms, were detected in the same population of Cal and in the nearby population of Honaz. It is worth mentioning that two Turkish haplotypes, H1 and H4, that were harbored by samples 1 and 5 from Bozkurt, respectively, showed an exceptionally high phylogenetic distance from all others to the extent that they were grouped in a cluster of their own (FIGS. 2 and 3). Also, H1 and H4 were placed in a separate clade with an intermediate position between the European/Turkish *T. aestivum* and the Chinese specimens in the phylogenetic tree built including taxa from China, namely, *Tuber sinoaestivum*, and *T. aestivum* sensu lato (Zhang et al. 2012; Zambonelli et al. 2012) (SUPPLEMENTARY FIG. 6).

A phylogenetic link was suggested between Turkey and southern Europe, since five of the Turkish haplotypes were very close to the highly frequent H36 and vice versa six haplotypes from Italy, France, and Spain were close to the Turkish haplotypes (FIGS. 2 and 3).

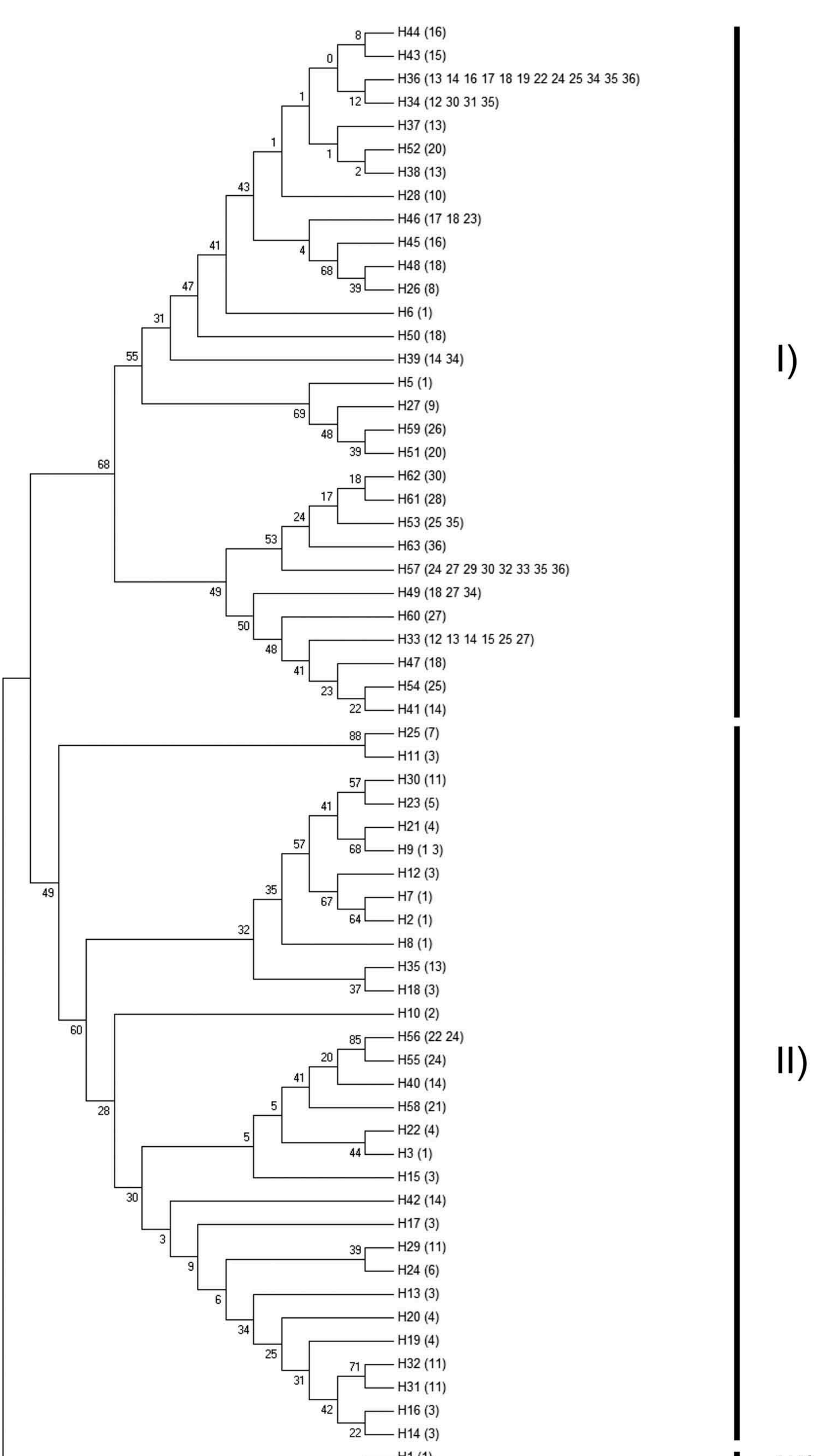


Figure 2. Phylogenetic tree of the haplotypes observed. Numbers near the branches indicate the bootstrap values (percentage over 100 replicates). Haplotypes are indicated as H1 to H63 as in SUPPLEMENTARY FIG. 1. Populations where each haplotype is present are indicated in brackets and numbered as in TABLE 1.

The Bayesian clustering performed with BAPS detected one minor and three major clusters showing a clear link with the already cited geographic areas (FIG. 4A). In fact, cluster A grouped most of the Turkish samples, together with the same few specimens that were grouped by phylogenetic analyses from other geographic areas (Italy, France, and Spain); cluster B grouped the only two Turkish samples from Bozkurt (population 1), confirming their divergence from all other haplotypes (FIGS. 2, 3, and 4A); cluster C mainly included Italian samples and cluster D, northern and eastern European samples. Moreover, the four clusters perfectly matched the pattern of the haplotype network (FIG. 4B).

To test for departure from neutral selection, we performed the Tajima's D and Fu and Li's D* and F* neutrality tests for northeastern Europe, southern Europe, and Turkey, together with the mismatch distribution analysis (SUPPLEMENTARY FIG. 3). Negative values were observed for all neutrality tests, although only D* and F* were significant for southern Europe (SUPPLEMENTARY FIG. 3B). In the northern and eastern European populations, the mismatch distribution analysis showed that sequence pairs differing for low numbers of nucleotide sites (0 and 1) had a particularly high frequency, whereas in the southern European and Turkish populations sequences differing for a low (0, 1) and a higher (5, 10) number of nucleotide sites had similar frequencies.

The analysis of molecular variance (AMOVA) revealed a strong genetic differentiation between populations (Fst = 0.44; $P < 0.001$). We performed the SAMOVA analysis to define groups of populations that were geographically homogeneous and maximally differentiated from each other. Quite similar Fct values were obtained when the number of groups of populations (K) ranged from 3 to 8 (SUPPLEMENTARY FIG. 4A). When K = 3 was considered, we identified groups of populations basically corresponding to the geographic areas of northeastern Europe, southern Europe, and Turkey. With the increase in K, this geographic pattern was basically confirmed and the tendency to split Turkish populations into further groups emerged (SUPPLEMENTARY FIG. 4B). The Mantel test showed that among-population differentiation significantly increased with geographic distance (R^2 = 0.109; $P < 0.01$), revealing a pattern of isolation by distance (SUPPLEMENTARY FIG. 5).

In order to evaluate the presence of cryptic species among *T. aestivum* samples, we calculated the divergence levels in terms of sequence similarity between the four BAPS clusters. The average nucleotide distances ranged from 0.81% to 2.14% between clusters A–D, A–C, and D–C and from 3.9% to 4.44% between clusters A–B, D–B, and C–B (TABLE 3), suggesting the possible presence of cryptic species within cluster B.

DISCUSSION

In the present study, we carried out the first ITS sequence analysis of populations of *T. aestivum* from Turkey, which, to the best of our knowledge, represents one of the southernmost areas of the distributional range of this truffle species. We further compared the ITS profiles of the Turkish specimens with ITS profiles of those collected from other regions of Europe, with the aim of performing a large-scale population genetics study and of assessing the possible presence of phylogeographic signals. Although it is highly unlikely that our data set reflects the whole genetic diversity of the species, the acquired data were enough to demonstrate the presence of a strong genetic structure among populations of *T. aestivum*, with those from Turkey displaying a higher rate of genetic diversity and private haplotypes compared with those from Europe. A phylogeographic pattern with three main spatially distinct genetic clusters (located in northeastern Europe, southern Europe, and Turkey) and the possible existence of cryptic species also emerged from our data set.

Our study sheds light on the historical population dynamics of this species. These results are also of practical relevance for the marketing of *T. aestivum* truffles and mycorrhizal seedlings and for the preservation of the biodiversity of this species.

ITS analysis suggests that geographic and genetic barriers shaped the spatial structure of T. aestivum.—Considering the polymorphisms of the ITS region, 63 ITS haplotypes were identified among the 189 *T. aestivum* samples analyzed. The level of *T. aestivum* genetic diversity, which emerged from our data set, was much higher than that of other *Tuber* species, as previously reported in analogous large-scale studies. In fact, in two independent screenings of 188 and 205 *T. melanosporum* European samples, only 10 and 13 ITS haplotypes were identified, respectively (Murat et al. 2004; Riccioni et al. 2008). Likewise, only 13 ITS haplotypes were found among the 136 samples of *T. brumale* analyzed by Merényi and colleagues (2014).

The phylogenetic analysis performed to evaluate the relationship between haplotypes showed a clear tendency of the Turkish and European haplotypes to be different from each other. The network analysis

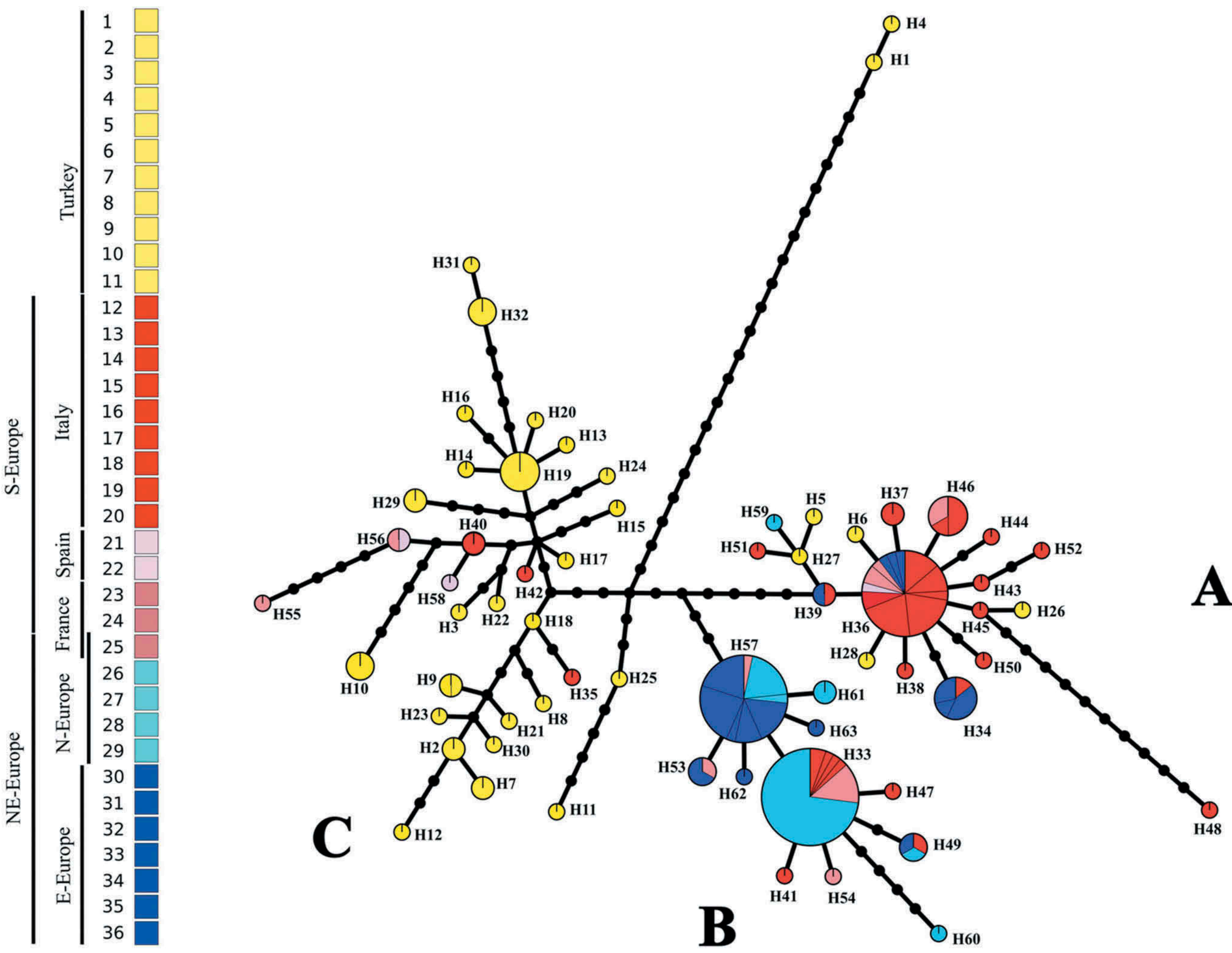


Figure 3. Haplotype network. Circles are shaded according to the percentage of samples belonging to the different populations numbered according to TABLE 1. The sizes of the circles are proportional to the haplotype frequency. Haplotypes are indicated as H1 to H63. Points along the lines indicate intermediate mutational steps between haplotypes.

conducted was even more effective in highlighting the presence of a phylogeographic structure, which also showed the tendency of northeastern and southern European samples to cluster in two distinctive groups. This pattern was still consistent even when the Turkish populations were excluded from the analysis (data not shown). The finding of two genetic clusters in Europe calls for additional studies aimed at verifying whether or not samples of the two clusters differ in morphology, ripening time, and/or habitat requirement.

Bayesian analysis and SAMOVA confirmed the presence of three main genetic clusters, corresponding to the three major phylogeographic areas under study, and of a fourth cluster, which only included two samples from Turkey.

High levels of genetic structure are usually associated with limited gene flow between populations as a consequence of either reproductive barriers or geographic isolation (Slatkin 1987). Reproductive isolation plays an important role in sympatric speciation processes (Giraud et al. 2008). Our analysis suggests that geographic isolation likely played a major role in limiting gene flow among populations of *T. aestivum*, as the isolation by distance test showed a significant correlation between genetic and geographic distances among the populations.

Conversely, at a small-scale level, reproductive genetic barriers might be evoked to explain the presence of strongly differentiated samples within a given population such as the case of samples 1 and 5 from Bozkurt. These samples were in fact not only differentiated from all other samples but also from the other nine samples belonging to the same population and formed a distinct genetic group (cluster B of BAPS analysis). The average distance of cluster B from the others was much higher than the average distances between all

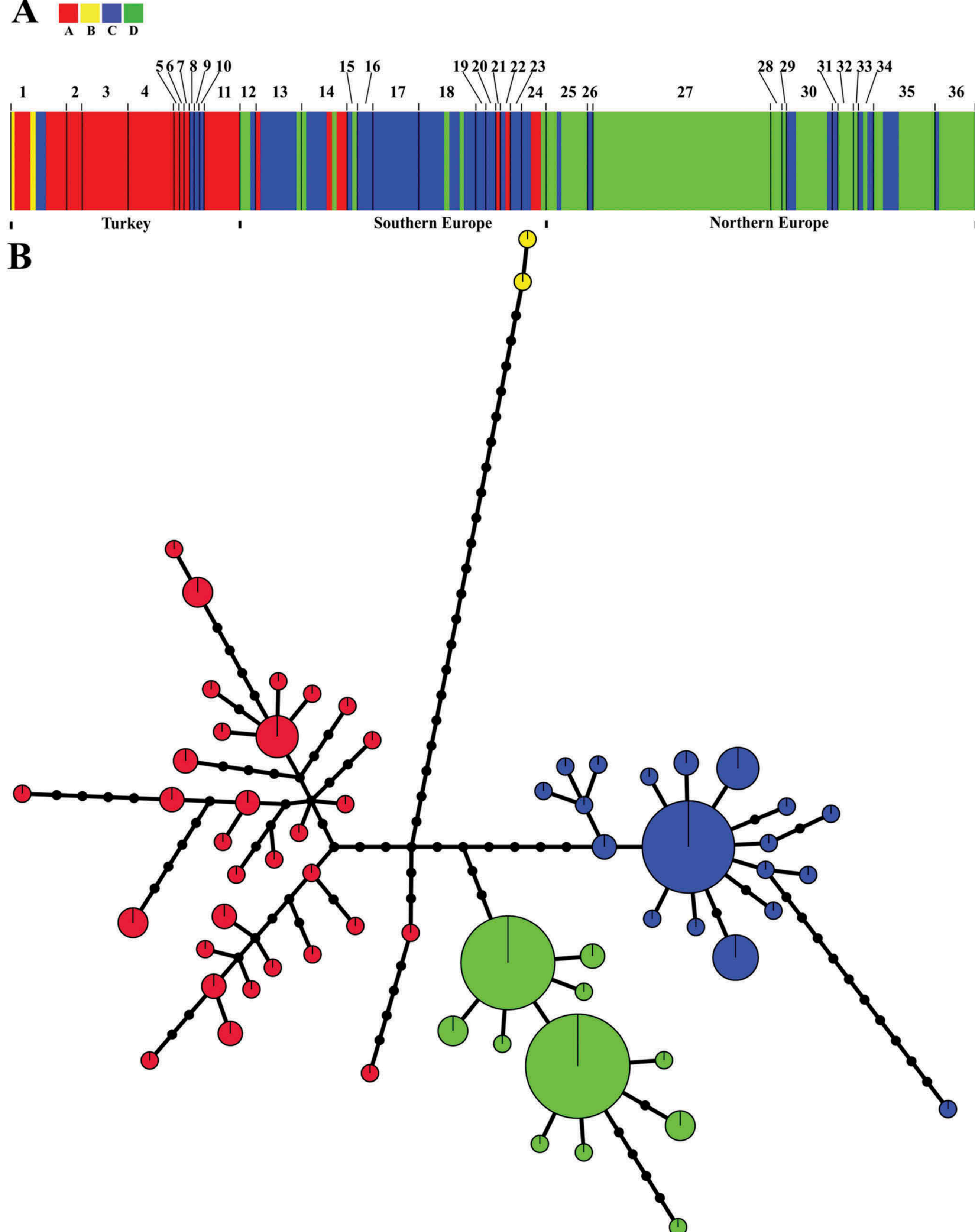


Figure 4. A. Genetic structure as estimated by BAPS software for K = 4. Each individual is represented by a vertical line shaded according to one of the four K groups. Numbers and black marks indicate the different populations. B. Haplotype network with overlapped BAPS clusters.

Table 3. Divergence between the groups identified by BAPS.

BAPS group	Pairwise p-distance
A-D	1.99
A-C	2.14
A-B	4.44
D-C	0.81
D-B	3.90
C-B	4.04

other clusters. The divergence values of cluster B was higher than the 3% threshold commonly used in fungal community studies to delineate species boundaries (Smith et al. 2007; Peay et al. 2008; Hughes et al. 2009) and the 4% threshold suggested by Bonito et al. (2010) for *Tuber* species. According to our phylogenetic analysis, samples of cluster B represent an intermediate taxon between *T. aestivum* and the related Chinese taxa *T. aestivum* sensu lato and *T. sinoaestivum*. To establish whether or not this intermediate taxon represents a cryptic species, further morphological and molecular analyses, based on additional genetic markers and a more representative number of fruit bodies from Bozkurt as well as from other Eastern countries, such as Iran and Azerbaijan, will be needed.

The study of the mating type (*MAT)* locus would be particularly informative to disclose inter- and intraspecific genetic barriers in *T. aestivum*. This locus has been identified in *Tuber* species of economic relevance, including *T. aestivum* (Martin et al. 2010, 2012; Rubini et al. 2011; Belfiori et al. 2013, 2016; Payen et al. 2014). As an example, in *T. indicum*, the sequencing of this locus revealed an extensive polymorphism and rearrangements between samples belonging to different ITS clades, suggesting the presence of cryptic species (Belfiori et al. 2013).

Genetic diversity and phylogeography suggest different demographic histories of T. aestivum in Europe and Turkey.—About 50% (32 out of 63) of the total number of haplotypes detected in this study were from Turkey. Interestingly, almost all of these haplotypes (31) were not shared among the Turkish populations. This scenario changed when European samples were considered: three haplotypes were largely shared between populations; two (H33 and H57) only differed by one SNP, and there were many rare haplotypes that only differed from the three most common by one to three polymorphisms (FIG. 3). Such a star-shaped network of the European haplotypes is consistent with the hypothesis of a recent population expansion. In fact, the few highly frequent haplotypes may represent ancestral haplotypes from which many rare ones likely derived in recent times (McCormack et al. 2008; Merényi et al. 2014). The negative values of Tajima's D and Fu and Li's D* and F* neutrality tests also point to a possible population expansion following a recent population bottleneck. The mismatch distribution analysis, in addition, highlighted a different trend between the populations from northeastern Europe and those from Turkey/southern Europe, since a large prevalence of haplotypes with few pairwise differences only emerged in the former group. Also, the haplotype diversity was shown to increase from north to south. These results can be explained by the fact that *T. aestivum* experienced a population bottleneck, during the last glacial age, which likely was more severe in northern and eastern Europe than in southern Europe and Turkey. These latter areas were probably refugia of the species, due to the favorable climatic conditions. In contrast, according to Molinier and colleagues (2016b), *T. aestivum* has not experienced a recent population bottleneck, although it must be underlined that no Turkish specimens were considered in their investigation. The occurrence of a population bottleneck and the presence of refugia in the southernmost distributional ranges were already envisaged for other *Tuber* species, such as *T. melanosporum* and *T. magnatum* (Bertault et al. 1998; Murat et al. 2004; Rubini et al. 2005; Riccioni et al. 2008).

Since *Tuber* species are mycorrhizal fungi, their postglacial population dynamics should track those of their host plants. In this respect, we note that Turkey was the refugium for several plant species (Médail and Diadema 2009) and, among them, for *T. aestivum* host species, such as *Quercus cerris* (Brewer et al. 2002; Bagnoli et al. 2016) and *Pinus sylvestris* (Cheddadi et al. 2006; Naydenov et al. 2007).

The very high level of genetic diversity and haplotype endemism of *T. aestivum* in Turkey may also be explained by the presence of geographic barriers to gene flow, such as mountain chains and the Central Anatolian Lake System, whose formation can be traced back to the Neogene (Bilgin 2011). The limited truffle knowledge and tradition in Turkey, where truffle cultivation and marketing are still at their infancy, might have also concurred to preserve such a local truffle biodiversity. Although there are only a few molecular investigations on fungal biodiversity in Turkey, the presence of diversity hot spots in this area was already hypothesized for other mushrooms belonging to the genus *Morchella* (Taşkın et al. 2012).

The haplotype network highlighted a clear-cut separation between European and Turkish strains in different clusters, with at least eight mutational steps from each other (FIG. 3). Nevertheless, a few Turkish strains clustered within the "European lineage" and vice versa some strains from southern Europe were detected

in the Turkish lineage, although without any haplotype sharing. We also note that the ITS sequences of a few *T. aestivum* samples from Iran recently published by Jamali (2017) show the same haplotype, H36, of some European samples. These pieces of evidence suggest an effect of either sequence homoplasy or the recent human-mediated introduction of allocthonous strains in non-native areas. Indeed, both *T. aestivum* ascocarps and mycorrhizal seedlings have been intensively marketed for decades, and *T. aestivum* is considered to be one of the few *Tuber* species that has become a non-native colonizer of regions distant from its habitat of origin (Vellinga et al. 2009; Bonito et al. 2010). Most of the Turkish samples were collected from natural *Pinus* forests, with the exception of samples from the Black Sea region where extensive cultivations of hazelnut trees are present. Interestingly, some of the Turkish samples of *T. aestivum*, holding haplotypes similar to those of the European samples, came from the latter region. It is therefore conceivable that non-native *T. aestivum* strains were introduced in Turkey as a consequence of the cultivation of hazelnut trees. However, additional Turkish samples from this area should be analyzed to test and confirm this hypothesis.

Potential impact of the current findings on truffle marketing and biodiversity conservation.—The rising truffle demand in a globalized market and the decrease in harvesting of the premium black and white truffles, coupled with the wider ecological range and higher cultivation potential of *T. aestivum* compared with other *Tuber* species, are reasons that have increased the scientific and commercial interest for *T aestivum* over the last few years (Stobbe et al. 2013). As an example, in Italy, where 70–80% of the national truffle production reaches the processing industry, about 65% of the processed truffles are *T. aestivum* (Pampanini and Martino 2006).

The pronounced genetic variability among populations of different geographic regions, which has emerged from the present study, opens the way to further efforts to trace natural populations of *T. aestivum* according to their geographic origin. This goal is of particular interest for the socioeconomic development not only of areas traditionally known for the presence of *T. aestivum*, but also of those in countries, such as Turkey, having a productive capacity that is not yet fully exploited. Such genetically distinct local productions might gain further value if specific aromatic profiles also could be assigned to them (Molinier et al. 2015). Ecological implications also emerge from our results. For instance, priority should be given to the protection of truffle resources from the areas, such as Turkey, that have a relatively short harvesting history, harbor autochthonous strains, and have limited to null tradition concerning truffle cultivation. For the biodiversity conservation of this species in these areas, in fact, the use of native strains as spore inocula to produce mycorrhizal plants to promote *T. aestivum* cultivation should be recommended. Ecological threats due to the undiscriminated use of allochtonous strains are unpredictable in terms of biodiversity erosion as well as adaptability of the newly introduced strains to new environmental conditions. Finally, although the effects of climate change on productivity of natural and man-made truffle orchards is a controversial topic (Büntgen et al. 2012; Splivallo et al. 2012a), comparative genetics and genomics studies on *T. aestivum* strains adapted to different ecological conditions may allow us in the very near future to identify traits related to tolerance and strains with higher adaptability to the ongoing climatic changes. The present study suggests that populations and strains of *T. aestivum* from Turkey are of outstanding relevance to approaching such a goal.

FUNDING

Dr. Aziz Türkoğlu received funding from the Scientific and Technological Research Council of Turkey (project number T-BAG-111T530). This study was also supported by a CNR-Tubitak bilateral project 2014–2015 (number TBAG-113Z893) entitled "Gaining insight into genetic diversity and sexual propagation patterns of truffles belonging to the *T. aestivum*/*T. uncinatum* species complex."

ORCID

Claudia Riccioni http://orcid.org/0000-0003-1069-8943
Andrea Rubini http://orcid.org/0000-0003-3988-6294
Beatrice Belfiori http://orcid.org/0000-0002-5366-8579
Francesco Paolocci http://orcid.org/0000-0002-9394-876X

LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403–410, doi:10.1016/S0022-2836(05)80360-2
Bagnoli F, Tsuda Y, Fineschi S, Bruschi P, Magri D, Zhelev P, Paule L, Simeone MC, González-Martínez SC, Vendramin GG. 2016. Combining molecular and fossil data to infer demographic history of *Quercus cerris*: insights on European eastern glacial refugia. Journal of Biogeography 43:679–690, doi:10.1111/jbi.12673
Belfiori B, Riccioni C, Paolocci F, Rubini A. 2013. Mating type locus of Chinese black truffles reveals heterothallism and the presence of cryptic species within the *Tuber indicum* species complex. PLoS ONE 8:e82353.

Belfiori B, Riccioni C, Paolocci F, Rubini A. 2016. Characterization of the reproductive mode and life cycle of the whitish truffle *T. borchii*. Mycorrhiza 26:515–527, doi:10.1007/s00572-016-0689-0

Bertault G, Raymond M, Berthomieu A, Callot G, Fernandez D. 1998. Trifling variation in truffles. Nature 394:734, doi:10.1038/29428

Bilgin R. 2011. Back to the suture: the distribution of intraspecific genetic diversity in and around Anatolia. International Journal of Molecular Sciences 12:4080–4103, doi:10.3390/ijms12064080

Bonito G, Smith ME, Nowak M, Healy RA, Guevara G, Cázares E, Kinoshita A, Nouhra E, Domínguez LS, Tedersoo L, Murat C, Wang Y, Moreno BA, Pfister DH, Nara K, Zambonelli A, Trappe JM, Vilgalys R. 2013. Historical biogeography and diversification of truffles in the *Tuberaceae* and their newly identified Southern Hemisphere sister lineage. PLoS ONE 8:e52765, doi:10.1371/journal.pone.0052765

Bonito GM, Gryganskyi AP, Trappe JM, Vilgalys R. 2010. A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Molecular Ecology 19:4994–5008, doi:10.1111/j.1365-294X.2010.04855.x

Brewer S, Cheddadi R, De Beaulieu JL, Reille M. 2002. The spread of deciduous *Quercus* throughout Europe since the last glacial period. Forest Ecology and Management 156:27–48, doi:10.1016/S0378-1127(01)00646-6

Büntgen U, Egli S, Camarero JJ, Fischer EM, Stobbe U, Kauserud H, Tegel W, Sproll L, Stenseth NC. 2012. Drought-induced decline in Mediterranean truffle harvest. Nature Climate Change 2:827–829, doi:10.1038/nclimate1733

Chatin MA. 1887. Une nouvelle espécé de truffe (*Tuber uncinatum*). Bulletin de la Société Botanique de France 34:246–248.

Cheddadi R, Vendramin GG, Litt T, François L, Kagey-ama M, Lorentz S, Laurent JM, De Beaulieu JL, Sadori L, Jost A, Lunt D. 2006. Imprints of glacial refugia in the modern genetic diversity of *Pinus sylvestris*. Global Ecology and Biogeography 15:271–282, doi:10.1111/j.1466-8238.2006.00226.x

Chen J, Murat C, Oviatt P, Wang Y, Le Tacon F. 2016. The black truffle *Tuber melanosporum* and *Tuber indicum*. In: Zambonelli A, Iotti M, Murat C, eds. True truffle (*Tuber* spp.) in the world. Soil ecology, systematic and biochemistry. Switzerland: Springer International Publishing. p. 19–32.

Chevalier G. 2008. Truffes et trufficulture en Europe. In: Atti del terzo congresso internazionale di Spoleto sul tartufo, Spoleto, Italy, 25th–28th November 2008. Perugia, Italy: Ed. Comunità Montana dei Monti Martani Serano e Subasio. p. 65–72.

Chevalier G, Desmas C, Frochot H, Riousset L. 1979. L'espèce *Tuber aestivum* Vitt. I. Definition. Mushroom Science X (Part 1):957–975.

Chevalier G, Sourzat P. 2012. Soils and techniques for cultivating *Tuber melanosporum* and *Tuber aestivum* in Europe. In: Zambonelli A, Bonito GM, eds. Edible ectomycorrhizal mushrooms. Springer Berlin and Heidelberg, Germany: Springer. p. 163–189.

Corander J, Marttinen P, Sirén J, Tang J. 2008. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. BMC Bioinformatics 9:539, doi:10.1186/1471-2105-9-539

Corander J, Waldmann P, Sillanpää MJ. 2003. Bayesian analysis of genetic differentiation between populations. Genetics 163:367–374.

Douhan GW, Vincenot L, Gryta H, Selosse MA. 2011. Population genetics of ectomycorrhizal fungi: from current knowledge to emerging directions. Fungal Biology 115:569–597, doi:10.1016/j.funbio.2011.03.005

Dupanloup I, Schneider S, Excoffier L. 2002. A simulated annealing approach to define the genetic structure of populations. Molecular Ecology 11:2571–2581, doi:10.1046/j.1365-294X.2002.01650.x

Excoffier L, Lischer HE. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564–567, doi:10.1111/j.1755-0998.2010.02847.x

Felsenstein J. 1989. PHYLIP 3.2 manual. Berkeley, California: University of California Herbarium.

Gandeboeuf D, Dupré C, Chevalier G, Roeckel-Drevet P, Nicolas P. 1997. Grouping and identification of *Tuber* species using RAPD markers. Canadian Journal of Botany 75:36–45, doi:10.1139/b97-005

García-Cunchillos I, Sánchez S, Barriuso JJ, Pérez-Collazos E. 2014. Population genetics of the westernmost distribution of the glaciations-surviving black truffle *Tuber melanosporum*. Mycorrhiza 24:89–100, doi:10.1007/s00572-013-0540-9

Giraud T, Refrégier G, Le Gac M, de Vienne DM, Hood ME. 2008. Speciation in fungi. Fungal Genetics and Biology 45:791–802, doi:10.1016/j.fgb.2008.02.001

Gryndler M, Hršelová H, Soukupová L, Streiblová E, Valda S, Borovička J, Gryndlerová H, Gažo J, Miko M. 2011. Detection of summer truffle (*Tuber aestivum* Vittad.) in ectomycorrhizae and in soil using specific primers. FEMS Microbiology Letters 318:84–91, doi:10.1111/j.1574-6968.2011.02243.x

Hughes KW, Petersen RH, Lickey EB. 2009. Using heterozygosity to estimate a percentage DNA sequence similarity for environmental species' delimitation across basidiomycete fungi. New Phytologist 182:795–798, doi:10.1111/j.1469-8137.2009.02802.x

Jamali S. 2017. First report of identification and molecular characterization of *Tuber aestivum* in Iran. Agroforestry Systems 91:335–343, doi:10.1007/s10457-016-9932-0

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452, doi: 10.1093/bioinformatics/btp187

Martin F, Kohler A, Murat C, Balestrini R, Coutinho PM, Jaillon O, Montanini B, Morin Emmanuelle, Noel B, Percudani R, Porcel B, Rubini A, Amicucci A, Amselem A, Anthouard V, Arcioni S, Artiguenave F, Aury JM, Ballario P, Bolchi A, Brenna A, Brun A, Buée M, Cantarel B, Chevalier G, Couloux A, Da Silva C, Denoeud F, Duplessis S, Ghignone S, Hilselberger B, Iotti M, Mello A, Miranda M, Pacioni G, Quesneville H, Riccioni C, Ruotolo R, Splivallo R, Tisserant E, Stocchi V, Zambonelli A, Zampieri E, Viscomi AR, Henrissat B, Paolocci F, Bonfante P, Ottonello S, Wincker P. 2010. Périgord black truffle genome uncovers evolutionary

origins and mechanisms of symbiosis. Nature 464:1033–1038, doi:10.1038/nature08867

Martin F, Murat C, Paolocci F, Rubini A, Riccioni C, Belfiori B, Arcioni S. 2012. Molecular method for the identification of mating type genes of truffles species. European Patent Application EP2426215; 2010.

McCormack JE, Bowen BS, Smith TB. 2008. Integrating paleoecology and genetics of bird populations in two sky island archipelagos. BMC Biology 6:28, doi:10.1186/1741-7007-6-28

Médail F, Diadema K. 2009. Glacial refugia influence plant diversity patterns in the Mediterranean Basin. Journal of Biogeography 36:1333–1345, doi:10.1111/j.1365-2699.2008.02051.x

Mello A, Cantisani A, Vizzini A, Bonfante P. 2002. Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Environmental Microbiology 4:584–594, doi:10.1046/j.1462-2920.2002.00343.x

Merényi Z, Varga T, Geml J, Orczán ÁK, Chevalier G, Bratek Z. 2014. Phylogeny and phylogeography of the *Tuber brumale* aggr. Mycorrhiza 24:101–113, doi:10.1007/s00572-014-0566-7

Molinier V, Murat C, Baltensweiler A, Büntgen U, Martin F, Meier B, Moser B, Sproll L, Stobbe U, Tegel W, Egli S, Peter M. 2016a. Fine-scale genetic structure of natural *Tuber aestivum* sites in southern Germany. Mycorrhiza 26:895–907, doi:10.1007/s00572-016-0719-y

Molinier V, Murat C, Frochot H, Wipf D, Splivallo R. 2015. Fine-scale spatial genetic structure analysis of the black truffle *Tuber aestivum* and its link to aroma variability. Environmental Microbiology 17:3039–3050, doi:10.1111/1462-2920.12910

Molinier V, Murat C, Peter M, Gollotte A, De la Varga E, Meier B, Egli S, Belfiori B, Paolocci F, Wipf D. 2016b. SSR-based identification of genetic groups within European populations of *Tuber aestivum* Vittad. Mycorrhiza 26:99–110, doi:10.1007/s00572-015-0649-0.

Molinier V, Van Tuinen D, Chevalier G, Gollotte A, Wipf D, Redecker D. 2013. A multigene phylogeny demonstrates that *Tuber aestivum* and *Tuber uncinatum* are conspecific. Organisms Diversity & Evolution 13:503–512, doi:10.1007/s13127-013-0146-2

Morcillo M, Sanchez M, Vilanova X. 2015. Truffle farming today—a comprehensive world guide. Barcelona, Spain: Micologia Forestal & Aplicada. p. 270–272.

Murat C, Díez J, Luis P, Delaruelle C, Dupré C, Chevalier G, Bonfante P, Martin F. 2004. Polymorphism at the ribosomal DNA ITS and its relation to postglacial re-colonization routes of the Perigord truffle *Tuber melanosporum*. New Phytologist 164:401–411, doi:10.1111/j.1469-8137.2004.01189.x

Murat C, Riccioni C, Belfiori B, Cichocki N, Labbé J, Morin E, Tisserant E, Paolocci F, Rubini A, Martin F. 2011. Distribution and localization of microsatellites in the Perigord black truffle genome and identification of new molecular markers. Fungal Genetics and Biology 48:592–601, doi:10.1016/j.fgb.2010.10.007

Naydenov K, Senneville S, Beaulieu J, Tremblay F, Bousquet J. 2007. Glacial vicariance in Eurasia: mitochondrial DNA evidence from Scots pine for a complex heritage involving genetically distinct refugia at mid-northern latitudes and in Asia Minor. BMC Evolutionary Biology 7:233, doi:10.1186/1471-2148-7-233

Pampanini R, Martino G. 2006. L'importanza del tartufo nell'economia della montagna. Micologia Italiana 3:3–17

Paolocci F, Rubini A, Granetti B, Arcioni S. 1999. Rapid molecular approach for a reliable identification of *Tuber* spp. ectomycorrhizae. FEMS Microbiology Ecology 28:23–30, doi:10.1111/j.1574-6941.1999.tb00557.x

Paolocci F, Rubini A, Riccioni C, Topini F, Arcioni S. 2004. *Tuber aestivum* and *Tuber uncinatum*. two morphotypes or two species? Fems Microbiology Letters 235:109–115, doi:10.1111/j.1574-6968.2004.tb09574.x

Payen T, Murat C, Bonito G. 2014. Truffle phylogenomics: new insights into truffle evolution and truffle life cycle. Advances in Botanical Research 70:211–234.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539.

Peay KG, Kennedy PG, Bruns TD. 2008. Fungal community ecology: a hybrid beast with a molecular master. BioScience 58:799–810, doi:10.1641/B580907

Riccioni C, Rubini A, Belfiori B, Gregori G, Paolocci F. 2016. *Tuber magnatum*: the special one. What makes it so different from the other *Tuber* spp.? In: Zambonelli A, Iotti M, Murat C, eds. True truffle (*Tuber* spp.) in the world. Soil ecology, systematic and biochemistry. Switzerland: Springer International Publishing. p. 87–103

Riccioni C, Belfiori B, Rubini A, Passeri V, Arcioni S, Paolocci F. 2008. *Tuber melanosporum* outcrosses: analysis of the genetic diversity within and among its natural populations under this new scenario. New Phytologist 180:466–478, doi:10.1111/j.1469-8137.2008.02560.x

Rousset F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. Genetics 145:1219–1228.

Rubini A, Belfiori B, Riccioni C, Tisserant E, Arcioni S, Martin F, Paolocci F. 2011. Isolation and characterization of *MAT* genes in the symbiotic ascomycete *Tuber melanosporum*. New Phytologist 189:710–722, doi:10.1111/j.1469-8137.2010.03492.x

Rubini A, Paolocci F, Granetti B, Arcioni S. 1998. Single step molecular characterization of morphologically similar black truffle species. FEMS Microbiology Letters 164:7–12, doi:10.1111/j.1574-6968.1998.tb13060.x

Rubini A, Paolocci F, Riccioni C, Vendramin GG, Arcioni S. 2005. Genetic and phylogeographic structures of the symbiotic fungus *Tuber magnatum*. Applied and Environmental Microbiology 71:6584–6589, doi:10.1128/AEM.71.11.6584-6589.2005

Rubini A, Topini F, Riccioni C, Paolocci F, Arcioni S. 2004. Isolation and characterization of polymorphic microsatellite loci in white truffle (*Tuber magnatum*). Molecular Ecology Notes 4:116–118, doi:10.1111/j.1471-8286.2004.00587.x

Slatkin M. 1987. Gene flow and the geographic structure of natural populations. Science 236:787–792, doi:10.1126/science.3576198

Smith ME, Douhan GW, Rizzo DM. 2007. Intra-specific and intra-sporocarp ITS variation of ectomycorrhizal fungi as assessed by rDNA sequencing of sporocarps and pooled

ectomycorrhizal roots from a *Quercus* woodland. Mycorrhiza 18:15–22, doi:10.1007/s00572-007-0148-z

Smith SE, Read DJ. 2010. Mycorrhizal symbiosis. London: Academic Press. 800 p.

Splivallo R, Rittersma R, Valdez N, Chevalier G, Molinier V, Wipf D, Karlovsky P. 2012a. Is climate change altering the geographic distribution of truffles?. Frontiers in Ecology and the Environment 10:461–462, doi:10.1890/12.WB.020

Splivallo R, Valdez N, Kirchhoff N, Ona MC, Schmidt JP, Feussner I, Karlovsky P. 2012b. Intraspecific genotypic variability determines concentrations of key truffle volatiles. New Phytologist 194:823–835, doi:10.1111/j.1469-8137.2012.04077.x

Stobbe U, Egli S, Tegel W, Peter, M, Sproll L, Büntgen U. 2013. Potential and limitations of Burgundy truffle cultivation. Applied Microbiology and Biotechnology 97:5215–5224, doi:10.1007/s00253-013-4956-0

Tabouret P. 2011. Exclusif! Description d'un site français producteur de truffe blanche d'Italie. Le Trufficulteur 78:18.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30:2725–2729, doi:10.1093/molbev/mst197

Taşkın H, Büyükalaca S, Hansen K, O'Donnell K. 2012. Multilocus phylogenetic analysis of true morels (*Morchella*) reveals high levels of endemics in Turkey relative to other regions of Europe. Mycologia 104:446–461, doi:10.3852/11-180

Türkoğlu A, Castellano MA, Trappe JM, Yaratanakul Güngör M. 2015. Turkish truffles I: 18 new records for Turkey. Turkish Journal of Botany 39:359–376, doi:10.3906/bot-1406-42

Vellinga EC, Wolfe BE, Pringle A. 2009. Global patterns of ectomycorrhizal introductions. New Phytologist 181:960–973, doi:10.1111/j.1469-8137.2008.02728.x

Wedén C, Danell E, Camacho FJ, Backlund A. 2004. The population of the hypogeous fungus *Tuber aestivum* syn. *T. uncinatum* on the island of Gotland. Mycorrhiza 14:19–23, doi:10.1007/s00572-003-0271-4

Wedén C, Danell E, Tibell L. 2005. Species recognition in the truffle genus *Tuber*—the synonims *Tuber aestivum* and *Tuber uncinatum*. Environmental Microbiology 7:1535–1546, doi:10.1111/j.1462-2920.2005.00837.x

Zambonelli A, Iotti M, Piattoni F. 2012. Chinese *Tuber aestivum* sensu lato in Europe. The Open Mycology Journal 6:22–26.

Zhang JP, Liu PG, Chen J. 2012. *Tuber sinoaestivum* sp. nov., an edible truffle from southwestern China. Mycotaxon 122:73–82.