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The Asian black truffle *Tuber indicum* can form ectomycorrhizas with North American host plants and complete its life cycle in non-native soils

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ABSTRACT

The Asian black truffle *Tuber indicum* is morphologically and phylogenetically similar to the European black truffle *Tuber melanosporum*. *T. indicum* is considered a threat to *T. melanosporum* trufficulture due to its presumed competitiveness and broad host compatibility. Recently, in independent events, *T. indicum* was found fruiting in a forest in Oregon, USA, and was detected as ectomycorrhizas within a truffle orchard established with trees believed to have been inoculated with *T. melanosporum*. We used haplotype networking to assess intraspecific ITS rDNA diversity among Asian and North American *T. indicum* group B isolates. To further assess the potential of *T. indicum* to spread onto native host plants it was inoculated onto seedlings of loblolly pine (*Pinus taeda*) and pecan (*Carya illinoensis*, Juglandaceae), species endemic to North America. *T. indicum* formed ectomycorrhizas on both host species examined. This supports previous studies from Europe and Asia that indicate *T. indicum* has a broad host spectrum, an ecological trait that may be important to its invasion ecology. This is the first report of *T. indicum* introductions in North America and of this species fruiting outside of its native range. To help prevent further unintended truffle introductions we recommend that fruitbodies used by the truffle industry for inoculating seedlings first be identified with DNA methods.

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Introduction

Species of truffles in the genus *Tuber* are eagerly sought due to their culinary qualities and highly valued fruit bodies (Mello *et al.* 2006). Among them is *Tuber melanosporum*, one of the few ectomycorrhizal fungal species successfully cultivated by humans. This has led to the establishment of truffières (truffle orchards) on several continents (Wang & Hall 2004; Dominguez *et al.* 2006). Unwanted exotic or competitor species introduced during the establishment phase of a truffière could have economic and ecological consequences.

Whereas invasions of fungal pathogens such as chestnut blight (*Cryphonectria parasitica*) and Dutch elm disease (*Ophiostoma novo-ulmi*) resulting in devastating effects are well documented (Desprez-Loustau *et al.* 2007; Loo 2009), invasions by mutualistic fungi are less understood and more easily overlooked (Vellinga *et al.* 2009). Because so little is known about the geographic ranges fungi inhabit, a major aim of current taxonomic research is to use molecular phylogenetic approaches to better assess species distributions and their changes associated with human activity (Pringle *et al.* 2009; Bonito *et al.* 2010; Wolfe *et al.* 2010).

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The European black truffle *T. melanosporum* is endemic to Mediterranean regions of southern Europe including Spain and France (Hall et al. 2007; Agueda et al. 2010). It grows in calcareous soils forming symbiotic ectomycorrhizal association particularly with deciduous trees (e.g. *Quercus* spp. and *Corylus avellana*) (Murat et al. 2004; Hall et al. 2007). Since the 1970's *T. melanosporum* has been cultivated in Europe by planting seedlings produced with ectomycorrhizas of *T. melanosporum* into prepared fields (Grente et al. 1972; Boutekrabort et al. 1990).

Evidence of black truffles fruiting in North America was absent prior to the 1980's when the cultivation of *T. melanosporum* in California was first reported (Rigdon 1994). However, recently a native black truffle species, *Tuber regimontanum*, was described from an undisturbed montane oak forest near Monterrey, Mexico, providing the first evidence of the *T. melanosporum* lineage in North America (Guevara et al. 2008). *T. regimontanum* is phylogenetically distinct and basal to its closest known relatives, *T. melanosporum* and *Tuber indicum*, and is distinguished morphologically by having larger spores ornamented with spines connected by low reticulations.

The Asian black truffle *T. indicum* was originally described from the Himalayas (Cooke & Masee 1892) and is harvested commercially in the Yunnan and Sichuan provinces of China (Zhang et al. 2005; Wang et al. 2006; Wang & Liu 2009). However, recent molecular analysis indicates that *T. indicum* is composed of at least two phylogenetic species, referred to as *T. indicum* A & *T. indicum* B; their taxonomy is still in flux (Hu 1992; Zhang et al. 2005; Wang et al. 2006; Huang et al. 2009; Bonito et al. 2010; Garcia-Montero et al. 2010). Analysis of 87 *T. indicum* ITS rDNA sequences accessioned in Genbank showed >7.0% interspecific sequence divergence between *T. indicum* A and B and <2.5% intraspecific variation within each clade (Bonito et al. 2010). However, there are no apparent morphological characters that distinguish these two phylogenetic species from each other and both show high morphological intraspecific variation, broad geographic distribution, and both species fruit across a wide variety of soil types in association with host species in the Betulaceae (*Corylus* spp., *Alnus* spp.), Fagaceae (*Quercus* spp., *Castanea* spp., *Castanopsis* spp., *Lithocarpus* spp.) and Pinaceae (*Pinus* spp., *Keteleeria* spp.) (Wang et al. 2006; Garcia-Montero et al. 2008; Geng et al. 2009; Wang & Liu 2009; Garcia-Montero et al. 2010).

Organoleptic (culinary) properties of *T. indicum* are similar, if more subtle than that of *T. melanosporum*. Levels of natural *T. indicum* production are relatively high (>300 tons from Yunnan Province, China, in 2006) and *T. indicum* is sold at a significantly lower price than is *T. melanosporum* (Wang et al. 2006; Murat et al. 2008). Since the late 1990's *T. indicum* has been exported from China to Europe, North America and Australia (Garcia-Montero et al. 2010). It can be challenging to distinguish *T. indicum* from *T. melanosporum* because their morphology is similar (Fig 1). Concerns of *T. indicum* being sold as *T. melanosporum* and unintentionally used as seedling inoculum have been articulated previously (Paolocci et al. 2000; Sejalon-Delmas et al. 2000; Murat et al. 2008).

Murat et al. (2008) reported on the occurrence of *T. indicum* ectomycorrhizas in an Italian *T. melanosporum* truffière. They suggest that fruitbodies of *T. indicum*, misidentified as *T. melanosporum*, were used to inoculate seedlings. *T. indicum* has been shown to form ectomycorrhizas with a variety of

European tree hosts including *Quercus* spp. and *Pinus pinea* indicating the potential of this species to invade European ecosystems (Zambonelli et al. 1998; Garcia-Montero et al. 2008).

Here we document the occurrence of *T. indicum* B from two locations in North America based on ITS rDNA sequence data generated from fruit bodies and ectomycorrhizas. We verify that six different ITS haplotypes of *T. indicum* B have been introduced to North America and demonstrate that *T. indicum* B forms ectomycorrhizas on North American angiosperm and gymnosperm hosts.

Methods

Truffle collections

Members of the North American Truffling Society (NATS) have been collecting truffles in Oregon since its founding in 1978. The basic technique to search for truffles involves the use of a multi-tined garden cultivator or hand rake for removing leaf litter and scratching into the mineral as deep as 15 cm. Forests composed of appropriate ectomycorrhizal hosts and animal diggings are targeted. Because of the difficulty in identifying truffle species such as *Tuber* based on morphology alone, molecular methods are often needed to identify taxa and to place them in the fungal phylogeny (Bonito et al. 2009).

Sampling orchard ectomycorrhizas

To determine whether *T. melanosporum* persisted as ectomycorrhizas on roots of trees inoculated with *T. melanosporum*, root samples were taken from seven private truffle orchards during the summers of 2006–2008. Permissions were granted with the request of anonymity. Orchards were located in North Carolina, Tennessee and California, USA, and had been established between 4 and 15 yr prior to sampling with trees purchased from companies specializing in the production of *T. melanosporum* infected seedlings. Inoculation methods are often regarded as proprietary or trade secrets. In one case, however, trees had been inoculated by the orchard owner using a slurry of spores, similar to the method described below. At least ten trees were sampled from each orchard by harvesting one to three 30 cm sections of fine roots from each tree. In the orchard where *T. indicum* was detected a second broader sampling of 150 trees was conducted to assess the prevalence of *T. indicum* within the orchard. Collected roots were soaked in tap water for 1 hr to loosen soil debris, rinsed on a 1 mm sieve and examined under a stereoscope. First, ectomycorrhizas were screened morphologically and sorted as *Tuber* and non-*Tuber*. The identity of *Tuber* morphotypes and a subset of non-*Tuber* morphotypes were determined through specific-primer PCR assays and DNA sequence analyses on one to eight single root tips as described below.

Inoculating North American pine and pecan seedlings with Asian truffles

A greenhouse study was conducted to determine the propensity of *T. indicum* to form ectomycorrhizas with

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