Mycorrhiza (2023) 33:221–228
https://doi.org/10.1007/s00572-023-01114-8

RESEARCH

Accidental cultivation of the European truffle *Tuber brumale* in North American truffle orchards

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Received: 13 January 2023 / Accepted: 25 May 2023 / Published online: 18 June 2023

Abstract

Tuber brumale is a European edible truffle species that is often viewed as a contaminant in truffle orchards, as it visually resembles more valuable black truffles such as *T. melanosporum*, but differs in aroma and flavor and sells for a much lower price. Although *T. brumale* is not native to or intentionally cultivated in North America, it was reported to have been accidently introduced into British Columbia in 2014 and North Carolina in 2020. However, in winter of 2021, various truffle orchards in eastern North America produced truffles that differed from the anticipated harvest of *T. melanosporum*. Molecular analysis of these specimens confirmed *T. brumale* truffle fruiting bodies from ten orchards distributed across six eastern USA states. Phylogenetic analysis of nuclear ribosomal ITS and 28S DNA sequences indicated that all samples belong to the *T. brumale* A1 haplogroup, the genetic subgroup of *T. brumale* that is more common in western Europe. This pattern of widespread fruiting of *T. brumale* in North American truffle orchards is likely the result of *T. brumale* being introduced in the initial inoculation of trees used as hosts in *T. melanosporum* truffle cultivation. We review other examples of introduced non-target truffle species and strategies for limiting their impact on truffle cultivation.

Keywords Edible ectomycorrhizal fungi · Introduced species · Quality control

Introduction

Truffle fungi are among the world's most expensive food products. With retail prices ranging from hundreds to thousands of US dollars per pound, depending on the species, quality, harvest location, and year, there is widespread interest in the commercial cultivation of truffles (Zambonelli et al. 2005; Ho et al. 2008; García-Montero et al. 2009; Zambonelli and Bonito 2012; Reyna and Garcia-Barreda 2014; Zambonelli and Bonito 2012; Bach et al. 2021). Truffles in the genus *Tuber* have been the focus of cultivation efforts for many years, particularly in southern and central Europe where regionally endemic species such as *Tuber melanosporum*, *T. aestivum*, and *T. magnatum* have a long tradition of culinary use (Hall et al. 2007; Zambonelli et al. 2016). Insights into the biology of these fungi in recent decades have allowed for greater control and standardization of cultivation practices, and as a result, several truffle species are now cultivated far beyond their native ranges (Le Tacon et al. 2016; Reyna and Garcia-Barreda 2014; Riccioni et al. 2008). However, given its relatively high market value and track-record of successful cultivation, *T. melanosporum* (the Périgord black truffle) has become the most widely cultivated truffle species. This species is cultivated throughout southern and central Europe as well as in countries beyond its native range such as Australia, Canada, Chile, China, New Zealand, South Africa, and the USA (Berch and Bonito 2014; Guerin-Laguette et al. 2013; Reyna and Garcia-Barreda 2014).

Despite these advances, there are many obstacles to successful cultivation of truffles, including the unintended introduction of less desirable species and competition between intended species and other ectomycorrhizal fungi. Although *Tuber brumale*, also known as the "winter truffle" or the "black musk truffle," is an economically important truffle species, it is considered by many to be a nuisance species in truffle orchards where it can occur along with the more valuable *T. melanosporum* or *T. aestivum* (Martin-Santafe et al. 2014; Merényi et al. 2016). All three of these species are native to Europe and often occur in similar habitats with a

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range of host plant genera such as *Carpinus*, *Corylus*, *Fagus*, *Pinus*, and *Quercus* (Chevalier and Sourzat 2012; Merényi et al. 2014). Although all three species have a dark-colored peridium (exterior) and are highly aromatic at maturity, *T. brumale* is morphologically much more similar to *T. melanosporum* than to *T. aestivum*, especially when mature. Notably, both *T. brumale* and *T. melanosporum* have a dark-colored gleba marbled with white veins when mature and contain dark-colored, spiny spores. *Tuber aestivum*, on the other hand, has a brown-colored gleba (rather than brown-black or black) and alveolate-reticulate spores (Molinier et al. 2016). Furthermore, *T. aestivum* is typically collected in either summer or fall, while *T. melanosporum* and *T. brumale* are both typically found in the winter (Hall et al. 2007; Molinier et al. 2016). Therefore, *T. brumale* is most likely to be confused with *T. melanosporum*, since these species are similar in both habitat, morphology, and phenology.

There are subtle morphological differences between *T. brumale* and *T. melanosporum*. For instance, the gleba of *T. melanosporum* fruiting bodies are typically darker in color than in *T. brumale*, and the sterile veins are often narrower (Fig. 1A). Additionally, spiny ornamentations on mature ascospores of *T. brumale* are longer than those of *T. melanosporum* (Fig. 1B, C) (Montecchi and Sarasini 2000). In the vegetative ectomycorrhizal stage, cystidia can be used to distinguish these two species from each other. *Tuber brumale* ectomycorrhizas form yellowish, unbranched, needle-like cystidia, while cystidia of *T. melanosporum* often form right angle branches (Fig. 1D, E) (Marozzi et al. 2017). Perhaps most importantly for growers, the aroma of *T. brumale* is considered to be more musk-like and sharper compared to *T. melanosporum* or *T. aestivum* and therefore sells for a much lower price (Hall et al. 2007; Merényi et al. 2016; Strojnik et al. 2020). However, neither aroma nor morphology are failsafe tools for separating these species. Accurate identification of morphologically similar *Tuber* species such as *T. brumale* and *T. melanosporum* is best achieved with molecular methods such as species-specific PCR or DNA sequencing of the ribosomal DNA (Benucci et al. 2011; Bonito 2009). Methods for species identification using molecular tools are well established and relatively low-cost, although they require a laboratory equipped for molecular analyses.

In its native range, *T. brumale* can occur naturally in *T. melanosporum* and *T. aestivum* orchards, either due to prior presence in the soil or rhizosphere, movement of soil or plants through human activity, natural dispersal of spores by mycophagous animals, or possibly through vegetative expansion of extraradical mycelium from nearby plants (Merényi et al. 2016; Ori et al. 2018; Parladé et al. 2013; Valverde-Asenjo et al. 2009). Due to its similarity to other cultivated species, *T. brumale* can also be accidentally included in inoculum used to establish truffle-producing trees (Linde and Selmes 2012). Where multiple species are present, *T. brumale* may interact and compete with other target *Tuber* spp. to colonize root tips of host trees, although the dynamics of these interactions are not well understood. One greenhouse study suggested that high irrigation levels may particularly favor *T. brumale* colonization on seedlings co-established with *T. melanosporum* and *T. brumale* mycorrhizas (Mamoun and Olivier 1993). However, a recent 14-year study of the direct competitiveness of *T. brumale* with adjacent *T. aestivum* and *T. melanosporum* plots found that *T. brumale* does not readily outcompete or displace either species on host roots (Ori et al. 2018). This suggests that issues with inoculum quality and nursery contamination are likely the most important vectors for introductions of *T. brumale* into truffle orchards. Outside of its native range in Europe, the only reasonable pathway for accidental introduction of *T. brumale* into truffle orchards is via human cultivation practices (Bonito et al. 2010; Guerin-Laguette et al. 2013; Linde and Selmes 2012).

Tuber brumale is not native to North America and has never been purposely cultivated on the continent, and there are no nurseries that advertise the sale of *T. brumale*-inoculated seedlings. However, there are two previously documented occurrences of *T. brumale* in North America, first from ectomycorrhizal root tip sequences obtained in a truffle orchard in British Columbia (Berch and Bonito 2014) and subsequently as an ascocarp collected in a truffle orchard in North Carolina that was established with plants said to have been inoculated with *T. melanosporum* (Meadows et al. 2020).

As part of ongoing efforts to document truffle fungi in North America, we obtained numerous *Tuber* specimens from ten *T. melanosporum* orchards that were identified by orchard owners and/or collectors as unusual. These samples were not collected as part of any systematic sampling and were sent to labs at the University of Florida or Michigan State University for identification at the request of growers and collectors. We hypothesized that truffles were of a non-target species, *T. brumale*. We tested this hypothesis by sequencing the ITS and 28S rDNA regions of these samples and predicted they will match sequences generated from European truffles (Merényi et al. 2014). We also review other instances where *Tuber* species of lesser economic value have been unintentionally introduced outside their native ranges and discuss strategies to reduce problems with the cultivation of non-target species.

Methods

Between December 2021 and December 2022, a total of 36 black truffle samples were sent for testing by growers and collectors from ten orchards intending to produce *T.*

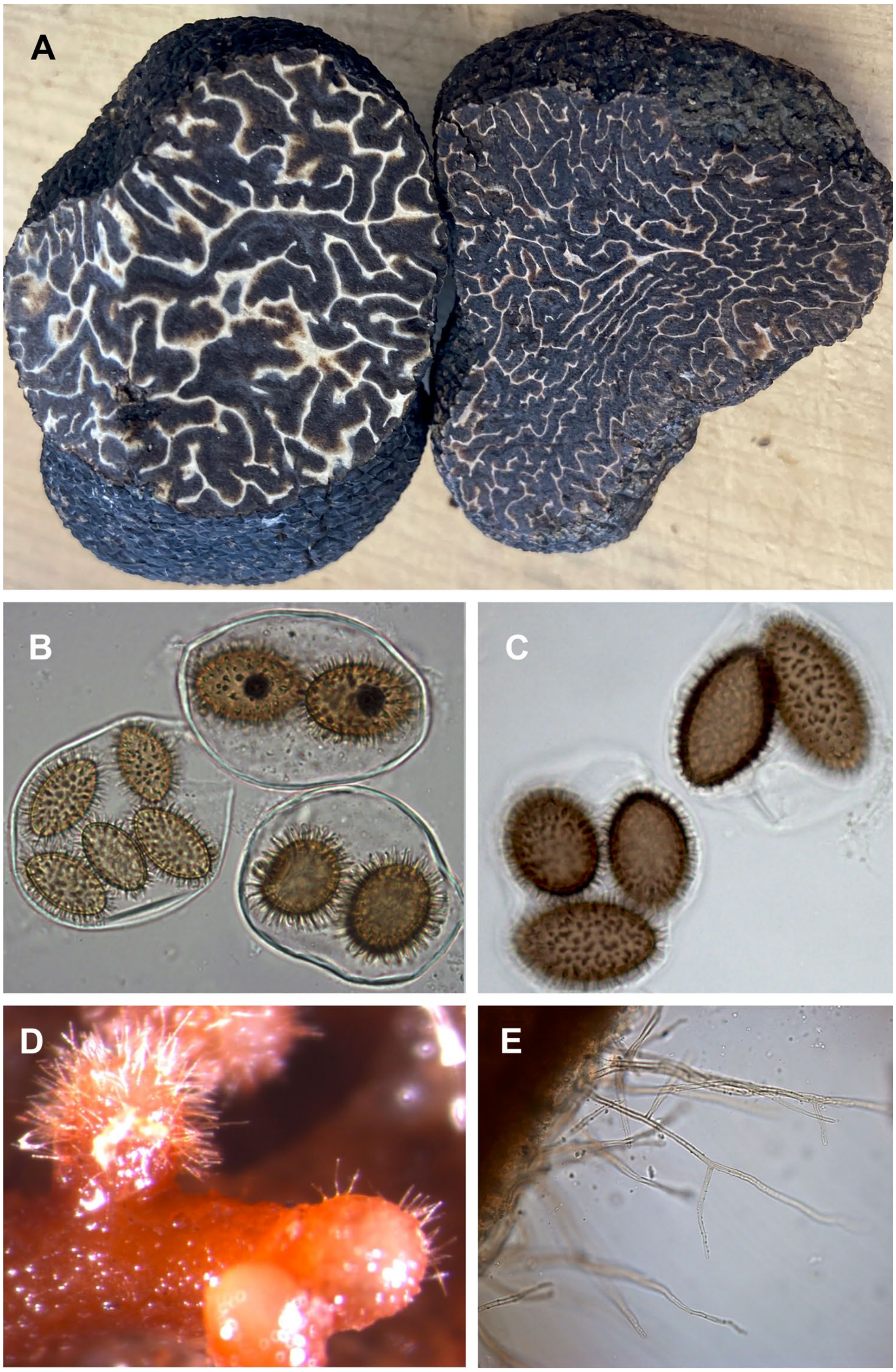

Fig. 1 Morphological similarity between *T. brumale* (**A**, left; **B**, and **D**) and *T. melanosporum* (**A**, right; **C**, and **E**) in gleba (**A**), spores (**B**, **C**), and emanating cystidia on ectomycorrhizal root tips (**D**, **E**)

melanosporum. Samples were air dried and sent to laboratories at either the University of Florida or Michigan State University by orchard owners or truffle collectors for analysis. These samples were collected and sent for testing as part of regular survey and harvesting activities conducted by individual growers and truffle collectors. These samples were not collected through any systematic sampling effort designed by the authors of this study. This analysis was provided as an identification service to growers and collectors and is part of a continuing effort by both laboratories to document truffle diversity and preserve diverse truffle specimens for future study (Lemmond et al. 2022b). Dried samples were deposited into the University of Florida Herbarium (FLAS-F) with precise location information obscured (S1).

Orchard names and exact locations are not disclosed to protect the confidentiality of growers and collectors. Orchards represented in this study ranged from 7 years to approximately 14 years since establishment. All orchards included in this study were established with trees purchased from commercial sources. Orchards were planted with either hazelnut (*Corylus*) or a mixture of hazelnut and oak (*Quercus*) hosts. Dried truffle samples were examined microscopically, and DNA was extracted from a small piece of clean internal gleba tissue using an alkaline DNA extraction buffer (Vandepol et al. 2020). The nuclear rDNA internal transcribed spacer (ITS) region ITS1-5.8S-ITS2 and the nuclear ribosomal 28S large subunit region were amplified using polymerase chain reaction (PCR) with some combination of fungal-specific primer pairs ITS1f-ITS4, ITS1f-LR3, and LROR-LR5 (Gardes and Bruns 1993; White et al. 1990) or Tuberaceae-specific primers TubITS1-TubITS4 (Bonito et al. 2013). PCR products were visualized on 1.5% agarose gels and Sanger sequenced with the same primers by Eurofins Genomics (Louisville, Kentucky). Sequences generated from these samples were deposited in Genbank (S1).

Reference ITS and 28S sequences of representative species in the Melanosporum clade were downloaded from Genbank to construct a phylogenetic analysis of the orchard samples included in this study (Fan et al. 2022; Lemmond et al. 2022a) (S1). *Tuber spinoreticulatum* was selected as an outgroup based on its phylogenetic position relative to the Melanosporum clade in Bonito et al. (2013). ITS and 28S sequences were concatenated for all samples that had separate ITS and 28S sequences. Sequences were aligned with MUSCLE 3.8.425 (Edgar 2004) using default settings in Geneious 2020.2.4 (Auckland, New Zealand) and checked manually. A large indel region (approximately 150 bp) present in the ITS1 region of several taxa in the Melanosporum clade was manually excluded from the analysis (Lemmond et al. 2022a; Merényi et al. 2016). Subsequently, other ambiguously aligned regions were removed from all alignments with Gblocks (Talavera and Castresana 2007) using the least stringent settings. The final alignment contained 83 sequences with 920 characters. The TIM2 + I + G model of nucleotide substitution was selected for the alignment with jModelTest2 (Darriba et al. 2012; Guindon and Gascuel 2003). Phylogenetic analysis was conducted with maximum likelihood (ML) using RAxML-NG on the CIPRES science portal (Miller et al. 2010) and 1000 bootstrap replicates to evaluate support for nodes. The resulting tree was visualized and rooted in FigTree 1.4.4 (Rambaut 2018).

Results

Between December 2021 and December 2022, we received 36 unidentified black truffle samples from ten orchards. The majority of samples were immature and had a whitish gleba, and only a few samples had brown gleba and mature spores. Since all specimens were received dried, aroma characteristics were not reported or compared from these truffles. Spore characteristics alone were not used as identifying factors due to the overlap in morphological characteristics among *Tuber* species in the Melanosporum clade, which includes both *T. melanosporum* and *T. brumale*, and the fact that most of the collections were so immature that they lacked spores (Merényi et al. 2017).

DNA sequencing and phylogenetic analysis identified 35 samples from ten orchards as *T. brumale* (Fig. 2). Only one sample included in this study was identified as *T. melanosporum* (FLAS-F-71144). The orchard that produced this sample, located in the Greater Philadelphia region, also produced four samples identified as *T. brumale*. A map depicting generalized locations of orchards with confirmed detection of *T. brumale* is included in Fig. 2. The geographic regions indicated on this map are broadly defined to protect the anonymity of the orchards where the samples originated. *Tuber brumale* was detected in four orchards in the North Carolina Piedmont region ($n = 4$ truffles tested), two orchards in the North Carolina Appalachian region ($n = 20$), one orchard in central Virginia ($n = 2$), one orchard in central Tennessee ($n = 3$), one orchard in central Kentucky ($n = 2$), and one orchard in the greater Philadelphia region ($n = 5$). The phylogenetic analysis placed all *T. brumale* samples within the Haplogroup A1 identified by Merényi et al. (2014). Haplogroup 1A is the *T. brumale* haplotype most common in western Europe, whereas Haplogroup A2 is more common in the Balkan region of eastern Europe. These results confirm the establishment and accidental cultivation of European *T. brumale* in six broadly defined regions in the eastern USA: central Virginia, central Tennessee, central Kentucky, the Greater Philadelphia region, and the Piedmont and Appalachians regions in North Carolina.

Discussion

This report documents the accidental cultivation of *T. brumale* in ten North American truffle orchards in six regions across the eastern USA, including several states where *T. brumale* has not been previously reported. Prior to this study, *T. brumale* had only been detected twice in North America, once from British Columbia (Berch and Bonito 2014), and once from North Carolina (Meadows et al. 2020). Thus far, all known records of *T. brumale* in North America are from orchards intending to produce *T. melanosporum*. This report of widespread occurrence of *T. brumale* in the eastern USA represents an escalation of the problem of orchard contamination from previous isolated reports, demonstrating that this is a more widespread and regional problem. As such, these findings are significant

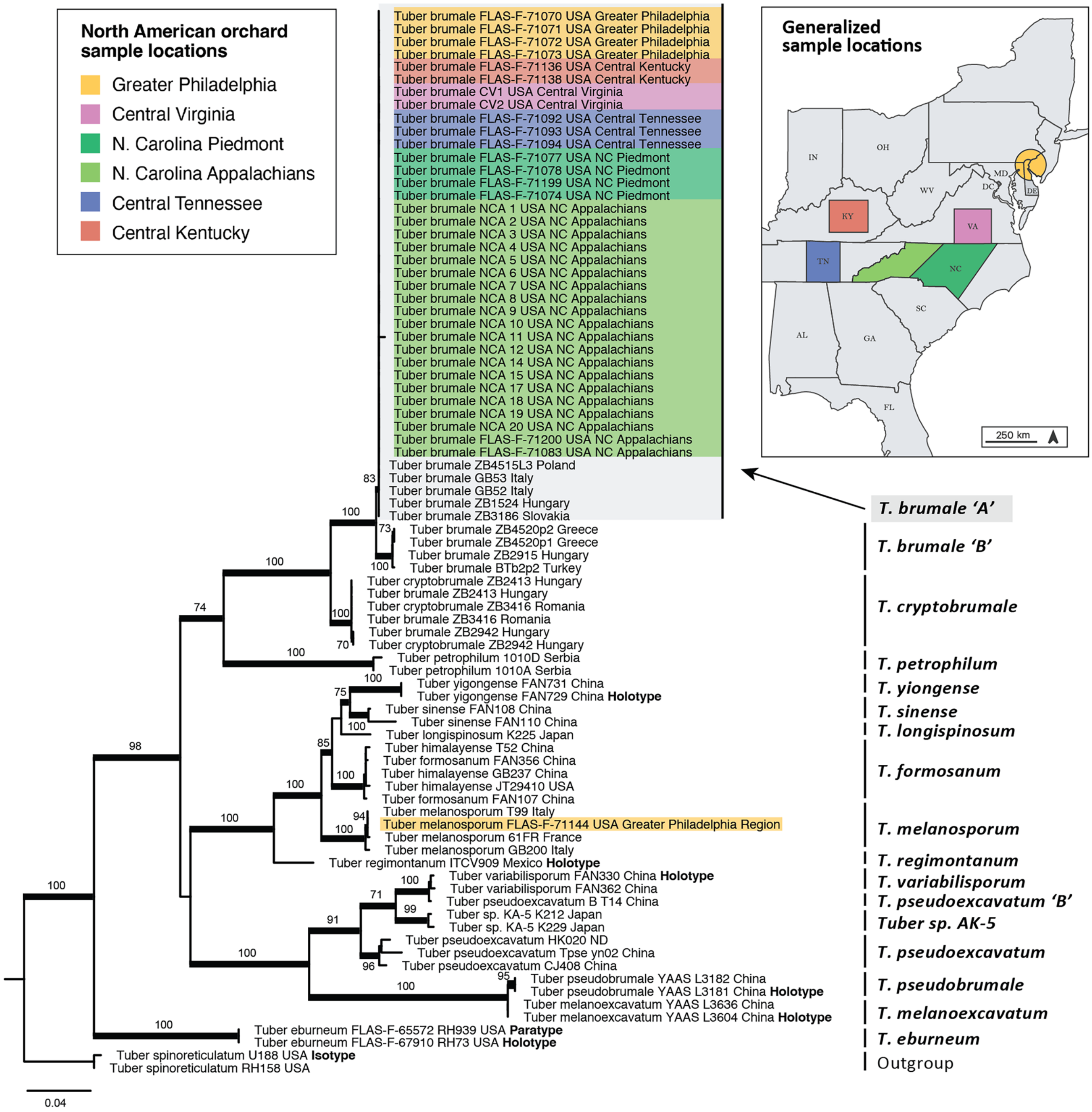

Fig. 2 Maximum likelihood phylogeny of concatenated ITS + 28S ribosomal DNA sequences showing placement of orchard samples (indicated by colored bars) either among European specimens of the *T. brumale* Haplogroup “A1” clade or among other specimens of *T. melanosporum*. Branches are considered supported when bootstrap values ≥ 70% (indicated by thickened lines). A map of the Eastern USA (top right) shows generalized regions of orchards with confirmed detection of *T. brumale*. Precise locations are obscured to protect the anonymity of the orchard owners. Regions indicated in the map are artificial and region boundaries do not correspond to any precise geographic extent of confirmed *T. brumale* presence

both for the individual truffle cultivators affected and for the North American truffle cultivation community at large. Thus far, management strategies to contain or eliminate *T. brumale* in orchards have not proven effective, and therefore the presence of *T. brumale* on contaminated orchards is likely to be a perennial issue (García-Montero et al. 2009; Valverde-Asenjo et al. 2009).

Given the improbability of eradicating *T. brumale*, it is also possible that this species may become naturalized in some areas outside of orchard settings. Naturalization of non-native

Tuber species has been observed many times globally and has already occurred in North America with *T. formosanum* (then identified as *T. indicum*), another unintentionally introduced edible black truffle (Berch and Bonito 2014, 2016; Bonito et al. 2011, 2013). However, the absence thus far of any documented naturalization of *T. brumale* in North America, the widespread geographic occurrence of *T. brumale* in truffle orchards, and the evidence suggesting that *T. brumale* does not readily displace *T. melanosporum* or *T. aestivum* in orchard settings (Ori et al. 2018) all make it highly unlikely that naturalized *T. brumale* is the source of the orchard contamination observed in this study. Given that there are no truffle nursery companies in North American that sell seedlings inoculated with *T. brumale*, the most likely explanation for the occurrence of *T. brumale* in multiple orchards across a large area is that *T. brumale* was unintentionally included in the original inoculum used for inoculating seedlings that were purchased to establish these orchards.

Despite its reputation as a contaminant truffle, *T. brumale* remains a valuable edible truffle that is processed and sold for commerical purposes (Hall et al. 2007). However, prices for *T. brumale* are typically lower than those for *T. melanosporum* and *T. aestivum* (Bonito et al. 2013). Numerous studies have demonstrated that the aroma of *T. brumale* is distinct from other black truffles (Kiss et al. 2011; Strojnik et al. 2020; Vahdatzadeh et al. 2015). Therefore, the detection of *T. brumale* on an orchard will likely impact the orchard's overall potential to return profit, though the exact impacts are difficult to estimate. In addition to differences in price between *T. brumale* and other black truffles, individual impacts of cultivating *T. brumale* will vary depending on the amount of *T. brumale* produced in comparison with *T. melanosporum* in any orchard where both are present. Regardless, orchard owners with confirmed *T. brumale* production may have to invest additional time and effort to differentiate species by testing truffles harvested from their orchard and will certainly need to take extra care to avoid contaminating future batches of *T. melanosporum* inoculum produced from their truffles.

The introduction of undesirable or non-target truffle species has occurred in several additional regions outside of Europe where cultivation of European black truffles has been attempted. For instance, *T. brumale* has been detected in New Zealand (Zambonelli et al. 2005; Ho et al. 2008; Guerin-Laguette et al. 2013), Australia (Linde and Selmes 2012), and Canada (Berch and Bonito 2014, 2016). All three countries lacked quality standards or testing requirements for inoculated seedlings at the time when truffle cultivation efforts first began. Currently, inoculum testing and certification processes are either mandated or at least available in some places where *T. brumale* and other non-target species are an issue, including most major European truffle-producing countries as well as Australia and New Zealand (Andrés-Alpuente et al. 2014; Australian Truffle Industry Association 2021; New Zealand Truffle Association n.d.). However, at present, there are no testing requirements or industry standards in place for truffles and truffle-inoculated seedlings in North America. Given that *T. brumale* is most likely introduced at the inoculum stage, a certification program with an accurate and reliable testing process is the best method for preventing unintended introductions of non-target *Tuber* species into cultivation systems (Murat 2015).

In conclusion, we used molecular approaches to confirm that *T. brumale* truffles are fruiting across multiple states and orchards in the eastern USA. Since these orchards were set up for cultivation of *T. melanosporum* and no seedling producers sell seedlings deliberately inoculated with *T. brumale*, it is most likely that *T. brumale* was introduced to seedlings during the inoculation stage, as has occurred on other continents. Given the limited and passive sampling that was undertaken here, it is likely that a broader, systematic sampling of North American truffle orchards would reveal additional sites where *T. brumale* has been introduced. While it is not feasible to eradicate this species once it has been introduced without destroying the orchard, it may be worthwhile to sell *T. brumale* in North America since these truffles are now being produced. However, if *T. brumale* is sold, it is essential that correct species names are used at all times instead of vague terms such as "black truffle" or "winter truffle" to avoid confusion with other, similar species. Establishing a certification program for truffle-inoculated seedlings could help prevent further unintended introductions or the sale of one truffle species for another. Truffle farmers and individuals planning to cultivate truffles could protect themselves from such mistakes by purchasing seedlings from reputable sources, having their trees tested with molecular tools prior to planting, and testing any other truffle inoculum that is used in their truffle orchard.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00572-023-01114-8.

Acknowledgements The authors wish to thank several anonymous truffle orchard owners and truffle collectors and their dogs for contributing specimens and information that made this work possible and to Alexis Guerin and Gian Maria Nico Benucci for providing images and discussion on *Tuber melanosporum* and *T. brumale*. We are also grateful to Jud Van Wyk and Marc Friedman for laboratory assistance and mentorship of AS.

Author contribution BL, MS, and GB conceived the project. BL and AS obtained samples and conducted labwork. BL and GB compiled data, conducted analyses, and prepared figures. GB and MS provided resources to facilitate this work. All authors contributed to writing and editing the final manuscript.

Funding This work was supported by the US National Science Foundation grant DEB-1946445 (to GB and MES) and US National Science Foundation Graduate Research Fellowship (Fellowship no. 2019277707) (to BL). AS was supported through US National Science Foundation DEB 1737898 to GB.

Availability of data and materials All fungi specimens are deposited at the Florida Museum of Natural History (FLAS-F), and DNA sequences are deposited at NCBI GenBank.

Declarations

Competing interests The authors declare no competing interests.

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