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Short note

Discovery of spontaneously fruiting Chinese black truffles in a hazelnut plantation in Bhutan

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Abstract
Chinese black truffle is commercially known as *Tuber indicum* but it represents a complex to which belong at least 6 species. The members of the *T. indicum* complex are reported from some Chinese provinces, as well as from India, Japan and Corea, where they grow in association with various local host trees in acidic soils characteristic of montane forests. The recent discovery of Chinese black truffles fruiting spontaneously beneath cultivated hazelnut (*Corylus avellana*) trees in Bhutan represents a remarkable and unexpected finding for this country. Multilocus phylogenetic analyses conducted in this study revealed that these truffles belong to *Tuber yunnanense*, a member of the *T. indicum* species complex. Although *T. yunnanense* is native to the Yunnan region of China, its natural occurrence in Bhutan (particularly within an agricultural *Corylus* orchard) extends its known ecological range and suggests potential implications for both regional biodiversity and local truffle production.

Keywords
Corylus avellana, *Tuber yunnanense*, *Tuber indicum* complex, multilocus phylogenetic analyses, morphology

Introduction

The ascomycetes of the genus *Tuber*, the true truffles, are among the most economically valuable edible fungi worldwide, and their demand continues to rise (Podadera-Rivera and Calderón-Vázquez, 2025). *Tuber melanosporum* Vittad., the Périgord black truffle, is the most prized black truffle species on the international market (Hall et al., 2007). It is widely cultivated across Europe and in several regions outside its native range (Zambonelli et al., 2015). However, despite advances in cultivation techniques, production of *T. melanosporum* has shown a general decline in its native countries over recent decades (Thomas and Büntgen, 2019). In France where this truffle is highly appreciated, domestic production is insufficient to meet market demand, and the deficit is increasingly compensated by imports of the Chinese black truffles. Unlike in Italy, in France and other European countries (e.g. Germany and Spain) Chinese black truffles are legally sold under the commercial name *Tuber indicum* Cooke & Massee, although this designation encompasses several distinct species (Chen et al., 2016; DLMBK, 2020; MIPAAF, 1985).

Indeed, *T. indicum* includes a complex of Asian truffle species (Wang et al., 2006; Chen et al., 2011; Kinoshita et al., 2018; Qiao et al., 2018), that are morphologically similar to *T. melanosporum* and belong to the same phylogenetic clade (Bonito et al., 2013). Currently, at least six distinct but closely related species are recognized within the *T. indicum* complex (Wang et al., 2024). They have been primarily reported from the Chinese provinces of Yunnan, Sichuan, and Tibet, as well as from India, Japan and Corea, where they grow in association with various local host trees in acidic soils characteristic of montane forests (Wang, 2012; Le Tacon et al., 2016).

The ecological adaptability of *T. indicum* has long been noted. Experimental studies have demonstrated its ability to form mycorrhizae with several European host plants (Comandini and Pacioni, 1997; Zambonelli et al., 1997; Garcia-Montero et al., 2008). Beyond controlled conditions, *T. indicum* mycorrhizae have been found in cultivated *T. melanosporum* orchards in Europe and the United States (Murat et al., 2008; Bonito et al., 2011), and fruiting bodies have even been found in a natural American forest (Bonito et al., 2011). These findings have raised concerns in non-Asian countries regarding its potential to behave as an invasive alien species capable of establishing and spreading by cultivation practices and replacing the endemic truffle species (Murat et al., 2008). On the other hand, *T. indicum* represents an important economic resource for Asian countries, where its cultivation offers promising opportunities for rural development and income generation (Wang, 2012).

In this study, we report the unexpected appearance of Chinese black truffles in Bhutan within a hazelnut orchard. We describe the ecological conditions of the fruiting site and assess the identity of these truffles through a multilocus phylogenetic analysis.

Materials and Methods

Study site

The hazelnut orchard where the fruiting bodies were found (Fig. 1a, b) is in Yakpogang, Mongar district, Bhutan (27°15'45" N; 91°16'25" E) at 1836 m a.s.l. The orchard, covering approximately 1.5 ha, consists of *Corylus avellana* L. plants of mixed cultivars (Ennis, Barcelona, Epsilon, Theta, Seedling, and Yamhill) planted at a spacing of 2 × 4 m between 2013 and 2017. The study site lies within a temperate highland Cwb climate (Köppen–Geiger), characterized by mild summer temperatures (maximum 23.5–28.3 °C), cool winters (minimum 5.4–7.1 °C), and a strongly monsoonal precipitation regime with extremely dry winters (0–6 mm) and a rainfall peak between June and September, for an average annual precipitation of 881.8 mm (839.5 mm in 2025, National Center of Hydrology and Meteorology, Royal Government of Bhutan). Monthly precipitation and temperature data (maximum, and minimum), summer days of the period 2019–2023 were obtained from the meteorological station located at Mongar near hazel orchard (Supplementary Table S1).

The soil at the fruiting site is characterized by near-neutral pH, very low salinity with a higher organic matter, organic carbon, and nitrogen in the topsoil than the sub-soil. The surface horizon is clay loam, while the sub-soil is heavy clay, indicating a more compact and poorly aerated deeper layer. Available phosphorus and potassium are low in both horizons (Supplementary Table S2). Black truffle fruiting bodies were accidentally discovered while digging the soil inside the hazelnut plantation at the end of December 2025.

Morphological and Phylogenetic analyses

Two of the collected black truffles were photographed macroscopically and then each specimen was cut in half. One half was dried and deposited in the Mycological Herbarium of the University of Bologna (CMI-Unibo 5501 and 5503), while the other half was preserved in pure ethanol pending molecular analyses.

Microscopic analyses were performed on dried material rehydrated in 5% potassium hydroxide (KOH). Observations were conducted using a Nikon Eclipse TE2000-U inverted microscope (Nikon Corporation, Tokyo, Japan). Images were captured with a Nikon DS-Fi3 camera (Nikon Corporation, Tokyo, Japan), and measurements were taken using the NIS Elements BR software (version 4.6, Nikon Corporation, Tokyo, Japan). At least 40 measurements for 4-spored asci of each fruiting body were taken (Gross, 1987). Microscopic measurements are presented according to the method used by Breitenbach and Kränzlin (1984). The 95% population limits for the mean were calculated, and the lower and upper values are given. The average spore quotient (length/width ratio, Qm) was also calculated and reported as mean ± standard deviation.

To identify truffles collected in this study, genomic DNA was extracted from the lyophilized ascoma gleba of samples 5501 and 5503 using the Norgen Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Canada), following the manufacturer's instructions. The ITS, RPB2, and TEF1-α regions were amplified by PCR using the primers and amplification conditions listed in the Supplementary Table S3. The resulting amplicons were sequenced using Sanger sequencing at Eurofins Genomics (Köln, Nordrhein-Westfalen, Germany) using both forward and reverse primers, and the corresponding NCBI accession numbers are listed here: PZ142840 and PZ142841 (ITS), PZ149757 and PZ149758 (RPB2), PZ149755 and PZ149756 (TEF1-α). The taxonomic placement of samples 5501 and 5503 was assessed using multilocus sequence analysis (MLSA) based on a concatenated alignment of the ITS2 (obtained from Genbank ITS sequences), RPB2, and TEF1-α sequences generated in this study together with those reported in the Supplementary Table S4. Due to the high variability in the size of ITS1 region in the Melanosporum clade, ITS1 sequences were excluded from the concatenated dataset to make alignment and phylogenetic analysis more solid and supported. The phylogenetic reconstruction was carried out with maximum likelihood (ML) implemented in MEGA11 software (Tamura et al., 2021). ML analyses were performed with 1000 throughout bootstrap replicates (100 runs), applying the models of nucleotide substitution T92+G. ML tree was edited using MEGA11 (Tamura et al., 2021). Only bootstrap values greater than 75% were shown on branches.

Results and discussion

Hazelnut trees are well-known hosts for several truffle species, particularly the European black truffle (*T. melanosporum*) and the Burgundy truffle (*T. aestivum* Vittad.) (Hall et al., 2007; Vlahova, 2021; Habtemariam et al., 2025). The spontaneous formation of Chinese black truffles beneath *C. avellana* in Bhutan expands the list of potential host species for members of the *T. indicum* complex and highlights the ecological flexibility of these Asian truffles.

Previous studies have shown that Chinese black truffles can colonize the roots of various European tree species (Comandini and Pacioni, 1997; Zambonelli et al., 1997; Garcia Montero et al., 2008), which is why they are considered potentially prone to spread beyond their native range (Murat et al., 2008). Indeed, fruiting bodies belonging to the *T. indicum* complex have even been reported in the United States, in a natural forest where a native *Corylus* species was present (Bonito et al., 2011). That has raised concerns about the possibility that these truffles could establish themselves in European environments, potentially competing with the most valuable truffle *T. melanosporum*.

The fruiting site in Bhutan is characterized by a subtropical montane climate, which appears suitable for the production of Chinese black truffles. The soil pH at the site, however, is higher than what has typically been reported as optimal for *T. indicum,* particularly in the subsoil (Wang, 2012; Le Tacon et al., 2016). It is important to consider that *T. indicum* is currently recognized as a species complex containing multiple morphologically similar species, many of which closely resemble *T. melanosporum* (Wang et al., 2006; Chen et al., 2011; Kinoshita et al., 2018; Qiao et al., 2018). These species may differ in their ecological requirements, some of which are still poorly understood.

In our study, multilocus analyses revealed that the Bhutanese specimens belong to *Tuber yunnanense* S.P. Wan, Rui Wang & F.Q. Yu, forming a genetically distinct and well supported clade within the recently described species (Fig. 2) (Wang et al., 2024). This lineage may possess specific ecological traits that facilitate its establishment under the environmental conditions found at the Bhutanese site. From a morphological perspective, the collected fruiting bodies also show slight micromorphological discrepancies compared with the published description of *T. yunnanense* (Fig. 1). In particular, the spores are shorter [(14.9–) 23.5–25.2 (–33.1) × (13.9–) 16.57–17.18 (–19.7) µm], and their shape is broadly ellipsoid (Qm = 1.44 ± 0.2). In contrast, *T. yunnanense* is reported to have slightly longer spores, since the upper limit of its main range is 31.6 µm. Moreover, the spores in our material are dark brown, whereas *T. yunnanense* is described as having yellowish spores. That could reflect a partial immaturity of the described specimens by Wang et al. (2024). The spore ornamentation consists of highly irregular, large spines, a character state consistent with the morphology typically observed in most species of the *T. indicum* complex. All other morphological characters, including peridial and glebal features and the number of spores per ascus (1 to 6), conform to the description of *T. yunnanense* provided by Wang et al. (2024).

The detection of *T. yunnanense*, a species of *T. indicum*-complex, in a hazelnut cultivated orchard in Bhutan, is particularly interesting and carries noteworthy implications. Economically, the presence of *T. yunnanense* suggests that truffle production could become a complementary activity within existing hazelnut orchards, potentially providing new opportunities for local farmers. At the same time, its ability to form mycorrhizae and fruit under *C. avellana* highlights the species' suitability for intentional cultivation, indicating promising prospects for applied mycorrhizal biotechnology co-cropping truffles and hazelnuts in the region. From an ecological standpoint, its establishment in Bhutan offers an opportunity to better understand the dispersal capacity and adaptive traits of the *T. indicum* complex, contributing to a clearer picture of how these truffles expand into new environments. Moreover, further research is needed to investigate the presence and the distribution of *T. yunnanense* in natural ecosystem of Bhutan, with the particular attention to the host plant range and overall ecological characteristics of the growing environments.

Fig. 1 – Excavation pit showing the location where fruiting body no. 5501 was recovered. (a) Hazelnut orchard where the truffles were collected; (b) fruiting body no. 5501; (c) fruiting body no. 5503; (d) four-spored ascus; (e) two-spored ascus; (f) scale bar = 10 µm.

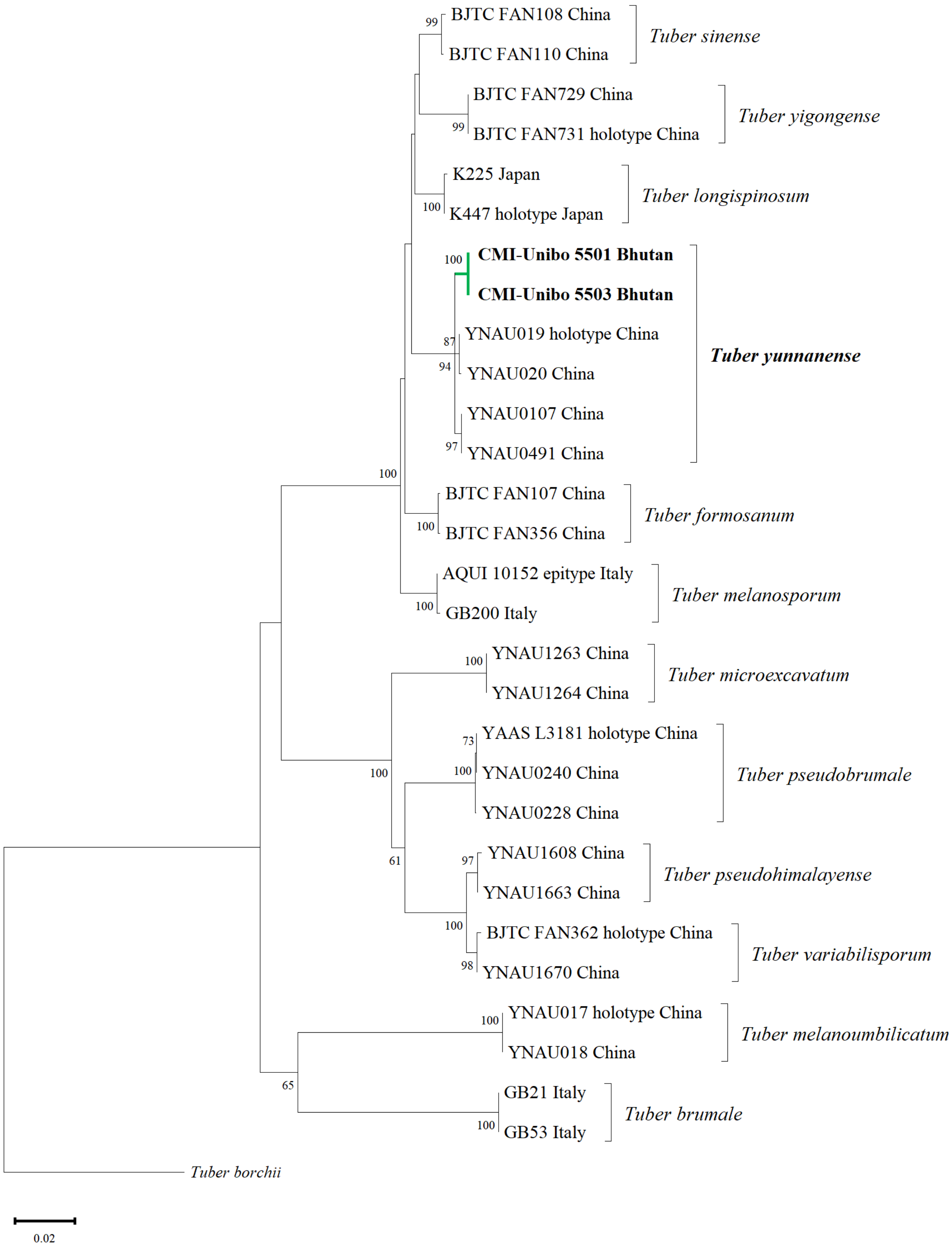

Fig. 2 – Maximum likelihood phylogeny based on concatenated ITS2, RPB2, and TEF1-α genetic region sequences, showing the relationships among truffle species included in the Melanosporum clade, together with the samples investigated in this study. Herbarium numbers are indicated before the location. Bootstrap values >75% are indicated at the nodes. Scale bar = 2 substitutions per 100 positions.

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Author Contributions

Conceptualization, A.Z. methodology, S.G. and M.I.; formal analysis, S.G.; investigation, D.P and P.L.; resources, A.Z.; data curation, M.I.: writing—original draft preparation, AZ. and S.G.; writing—review and editing, M.I; visualization, S.G. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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