Chinese *Tuber aestivum sensu lato* in Europe

Alessandra Zambonelli*, Mirco Iotti and Federica Piattoni

Department of Food Protection and Valorization, Faculty of Agriculture, University of Bologna, Viale Fanin 46, 40127 Bologna, Italy

Abstract: Two specimens of *Tuber aestivum sensu lato* from China were found between *T. aestivum* ascomata for sale in Italy. The morphological features of these ascomata were intermediate between those of *T. aestivum* and *Tuber mesentericum*. The spores were roundish and smaller than those of *T. mesentericum* and *T. aestivum*. Phylogenetic analyses showed that the Chinese specimens are placed in a separate clade to *T. aestivum* and *T. mesentericum*. This, together with the supporting morphological differences, strongly suggests that the two Chinese truffles are a separate taxon.

Keywords: Chinese truffles, European market, ITS sequences, morphology, *Tuber aestivum sensu lato*.

INTRODUCTION

For many years *Tuber uncinatum* Chatin and *Tuber aestivum* Vittad. were considered two different species with different geographical distributions, with *T. aestivum* only growing in the southernmost parts of Europe [1]. Morphological and molecular investigations in the mid 2000s suggested that the two are the same species and hence the older name, *T. aestivum*, should be used [2, 3]. Consequently, *T. aestivum* has the widest distribution of any of the edible truffles [1], being found from Spain to eastern Europe and from Gotland, Sweden, to North Africa [4] and has a very broad genetic diversity [2, 3, 5]. *T. aestivum* has also been reported to have been found in south-western China [6].

Chinese *T. aestivum* was first reported by Mao [7, 8] but without any indication of its location. Moreover, there are no collections deposited in any Chinese herbarium and this taxon was not mentioned in a more recent publication of the same author [9]. The presence of *T. aestivum* in China was confirmed only recently by Song *et al.,* [10] who gave a morphological description of the Chinese ascomata and compared them with European collections of *T. aestivum* and related species (*Tuber mesentericum, Tuber melanosporum* and *Tuber brumale*). They indicated that although there are a few differences between the Chinese and European collections (peridial warts are blunt and also lower than those of European collections) they can be considered to be conspecific.

The only two ITS sequences of *T. aestivum* from China deposited in GenBank were obtained from ascomata collected from Sichuan Province [11]. However, in this paper only the phylogenetic position of species within the *Tuber indicum* complex was critically analysed. In 1989, a small quantity of Chinese black truffles was exported to Germany for appraisal [6]. Since then, increasing quantities have been exported to the international market, which has created concern and also interest in the study of these Chinese *Tuber* species [12]. Chinese truffles have been regularly shipped to Europe, Japan, United States and Australia [7, 12, 13], due in part to the fact that they are much cheaper than European truffles. *T. indicum sensu lato* is the predominant Asiatic species exported to the international market and a small quantity of *Tuber pseudoexcavatum* has been found to be mixed with *T. indicum sensu lato* in shipments [6, 14-16]. In 1997, García-Montero [7] was the first to recognize *T. aestivum* ascomata in shipments imported from China but only recently *T. aestivum* is also being sold on the international market [17, 18].

In this paper two Chinese *T. aestivum sensu lato* samples found in the Italian market were morphologically described and molecularly characterized by sequencing the ITS regions of rDNA.

MATERIAL AND METHODS

Source and Analysis of Ascomata

In December 2010 Dr. Lucio Pierantoni, president of the Italian association "truffle cultivation and environment", examined a small sample of *T. aestivum* fruiting bodies coming from a truffle seller of Central Italy and found two ascomata with anomalous morphological features. These truffles were sent to our laboratory with a request for a confidential identification.

A small fraction of each ascomata was conserved both frozen at – 80° C and in FAA (formaldehyde : acetic acid : 70% ethanol, 5 : 5 : 90) for further molecular and morphological analyses with the remainder dried and collected in CMI-Unibo herbarium (collection numbers 4178a and 4178b).

Morphological Analyses

Sections of the ascomata fixed in FAA were made and their morphology and anatomy photographed and described. Sections between 8 μm and 20 μm thick from each fruiting

*Address correspondence to this author at the *DIPROVAL*, Facoltà di Agraria, Università di Bologna, Viale Fanin 46, 40127 Bologna, Italy; Tel: +39 0512096579; E-mail: zambonel@agrsci.unibo.it

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body were either cut by hand, or with a rotary cryomicrotome (Tissue Tek II, Naperville, IL, USA) after embedding in Tissue Tek OCT compound. Serial sections were mounted in lactic acid and observed under a Nikon ECLIPSE TE 2000-E microscope at 600 X and 1000 X magnification. Measures were made using NIS-Elements AR 2.20 software (Nikon) from images captured with a Nikon DXM1200F digital camera. The following parameters were measured: total thickness of peridium; thickness of external peridium; dimensions of external peridium cells (area, perimeter, equivalent diameter, maximum and minimum Feret's diameter); number of spores per ascus; dimensions of spores in 4 – spore asci – without ornamentations (area, perimeter, equivalent diameter, maximum and minimum Feret's diameter); spore ornamentation length; number of spore meshes along the major axes. Mean values of morphological parameters for each specimen were calculated and compared with those of *T. aestivum* - *T. uncinatum* and *T. mesentericum* reported in the literature [1, 14, 19, 20].

Molecular Analyses

Genomic DNA of the fruiting bodies was isolated from 100 mg of frozen gleba for each sample using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol for fungi. Extracts were eluted in 50 µl of sterile water and DNA concentration was estimated using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Amplifications were performed using 50 ng of template DNA in a final volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM for each dNTP, 400 nM for each primer and 1.5 U of TaKaRa^{TM} rTaq DNA polymerase (Takara, Otsu, Japan). The primer pair ITS1F-ITS4 [21] were used to amplify nuclear rDNA-ITS regions. PCR products were first purified by the NucleoSpin Extract II kit (Machery-Nagel, Düren, Germany) and were then sequenced in both directions using primers ITS1F and ITS4. Sequences were submitted to GeneBank with the following accession numbers: JN975879 and JN975880. Sequences generated in this study were compared with those downloaded from GenBank database obtained from *T. aestivum*-*T. uncinatum* and *T. mesentericum* fruiting bodies of known geographical origin [2, 3, 5, 11, 22].

Sequences were aligned by ClustalW [23] and phylogenetic trees were generated by the neighbour joining (NJ) and maximum parsimony (MP) methods using Mega 5.0 [24] and Paup 4.0b [25], respectively. *Tuber maculatum* Vittad. was used as outgroup in the phylogenetic tree. Branch support values (1000 bootstrap replicates) are expressed as percentages.

RESULTS AND DISCUSSION

The examined truffles had a weak aroma and external morphological features intermediate between those of *T. aestivum* and *T. mesentericum*. Peridial warts were blunt and smaller than those of *T. aestivum* but an orifice, typical of *T. mesentericum*, was lacking. The colour of the spore-bearing tissue was greyish, with narrow and numerous veins (Fig. **1a**). The peridium was composed by a thin outer layer, almost opaque in section, composed by roundish, irregular pseudoparenchimatic cells with a red-brown wall and by a thick inner layer made of interwoven hyphae merging with the glebal tissue (Fig. **1b** and **1c**). The spores (1-6 per ascus) were globose or subglobose, rarely ellipsoid with a reticulum with entire nets (Fig. **1d**).

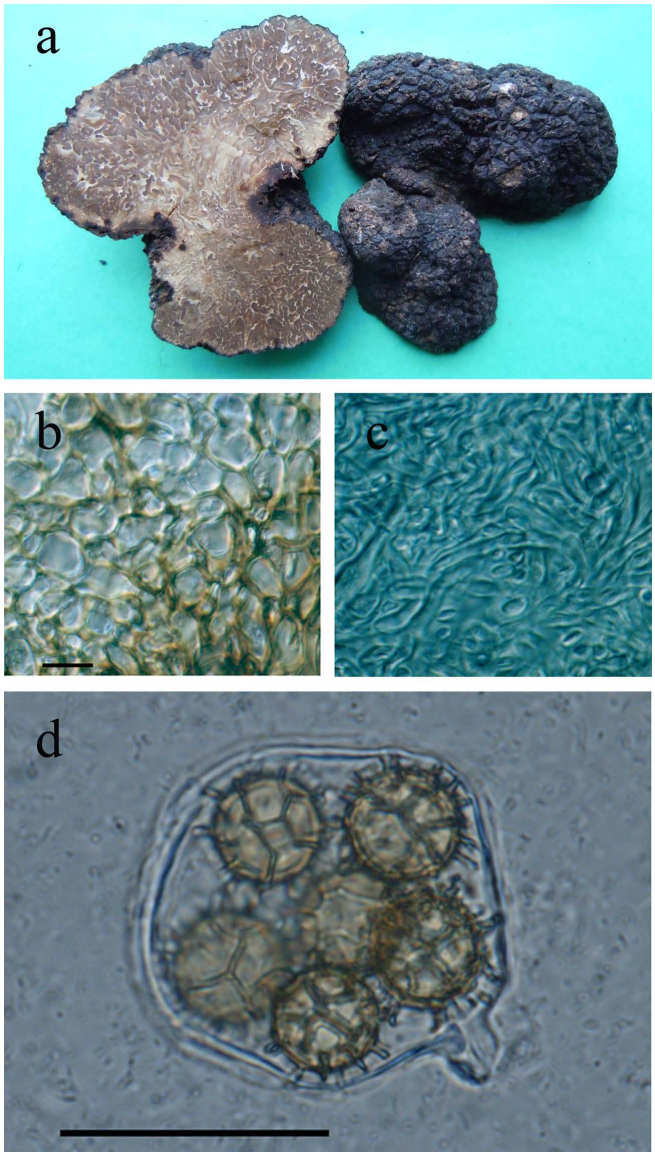

Fig. (1). Morphological features of the Chinese *T. aestivum sensu lato* **a**) Ascoma; **b**) external peridium cells, bar = 10 µm **c**) hyphal internal peridium **d**) ascus containing 6 spores, bar = 50 µm.

The blunt small warts and the greyish gleba of the specimens resembled *T. mesentericum* but the form and the ornamentations of the spores were more similar to *T. aestivum* (Fig. **2**). However, the spore dimensions were somewhat smaller than those of the European *T. aestivum* (29-43 x 25-28 µm) [1] and were more globose (Table **1** and Fig. **2**) resembling those of *Tuber bellonae* Quél, a rare species not always recognized by mycologists [14]. However, the spores of *T. bellonae* are consistently larger (22,5-55 µm) [26].

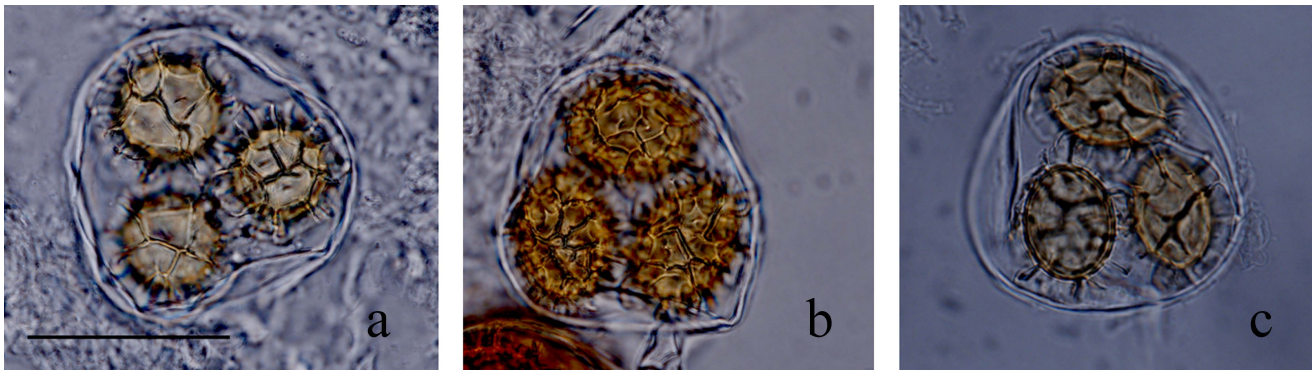

Fig. (2). Comparison between the spores in three-spored asci of the Chinese *T. aestivum sensu lato* (**a**) and those of *T. mesentericum* (**b**) and European *T. aestivum* (**c**), bar = 50 µm.

Table 1. Mean Values of Morphological Measurements of Peridium and Spore Characters of the Two Chinese Specimens

Morphological Characters		Chinese Specimens
		Mean
Total thickness of peridium (µm)[1]		282 (90)
Thickness of external peridum (µm)[1]		95 (22)
External peridium cells[1]	Area (µm^2)	39 (19)
	Perimeter (µm)	26 (8)
	Equivalent diameter (µm)	6.9 (1.8)
	Maximum Feret diameter (µm)	9.5 (2.9)
	Minimum Feret diameter (µm)	6.2 (1.7)
Spores[1]	Area (µm^2)	377 (93)
	Perimeter (µm)	71 (9)
	Equivalent diameter (µm)	22 (2.5)
	Maximum Feret diameter (µm)	25 (3.3)
	Minimum Feret diameter (µm)	21 (2.4)
Number of ornamentations across width of spore		2-3 (4)
Number of spores per ascus		(1) 2-6 (7)

[1]The data are the mean of 50 measures taken from each of the two Chinese ascomata. Between brackets the standard deviation (STD) is reported.

As the topology of the resulting phylogenetic trees was congruent at all supported nodes, only the NJ tree is shown (Fig. **3**). Phylogenetic analyses showed that the two analysed specimens are placed in a separate clade with respect to the European *T. aestivum* and *T. mesentericum*.

Similarity between ITS1-5.8S-ITS2 sequences obtained in this study and those of European *T. aestivum* deposited in GenBank is lower than 92%. Indeed the sequences of the two specimens examined are similar to the two sequences of Chinese *T. aestivum* previously deposited in GenBank by Chen *et al.,* [11] (Table **2**). This, together with the supporting morphological differences, suggests that the two analysed specimens originate from China and belong to a separate taxon closely related with the European *T. aestivum*. After our morphological and molecular identification the Italian seller eventually confirmed that the specimens originated from China.

Table 2. Comparison between ITS1-5.8S-ITS2 Chinese *T. aestivum* Sequences Obtained in this Study and those from GenBank Database (GQ217542 and GU979038)

Similarity (whole ITS1-5.8S-ITS2)		Coverage	Similarity (ITS1 region)		Similarity (ITS2 region)	
%	nt	%	%	nt	%	nt
98.5	603/612	100	98.7	230/233	97.3	219/225

Further morphological and molecular analyses on a most representative number of ascomata and using additional genetic markers are necessary to confirm whether this Chinese taxon is a separate species from *T. aestivum*.

In China, truffles of the *T. indicum* complex, the Chinese *T. aestivum* and *T. pseudoexcavatum,* share the same habitats. They are found predominately in coniferous forests of *Pinus yunnanensis*, *Pinus armadii* and *Keteleeria evelyniana*; in plantations of these species 20-40 years old and secondary-growth coniferous forests developed following the destruction of tropical evergreen broad-leaved forests of *Castanopsis*, *Lithocarpus*, and *Cyclobalanopsis* (1,400–2,500 m a.s.l.). Chinese black truffles also grow in evergreen broad-leaved forests but fruiting is lower in these areas [6]. However, Chinese truffles of *T. indicum* complex can also form ectomycorrhizas with European oaks and pine [27-30]. They have also been found in cultivated *T. melanosporum* truffières in Italy [31] and in American *T. melanosporum* orchards, probably as a result of the use of contaminated spore inoculum in the nursery [32]. *T. indicum* has also been found in the McDonald-Dunn Research Forest fruiting among host plants native to North America, which again demonstrates the ability of this species to colonise non-traditional host plants in an atypical soil [32].

We are concerned that *Tuber aestivum sensu lato* from China could be unintentionally used as inoculum for the production of mycorrhizal plants for truffle cultivation in Italy and in other European regions. In fact we found Chinese *T. astivum* mixed to European truffles in the Italian market and these Chinese truffles might escape detection during inoculum quality control. In conclusion, similarly to

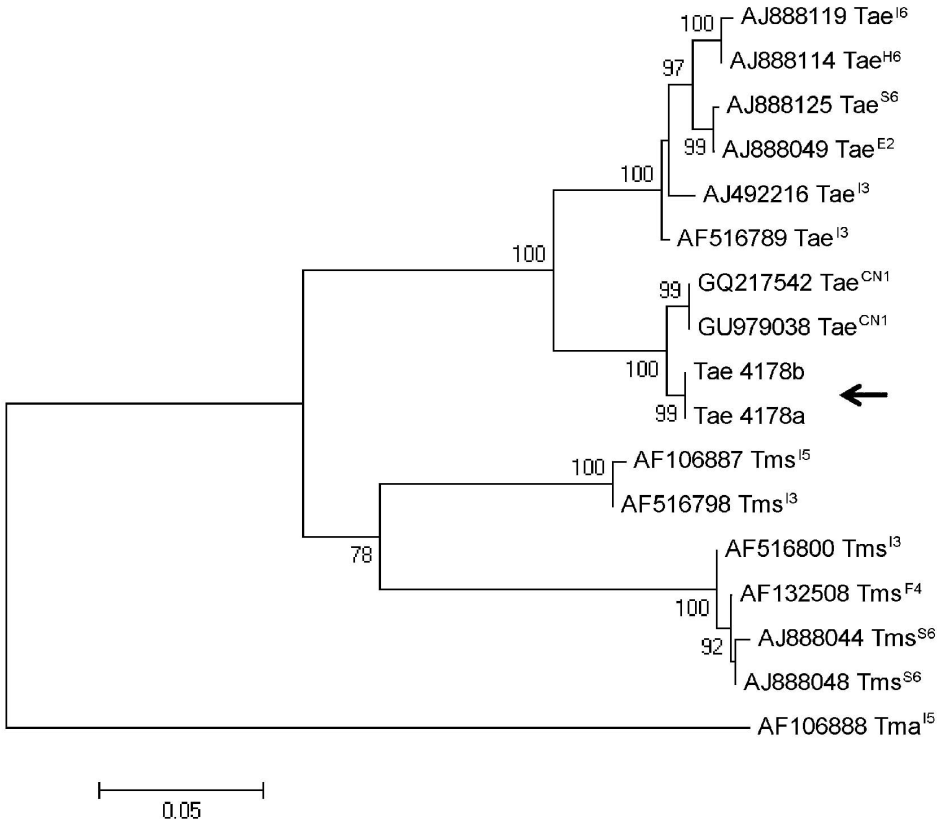

Fig. (3). Neighbour-Joining dendrogram based on ITS1-ITS2 rDNA sequences of species of *T. aestivum* group. Taxa are labelled by GenBank accession number, species abbreviation (Tae, *T. aestivum*; Tms, *T. mesentericum*; Tma, *T. maculatum*), geographic origin: (I, Italy; F, France; H, Hungary; E, Spain; S, Sweden; CN, China) and the references: 1, [11]; 2, [5]; 3, [3]; 4, [22]; 5, Rubini, unpublished; 6, [2]. The two specimens analysed in this study are labelled with species abbreviation and herbarium numbers and they are indicated with an arrow.

that claimed for *T. indicum*, economical and ecological damages may derive from the introduction in Europe of this non autochthonous truffle.

CONFLICT OF INTEREST

None declared.

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