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Two new truffle species, *Tuber japonicum* and *Tuber flavidosporum* spp. nov. found from Japan

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ABSTRACT

We describe and illustrate two new species, *Tuber japonicum* and *Tuber flavidosporum*, based on molecular and morphological analyses. *Tuber japonicum* is characterized by its two-spored asci and pale yellow irregularly reticulate ascospores. *Tuber flavidosporum* has one ascospore per ascus, which is similar to some related species in China, but is distinguishable by its large reticulate ornamentation. Molecular phylogenetic analysis also supports *T. japonicum* and *T. flavidosporum* as new species.

1. Introduction

The genus *Tuber* F.H. Wigg. belongs to the Tuberaceae in Pezizales, Ascomycota. Fruit bodies of some *Tuber* species are known as "truffles", which are highly prized due to their aromatic qualities. Two European species in particular, *Tuber melanosporum* Vittad. (Perigord black truffle) and *Tuber magnatum* Pico (Piedmont white truffle), are among the most famous truffles in the world. The genus is also known to form ectomycorrhizal associations and help the growth of major forest tree species such as the Betulaceae, the Fagaceae and the Pinaceae (Zambonelli and Bonito 2013). Because of its economic and ecological importance, the taxonomy of *Tuber* has attracted much attention.

Index Fungorum lists 290 species, subspecies and varieties, but these could potentially include many synonyms. A recent molecular phylogenetic study suggests that the genus comprises at least 180 species (Bonito et al. 2010). *Tuber* diversity is relatively well documented in Europe (Ceruti et al. 2003), North America (Trappe et al. 2009) and China (e.g., García-Montero et al. 2010), but the species diversity in other regions is poorly understood. In Japan, the first public report on *Tuber* was released in 1976 (Trappe 1976), and described *T. californicum*

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Harkn. Later, a few local reports noted the records of European and North American species (e.g., *T. aestivum* (Wulfen) Spreng., *T. californicum* and *T. magnatum*; e.g., Yoshimi 2008) in Japan, although their taxonomical identity is uncertain. Recently, we have conducted molecular phylogenetic analyses for the 186 ascomata collections newly sampled in Japan from 1999 to 2008. This study demonstrated that the Japanese truffles include at least 20 species, most of which are different from European and North American species and likely to be new species (Kinoshita et al. 2011). Moreover, we found a new phylogroup, the Japonicum group, composed of only two Japanese species (*Tuber* sp. 8 and sp. 9) and distantly related to other known phylogroups. Common morphological characteristics of the phylogroup are whitish to pale yellow colored ascocarp, and one or two ascospores per ascus. Here, we formally describe these two Japanese species based on detailed morphological and molecular phylogenetic analyses.

2. Materials and methods

2.1. Morphological observations

Morphological observation was performed for the collections of fresh and dried ascocarp specimens used in our phylogenetic study (Kinoshita et al. 2011) and a few additional samples. For macroscopic characteristics, we observed ascocarp size, ornamentation, and colors following the Munsell system, mostly using fresh specimens. Microscopic features of fresh and dried specimens were observed in water, 3% (w/v) KOH solution, and Melzer's reagent. Photographs were taken under a light microscope and then we measured the size of fully matured ascospores and asci, peridium thickness, and other microscopic features using PhotoRuler 1.1 (http://hyogo.inocybe.info/_userdata/ruler/help-eng.html). For scanning electron microscopy (SEM), spores were scraped from the dried gleba and mounted in distilled water on a cover glass. After drying, the cover glass was pasted directly onto an SEM stub with double-sided tape, coated with gold-palladium, and photographed with a HITACHI S-4800. The examined specimens were deposited in the Mycological Herbarium of Forestry and Forest Products Research Institute Herbarium (TFM).

2.2. Phylogenetic analyses

In the previous study, we demonstrated that the two *Tuber* species were phylogenetically distinct from the known species based on four nuclear loci (Kinoshita et al. 2011). To include recent sequence data of potentially related species described later (e.g., Fan et al. 2015) in our phylogenetic analyses, we conducted BLAST searches (blastn; Altschul et al. 1997) and retrieved related sequences from the International Nucleotide Sequence Database (INSD, DDBJ/EMBL/GenBank databases; Table 1). In the internal transcribed spacer (ITS) data set, we included all sequences for each of the two species and their closest match sequences found in the INSD. We aligned the ITS data set using MAFFT 7 (Katoh and Standley 2013) with default settings, and poorly aligned sites were identified using Gblocks 0.91B (Castresana 2000) with a minimum block-length parameter of 5 and with gaps allowed in conserved blocks, with all other parameters left at default values. All identified ambiguous sites were excluded before phylogenetic analyses. Maximum likelihood (ML) analyses were conducted with PhyML 3.0 (Guindon et al. 2010) using a GTR + G6 + I model, which was selected by Smart Model Selection (SMS) implemented in PhyML to determine the best substitution model. The approximate likelihood ratio test (aLRT), SH-like fast likelihood-based optimization method was used to evaluate branching support. The ML tree was visualized with MEGA 6 (Tamura et al. 2013).

To evaluate the effect of different phylogenetic approaches, we also conducted Bayesian phylogenetic analyses with MrBayes 3.2.1 (Ronquist et al. 2012). In the Bayesian analysis, after selecting the best substitution models as determined by SMS (GTR + G + I), we ran two independent MCMC chains, sampling every 100th tree until the standard deviation of the split frequency (ASDSF) became <0.01. The log files of MrBayes were further analyzed using Tracer 1.6 (Rambaut et al. 2014) to check the effective sample sizes, which were always >100, indicating sufficient independent sampling to estimate the posterior distribution of each parameter. The first 10% of the sampled trees were discarded as burn-in. The remaining 1502 trees (751 trees from each run) were used to construct a 50% majority rule consensus tree and the consensus trees were visualized with FigTree 1.4 (Rambaut 2014). The ITS alignment file was deposited in TreeBASE (Accession No. 18938).

3. Results

3.1. Taxonomy

Tuber japonicum H. Sasaki, A. Kinosh. & Nara, sp. nov. Fig. 1. MycoBank no.: MB815829.

Ascomata subglobose or lobed, firm, whitish to pale yellow, 10–40 mm in diam. Peridium smooth, two layers, the outer layer pseudoparenchymatous and composed of irregular cells, the inner layer composed of interwoven hyphae. Gleba whitish to pale yellow marbled with white sterile veins. Asci (1–)2(–3)-spored, 75–133 × 47–106 μm, spindle-to obovate-shaped. Ascospores globose, whitish to yellow, 25–50 μm in diam excluding reticulate ornamentation.

Type: JAPAN, Miyagi Prefecture, Sendai, under *Pinus densiflora* Siebold et Zucc. and *Quercus serrata* Murray trees, 6 Nov 2007, collected by Hiromi Sasaki and Kazuhide Nara, N88 (holotype, TFM: S16001). Paratype, TFM: S16002–S16011.

DNA sequences: holotype AB553444 (ITS); paratype AB553433–AB553434, AB553436–AB553441, AB553443, AB553445 (ITS), AB553519 (LSU), AB553539 (TEF-1α), AB553559 (RPB2).

Etymology: *japonicum* (Lat.), referring to the species' occurrence in Japan only (Japanese name "*Honseiyoshoro*" from *Hon-* = Japan, the type locality, *seiyoshoro* = Japanese name for the genus *Tuber*).

Ascomata hypogeous, subglobose to irregular and lobed, whitish or pale straw yellow when young, brown to dark brown at maturity, 10–40 mm in diam. Odor garlicky. Peridium smooth, 240–280 μm thick, composed of two layers: