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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

Gregory BONITO[a,*], *James M. TRAPPE*[b], *Sylvia DONOVAN*[c], *Rytas VILGALYS*[a]

[a]*Department of Biology, Duke University, Durham, NC 27708 0338, USA*
[b]*Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR 97331 5752, USA*
[c]*North American Truffling Society, PO Box 296, Corvallis, OR 97339, USA*

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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 illinoinensis*, 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.

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).

* *Corresponding author.*
E-mail address: gregory.bonito@duke.edu (G. Bonito).
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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; Boutekrabt *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 & Massee 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

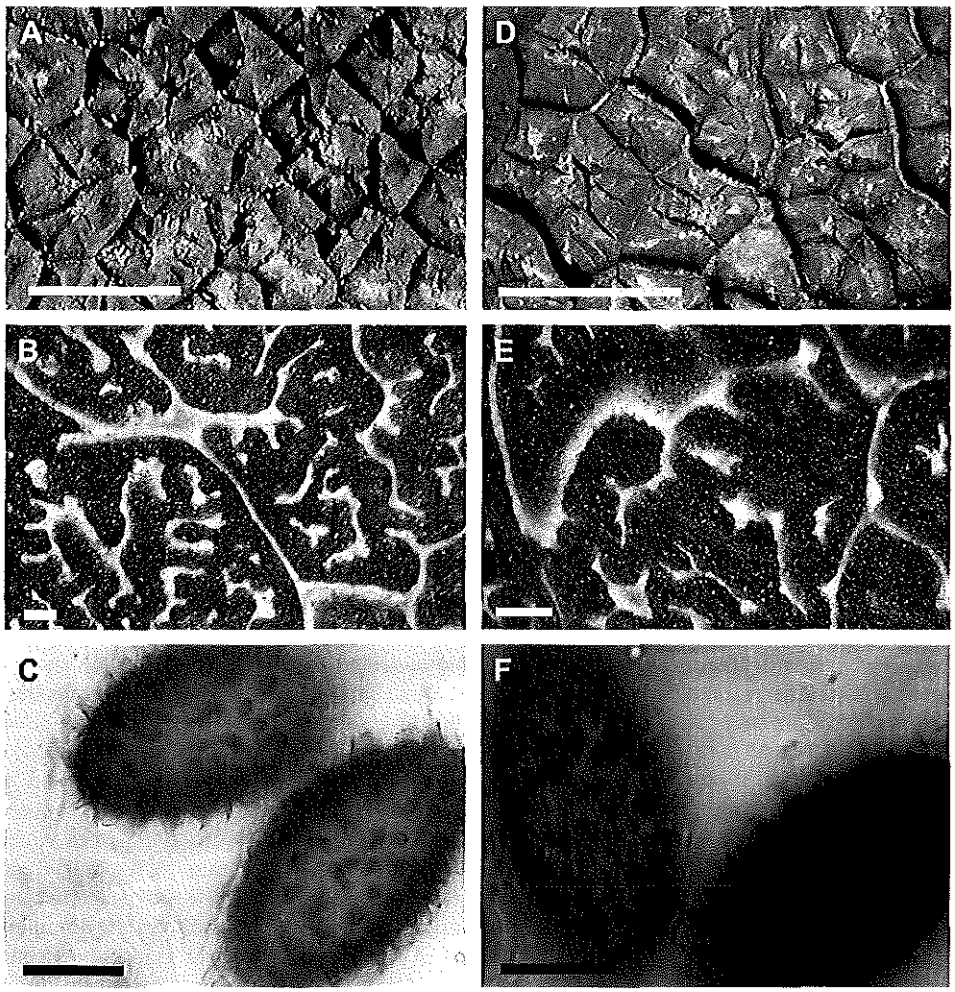

Fig 1 – Fruitbodies of the Asian black truffle species *Tuber indicum* group B (A–C) and the European black truffle species *Tuber melanosporum* (D–F) are morphologically similar. Flat to angular polygonal warts ornament their peridium (A & D). Fertile glebal tissue is reddish-brown to black in color with narrow white veins of sterile tissue weaving throughout (B, E). Their spores are ellipsoid in shape and are ornamented with spines (C, F), however there is some variation in these characters and in some cases spores of *T. indicum* may have blunt spines or reticulated ridges connecting the spines. On average, *Tuber melanosporum* spores have a higher density of spines than do spores of *T. indicum*. Photos are of the following collections: A, B = GB230, C = JT29410, D–F = GB253. Scale bars: A, D = 1 cm; B, E = 1 mm; C, F = 10 μm.

angiosperm (pecan) and gymnosperm (loblolly pine) hosts. These North American host species are economically important as nut and timber crops, respectively, particularly in the Eastern US where a majority of the *T. melanosporum* orchards in North America have been established. These tree species are now grown in exotic locations because of their economic importance (Wakeling *et al.* 2001; Giachini *et al.* 2004; Poorbabaei & Poorrahmati 2009). Consequently, their ability to form ectomycorrhizas with non-native fungi is of practical interest.

Pecan seeds were collected from a pecan orchard in North Carolina and were stored at 4 °C prior to stratification. Pecan seeds were stratified by soaking in tap water for 10 d at room temperature, changing the water daily to maintain aerobic conditions (Adams & Thielges 1978). Pine seeds were obtained through the North Carolina State Cooperative Tree Improvement Program. For stratification, seeds were soaked for 24 hr in tap water and were then placed in a zip-lock bag and stored at 4 °C for 30 d. After stratification, seeds were surface sterilized in a 6 % H_2O_2 for 10 min and set in sterile perlite. Bottom heat (95 °C) was provided until germination. Germinants were placed under florescent lights once the apical meristem emerged.

T. indicum sporocarps were purchased from China early in 2009. Because they were obtained from a commercial source their specific geographic origin remains unknown. Upon arrival the truffles were soaked in cold water for 1 hr to remove insect larvae. They were then scrubbed with a brush to remove surface contaminants. A small sample from each specimen was taken as a voucher and for DNA analysis. The truffles were then frozen until use. Species identifications were determined by DNA sequence analysis of ITS and LSU rDNA.

For inoculations, truffles were sterilized in 6 % H_2O_2 for 10 min and rinsed three times in tap water. A spore-slurry was made by blending chopped truffles for 3–5 min in deionized water and crushed ice, to prevent overheating the spores. The spore-slurry was thoroughly mixed into double autoclaved soil-less media composed of peat, vermiculite, perlite and crushed limestone (1:2:2:0.5) (Michaels 1982; Hall *et al.* 2007). Each tree was inoculated at a concentration of 1 g of crushed truffle fruitbody. Uninoculated control seedlings were grown along-side inoculated seedlings. Crushed limestone was placed on top of the potting media to keep the soil in place during watering. Seedlings were watered 3 times per week with 1/10 Hoagland's solution (Hoagland & Snyder 1933) modified to include a chelated iron source (Supplementary Table 2). Seedlings were checked for mycorrhization after five months of growth.

Molecular analyses of truffles and ectomycorrhizas

DNA from dried fruit bodies and truffle orchard mycorrhizas were extracted with CTAB 2× and chloroform:isoamyl alcohol (24:1). Genomic DNA from ectomycorrhizas with *Tuber* morphology was amplified first with *T. melanosporum* specific primers designed to amplify the nuclear ITS rDNA (ITS) of this species (forward primer TTG CTT CCA CAG GTT AAG TGA; reverse primer TAA AGT CCG TCG TTC ATG C) (Bonito 2009). Samples that failed to amplify with *T. melanosporum* specific primers were amplified along with DNA extracts from dried fruit bodies and non-*Tuber* ectomycorrhizal morphotypes using the primer set ITS1f and LR5. For the ectomycorrhizal synthesis experiment, DNA of *T. indicum* fruit bodies was amplified with the primer set ITS1f & LR5 by direct PCR as described in Bonito (2009). Sections from the root system of mycorrhized plants were observed under a stereoscope. DNA was extracted from individual ectomycorrhizas with the Extract-N-Amp kit (Sigma–Aldrich). DNA extracts were amplified with the primer set ITS1f and ITS4.

PCR products were cleaned on Quiagen Quick-Clean columns. Sanger sequencing was performed by Big Dye chemistry v3.1 (Applied Biosystems, Foster City, CA) with the forward primer ITS5 or LROR, and reverse primers ITS4 or LR5. DNA sequences were determined on an ABI 3700 (Applied Biosystems, Foster City, CA). Sequences were manually edited in Sequencher4.0 (Gene Codes, Ann Arbor, MI) and ambiguous regions at the ends were trimmed. Sequences were queried against the NCBI public database Genbank with the BLASTN algorithm to assess their identification.

For phylogenetic analyses, DNA sequences of *Tuber* were manually aligned against other *Tuber* species with MacClade 4.0 (Maddison & Maddison 2002). Ambiguous regions were excluded from the alignment. Heuristic searches with unweighted parsimony were conducted in PAUP* 4.0b10 with 1000 random addition sequences and 5000 bootstrap replicates (Swofford 2002). *Tuber excavatum* was chosen as an outgroup based on previous phylogenetic studies on *Tuber* (Jeandroz *et al.* 2008; Bonito *et al.* 2009). The computer program TCS (Clement *et al.* 2000) was used to examine ITS rDNA haplotype diversity of *T. indicum* B from both inside and outside of Asia. Sequences generated in this study have been deposited in Genbank under the accession numbers FJ48899–FJ748914 and HM849635–HM849658. Collection information is provided in Table 1.

Results

In 2004, in the McDonald-Dunn Research Forest of Oregon State University, a single black truffle (Trappe 29410, leg. Sylvia Donovan) was collected from a *Pseudotsuga menziesii* (Douglas-fir) forest with an understory of *Corylus cornuta* var. *californica*. The only truffières from this vicinity are over 10 km away and are too young (<5 yr) to produce truffles yet. Members of the North American Truffling Society have collected hypogeous fungi in this forest for more than 25 yr, but no other black truffles have ever been found. Subsequent visits to the collection site since 2004 have not turned up black truffles. The ITS sequence of this collection matched over 99 % to other Genbank accessions of *T. indicum* B but appears to be a unique ITS haplotype from those in Genbank (Fig 2).

In 2006, a survey of truffle orchard roots revealed *T. indicum* B ectomycorrhizas on eight of 150 sampled trees, which included both *Corylus* and *Quercus* species. *T. melanosporum* was not detected on any of the trees colonized by *T. indicum*. Five distinct haplotypes of *T. indicum* B were detected in the orchard (Fig 3). Haplotype network analysis (Fig 3) demonstrates ITS haplotype diversity for *T. indicum* B is high. For instance, 19 distinct haplotypes were recovered from 41 *T. indicum* B sequences analyzed. Asia showed the greatest haplotype diversity and most divergent haplotypes, which were five steps removed from the dominant haplotype positioned at the network hub. In contrast, haplotypes from North America were at most two steps removed from the dominant haplotype (Fig 3). A single haplotype was shared between North America and Asia. The haplotype recovered from Europe by Murat *et al.* (2008) was identical to the most common Asian haplotype.

Although ectomycorrhizas were prevalent in sampled roots, *T. melanosporum* was not always detected. Non-target ectomycorrhizal species identified in the samples from North American truffle orchards included other (even novel) species of *Tuber* (Fig 2), *Thelephora/Tomentella* spp., *Hebeloma* spp., *Tarzetta* spp., *Pachyphloeus* spp., *Cortinarius* spp. and *Inocybe* spp. (Supplementary Table 1).

DNA sequencing of sporocarps purchased from China revealed that they consisted of a mixture of *T. indicum* A (FJ748906) and *T. indicum* B (FJ748907). Because the presumed introductions of *T. indicum* in Europe and North America involved *T. indicum* B, sporocarps of this species were used in inoculations. After five months *T. indicum* ectomycorrhizas had formed on both *Pinus taeda* (Pinaceae) and *Carya illinoinensis* (Juglandaceae) seedlings (Fig 4). The identification of ectomycorrhizas was confirmed by morphology (Fig 4) and ITS sequence data (Fig 2). Ectomycorrhizas produced by *T. indicum* B on *C. illinoinensis* were unramified to irregularly pinnate, and on *P. taeda* were unramified to dichotomously pinnate. On both hosts, *T. indicum* ectomycorrhizas were dark amber in color and showed characteristic puzzle-like pseudoparenchyma cells in the outer mantle with emanating right-angle branching cystidia, as reported from other hosts (Garcia-Montero *et al.* 2008; Geng *et al.* 2009). Few cystidia were observed on *C. illinoinensis* ectomycorrhizas.

Table 1 – Collection information for specimen and sequences used in this study

Species name	Source	Voucher	Location	Genbank #	Citation
T. brumale	Fruitbody	GB53	Emilia-Romagna, Italy	FJ748900	[a]
T. brumale	Fruitbody		Marche, Italy	AF106880	Zhang *et al.* 2005
T. candidum	Mycorrhiza		CA, USA	AY634170	Bidartondo *et al.* 2004
T. excavatum	Fruitbody	BM100	Spain	FJ748899	[a]
T. ferrugineum	Fruitbody		France	AF132506	Roux *et al.* 1999
T. indicum	Mycorrhiza		Piedmont, Italy	AM932205	Murat *et al.* 2008
T. indicum	Mycorrhiza		USA	FJ748901	[a]
T. indicum	Fruitbody	JT29410	OR, USA	FJ748902	[a]
T. indicum	Fruitbody		Sichuan, China	DQ375501	Wang *et al.* 2006
T. indicum	Fruitbody		China	Y09791	Zhang *et al.* 2005
T. indicum	Fruitbody	GB236	China	FJ748906	[a]
T. indicum	Fruitbody	GB237	China	FJ748907	[a]
T. indicum	Fruitbody	GB240	China	FJ748908	[a]
T. indicum	Fruitbody		China	U89361	Paolocci *et al.* 1997
T. indicum	Fruitbody		China	DQ375524	Wang *et al.* 2006
T. lyonii	Fruitbody	GB108	GA, USA	FJ748910	[a]
T. lyonii	Fruitbody	GB119	VA, USA	FJ748911	[a]
T. melanosporum	Mycorrhiza		NC, USA	FJ748903	[a]
T. melanosporum	Fruitbody		Spain	AJ583657	Murat *et al.* 2004
T. melanosporum	Fruitbody	GB120	VA, USA	FJ748904	[a]
T. melanosporum	Mycorrhiza		CA, USA	FJ748905	[a]
T. melanosporum	Fruitbody		Spain	AJ583631	Murat *et al.* 2004
T. pseudoexcavatum	Fruitbody		Yunnan, China	AY514310	Zhang *et al.* 2005
T. regimontanum	Fruitbody		Monterrey, Mexico	EU375838	Guevara *et al.* 2008
T. rufum	Fruitbody		Vaucluse, France	DQ329375	Wang *et al.* 2006
T. spinoreticulatum	Fruitbody	Uecher188	MD, USA	FJ748913	[a]
T. spinoreticulatum	Fruitbody	RH721	IA, USA	FJ748914	[a]
Tuber spp. 54[b]	Mycorrhiza		Perugia, Italy	DQ402504	Baciarelli-Falini *et al.* 2006
Tuber spp. 57[b]	Mycorrhiza		USA	FJ748909	[a]
Tuber spp. 57[b]	Mycorrhiza		CA, USA	AY634169	Bidartondo *et al.* 2004
Tuber spp. 61[b]	Mycorrhiza		NC, USA	FJ748912	[a]

a This study.
b Represent undescribed species.

Discussion

Discovery of the Asian truffle species T. indicum *in North America*

Demonstrating whether species have been introduced into exotic locations is not trivial. It is even more challenging when distribution and biogeography data are incomplete. Although mutualists are more likely to go undetected when introduced compared with pathogens, which may cause landscape-scale die-off, introductions of mutualists can also impact carbon and nutrient cycling (Chapela *et al.* 2001; Desprez-Loustau *et al.* 2007). To develop prevention or eradication measures, therefore, it is important to document introduction events early.

In this study, we infer from ITS rDNA that at least two independent introductions of the Asian black truffle *T. indicum* B have occurred in North America. *Tuber* is a diverse ectomycorrhizal lineage characterized by high levels of continental endemism and few shared taxa between continents (Bonito *et al.* 2010). Most *Tuber* species differ by at least 4 % in the ITS rDNA (Bonito *et al.* 2010). All ITS sequences of *T. indicum* from North American isolates shared >99 % sequence similarity to sequences from Asian isolates. We hypothesize that *T. indicum* in North America is the result of recent (human-mediated) long distance dispersal as opposed to a relic geographic disjunct. This is also supported by observations that: (1) ITS sequences of other *Tuber* species show higher levels of divergence at the *plot level* (Smith *et al.* 2007; Jumpponen *et al.* 2010); and (2) isolates of *T. indicum* B from mainland China and Taiwan differ at their ITS by >1 % and show some geographic structure (Huang *et al.* 2009). Further, one haplotype was shared between North America and Asia and haplotype diversity was greatest in Asia, as would be predicted for a source population. We expect additional haplotype diversity will be discovered as more Asian isolates are sequenced.

In the first case, *T. indicum* was found in the McDonald-Dunn Research Forest fruiting among host plants native to North America (e.g. *Pseudotsuga menziesii* and *C. cornuta*), although ectomycorrhizal evidence is still needed to verify the host plant. Despite 25 yr of active collecting in the McDonald-Dunn Research Forest it has been found only once. If it was introduced, where did it come from, and how did it get to the collection site?

We hypothesize that this species was introduced through the Peavy Arboretum, located in the McDonald-Dunn Research Forest, about 2 km from the *T. indicum* collection site. Established in 1926, the arboretum has a few ectomycorrhizal Asian tree species from within the distribution of *T. indicum* (and probably

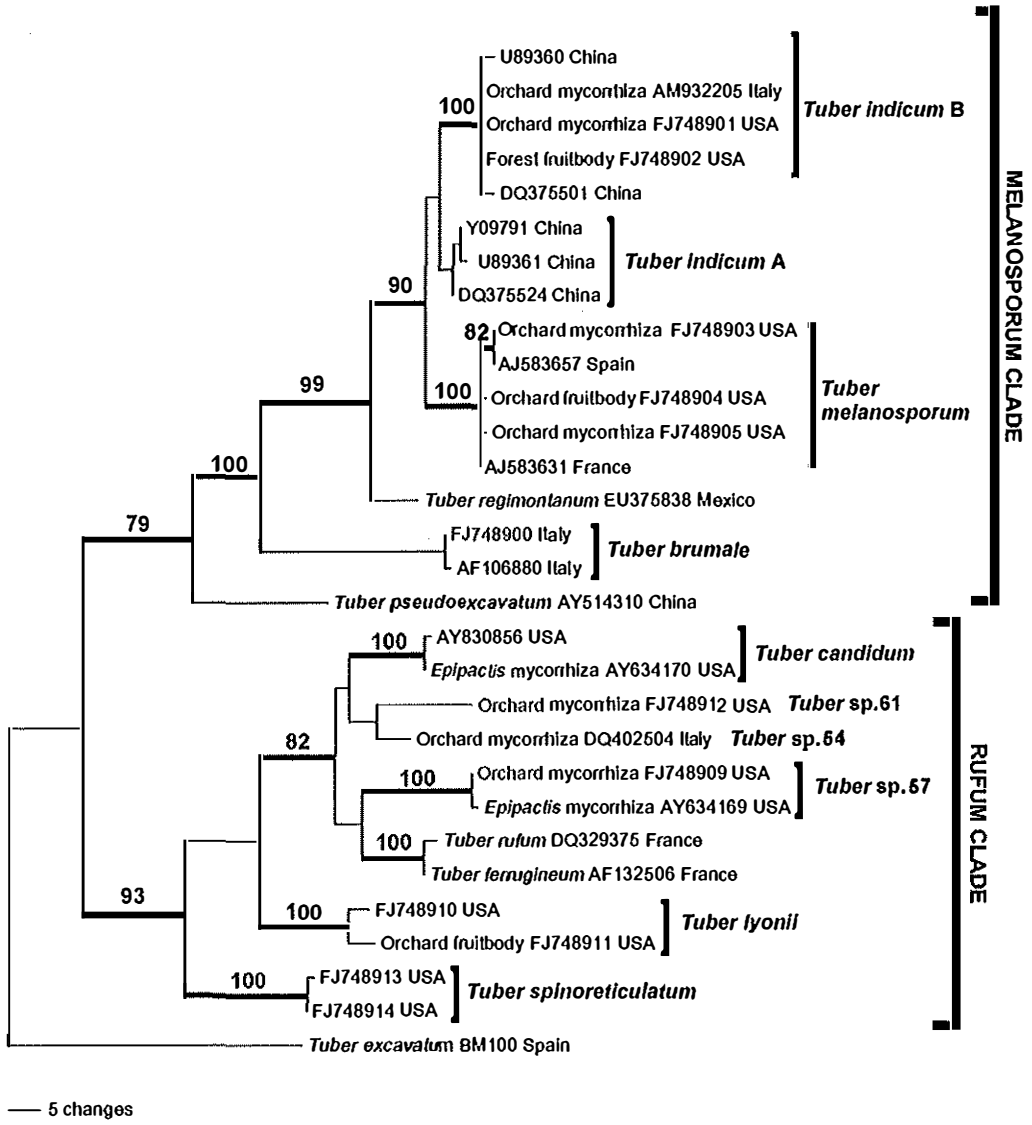

Fig 2 – One of 40 most parsimonious (MP) trees of spiny-spored truffles (Rufum and Melanosporum clades) obtained after 1000 random sequence additions by use of internal transcribed spacer ribosomal DNA (rDNA) for 31 taxa and with 113 parsimony-informative characters (332 steps; CI = 0.657; RI = 0.869). Bootstrap support based on 1000 replications is shown at the nodes. Thickened branches represent nodes having high statistical support (> 70 %). Taxa are labeled with their Latin binomial (or environmental source), Genbank number and location. Colonies of *Tuber melanosporum* and *T. indicum* (group B) mycorrhiza have been established in North America and fruit bodies have been collected. Mycorrhizas and fruit bodies from other native *Tuber* species in the Rufum clade have also been found in North American truffle orchards. These include *Tuber lyonii* (a commercial species with an established market in the Southeastern US) and 2 species that have yet to be described (*Tuber* sp. 57 and *Tuber* sp. 61) are only known from mycorrhizas.

others that are no longer present). Some of the early plantings could have been of *T. indicum*-bearing seedlings imported from Asia prior to plant quarantine measures. Unfortunately records for those early arboretum years were not kept.

In a separate case we detected ectomycorrhizas of *T. indicum* in a truffle orchard established with seedlings of European *Corylus* and *Quercus* spp. inoculated with what was thought to be *T. melanosporum*. In Asia *T. indicum* (A and B) are associated with a broad diversity of ectomycorrhizal gymnosperm and angiosperm hosts (Riousset *et al.* 2001; Ceruti *et al.* 2003; Geng *et al.* 2009). *T. indicum* has also been shown to form ectomycorrhizas with European species of *Pinus* and *Quercus* (Zambonelli *et al.* 1998; Garcia-Montero *et al.* 2008). In our inoculation experiment, ectomycorrhizas of *T. indicum* B formed on both *C. illinoinensis* and *P. taeda* (Fig 4). As far as we know this is the first time mycorrhizas of this species have been synthesized with North American hosts or with a member of the Juglandaceae. Taken together these data support previous assertions of

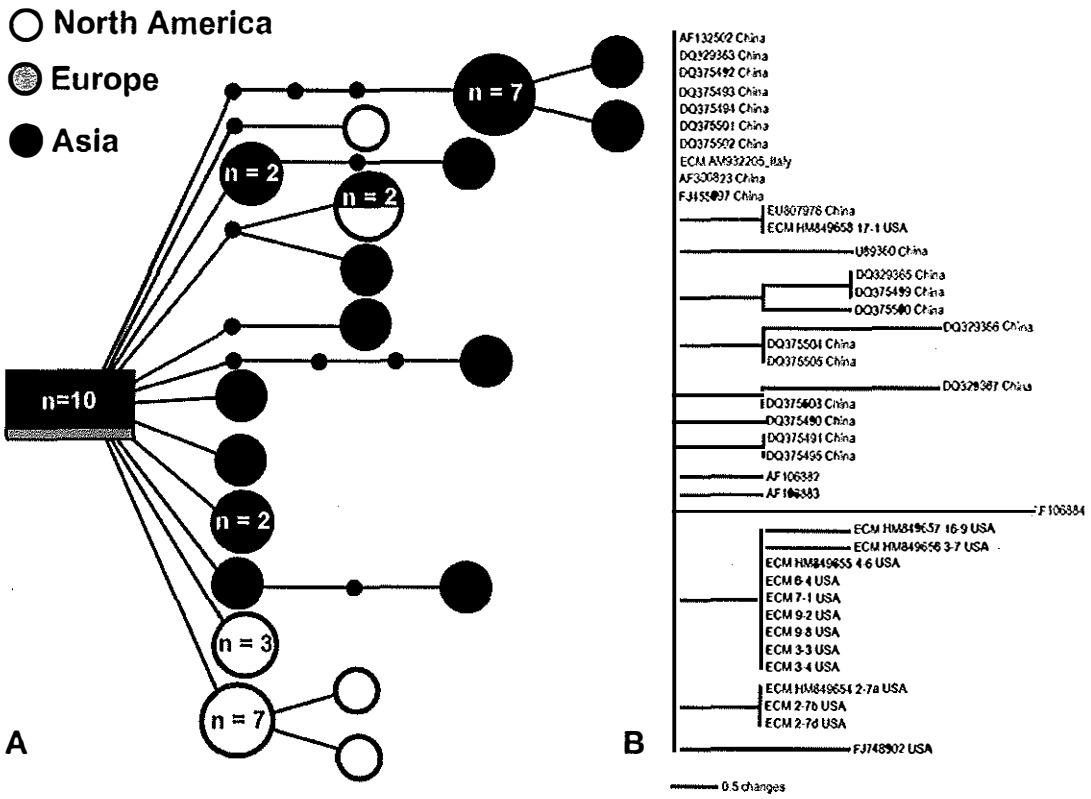

Fig 3 – A, Haplotype network of *Tuber indicum* B based on parsimony analysis of 41 ITS rDNA sequences from this study and others available through GenBank. Nineteen distinct haplotypes were detected. A total of 14 haplotypes were from Asian isolates, six from North American isolates, and one from Europe. One haplotype was shared between Asia and North America samples and one was shared between Asia and Europe. The area of each circle relates to the number of sequences represented in that haplotype cluster, as shown inside the circle for haplotypes represented by more than a single sequence. Haplotypes from Europe and North America are putatively introduced. In the limited sampling from Asia, *T. indicum* shows considerable intraspecific ITS variation. B, An alternative tree view of the *T. indicum* B ITS haplotype network. Taxa included in this most parsimonious tree are labeled by their geographic origin (when known) and Genbank number. Sequences from ectomycorrhiza are labeled ECM.

T. indicum's broad host spectrum, an ecological trait that may be important to its invasion ecology.

Whether or not *Tuber* species are more predisposed to invasiveness than are other mycorrhizal fungi is not known, but we have provided evidence that *T. melanosporum* and *T. indicum* have become established and have completed their life cycle in North American soils. In some instances ectomycorrhizal colonization by *T. melanosporum* persisted for more than 8 yr after planting, demonstrating the ability of this species to perennate on roots in non-native soils. In other instances *T. melanosporum* was not detected at all and may have been displaced by native ectomycorrhizal species. There is no indication that *T. melanosporum* has spread into North American forests, but *T. melanosporum* is capable of forming ectomycorrhizal associations with the North American hosts *Quercus douglasii* and *Quercus garryana* (Michaels 1982). *T. melanosporum* has also been shown to form ectomycorrhizas with Southern hemisphere hosts *Nothofagus obliqua* and *Nothofagus glauca* and Asian hosts *Quercus aliena* and *Castanea mollissima* (Pérez *et al.* 2007; Wang & Liu 2009). We detected non-target *Tuber* species and a diversity of Thelephoraceae species in the ectomycorrhizal community of truffle orchards, consistent with previous studies that have used DNA sequencing to identify non-target ectomycorrhizal species in truffle orchards (Murat *et al.* 2005; Baciarelli-Falini *et al.* 2006; Pruett *et al.* 2008; Napoli *et al.* 2010).

DNA testing of truffle inoculum to prevent unintended Tuber *introductions*

Establishing *T. melanosporum* truffières is a costly, long-term investment. Growth of other truffle species at the expense of *T. melanosporum* could lead to substantial economic losses. *T. melanosporum* has a long-standing reputation of superior culinary quality and its economic value far exceeds that of the Asian species *T. indicum*. Still, it can be difficult to distinguish black truffle species by morphology alone and without use of molecular tools truffle seedling inoculators risk using misidentified truffles as tree inoculum. Thereby, unwanted species may inadvertently be planted into non-native habitats.

Truffles used as inoculum are often obtained from a commercial supplier, and as we discovered, may consist of

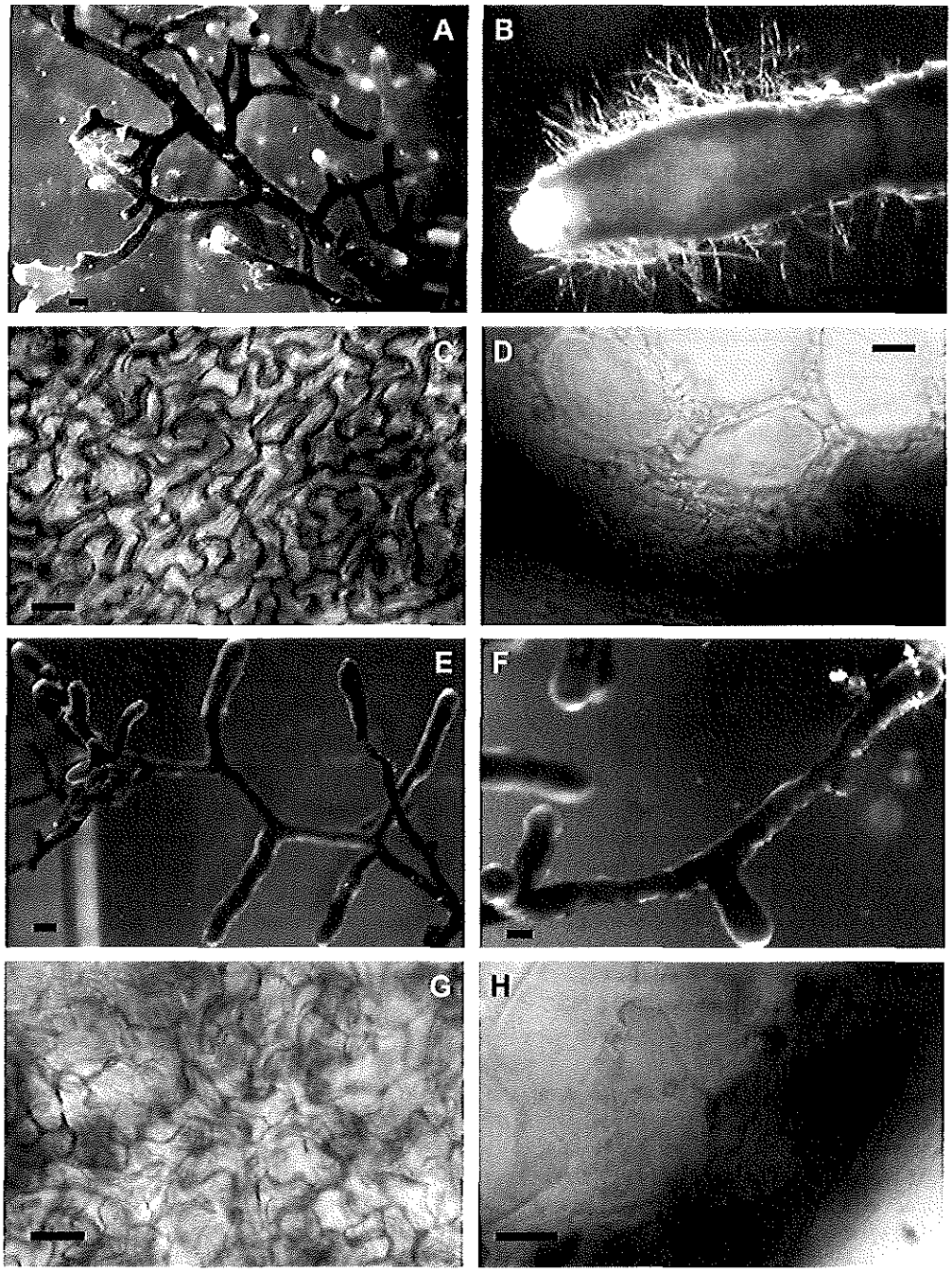

Fig 4 – Morphological–anatomical characteristics of *Tuber indicum* B ectomycorrhizas (ECM) synthesized on the North American host plants loblolly pine *Pinus taeda* (A–D) and pecan *Carya illinoinensis* (E–H). A. dichotomous and irregularly pinnate ECM; B. ECM with emanating hyphae; C. & G. puzzle-like pseudoparenchyma cells of the outer mantle layer; D. & H. transverse section of ECM showing the outer mantle layer and inner Hartig net; E. & F. simple and irregularly pinnate ECM. Scale bars: A, E = 1 mm; B, F = 500 μm; C, D, G, H = 10 μm.

a mix of morphologically similar species. A standard practice to help prevent further accidental or unwanted introductions of *T. indicum* (or other species, such as *Tuber brumale*) into truffières would be for truffle inoculators to employ DNA testing of each fruit body used for inoculations. Such analyses are relatively inexpensive now, owing to the development of species-specific primers, direct and multiplex PCR methods (Paolocci *et al.* 1997; Iotti & Zambonelli 2006; Iotti *et al.* 2007; Bonito 2009). We also recommend that truffle inoculators archive a small, dried voucher from each fruitbody, record which trees were inoculated with which fruitbodies, and provide DNA certification of their rootstock and inoculum.

Recent evidence indicates *T. melanosporum* is a bipolar outcrossing species (Riccioni *et al.* 2008; Martin *et al.* 2010). With the release of the *T. melanosporum* genome (Martin *et al.* 2010) it may soon be possible to provide molecular diagnoses of the mating type(s) of ectomycorrhizas on seedlings. Such diagnoses could help growers ensure that their orchards are fertile (e.g. consisting of both mating types), and not sterile due to the absence of one mating type. However, there is still a need for independent or government entities to provide these services.

The phenomenon of ectomycorrhizal introductions

To assess global patterns of ectomycorrhizal introductions Vellinga *et al.* (2009) compiled a database of over 200 species of ectomycorrhizal fungi (including eight *Tuber* species) that have been introduced around the world, largely through forestry and plant nursery trades. They propose a conceptual model on the invasion process of ectomycorrhizal fungi involving 4 phases: (1) transport; (2) establishment; (3) spread; and (4) ecological impacts. It is certain that some species of *Tuber*, *Rhizopogon*, *Scleroderma*, *Suillus* and *Amanita* can establish reproductive genets in non-native soils (Chu-Chou & Grace 1983; Wang & Hall 2004; Pringle & Vellinga 2006; Pringle *et al.* 2009; Bulman *et al.* 2010). In fact, 16 species of *Tuber* (>13 % of the described species) have putatively been introduced outside of their native range (Bonito *et al.* 2010). In some cases introduced mycorrhizal species have made evolutionary jumps onto resident hosts forming unforeseen fungus–host species combinations, as in the case of *Amanita muscaria* and *Chalciporus piperatus* on *Nothofagus* (Orlovich & Cairney 2004). Ectomycorrhizal fungi may also facilitate the spread of invasive plants into non-native soils, as has occurred with introductions of *Pinus* in the Southern hemisphere and *Eucalyptus* in Europe (Higgins & Richardson 1988; Diéz 2005; Dickie *et al.* 2010), and contrary, a lack of suitable ectomycorrhizal fungi may suppress plant invasions (Nuñez *et al.* 2009).

Plantations of trees inoculated with *T. melanosporum* continue to be established around the world (e.g. Israel, Chile, Argentina, United States, Canada, Australia, New Zealand, South Africa) with the hope of cultivating these valuable fruit bodies. Having been found in truffières in the United States and Italy *merely by accident*, it is likely that *T. indicum* will be (or has already been) introduced into other countries (Murat *et al.* 2008).

The current establishment of truffles (*Tuber*) and other mycorrhizal fungi into exotic habitats provides an opportunity to study factors involved in the establishment of mycorrhizal species, such as how exotic mycorrhizal species interact with local flora and fauna, and their rate of spread across the landscape and ecological impact (Pringle *et al.* 2009; Wolfe *et al.* 2010). Although we have focused on *T. melanosporum* and *T. indicum*, other truffle (e.g. *Tuber borchii*, *T. brumale*, *Tuber aestivum*) and mycorrhizal species continue to be introduced both deliberately and unintentionally around the world (Schwartz *et al.* 2006; Pruett *et al.* 2008; Guerin-Laguete *et al.* 2009; Vellinga *et al.* 2009). With plantings of many thousands of hectares of *T. melanosporum*-inoculated seedlings in more than a dozen countries, the experiment has begun. Long-term studies are needed to document and understand the ecological and legacy effects of these mycorrhizal introductions (Richter *et al.* 2007). Here is an opportunity for increased collaboration between international governmental agencies, researchers, industry and farmers. We may yet learn much from truffles about the ecology of mycorrhizal fungi.

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Supplementary material

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