Letters

Is the Perigord black truffle threatened by an invasive species? We dreaded it and it has happened!

Invasive alien species are species introduced deliberately or unintentionally to areas outside their natural habitats. They can cause a significant irreversible environmental and socio-economic impact at genetic, species and ecosystem levels, and as claimed by Moore (2000), 'throughout the world, exotic aliens are wreaking havoc'. The control of these invasive alien species has been discussed at international conventions, such as the Bern convention in 1979. Research on biological introductions has primarily focused on plant and animal invaders. Until now only a few studies have dealt with the spread of fungi through different continents and countries (Desprez-Loustau *et al.*, 2007), largely because of a lack of scientific knowledge of fungal biodiversity and ecology. Reports have mostly focused on the introduction of agronomically important plant pathogenic fungi (above all, rusts and Ascomycetes) and pseudofungi (Stramenopila and Peronosporomycetes), and their invasion routes (Desprez-Loustau *et al.*, 2007). The most common way of introducing fungal pathogens is through the movement of infected planting stock or infested wood (Coetzee *et al.*, 2001). The success of invasive fungal pathogens in these new environments might be explained by an increased aggressiveness towards new host species that have not had an opportunity to evolve resistance.

The ecological impacts of the intentional/unintentional introduction of fungal species, apart from plant pathogens, have been largely ignored (Schwartz *et al.*, 2006). Regarding saprotrophic taxa, in-depth studies have only been carried out with a few species, for example *Coprinopsis stangliana* (Bougher, 2006), and some woodchip fungi (Shaw *et al.*, 2004). As far as ectomycorrhizal fungi are concerned, the accidental introduction and spread of *Amanita phalloides* (Pringle & Vellinga, 2006), *Boletus edulis* s.l. (Hall *et al.*, 1998) and *Eucalyptus*-associated species, such as *Pisolithus* spp. (Martin *et al.*, 2002), have been carefully studied and monitored. These symbiotic fungi were accidentally introduced when allochthonous trees were planted for agriculture and reforestation (Hall *et al.*, 1998; Martin *et al.*, 2002). The deliberate movement of nonindigenous mycorrhizal fungal species and strains is a phenomenon occurring with increasing frequency as a consequence of the use of beneficial soil organisms to improve horticulture (Azcon-Aguilar & Barea, 1997), bioremediation (Leyval *et al.*, 2002), reforestation (Duponnois *et al.*, 2005) and edible fruit body production (Hall *et al.*, 1998; Hall *et al.*, 2003).

The impact of all these introductions on the overall native resident fungal communities is not well understood, and scant attention has been paid to the ecological impact of transporting nonpathogenic fungi across continents (with a few exceptions, e.g. Selosse *et al.*, 1998). Monitoring efforts are therefore urgently needed to track the spreading pattern of introduced fungi.

Spreading the truffles: their distribution and genetic variability

Since the 1970s, humans have played an important role in the dissemination of some *Tuber* species, for example, by using inoculated seedlings to create artificial truffle grounds (Chevalier & Grente, 1979). One of the species most commonly inoculated is the famous Perigord black truffle (*Tuber melanosporum* Vittad.), which lives in symbiosis with several trees and shrubs. Besides its natural range (France, Italy and Spain), the Perigord black truffle is currently produced in Morocco, Australia and New Zealand, and it has recently been implanted in Canada, the USA, Argentina, Chile and Israel. However, in spite of this successful seedling production, the amount harvested annually has declined over the last century (Hall *et al.*, 2003).

The name 'truffle' is a broad term that comprises fungal species of the symbiotic hypogeous genus *Tuber*, belonging to the *Tuberaceae* (*Ascomycota*, *Pezizomycetes* and *Pezizales*) family. Recently, Jeandroz and colleagues (2008) have highlighted the history and the biogeography of the *Tuber* genus, which seems to have originated in Laurasia during the Trias/Jurassic periods. *Tuber* currently displays a widespread geographic distribution over the northern hemisphere. It is present throughout all of Europe (Riousset *et al.*, 2001), and five species have been found in North Africa (Kahbar *et al.*, 2007). It is widespread in Asia (India, China and Mongolia) and is present in North America (Gilkey, 1954). The distribution of *Tuber* species depends on several factors: the distribution and migration of the host trees; dispersion by underground spores; dispersion via mammals; climatic conditions; and the existence of geographical barriers (Murat *et al.*, 2004).

Bertault and colleagues (1998) found an extremely low level of polymorphism for *T. melanosporum* using random amplification of polymorphic DNA (RAPD) and microsatellite analysis. Its low genetic diversity casts doubt on its survival when faced with climatic changes or invasive alien species. On the other hand, Bertault and colleagues (1998) claimed that there was no genetic structure for *T. melanosporum* because they did not find an increase in genetic differentiation with geographic distance using the Mantel test. A strong geographic pattern for *T. melanosporum* has instead been identified more recently (Murat *et al.*, 2004) using moderate variations of the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS). None of the analyses, conducted using microsatellites and rDNA ITS sequencing, identified heterozygotes, suggesting that *T. melanosporum* has a very closed mating system, such as homothallism, pseudohomothallism or even exclusively selfing (Bertault *et al.*, 1998; Murat *et al.*, 2004). However, Paolocci and colleagues (2006) showed that recombination occurred in the white truffle *Tuber magnatum*; they explain the lack of heterozygosis by pointing out that the 'DNAs extracted from the ascocarps are almost exclusively that of the maternal parent'. This hypothesis gives new ideas on the *T. magnatum* life cycle and opens the question of whether the same could be true for *T. melanosporum* (Rubini *et al.*, 2007).

Tuber melanosporum and *Tuber indicum* are close relatives

The phylogenetic analysis of the genus *Tuber* showed that the Perigord black truffle is closely related to the Chinese truffle, *Tuber indicum* Cook & Massee (Wang *et al.*, 2006). Moreover, both species are morphologically similar and confusion between them is possible (Paolocci *et al.*, 2000; Riousset *et al.*, 2001). Unlike *T. melanosporum*, *T. indicum* has a high level of genetic diversity, and a strong phylogeographic structure has recently been identified in China (Wang *et al.*, 2006). The natural production of this species is high (at least 300 tons in 2006, according to Knapp, 2006). Even though the total production is increasing each year, the local production is declining, mostly as a result of destructive harvesting methods. For example, before 1993 more than 20 tons were harvested in Huidong county (Sichuan, China) whereas only 4–5 tons were recorded as being harvested in 2003 (Wang *et al.*, 2007).

Importation of the Chinese truffle, instead of the Perigord truffle, for sale in Europe has been known about since the middle of the 1990s and, as claimed by Hannah Beech (TIME Magazine, April 17, 2005): 'Chinese fungi are flooding gourmet-markets, and Europeans are not amused'. However, *T. indicum* does not have the same organoleptic qualities as its European cousin, which can cost 1000 Euro kg^{-1}. Because of the absence of specific regulations in France and Spain, *T. indicum* can be sold in these countries, unlike in Italy where the sale of *T. indicum* is not permitted (law n°752, December 16, 1985, modified May 17, 1991). *In vitro* experiments have displayed that *T. indicum* is dominant, competitive and more aggressive than *T. melanosporum* (Gérard Chevalier and Alessandra Zambonelli, pers. comm.). It is therefore of concern that *T. indicum* could be used (mixed with *T. melanosporum*) to inoculate seedlings, which could thereafter be disseminated in truffle ground and then threaten the indigenous *T. melanosporum* populations (Paolocci *et al.*, 1999; Mabru *et al.*, 2001). To investigate the accidental introduction of *T. indicum* in Europe, molecular tools for discriminating both are already available (Paolocci *et al.*, 1999; Mabru *et al.*, 2001).

From China to Piedmont

The potential introduction of *T. indicum* received its first experimental confirmation during a survey in a truffle plantation in Piedmont (Italy), where by chance we identified the Chinese black truffle, *T. indicum*, in addition to the expected Perigord black truffle. In this plantation the owner introduced hazel and hornbeam seedlings in 1997 that were expected to be inoculated with *T. melanosporum*, but he never harvested any truffle fruiting bodies. To establish the presence of *T. indicum*, we randomly sampled root tips and soil samples from six trees (five hornbeams and one hazel) in the plantation (for methodology details see the Fig. 1 legend). The molecular analysis, based on the rDNA ITS sequencing, enabled us to identify two *Tuber* species (*Tuber borchii* and *T. indicum*) within genotyped ectomycorrhizal tips (data not shown). In a second step, fungal DNA was extracted directly from the soil samples and specific primers were used to genotype *T. melanosporum* and *T. indicum.* The Perigord black truffle was identified in two soil samples, and *T. indicum* was identified in three soil samples (data not shown). The four *T. indicum* sequences obtained from the Piedmont mycorrhizae and soil samples were identical and clustered in the *T. indicum* clade II (Fig. 1). This clade comprises all the samples from Gong-shan (Yunnan), Miyi and Panzhihua (Sichuan) (Wang *et al.*, 2006). As a result of the high phylogeographic structure of *T. indicum*, we suggest that the fruit bodies used to inoculate the seedlings were probably imported from these regions of China. The present report is therefore the first evidence that *T. indicum* has been used to inoculate seedlings implanted in Europe and that this species can spread in European ecosystems. *Tuber indicum* is a novel invasive alien species in Italy. More than a gourmet-market problem, it constitutes an ecological threat for the Perigord truffle, in particular because *T. indicum* can be found in China in ecosystems that are similar to those in central Italy (Wang *et al.*, 2007).

Truffles: indigenous vs alien species

The replacement of *T. melanosporum* in productive plantations with autochthonous species, such as *Tuber brumale* Vittad. and *Hebeloma* sp., has been well documented (Chevalier *et al.*, 1982; Granetti & Angelini, 1992). One of the main reasons

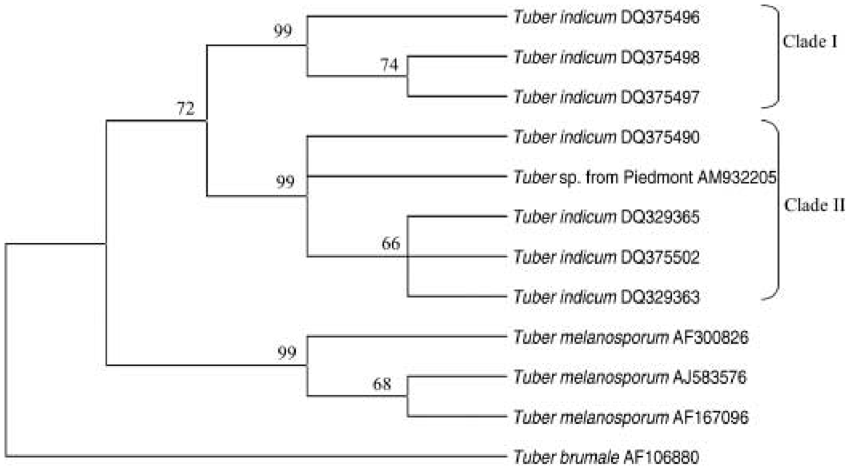

Fig. 1 Maximum parsimony bootstrap consensus tree obtained from internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequences of three black *Tuber* species belonging to the *Melanosporum* group (Riousset *et al*., 2001; Jeandroz *et al*., 2008). The alignment of 553 bp and the maximum parsimony phylogenetic trees were obtained with MEGA 4.0 (Tamura *et al*., 2007) using default parameters. Bootstrap values corresponding to 1000 repeats are shown. Clades I and II correspond to the two *Tuber indicum* clades defined by Wang *et al.* (2006). None of the four sequences related to *T. indicum* and originating from the truffle ground soil samples and mycorrhizae in Piedmont (Italy) showed polymorphism. For that reason, they are represented as a single sequence in the tree (AM932205). The truffle ground is located near Torino (Piedmont, Italy). In this plantation, 50 hazel and 30 hornbeam plants expected to be mycorrhized by *Tuber melanosporum* were implanted in June 1997. Until now, the owner has never harvested truffle fruiting bodies in the plantation. Six trees (five hornbeams and one hazel) were chosen to verify the presence of truffle in the plantation. These trees were selected because they were surrounded by a scanty vegetation, suggesting the potential presence of *T. melanosporum* (Splivallo *et al*., 2007). In November 2006, for each tree, root tips and soil samples were harvested at four points (North, East, South and West), 1 m from the tree. A total of 150 root tips were morphologically analysed and sorted into 12 morphotypes. Six morphotypes were excluded for further analyses because they could not be attributed to *Tuber* spp. The DNA from the remaining six morphotypes was extracted using the DNA Plant Minikit (Qiagen, Milan, Italy), following the manufacturer's instructions. The ITS region was amplified using the universal primers ITS1f (Gardes & Bruns, 1993) and ITS4 (White *et al*., 1990) and cloned in vector pGEM-T (Promega, Milan, Italy). Five clones for each morphotype were sequenced. Similarities of the cloned sequences to sequences in the GenBank database were determined using BLAST (NCBI, Bethesda, MD, USA). The FastDNA SPIN Kit for Soil (Q-Biogene, Rome, Italy) was used to extract DNA from soil samples following a modified protocol described by Luis *et al*. (2004). The ITS regions were amplified with specific primers for *T. indicum* and *T. melanosporum* (ITSCHCH, ITSML and ITS4LNB; Paolocci *et al*., 1999). Similarities of the cloned sequences to sequences in the GenBank database were determined using BLAST (NCBI). All the clones were sequenced by DiNAMYCODE s.r.l. (Torino, Italy).

for these substitutions is the low competitiveness of *T. melanosporum* regarding these species. This trait is probably one of the reasons for its progressive decline. However, the ecological consequences of the introduction of *T. indicum* into a *T. melanosporum*-producing area in Europe are still unknown and their close phylogenetic relationship opens new questions of scientific relevance.

- Are both species able to inbreed?
- Is *T. indicum* able to spread over long distances?
- Is *T. indicum* really capable of replacing *T. melanosporum* in truffle grounds?

Our findings point out the need for local agencies to control not only the importation of *T. indicum* fruiting bodies but also the production of mycorrhizal seedlings. Moreover, it is of vital importance to draw attention to *T. indicum* as an invasive alien species in Europe and to prevent its dissemination in *T. melanosporum* production areas. It is recommended that *T. melanosporum* plantations in France, Italy and Spain are investigated for the presence of *T. indicum*. How can we control or eliminate *T. indicum* in Europe? In France, Gérard Chevalier advised pulling out trees and disinfecting soils. However, this is a difficult option, particularly because owners have invested money in their plantations, and this option may need to be a political decision, perhaps with incentives for the owners, such as financial support.

In conclusion, we would like to point out the importance of conserving truffle biodiversity, above all for species such as *T. melanosporum* that has a low genetic diversity and, like other fungi (Gange *et al*., 2007), might be influenced by climatic changes (Bertault *et al*., 1998).

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