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Genetic Diversity and Mating Type Distribution of *Tuber melanosporum* and Their Significance to Truffle Cultivation in Artificially Planted Truffiéres in Australia

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***Tuber melanosporum* is a truffle native to Europe and is cultivated in countries such as Australia for the gastronomic market, where production yields are often lower than expected. We assessed the genetic diversity of *T. melanosporum* with six microsatellite loci to assess the effect of genetic drift on truffle yield in Australia. Genetic diversity as assessed on 210 ascocarps revealed a higher allelic diversity compared to previous studies from Europe, suggesting a possible genetic expansion and/or multiple and diverse source populations for inoculum. The results also suggest that the single sequence repeat diversity of locus ME2 is adaptive and that, for example, the probability of replication errors is increased for this locus. Loss of genetic diversity in Australian populations is therefore not a likely factor in limiting ascocarp production. A survey of nursery seedlings and trees inoculated with *T. melanosporum* revealed that <70% of seedlings and host trees were colonized with *T. melanosporum* and that some trees had been contaminated by *Tuber brumale*, presumably during the inoculation process. Mating type (MAT1-1-1 and MAT1-2-1) analyses on seedling and four- to ten-year-old host trees found that 100% of seedlings but only approximately half of host trees had both mating types present. Furthermore, MAT1-1-1 was detected significantly more commonly than MAT1-2-1 in established trees, suggesting a competitive advantage for MAT1-1-1 strains. This study clearly shows that there are more factors involved in ascocarp production than just the presence of both mating types on host trees.**

Tuber melanosporum (Perigord or Black truffle) is a subterranean ascomycete ectomycorrhizal fungus that develops a symbiotic relationship with host trees such as oaks (e.g., *Quercus ilex* and *Q. robur*) and hazels (e.g., *Corylus avellana*). It is prized for the ascocarps, known as truffles, produced in winter. *Tuber melanosporum* is found growing naturally in the calcareous soils of Spain, France, and Italy. Through the inoculation of young trees, truffles are now cultivated in other areas such as Australia, New Zealand, and North America (2, 3). The first truffiére in Australia was established in Tasmania in 1993 with the first truffle produced in 1999. Unfortunately for many Australian growers, the yields harvested are low and highly variable, with only 10% of the 150 truffiéres established producing commercial quantities of truffle and over 100 truffiéres yet to produce even a single truffle (Graham Duell, president of the Australian Truffle Association, unpublished data). There are a number of factors that might be limiting truffle production in Australia, including climate and edaphic factors. Factors we investigate here include the low genetic diversity of truffles (founder effect) when imported from Europe and used in tree inoculations, the quality of the inoculum on nursery trees, and the distribution of *T. melanosporum* mating types, i.e., the potential for sexual reproduction to occur.

Genetic diversity plays a major role in species survival and adaptability to changing environments. Previous studies on *T. melanosporum* in Europe revealed strikingly low genetic variation, most likely due to the effects of a bottleneck that occurred during the last ice age (ca. 10,000 years ago) (1, 2, 14). During this time the broadleaved forests of Europe, including oaks, the main host of *T. melanosporum*, were considerably reduced and persisted in refuges in southern Europe, mainly in the Mediterranean coastal zone (1, 15). Since *T. melanosporum* ascocarps are susceptible to frost and cannot tolerate the cold temperatures of high mountains or northern Europe, a bottleneck this severe as a result of an ice age may have driven many loci to fixation, thus severely reducing the amount of genetic diversity within the species (14). As an import to establish the truffle industry, *T. melanosporum* in Australia would have undergone a further bottleneck and most likely has a further reduced genetic diversity in comparison to the European population, which may impact its ability to adapt to Australian environmental and soil conditions.

Until recently, it was widely believed that *T. melanosporum* was homothallic, or an exclusively selfing species (1). Riccioni et al. (24) found evidence that *T. melanosporum* undergoes sexual reproduction when additional alleles in the asci were found, beside those present in the surrounding gleba. A study by Martin et al. (12) has confirmed that *T. melanosporum* is a heterothallic and outcrossing species through the identification of genes related to sexual components such as pheromone response and meiosis. The presence of mating type (MAT) genes known to establish sexual compatibility in other ascomycete filamentous fungi were also identified (12). Therefore, strains of opposite mating type of this obligate outcrossing species must come into contact for sexual reproduction, i.e., truffle formation, to occur (15, 26). By identifying the mating type of *T. melanosporum* present as mycorrhiza on host trees, it is possible to investigate the potential for reproduction to occur. If either MAT strain is absent from a host tree, i.e., if opposite MAT strains are unable to make contact, then

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TABLE 1 Genetic diversity within ascocarps collected from seven Australian populations based on six SSR markers

Population	No. of ascocarps	No. of haplotypes	Mean no. alleles per locus (SE)[a]	Gene diversity (*H*) (SE)
WA1	24	7	1.62 (0.211)	0.18 (0.082)
TAS1	23	8	1.99 (0.258)	0.27 (0.085)
TAS2	27	6	1.62 (0.333)	0.15 (0.090)
NSW	37	10	1.97 (0.307)	0.21 (0.078)
WA2	13	7	1.83 (0.307)	0.19 (0.064)
VIC	30	9	2.23 (0.477)	0.17 (0.079)
TAS3	56	9	1.94 (0.494)	0.16 (0.075)
Overall	210	34	3.67 (0.760)	0.25 (0.090)

[a] That is, the mean number of alleles rarefactioned to a sample size of 13 for individual populations.

TABLE 2 Presence of *Tuber melanosporum* and *Tuber brumale* mycorrhiza on *T. melanosporum*-inoculated seedlings

Nursery	No. of seedlings analyzed	No. of seedlings colonized with:		
		T. melanosporum	*T. brumale*	Neither
Q. robur				
Nursery 1	20	19	0	1
Nursery 3	26	9	2	15
Nursery 4	3	2	0	1
Nursery 5[a]	2	2	1	0
Total (%)	51	32 (62.8)	3 (5.9)	17 (33.3)
Q. ilex				
Nursery 2	20	20	0	0
Nursery 3	8	3	4	1
Nursery 5	3	1	0	2
Total (%)	31	24 (77.4)	4 (12.9)	3 (9.7)
C. avellana				
Nursery 5	3	2	0	1
Total (%)	3	2 (66.7)	0 (0.0)	1 (33.3)
Total overall (%)	85	58 (68.2)	7 (8.2)	21 (24.7)

[a] One seedling had both *T. melanosporum* and *T. brumale* present.

sexual reproduction cannot occur and ascocarps will not be produced.

A recent study has found that competition may be occurring within genetically different *T. melanosporum* strains on host plants. Rubini et al. (26) analyzed the MAT distribution of seedlings 6 months after inoculation with *T. melanosporum* and again 13 months later. These researchers found that, initially, 11 of 12 seedlings had mycorrhiza of both MAT strains, while 13 months later the mycorrhiza of 7 of 12 of these seedlings had only one MAT strain present with another 2 seedlings showing a strong prevalence for one MAT strain. Only three seedlings had both mating types present in approximately equal proportions (26). These authors also studied the distribution of MAT strains in natural truffle soil and found that the MAT distribution was patchy. The mycorrhizae from productive host trees sampled 3 to 30 m apart all shared the same MAT strain and the closest samples with opposite MAT strains were 50 m apart (25). This unequal distribution of mating types may have occurred due to one mating type being more competitive than the other. This idea is consistent with vegetative spread of a single fungal strain that is able to outcompete and displace all other strains (25). However, the trees were still producing; therefore, the other mating type was either present but not sampled or present as soil inoculum only.

The aims of the present study were to compare the genetic diversity of *T. melanosporum* in Europe and Australia, using microsatellite markers, and to determine whether founder effects may have negatively affected truffle production in Australia through a reduction in genetic diversity. To address the issue of low truffle yields in Australia, we analyzed *T. melanosporum*-inoculated seedlings with species-specific primers prior to being planted on the truffiére to assess whether seedlings are colonized by *T. melanosporum*. In the absence of a tree certification program in Australia, there is effectively no control on the quality of seedlings sold to potential truffle growers and whether these seedlings have the correct truffle species present. We also analyzed host trees that had been planted on truffiéres between 2003 and 2007 to assess whether *T. melanosporum* was able to persist on host trees. The potential for sexual reproduction to occur was assessed by analyzing the mycorrhiza from a subset of inoculated seedlings and host trees for the presence of *T. melanosporum* mating types: MAT1-1-1 and MAT1-2-1.

MATERIALS AND METHODS

Single sequence repeat (SSR) and data analysis. To assess the genetic diversity of *T. melanosporum* in Australia, 210 ascocarps were sourced from seven truffiéres spanning locations in New South Wales (NSW), Australian Capital Territory (ACT), Victoria (VIC), Tasmania (TAS), and Western Australia (WA), in 2009 and 2010 (Table 1). These truffiéres were established with inoculated trees from at least four nurseries. DNA was extracted from the gleba with a DNeasy plant minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Seven previously described nonlinked SSR loci—ME2, ME4, H1b, F12I, 07ISSR9, and 07ISSR14—were selected for analyses (Riccioni et al. [24]). To compare SSR alleles found in the present study to a previous study of *T. melanosporum* in Europe (Riccioni et al. [24]), 17 representative European samples were included in our analyses to calibrate allele sizes (the DNA was kindly provided by Francesco Paolocci and Claudia Riccioni, CNR-Institute of Plant Genetics, Perugia, Italy).

The SSR PCR cycles and reaction mixtures followed those of Riccioni et al. (24). Annealing temperatures were optimized for each locus on an Eppendorf PCR cycler (Applied Biosystems) and was increased for 07ISSR9 from 54 to 59°C and decreased for H1b to 43°C. Forward primers were labeled with a fluorescent dye (FAM, VIC, NED, or PET), and the resulting amplicons were analyzed by capillary electrophoresis on an ABI 3100 sequencer (Applied Biosystems) with a Genescan 500 LIZ size standard (Applied Biosystems). Sizing of amplicons was performed using GeneMapper software version 3.7 (Applied Biosystems).

Estimations of allele frequency and gene diversity (17) were performed using GenAlEx v 6.41 (21) for individual populations and the combined data set. Allele richness for each population was calculated in FSTAT v2.9 (6) based on the rarefaction method (22) to a minimum sample size of 13.

Root sample collection and DNA extraction. As part of a survey to determine the presence and mating type of *T. melanosporum* as mycorrhizae on roots, 85 2-year-old seedlings purchased in 2010 and 2011 from a range of Australian nurseries (Table 2) and 91 host trees sampled in 2011 across 12 truffiéres which had been planted between 2003 and 2007 (Table 3) were analyzed. All plants were reportedly inoculated with *T. melanosporum*. The host trees analyzed included *Q. robur*, *Q. ilex*, and *C. avellana* (Table 2). Mating type analyses of host trees were conducted on productive (have produced at least one ascocarp since establishment), as well as unproductive trees (an ascocarp has not been detected). Six *Q. ilex* and four *Q. robur* 2-year-old nursery seedlings were also included for mating type analyses.

Four soil and root samples were collected at a depth of ca. 15 to 20 cm

TABLE 3 Presence of *Tuber melanosporum* and *Tuber brumale* mycorrhizae on host trees

Truffière	No. of trees analyzed	No. of trees that had:		
		T. melanosporum	*T. brumale*	Neither
Q. robur, nonproducing				
Truffière 1	14	13	1	0
Truffière 2	3	1	1	1
Truffière 3	2	2	0	0
Truffière 4	10	8	0	2
Truffière 5	1	0	1	0
Truffière 6	4	1	2	1
Truffière 7	3	1	0	2
Truffière 8	2	2	0	0
Truffière 9	14	3	2	9
Truffière 10	2	1	0	1
Truffière 11	1	0	1	0
Truffière 12	1	1	0	0
Total (%)	57	33 (57.9)	8 (14.0)	16 (26.3)
Q. robur, producing				
Truffière 1	8	8	0	0
Truffière 2	4	4	0	0
Total (%)	12	12 (100.0)	0 (0.0)	0 (0.0)
Q. ilex, nonproducing				
Truffière 3	2	2	0	0
Truffière 5	2	2	0	0
Truffière 12	2	1	0	1
Total (%)	6	5 (83.3)	0 (0.0)	1 (16.7)
C. avellana, producing				
Truffière 2	4	4	0	0
Total (%)	4	4 (100.0)	0 (0.0)	0 (0.0)
C. avellana, nonproducing				
Truffière 2	3	1	0	2
Truffière 3	2	1	0	1
Truffière 5	6	5	0	1
Truffière 12	1	0	1	0
Total (%)	12	7 (58.3)	1 (8.3)	4 (33.0)
Total overall (%) ($n = 91$)	91	61 (67.0)	9 (9.9)	21 (23.1)

from each tree at the corners of a square, 0.5 to 1.5 m from the tree trunk, depending on tree age. The root samples were soaked in water to separate roots from soil. From each sample, 30 root tips colonized with *Tuber* were collected randomly using a stereomicroscope. Identification of *Tuber* ectomycorrhizae, followed morphological characteristics as previously described (23). The samples were lyophilized, and tissue was disrupted in a Fast Prep (Thermo Electron Corp., Milford, MA) machine, before DNA was extracted using a DNeasy plant minikit (Qiagen) according to the manufacturer's instructions.

Species-specific and MAT PCR analysis. A species-specific PCR analysis of the internal transcribed spacer (ITS) region was performed to determine whether *T. melanosporum* and/or *Tuber brumale* and *Tuber indicum* were present on roots from the 85 seedlings and 91 host trees. A PCR diagnostic test for *T. brumale* was included since cystidia typical of *T. brumale* (4) were observed in preliminary observations. Furthermore, MAT amplicons were occasionally produced from root samples containing *T. brumale* DNA (data not shown), and therefore these samples needed to be excluded from mating type analyses for *T. melanosporum*. The analysis was carried out on all samples using *T. melanosporum*, *T. brumale*, and *T. indicum* species-specific primers, i.e., ITSML, ITSB, and ITSCHCH in combination with the universal primer ITS4LNG (19). The PCR cycling parameters were as follows: 6°C for 10 min and 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s, and 45°C for 30 s, followed by 72°C for 7 min, 5°C for 5 min, and 25°C for 1 min. The PCRs were performed in a 55-μl reaction mixture containing 1× PCR buffer (Mango *Taq* buffer; Bioline), 2.2 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphates (dNTPs), 10 pmol of each primer (ITSB, ITS4LNG, and ITSML), 375 mg of bovine serum albumin (BSA; Sigma catalog no. A7906), 2.5 U of *Taq* (Mango *Taq*; Bioline), and 20 to 50 ng of template DNA.

Mating type analysis was conducted on samples where at least three samples per tree were found to contain *T. melanosporum* in the ITS analysis. In total, 38 trees (33 *Q. robur*, 4 *C. avellana*, and 1 *Q. ilex*) and 10 seedlings (6 *Q. ilex* and 4 *Q. robur*) were analyzed for mating type. Primers P_19 and P_20 to amplify MAT1-1-1 and primers P_1 and P_2 for MAT1-2-1 were used (26) with the following PCR cycling parameters: 6°C for 10 min and 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 65 or 69°C (for MAT1-2-1 or MAT1-1-1, respectively) for 30 s, and 72°C for 30 to 45 s, followed by 72°C for 7 min, 5°C for 5 min, and 25°C for 1 min. The PCRs were performed separately for each mating type in a 55-μl reaction mixture containing 1× PCR buffer (Mango *Taq* buffer; Bioline), 4 mM $MgCl_2$, 0.1 mM dNTPs, 10 pmol of each primer, 375 mg of BSA (Sigma catalog no. A7906), 2.5 U of *Taq* (Mango *Taq*; Bioline), and 20 to 50 ng of template DNA.

PCR products were electrophoresed in 1.2 and 1.6% (wt/vol) agarose gels for ITS PCR products and MAT PCR products, respectively, and visualized by post-staining with Gel Red (Biotium, Hayward, CA) according to the manufacturer's directions. The PCR gels were viewed and photographed with a UV transilluminator. Where a band of the expected DNA fragment length was observed, the sample was scored as positive for the mating type or *Tuber* species present. Differences in the numbers of root samples and trees where MAT1-1-1 and MAT1-2-1 were identified were compared by chi-square analysis.

RESULTS AND DISCUSSION

This study provides an assessment of *T. melanosporum* colonization of inoculated host trees, as well investigating two genetic factors that potentially influence ascocarp production: mating type distribution and genetic diversity.

Genetic diversity of *T. melanosporum*. All six loci were polymorphic with a mean number of alleles per locus of 3.67 among the 210 ascocarps examined (Table 1). Allele richness was similar across truffières and ranged from 1.62 to 2.23. Also, gene diversity (H) across truffières was similar with a mean for the Australian population of $H = 0.25$ (Table 1). Similar to the findings of Riccioni et al. (24) for European *T. melanosporum*, the SSR genetic diversity observed in Australia is low, and one allele per locus was always found at a significantly higher frequency (fixed alleles) within a population, with the exception of ME2 (Table 4). However, although the allele richness estimates between the Australian and European populations are comparable, we found more alleles for loci 07ISSR9 (Australia = 4, Europe = 2), ME2 (Australia = 7, Europe = 2), and ME4 (Australia = 4, Europe = 2) and one allele less for loci 07ISSR14, F12I, and H1b, but one allele more at locus F121 than found by Bertault et al. (2) (Table 4). In addition, Riccioni et al. (24) observed only 22 haplotypes across 206 individuals from 13 populations across Spain, Italy, and France, whereas we identified here 34 haplotypes out of 210 samples (Table 1). The numbers of individuals sampled by Riccioni et al. (24) and Bertault et al. (2) of 206 and 197 individuals, respectively, were only slightly smaller than the sample size used in the present study, so the differences in allelic diversity are unlikely to be a factor of sample size. Therefore, it can be assumed that there was no significant loss of genetic diversity within *T. melanosporum* in Australia as a result of founder effects.

It is possible that the higher allelic and haplotype diversity observed in the Australian populations may be a result of ascocarps being sourced from many populations across Europe to be used as

TABLE 4 Allele frequencies at each locus per population and for the combined data set[a]

Locus	Allele[b]	Allele frequency							
		WA1	TAS1	TAS2	NSW	WA2	VIC	TAS3	Australia
07ISSR9	234	0	0	0	0	0	0.07	0	0.010
	245	0	0	0	0	0	0.04	0.04	0.015
	246	**0.96**	0.26	**0.90**	**0.77**	**0.75**	**0.75**	**0.69**	**0.722**
	247	0.04	**0.74**	0.10	0.23	0.25	0.14	0.27	0.253
ME2	230	0	0	0	0.03	0	0	0	0.005
	240	0	0	0	0	0.08	0	0	0.005
	242	**0.50**	0.29	**0.48**	**0.97**	**0.84**	**0.97**	0	**0.507**
	244	0	0	0.08	0	0.08	0.03	0.02	0.025
	246	**0.50**	**0.52**	0.44	0	0	0	**0.94**	0.429
	248	0	0	0	0	0	0	0.04	0.010
	250	0	0.19	0	0	0	0	0	0.020
ME4	196	0	0	0	0	0	0	0.09	0.025
	198	0.21	0.13	0.07	0.29	0.15	0.29	0.09	0.176
	200	**0.79**	**0.87**	**0.93**	**0.71**	**0.85**	**0.71**	**0.80**	**0.794**
	202	0	0	0	0	0	0	0.02	0.005
07ISSR14	***210***	**0.92**	**0.86**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**0.975**
	212	0.08	0.14	0	0	0	0	0	0.025
F12I	208	0	0	0	0.13	0	0	0	0.019
	215	0	0.09	0	0.06	0.15	0.07	0.05	0.053
	219	**1.00**	**0.91**	**1.00**	**0.81**	**0.85**	**0.93**	**0.95**	**0.928**
H1b	85	0	0	0	0.03	0	0	0	0.005
	90	**1.00**	**1.00**	**1.00**	**0.97**	**1.00**	**1.00**	**1.00**	**0.995**

[a] The most common allele at each locus for each population is indicated in boldface.
[b] Alleles identified in Europe are indicated in italics.

inocula. Furthermore, if such a source population contains a rare SSR allele, that allele will become artificially abundant in the Australian population. An example may be allele 246 at locus ME2. However, it is also likely that the higher allele diversity observed in Australia represents a population genetic expansion due to rapid evolution of SSR loci. The evolution of noncoding, tandemly repetitive DNAs is driven by slippage during DNA replication (10, 29), unequal crossing over (8), or gene conversion (28). Among these, replication slippage is reported to play a more important role in producing new alleles at SSR loci (10, 32). Furthermore, Stephan and Cho (27) suggest that natural selection plays an essential role in controlling the length of a repeat. It was also shown that stress, e.g., edaphic stress, increases replication error (9, 11). The many new alleles observed at ME2 suggest that this locus is particularly prone to accelerated evolution, perhaps due to a combination of factors, which may include stress, different edaphic factors (soil in Australia is ameliorated with lime to attain pH 8), or gene targets of selection that may be linked to this locus.

Problems with seedling quality. The ability of *T. melanosporum* to persist on a host tree is influenced by a number of environmental factors, including climate, soil chemical characteristics, the presence of competing fungi and the relationship between the mycorrhiza and the host tree (5, 7, 13, 30, 31). In Australia, *T. melanosporum* could not be detected in 25.6% of nursery seedlings (Table 2), indicating that the absence of a tree certification program in Australia results in poorly colonized seedlings produced by some nurseries. A total of 280 root samples (each containing 30 root rips) from 12 commercial truffiéres across Australia were collected from 91 host trees inoculated with *T. melanosporum*. Overall, 68.1% of host trees had *T. melanosporum*, whereas 9.9% of host trees had *T. brumale* present as mycorrhizae (Table 3). This suggests that *T. melanosporum* is able to persist in truffiéres in most cases and that the 22.0% of established host trees which did not show *Tuber* colonization most likely were poorly colonized seedlings.

No *T. indicum* was detected in the present study (data not shown), but *T. brumale* was detected in 8.2% of the seedlings analyzed. This indicates that ascocarps of *T. brumale* have been used as inoculum instead of *T. melanosporum* ascocarps, a situation that is easily prevented by PCR testing (19, 20). Contamination of truffiéres is also a problem in Europe, where contaminated *T. melanosporum* inoculum led to the more competitive *T. brumale* spreading through French truffiéres and through natural *Quercus pubescens* forests at the expense of *T. melanosporum* (7). More recently, *Tuber indicum* has also been found contaminating *T. melanosporum* inoculum and on the roots of *T. melanosporum* inoculated plants on commercial truffiéres in Europe (7). Since *T. indicum* and *T. brumale* are regarded as inexpensive but more competitive than *T. melanosporum*, there is the potential for considerable economic and ecological impacts for cultivated truffiéres (16, 19). Ascocarp identification methods need to be used to ensure that contamination of inoculum does not continue to occur.

Mating type distribution. Mating type PCRs were undertaken on a subset of 10 seedlings and 37 host trees which tested positive for *T. melanosporum* in at least three of the four root samples per tree analyzed. Samples which tested positive for *T. brumale* were excluded from the mating type analyses. All of the seedlings examined had both mating types present among the four samples analyzed. All truffiéres in the mating type study had host trees with both mating strains of *T. melanosporum* present. However, only 50.0% of productive and 42.9% of unproductive host trees had both mating types present (Table 5). Although Rubini et al. (25) also found both mating types in all seedlings analyzed, these authors also found that all host trees analyzed had only either mating type present. These findings were interpreted as being due to competition that may be occurring between *T. melanosporum* strains on the host tree. The question is, how can a truffle be produced on trees with one mating type only, or were the other mating type present but unsampled and/or present in the soil only? In its natural distribution, truffle inoculum is present in the soil, and trees in natural stands have root contact, thereby exchanging mating types. This is impossible in Australia since natural truffle inoculum is absent in the soil, and in most cases trees have not made root contact with neighboring trees yet. The most likely explanation for failure of detecting both mating types in 50% of productive trees in Australia is sampling error. Distribution of mating types on an established tree is irregular, rendering four samples insufficient to detect both mating types on an established tree.

There is evidence for the irregular distribution of mating types on a tree and further suggesting that the four samples analyzed are insufficient for studying the distribution of mating types. Of the 18 trees where both mating types were identified (Table 5), only one tree had both mating types present in as many as 75% of the samples per tree taken. Another tree had both mating types in two of the four samples, 11 trees had both mating types in only 25% of the samples, and 4 trees did not have both mating types identified within an individual sample (data not shown). Furthermore, in

TABLE 5 Presence of MAT1-1-1 and MAT1-2-1 on nursery seedlings and host trees

Source	Host	No. trees analyzed	No. of seedlings/trees		
			MAT1-1-1 only	MAT1-2-1 only	Both MATs
Seedling suppliers					
Nursery 1	*Q. robur*	4	0	0	4
Nursery 2	*Q. ilex*	6	0	0	6
Total (%)		10	0 (0.0)	0 (0.0)	10 (100.0)
Truffiéres					
Productive trees					
Truffiére 1	*Q. robur*	8	4	0	4
Truffiére 2	*Q. robur*	4	1	1	2
Truffiére 2	*C. avellana*	4	1	1	2
Total (%)		16	6 (37.5)	2 (12.5)	8 (50.0)
Unproductive trees					
Truffiére 1	*Q. robur*	13	5	3	5
Truffiére 2	*Q. robur*	1	1	0	0
Truffiére 3	*Q. robur*	2	1	0	1
	Q. ilex	1	0	0	1
Truffiére 4	*Q. robur*	4	1	1	2
Total (%)		21	8 (38.1)	4 (19.0)	9 (42.9)

only 16 of the 148 (10.8%) samples from 37 trees were both mating types identified.

Despite these findings, the truffiéres analyzed here more often had both mating types present than did the natural truffle stands in Europe studied by Rubini et al. (25). Again, the differences observed in mating type distribution may be a result of the sampling methodology since Rubini et al. (25) sampled the mycorrhiza only from productive trees, and it is possible that nearby unproductive trees at the site may have had the opposite mating type. In addition, the small number of samples collected over a large sampling area may also be influencing mating type distribution observed by Rubini et al. (25).

Another interesting finding was that MAT1-1-1 was identified in a significantly ($\chi^2 = 8.000$; $P < 0.01$) higher number (99 of 148 [66.9%]) of samples than MAT1-2-1 (63 of 148 samples [42.6%]) on the host trees. Overall, when only one mating type was present on a tree, MAT1-1-1 was identified on 14 trees compared to only 6 trees for MAT1-2-1 (Table 5). This skewed mating type frequency was not observed in the 10 nursery seedlings analyzed, suggesting that the mating type distribution observed occurred as a result of competition on the host trees due to, for example, environmental factors. The only scenario where competition would be likely is if MAT1-1-1 is associated with a functional trait (linked to a gene under selection), rendering MAT1-1-1 strains with a higher fitness. Mating type genes in heterothallic fungi, in addition to their primary functions in sexual compatibility, rarely are associated with ecological and life history traits (18). However, none of these associations were ever shown to impact mating type distribution, i.e., they do not provide a competitive advantage. If MAT1-1-1 is indeed linked to a functional trait providing it with a fitness advantage, this would imply that the selection force is the same across all four truffiéres sampled here, spanning a large geographic area in Australia, but that the selection force is absent in Europe, since a particular mating type was not found to always occur at a higher frequency (25), although very small sample sizes were analyzed in the European study. An alternative explanation to MAT1-1-1 frequency bias could be selective amplification of MAT1-2-1 due to, for example, polymorphism in the priming site. We sequenced the mating type loci of seven ascocarps but did not identify any polymorphism within the primer-binding site for either mating type (data not shown). Sequencing of a larger sample size is needed to confirm that the mating type loci are highly conserved and unlikely to have polymorphisms that may affect results using these primers.

Influence of mating type distribution on truffle production. In *T. melanosporum*, truffles are the product of sexual reproduction between two mating types. Therefore, an interaction between the two mating types present is essential for ascocarp production. In the present study, we did not always find both mating types present on productive trees, most likely due to insufficient sampling. We also showed that 42.9% of the unproductive trees had both mating types present as mycorrhiza but still did not produce any truffles. Therefore, close proximity of the two mating types leading to an interaction between them may be essential but does not guarantee ascocarp formation. These findings clearly show that there are more factors involved in ascocarp production than just the presence of both mating types on host trees.

In conclusion, the identification of new alleles and equal or greater allelic diversity in Australian *T. melanosporum* populations compared to European populations indicates that genetic loss from founder effects is not a significant factor in the observed low yields of Australian harvests. The results indicate that the quality of nursery purchased inoculated seedlings needs to be improved, particularly to minimize the spread of *T. brumale*. We also found that the mating type distribution on host trees and seedlings is an area that needs further study to better understand the potential for competition between *T. melanosporum* strains and to assess whether mating type distribution is limiting truffle reproduction.

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