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Species diversity, phylogeny, endemism and geography of the truffle genus *Tuber* in China based on morphological and molecular data

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Key words

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Abstract The genus *Tuber* (*Tuberaceae*, *Pezizales*) is an important fungal group of *Ascomycota* both economically and ecologically. However, the species diversity, phylogenetic relationships, and geographic distribution of *Tuber* species in China remains poorly understood, primarily because descriptions of many new species relied heavily on morphological features with molecular data either not sought or ignored. The misapplication of European and North American names further added to confusion regarding the taxonomy of Chinese *Tuber* species. In this study, we examined more than 1 000 specimens from China, and performed a comprehensive phylogenetic analysis for Chinese *Tuber* species using ITS sequences and multilocus sequence data. To infer the phylogeny of Chinese *Tuber* spp., 11 molecular datasets were assembled, including a concatenated internal transcribed spacers of the nuc rDNA (ITS), nuc rDNA 28S subunit (LSU), translation elongation factor 1-alpha (*tef1-α*), and RNA polymerase II subunit (*rpb2*) dataset as well as 10 ITS datasets (totally including 1 435 sequences from 828 collections with 597 newly generated sequences, and 168 sequences from the types of 63 species). Our phylogenetic tree based on a concatenated multilocus dataset revealed that all Chinese *Tuber* species nested in nine phylogenetic clades (phylogroups), including *Aestivum*, *Excavatum*, *Latisporum*, *Macrosporum*, *Maculatum*, *Melanosporum*, *Puberulum*, *Rufum* and *Turmericum*. Of these, five phylogroups (*Macrosporum*, *Maculatum*, *Melanosporum*, *Puberulum* and *Rufum*) are shared across the continents of Asia, Europe and North America; two phylogroups (*Aestivum* and *Excavatum*) are shared by Europe and Asia; and the phylogroups *Turmericum* and *Latisporum* are endemic only to Asia. Phylogenetic trees based on 10 ITS datasets confirmed the presence of at least 82 phylogenetic species in China. Of these, 53 are identified as known species, including three new records for China, and 25 species are identified as new to science. Of the new species, nine are described and illustrated in this paper, and the others remain unnamed due to the paucity or absence of ascomatal materials. Accordingly, the confirmed, excluded and doubtful *Tuber* species in China are discussed. *Tuber* species showed high endemism. Of the 82 phylogenetic species found in China, 68 species occur only in China, six species are also found in other regions in Asia, and only eight species (*T. anniae*, *T. excelsum-reticulatum*, *T. formosanum*, *T. maculatum*, *T. wenchuanense*, *Tuber* sp. CHN-3, *Tuber* sp. CHN-10 and *Tuber* sp. CHN-11) are shared with other continents. Most *Tuber* species have a small and limited distribution in China, but a few, such as *T. formosanum* and *T. parvomurphium*, are widely distributed across China. Some phylogenetically closely related species, such as *T. liaotongense* and *T. subglobosum*, as well as *T. xuanhuaense* and *T. lijiangense*, show a pattern of allopatric distribution.

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INTRODUCTION

The truffle genus *Tuber* (*Tuberaceae*, *Pezizales*, *Pezizomycotina*, *Ascomycota*) is economically and ecologically one of the most important fungal genera. All species in this genus are ectomycorrhizal forming mutually beneficial symbiotic associations with a wide range of host plants including *Pinus* spp., *Picea* spp., *Larix* spp., *Pseudotsuga* spp., *Populus* spp., *Fagus* spp., *Corylus* spp., *Quercus* spp., which are dominant trees in North temperate forests (Hall et al. 2007, Trappe et al. 2009, Deng et al. 2014). These fungi play a very important role in forest development and ecological balance (Maser et al. 2008, Courty et al. 2010). *Tuber* is also the most widely known and studied fungal genus because *T. aestivum*, *T. borchii*, *T. melanosporum* and *T. magnatum*, the culinarily famous and expensive summer truffle, bianchetto truffle, French black truffle and Italian white truffle, are nested in this genus.

The scientific classification of *Tuber* has a long history in Europe (Vittadini 1831, Tulasne & Tulasne 1851, Ceruti 1960, Riousset et al. 2001, Ceruti et al. 2003, Jeandroz et al. 2008), and in North America (Harkness 1899, Gilkey 1939, 1954, Trappe et al. 2009, Bonito et al. 2010, 2013, Guevara et al. 2013). However, the knowledge of this genus is absent from China before 1985s. The first scientific record of *Tuber* in China is *T. taiyuanense*, a domestic species, harvested under *Pinus* sp. from Shanxi Province in North China (Liu 1985). Later, Wang (1988) reported seven *Tuber* species from Liaoning Province in north-eastern China including the new species *T. liaotongense*. Published information on truffles in southern China began with the publication of *T. sinense* (Tao et al. 1989), which currently is an important commercial black truffle on the international market although often this is mistakenly called *T. indicum*.

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Since 1991, considerable effort has been focused on *Tuber* in China, and many species have been added to the list of Chinese species, many of which were based on morphology (mostly earlier than 2005) or both morphology and molecular data (Wang & Li 1991, Hu 1992, Wang et al. 1998, Xu 1999, Wang & He 2002, Chen et al. 2005, Hu & Wang 2005, Chen & Liu 2007, Fan et al. 2011a, b, 2012a–e, 2013a, b, 2014, 2015, 2016a, b, 2018, Fan & Cao 2012, Zhang et al. 2012, Fan & Yue 2013, Qiao et al. 2013, Su et al. 2013, Qing et al. 2015, Huang et al. 2017, Wan et al. 2017a, b, Xu et al. 2017, Fan 2021). Three doctoral theses were focused on phylogenetic and taxonomic work on this genus with evidence from both morphological and molecular data (Song 2005, Chen 2007, Cao 2010), and these contributed much to our understanding of *Tuber* in China, particularly over the past decade. Despite this we still know little about their phylogenetic relationships and geographic distribution. There is also an urgent need to re-examine species erected based on morphological features with molecular methods. There is also a need to explore the taxonomic positions of some taxa known only from ectomycorrhizal (ECM) root tips.

Identifying *Tuber* species based on morphology alone is often very difficult due to the limited number of morphological features (Kinoshita et al. 2011, 2018, Fan et al. 2018), and the occurrence of cryptic species (Chen et al. 2011, Kinoshita et al. 2011). These factors contribute to confuse species delimitations and definitions, which has led to confusion in the Chinese literature. The taxonomic confusion is more common in the literature written before 2010 when an ITS-based DNA delimitation of *Tuber* species was proposed (Bonito et al. 2010). Molecular analyses based on DNA sequence data provide powerful tools to accurately identify *Tuber* species (Wang et al. 2006a, b, 2007, Bonito et al. 2010, Kinoshita et al. 2011), and facilitate the study of *Tuber* species not only from ascomata but also from environmental DNA sequences, thereby increasing our knowledge of the ecology as well as diversity of these truffles.

In our current study, morphological features and DNA-based molecular analysis were conducted based on more than 1 000 collections garnered in both North and South China over the past two decades. The aim of this paper is to clarify the species diversity, phylogenetic relationships, endemism and geographic distribution of this important truffle genus *Tuber* in China.

MATERIALS AND METHODS

Sampling

Samples collected in China over the past two decades were examined. Voucher specimens have been accessioned in the Herbarium Biology Department, Capital Normal University (BJTC). Additional specimens on loan from other fungaria were also studied. The principal herbaria were the Herbarium Mycologicum Academiae Sinicaea at the Institute of Microbiology, Chinese Academy of Sciences (HMAS) and the Herbarium of Cryptogams at the Kunming Institute of Botany, Chinese Academy of Sciences (HKAS).

DNA extraction, PCR amplification, sequencing and nucleotide alignment

Gleba tissue sampled from fungarium specimens were crushed by shaking for 3 min at 30 Hz (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 mL tube together with one 3-mm-diameter tungsten carbide ball, and total genomic DNA was extracted using the E.Z.N.A. Fungal DNA kit (Omega Bio-Tek Inc. Norcross, Georgia, USA) following the manufacturer's protocol.

The following primer pairs were used for PCR amplification and sequencing: ITS1f / ITS4 (White et al. 1990, Gardes & Bruns 1993) were used for the internal transcribed spacers of the nuc rDNA (ITS1-5.8S-ITS2 = ITS); LR0R/LR5 (Vilgalys & Hester 1990) for the nuc rDNA 28S subunit (nrLSU); EF1Tub_for, 2/EF1Tub_rev1 (Bonito et al. 2013) for the translation elongation factor 1-alpha (*tef1-α*); fRPB2_5f/RPB2_7r (Bonito et al. 2013) for the RNA polymerase II subunit (*rpb2*). PCRs were performed in 50 µL reactions containing 4 µL DNA template, 2 µL of each primer (10 µM/L), 25 µL 2× Master Mix (Tiangen Biotech (Beijing) Co. Ltd.), and 17 µL ddH_2O. PCR reactions were run as follows: for the ITS gene, initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 56 °C for 50 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min; for the LSU gene, initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min; for the *tef1-α* gene, initial denaturation at 94 °C for 90 s, followed by 35 cycles at 94 °C for 30 s, 60 °C for 35 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min; for the *rpb2* gene, initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 40 s, with step down starting at 50 °C, decreasing by 1 °C per cycle to 45 °C for 45 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR products were sent to Beijing Zhongkexilin Biotechnology Co. Ltd. (Beijing, China) for purification and sequencing. The newly generated sequences were assembled and edited using SeqMan (DNA STAR package; DNAStar Inc., Madison, WI, USA) with generic-level identities for sequences confirmed via BLAST queries of GenBank.

A total of 1 344 sequences from 957 collections of *Tuber* were used in the molecular phylogenetic analyses. Specimen information is provided in Table S1–S3. Sequences of all DNA regions generated in this study were deposited in GenBank. The sequences obtained from GenBank are based on published literature (Kinoshita et al. 2011, Fan et al. 2011a, b, 2012a–e, 2013a, b, 2014, 2015, 2016a, b, 2018, Fan & Cao 2012, Bonito et al. 2013, Huang et al. 2017, Yan et al. 2018), or selected by using the BLASTn search function of the NCBI database to find similar matches with taxa in *Tuber*. For ITS sequence data, the genus search tool in UNITE database and emerencia (Nilsson et al. 2005) was used for retrieving *Tuber*-related ITS sequences, and the ITS sequences with short lengths (≤ 300 bp) were excluded from the final analyses.

Eleven datasets were assembled for this study. Dataset I (ITS/nrLSU/*tef1-α*/*rpb2*) contains the backbone species and all phylogroups of *Tuber*, which were used to infer the phylogenetic placement of Chinese *Tuber* species. This dataset was concatenated and included one to two representative samples per species, with three species of *Choiromyces* and *Labyrinthomyces* as outgroup taxa. Datasets II–X (ITS) correspond to nine phylogroups, and include all available sequences of Chinese *Tuber* species, and the sequences of representative (non-Chinese) species (one to two samples per species) for each *Tuber* phylogroup. These nine ITS-datasets were employed to delimit the Chinese *Tuber* species. Moreover, for making each sequence employed in Datasets II–X to be classified in the homologous phylogroup, we selected the representative sequences of each species using the threshold of 98 % ITS sequence similarity as species delimitation according to our research experiences. Sequence similarity is obtained using Mothur v. 1.39.5 (Schloss et al. 2009) with minimum match set to 98 %, which is less than the species delimitation (95–96 %) proposed by Kinoshita et al. (2011) and Bonito et al. (2013), and similar to the threshold (97–98 %) usually used for ectomycorrhizal fungal community studies (Smith et al. 2007, Peay 2008, Brock et al. 2009, Hughes et al. 2009), and iteratively constructed the phylogenetic analyses for the representative sequence dataset using the maximum likelihood (ML) method. Dataset XI (ITS) contains all Chinese *Tuber* species that were described

in previous studies or discovered in this study except for three species that lack available molecular data. These 10 alignments (Dataset II–XI) were used to infer the phylogenetic relationships between Chinese *Tuber* species. Three species that lack molecular data are *T. gigantosporum*, *T. polyspermum*, and *T. xizangense*. We cannot obtain the specimens of *T. gigantosporum* and *T. xizangense* as they may be lost according to the original authors. The molecular data of *T. polyspermum* is not successfully sequenced.

Sequences were aligned and edited in MAFFT (Katoh & Frith 2012) under default parameters, and manually adjusted for maximum sequence similarity in Se-Al v. 2.03a. (Rambaut 2000). Ambiguously aligned regions and gaps in the alignment were excluded before the analyses. For the concatenated dataset (Dataset I), alignments were constructed separately for each of the gene fragments using MAFFT (Katoh & Frith 2012), optimised using BioEdit v. 7.0.9 (Hall 1999), then concatenated using SequenceMatrix v. 1.7.8 (Vaidya et al. 2011). Unsampled gene regions were coded as missing data and all introns of *rpb2*, *tef1-α* were excluded because of the difficulty in alignment. Poorly aligned sites were identified by Gblocks 0.91b (http://www.phylogeny.fr/one_task.cgi?task_type=gblocks&tab_index=2; Castresana 2000; using default options except ALLOWED GAP POSITIONS 5 half) with default parameters. All identified ambiguous sites were excluded before the analyses. Alignments of all datasets used in this study were submitted to TreeBASE (No. 29218).

Phylogenetic analyses

Maximum likelihood (ML) analyses on all eleven datasets in this study were conducted with RAxML v. 8.0.14 (Stamatakis et al. 2005, Stamatakis 2006, 2014) and the GTRGAMMAI substitution model (for all 11 datasets) with parameters unlinked. ML bootstrap replicates (1 000) were computed in RAxML with a rapid bootstrap analysis and search for the best-scoring ML tree.

Bayesian inference (BI) was performed with MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) as an additional method determining branch support. The best substitution model that fit the data at each locus was evaluated using MrModeltest v. 2.3 (Nylander 2004). For the concatenated analysis (Dataset I), each locus is considered a partition and assigned its own best-fitting substitution model, that is HKY+I+G for ITS, *rpb2* and *tef1-α*, GTR+I+G for nrLSU. We used two independent runs with four Markov chains Monte Carlo (MCMC) for 645 000 generations under the default settings. For single gene analysis (Dataset II–XI), the best substitution model for each dataset respectively was HKY+I (Dataset II), HKY+G (Dataset III), GTR+G (Datasets IV–VII), GTR+I+G (Datasets VIII–IX), SYM+I+G (Dataset X, Phylogroup *Excavatum*), and GTR+I+G (Dataset XI). The MCMC analysis was run for 10 M generations. Average standard deviations of split frequency (ASDSF) values were far lower than 0.01 at the end of the generations. Trees were sampled every 100 generations after burn-in (25 % of trees were discarded as the burn-in phase of the analyses, set up well after convergence), and 50 % majority-rule consensus trees were constructed.

Clades with bootstrap support (BS) ≥ 70 % and Bayesian posterior probability (PP) ≥ 0.99 were considered as significant-support (Hillis & Bull 1993, Alfaro et al. 2003). All phylogenetic trees were viewed with TreeView32 (Page 2001).

RESULTS

Phylogenetic analyses

Phylogeny of Chinese Tuber at phylogroup level

The phylogenetic trees of *Tuber* based on individual loci (including ITS, nrLSU, *tef1-α*, *rpb2*) showed the same major clades as that of the combined Dataset I (Fig. 1). There was no strongly supported conflict between single gene phylogenies, except for the nrLSU phylogeny does not resolve *Puberulum* phylogroup or place *T. magnatum* within *Aaestivum* phylogroup (similar to Bonito et al. 2013). So here the combined dataset was used to infer the phylogenetic placement of Chinese *Tuber* species.

Dataset I (ITS/nrLSU/*tef1-α*/*rpb2*) contained 532 sequences from 96 species, including 278 novel sequences of all four genes from the Chinese collections. *Choiromyces alveolatus* (HM485332), *C. meandriformis* (HM485330) and *Labyrinthomyces* sp. 3 (HM485335) were selected as the outgroup (Bonito et al. 2013). The length of the aligned dataset was 2 793 bp after exclusion of poorly aligned sites, with 374 bp for ITS, 828 bp for nrLSU, 791 bp for *tef1-α*, and 800 bp for *rpb2*. The tree inferred from the ML analysis is illustrated with strong statistical bootstrap from ML and posterior probability from BI support values shown (Fig. 1).

This multilocus phylogeny on ML tree resolved 12 clades of *Tuber* species with high statistical support. Of them, 11 clades respectively corresponded to the phylogroup *Aestivum*, *Excavatum*, *Genadii*, *Gibbosum*, *Macrosporum*, *Maculatum*, *Melanosporum*, *Multimaculatum*, *Puberulum*, *Rufum* and *Turmericum* (= *Japonicum*; Kinoshita et al. 2011, Fan et al. 2015) proposed by Bonito et al. (2013), and the remaining one corresponded with the *Latisporum* phylogroup proposed by Fan et al. (2016a, b). Chinese samples were nested in 9 phylogroups, i.e., *Aestivum*, *Excavatum*, *Latisporum*, *Macrosporum*, *Maculatum*, *Melanosporum*, *Puberulum*, *Rufum* and *Turmericum*, and five phylogroups (*Macrosporum*, *Maculatum*, *Melanosporum*, *Puberulum*, *Rufum*) were distributed in Asia, Europe and North America, two phylogroups (*Aestivum*, *Excavatum*) evolved in Asia and Europe, and *Latisporum* and *Turmericum* were endemic to Asia (Kinoshita et al. 2011, Bonito et al. 2013, Fan et al. 2016a, b). The Bayesian tree reconstructed using Bayesian patitional analysis showed significant support for each phylogroup resulted from ML analysis. However, the BI tree exposed several discrepancies with the ML tree (Fig. 1). It showed that Phylogroup *Genndii* was positioned at the base in BI tree rather than in the middle in ML tree, adjacent to Phylogroup *Macrosporum*. Phylogroup *Turmericum* well clustered together with Phylogroups *Excavatum* and *Aestivum* and was adjacent to Phylogroup *Genndii* rather than an independent clade at the base in ML tree, adjacent to Phylogroup *Aestivum*.

Phylogeny of Chinese Tuber at species level

The ITS rDNA barcode marker was employed for the analysis of Chinese *Tuber* species diversity in this study, because ITS works well for the delimitation of species of *Tuber* (except for species complexes) (Smith et al. 2007, Peay 2008, Brock et al. 2009, Hughes et al. 2009, Kinoshita et al. 2011, Bonito et al. 2013, Fan et al. 2016a, b). A total of 1 031 ITS sequences, including 358 novel sequences from Chinese collections, were obtained for this study. Our multigene phylogenetic analysis revealed that Chinese *Tuber* species were classified in nine clades (Fig. 1), therefore ITS sequences were split into nine datasets representing each clade (Datasets II–X). This parsing of species facilitate the molecular phylogenetic analyses by delimiting the large number of Chinese *Tuber* species and detecting the phylogenetic relationships between the *Tuber* species from China and those from other geographic areas,

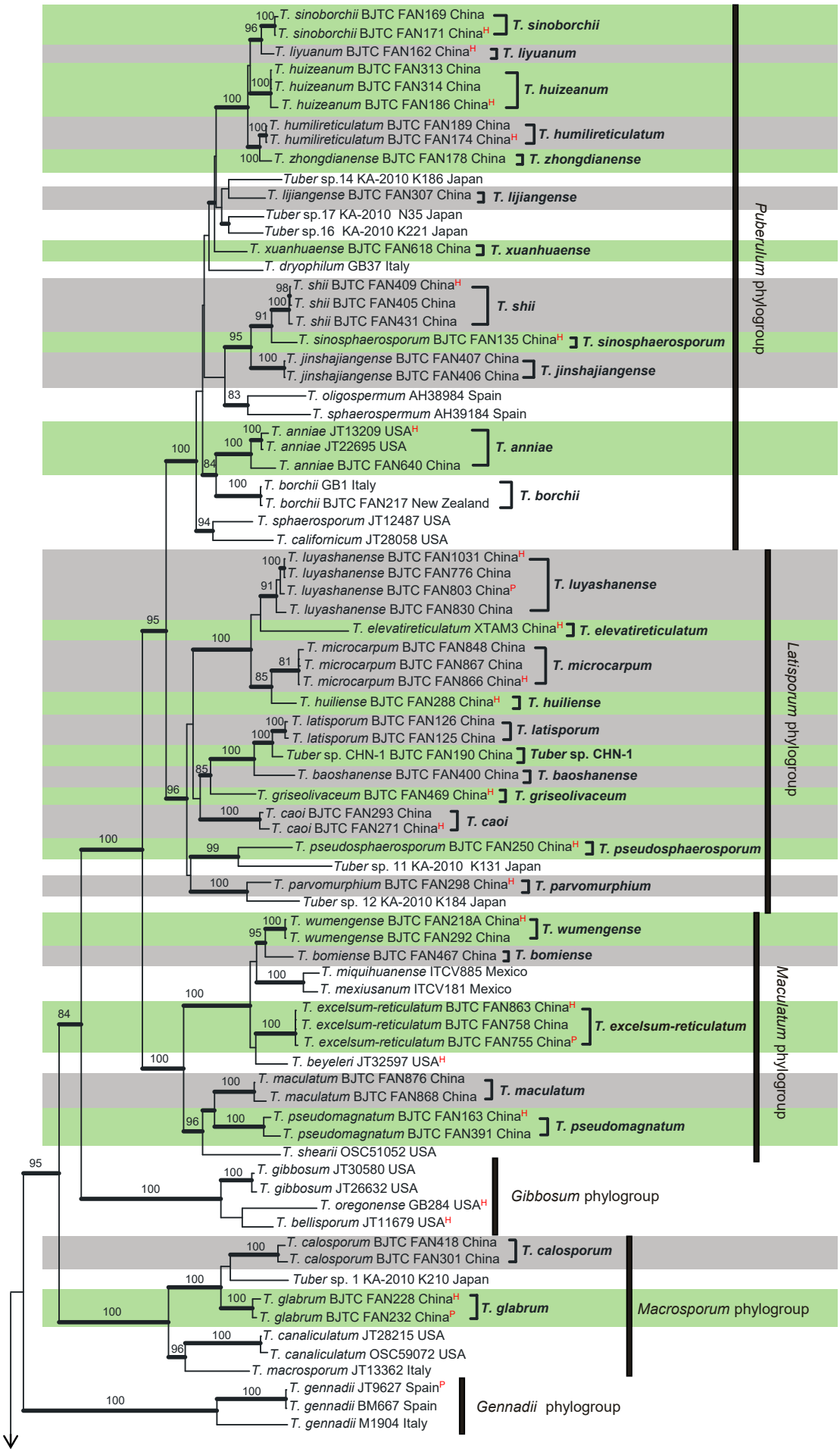

Fig. 1 Phylogeny of Chinese *Tuber* inferred from the Dataset I (ITS/nrLSU/*tef1-α*/*rpb2*) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. Chinese *Tuber* species are printed in **bold**.

including Japan, Europe and North American. The phylogenetic analyses for all nine datasets are documented below.

1. *Phylogroup Aestivum*

Dataset II (ITS) comprised 30 sequences from the taxa belonging to phylogroup *Aestivum*, in which 19 sequences were isolated from Chinese *Tuber* specimens and ECM root tips of *Pinus armandii* in China. The length of the dataset was 601 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analysis is shown (Fig. S1) with statistical support values. Only one species *T. sinoaestivum* is recognised from China for this lineage. *Tuber sinoaestivum* is sister to the European *T. aestivum* with strong support (Fig. S1).

2. *Phylogroup Excavatum*

Dataset III (ITS) comprised 75 sequences from the phylogroup *Excavatum*, in which 38 sequences were isolated from Chinese *Tuber* species. The length of the dataset was 583 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analysis is shown. In this tree, the Chinese taxa were clearly placed in seven well-supported clades, represented by five known species and two new species (Fig. S2). The five

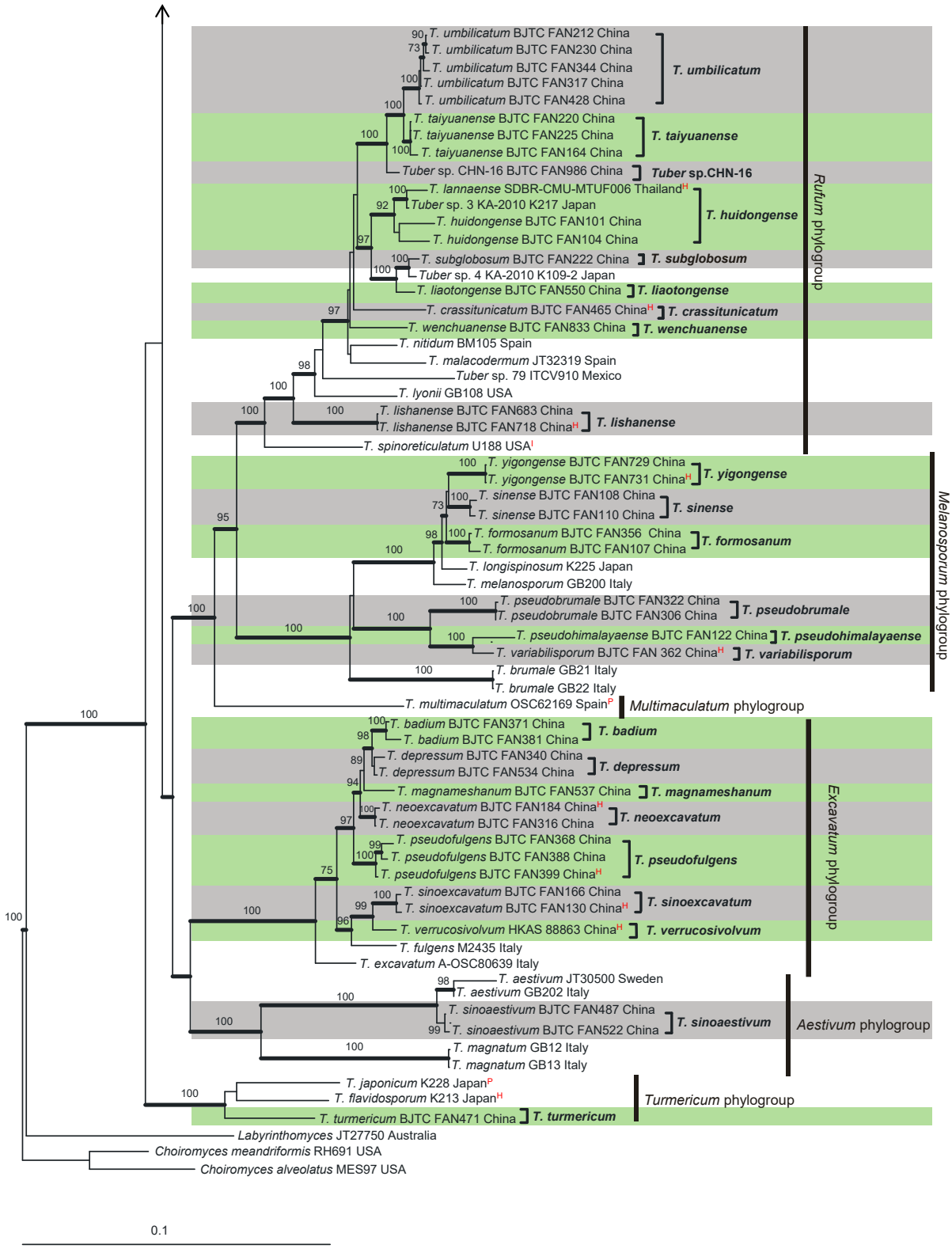

Fig. 1 (cont.)

known species are *T. badium*, *T. depressum*, *T. neoexcavatum*, *T. sinoexcavatum* and *T. verrucosivolvum.* The two new species are described as *T. magnameshanum* and *T. pseudofulgens* in this study.

3. *Phylogroup Latisporum*

Dataset IV (ITS) comprised 162 sequences from the phylogroup *Latisporum*, in which 139 sequences were isolated from Chinese *Tuber* species. The length of the dataset was 550 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analysis is shown (Fig. S3). Our phylogenetic analysis resolved 31 clades with strong support in this phylogroup. Eleven clades correspond to known species, and 20 are putatively new species (Fig. S3). The 11 known species include *T. alboumbilicum*, *T. baoshanense*, *T. caoi*, *T. elevatireticulatum*, *T. griseolivaceum*, *T. latisporum*, *T. panzhihuanense*, *T. parvomurphium*, *T. polymorphosporum*, *T. pseudosphaerosporum* and *T. thailandicum*. Of the 20 clades that are putatively new species, 12 clades are from China, and eight are from Japan (Kinoshita et al. 2011). Of the 12 Chinese clades, three are described as new species in this study, i.e., *T. huiliense*, *T. luyashanense*, *T. microcarpum*. The remaining nine clades are not described in this study although they may represent new species. We do not describe them because *Tuber* sp. CHN-2, *Tuber* sp. CHN-4, *Tuber* sp. CHN-5, *Tuber* sp. CHN-6 and *Tuber* sp. CHN-9 are known only from ECM, *Tuber* sp. CHN-1 only have a single ascoma, and ascomata of *Tuber* sp. CHN-3, *Tuber* sp. CHN-7 and *Tuber* sp. CHN-8 was not available for this study.

4. *Phylogroup Macrosporum*

Dataset V (ITS) comprised 22 sequences from the taxa of phylogroup *Macrosporum*, in which 14 sequences were isolated from Chinese *Tuber* species. The length of the dataset was 647 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analysis is shown (Fig. S4). This phylogenetic analysis resolved three clades of Chinese samples in this phylogroup, which respectively correspond to *T. calosporum*, *T. glabrum* and *T. sinomonosporum*. Two species are resolved from Japan (*Tuber* sp. 1KA-2010 and *Tuber* sp. 2KA-2010; Kinoshita et al. 2011) in this lineage, possibly native to the Japanese islands.

5. *Phylogroup Maculatum*

Dataset VI (ITS) comprised 116 sequences of the phylogroup *Maculatum*, in which 61 sequences were isolated from Chinese *Tuber* species. The length of the dataset was 438 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analysis is shown (Fig. S5). In this tree, the Chinese samples were resolved in six clades, representing six known species and one new species. The known species are *T. bomiense*, *T. hubeiense*, *T. maculatum*, *T. microverrucosum*, *T. pseudomagnatum* and *T. wumengense*. It is interesting that the sequence (JN870099) isolated from the type specimen of *T. microverrucosum* is resolved into the clade of *T. pseudomagnatum* and shared 97–99.7 % similarity in the ITS region, which suggests they may be conspecific although they have distinctive ascomatal colour differences (yellow for *T. pseudomagnatum* and brown for *T. microverrucosum*). However, we hesitate to synonymize the two species formally before more specimens become available for study. The new species is described here as *T. excelsum-reticulatum.*

6. *Phylogroup Melanosporum*

Dataset VII (ITS) comprised 210 sequences from the phylogroup *Melanosporum*, in which 198 sequences were isolated from Chinese *Tuber* species. The length of the dataset was 500 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analysis is shown (Fig. S6). This ITS-based phylogenetic analysis resolved seven clades of Chinese samples from this phylogroup. Of these, five clades are known species: *T. formosanum*, *T. pseudobrumale*, *T. pseudohimalayense*, *T. sinense* and *T. yigongense*. One of the remaining two clades is described here as *T. variabilisporum* sp. nov., and another is designated as *Tuber* sp. CHN-10. *Tuber* sp. CHN-10 is supported by two ITS sequences (JQ639006 from ECM, FM205595) downloaded from GenBank, they shared less than 95.8 % similarity in the ITS region with other *Tuber* species and group in a well-supported clade. We are not able to treat this taxon taxonomically until the specimens represented by these sequences become available or new collections are obtained.

7. *Phylogroup Puberulum*

Dataset VIII (ITS) comprised 217 sequences from the phylogroup *Puberulum*, in which 155 sequences were isolated from Chinese *Tuber* species. The length of the dataset was 561 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analysis is shown (Fig. S7). A total of 19 clades having strong bootstrap support were recognised from the Chinese samples in this phylogroup. Of them, 12 clades contain 15 previously described species. Including *T. anniae*, *T. huizeanum*, *T. jinshajiangense*, *T. lijiangense*, *T. liui*, *T. liyuanum*, *T. microsphaerosporum*, *T. shidianense*, *T. shii*, *T. sinoniveum*, *T. sinopuberulum*, *T. sinosphaerosporum*, *T. vesicoperidium*, *T. xuanhuaense* and *T. zhongdianense*. The other seven clades are putatively undescribed species as they do not correspond to any described species. Of these seven clades, four clades consist of sequences from ECM root tips or environmental sequences, and thus remain undescribed until ascomatal specimens are collected. Here we temporarily designate them as *Tuber* sp. CHN-11, *Tuber* sp. CHN-12, *Tuber* sp. CHN-13 and *Tuber* sp. CHN-15. DNA analyses revealed that they respectively shared less than 96.9 % (for *Tuber* sp. CHN-11), 95.2 % (for *Tuber* sp. CHN-12), 97.6 % (for *Tuber* sp. CHN-13) and 92.9 % (for *Tuber* sp. CHN-15) similarity in the ITS region to other *Tuber* species. The remaining three clades with ascomatal sequences, *Tuber* sp. CHN-14 is represented by three sequences (DQ898182, HXZE1762, HXZE1716). Unfortunately, all three specimens represented by these sequences have been lost or were unavailable, and consequently, we were not able to describe them. Only two clades are here described as *T. humilireticulatum* and *T. sinoborchii.* It is interesting that the three morphologically distinct species *T. microsphaerosporum*, *T. sinopuberulum* and *T. vesicoperidium* are all resolved in the *T. lijiangense* clade with strong support (Fig. S7), and DNA analysis revealed there was the 97–99 % ITS similarity between them. These results suggest that these four species are phylogenetically conspecific. However, since there are distinct morphological differences between each of them, and the name *T. lijiangense* has precedence, we prefer to treat them as the *T. lijiangense* complex until more samples become available for examination.

8. *Phylogroup Rufum*

Dataset IX (ITS) comprised 184 sequences from the phylogroup *Rufum*, in which 159 sequences are isolated from Chinese *Tuber* species. The length of the dataset was 493 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses

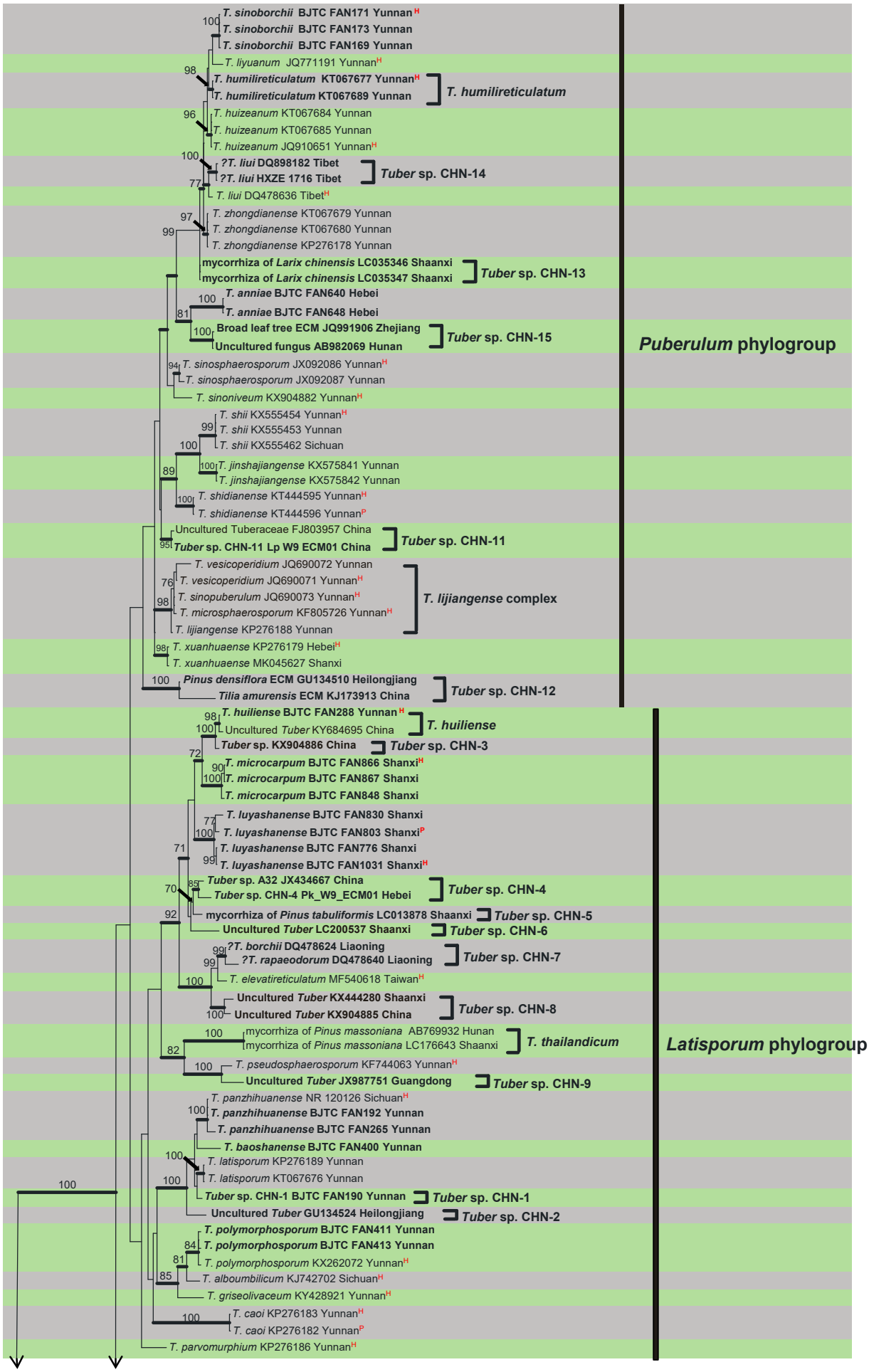

Fig. 2 Phylogeny of Chinese *Tuber* species inferred from the Dataset XI (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species are printed in **bold**.

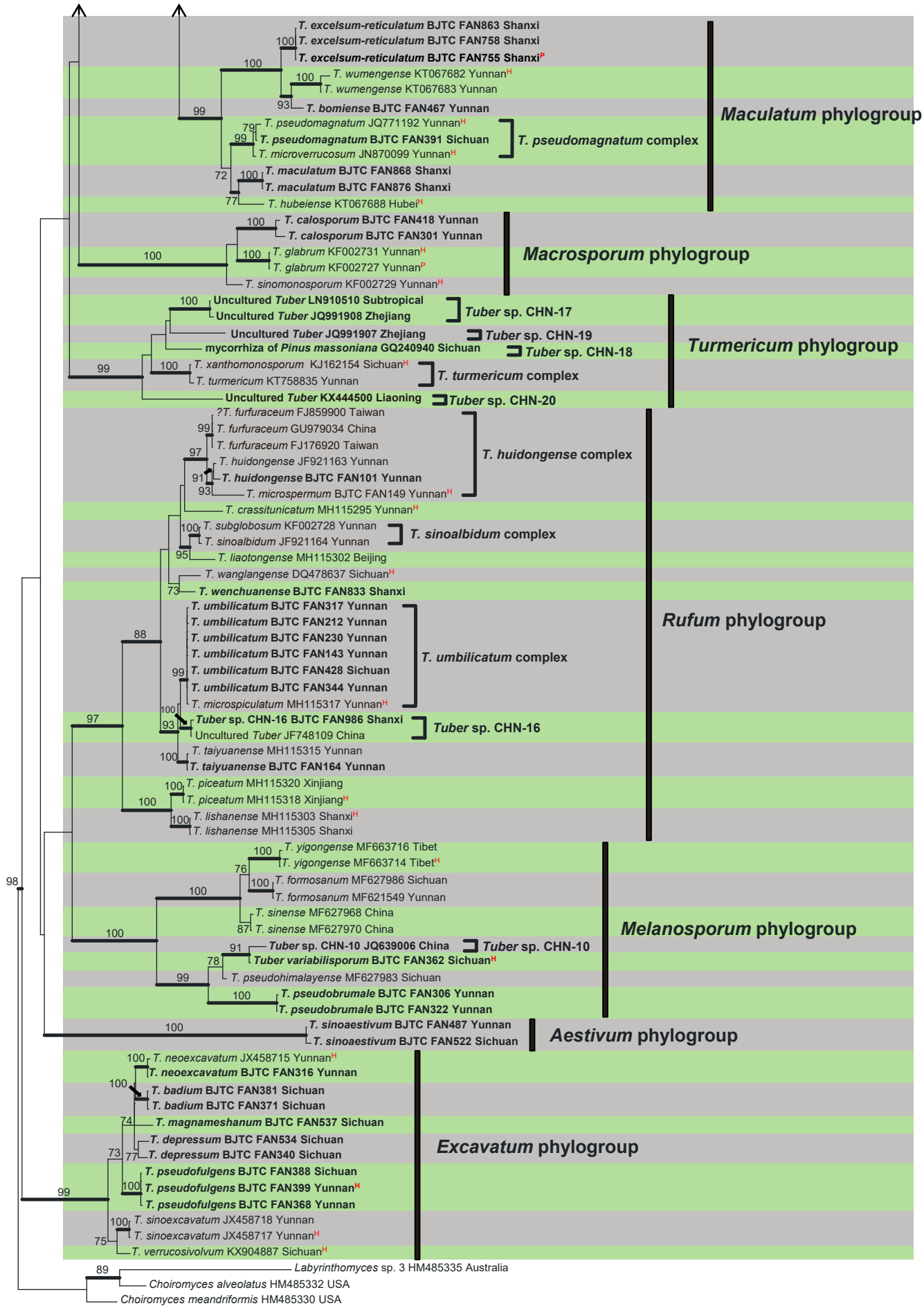
T. excelsum-reticulatum BJTC FAN863 Shanxi
T. excelsum-reticulatum BJTC FAN758 Shanxi
T. excelsum-reticulatum BJTC FAN755 Shanxi P
T. wumengense KT067682 Yunnan H
T. wumengense KT067683 Yunnan
T. bomiense BJTC FAN467 Yunnan
T. pseudomagnatum JQ771192 Yunnan H
T. pseudomagnatum BJTC FAN391 Sichuan
T. microverrucosum JN870099 Yunnan H
T. pseudomagnatum complex
T. maculatum BJTC FAN868 Shanxi
T. maculatum BJTC FAN876 Shanxi
T. hubeiense KT067688 Hubei H
Maculatum phylogroup
T. calosporum BJTC FAN418 Yunnan
T. calosporum BJTC FAN301 Yunnan
T. glabrum KF002731 Yunnan H
T. glabrum KF002727 Yunnan P
T. sinomonosporum KF002729 Yunnan H
Macrosporum phylogroup
Uncultured Tuber LN910510 Subtropical
Uncultured Tuber JQ991908 Zhejiang
Tuber sp. CHN-17
Uncultured Tuber JQ991907 Zhejiang
Tuber sp. CHN-19
mycorrhiza of Pinus massoniana GQ240940 Sichuan
Tuber sp. CHN-18
T. xanthomonosporum KJ162154 Sichuan H
T. turmericum KT758835 Yunnan
T. turmericum complex
Uncultured Tuber KX444500 Liaoning
Tuber sp. CHN-20
Turmericum phylogroup
?T. furfuraceum FJ859900 Taiwan
T. furfuraceum GU979034 China
T. furfuraceum FJ176920 Taiwan
T. huidongense JF921163 Yunnan
T. huidongense BJTC FAN101 Yunnan
T. microspermum BJTC FAN149 Yunnan H
T. huidongense complex
T. crassitunicatum MH115295 Yunnan H
T. subglobosum KF002728 Yunnan
T. sinoalbidum JF921164 Yunnan
T. sinoalbidum complex
T. liaotongense MH115302 Beijing
T. wanglangense DQ478637 Sichuan H
T. wenchuanense BJTC FAN833 Shanxi
T. umbilicatum BJTC FAN317 Yunnan
T. umbilicatum BJTC FAN212 Yunnan
T. umbilicatum BJTC FAN230 Yunnan
T. umbilicatum BJTC FAN143 Yunnan
T. umbilicatum BJTC FAN428 Sichuan
T. umbilicatum BJTC FAN344 Yunnan
T. microspiculatum MH115317 Yunnan H
T. umbilicatum complex
Tuber sp. CHN-16 BJTC FAN986 Shanxi
Uncultured Tuber JF748109 China
Tuber sp. CHN-16
T. taiyuanense MH115315 Yunnan
T. taiyuanense BJTC FAN164 Yunnan
T. piceatum MH115320 Xinjiang
T. piceatum MH115318 Xinjiang H
T. lishanense MH115303 Shanxi H
T. lishanense MH115305 Shanxi
Rufum phylogroup
T. yigongense MF663716 Tibet
T. yigongense MF663714 Tibet H
T. formosanum MF627986 Sichuan
T. formosanum MF621549 Yunnan
T. sinense MF627968 China
T. sinense MF627970 China
Tuber sp. CHN-10 JQ639006 China
Tuber sp. CHN-10
Tuber variabilisporum BJTC FAN362 Sichuan H
T. pseudohimalayense MF627983 Sichuan
T. pseudobrumale BJTC FAN306 Yunnan
T. pseudobrumale BJTC FAN322 Yunnan
Melanosporum phylogroup
T. sinoaestivum BJTC FAN487 Yunnan
T. sinoaestivum BJTC FAN522 Sichuan
Aestivum phylogroup
T. neoexcavatum JX458715 Yunnan H
T. neoexcavatum BJTC FAN316 Yunnan
T. badium BJTC FAN381 Sichuan
T. badium BJTC FAN371 Sichuan
T. magnameshanum BJTC FAN537 Sichuan
T. depressum BJTC FAN534 Sichuan
T. depressum BJTC FAN340 Sichuan
T. pseudofulgens BJTC FAN388 Sichuan
T. pseudofulgens BJTC FAN399 Yunnan H
T. pseudofulgens BJTC FAN368 Yunnan
T. sinoexcavatum JX458718 Yunnan
T. sinoexcavatum JX458717 Yunnan H
T. verrucosivolvum KX904887 Sichuan H
Excavatum phylogroup
Labyrinthomyces sp. 3 HM485335 Australia
Choiromyces alveolatus HM485332 USA
Choiromyces meandriformis HM485330 USA
0.1

Fig. 2 (cont.)

yielded identical tree topologies; only the tree inferred from the ML analysis is shown (Fig. S8). This phylogenetic analysis resolved 11 clades of Chinese taxa in this *Tuber* phylogroup. Of them, 10 clades are of previously described species: *T. crassitunicatum*, *T. furfuraceum*, *T. huidongense*, *T. liaotongense*, *T. lishanense*, *T. microspermum*, *T. microspiculatum*, *T. piceatum*, *T. subglobosum*, *T. sinoalbidum*, *T. taiyuanense*, *T. umbilicatum*, *T. wanglangense* and *T. wenchuanense*. The other one (designated as *Tuber* sp. CHN-16) did not correspond to any of the described species, suggesting that it is probably new to science. *Tuber* sp. CHN-16 contains one sequence from ascoma (BJTC FAN986) and an ECM-sequence of *Quercus liaotungense* from China, however, morphological examination revealed that the present ascomata lack mature asci and ascospores, it thus remains undescribed until additional ascomata are collected. Moreover, four species described in this lineage from China in previous studies, including *T. furfuraceum*, *T. microspermum*, *T. microspiculatum*, *T. sinoalbidum*, were not supported by our ITS-based analysis. Of them, both the sequences from the authentic specimens of *T. furfuraceum* and *T. microspermum* were grouped together with *T. huidongense*; *T. microspiculatum* clustered with *T. umbilicatum*; and *T. sinoalbidum* was resolved in the *T. subglobosum* clade. However, because all four species mentioned above are morphologically distinct from the species they are molecularly supported with, we treat these clades as species complexes until more specimens are available. They are the *T. huidongense* complex, *T. sinoalbidum* complex and *T. umbilicatum* complex.

9. *Phylogroup Turmericum*

Dataset X (ITS) comprised 20 sequences from the phylogroup *Turmericum*, in which 17 sequences were isolated from Chinese *Tuber* species. The length of the dataset was 462 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analysis is shown (Fig. S9). Our phylogenetic analysis resolved five clades of Chinese samples in this phylogroup. Of them, one clade corresponds to *T. turmericum*, and the other four clades are composed only of sequences from ECM root tips. The four clades of ECM sequence respectively clustered in their own independent clades with strong support and shared lower ITS similarity (less than 95 % to other *Tuber* species), thus they may represent undescribed species. Here we temporarily designate them respectively as *Tuber* sp. CHN-17, *Tuber* sp. CHN-18, *Tuber* sp. CHN-19 and *Tuber* sp. CHN-20. *Tuber xanthomonosporum*, newly described from southwestern China, was resolved in the *T. turmericum* clade, and shares more than 98 % ITS similarity with *T. turmericum*. We are not able to examine the specimens of this species in this study, so its relationship to *T. turmericum* needs further study.

The species diversity of Tuber in China

Dataset XI (ITS) comprised 161 sequences of Chinese collections, including all Chinese *Tuber* species determined based on the phylogenetic analyses for Dataset II–X outlined above. *Labyrinthomyces* sp. 3 (HM485335), *Choiromyces alveolatus* (HM485332) and *C. meandriformis* (HM485330) were selected as the outgroups. The length of the dataset was 431 bp after alignment and exclusion of poorly aligned sites. ML and BI analyses yielded identical tree topologies; only the tree inferred from the ML analyses is shown (Fig. 2). This phylogenetic analysis resolved all Chinese *Tuber* samples into nine clades and 82 phylogenetic species, which corresponded with the results of the multilocus phylogenetic analyses (Fig. 1) and ITS phylogenetic analyses for phylogroups in this study (Fig. 1, S1–S9).

Consequently, our phylogenetic analyses revealed 82 phylogenetic species in nine *Tuber*-phylogroups from the Chinese samplings in this study (Fig. 1, 2, S1–S9). Of them, 53 clades correspond well to 53 known species (or species complexes) (Liu 1985, Wang 1988, Tao et al. 1989, Tao & Liu 1990, Wang et al. 1998, Xu 1999, Chen & Liu 2007, Fan et al. 2011a, b, 2012a–e, 2013a, b, 2014, 2015, 2016a, b, Fan & Cao 2012, Zhang et al. 2012, Deng et al. 2014, Li et al. 2014, Huang et al. 2017, Wan et al. 2017a, b, Lin et al. 2018, Yan et al. 2018), including three species new to China, i.e., *T. anniae* (Fig. 2, S7), *T. maculatum* (Fig. 2, S5) and *T. thailandicum* (Fig. 2, S3). Of the remaining 29 clades, nine clades are proposed as new species that are described and illustrated in this paper (see Taxonomy), and 20 represent possibly undescribed species that are not treated taxonomically due to the absence or poor condition of ascomata (see hypothesized species of *Tuber* in China in Discussion).

TAXONOMY

Based on our phylogenetic, morphological and ecological data, nine new species and two new records of *Tuber* from China are described and illustrated here.

In the taxonomic descriptions of species, 'Q (L/I)' refers to the length/width ratio of ascospores in side-view; 'Qm' refers to the average Q of all ascospores ± standard deviation.

Tuber excelsum-reticulatum L. Fan & T. Li, *sp. nov.* — MycoBank MB 842427; Fig. 3a–c

Etymology. *excelsum-reticulatum*, referring to the prominently high reticulum of the ascospores.

Ascomata sub-globose, 0.5–1.5 cm diam, yellow-white, pale yellow-brown to yellow-brown, often with several superficial furrows, surface smooth, glabrous. *Odour* light, flavour not recorded. *Gleba* pale when young, dark brown at maturity, marbled with white vines. *Peridium* 320–430 µm thick, two-layered: outer layer 190–230 µm thick, pseudoparenchymatous, composed of sub-globose cells of 8.5–15(–20) µm wide, hyaline, slightly thick-walled, walls 2.5–5 µm thick; inner layer 130–200 µm thick, composed of interwoven hyphae 3–5 µm wide, hyaline, thin-walled, septate. *Asci* 1–3-spored, occasionally 4-spored, sub-globose to ellipsoid, thin-walled, sessile, 60–80 × 40–57.5 µm. *Ascospores* oblong-ellipsoid, ellipsoid to broadly ellipsoid, yellow-brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 40–50 × 27.5–30 µm, Q (L/I) = 1.33–1.82 (Qm = 1.54 ± 0.14) (n = 30), in 2-spored asci 27.5–37.5 × 20–25 µm, Q (L/I) = 1.22–1.50 (Qm = 1.37 ± 0.11) (n = 30), in 3-spored asci 20–32.5 × 15–25 µm, Q (L/I) = 1.10–1.50 (Qm = 1.28 ± 0.12) (n = 30), reticulum (4–)5–7 µm high, mostly 4–5(–6) meshes across spore width.

Habitat — Hypogeous, in soil under forest of *Picea* sp. and *Larix* sp. mixed with *Betula* sp.; ascoma occurring in autumn.

Distribution — Known only from Shanxi Province, northern China.

Specimens examined. China, Shanxi Province, Lvliang City, Jiaocheng County, Shenweigou, in soil under *Betula platyphylla*, alt. 1853 m, N37°51'30" E111°27'22", 8 Sept. 2017, *X.Y. Yan* (holotype BJTC FAN863, GenBank Acc. No.: ITS = OM265267, nrLSU = OM366224, *tef1-α* = OM649631, *rpb2* = OM584281, BJTC FAN864); ibid., in soil under *Betula platyphylla* of a coniferous and broad-leaf mixed forest, alt. 2003 m, N37°52'16" E111°30'25", 7 Sept. 2017, *X.Y. Yan* (BJTC FAN849), *K.B. Huang* (BJTC FAN851); ibid., in soil under *Larix principis-rupprechtii*, *K.B. Huang* (BJTC FAN853, BJTC FAN859, BJTC FAN860); ibid., in soil of *Larix principis-rupprechtii* of a coniferous and broad-leaf mixed forest; ibid., in soil under *Picea asperata* (*Picea asperata*) of a coniferous and broad-leaf mixed forest, alt. 2160 m, N37°53'5" E111°25'53", 6 Sept. 2017, *K.B. Huang* (BJTC FAN834, BJTC FAN835); ibid., in soil under *Larix principis-rupprechtii*, *Y.Y. Xu* (BJTC FAN871), *K.B. Huang* (BJTC FAN872); Shanxi Province, Xinzhou City, Nangoumiao, in soil under *Larix principis-rupprechtii* of a coniferous and broad-leaf mixed forest,

alt. 2184 m, N38°48'31" E111°58'19", 23 Aug. 2017, *K.B. Huang* (BJTC FAN755, BJTC FAN757, BJTC FAN758).

Notes — *Tuber excelsum-reticulatum* is characterised by its higher ornamentation of ascospores reaching 5–7 µm on average. The closely similar and related species in China is *T. wumengense* that also has a deep reticulum on its ascospores, but is differentiated from this new species by its long ellipsoid ascospores (Fan et al. 2016b). Moreover, the ascomata of *T. wumengense* are more brownish, while that of *T. excelsum-reticulatum* are more whitish.

This species is frequently encountered in Lvliangshan Mts under *Picea-Larix* trees, Shanxi Province, North China. Twenty-nine ITS ECM-sequences (three from Shaanxi Province, China, one from North America, 25 from Europe) match this species in our analysis (Fig. S5). The host includes *Alnus glutinosa*, *Epipactis fibri*, *Pinus contorta*, *Pinus tabuliformis*, *Populus alba*, *Populus simonii*, *Quercus ilex*, *Salix alba*, *Salix caprea*, *Salix fragilis* (Fig. S5). These indicate *T. excelsum-reticulatum* also occurs in Shaanxi Province, North China, Europe and North America, and has a wide range of hosts. *Tuber excelsum-reticulatum* shares less than 96.7 % similarity in the ITS region with other *Tuber* species.

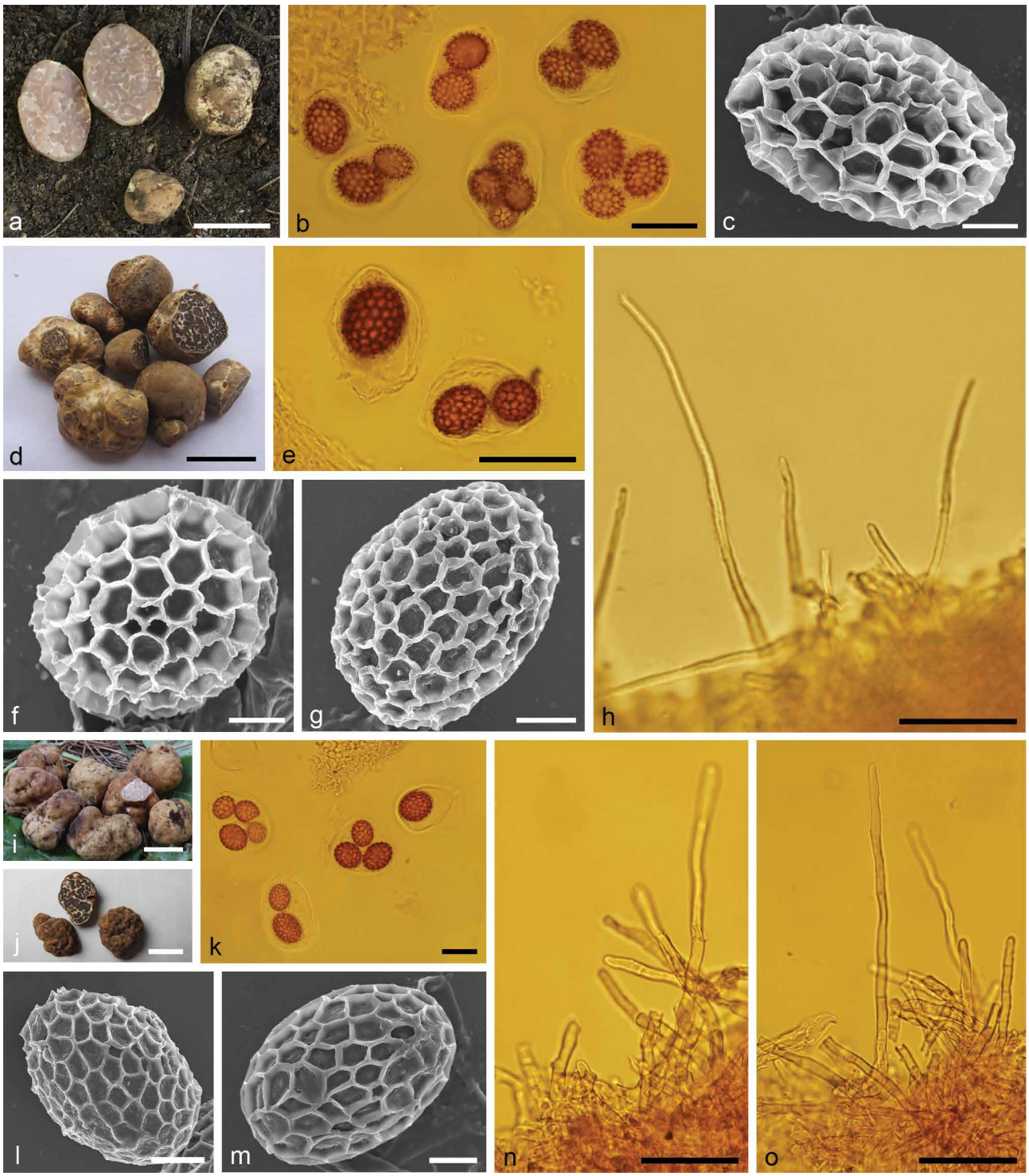

Fig. 3 a–c. *Tuber excelsum-reticulatum*. a. Ascomata (BJTC FAN863); b. asci and ascospores under LM; c. ascospores under SEM. — d–h. *Tuber huiliense*. d. Ascomata (BJTC FAN288); e. asci and ascospores under LM; f–g. ascospores under SEM; h. hairs. — i–o. *Tuber humilireticulatum*. i–j. Ascomata (BJTC FAN174); k. asci and ascospores under LM; l–m. ascospores under SEM; n–o. hairs. — Scale bars: a, d, i–j = 1 cm; b, e, h, k, n–o = 50 µm; c, f, g, l–m = 10 µm.

Tuber huiliense L. Fan, *sp. nov.* — MycoBank MB 842428; Fig. 3d–h

Etymology. *huiliense*, referring to the type locality.

Ascomata sub-globose, more or less irregular, 0.8–1.6 cm diam, yellow-white, yellow-brown to grey-brown, occasionally dark brown, typically with grey-olive tints when fresh, often with several superficial white furrows, surface smooth, puberulent when young, sometimes abundant in furrows. *Odour* light, flavour not recorded. *Gleba* pale when young, and changing to blackish at maturity, marbled with white vines. *Peridium* 175–225 µm thick, two-layered: outer layer 80–110 µm thick, pseudoparenchymatous, composed of sub-globose cells of 5–12.5(–15) µm wide, hyaline, thin-walled; inner layer 85–120 µm thick, composed of interwoven hyphae of 3–5 µm wide, hyaline, thin-walled, septate. *Hairs* arising from the outermost cells of peridium, hyphoid, abundant at furrows, tapered at apex, usually with 1 septa, 30–75 × 2.5–3 µm. *Asci* 1–2-spored, occasionally 3–4-spored, sub-globose to ellipsoid, thin-walled, sessile or with a very short stipe, 67.5–92.5 × 55–75 µm. *Ascospores* sub-globose to very broadly ellipsoid, brown to dark brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 35–47.5 × 25–36.25 µm, Q (L/I) = 1.14–1.46 (Qm = 1.28 ± 0.11) (n = 30), in 2-spored asci 25–40 × 22.5–27.5 µm, Q (L/I) = 1.10–1.32 (Qm = 1.17 ± 0.08) (n = 30), reticulum of ornamentation 2.5–5 µm high, mostly 4–6(–7) meshes across spore width.

Habitat — Hypogeous, in soil of a forest dominated by *Pinus armandii*; ascoma occurring in autumn.

Distribution — Known from Sichuan Province, southwestern China.

Specimens examined. China, Sichuan Province, Huili County, in soil of a forest dominated by *Pinus armandii*, 4 Sept. 2013, *J.Z. Cao* (holotype, BJTC FAN288, GenBank Acc. No.: ITS = OM256781, nrLSU = OM366181, *tef1-α* = OM649585, *rpb2* = OM584238).

Notes — The ascomatal appearance of *T. huiliense* is similar to *T. caoi* because its ascomata also has olive tints (Fan et al. 2016a), but the ascospores in *T. caoi* are globose rather than sub-globose to broadly ellipsoid in *T. huiliense*. *Tuber latisporum* is similar to *T. huiliense* in ascospore shape, but the colour of its ascospores is red-brown to reddish, meshes of ascospore ornament reticulum large (usually with 3–4 meshes/spore width) (Chen & Liu 2007). Analogously, the European *T. puberulum* is similar to *T. huiliense* in ascospore shape and peridium hairs. However, the gleba of *T. puberulum* is brown, never blackish, and its ascomata lack olive tints (Pegler et al. 1993). Phylogenetically, *T. puberulum* is nested in the *Puberulum* phylogroup, while *T. huiliense* is in the *Latisporum* phylogroup. *Tuber huiliense* is closely related to *T. luyashanense* and *T. microcarpum* (Fig. 2, S3), but both *T. luyashanense* and *T. microcarpum* have white ascomata completely lacking olive tints. A sequence (KY684695) from ECM root tip of *Quercus* sp. in Shaanxi Province matches this species (Fig. S3). That indicates this species may have a wide host range. *Tuber huiliense* shares less than 96.7 % similarity in the ITS region with other *Tuber* species.

Tuber humilireticulatum L. Fan, *sp. nov.* — MycoBank MB 842429; Fig. 3i–o

Etymology. *humilireticulatum*, referring to the prominently low reticulum on the ornaments of the ascospores.

Ascomata sub-globose, 0.7–2 cm diam, yellow-white, pale yellow-brown to yellow-brown, surface smooth, glabrous. *Odour* light, flavour not recorded. *Gleba* pale when young, brown to dark brown at maturity, marbled with white vines. *Peridium* 240–340 µm thick, two-layered: outer layer 115–220 µm thick, pseudoparenchymatous, composed of sub-globose cells of 7–15(–20) µm wide, hyaline, thin-walled; inner layer 120–170 µm thick, composed of interwoven hyphae 3–5(–7.5) µm wide, hyaline, thin-walled, septate. *Hairs* arising from the outermost cells of the peridium, hyphoid, 25–215 × 2.5–5 µm, walls thin to slightly thickened, usually with 1–4-septa. *Asci* 1–3-spored, occasionally 4-spored, sub-globose to ellipsoid, slightly thick-walled, walls 2–3 µm, sessile, 87.5–105 × 60–75 µm. *Ascospores* broadly ellipsoid, yellow-brown to brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 42.5–57.5 × 32.5–50 µm, Q (L/I) = 1.15–1.50 (Qm = 1.35 ± 0.11) (n = 30), in 2-spored asci 30–47.5 × 25–35 µm, Q (L/I) = 1.15–1.46 (Qm =1.34 ± 0.10) (n = 30), in 3-spored asci 32.5–40 × 25–30 µm, Q (L/I) = 1.12–1.33 (Qm = 1.27 ± 0.07) (n = 30), reticulum 1.5–2.5 µm high, mostly (4–)5–6 meshes across spore width.

Habitat — Hypogeous, in soil under *Pinus* sp.; ascoma occurring in winter.

Distribution — Known from Yunnan Province, southwestern China.

Specimens examined. China, Yunnan Province, Kunming city, in soil under *Pinus* sp., 28 Nov. 2011, *S.P. Li* (holotype, BJTC FAN174, GenBank Acc. No.: ITS = KT067677, nrLSU = OM366168, *tef1-α* = KT067724, *rpb2* = OM584224); ibid., *S.P. Li* (BJTC FAN175); ibid., *J.Z. Cao* (BJTC FAN189); Yunnan Province, Huize County, in soil under *Pinus* sp., 12 Nov. 2015, *J.Z. Cao* (BJTC FAN485, BJTC FAN486).

Notes — *Tuber humilireticulatum* is recognised by its lower (< 2.5 µm high) reticulum on the surface of ascospores. *Tuber lijiangense* is reported with extremely low ascospore ornamentations (Fan et al. 2011a), but the shape of its ascospores is globose or sub-globose. *Tuber zhongdianense* is also described with the same pattern of ascospore ornamentation, but its ascomata are covered with two types of hairs (He et al. 2004).

This new species is morphologically difficult to distinguish from others in the *Puberulum* phylogroup, especially *T. borchii*, *T. huizeanum*, *T. sinoborchii* and *T. zhongdianense*. However, phylogenetic analysis distinguishes this species (Fig. 2, S7). DNA analysis reveals it shares less than 97 % similarity in the ITS region with other *Tuber* species.

Tuber luyashanense L. Fan, *sp. nov.* — MycoBank MB 842430; Fig. 4a–f

Etymology. *luyashanense*, referring to type locality.

Ascomata sub-globose, 0.6–2.3 cm diam, pure white, whitish or yellow whitish, puberulent, surface smooth or with a few superficial furrows. *Odour* light, flavour not recorded. *Gleba* pale when young, and changing to dark grey to dark brown at maturity, marbled with white vines. *Peridium* 150–200 µm thick, two-layered: outer layer 65–120 µm thick, pseudoparenchymatous, composed of sub-globose cells of 8–25 µm diam, hyaline, thin walled; inner layer 65–110 µm thick, composed of interwoven hyphae of 3–5(–7.5) µm wide, often with swollen cells of 5–15 µm, hyaline, thin-walled, septate. *Hairs* arising from the outermost cells of peridium, seta-like, abundant when young, tapered at apex, hyaline, slightly thickened walls, usually with 1–3 septa, reaching 37.5–150 × 2.5–5 µm. *Asci* 1–2-spored, occasionally 3-spored, sub-globose to ellipsoid, thin-walled, sessile, 80–105 × 72.5–85 µm. *Ascospores* very broadly ellipsoid to sub-globose, brown to dark brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 42.5–50 × 35–42.5 µm, Q (L/I) = 1.09–1.36 (Qm = 1.22 ± 0.09) (n = 30), in 2-spored asci 32.5–41.25 × 27.5–37.5 µm, Q (L/I) = 1.07–1.23 (Qm = 1.15 ± 0.05) (n = 30), reticulum 2.5–4 µm high, mostly (4–)5–7(–8) meshes across spore width.

Habitat — Hypogeous, in soil under forest of *Picea* sp. and *Larix* sp. mixed with *Betula* sp.; ascoma occurring in autumn.

Distribution — Known only from Shanxi Province, northern China.

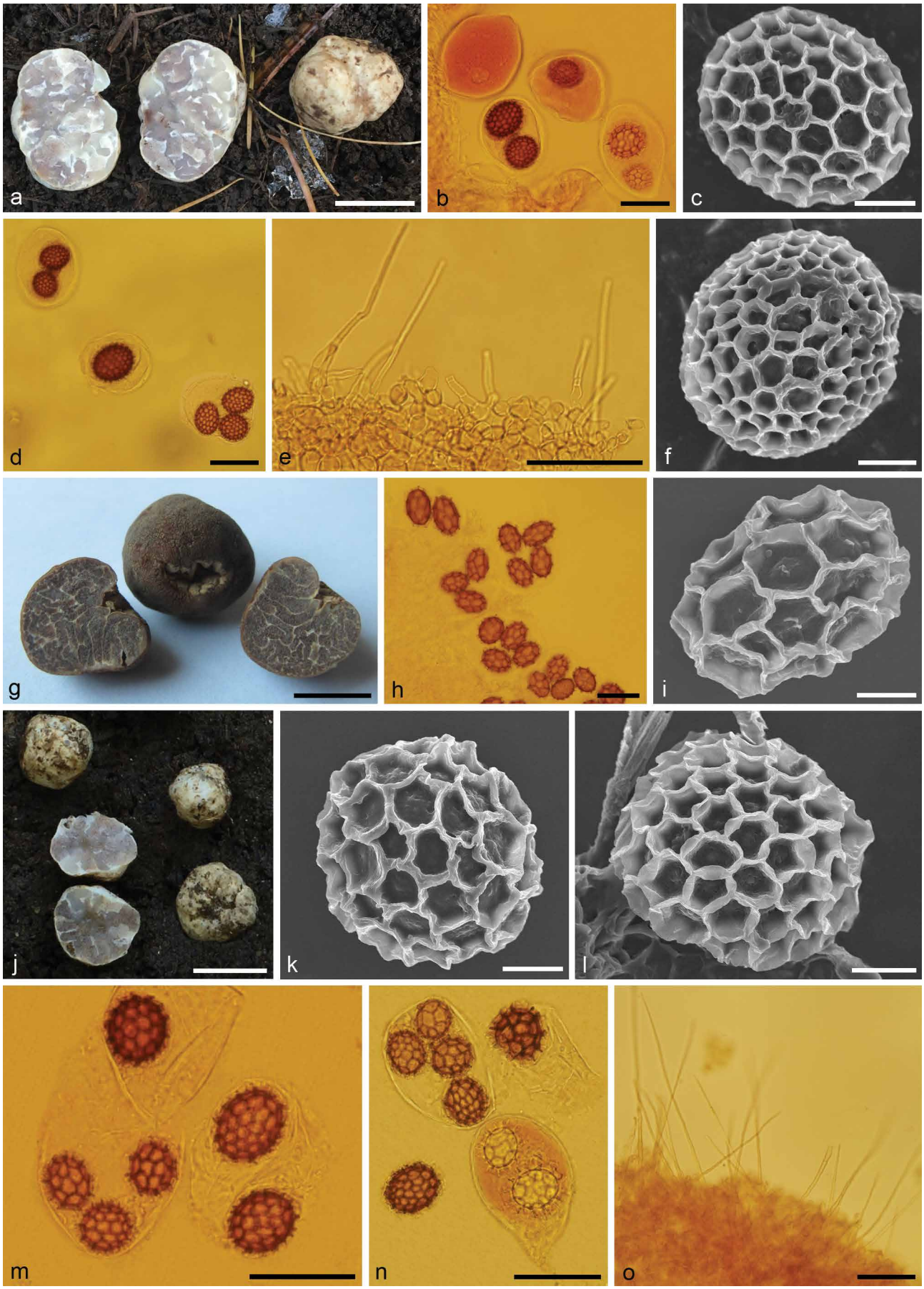

Fig. 4 a–f. *Tuber luyashanense*. a. Ascomata (BJTC FAN1031); b, d. asci and ascospores under LM; c, f. ascospores under SEM; e. hairs. — g–i. *Tuber magnameshanum*. g. Ascomata (BJTC FAN537); h. asci and ascospores under LM; i. ascospores under SEM. — j–o. *Tuber microcarpum*. j. Ascomata (BJTC FAN866); k–l. ascospores under SEM; m–n. asci and ascospores under LM; o. hairs. — Scale bars: a, g = 1 cm; j = 0.5 cm; b, d–e, h, m–o = 50 µm; c, f, i, k–l = 10 µm.

Specimens examined. China, Shanxi Province, Ningwu Cunty, Luyashan Mt., in soil under a plantation of *Larix principis-rupprechtii*, alt. 2200 m, 12 Oct. 2017, *X.Y. Yan* (holotype, BJTC FAN1031, GenBank Acc. No.: ITS = OM256769, nrLSU = OM366157, *tef1-α* = OM649637); Shanxi Province, Xinzhou city, Qiuqiangou, in soil under *Larix principis-rupprechtii* of a coniferous and broad-leaf mixed forest, alt. 2099 m, N38°51'22" E112°0'31", 25 Aug. 2017, *K.B. Huang* (BJTC FAN776); Xinzhou city, Dashidong, in soil under *Picea asperata*, alt. 2200 m, N38°24'7" E112°08'3", 24 Aug. 2017, *X.Y. Yan* (BJTC FAN783); ibid., in soil under *Larix principis-rupprechtii*, *Y.Y. Xu* (BJTC FAN784); Lvliang City, Jiaocheng County, Pangquangou, alt. 2160 m, N37°53'5" E111°25'5", 6 Sept. 2017, *Y.Y. Xu* (BJTC FAN801, BJTC FAN802, paratype BJTC FAN803, BJTC FAN804); ibid., in soil under *Betula platyphylla*, *X.Y. Yan* (BJTC FAN810, BJTC FAN811, BJTC FAN813); ibid., in soil under *Larix principis-rupprechtii*, *X.Y. Yan* (BJTC FAN814, BJTC FAN815, BJTC FAN816); ibid., *T Li* (BJTC FAN822, BJTC FAN823, BJTC FAN824, BJTC FAN825, BJTC FAN826); ibid., alt. 2160 m, N37°53'5" E111°25'5", 6 Sept. 2017, *T Li* (BJTC FAN829, BJTC FAN830, BJTC FAN831); ibid., in soil under *Picea asperata*, alt. 2160 m, N37°53'5" E111°25'5", 6 Sept. 2017, *K.B. Huang* (BJTC FAN837, BJTC FAN838, BJTC FAN839, BJTC FAN840, BJTC FAN844, BJTC FAN845).

Notes — *Tuber luyashanense* is characterised by its white ascomata with puberulent surface and the very broadly ellipsoid ascospores with reticulum of (4–)5–7(–8) meshes across spore width. *Tuber latisporum*, which is very common in Southwest China, looks similar to this new species when young, but its white ascomata are usually stained red-brown and change to grey brown at maturity according to our observations. Also, its gleba is blackish and ascospores are typically red-brown to reddish (Chen & Liu 2007). *Tuber microcarpum*, found in the same region, is closely related and similar to *T. luyashanense* (Fig. 2, S3), but *T. microcarpum* is distinguished by its small ascomata, seta-like and hyphoid hairs and spore ornamentations with sparse meshes (4–5/spore width). Three previous sequences extracted from the specimens of north-eastern China, respectively labelled as *T. borchii* (DQ478623), *T. puberulum* (DQ478638) and *T. rapaeodorum* (DQ478641), match *T. luyashanense* in this study (Fig. S3), extending its distribution from north region to north-eastern region in China. This new species shares less than 92.8 % similarity in the ITS region with other *Tuber* species.

Tuber magnameshanum L. Fan, *sp. nov.* — MycoBank MB 842431; Fig. 4g–i

Etymology. *magnameshanum*, referring to the large meshes of spore ornamentations.

Ascomata sub-globose, 1.2–1.7 cm diam, red-brown to brown, surface minutely verrucose, glabrous, with basal cavity. *Odour* sharp and smelly, flavour not recorded. *Gleba* pale when young, dark brown to blackish at maturity, marbled with white vines. *Peridium* 200–300 µm thick, two-layered: outer layer 100–150 µm thick, pseudoparenchymatous, composed of sub-globose cells of 5–12.5 µm diam, hyaline, thin-walled; inner layer 100–180 µm thick, composed of interwoven hyphae of 2.5–7.5 µm wide, hyaline, thin-walled, septate. *Asci* 1–4-spored, sub-globose to ellipsoid, more or less thick-walled, walls 2.5–7.65 µm thick, with a stipe of 12.5–31 µm long, 87–115 × 67–87.5 µm. *Ascospores* broadly ellipsoid, ellipsoid to oblong-ellipsoid, yellow-brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 42.5–50 × 27.5–30 µm, Q (L/l) = 1.55–1.73 (Qm = 1.63 ± 0.06) (n = 30), in 2-spored asci 33.5–42.5 × 22.5–30 µm, Q (L/l) = 1.22–1.89 (Qm = 1.41 ± 0.19) (n = 30), in 3-spored asci 27.5–47.5 × 22.5–27.5 µm, Q (L/l) = 1.20–1.73 (Qm = 1.47 ± 0.17) (n = 30), in 4-spored asci 32.5–37.5 × 22.5–27.5 µm, Q (L/l) = 1.27–1.50 (Qm = 1.39 ± 0.08) (n = 30), reticulum 3.5–6 µm high, mostly 2–3 meshes across spore width.

Habitat — Hypogeous, in soil under *Pinus armandii* forest; ascoma occurring in winter.

Distribution — Known from Sichuan Province, southwestern China.

Specimens examined. China, Sichuan Province, Huidong County, in soil under a forest of *Pinus armandii*, 4 Jan. 2016, *J.Z. Cao* (holotype, BJTC FAN537, GenBank Acc. No.: ITS = OM256767, nrLSU = OM366212, *tef1-α* = OM649617).

Notes — *Tuber magnameshanum* is characterised by its brown smooth ascomata, oblong ascospores and spore ornamentations with large meshes. This species is composed by a strongly supported clade of three Chinese *Tuber-excavatum* phylogroup species in the phylogenetic analyses (Fig. 2, S2), including *T. badium*, *T. depressum* and *T. neoexcavatum*, but the latter three species can be separated from *T. magnameshanum* by their ascomata with verrucose warts and yellow-brown or brown gleba. Also, the ascospore number per ascus is 2–4-spored in *T. neoexcavatum*, and the ascospore reticulate ornamentations are higher (2–9 µm for *T. depressum* and 2–13 µm for *T. badium*; Wan et al. 2017b). *Tuber magnameshanum* shares less than 94.2 % similarity in the ITS region with other *Tuber* species.

Tuber microcarpum L. Fan & T. Li, *sp. nov.* — MycoBank MB 842432; Fig. 4j–o

Etymology. *microcarpum*, referring to the small size of the ascomata.

Ascomata globose to sub-globose, 0.5–0.8 cm diam, white, whitish or yellow whitish, puberulent when young, slightly convolute or with several superficial furrows. *Odour* light, flavour not recorded. *Gleba* pale when young, and changing to dark grey to dark brown at maturity, marbled with white vines. *Peridium* 50–100 µm thick, single-layered, composed of interwoven hyphae of 3–5(–7.5) µm wide, hyaline, thin-walled, septate, and mixed with sub-globose cells of 5–12.5(–15) µm. *Hairs* arising from the outermost cells of peridium, seta-like, abundant when young, 60–215 × 2.5–5 µm, tapered at apex, walls slightly thickened, usually with 1–4-septa. *Asci* 1–2(–3)-spored, occasionally 4-spored, sub-globose to elliptic, thin-walled, sessile, 72.5–90 × 57.5–80 µm. *Ascospores* very broadly ellipsoid to sub-globose, brown to dark brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 40–47.5 × 35–42.5 µm, Q (L/l) = 1.06–1.14 (Qm = 1.10 ± 0.03) (n = 30), in 2(–3)-spored asci 27.5–37.5 × 25–35 µm, Q (L/l) = 1.07–1.20 (Qm = 1.12 ± 0.04) (n = 30), reticulum 2.5–3 µm high, mostly (3–)4–5 meshes across spore width.

Habitat — Hypogeous, in soil under a forest dominated by *Picea* sp. and/or *Larix* sp.; ascoma occurring in autumn and winter.

Distribution — Known from northern China, Shanxi Province.

Specimens examined. China, Shanxi Province, Guandishan Mt., in soil under forest dominated by *Picea* sp., alt. 1820 m, 8 Sept. 2017, *Y.Y. Xu* (holotype, BJTC FAN866, GenBank Acc. No.: ITS = OM256770, nrLSU = OM366225, *tef1-α* = OM649632, *rpb2* = OM584282); Shanxi Province, Lvliang City, Jiaocheng County, Shenweigou, in soil under *Picea* sp., alt. 2003 m, N37°52'16" E111°30'25", 7 Sept. 2017, *X.Y. Yan* (BJTC FAN848); Lvliang City, Jiaocheng County, Pangquangou, in soil under *Picea* sp., alt. 1853 m, N37°51'3" E111°27'22", 8 Sept. 2017, *Y.Y. Xu* (BJTC FAN865, BJTC FAN867); ibid., in soil under *Larix* sp., *T. Li* (BJTC FAN880); ibid., *X.Y. Yan* (BJTC FAN881).

Notes — *Tuber microcarpum* is characterised by its small, puberulent, white to whitish ascomata, sub-globose to broadly ellipsoid ascospores, and spore ornamentations with a small number (4–5) of meshes. *Tuber luyashanense* is closely related and similar to *T. microcarpum* (Fig. 2, S3), and both occur in the same geographic region. The main differences between them are the following: i) the size of ascomata of *T. luyashanense* are larger than 10 mm diam on average and reach to 23 mm, but all the individuals of *T. microcarpum* examined in

this study are less than 8 mm diam; ii) the peridium is thick in *T. luyashanense*, ranging from 150–200 µm, but clearly thinner in *T. microcarpum*, ranging from 50–100 µm; iii) the reticulum of ascospores is mostly 5–7 meshes/width in *T. luyashanense* but 4–5 meshes/width in *T. microcarpum.*

All the ascomatal specimens of *T. microcarpum* are collected from the type locality, where it is frequently encountered. However, one ITS sequence (Pk_W6_ECM06) isolated from the ectomycorrhizal root tip of *Populus koreana* from Hebei Province matches this species (Fig. S3), extending the known distribution of *T. microcarpum* to that area, too. *Tuber microcarpum* shares less than 91.4 % similarity in the ITS region with other *Tuber* species.

Tuber pseudofulgens L. Fan & X.Y. Sang, *sp. nov.* — MycoBank MB 842433; Fig. 5a–e

Etymology. *pseudofulgens*, referring to its similarity with *Tuber fulgens*.

Ascomata sub-globose, 0.9–1.6 cm diam, brown, with several superficial furrows, surface polylateral verrucose, glabrous, with basal cavity. *Odour* light, flavour not recorded. *Gleba* pale when young, and changing to dark brown at maturity, marbled with white vines. *Peridium* 300–400 µm thick, two-layered: outer layer 150–200 µm thick, pseudoparenchymatous, composed of sub-globose cells of 7.5–12.5 µm wide, hyaline, thin-walled; inner layer 180–200 µm thick, composed of interwoven hyphae of 3–6.5 µm wide, hyaline, thin-walled, septate. *Asci* 1–4-spored, sub-globose to ellipsoid, thin-walled, sessile or with a short stipe, 65–87.5 × 52.5–67.5 µm. *Ascospores* ellipsoid, broadly ellipsoid, yellow-brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 30–42.5 × 22.5–27.5 µm, Q (L/I) = 1.20–1.70 (Qm = 1.44 ± 0.19) (n = 30), in 2-spored asci 27.5–37.5 × 22.5–28.75 µm, Q (L/I) =1.18–1.40 (Qm = 1.28 ± 0.08) (n = 30), in 3-spored asci 27.5–32.5 × 22.5–25 µm, Q (L/I) = 1.22–1.33 (Qm = 1.29 ± 0.05) (n = 30), in 4-spored asci 22.5–30 × 20–22.5 µm, Q (L/I) = 1.10–1.50 (Qm = 1.24 ± 0.16) (n = 30), reticulum 3–5 µm high, mostly 2–3 meshes across spore width.

Habitat — Hypogeous, in soil of a mixed forest dominated by *Pinus* spp., ascoma occurring in autumn and winter.

Distribution — Known from southwestern China.

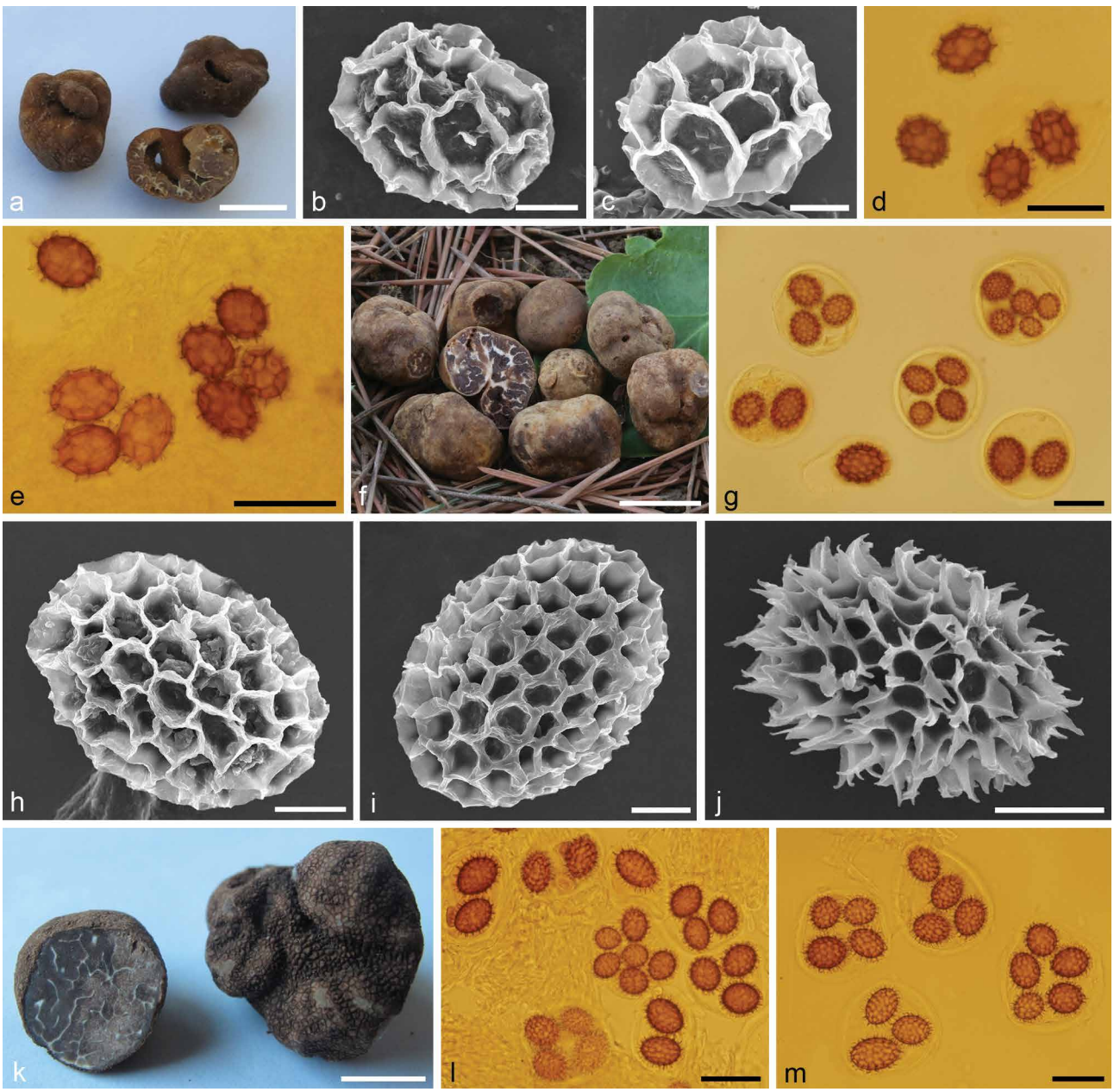

Fig. 5 a–e. *Tuber pseudofulgens*. a. Ascomata (BJTC FAN399); b–c. ascospores under SEM; d–e. asci and ascospores under LM. — f–i. *Tuber sinoborchii*. f. Ascomata (BJTC FAN171); g. asci and ascospores under LM; h–i. ascospores under SEM. — j–m. *Tuber variabilisporum*. j. Ascospores under SEM; k. ascomata (BJTC FAN362); l–m. asci and ascospores under LM. — Scale bars: a, f, k = 1 cm; d–e, g, l–m = 50 µm; b–c, h–j = 10 µm.

Specimens examined. China, Yunnan Province, Baoshan City, in soil of a forest dominated by *Pinus* sp., 27 Nov. 2014, *J.Z. Cao* (holotype, BJTC FAN399, GenBank Acc. No.: ITS = OM256757, nrLSU = OM366196, *tef1-α* = OM649601, *rpb2* = OM584259); Yunnan Province, Kunming City, Dongchuanqu, in soil under *Pinus* sp., 22 Sept. 2014, *J.Z. Cao* (BJTC FAN368); Sichuan Province, Huidong, in soil under *Pinus* sp., Sept. 2014, *J.Z. Cao* (BJTC FAN388).

Notes — *Tuber pseudofulgens* is characterised by its small brown ascomata with a central cavity opening by a basal hole. *Tuber fulgens*, a European native species, is similar to *T. pseudofulgens* in its basal hole leading to the inner cavity, but differs by its bright yellow-brown ascomata and globose to sub-globose ascospores (Riousset et al. 2001). This small brown species is possibly rare. There are only three specimens collected since 2014. It shares less than 91.2 % similarity in the ITS region with other *Tuber* species.

Tuber sinoborchii T. Li & L. Fan, *sp. nov.* — MycoBank MB 842434; Fig. 5f–i

Etymology. *sinoborchii*, referring to its similarity with *Tuber borchii* in ascomata and ascospores.

Ascomata sub-globose, 0.8–1.2 cm diam, yellow-whitish, yellow-brown to brown, surface smooth, glabrous. *Odour* light, flavour not recorded. *Gleba* pale brown when young, brown to black-brown at maturity, marbled with white vines. *Peridium* 200–250 µm thick, two-layered: outer layer 70–100 µm thick, pseudoparenchymatous, composed of sub-globose cells of 5–12.5 µm wide, hyaline, thin-walled; inner layer 110–170 µm thick, composed of interwoven hyphae of 3–5 µm wide, hyaline, thin-walled, septate. *Asci* 1–4-spored, occasionally 5-spored, sub-globose to ellipsoid, more or less thick-walled, walls 2.5–5 µm, sessile, 82.5–117.5 × 62.5–80 µm. *Ascospores* ellipsoid, broadly ellipsoid, sub-globose, yellow-brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 40–57.5 × 27.5–40 µm, Q (L/I) = 1.22–1.45 (Qm = 1.33 ± 0.09) (n = 30), in 2-spored asci 30–47.5 × 25–37.5 µm, Q (L/I) = 1.09–1.58 (Qm = 1.27 ± 0.14) (n = 30), in 3-spored asci 27.5–40 × 25–32.5 µm, Q (L/I) = 1.10–1.36 (Qm = 1.22 ± 0.09) (n = 30), in 4-spored asci 25–37.5 × 22.5–30 µm, Q (L/I) = 1.09–1.44 (Qm = 1.21 ± 0.12) (n = 30), reticulum 2.5–5 µm high, mostly 4–5 meshes across spore width.

Habitat — Hypogeous, in soil of pine forest dominated by *Pinus armandii*; ascoma occurring in winter.

Distribution — Known from southwestern China, Yunnan Province.

Specimens examined. China, Yunnan Province, Huize County, in soil under mixed forest with *Pinus armandii* as dominant, 12 Nov. 2011, *S.P. Li* (holotype BJTC FAN171, GenBank Acc. No.: ITS = OM286802, nrLSU = OM366167, *tef1-α* = OM649573, *rpb2* = OM584223); Yunnan Province, Huize County, in soil under mixed forest with *Pinus armandii* as dominant, 12 Nov. 2011, *S.P. Li* (BJTC FAN169, BJTC FAN173).

Notes — *Tuber sinoborchii* is similar to the European *T. borchii* in ascomatal appearance and ascospores, but differs by its glabrous ascoma surface (Pegler et al. 1993, Riousset et al. 2001). *Tuber sinoborchii* is very similar to several species in morphology, including *T. liui*, *T. liyuanum*, *T. huizeanum*, *T. zhongdianense*. They are difficult to separate by morphological evidence alone, and even may be recognized as a morphological species complex of *T. liui*. DNA analysis is needed for their accurate examination. *Tuber borchii* is previously reported from China (Wang 1988, Song 2005, Chen 2007), but a recent study (Fan et al. 2016b) reveals that no DNA data support the occurrence of this European species in China. This new species shares less than 97.2 % similarity in the ITS region with other *Tuber* species.

Tuber variabilisporum L. Fan & T. Li, *sp. nov.* — MycoBank MB 842435; Fig. 5j–m

Etymology. *variabilisporum*, referring to the variation of ascospore size.

Ascomata sub-globose, 1.2–2.7 cm diam, dark brown to black-brown, verrucose, sometimes with several superficial furrows. *Odour* light, flavour not recorded. *Gleba* brown when young, dark brown to black-brown at maturity, marbled with sparsely white vines. *Peridium* 190–340 µm thick, two-layered: outer layer 120–280 µm thick, pseudoparenchymatous, composed of sub-globose cells of 7–15 µm wide, hyaline, thin-walled; inner layer 60–90 µm thick, composed of interwoven hyphae of 3–7.5 µm wide, hyaline, thin-walled, septate. *Asci* 1–5-spored, occasionally 6-spored, sub-globose to ellipsoid, more or less thick-walled, walls 2–3 µm, sessile, 60–72.5 × 52.5–62.5 µm. *Ascospores* ellipsoid, broadly ellipsoid, sub-globose, yellow-brown at maturity, reticulate, excluding ornamentations, in 1-spored asci 30–37.5 × 21.75–27.5 µm, Q (L/I) = 1.36–1.44 (Qm = 1.41 ± 0.03) (n = 30), in 2-spored asci 27.5–32.5 × 20–23.5 µm, Q (L/I) = 1.20–1.41 (Qm = 1.35 ± 0.08) (n = 30), in 3-spored asci 22.5–32.5 × 18.75–21.25 µm, Q (L/I) = 1.07–1.43 (Qm = 1.29 ± 0.19) (n = 30), in 4-spored asci 17.5–25.5 × 16.5–20 µm, Q (L/I) = 1.06–1.33 (Qm = 1.19 ± 0.09) (n = 30), in 5-spored asci 16.5–22.5 × 13.25–17.5 µm, Q (L/I) = 1.14–1.42 (Qm = 1.25 ± 0.08) (n = 30), spino-reticulum 3–5 µm high, mostly 4–5 meshes across spore width.

Habitat — Hypogeous, in soil of pine forest containing *Pinus armandii*; ascoma occurring in winter.

Distribution — Known from southwestern China.

Specimens examined. China, Sichuan Province, Panzhihua City, in soil under *Pinus armandii*, 16 Jan. 2014, *J.Z. Cao* (holotype BJTC FAN362, GenBank Acc. No.: ITS = OM287845, nrLSU = OM366190, *tef1-α* = OM649595, *rpb2* = OM584253; BJTC FAN330).

Notes — *Tuber variabilisporum* is characterised by its verrucose ascomata without basal cavity, and yellow-brown ascospores with spino-reticulate ornamentations. Phylogenetically, this new species is placed in *Melanosporum* phylogroup and closely related to *T. pseudohimalayense* (Fig. 1, 2, S6). Morphologically *T. pseudohimalayense*, *T. pseudobrumale* and *T. indicum* complex (including *T. formosanum*, *T. indicum*, *T. yigongense* and *T. sinense*) may be confused with this new species. Of them, both *T. pseudohimalayense* and *T. pseudobrumale* are separated by their ascomata with distinctly basal cavity (Wang et al. 1998, Li et al. 2014). *Tuber indicum* complex differs from this species by their ascomata covered with pyramidal warts and their red-brown ascospores with isolated spines or irregularly reticulate ornamentations (Tao et al. 1989, Hu 1992, Fan et al. 2018). *Tuber variabilisporum* shares less than 95.5 % similarity in the ITS region with other *Tuber* species. This species seems not uncommon. Only two specimens from Sichuan are available. Other sequences are downloaded from GenBank. This species is known from southwestern China (Sichuan and Yunnan provinces).

Tuber anniae W. Colgan & Trappe, Mycotaxon 64: 438. 1997 — Fig. 6a–c

Habitat — Hypogeous, in soil under *Larix principis-rupprechtii*; ascoma occurring in autumn.

Distribution — Known from the northern area in China.

Specimens examined. China, Hebei Province, Xinglong County, Wuling Mts, in soil under *Larix principis-rupprechtii*, alt. 1801 m, N40°35' E117°28', 5 Oct. 2016, *T. Li* (BJTC FAN640); ibid., *X.Y. Yan* (BJTC FAN644, BJTC FAN648); Shanxi Province, Jiaocheng County, Guandishan Mts, in soil under *Larix principis-rupprechtii*, alt. 1879 m, N37°51' E111°27', 6 Sept. 2017, *X.Y. Yan* (BJTC FAN818, BJTC FAN173); ibid., 8 Sept. 2017, *K.B. Huang* (BJTC FAN875).

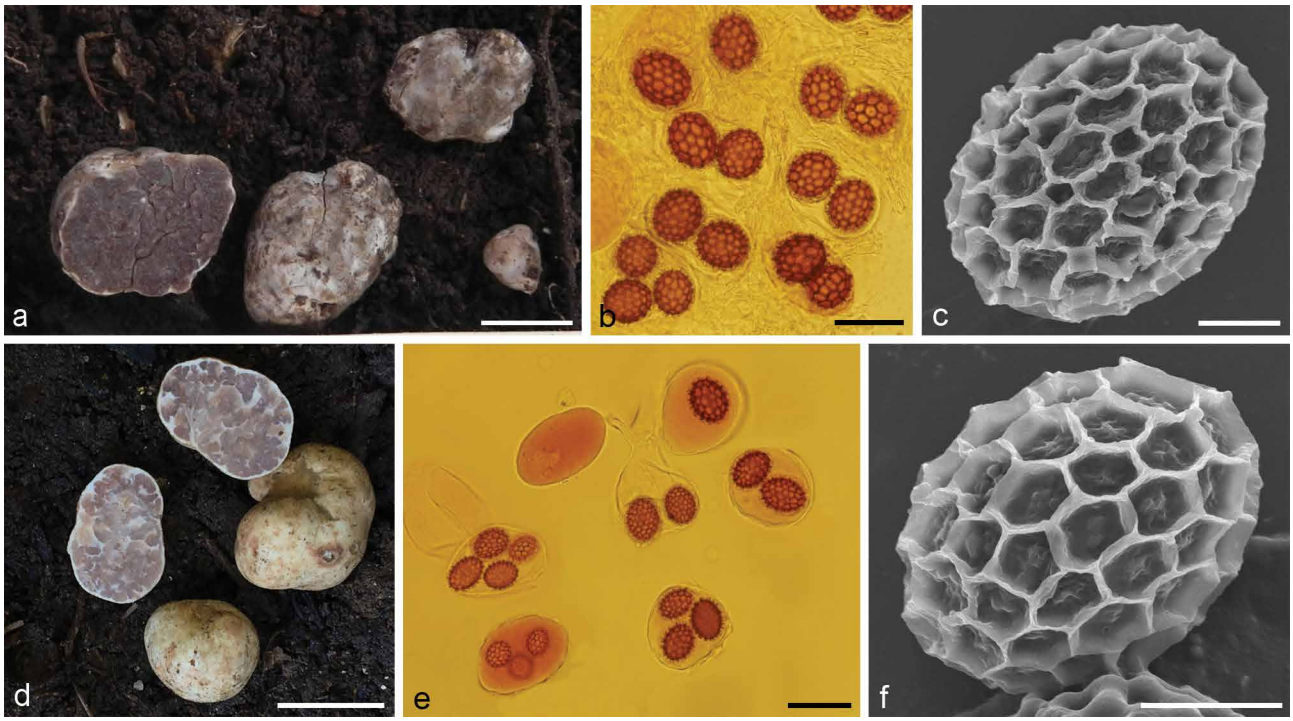

Fig. 6 a–c. *Tuber anniae*. a. Ascomata (BJTC FAN640); b. asci and ascospores under LM; c. ascospores under SEM. — d–f. *Tuber maculatum*. d. Ascomata (BJTC FAN869); e. asci and ascospores under LM; f. ascospores under SEM. — Scale bars: a, d = 1 cm; b, e = 50 µm; c, f = 10 µm.

Notes — *Tuber anniae* is confirmed from Hebei and Shanxi provinces under *Larix principis-rupprechtii* based on morphological and DNA evidence. Wang et al. (2013) treats *T. anniae* as a species complex, but our present analysis indicates that it should be treated as a distinct species with a global distribution (Fig. S7). Geographically, like its pattern in Europe and North America (Colgan & Trappe 1997, Wang et al. 2013, Healy et al. 2016), it is also distributed as a cold climate zone species in North China.

Tuber maculatum Vittad., Monogr. Tuberac. (Milano): 45. 1831 — Fig. 6d–f

Habitat — Hypogeous, in soil under mixed forest of *Larix principis-rupprechtii* and *Picea* sp.; ascoma occurring in autumn.

Distribution — Known from northern China.

Specimens examined. China, Shanxi Province, Lvliang City, Jiaocheng County, in soil under *Larix principis-rupprechtii*, alt. 1853 m, N37°51' E111°27', 8 Sept. 2017, *Y.Y. Xu* (BJTC FAN868, BJTC FAN869); ibid., under *Picea* sp., *T. Li* (BJTC FAN876, BJTC FAN877, BJTC FAN878).

Notes — The occurrence of *T. maculatum* in China is confirmed from Shanxi Province under *Larix*/*Picea* dominant forest based on molecular and morphological evidence, and an ITS sequence (GU134516) from the ECM of *Pinus densiflora* in Shaanxi Province also matches this species. *Tuber maculatum* is originally described from Europe, and also reported from South America, Australia and New Zealand (Bonito et al. 2010, Bulman et al. 2010). In China it is not certain whether *T. maculatum* is naturally occurring or has been accidentally introduced by man.

DISCUSSION

Asian China is a diverging centre of Tuber in the world

Our study revealed that *Tuber* is highly represented in, and many species radiate extensively in, China. Of the 12 resolved clades or phylogroups of *Tuber* worldwide (Kinoshita et al. 2011, Bonito et al. 2013, Fan et al. 2015, 2016a, b), nine phylogroups diversified in China, including species in the *Aestivum*, *Excavatum*, *Latisporum*, *Macrosporum*, *Maculatum*, *Melanosporum*, *Puberulum*, *Rufum* and *Turmericum* phylogroups; and we have presented evidence that two phylogroups, *Latisporum* and *Turmericum*, are endemic to Asia, because all molecular signatures of these species are from Asia alone with the only exception of sequence (UDB0752113) isolated from the ECM root tip of *Tilia amurensis* in Estonia (Fig. 1). However, *Tilia amurensis* is a species native to north-eastern Asia (China, Russian Far East, North Korea and South Korea) according to Species 2000 (https://sp2000.org/), thus we strongly believe that the presence of UDB0752113 in Estonia is a consequence of human activity. Phylogroup *Latisporum*, the most diverse lineage in Asia, includes 31 species, 23 in China (Fig. S3). Phylogroup *Turmericum* includes seven species in Asia, five in China (Fig. S9). In general, most of the phylogroups in the genus *Tuber* successfully radiate in China, but Phylogroup *Aestivum*, a basal evolving lineage (Bonito et al. 2013) with mostly variable morphology, is an exception, as that is poorly represented in this region with only one species *T. sinoaestivum* (Fig. 1, 2, S1). It is notable that the European species *T. magnatum* was recently found in northern Thailand based on morphological and molecular data (Suwannarach et al. 2017), and is the first record of this lineage in Asia, but it has not been found in China. Moreover, the other six phylogroups also represent many species in China as revealed in this study. It is remarkable that there is no species to be mentioned from Japan by Kinoshita et al. (2011) and this study from either of the *Excavatum* and *Maculatum* phylogroups, which are well represented in China. The reason why these phylogroups are missing from Japan remains unknown. China is the third largest country in the world in terms of geographic mass, across wide altitudinal and latitudinal gradients. The climate includes tropical to frigid zones. The truffle host plant ranges include a rich diversity of trees like *Larix principis-rupprechtii*, *Pinus armandii*, *P. tabulaeformis*, *P. yunnanensis*, *Quercus liaotungensis*. The genus *Tuber* therefore evolved a rich diversity in China, including 82 phylogenetic species within nine phylogroups. Compared to Europe (c. 51 accepted species) and North America (c. 54 accepted species), China appears to be the global centre of diversification of the truffle genus *Tuber*.

The high endemism and narrow geographic area of Tuber in China

The extremely high endemism of *Tuber* species is revealed in China in this study. Among the total 82 *Tuber* species in China, 68 species are endemic, which accounts for 82.9 % of Chinese *Tuber* species. Only eight species shared their distribution with other continents, including *T. anniae*, *T. excelsum-reticulatum*, *T. formosanum*, *T. maculatum*, *T. wenchuanense*, *Tuber* sp. CHN-3, *Tuber* sp. CHN-10 and *Tuber* sp. CHN-11. *Tuber anniae* is a species originally described from North America and also occurs in Europe, but now it is also known under *Larix* sp. in Hebei and Shanxi provinces, northern China (Fig. S7). *Tuber maculatum* is a common European species. There is a report from Taiwan, but no record from mainland China (Chen 2007). Recently this species is collected several times under mixed woods of *Picea* sp. and *Larix* sp. from Shanxi Province of northern China (Fig. S5). *Tuber wenchuanense*, a Chinese species from Sichuan and Shanxi provinces, is detected in the ECM root tips from both Canada (North America) and Poland (Europe) (Fig. S8). *Tuber excelsum-reticulatum*, a new Chinese species in this study, is detected in the ECM root tips of different plants from North America and Europe (Fig. S5, and see the comments about this species in Taxonomy). The most outstanding sample is the Asian *T. formosanum*, which had been found sporulating in natural forests of North America (as *Tuber indicum*-B, Bonito et al. 2011), and the same situation is also observed in isolated pockets of *T. melanosporum* in southern Europe (Riousset et al. 2001, Hall et al. 2007). However, the reasons for this biogeographic pattern of distribution for these eight species have not been resolved. They may be naturally distributed, or they might be a deliberate introduction by human activity.

Our analyses also showed that, for most species of *Tuber* from China, each species is limited in its distribution to a relatively narrow region. For example, *T. yigongense*, a newly described black truffle species, only known from Yigong County in Tibet (Fan et al. 2018), and *T. piceatum* is probably limited in Tianshan region in Xinjiang Province. More samples include *T. caoi*, *T. glabrum*, *T. latisporum*, *T. panzhihuanense*, *T. pseudobrumale*, *T. pseudomagnatum*, *T. pseudosphaerosporum*, *T. sinoaestivum*, *T. sinosphaerosporum*, which all are limited to Yunnan Province and/or southern Sichuan Province. Also, there are some northern Chinese species including *T. liaotongense*, *T. lishanense* and *T. xuanhuaense*, that are distributed only in the north region of China. The most extreme examples are probably the species *T. luyashanense*, *T. microcarpum* and *T. excelsum-reticulatum*, which are limited to the Lvliangshan Mts in Shanxi Province.

On the other hand, there are a few species with a broad area of distribution. The best examples are *T. formosanum* from the phylogroup *Melanosporum* and *T. parvomurphium* from the phylogroup *Latisporum*. These species occurred across East Asia including the Far East region of Russia as revealed in this study (Fig. S3, S6). The distinct geographic pattern is helpful for species recognition of the Chinese black truffles, i.e., *T. sinense* is known from Yunnan and Sichuan provinces, *T. yigongense* is limited to Tibet only, while *T. formosanum* is widely distributed throughout mainland China, from southwest to northeast, and the Taiwan islands. The geographic pattern of some sister species is variable in China. For example, the sister species *T. jinshajiangense* and *T. shii* share the same region in the southwest area of China (Fig. S7), but the distribution of the sister species *T. subglobosum* and *T. liaotongense* are exclusive with *T. subglobosum* in the southwest and *T. liaotongense* in the north regions (Fig. S8).

One reason for the relatively narrow distribution of *Tuber* species may be their adaptation to belowground sporulation in which they lost the ability to forcibly discharge their spores and become dependent on animals for the dispersal of their spores. On the other hand, all *Tuber* species are obligate ectomycorrhizal fungi with a broad diversity of trees (Hall et al. 2007, Maser et al. 2008, Trappe et al. 2009). Thus, the movement or migration of the mycophagous animals and ectomycorrhizal host plants would greatly influence the migration and distribution of *Tuber* species. Geographic and climatic factors can affect the migration of both animals and host plants, such as large bodies of water (oceans), high mountain ranges, desert zones, climate zones. These factors can furthermore inhibit the spore dispersal and the gene flow of *Tuber* spp., and help to drive speciation. The fact that most of the *Tuber* species had a narrow distribution range can explain their high endemism for different geographic regions, and may be why the present *Tuber* flora in Asia is so different from both Europe (c. 51 accepted species) and North America (c. 54 accepted species).

The congruence and conflict between morphology and DNA molecular data

Our present study revealed that most species of *Tuber* in China are supported by both morphology and molecular phylogeny. Species with few differences from each other in morphological features are well separated by molecular data. For example, the species *T. jinshajiangense* and *T. shii* are closely related and highly similar to each other. The only major morphological difference between them is the ascospore shape, which is globose in the former, while globose to sub-globose in the latter, but ITS-based phylogenetic analysis separated them well (Fig. S7) (Wang et al. 2016). These indicate there is the strong consistency between morphology and DNA molecular data on the species delimitation of the genus *Tuber*. However, there are some species supported by molecular data but not by morphology, among which almost no morphological differences are observed. These are called cryptic species. Cryptic species are not uncommon in *Tuber* in China. The outstanding example is a pair of commercial black truffle species, *T. formosanum* and *T. sinense*, both of which are commonly called *T. indicum* in China. These two black truffle species are not distinguishable morphologically, which resulted in the synonymization of the two species by some authors (Song 2005, Chen 2007), but DNA analysis can separate them well (Fig. S6), and a recent study revealed their great difference in geographic distribution that is limited to Yunnan and Sichuan provinces for *T. sinense* and widely mapped in northeast Asia for *T. formosanum* (Fan et al. 2018). In contrast, the more challenging problem for species delimitation of Chinese *Tuber* is that some species have distinct morphological features but are not supported by molecular analysis. Good examples are *T. vesicoperidium* and *T. microspiculatum*. *Tuber vesicoperidium* is a rare and morphologically distinct species from Yunnan Province in southwestern China, that is characterised by large, thick-walled, swollen cells in the ascomatal peridium (Fan et al. 2012c), but is not differentiated from *T. lijiangense* by molecular data in this study (Fig. S7). According to our observation for more than 1 000 specimens in China, the feature of large swollen cells with thickened walls in the peridium is uncommon in *Tuber* species. Similarly, *T. microspiculatum* differs from *T. umbiliculatum* in its spiny-reticulate spore ornamentation with very minute and numerous meshes, but molecular analysis did not support their difference in this study (Fig. S8). Ascomatal colour in *Tuber* varies greatly for most species (for example, the colour of ascomata changes from white to blackish brown in age in *T. latisporum*, and from red-brown to blackish in *T. sinense*). Ascospore ornamentation, on the other hand, is a stable and important feature although with some exceptions, for example, the workable morphological trait to separate *T. taiyuanense* from *T. huidongense* is the mesh

number of ascospore ornamentations across the spore width, which is usually numbered 5–8 meshes/width in the former while mostly 3–5 in the latter. A new sample in this study, the specimen (BJTC FAN297), is identified as *T. pseudohimalayense* by DNA analysis (Fig. S6). However, its ascomata had no excavated cavities at the ascomatal base, a feature that diagnoses *T. pseudohimalayense*, but instead had several deep furrows. The question then, is whether these morphological variations come from environmental factors or actually signal that they are different taxa? In fact, it is very difficult to treat some of these samples because the current DNA data from the selected loci cannot solve taxonomic problems for 100 % of the species.

Host specificity and Chinese Tuber species

In general, *Tuber* species are hosted by a wide range of plants including both conifers and broad-leaf trees (Hall et al. 2007). However, some individual species are associated with specific host plants according to our observation. Our investigations show that *T. xuanhuaense* (14 collections), a commonly encountered species in North China, is harvested under *Betula* spp., *Quercus* spp., *Pinus* spp., *Populus* spp., but never under *Picea* spp. and *Larix* spp., while *T. liaotongense* (15 collections), another widely distributed species in this region, can be found under all of the trees mentioned above. *Tuber lishanense* (22 collections) is known from *Pinus armandii* only in North China. Both *T. luyashanense* (30 collections) and *T. microcarpum* (six collections) only came from the *Picea-Larix* forest in Shanxi Province. *Tuber taiyuanense* (11 collections), a very common species in the phylogroup *Rufum* in China, seems to be associated only with *Pinus* spp. including 2-needled pine (*Pinus tabulaeformis*), 3-needled pine (*Pinus yunnanensis*) and 5-needled pine (*Pinus armandii*), but *T. huidongense* (42 collections) closely related to *T. taiyuanense* is hosted by a wide range of plants including both conifers and broad-leaf trees. *Tuber sinoaestivum* (19 collections) seems to be associated only with 5-needled pines (*Pinus armandii* and *Pinus wallichiana*). The host range may be related to the geographic map of *Tuber* species. A good example is two sister species of black truffle, *T. sinense* (90 collections) and *T. formosanum* (65 collections). The former is limited to the southwest area of China and probably hosted by only conifers, but the latter is associated with a wide range of trees including both conifers (2-, 3- and 5-needled pines) and broad-leaved trees (*Castanea mollissima*, *Quercus* spp., *Populus* spp.). Accordingly, *T. formosanum* spread across the southwest and to the northeast regions in East Asia (Fan et al. 2018). In spite of many observations, our knowledge on the host specificity of *Tuber* species in China remains poor. Also, there is no reliable work that whether the host specificity is used as species delimitation in *Tuber*. How the truffles co-evolute with their hosts is not known. More evidence is therefore needed before we can outline a pattern of the relationship between these truffles and their hosts.

CONFIRMED SPECIES OF TUBER IN CHINA

Phylogroup Aestivum

Tuber sinoaestivum J.P. Zhang & P.G. Liu, Mycotaxon 122: 75. 2012

Tuber sinoaestivum is a phylogenetically distinct species (Fig. 2, S1). It shares less than 93.6 % similarity in the ITS region with other *Tuber* species. Morphologically it is easily confused with the European *T. aestivum* (Vittadini 1831, Zhang et al. 2012), and is treated as that species in earlier Chinese works (Song 2005, Cao 2010). Its sub-globose ascospores helps to separate it from *T. aestivum* that usually has ellipsoidal ascospores (Riousset et al. 2001). *Tuber sinoaestivum* is only found in Sichuan and Yunnan provinces in China. A sequence (KP222539) from the ECM root tips of *Pinus wallichiana* in Kashmir (India) well matches the species *T. sinoaestivum* (Fig. S1). This indicates *T. sinoaestivum* also occurs in India.

Phylogroup Excavatum

Tuber badium S.P. Wan, Phytotaxa 296: 233. 2017

This species is described from Yunnan Province based on a single specimen (Wan et al. 2017b). We collected six specimens from southwestern China that phylogenetically and morphologically matches this species in this study (Fig. 2, S2). *Tuber badium* is morphologically similar to *T. neoexcavatum* and its allies in China, all of which are probably limited in their geographic regions in the southwest region of China. The diagnoses for this species include the pale ascomata that are completely absent from olive tints, and ellipsoidal to broadly ellipsoidal ascospores. *Tuber badium* shares less than 96.2 % similarity in the ITS region with other *Tuber* species.

Tuber depressum S.P. Wan, Phytotaxa 296: 233. 2017

This species was recently described from Yunnan Province, China (Wan et al. 2017b), and is very similar to *T. neoexcavatum* according to the original description. We were not able to examine the type specimen in this study, but our phylogenetic analyses support it as a distinct species (Fig. 2, S2). Morphological features of our collections match that written for the description of the type of *T. depressum. Tuber depressum* is hardly separable from *T. neoexcavatum* in morphology alone, but its narrowly ellipsoid ascospores differ from the latter. This species shares less than 95.1 % similarity in the ITS region with other *Tuber* species.

Tuber magnameshanum L. Fan (see Taxonomy)

Tuber neoexcavatum L. Fan & Y. Li, Mycotaxon 124: 159. 2013

This species is the most frequently encountered *T. excavatum*-like species in China. The medium-sized, olive ascomata covered with fine warts is probably a distinctive feature to diagnose it. To our knowledge, this species is mainly distributed along the Jinshajiang Valley. *Tuber neoexcavatum* shares less than 96.5 % similarity in the ITS region with other *Tuber* species.

Tuber pseudofulgens L. Fan (see Taxonomy)

Tuber sinoexcavatum L. Fan & Y. Li, Mycotaxon 116: 352. 2011

This species looks like the European *T. excavatum* in ascomatal appearance, but its ascospores are very similar to the sub-globose ascospores of another European native species *T. fulgens* (Riousset et al. 2001). It shares less than 96.3 % similarity in the ITS region with other *Tuber* species. Not common.

Tuber verrucosivolvum S.P. Wan, Phytotaxa 296: 235. 2011

Tuber verrucosivolvum is diagnosed by the yellow-brown ascomata covered with pyramidal warts (Wan et al. 2017b). Our phylogenetic analyses show this species is distinct and closely related to *T. sinoexcavatum* (Fig. 2, S2). This species shares less than 93.9 % similarity in the ITS region with other *Tuber* species.

Phylogroup Latisporum

Tuber alboumbilicum Y. Wang & Shu H. Li, Mycol. Progr. 13: 1160. 2014

This species was established based on a single specimen (Li et al. 2014). According to the authors, this species was diagnosed by its whitish ascomata with basal cavity and broadly ellipsoidal ascospores with reticulate ornaments. The type collection came from southwestern China. Our analyses suggest that IF89261 (DQ478639) from North China is the same species (Fig. 2, S3). *Tuber alboumbilicum* shares less than 94.2 % similarity in the ITS region with other *Tuber* species.

Tuber baoshanense S.P. Wan, Mycoscience 58: 316. 2017

This species is phylogenetically closely related to *T. latisporum* (Fig. 2, S3), and can hardly be separated from *T. latisporum* in morphology. According to the authors, *T. baoshanense* is diagnosed by the dark ascomata (Wan et al. 2017a). However, the dark brown ascomata and even blackish brown individuals are not uncommon for fully mature *T. latisporum* according to our observation. DNA examination is necessary for the separation of the two species. *Tuber baoshanense* shares less than 94.8 % similarity in the ITS region with other *Tuber* species. This species is currently only known from Yunnan Province.

Tuber caoi L. Fan, Mycologia 108: 345. 2016

This is a frequently encountered truffle species in southwestern China (Fan et al. 2016a). Morphologically, it is diagnosed by the combination of grey ascomata often with olive tints, the blackish grey gleba at maturity and the globose ascospores. Molecular analyses support it as a distinct species (Fig. 2, S3). *Tuber caoi* shares less than 87.5 % similarity in the ITS region with other *Tuber* species.

Tuber elevatireticulatum K.F. Wong & H.T. Li, Bot. Studies (Taipei) 59 (no. 25): 4. 2018

This species was recently described from Taiwan Islands based on specimens found in soil under *Keteleeria fortunei* var. *cyclolepis* (Lin et al. 2018). We were not able to examine the type specimen of this white truffle species, but our DNA analyses support it as a good species. *Tuber elevatireticulatum* shares less than 96.2 % similarity in the ITS region with other *Tuber* species. This is the third *Tuber* species found in Taiwan, and phylogenetically it is closely related to the Chinese *Tuber* sp. CHN-7 (from Liaodong peninsula) and the Japanese *Tuber* sp. 10 (Kinoshita et al. 2011) in the phylogroup *Latisporum* (Fig. 2, S3).

Tuber griseolivaceum L. Fan & K.B. Huang, Phytotaxa 309: 168. 2017

Morphologically, *T. griseolivaceum* is diagnosed by the combination of olive-grey ascomata, blackish gleba at maturity and globose ascospores. *Tuber caoi* is somewhat similar in ascomatal colour, but its size is usually smaller, with grey gleba (Huang et al. 2017). This species is found in Yunnan Province, but seems rare. Phylogenetically it is closely related to *T. alboumbilicum* and *T. polymorphosporum* in the *Latisporum* phylogroup (Fig. 2, S3). This species shares less than 89.6 % similarity in the ITS region with other *Tuber* species.

Tuber huiliense L. Fan (see Taxonomy)

Tuber latisporum J. Chen & P.G. Liu, Mycologia 99: 476. 2007

This is one of the most frequently encountered truffle species in southwestern China, and is both morphologically and phylogenetically distinct (Fig. 2, S3). The diagnosed features of *T. latisporum* included: ascomata medium to large in size, usually from white to grey-brown or even dark brown in age, gleba blackish to black at maturity, ascospores broad ellipsoid or sub-globose, red-brown and covered with roughly reticulate ornaments, odour usually very pungent but pleasant (Chen & Liu 2007). This species is easily confused with its sister species *T. panzhihuanense* in morphology, but according to our observation, the larger size of ascomata and the dark grey gleba (never black) could potentially help to separate *T. panzhihuanense* from *T. latisporum*. This species shares less than 96.5 % similarity in the ITS region with other *Tuber* species.

Tuber luyashanense L. Fan (see Taxonomy)

Tuber microcarpum L. Fan & T. Li (see Taxonomy)

Tuber parvomurphium L. Fan, Mycologia 108: 349. 2016

This species is characterised by the combination of the small-sized, yellow-white to yellow-brown ascomata, blackish gleba and globose ascospores. Our DNA analyses support *T. parvomurphium* is a distinct species (Fig. 2, S3). It shares less than 95.7 % similarity in the ITS region with other *Tuber* species. This species is originally described from southwestern China (Fan et al. 2016a), but DNA sequences obtained later from both ascomata and ECM tips harvested from Beijing city, Shanxi, Hebei, Liaoning provinces of North China, Japan and Far East of Russia (Fig. S3, Table S3), indicate that *T. parvomurphium* is widely distributed in East Asia.

Tuber panzhihuanense X.J. Deng & Y. Wang, Mycol. Progr. 12: 558. 2013

This is a large white truffle species that mainly occurs under *Pinus* spp. along the Jinshajiang Valley. Not uncommon. The largest individuals reach 10 cm diam or more (Deng et al. 2013, this study). *Tuber panzhihuanense* shares less than 97 % similarity in the ITS region with other *Tuber* species.

Tuber polymorphosporum S.P. Wan, Mycoscience 58: 313. 2017

This is a commonly encountered species in southwestern China, mainly from north-western Yunnan Province and southwestern Sichuan Province (Wan et al. 2017a, this study). According to the authors, the ascospores that vary in shape from broad ellipsoid to long ellipsoid probably help to diagnose it. Our analyses reveal that *T. polymorphosporum* is closely related to *T. alboumbilicum* in the phylogroup *Latisporum* (Fig. 2, S3). This species shares less than 94 % similarity in the ITS region with other *Tuber* species.

Tuber pseudosphaerosporum L. Fan, Mycotaxon 125: 286. 2013

This species resembles *T. panzhihuanense* in ascomatal appearance, both being large and white, and in odour, both being pungent. The ascospore shape differentiates them well, with globose ascospores in *T. pseudosphaeosporum* and ellipsoid ascospores in *T. panzhihuanense* (Deng et al. 2013, Fan & Yue 2013). Phylogenetically these two large white truffles are not so closely related although they evolved within the same lineage (Fig. 2, S3). *Tuber pseudosphaerosporum* shares less

than 94.7 % similarity in the ITS region with other *Tuber* species. It is only known from the Jinshajiang Valley (Fan &Yue 2013, this study).

Tuber thailandicum Suwannar. et al., Mycol. Progr. 14: 3. 2015

Tuber thailandicum was described from Thailand (Suwannarach et al. 2015). We could not harvest its sporocarps in China, but two ITS sequences (AB769932, LC176643) from the ECM root tips of *Pinus massoniana* in Hunan and Shaanxi provinces revealed its occurrence in China (Fig. 2, S3, Table S3). The Japanese *Tuber* sp. 11 (Kinoshita et al. 2011) should be the same species (Fig. 2, S3). Our phylogenetic analyses revealed *T. thailandicum* was closely related to Chinese *T. pseudosphaerosporum* in the *Latisporum* phylogroup (Fig. 2, S3). Morphologically they were different in ascospore shape, which was broad ellipsoid in *T. thailandicum*, while globose in *T. pseudosphaerosporum*. This species shares less than 87.3 % similarity in the ITS region with other *Tuber* species.

Phylogroup Macrosporum

Tuber calosporum S.P. Wan, Mycoscience 57: 396. 2016

This species was described from Yunnan Province (Wan et al. 2016). We were not able to examine the type specimen, but two specimens collected from Yunnan Province by us phylogenetically match this species with strong support (Fig. 2, S4), and morphologically are in accordance with the description. *Tuber calosporum* is confused with *T. glabrum* in morphology, the difference probably is the shape of ascospores that is broadly ellipsoid in *T. calosporum* and ellipsoid in *T. glabrum* (Fan et al. 2014, Wan et al. 2016). Moreover, we note a peculiar pungent odour from our mature ascomata collections, which is completely absent from *T. glabrum*. *Tuber calosporum* shares less than 89.7 % similarity in the ITS region with other *Tuber* species.

Tuber glabrum L. Fan & S. Feng, Mycol. Progr. 13: 244. 2014

There are three species from the *Macrosporum* phylogroup in China. They are very similar in morphology. Of them, *T. glabrum* is the most frequently encountered along the Jinshajiang Valley. It shares less than 85.6 % similarity in the ITS region with other *Tuber* species.

Tuber sinomonosporum J.Z. Cao & L. Fan, Mycol. Progr. 13: 245. 2014

This species is originally described as *Paradoxa sinensis* (Fan et al. 2012a), but later transferred to *Tuber* as *T. sinomonosporum* (Fan et al. 2014). The European *T. monosporum* is easily confused in the ascomatal appearance and ascospore shape. The potential difference between them is probably the size of ascospores, that is (55–)62.5–72.5 µm in *T. sinomonosporum* and 50–60 µm diam in *T. monosporum* according to Fan et al. (2012a). To our knowledge, no DNA data are available for *T. monosporum*, thus the phylogenetic relationship between the two species remains unknown. *Tuber sinomonosporum* shares less than 87.9 % similarity in the ITS region with other *Tuber* species. It is only known from Yunnan Province, China.

Phylogroup Maculatum

Tuber bomiense K.M. Su & W.P. Xiong, Mycotaxon 126: 129. 2013

This species is recognised by the red-brown ascomata covered with fine warts, brown gleba and broadly ellipsoidal ascospores ornamented with a deep reticulum (Su et al. 2013). The type specimen is collected from Tibet, and the second collection is from Yunnan. This species is probably not common. It shares less than 96.1 % similarity in the ITS region with other *Tuber* species.

Tuber excelsum-reticulatum L. Fan & T. Li (see Taxonomy)

Tuber hubeiense L. Fan, Mycologia 108: 355. 2016

This species was described from Hubei Province, central China (Fan et al. 2016b). Although the type specimen is available, it is not clear whether it is a rare species or not, because no more truffle investigations were conducted for this region since Dr Zhang collected this specimen thirty years ago. *Tuber hubeiense* is morphologically difficult to distinguish, and easily confused with *T. maculatum* and *T. puberulum*. This specimen was originally determined as *T. puberulum* (Zhang 1990, Song 2005), and later as *T. maculatum* (Chen 2007). It shares less than 96 % similarity in the ITS region with other *Tuber* species.

Tuber maculatum Vittad., Monogr. Tuberac. (Milano): 45. 1831

This is the first molecularly confirmed report of the European *T. maculatum* in China from ascomatal materials collected from Shanxi Province, North China. This name is misapplied to a specimen (HMAS60233, Zhang 1990) from Hubei Province, central China, but that specimen has since been described as *T. hubeiense* (Fan et al. 2016b). Two ITS sequences from Qinling Mts, Shaanxi Province, about 500 km southwest of where *T. maculatum* was collected in Shanxi Province, also match this species. Whether the occurrence of *T. maculatum* in China is natural or is due to human activity as it is in Australia and New Zealand (Hall et al. 2007) remains unknown. *Tuber maculatum* shares less than 96.4 % similarity in the ITS region with other *Tuber* species.

Tuber pseudomagnatum L. Fan (complex), Mycotaxon 121: 300. 2012

This is a frequently encountered truffle species in Jinshajiang Valley, southwestern China. The colour of its ascomata is yellow or bright yellow when immersed in soil, then when open to air, it quickly fades to yellow-whitish and finally yellow-brown. Moreover, the pungent and special odour is unique to this species according to our observations. Our molecular analysis places the sequence of *T. microverrucosum* into the *T. pseudomagnatum* clade with strong support (Fig. S5), and DNA analysis reveals that there is more than 97 % ITS similarity between them. *Tuber microverrucosum* is a brown species and nearly completely absent from yellow tints, but its ascospores are highly similar (Fan & Cao 2012, Fan et al. 2012e). The two species are probably conspecific, but there is only one collection of *T. microverrucosum*, so more samplings will be needed to clarify the taxonomic relationship between the two species. The *T. pseudomagnatum* complex shares less than 95.2 % similarity in the ITS region with other *Tuber* species.

Tuber wumengense L. Fan, Mycologia 108: 359. 2016

This species was originally described from Yunnan and Hubei provinces (Fan et al. 2016b). Our recent investigations in southern Shanxi Province of North China show this species is frequently collected in the Zhongtiaoshan Mts under *Pinus armandii*. One ECM-sequence of *Quercus* sp. in Shaanxi, North China matches this species. These indicate *T. wumengense* can occur with both conifers and broadleaved trees. Morphologically the long ellipsoidal ascospores covered with a deep

reticulum are diagnostic for this species. *Tuber wumengense* shares less than 96 % similarity in the ITS region with other *Tuber* species.

Phylogroup Melanosporum

Tuber formosanum H.D. Hu & Y. Wang, Mycotaxon 123: 296. 2013

Originally, *T. formosanum* was described from Taiwan islands, but a recent study revealed this species widely distributed across East Asia (Fan et al. 2018). Morphologically, it is almost indistinguishable from its sister *T. sinense* in China, both of which are sold as Asian black truffles. This species shares less than 94 % similarity in the ITS region with other *Tuber* species.

Tuber pseudobrumale Y. Wang & Shu H. Li, Mycol. Progr. 13: 1160. 2014

Although named as *T. pseudobrumale*, this species looks more similar to *T. mesentericum* as it has the same ascomatal appearance. Feng has proposed *T. pseudomesentericum* based on several collections from southwestern China in her Master Thesis, but this is not validly published (Feng 2014). This not uncommon species shares less than 89.2 % similarity in the ITS region with other *Tuber* species.

Tuber pseudohimalayense G. Moreno et al., Mycotaxon 63: 218. 1997

Tuber pseudohimalayense is one of the most frequently encountered truffle species in China, with the local name 'female truffle', it has been harvested yearly in commercial quantities. This species shares less than 90.8 % similarity in the ITS region with other *Tuber* species.

Tuber sinense K. Tao & B. Liu, Shanxi Univ. J., Nat. Sci. Ed. 12: 215. 1989

A recent publication showed that *T. sinense* is limited to southwestern China (Fan et al. 2018) and is particularly common in Yunnan and Sichuan provinces. *Tuber sinense* shares less than 94 % similarity in the ITS region with other *Tuber* species.

Tuber variabilisporum L. Fan (see Taxonomy)

Tuber yigongense L. Fan & W.P. Xiong, Mycotaxon 133: 190. 2018

This black truffle species was recently recorded (Fan et al. 2018), and probably only grows in a small, isolated region in Tibet. This species shares less than 93 % similarity in the ITS region with other *Tuber* species.

Phylogroup Puberulum

Tuber anniae W. Colgan & Trappe, Mycotaxon 64: 438. 1997

Tuber anniae was originally described from North America (Colgan & Trappe 1997). Our study is the first to confirm the occurrence of this species in China. This species was first detected by ITS-sequencing from ECM root tips of *Pinus tabuliformis* and *Larix principis-rupprechtiii* in Hebei Province, and subsequently the ascomata were harvested from Hebei and Shanxi provinces in 2016 and 2017. This species shares less than 97 % similarity in the ITS region with other *Tuber* species.

Tuber huizeanum L. Fan & C.L. Hou, Mycotaxon 122: 166. 2012

This species, like *T. liyuanum*, is most frequently encountered white truffle species in Jinshajiang Valley. According to our analysis, *T. huizeanum* and *T. liyuanum* are closely related and morphologically very similar to each other (Fig. 2, S7) (Fan & Cao 2012, Fan et al. 2012e), and share the same geographic region. The two species differ in the shape of ascospores that are sub-globose to broad ellipsoid in *T. huizeanum*, and ellipsoid in *T. liyuanum*. *Tuber huizeanum* shares less than 97.3 % similarity in the ITS region with other *Tuber* species.

Tuber humilireticulatum L. Fan (see Taxonomy)

Tuber jinshajiangense L. Fan, Mycologia 108: 349. 2016

This species was originally described from Jinshajiang Valley (Fan et al. 2016a), although it had previously been named as *Tuber californicum* by Cao (2010). Morphologically, this small white truffle is difficult to distinguish from several globose-spored species including Chinese *T. xuanhuaense*, and the North American *T. californicum*, but the phylogenetic analysis separates them well (Fig. 2, S7). Moreover, geographically, *T. californicum* is probably a native species to North America, and *T. xuanhuaense* is clearly limited to North China, but *T. jinshajiangense* is found only in southwestern China according to our study. Not uncommon. *Tuber jinshajiangense* shares less than 93.4 % similarity in the ITS region with other *Tuber* species.

Tuber lijiangense L. Fan & J.Z. Cao (complex), Mycotaxon 116: 350. 2011

Our present analyses revealed that *T. lijiangense* (designated as *Tuber* sp. 33 in Bonito et al. 2010), *T. microsphaerosporum*, *T. sinopuberulum* and *T. vesicoperidium* cluster in the same clade with strong support (Fig. 2, S7), and share more than 98 % similarity in ITS sequence between them, strongly suggesting that phylogenetically they are the same species whereas they are morphologically four distinct species. *Tuber lijiangense* is separated from the others by its sub-globose to globose ascospores; *T. microsphaerosporum* is recognised by its globose ascospores and asci that contain 1–7 spores. In contrast *T. sinopuberulum* has sub-globose ascospores; and *T. vesicoperidium* has huge cells with thickened walls in the peridium. *Tuber lijiangense* is a frequently encountered truffle species, but the other three species are rare. More collections are needed to clarify the relationships between the four species. *Tuber lijiangense* shares less than 95.1 % similarity in the ITS region with other *Tuber* species.

Tuber liui A-S. Xu, Mycosystema 18: 361. 1999

This species is recognised by the large ascospores reaching 70 µm in length (Xu 1999). Our phylogenetic analyses show that it is a good species (Fig. 2, S7). *Tuber liui* is probably native to Tibet. It shares less than 96.1 % similarity in the ITS region with other *Tuber* species.

Tuber liyuanum L. Fan & J.Z. Cao, Mycotaxon 121: 301. 2012

This species is a common truffle species under *Pinus* spp. in Jinshajiang Valley, southwestern China. Morphologically, this species is diagnosed by pale small to medium sized ascomata, brown gleba and ellipsoidal ascospores with reticulate ornaments. *Tuber huizeanum*, another frequently encountered white truffle species from the same region, is similar and closely related both morphologically and phylogenetically (Fig. 2, S7).

However, the ascospores in *T. huizeanum* are shaped broadly ellipsoid to sub-globose. *Tuber liyuanum* shares less than 97.6 % similarity in the ITS region with other *Tuber* species.

Tuber shidianense S.P. Wan & F.Q. Yu, Mycoscience 57: 394. 2016

Phylogenetically, *T. shidianense* is a distinct species and closely related to both *T. jinshajiangense* and *T. shii* (Fig. 2, S7). *Tuber shidianense* has small white ascomata and globose to sub-globose ascospores with reticulated ornaments (Wan et al. 2016), which make it difficult to distinguish from *T. shii* morphologically. The spore size may be the potentially difference between the two species, which is larger in *T. shii* (29–56.5 µm in length) than in *T. shidianense* (24–43 µm in length). Also, the depth of meshes in spore ornaments is lower in *T. shii* (2.5–5 µm) than in *T. shidianense* (3–7.7 µm). *Tuber shidianense* shares less than 89.3 % similarity in the ITS region with other *Tuber* species.

Tuber shii L. Fan & Y.W. Wang, Phytotaxa 269: 282. 2016

This species is sister to *T. jinshajiangense* in the phylogenetic trees (Fig. 2, S7), but morphologically they are distinguished from each other by the shape of ascospores, that is globose in *T. jinshajiangense*, but globose to sub-globose in *T. shii* (Wang et al. 2016). This species is rare, only known from Yunnan Province. It shares less than 93.5 % similarity in the ITS region with other *Tuber* species.

Tuber sinoborchii T. Li & L. Fan (see Taxonomy)

Tuber sinoniveum S.P. Wan, Phytotaxa 298: 257. 2016

According to the original description (Xu et al. 2017), this species is diagnosed by small white ascomata and globose ascospores with reticulate ornaments. Currently, this is a poorly understood species. Our analyses reveal it as a distinct species and closely related to *T. sinosphaerosporum* (Fig. 2, S7). It shares less than 92.8 % similarity in the ITS region with other *Tuber* species.

Tuber sinosphaerosporum L. Fan, J.Z. Cao & Y. Li, Mycotaxon 122: 350. 2012

This is a medium to large white truffle species (ascomata reaching 5 cm diam) with reticulate globose ascospores (Fan et al. 2012b). Other white truffle species with globose ascospores in China usually have smaller ascomata with the exception of *T. pseudosphaerosporum* (Fan & Yue 2013), which, we believe, evolved in the phylogroup *Latisporum* (Fig. 2, S3) rather than the *Puberulum* phylogroup (Fig. 2, S7). Morphologically *T. pseudosphaerosporum* is differentiated from *T. sinosphaerosporum* by its glabrous ascomata and grey-purple tinted gleba. *Tuber sinosphaerosporum* has never been harvested from Jinshajiang Valley although it is probably limited to southwestern China. *Tuber sinosphaerosporum* shares less than 93 % similarity in the ITS region with other *Tuber* species.

Tuber xuanhuaense L. Fan, Mycologia 108: 350. 2016

Tuber xuanhuaense is one of the most frequently encountered truffle species in North China and is found under both conifers and broad leaf trees. *Tuber xuanhuaense* was confused with the North American *T. californicum* in earlier Chinese works (Wang 1988, Tao 1988). It shares less than 95.5 % similarity in the ITS region with other *Tuber* species.

Tuber zhongdianense X.Y. He, H.M. Li & Y. Wang, Mycotaxon 90: 213. 2004

Tuber zhongdianense is morphologically difficult to distinguish in this phylogroup, and easily confused with the European *T. borchii* and both Chinese species *T. huizeanum* and *T. hubeiense*. Phylogenetic analyses distinguish this species (Fig. 2, S7). It shares less than 97.8 % similarity in the ITS region with other *Tuber* species.

Phylogroup Rufum

Tuber crassitunicatum L. Fan & X.Y. Yan, Mycologia 110: 774. 2018

Tuber crassitunicatum is similar to *T. taiyuanense* in the colour of both ascomata and gleba, but its ascospore ornaments are intermediate between having isolated spines and having a spinose-reticulate pattern, which distinguishes *T. crassitunicatum* from *T. taiyuanense*. *Tuber crassitunicatum* is probably a rare species in China, only known from Yunnan and Shanxi provinces. It shares less than 89.4 % similarity in the ITS region with other *Tuber* species.

Tuber huidongense Y. Wang (complex), Mycotaxon 83: 191. 2002

Our phylogenetic analyses show that *T. huidongense* (from Yunnan and Sichuan provinces), *T. furfuraceum* (from Taiwan), *T. lannaense* (from Thailand), *T. microspermum* (from Yunnan Province) and the Japanese *Tuber* sp. 3 (Kinoshita et al. 2011) are grouped in the same clade with strong support (Fig. 2, S8), and they share more than 97 % ITS similarity, which suggests these species are conspecific. However, there was only limited sampling available for these geographically or morphologically distinct 'species', and therefore further studies are needed before attempting a formal taxonomic treatment. *Tuber huidongense* shares less than 90 % similarity in the ITS region with other *Tuber* species.

Tuber liaotongense Y. Wang, Atti del Secondo Congresso Internazionale sul Tartufo, Spoleto, 24–27 Novembre 1988 (Perugia): 46. 1990

Originally, it was described from Liaoning Province (Wang 1988). The present study shows it is one of the most frequently encountered *Tuber* species in northern China. It shares less than 95.2 % similarity in the ITS region with other *Tuber* species.

Tuber lishanense L. Fan & X.Y. Yan, Mycologia 110: 776. 2018

Tuber lishanense is diagnosed by its sub-globose ascospores covered with isolated, narrow and densely positioned long spines. This is a very common truffle species under *Pinus armandii* in the Zhongtiaoshan Mts of Shanxi Province, North China (Yan et al. 2018). Two ITS sequences derived from the collections of Gansu Province match this species (Fig. 2, S8), indicating it is also distributed in that region. This species shares less than 94.4 % similarity in the ITS region with other *Tuber* species.

Tuber piceatum L. Fan, X.Y. Yan & M.S. Song, Mycologia 110: 777. 2018

Tuber piceatum and *T. lishanense* are sister species (Fig. 2, S8). They are the only truffle species with spiny ascospores currently known in China. They differ morphologically in the ascospores that are ellipsoid with short and rare spines in *Tuber piceatum*,

but sub-globose with long and dense spines in *T. lishanense*. *Tuber piceatum* is probably limited to the Xinjiang region under *Picea* sp. (Yan et al. 2018). It shares less than 94.4 % similarity in the ITS region with other *Tuber* species.

Tuber sinoalbidum L. Fan & J.Z. Cao (complex), Mycotaxon 118: 408. 2011

This species complex contains *T. sinoalbidum* and *T. subglobosum* (Fig. 2, S8). In morphology, the type specimen of *T. sinoalbidum* has whitish ascomata and ellipsoidal to broad ellipsoidal ascospores (Fan et al. 2011b), while *T. subglobosum* has brown ascomata and sub-globose ascospores (Fan et al. 2013a). *Tuber sinoalbidum* may be rare as only the type specimen is available, but *T. subglobosum* is one of the most frequently collected species in southwestern China. DNA analysis revealed that the two species share more than 97.3 % similarity in ITS sequence. More specimens are needed for their taxonomic treatment. The Japanese sample marked as *Tuber* sp. 3 (Kinoshita et al. 2011) grouped into the clade of *T. sinoalbidum* complex, which implied *Tuber* sp. 3 may be the same species. *Tuber sinoalbidum* complex shares less than 95.9 % similarity in the ITS region with other *Tuber* species.

Tuber taiyuanense B. Liu, Acta Mycol. Sin. 4: 84. 1985

Tuber taiyuanense is one of the most common truffles in China, and widely distributed in both northern and southern regions. It shares less than 97.6 % similarity in the ITS region with other *Tuber* species.

Tuber umbilicatum J. Chen & P.G. Liu (complex), Mycotaxon 94: 2. 2005

As shown in our analyses, *T. umbiliculatum* and *T. microspiculatum* fall into the same clade with strong support (Fig. 2, S8), implying they may be conspecific. However, the two species were separated from each other morphologically by the spiny-reticulate ornaments of ascospores, that is 6–8 meshes across the spore width in *T. umbiliculatum*, but 10–16 in *T. microspiculatum* (Cao et al. 2011, Fan et al. 2012d). *Tuber umbiliculatum* is very similar and closely related to *T. taiyuanense* (Fig. 2, S8), and hardly distinguishable based on morphology alone. Geographically, *T. umbilicatum* is common in the southwestern region, but absent from the northern region of China. *Tuber umbilicatum* shares less than 97.9 % similarity in the ITS region with other *Tuber* species.

Tuber wanglangense L. Fan, Mycologia 110: 777. 2018

This species is a rare and poorly understood species, only known from the Sichuan Province. It shares less than 94.8 % similarity in the ITS region with other *Tuber* species.

Tuber wenchuanense L. Fan & J.Z. Cao, Mycotaxon 123: 99. 2013

This is a rare truffle species with spinose-reticulate ornaments on the surface of ascospores (Fan et al. 2013a). The type specimen of *T. wenchuanense* is from the Sichuan Province. The Shanxi Province is the second locality in China where this species is recorded. The sequences downloaded from GenBank, JX630932 (from the ECM root tip of *Salix arctica* in Canada) and AY748863 (from the ECM root tip of *Salix caprea* in Poland) match *T. wenchuanense*, which indicate this species also occurs in North America and Europe (Fig. 2, S8). *Tuber wenchuanense* shares less than 90 % similarity in the ITS region with other *Tuber* species.

Phylogroup Turmericum

Tuber turmericum L. Fan (complex), Mycol. Progr. 14: 2. 2015

This is a very peculiar truffle species from southwest China, which possesses a turmeric coloured gleba in fully mature ascomata, and the asci invariably contain one ascospore (Fan et al. 2015). *Tuber xanthomonosporum* was described from the same region and published almost at the same time (Qing et al. 2015), and is very similar to *T. turmericum* in morphology according to its original description. Phylogenetically, the ITS sequences of *T. xanthomonosporum* are placed in the *T. turmericum* clade in our phylogenetic trees with strong support (Fig. 2, S9). DNA analysis showed both species shared more than 98.6 % similarity in ITS sequence. Therefore, these two 'species' may be conspecific. We failed to loan the type specimen so a formal taxonomic treatment is still pending. *Tuber turmericum* shares less than 88.8 % similarity in the ITS region with other *Tuber* species.

HYPOTHESIZED SPECIES OF TUBER IN CHINA

Phylogroup Latisporum

***Tuber* sp. CHN-1**

This taxon is represented by a sequence extracted from an ascomatal specimen (BJTC FAN190). Our phylogenetic analyses support its taxonomic position in *Tuber* (Fig. 2, S3). However, since only one poor ascoma is available for this taxon, we await additional collections prior to describing it. It shares less than 97 % similarity in the ITS region with its closest relative.

***Tuber* sp. CHN-2**

This taxon is known only from one ECM-sequence (GU134524) of *Pinus densiflora* from Heilongjiang Province, north-eastern China (Fig. 2, S3). Phylogenetic analyses reveal it as a well-supported species with an ITS sequence similarity less than 95.1 % to its closest relatives, *T. latisporum* and its allies. Currently, no fruitbodies are available for this species, so its taxonomic treatment remains to be done.

***Tuber* sp. CHN-3**

This taxon is represented by five sequences. Of those, three are extracted from the ascomata collected from the soil of *Pinus armandii* in Yunnan Province, China. Two are ECM-sequences, respectively from Estonia (UDB0752113, host unclear) and the root tips of *Abies sachalinensis* in Japan (LC556136). Our phylogenetic analyses position this species in Phylogroup *Latisporum* (Fig. 2, S3), and is closely related to *T. huiliense*. They share less than 97.6 % similarity in ITS sequence. We cannot obtain the ascomatal specimens, so we cannot describe it in this study.

***Tuber* sp. CHN-4**

This taxon is supported by an independent phylogenetic clade composed of ECM-sequences only (Fig. 2, S3). The known host plants are *Betula costata*, *Pinus tabuliformis*, *Populus koreana* and *Quercus liaotungensis*. The sequences in this clade share more than 98 % ITS similarity among them, less than 95 % to other *Tuber* species. These results support its position as a distinct species, but currently, no sporocarps are available for this species, so its taxonomic treatment is pending.

Tuber sp. CHN-5

This species is supported by an ECM-sequence (LC013878) of *Pinus tabuliformis* in Shaanxi Province, North China (Fig. 2, S3). Phylogenetic analyses support it as a distinct species and closely related to *T. luyashanense* and its allies. DNA analysis shows this taxon shares less than 96.4 % similarity in ITS sequence with *T. luyashanense* and its allies. Currently, no sporocarps are available for this species.

Tuber sp. CHN-6

This species is supported by an ECM-sequence (LC200537) of *Pinus bungeana* in Shaanxi Province, North China (Fig. 2, S3). Phylogenetic analyses support it as a distinct species and closely related to *T.* sp. CHN-4, *T.* sp. CHN-5. DNA analysis shows this taxon shares 94.3 % similarity in ITS sequence with *T.* sp. CHN-5 and less than 93.4 % similarity with *T.* sp. CHN-4. Currently, no sporocarps are available for study.

Tuber sp. CHN-7

This species is supported by the sequences derived from specimens IFS. Wang 89377, 89556 and 89719, collected from north-eastern China. These specimens were originally determined as either *T. borchii* or *T. rapaeodorum* (Wang 1988). Our phylogenetic analyses reveal these specimens represent a well-supported species with an ITS sequence similarity of 95.1 % to its closest relatives (Fig. 2, S3; Fan et al. 2016b). However, we were not able to examine the specimens in this study.

Tuber sp. CHN-8

This taxon is represented by three sequences. Of those, one was extracted from the ascocarp collected from the soil under *Pinus armandii* in Yunnan Province, China. Two were isolated from the ECM root tips of *Quercus* sp. in Shaanxi Province, China. Our phylogenetic analyses positions this species in the phylogroup *Latisporum* (Fig. 2, S3). The sequences in this clade share more than 98 % ITS similarity between them, less than 94.6 % to other *Tuber* species. We could not obtain ascomatal material, so further study is pending.

Tuber sp. CHN-9

This taxon is known only from one sequence (JX987751) from a soil sample in Guangdong Province, South China (Fig. 2, S3). Phylogenetic analyses reveal it as a well-supported species with an ITS sequence similarity of 95.6 % to its closest relative *T. pseudosphaerosporum*. Currently, no sporocarps are available for this species, so its taxonomic treatment awaits.

Phylogroup Melanosporum

Tuber sp. CHN-10

This species is recognised by two ITS sequences, one from a personal collection in the Balkan Peninsula (FM205595), another from an ECM root tip in Yunnan Province, China (JQ639006). We were not able to examine the specimen bearing FM205595 in this study, but our molecular analyses clearly support it as distinct species and sister to *T. variabilisporum* (Fig. 2, S6). It shares less than 95.8 % similarity in the ITS region with its closest relative.

Phylogroup Puberulum

Tuber sp. CHN-11

This species is represented by 19 ECM sequences, two from China (Hebei and Inner Mongolia) and 17 from Europe. The hosts include *Betula platyphylla*, *Larix principis-rupprechtiii*, *Picea abies*, *Salix alba* and *Salix fragilis*. No ascomata are available. Phylogenetic analyses (Fig. 2, S7) show that it is closely related to Japanese *Tuber* sp. 16 and *Tuber* sp. 17 (Kinoshita et al. 2011). It shares less than 96.9 % similarity in the ITS region with other *Tuber* species.

Tuber sp. CHN-12

This species is represented by two ECM sequences, respectively from *Pinus densiflora* and *Tilia amurensis* in Heilongjiang Province, north-eastern China. No ascomata are available. Our analyses reveal it as sister to the North American *T. californicum* (Fig. 2, S7). It shares less than 95.2 % similarity in the ITS region with its closest relative.

Tuber sp. CHN-13

This species is recognised from two ECM sequences from *Larix chinensis* in Shaanxi Province, North China (Fig. 2, S7). No ascomata are available. It shares less than 97.6 % similarity in the ITS region with its closest relative.

Tuber sp. CHN-14

This species is represented by three Tibetan specimens identified as *T. liui* respectively by Song (2005) and Chen & Liu (2007). We were not able to examine the specimens in this study, but we think they represent an undescribed species according to our analyses (Fig. 2, S7). It shares less than 95.9 % similarity in the ITS region with its closest relative.

Tuber sp. CHN-15

This species is recognised from four ITS sequences of ECM from a subtropical region of eastern China. Phylogenetic analyses reveal it as related to *T. anniae* and *T. borchii* (Fig. 2, S7). No ascomata are available for this species. It shares less than 92.9 % similarity in the ITS region with its closest relative.

Phylogroup Rufum

Tuber sp. CHN-16

This species is represented by a specimen (BJTC FAN986) collected under *Pinus tabuliformis* from Shanxi Province, China and an ECM-sequence of *Quercus liaotungensis* in Loess Plateau, China. Our phylogenetic analyses support its description as a new species (Fig. 2, S8). However, this specimen is not fully mature, thus more collections are needed before describing this species. This species shares less than 96.4 % similarity in the ITS region with other *Tuber* species.

Phylogroup Turmericum

Tuber sp. CHN-17

Two ITS sequences, one from soil in a subtropical mountain forest (LN910510), another from an ECM root tip in a subtropical evergreen broad-leaved forest (JQ991908), generated a distinct clade in our phylogenetic analyses (Fig. 2, S9), sup-

porting our hypothesis that these represent an undescribed species. However, no ascomata of this species were found in this study. It shares less than 88.8 % similarity in the ITS region with other *Tuber* species.

Tuber sp. CHN-18

The sequence (GQ240940) extracted from an ECM root tip of *Pinus massoniana* in Sichuan Province generated an isolated clade in the phylogroup *Turmericum* in our phylogenetic analyses (Fig. 2, S9), indicating this clade as a well-supported species with an ITS sequence similarity of 88.7 % to its closest relative. No ascomata of this species were found in this study.

Tuber sp. CHN-19

The ECM-derived sequence (JQ991907) from Zhejiang Province, north-eastern China, generated an isolated clade in the phylogroup *Turmericum* (Fig. 2, S9), supporting it as a potentially undescribed *Tuber* species with an ITS sequence similarity of 86.7 % to its closest relative. No ascomata of this species were found in this study.

Tuber sp. CHN-20

The sequence (KX444500) extracted from an ECM root tip of *Quercus liaotungensis* in Liaoning Province, north-eastern China generated an isolated clade in the phylogroup *Turmericum* (Fig. 2, S9), supporting this clade as an undescribed *Tuber* species. This species clustered with *T. flavidosporum* from Japan with a moderate support, that indicates they are phylogenetically related to each other. No ascomata of this species were found for this study. *Tuber* sp. CHN-20 shares less than 88.1 % similarity in the ITS region with its closest relative.

EXCLUDED AND DOUBTFUL TUBER SPECIES IN CHINA

Many European and North American species were recorded on the list of Chinese *Tuber* species before molecular phylogenetic analysis was used for the taxonomic treatment of *Tuber* in China, including *T. asa*, *T. borchii*, *T. borchii* var. *sphaerosporum*, *T. brumale*, *T. californicum*, *T. dryophilum*, *T. excavatum*, *T. foetidum*, *T. melanosporum*, *T. nitidum*, *T. oligospermum*, *T. puberulum* and *T. rufum* (Wang 1988, Zang et al. 1992, Xu 1999, Song 2005, Chen 2007, Cao 2010). More commonly, the Indian species *T. indicum* s.lat. and *T. himalayense* are usually listed (Fan et al. 2018). Moreover, some species originally described from China still lack DNA data, including *T. gigantosporum*, *T. polyspermum* and *T. xizangense* (Wang & Li 1991, Xu 1999, Fan et al. 2011b). It is doubtful whether the above species occur in China according to our present study. Here we present the results of investigations of these *Tuber* species in China as follows.

Tuber asa in China was originally reported from Liaoning Province (Wang 1988). A recent study showed those specimens bearing the name *T. asa* by Dr Y. Wang (1988) are more likely *T. xuanhuaense* (Fig. S7, Fan et al. 2016a).

Tuber borchii in China was originally recorded from Liaoning Province by Dr Y. Wang (Wang 1988), and later reported again by Song (2005) and Chen (2007). A previous study (Fan et al. 2016b) and this work showed that this European species does not occur in China, and the specimens bearing the name of *T. borchii* represent two different Chinese endemic species (Fig. 2, S3). One is *T. luyashanense*, a new species described in this study. Another composed of four sequences (including a sequence identified as *T. rapaeodorum*) is a putatively novel species, which is temporarily identified as *Tuber* sp. CHN-7, and remains undescribed until available specimens are examined or new ones collected.

Tuber borchii var. *sphaerosporum* (= *Tuber sphaerospermum*) in China was originally from Chen's PhD thesis (Chen 2007), and later this specimen was treated as the holotype of the Chinese endemic species *T. lijiangense* (Fan et al. 2011a). Also, *Tuber borchii* var. *sphaerosporum* probably is a species native to Europe (Alvarado et al. 2012).

Tuber brumale in China was mentioned by Prof. M. Zang (1992) based on a black truffle specimen (HKAS 20520). We were not able to examine this specimen but Song (2005) concluded that it is a young *T. indicum* after re-examining this specimen.

Tuber californicum in China dated from Dr Y. Wang's work (Wang 1988), in which he reported this North American species from Liaoning Province, and later this species was commonly mentioned by different authors (Tao 1988, Song 2005, Chen 2007, Cao 2010). A recent study revealed this species to be unconfirmed in China, and all available specimens bearing this North American species epithet are *T. parvomurphium*, a Chinese endemic species in the *Latisporum* phylogroup (Fan et al. 2016a).

Tuber dryophilum in China was reported originally by Dr Y. Wang (1988) from Liaoning Province. Song (2005) re-examined the specimens bearing this name for a European species in Y. Wang's Herbarium (IFS). She concluded that these specimens are *T. liaotongense* based on morphological features, which is further well supported by the DNA data from these specimens by Wang et al. (2007) (also see Fig. S8 in this study). Thus, the occurrence of *T. dryophylum* in China is not confirmed.

Tuber excavatum in China was first reported from Sichuan Province by B. Wang (Wang 1995). Song (2005) re-examined the specimen cited by B. Wang and concluded that it is *T. pseudoexcavatum*, a widely distributed species in both Sichuan and Yunnan provinces. Chen (2007) reported this species from Sichuan Province again based on a specimen (HKAS 52006), and followed by Cao (2010). Our present molecular analysis revealed that the specimen does not match the European *T. excavatum*, but nests within the *T. depressum* clade, a species newly described from China (Wan et al. 2017b). Thus, the occurrence of *T. excavatum* is not confirmed in China.

Tuber foetidum in China was originally reported from Sichuan Province based on the specimen HMAS 60230 (Zhang 1990). We were not able to examine this specimen but Song (2005) concluded it is *T. liaotongense* after re-examining this material.

Tuber gigantosporum was described from southwestern China (Wang & Li 1991). According to the author, the type of this species is lost, and no DNA data are available. This species may be distinct and probably closely related to both *T. glabrum* and *T. calosporum*, but DNA data are needed for confirmation.

Tuber indicum s.lat. and *T. himalayense*. *Tuber indicum* was originally reported in China by Zang (Zang et al. 1992). He treated the Chinese black truffle as an Indian species rather than *T. sinense* (Tao et al. 1989), a species based on the Chinese black truffle from Sichuan Province. A recent study showed that both *T. indicum* s.lat. and *T. himalayense* were misapplied to Chinese taxa, and no DNA evidence supports the occurrence of the two Indian species in China (Fan et al. 2018). According to our present study, most *Tuber* species

map to a narrow geographic area, and the two Indian species are therefore probably native only to India.

Tuber lyonii was reported in China by Song (2005) in her PhD thesis. However, the morphological and molecular examination for those specimens (HMAS 60239, HMAS 60241, HMAS 88575, HMAS 88577) cited by Song (2005) resulted in the description of both *T. wenchuanense* (Fan et al. 2013a) and *T. crassitunicatum* (Yan et al. 2018). Thus, the occurrence of the North American *T. lyonii* in China is not supported.

Tuber melanosporum in China was reported from Hunan Province (Li 1997) based on the specimen MHHNU4973. However, according to Song (2005), this specimen is *T. indicum* s.lat. rather than the European *T. melanosporum*. We were not able to study this specimen.

Tuber nitidum and *T. rufum* were originally described from Europe. Both species are reported from China, and *T. nitidum* was treated as a synonym of *T. rufum* (Song 2005, Chen 2007, Cao 2010). *Tuber nitidum* was reported from Sichuan Province based on the specimen HMAS60220 (Zhang 1990), but recently a new species *T. wanglangense* was described based on the same specimen (Yan et al. 2018). *Tuber rufum* was recorded from Gansu Province by Wang (1988), but a recent work showed that the two specimens (IFS Y. Wang 89245, IFS Y. Wang 89257) cited by Wang (1988) are conspecific with *T. lishanense*, a newly described species from northern China (Yan et al. 2018). Thus, currently no evidence supports the occurrence of either *T. nitidum* or *T. rufum* in China.

Tuber oligospermum in China was reported only from Tibet (Xu 1999). Song (2005) examined the specimen (HXZE851) cited by Xu (1999), and found the exoperidium of ascomata of this specimen is pseudoparenchymatous. She thus considered this specimen to not be *T. oligospermum*, but a species related to *T. californicum*. *Tuber oligospermum* is mainly distributed around the Mediterranean Sea (Moreno-Arroyo et al. 2000, Alvarado et al. 2012), and morphologically this Mediterranean species is highly similar to several Chinese species including *T. lijiangense* and *T. shii*.

Tuber polyspermum was described from China based on morphological evidence (Fan et al. 2011b). However, the molecular data of this species is confused, and we failed to re-extract any of the DNA from the type specimen in this study. Considering the fact that it is very similar to *T. taiyuanense* and its allies in morphology and occupies the same geographic region, we prefer to treat this species as a doubtful species.

Tuber puberulum in China was originally reported from Liaoning Province (Wang 1988), then from Sichuan Province (Zhang 1990) and Hubei Province (Song 2005). We could not study the specimen (IFS. Y. Wang 86748) cited by Wang (1988) from Liaoning. Zhang's specimen from Sichuan could not be traced, but some specimens (HMAS60225, HMAS60226) from the same locality at the same time represent *T. polymorphosporum* as revealed in this study. The specimen HMAS60233 from Hubei cited by Song (2005) was described as *T. hubeiense* (Fan et al. 2016b). Moreover, our present study revealed that two sequences (DQ478638 and DQ478639) based on the collections from Gansu Province and marked as *T. puberulum* by Wang (Wang et al. 2007) are respectively identified as *T. luyashanense* and *T. alboumbilicum* in this study (Fig. S3). Thus, the occurrence of *T. puberulum* is not confirmed in China.

Tuber rapaeodorum in China was originally reported by Dr Y. Wang from the eastern area of Liaoning Province (Wang 1988). However, our present analysis showed that one of his specimens (IFS Y. Wang 89719, ITS = DQ478640) represents an undescribed species together with three samples misidentified as *T. borchii* in the Asian clade of *Latisporum*, and closely related to *T. elevatireticulatum* from Taiwan (Lin et al. 2018) and *Tuber* sp. 10 from Japan (Kinoshita et al. 2011). Here it is temporarily identified as *Tuber* sp. CHN-7, and remains undescribed until additional specimens are collected (Fig. S3).

Tuber xizangense was described from Tibet (Xu 1999). Song (2005) re-examined the type specimen and treated it as a doubtful species as she could not find ascospores in the specimen. No DNA data are available for this species, so we follow Song and treat this species as doubtful.

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Supplementary material

Fig. S1 Phylogeny of Chinese *Tuber* species of *Aestivum* phylogroup inferred from the Dataset II (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Fig. S2 Phylogeny of Chinese *Tuber* species of *Excavatum* phylogroup inferred from the Dataset III (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Fig. S3 Phylogeny of Chinese *Tuber* species of *Latisporum* phylogroup inferred from the Dataset IV (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Fig. S4 Phylogeny of Chinese *Tuber* species of *Macrosporum* phylogroup inferred from the Dataset V (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Fig. S5 Phylogeny of Chinese *Tuber* species of *Maculatum* phylogroup inferred from the Dataset VI (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Fig. S6 Phylogeny of Chinese *Tuber* species of *Melanosporum* phylogroup inferred from the Dataset VII (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Fig. S7 Phylogeny of Chinese *Tuber* species of *Puberulum* phylogroup inferred from the Dataset VIII (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Fig. S8 Phylogeny of Chinese *Tuber* species of *Rufum* phylogroup inferred from the Dataset IX (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Fig. S9 Phylogeny of Chinese *Tuber* species of *Turmericum* phylogroup inferred from the Dataset X (ITS) using the maximum likelihood (ML) analysis. Bootstrap values over 70 % are shown above the individual branches. Thick black branches received Bayesian posterior probabilities ≥ 0.99. New species and new sequences are printed in **bold**.

Table S1 Taxa for multiple-gene analysis used in the presented study and GenBank accession numbers of their sequences. Sequences newly generated for this study are in **bold**.

Table S2 Chinese *Tuber* collections used in the presented study and GenBank accession numbers of their ITS sequences. Sequences newly generated and new species are both in **bold**. (? misidentified collections in previous study).

Table S3 International *Tuber* collections (except China) used in the presented study and GenBank accession numbers of their ITS sequences.