Mycorrhiza (2016) 26:781–792
DOI 10.1007/s00572-016-0713-4

ORIGINAL ARTICLE

Mycorrhizal detection of native and non-native truffles in a historic arboretum and the discovery of a new North American species, *Tuber arnoldianum* sp. nov.

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Received: 27 March 2016 / Accepted: 2 June 2016 / Published online: 10 June 2016

Abstract During a study comparing the ectomycorrhizal root communities in a native forest with those at the Arnold Arboretum in Massachusetts (USA), the European species *Tuber borchii* was detected on the roots of a native red oak in the arboretum over two successive years. Since *T. borchii* is an economically important edible truffle native to Europe, we conducted a search of other roots in the arboretum to determine the extent of colonization. We also wanted to determine whether other non-native *Tuber* species had been inadvertently introduced into this 140-year-old Arboretum because many trees were imported into the site with intact soil and roots prior to the 1921 USDA ban on these horticultural practices in the USA. While *T. borchii* was not found on other trees, seven other native and exotic *Tuber* species were detected. Among the North American *Tuber* species detected from ectomycorrhizae, we also collected ascomata of a previously unknown species described here as *Tuber arnoldianum*. This new species was found colonizing both native and non-native tree roots. Other ectomycorrhizal taxa that were detected included basidiomycetes in the genera *Amanita*, *Russula*, *Tomentella*, and ascomycetes belonging to *Pachyphlodes*, *Helvella*, *Genea*, and *Trichophaea.* We clarify the phylogenetic relationships of each of the *Tuber* species detected in this study, and we discuss their distribution on both native and non-native host trees.

Keywords *Tuber borchii* · Root mantle cystidia · Urban landscape · Fungal introduction

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Introduction

Truffles in the genus *Tuber* are among the most economically important edible fungi. The ascomata of many species in this genus are aromatic, flavorful, and gastronomically prized. However, due to their ectomycorrhizal (EcM) mode of nutrition, *Tuber* species are difficult to cultivate and grow slowly in pure culture away from their host plants. In particular, while the mating system of *Tuber* species has been demonstrated to be bipolar and heterothallic, the process and biology of truffle mating still remains somewhat mysterious (Rubini et al. 2007). Many of the gastronomically desirable *Tuber* species are native to southern Europe, where the soil is relatively basic in pH and the climate mild. These conditions are not easily reproduced in northeastern North America, but edible *Tuber* species have been successfully cultivated in the southeastern USA and the Pacific Northwest (Berch 2013, Bonito et al. 2010a, O'Connell 2010, Sharma et al. 2012, Smith et al. 2012). In New England, where the soil is relatively acidic and the climate prone to extremes of hot and cold, there are no reports of successful cultivation of *Tuber* species.

In a study comparing the fungal symbionts on EcM roots in a natural forest with those in an arboretum, the economically important edible European species *Tuber borchii* was detected on root tips of *Quercus rubra*, a red oak native to North America. This oak was growing in the Arnold Arboretum of Harvard University, near Boston, MA, USA (Healy unpublished). The Arnold Arboretum is a 281-acre botanical garden that was established in 1872 as stipulated by the will of James Arnold, a wealthy benefactor to Harvard University (Hay 1995). It was created to be a home for "all the trees [and] shrubs…either indigenous or exotic, which can be raised in the open air" (Hay 1995, Page 64). Though the diverse trees in the arboretum have been extensively studied by generations of Harvard researchers, the fungal biota in the soil has not been characterized. There have been recent attempts and success in cultivating *T. borchii* in North America in 2012 (Berch 2013), but there are no records of unintentional introduction of *T. borchii* in North America and this truffle has not been reported from North America prior to 2010 (Bonito et al. 2010a, Charles Lefevre personal communication). Vellinga et al. (2009) adapted the model for biotic introductions by Lockwood et al. (2007) for EcM fungi. Their model lists possible outcomes of introduced EcM fungi as follows: (1) death without establishment, (2) initial survival but replacement by native fungi, (3) survival on the original host taxon roots (including spread to other exotic hosts), but failure to establish on native tree roots, (4) survival on both the original host roots and spread with successful establishment on native host roots, or (5) failure to persist on original host roots but spread with successful establishment on native host(s) (Vellinga et al. 2009).

Due to the unexpected presence of the European species *T. borchii* in the arboretum, we explored the extent of colonization of this species as well as the presence of other *Tuber* species on other native and non-native trees in the arboretum. Specifically, we wanted to explore the five possible EcM fungus introduction outcomes outlined in Vellinga et al. (2009) with regard to the *Tuber* species we detected in the Arboretum. Based on preliminary data, we expected *T. borchii* to fit the fourth outcome of persisting on the original host roots and also establishing on native host roots.

During the course of our study, we also discovered the ascomata of a *Tuber* species that has previously only been detected from EcM root DNA sequences (Karpati 2010, Karpati et al. 2011, Bonito et al. 2010a). This species was referred to as *Tuber* sp. 46 by Bonito et al. (2010b) and is genetically distinct from other described *Tuber* species. This new *Tuber* has alveolate-reticulate spores and phylogenetic affinity with the /maculatum clade that is composed of pale-colored *Tuber* species. The collection of numerous mature ascomata enables us to morphologically describe this new species as *Tuber arnoldianum*. We also discuss *T. borchii* and other *Tuber* species detected from EcM root tips of native oaks as well as from non-native trees in the Arboretum with respect to species introductions and invasion biology.

Materials and methods

Sampling was conducted in the Arnold Arboretum, Harvard University, Boston, MA, USA, during the summer of 2014. We sampled a total of 13 trees for this study, including four native oak trees and nine exotic trees (including representative species of beech, hazel, oak, and pine—Table 1). Six of the selected European trees were brought to the Arboretum from Europe as intact plants prior to 1921, when USDA restrictions on imported trees with associated soil were enacted to avoid the introduction of pathogens and invasive species with their host plants (United States Bureau of Entomology and Plant Quarantine, 1921 Page 30). To locate the target trees, we used the Arboretum online plant inventory (http://arboretum.harvard.edu/plants/plant-inventory/) and Collection Researcher v 1.0 (http://map.arboretum.harvard.edu/). Trees denoted as "NAC" (not accessioned) were trees that were present before the establishment of the Arboretum and were included in the Arboretum design but do not have formal accession numbers. These unaccessioned trees were selected because they represented native EcM host plants originating on site. All target trees were sampled for ectomycorrhizae and associated ascomata. For harvesting roots, 15 × 15 × 10 cm cores were collected from the soil 1–2 m away from the base of the selected trees. Fine roots were sorted and stored at 4 °C for 7 days or less before analysis. Each tree was sampled 1–2 times. Ascomata were collected at the same time as roots were sampled (June–September 2014) with the aid of a short-handled three-pronged hand cultivator. Additional collections of *Tuber arnoldianum* were made in August and October of 2013.

Pooled roots from each tree were rinsed to remove soil, and morphology examined in tap water under an Olympus SZX9 dissecting microscope (Olympus America Inc., Center Valley, PA). Root tips were scanned, with special attention paid to those displaying the bristle-like EcM mantle characteristic of many *Tuber* species, including *T. borchii* (Agerer 2006) (Fig. 3a, b). Bristle-like root tips were placed in DNA extraction solution. Since bristles may be lost or crushed during harvesting and handling (Agerer 2006), additional root tips similar to described *Tuber* EcM as well as other representative morphotypes were also picked and isolated for extraction.

Ascomata were sectioned, mounted in water on slides, and examined under an Olympus BX40 compound microscope (Olympus America Inc., Center Valley, PA). Measurements were made of the peridium thickness, peridial cell length (l) × width (w), spore size (l × w) excluding ornamentation ($n = 50$ spores/ascoma), asci (l × w), inner hyphae (w), and hyphoid hairs (l × w). Images were taken using Microsuite Special Edition imaging software (Olympus America Inc., Center Valley, PA). Vouchers were air dried and deposited in the Farlow Herbarium (FH) at Harvard University.

Molecular Analysis: Genomic DNA of representative samples of each ascoma collection were extracted using a standard CTAB procedure (Gardes and Bruns 1993). PCR was used to amplify the complete internal transcribed spacer region (ITS1, 5.8 s, and ITS2, hereafter referred to as ITS) of the nuclear

Table 1 *Tuber* ascomata and ectomycorrhizal roots detected on accessioned non-native trees and unaccessioned native trees (NAC) in the Arnold Arboretum near Boston, Massachusetts during 2014. Locations correspond to those denoted on the Arboretum map[a]. Bolded text denotes exotic species

Tree accession and name	Location in Arboretum	*Tuber* species detected	Material collected	Geographic origin and year
7549*A *Corylus ×vilmorinii*	19 NW	*Tuber menseri* nom prov.; *Tuber arnoldianum*	Roots	Hybrid, received from France in 1911
14592*A *Fagus sylvatica* 'Grandidentata'	37 SE	***Tuber* sp. 37**; *Tuber arnoldianum*	Roots	Cultivated variety received from Germany in1912
14597*A *Fagus sylvatica* 'Pendula'	38 SW	***Tuber* sp. 37**; *Tuber arnoldianum*	Roots	Cultivated variety received from Holland in1903
253–80*A *Fagus sylvatica* 'Tortuosa'	45 NW	*Tuber arnoldianum*, *Tuber* sp. 36	Roots	Received from England in 1965 as whole plant with soil washed off.
2097–65*A *Fagus sylvatica*	58 SW	*Tuber arnoldianum*, *Tuber* sp. 36	Roots	Received from England in 1965 as whole plant with soil washed off.
5361*A Fagus sylvatica 'Laciniata'	53 NW	*Tuber* sp. 57	Roots	Cultivated variety received from Holland in 1903 as whole plant
5717*B *Pinus nigra* ssp. *Salzmannii*	44 SE	*T. anniae*; *Tuber menseri* nom prov.	Roots	Received from France 1907 as whole plant
901–60*B *Quercus petraea*	32 SW	*Tuber arnoldianum*	Roots and ascomata	Seed collected in France in 1959; received as a seedling in 1960 from USDA
347–2016*A *Quercus alba*	30 NE	*Tuber arnoldianum*	Ascomata	USA History unknown
NAC 268 *Quercus rubra*	52 NE	None	Roots	USA History unknown
NAC 270 *Quercus rubra*	52 NW	***Tuber borchii*** *Tuber* sp. 34	Roots	USA History unknown
NAC 272 *Quercus rubra*	52 NE	None	Roots	USA History unknown
Quercus sp.[b]	31 SW	***Tuber* sp. 37**; *Tuber* sp. 34, *Tuber arnoldianum*	Roots	Unknown

[a] Arnold Arboretum map available at: http://arboretum.harvard.edu/wp-content/uploads/ArnArb-Master-Grid-Map.pdf

[b] The sequenced host was *Quercus*, which was different from the host we sampled under. We do not know the species

ribosomal DNA (nrDNA) using primers ITS1f and ITS4 using the basic protocols of Gardes and Bruns (1993). The EcM root tips were extracted and the ITS region was amplified using Extract 'n Amp ready mix solution (Sigma-Aldrich, St. Louis, MO, USA) following protocols of Avis et al. (2003). Each 25-μl reaction included 1.04 μl sterile, filtered deionized water, 10 μl ready mix, 1.88 μl each of 10 μM ITS1f and ITS4, 1 μl BSA, and 4 μl of DNA extract. The thermocycler program was set for 94 °C for 3 min (initiation and denaturation) followed by 35 cycles of 94 °C for 1 min (denaturation), 50 °C for 45 s (annealing), 72 °C for 1.5 min (extension), with a final extension time of 10 min at 72 °C. To verify the host of the sampled EcM roots, the primers trnL-c and trnL-d were used to amplify a region of the chloroplast DNA (Taberlet et al. 1991) on amplified root tips for each tree sampled, following thermocycler conditions of Kennedy et al. (2011). Amplification of DNA was confirmed by electrophoresis on 1 % agarose gels and visualized with GelRed (Biotium, Hayward, CA) under UV light. Amplicons were cleaned and bi-directional Sanger sequencing performed in the Beckman Coulter Genomics Laboratory (Danvers, MA) using the same primers as for amplification. Sequences were trimmed and edited in Geneious Pro v 5.6.7 (Drummond et al. 2012). DNA was extracted from a total of 292 root tips of which 154 were successfully sequenced. Representative sequences were deposited in the National Center for Biotechnology Information (NCBI) nucleotide database under accession numbers KU186910-KU186954, KU238896-KU238922, and KX163994-KX163996.

For each sequence, a BLAST search was performed to identify the closest related sequences in GenBank. Highly similar sequences were downloaded and aligned with *Tuber* sequences from root tips and ascomata, along with sequences from phylogenetic analyses of the /puberulum and /maculatum clades of Berch and Bonito (2014), Bonito et al. (2010a), Bonuso et al. (2010), Deng et al. (2013), Fan et al. (2012a), Fan et al. (2012b), Fan and Yue (2013), Fan et al. (2013), Guevara et al. (2013), Su et al. (2013), and Wang et al. (2013). Alignments of ITS sequences for the /maculatum and /puberulum clades were generated and analyzed. The sequences were aligned using MAFFT v 7.058 (Katoh and Toh, 2010) and manually optimized in SeAl v 2.0a11 (Rambaut, 2007). No regions were excluded. For each alignment, a model of nucleotide substitution was selected by jModeltest (Posada, 2008), under the Aikake Information criterion. Bayesian analysis was run in MrBayes v 3.2.3 (Huelsenbeck and Ronquist, 2001) for 20,000,000 generations in two parallel runs, with trees sampled every 1000th generation, and the first 25 % of sampled trees discarded as burn in. To ensure that stationarity had been reached and chains had properly mixed during the Markov chain Monte Carlo runs, the trace files were checked in Tracer (Rambaut and Drummond, 2007). One 50 % majority rule consensus tree was recovered for each analysis. Significant support was considered to be ≥0.95. A maximum likelihood tree was inferred for each alignment using RAxML-HPC2 v 8.1.11 (Stamatakis 2014) with a GTR + gamma model of nucleotide substitution. One thousand bootstrap iterations were performed with rapid bootstrapping. Significant support was considered to be ≥70 %. MAFFT, RAxML, and MrBayes were run on XSEDE on the CIPRES Science Gateway v 3.3 (Miller et al., 2010). The datasets included 53 taxa and 674 sites for the /maculatum clade, and 72 taxa and 670 sites for the /puberulum clade analyses.

Results

A total of 25 molecular operational taxonomic units (mOTUs) based on ≥97 % sequence similarity were detected on sampled roots in the Arboretum (Table 1). Of these, eight mOTUs were phylogenetically placed in the genus *Tuber* (Tables 1 and 2). The *Tuber* roots comprised 103 of the 158 root tips sequenced (65 %). Other genera detected during this sampling included *Amanita*, *Genea*, *Helvella*, *Pachyphlodes*, *Russula*, *Tomentella*, and *Trichophaea*. Of these genera, only *Tomentella* and *Trichophaea* were mistaken for *Tuber* based on ectomycorrhizal root morphology. All but two of the 13 surveyed trees (85 %) were colonized by at least one *Tuber* mOTU, and seven trees had two *Tuber* mOTUs (Tables 1 and 2). Four mOTUs were placed in the /puberulum clade (Fig. 1), three mOTUs were placed in the /maculatum clade (Fig. 2), and one mOTU belonged to the /rufum clade. In the /puberulum clade, four mOTUs corresponded to *T. anniae*, *T. borchii* clade II of Bonuso et al. (2010), and two undescribed species, *Tuber* sp. 34 and *T. menseri* nom. prov. of Bonito et al. (2010a) (Fig. 2). Three mOTUs in the /maculatum clade corresponded to undescribed species designated as *Tuber* sp. 36, *Tuber* sp. 37, and *Tuber* sp. 46 in Bonito et al. (2010a). Two *Tuber* species were collected as ascomata: *Tuber* sp. 37 and *Tuber* sp. 46. All species in these clades have alveolate-reticulate spore ornamentation. One additional mOTU was 100 % similar in the ITS region to a spiny-spored species of the /rufum clade, *Tuber* sp. 57 of Bonito et al. (2010a). All of the roots with characteristic bristle cystidia had DNA sequences that resolved within the genus *Tuber* (Fig. 3a–b).

Tuber borchii was recovered for a second year from multiple root tips on the same *Quercus rubra* tree (NAC 270) as in 2013 (Tables 1 and 2, Fig. 3a). This species was not detected on any other tree sampled.

Tuber sp. 46 was one of the two most frequently sequenced mOTUs and was collected as both EcM roots (Fig. 3b) and ascomata (Fig. 3c). *Tuber* sp. 46 was found on native and non-native tree roots in the arboretum (Table 1). *Tuber borchii* and *Tuber* sp. 34 were the only other *Tuber* species detected on native trees in the Arboretum.

Another frequently sequenced mOTU had high similarity to a sequence labeled *Tuber scruposum* from Armenia (DQ011847 Table 1, Fig. 2). This mOTU is resolved in the /maculatum clade. The name *T. scruposum* has been applied to at least two phylogenetically distinct species in GenBank (Bonito et al., 2010a Fig. S2). We refer this taxon to "Tuber sp. 37" until study of the holotype can clarify the *T. scruposum* species concept. *Tuber* sp. 37 was detected on the roots of three trees of European origin in the Arboretum and one of European origin grafted onto native roots. Two of the trees were brought to the arboretum with roots intact and with residual soil (*F. sylvatica* 14597*A, and 14592*A). Another *F. sylvatica* (2097–65*A) was brought as a whole plant with soil washed off. *Fagus sylvatica* 253–80*A was grafted onto roots of *F. grandifolia*, a native beech. In this situation, *Tuber* sp. 37 was associated with the roots of native beech, although most of the above ground portion was European beech. We sequenced ITS of this species from a total of 29 root tips from these four trees. In addition, we collected ascomata with the same ITS sequence near *F. sylvatica* 2097–65*A (Table 1 and 2).

Tuber sp. 57 was detected on five root tips of *Fagus sylvatica* 'laciniata' (5361*A). The best sequence was 659 bp long and there was no variation among sequences from the five different root tips. GenBank sequences with 98–100 % coverage and similarity were from Germany on *Salix* (GU990358), *Epipactis* (AY634169), and *Populus* (GU990353) roots, and from a fruit body from West Virginia, USA (JQ925650). Shorter GenBank sequences with 99–100 % similarity were from New Zealand (AM900439), Italy (AY940646), France (JX135044), and from other sites in the eastern USA (FJ748909, HM485420).

Most non-*Tuber* sequences matched highly similar sequences from North America (98–100 % coverage with 99–100 % similarity) (Table 2). One notable exception was *Tomentella* sp. 3. We sequenced the ITS of this species from six root tips of a European accession of *Fagus sylvatica* (14, 592*A). The ten top hit sequences from GenBank with exact or highly similar sequences (99–100 % coverage and similarity) were from *F. sylvaticus* or *Q. robur* roots from five

Table 2 Ectomycorrhizal root tip ITS sequence mOTUs, number of tips with the same sequence, and their host(s) in the Arnold Arboretum (Table 1). Bolded text denotes exotic tree species. Closest matches to GenBank sequences shown

Putative ID	Representative accession number	Host	Total root tips	GenBank match and geographic locality	% identity	Seq. length
Amanita solaniolens	KU238896	*Q. rubra*	1	*Amanita solaniolens* JF313659 USA: TN	99 (630/631)	667
Genea hispidula	KU238897	*Q. rubra*	1	*Genea hispidula* AJ969622 Denmark	99 (417/419)	418
Helvella sp.	KU238898	***Corylus***	1	*Helvella* EcM KC110999 Pakistan	95 (511/539)	675
Pachyphlodes sp. 18	KU238899	*Q. rubra*	3	*Pachyphlodes* sp. 18 JN102404 USA: NC	98 (589/600)	618
Pezizaceae sp.	KU238900	*Q. rubra*	1	Pezizaceae JN102444 USA: NC	99 (606/607)	619
Russula pectinatoides	KU238906	*Q. rubra*	3	*Russula pectinatoides* EU598185 USA: TN	100 (574/574)	584
Russula cf. *pectinatoides*	KU238904	*Q. rubra*	3	*Russula pectinatoides* EU819493 USA: WI	99 (671/672)	672
Russula sp. 1	KU238902	*Q. rubra*	6	Uncultured *Russula* DQ493553 USA: MA	99 (703/705)	719
Russula sp. 2	KU238908	*Q. rubra*, ***F. sylvatica***	13	*Russula* sp. JX030256 USA: NY	97 (636/654)	699
Russula sp. 3	KU238910	*Q. rubra*	3	Uncultured *Russula* EcM FM999541 USA: Ohio	100 (411/411)	413
Russula sp. 4	KU238911	*Q. rubra*	1	Uncultured *Russula* EcM GU907803 USA: MA	99 (642/643)	646
Russula sp. 5	KU238901	***P. nigra***	1	Uncultured *Russula* EcM DQ777985 USA: MA	99 (657/659)	658
Tomentella sublilacina	KU238918	***F. sylvatica***	4	*Tomentella sublilacina* JQ272367 USA: NC	99 (644/645)	674
Tomentella ferruginea	KU238914	***F. sylvatica***	4	*Tomentella ferruginea* EU819497 USA: WI	99 (679/685)	683
Tomentella sp. 2	KU238912	***Corylus***	1	Uncultured Thellephoraceae EU516674 Austria	96 (432/450)	451
Tomentella sp. 3	KU238916	***F. sylvatica***	6	Uncultured *Tomentella* JX844770 Germany	100 (639/639)	639
Trichophaea sp.	KU238919	***F. sylvatica***	3	*Trichophaea* sp. KF742769 Canada: BC	100 (485/485)	485
Tuber anniae	KU186937	***P. nigra***	2	*Tuber anniae* JN207851 Finland	99 (625/627)	656
Tuber arnoldianum	KU186924	***F. sylvatica***, *Q. rubra*, ***Corylus x vilmorinii***	29	*Tuber* sp. 46 HM485415 USA: NY	100 (598/598)	598
Tuber borchii	KU186940	*Q. rubra*	8	*Tuber borchii* JN392228 Greece	99 (511/512)	528
Tuber menseri nom prov.	KU186933	***P. nigra, Corylus***	9	*Tuber* sp. GMB-2010b HM485376 USA: OR	100 (609/609)	651
Tuber sp. 34	KU238921	*Q. rubra*	13	Uncultured EcM GU907804 USA: NJ	99 (624/627)	665
Tuber sp. 36	KU186932	***F. sylvatica***, *Quercus*	3	*Tuber* sp. 36 JN033366 USA: NC	100 (508/508)	509
Tuber sp. 37	KU186930	***F. sylvatica, P. nigra***	33	*Tuber scruposum* DQ011847 Armenia	99 (553/555)	555
Tuber sp. 57	KX163994	***F. sylvatica***	5	*Tuber* sp. 57 JQ925650 USA: WV	100 (605/605)	605

European countries. *Genea hispidula* was another mOTU with high similarity (≥ 97 %) to a species from Europe, but this species is also known to occur in North America (Alvarado et al. 2016).

Most of the chloroplast trnL intron sequences from the sampled roots verified the expected host. However, sequences for *Corylus heterophylla* 7907*A came back as a *Quercus* sp., and gene amplification failed for *Quercus alba* (NAC), *and Q. petraea* (901–60*B).

Taxonomy

Tuber arnoldianum Healy, Zurier and Bonito Fig. 3.
MB 816348.

Holotype United States, Massachusetts, Suffolk County, Boston, Arnold Arboretum of Harvard University under *Quercus alba* (347–2016*A), 13 Oct 2013, coll. R. Healy RH1619 (FH 00377353) GenBank KU186913.

Diagnosis Ascomata globose to ovoid, and cream colored becoming mottled with dark reddish-brown areas when mature, smooth (not verrucose). Gleba is solid and dark reddish-brown, marbled with white veins. Peridium has occasional short individual septate hyphae that project from depressions on the surface. Spores in one-spored asci are 25–47 × 15–40 μm, Q = 1–1.2 (1.6), in two-spored asci (18) 24–32 × (16) 20–26 (28), Q = 1–1.2 (1.6), in three-spored asci 22–30 (32) × 18–22 (26), Q = 1–1.3 (1.5), in four spored asci 18–30 × 17–24, Q = 1–1.2. The spores are alveolate-reticulate, and the alveolae are larger than in similar species. About half

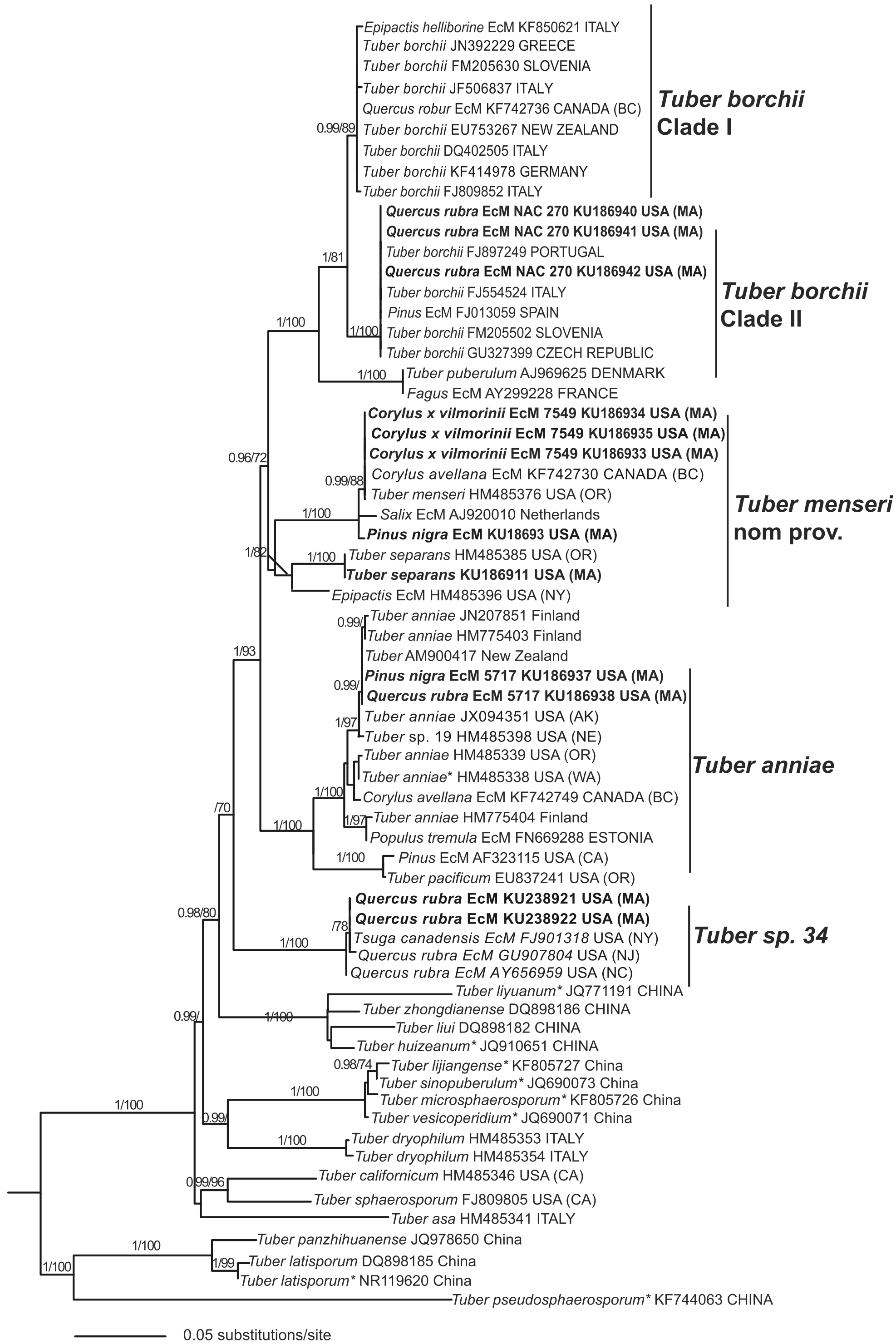

Fig. 1 Midpoint-rooted maximum likelihood phylogram of the /puberulum lineage of the genus *Tuber* based on ITS ribosomal DNA as generated with RAxML with 1000 bootstrap iterations. Bootstrap support >70 % on the *right*, Bayes probability >0.95 on the *left* at nodes. Branches thickened in proportion to support. *Asterisks* denote sequences from holotype specimens. *Double asterisk* denotes sequence from epitypes. *Bolded lettering* refers to sequences generated in this study. *EcM* denotes sequences obtained from ectomycorrhizal root tips. All other sequences from ascomata. Clades 1 and 2 of Tuber borchii were delimited in Bonuso et al. 2010

of spores have one to three internal ridges that are 1–5 μm long and 0.5–1.5 μm high. These intra-alveolar ornaments help to separate *T. arnoldianum* from most other species in the /puberulum or /maculatum clades. It most closely resembles *T. walkeri*, but the spores have fewer alveolae and lower reticula. *Tuber arnoldianum* is also genetically differentiated from other *Tuber* species based on ITS rDNA sequences.

Etymology In honor of James Arnold, a whaling merchant and benefactor to Harvard University who in his will

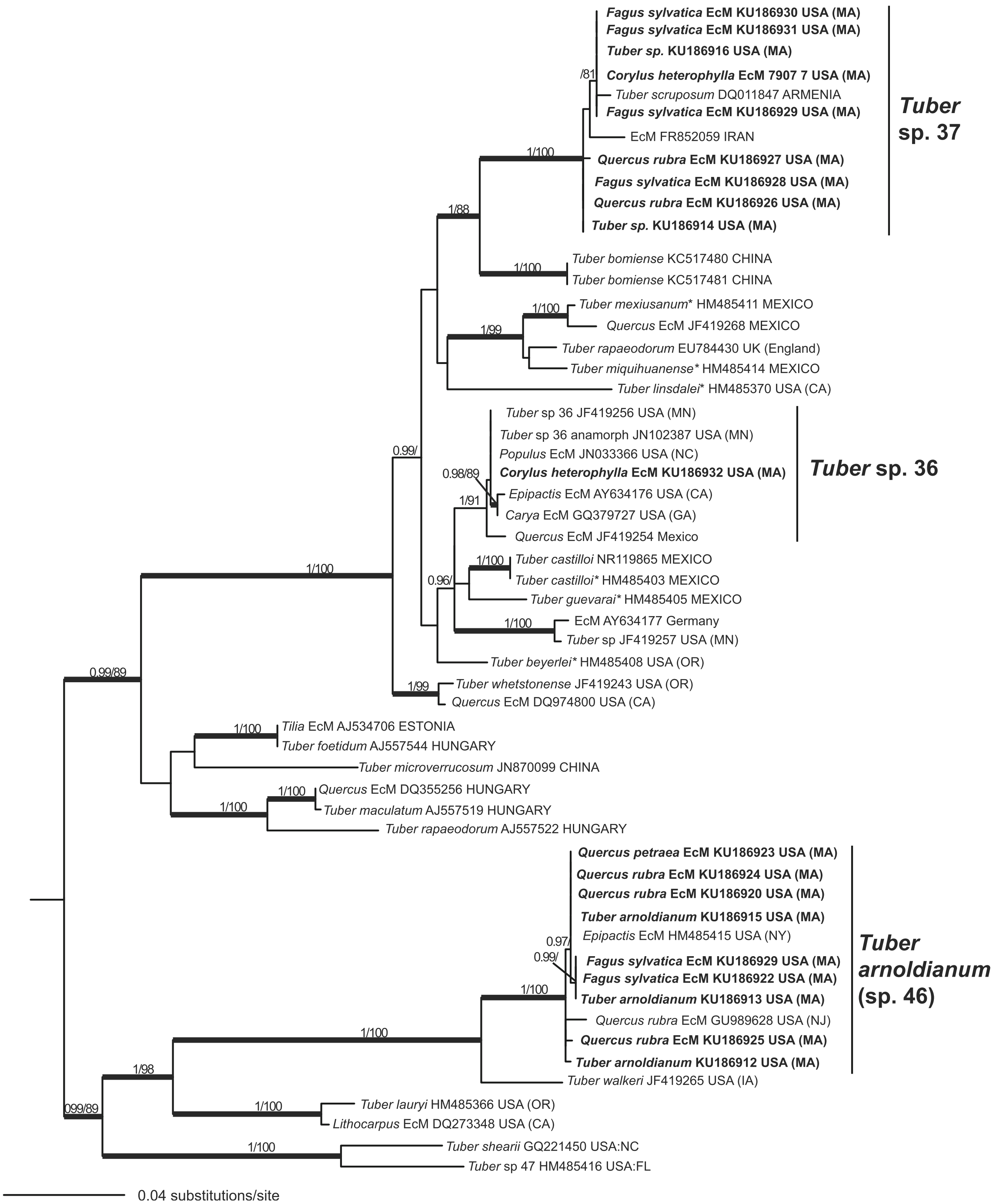

Fig. 2 Midpoint-rooted maximum likelihood phylogram of the /maculatum lineage of the genus *Tuber* based on ITS ribosomal DNA as generated with RAxML with 1000 bootstrap iterations. Bootstrap support >70 % on the *right*, Bayes probability >0.94 on the *left* at nodes. Branches thickened in proportion to support. *Asterisks* denote sequences from holotype specimens. *Double asterisk* denotes sequence from epitypes. *Bolded lettering* refers to sequences generated in this study. *EcM* denotes sequences obtained from ectomycorrhizal root tips. All other sequences from ascomata

stipulated that a portion of his estate go to "...the promotion of Agricultural, or Horticultural improvements". This money was used to establish the Arnold Arboretum of Harvard University, where the only known ascomata of *Tuber arnoldianum* species have been found.

Description Ascomata 8–9 × 9–11 mm, globose to ovoid, irregular. Peridium cream to dark reddish-brown, mottled. Odor pleasant, slightly nutty, flavor not recorded. Gleba solid, at maturity dark reddish-brown and marbled with white veins (Fig. 3c). Short hyphae (hyphoid hairs) projecting from the

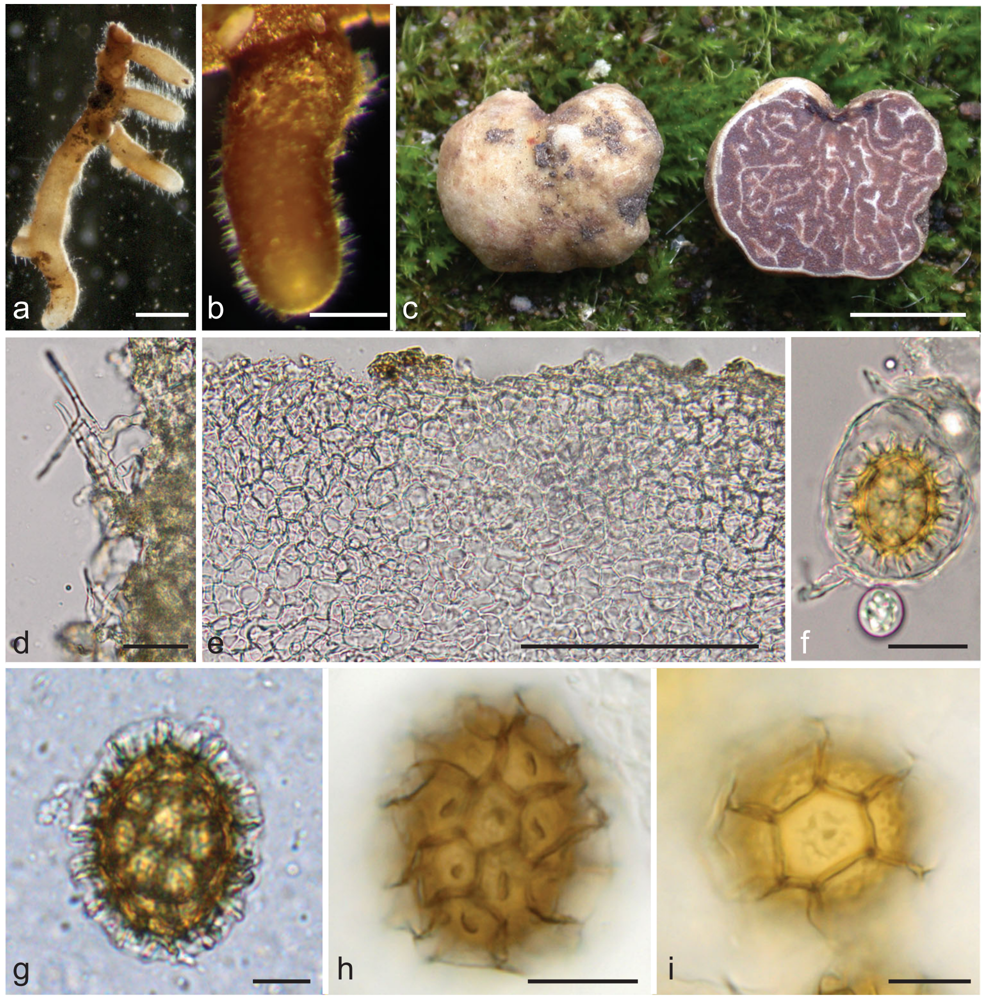

Fig. 3 **a** *Quercus rubra* root tip colonized by *Tuber borchii* (bar = 2 mm). **b** *Quercus alba* root tip colonized by *Tuber arnoldianum* (*bar* = 1 mm). **c** *Tuber arnoldianum* ascoma cut in half showing peridium (*left*) and gleba (*right*) (*bar* = 5 mm). **d**–**h** Light micrographs of *Tuber arnoldianum*. **d** Hyphoid hair (*bar* = 25 μm). **e** Peridium (*bar* = 100 μm). **f** Ascus in KOH (*bar* = 25 μm). **g** Spore showing microreticulum (*bar* = 10 μm). **h** Spore showing intra-alveolar ornaments (*bar* = 10 μm). **i** *Tuber walkeri* spore with intra-alveolar ornaments (*bar* = 10 μm)

peridial surface present in depressions, single to clustered, 5–7 × 20–30 μm at base, tapered to a point (Fig. 3d). Unless specified otherwise, all measurements are l x w.

Peridium 150–450 μm thick, pellis 100–200 μm thick, *textura angularis*, cells 10–20 × 14–25 μm. subpellis 50–250 μm thick, *textura intricata*, cells 5–10 μm broad (Fig. 3e). Inner hyphae interwoven, 4–8 μm broad. Sterile veins 7–12 μm broad.

Asci contain 1–5 spores/ascus, with 1–3 spores/ascus being most common. One-spored asci globose to slightly ellipsoid, 40–90 × 35–70 μm in water when fresh (Fig. 3f). Ascospores globose to ellipsoid, excluding alveolate-reticulate ornamentation, extreme values in parentheses, in one-spored asci 25–47 × 15–40 μm, with an average of 34 × 27, Q = 1–1.6, in two-spored asci 18–32 × 16–28, with an average of 26 × 22, Q = 1–1.2 (1.6), in three-spored asci 22–32 × 18–26, Q = 1–1.3 (1.5), in four spored asci 18–30 × 17–24, Q = 1–1.2; the walls reddish-brown in water, reticulum with 3–5 alveoli along length and 3–5 along width, the alveolar walls (2) 3–5 (8) μm high. In some spores, a microreticulum (a honey-comb mesh that is visible at the optical cross section of mature spores in some *Tuber* species) is visible (Fig. 3g). In addition, within the alveoli of 50 % of ascospores, there is one or more ridge(s) of wall material referred to here as intra-alveolar ornaments. These ornaments are 1–5 μm long and 0.5–1.5 μm high and can be present in one or more alveolae in any given spore. They can be either rounded or spiky; some are elongated ridges, while others are narrower spines. Individual alveolae may contain up to 3 intra-alveolar ornaments (Fig. 3h). Generally, single ornaments within alveolae are located in the center, while multiple ornaments are irregularly scattered. Single ornaments within an alveola are larger on average than multiple ornaments within an alveola.

Distribution, habitat, and season New York (Bonito et al. 2010a), New Jersey (Karpati et al. 2011), and Massachusetts (this study); EcM with the orchid *Epipactis* (Bonito et al. 2010a) and the trees *Fagus sylvatica*, *Pinus strobus*, *Quercus alba*, and *Q. rubra*. Ascomata solitary to gregarious, mature specimens are found from late August to October whereas immature specimens have been found as early as June.

Additional specimens examined *Tuber arnoldianum*—USA, Massachusets, Suffolk County, Boston, hypogeous under *Quercus rubra*, 24 Aug 2013, coll. R. Healy RH1605 (FH); hypogeous under *Quercus prinus*, 13 Oct 2013, coll. R. Healy RH1618 (FH); 18 June 2014, coll. Hannah Zurier RH1644 (FH); *Quercus alba*, 24 July 2014, col. R. Healy RH1647; *Quercus alba* 1 September 2014 RH1693 (FH). *Tuber walkeri*—USA, Iowa, Story County, Hickory Grove Park, hypogeous under *Q. macrocarpa*, 6 Aug 1999, coll. R. Healy RH521 (ISC).

Comments: *Tuber arnoldianum* most closely resembles *T. walkeri* Healy, Bonito and Guevara, its closest relative, but differs in that *T. walkeri* has more numerous alveolae on its spores (4–8 across the length × 3–7 across the width in *T. walkeri* vs. 3–5 across the length and 3–5 across the width in *T. arnoldianum*) and higher reticulum walls (7.5–10 μm in *T. walkeri* vs. (2) 3–5 (8) μm high μm in *T. arnoldianum*). While both species have hyphoid hairs, those on *T. walkeri* are longer (25–70 μm) and cells in the peridium are smaller on average (4–38 μm wide) (Guevara et al. 2013). *Tuber arnoldianum* differs from another close relative *T. lauryi* Trappe, Bonito and Guevara in that *T. lauryi* has rounder spores (Q = 1.12–1.33) with more numerous alveolae (4–10 across the length × 3–8 across the width) and a thicker peridium (300–1000 μm thick) with smaller cells (4–24 μm wide). *Tuber lauryi* is only known from Oregon (Guevara et al. 2013). *Tuber beyerlei* Trappe, Bonito, and Guevara is known from *Pseudotsuga* in Oregon, and *Corylus* in British Columbia (Berch and Bonito 2014).This species has larger spores (37–47 × 32–40 μm) than *T. arnoldianum*, with more numerous alveolae (6–10 across the length × 3–8 across the width), smaller peridial cells (7–30 μm wide), and longer, thinner hyphoid hairs (35–85 × 2–4 μm). The primary differences between *T. arnoldianum* and *T. mexiusanum* Guevara, Bonito, and Cázares are that *T. mexiusanum* has more numerous alveolae on its spores (4–8 across the length × 3–5 across the width) and a thinner inner peridium (30–125 μm thick). *Tuber shearii* Harkness has rounder spores than *T. arnoldianum* (Q = 1–1.2) and smaller, finer alveolae (3–7 across the length × 3–6 across the width). *Tuber linsdalei* Gilkey also has finer alveolae than *T. arnoldianum* and fewer spores per ascus (1–2 (3)). *Tuber* sp. 37, a European species in the /maculatum clade found in the Arnold Arboretum, has smaller spores than *T. arnoldianum.* The following three species in the /maculatum clade are morphologically similar to *T. arnoldianum* but are known only from Mexico. *Tuber castilloi* Guevara, Bonito, and Trappe has spores with a more pronounced elliptical shape (Q = 1.4–2.3) than *T. arnoldianum* and more numerous alveolae (3–10 × 3–6). *Tuber guevarai* Bonito and Trappe lacks the surface hyphae of *T. arnoldianum* and *T. guevarai* has larger spores (25–45 × 15–40 μm in *T. arnoldianum* vs. 36–55 × 28–42 μm in *T. guevarai*) with more numerous alveolae (3–9 across the length × 3–7 across the width). *Tuber miquihuanense* Guevara, Bonito, and Cázares has larger spores(40–50 × 30–39 μm) with more numerous alveolae (4–8 across the length × 3–5 across the width) than *T. arnoldianum*; additionally *T. miquihuanense* lacks surface hyphae and has smaller peridial cells (5–24 μm).

The ornaments within the alveolae of *T. arnoldianum* are unusual in the /maculatum and /puberulum clades. *Tuber walkeri*, the sister species to *T. arnoldianum*, has these unusual intra-alveolar ornaments in greater numbers. They are present in more than 50 % of spores and there are sometimes up to four intra-alveolar ornaments in a given alveola (Fig. 3i). In other major clades of *Tuber*, intra-alveolar ornaments are clearly present in a few species of the /aestivum and /excavatum lineages of *Tuber* (Bonito et al. 2010a). These include *Tuber mesentericum* Vitt., *T. aestivum* Vitt., *T. magnatum* Pico, *T*, *excavatum* forma *monticellianum* Vitt. (Ceruti et al. 2003), and *Tuber fulgens* Quél. (see scanning electron micrographs in Ławrynowicz 2009). Based on molecular analyses, none of these species is closely related to *T. arnoldianum*. However, this character may be taxonomically useful in distinguishing species of *Tuber*.

Discussion

Our limited and relatively small sampling effort detected a total of eight *Tuber* species in the Arnold Arboretum, six (*T. anniae*, *T. arnoldianum*, *T. menseri* nom prov., *Tuber* sp. 36, *Tuber* sp. 37, *Tuber* sp. 57) on exotic trees and four (*T. borchii*, *T. arnoldianum*, *Tuber* sp. 34, *Tuber* sp. 37) on native trees or native rootstock. Most of these were from the /maculatum and /puberulum clades, which consist mostly of small, pale-colored truffles. Many taxa in these groups are considered insufficiently described because we only know of them from EcM root sequences (Bonito et al. 2009). We detected at least two species in the arboretum that are exotic to North America, *T. borchii* and *Tuber* sp. 37. Both of these species are native to Europe and are reported from natural habitats in several countries (see below).

Tuber borchii was detected over two successive years from ITS sequences on root tips of a single native oak (*Quercus rubra* NAC 270) in the Arnold Arboretum. Efforts to find this species on other native and non-native EcM trees in the arboretum were not successful. Many EcM fungi are not dominant or are “rare” on any given host tree, and non-dominant mOTUs of EcM fungi have a patchy distribution on root tips (Richard et al. 2005, Gebhardt et al. 2009). Therefore, we could have missed this species in our sampling. Attempts to find ascomata or mitotic spore mats described for this species

(Urban et al. 2004) were also unsuccessful. It appears that this introduced EcM species did become established on a native host, but we do not know the identity of the original host species or if *T. borchii* was able to persist on the original host. It is very unusual for an introduced EcM species to persist only on native hosts (Vellinga et al. 2009), so we assume that if *T. borchii* was introduced on an exotic host in the Arboretum, it may still persist on that host species. All eight sequences matched only one of two phylogenetically resolved clades known for *T. borchii* (Clade II of Bonuso et al. 2010). To date, there are no other sequences for this clade from outside of Europe. In 2012, truffles of *T. borchii* Clade I were successfully harvested from inoculated trees in Idaho (Berch 2013) and this species was recently detected on roots in a truffière in British Columbia (Berch and Bonito 2014). The presence of *T. borchii* in British Columbia likely resulted from a mix-up of inoculated seedlings by the supplier (Berch and Bonito 2014). The mOTU of the ectomycorrhizae in British Columbia were phylogenetically resolved in Clade I of Bonuso et al. (2010). The only other countries where *T. borchii* has been detected outside of its native European range are Argentina (as determined through EcM morphology) and New Zealand, where it has been intentionally introduced for cultivation (Bonito et al. 2010a). The most likely origin of *T. borchii* in the Arnold Arboretum is colonized roots that were transplanted from Europe prior to 1921. The Arnold Arboretum has good records of European trees that were introduced with their native soil, including the trees that were sampled for this study (Table 1). Although less likely, it is also possible that *T. borchii* spread to the Arboretum via the bare roots of trees that were introduced after 1921 or via an unknown truffière or local garden with exotic EcM trees. A North American supplier sent hazelnut trees inoculated with *T. borchii* to a customer in Maine "some years ago," but there is no evidence that *T. borchii* has been successfully grown there or elsewhere in New England (Charles Lefevere, personal communication). Efforts to track down truffle production in New England have not been successful, and there are no New England states listed among members of the North American Truffle Growers Association. This is the first record of *T. borchii* on *Quercus rubra*, and the first time this species has been detected in North America outside of a truffiére.

The European truffle *Tuber* sp. 37 was prominent among *Tuber* EcM and ascomata because it was found on four of thirteen trees on abundant root tips (n = 33). It apparently has the capacity to colonize native beech roots and was also found on a *Quercus* root of unknown provenance. It is not economically valuable, so it was probably unintentionally introduced and may have arrived as a symbiont on the roots of one of the European trees imported prior to 1921. This pattern fits the fourth introduction outcome described by Vellinga et al. (2009), that introduced EcM fungi thrive on their original host taxa and spread to native tree roots where they become established. This is the first report of *Tuber* sp. 37 in North America.

Tuber anniae was detected on the roots of a European tree, *Pinus nigra*. *Tuber anniae* was described from Washington (Colgan and Trappe 1997) and has been reported from Alaska, Nebraska, Finland, Estonia, Eastern Canada, and non-native pine plantations in New Zealand (Wang et al. 2013; Berch and Bonito 2014). As pointed out by Wang et al. (2013), it is difficult to tell from the available data if this is a single species with a disjunct distribution or if there are two or more cryptic species. Therefore, we cannot infer the geographic origin of *T. anniae* in the Arboretum based on available data.

Tuber arnoldianum was dominant in terms of abundance on roots, number of host trees (four of the 13 studied host trees here), and diversity of hosts (*Corylus*, *Fagus*, and *Quercus*). It was found on both native (*Q. rubra*) and non-native (*Corylus* × *vilmorinii* and *Fagus sylvatica*) hosts. Two previous studies have detected *Tuber arnoldianum* on *Quercus* roots in the USA. In one of these studies focused on *Q. rubra* seedlings from a native plant nursery, seedlings were planted as bait for EcM fungi in a degraded woodland in New Jersey (Karpati 2010) and in a reconstructed woodland in New York (Karpati et al. 2011). The results of Karpati (2010) suggested that this *Tuber* species was acquired in the nursery rather than in the woodlands. Evidence for this interpretation included its dominance on ECM roots in all treatments, including the control treatment where nothing was added to roots of nursery grown seedlings. The second record of *T. arnoldianum* came from an *Epipactis* orchid root tip from New York (HM485415, T. Horton, unpublished). Together with our results, these reports are interesting for several reasons: (1) *Tuber arnoldianum* appears to be a strong competitor when exposed to other native EcM fungi in disturbed environments, (2) this species has the potential to be a good EcM inoculum for native and non-native trees in the Fagaceae that are planted in disturbed environments in eastern North America, and (3) due to its strong competitive ability it is likely that *T. arnoldianum* would directly compete with economically important truffles in any potential future truffières in New England.

Tuber sp. 34 is only known from the Eastern USA and is one of three *Tuber* sp. detected on native red oak in the Arboretum. *Tuber* sp. 36 is native to Eastern North America (Bonito et al. 2010a), but was detected on an exotic host. *Tuber menseri* nom. prov., another North American native, was detected on two exotic hosts, including a gymnosperm and an angiosperm. This species has a broad host range and has been introduced to Europe and New Zealand (Bonito et al. 2010a). It is surprising that it was not detected on any of the native trees. In North America, it has been previously reported from the Pacific Northwest and Quebec (Bonito et al. 2010a).

Tuber sp. 57 was the only representative of the /rufum clade of *Tuber* and was detected on multiple roots of a single *Fagus sylvatica* 'Laciniata' (5361*A). This species has a broad host range having been detected on *Populus*, plantation pine and truffle orchards in Europe and in pecan groves and natural woodlands in North America. This species has also been introduced to New Zealand (Bonito et al. 2010a). We are unable to infer the geographic origin of *Tuber* sp. 57 in the Arboretum.

Arboreta are established to display plants from different origins, with little attention given to belowground symbionts. While exotic forest plantations (Bahram et al. 2013, Barroetaveña et al. 2007, O'Hanlon and Harrington 2012, Tedersoo et al. 2007, Trocha et al. 2012) and urban landscapes (Lothamer et al. 2014) have been studied for their native and exotic mycorrhizal symbionts, there are few records of mycorrhizal fungi in arboreta explicitly reported as exotic. *Descolea*, a southern hemisphere mushroom, was introduced with *Nothofagus* roots into a Danish arboretum (Vellinga et al. 2009). To our knowledge, this paper is the first record of introduced EcM fungi in a North American arboretum. Here, we provide evidence for the accidental transport, establishment and persistence of two European species of truffles (*Tuber*) in North America. We infer these fungi were likely growing associated with roots of their host trees when the trees were transplanted from Europe to the USA over a century ago. We suspect this is not an uncommon incidence. It is probable that many fungi have escaped detection and have been introduced worldwide into other Arboreta, Botanic Gardens and nurseries established prior to mandatory quarantine restrictions (Vellinga et al. 2009). Our inability to determine the origin of *T. anniae*, *Tuber* sp. 57 or some of the non-*Tuber* EcM fungi highlights our limited knowledge on species delimitation and geographic distribution for many fungi, which hampers the tracking of their introduction to a given region. This lack of knowledge is a common problem for EcM and saprotrophic fungi, which can be alleviated by more extensive and methodical reporting of species occurrences, whether or not they are novel (Vellinga et al. 2009) and would improve understanding of fungal invasion ecology. A larger sampling effort that includes all EcM fungi in the Arnold Arboretum would be of interest to better assess introduction outcomes of EcM fungi. The numerous established and well-documented exotic trees alongside native trees makes the Arnold Arboretum a promising site for research on the potential outcomes of introduced species under similar conditions in northeastern North America. Along with the two introduced European *Tuber* species, we also detected a common but previously undescribed native species, *T. arnoldianum*. Based on our results and evidence in the literature, it appears that this newly described truffle species is an aggressive colonizer of native as well as non-native hosts and may have utility in forestry and restoration.

Acknowledgments Zurier received funding for this project from the Microbial Science Initiative at Harvard University. Healy received funding from the Sargent Award through the Arnold Arboretum. Matthew E. Smith received funding from the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number FLA-PLP-005289 and his participation was also made possible via support by the Institute of Food and Agriculture Sciences at University of Florida. Bonito thanks Michigan State University's AgBioResearch for research support. Charles Lefevre of New World Trufflieres Inc. is thanked for his helpful discussion regarding the likelihood of *Tuber borchii* introduction to New England through inoculated roots. Peter Avis is thanked for help with methods. Michael Dosmann, Kathryn Richardson, and Faye Rosin of the Arnold Arboretum are thanked for facilitating this project. The Farlow Herbarium curators are thanked for help with vouchers.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

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