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Population dynamics of the ectomycorrhizal fungal species *Tricholoma populinum* and *Tricholoma scalpturatum* associated with black poplar under differing environmental conditions

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Summary

Fungi combine sexual reproduction and clonal propagation. The balance between these two reproductive modes affects establishment dynamics, and ultimately the evolutionary potential of populations. The pattern of colonization was studied in two species of ectomycorrhizal fungi: *Tricholoma populinum* and *Tricholoma scalpturatum*. The former is considered to be a host specialist whereas *T. scalpturatum* is a generalist taxon. Fruit bodies of both basidiomycete species were mapped and collected over several years from a black poplar (*Populus nigra*) stand, at two different sites. Multilocus genotypes (= genets) were identified based on the analysis of random amplified polymorphic DNA (RAPD) patterns, inter-simple sequence repeat (ISSR) patterns and restriction fragment length polymorphisms (RFLPs) in the ribosomal DNA intergenic spacer (rDNA IGS). The genetic analyses revealed differences in local population dynamics between the two species. *Tricholoma scalpturatum* tended to capture new space through sexual spores whereas *T. populinum* did this by clonal growth, suggesting trade-offs in allocation of resources at the genet level. Genet numbers and sizes strongly differ between the two study sites, perhaps as a result of abiotic disturbance on mycelial establishment and genet behaviour.

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Introduction

Ectomycorrhizal fungi (ECM) are a group of soil organisms that form intimate symbiotic structures with roots of trees and shrubs (Smith and Read, 1997). The fungal symbionts are crucial to the functioning of forest ecosystem as they contribute to plant growth, mineral nutrition, water uptake and protection against root pathogens (Smith and Read, 1997). Large quantitative variation among isolates of the same species has been shown in physiological traits, which are putatively involved in formation of ectomycorrhiza or mobilization of soil nutrients (Smith and Read, 1997; Guidot *et al.*, 2005). Approximately 5000 species of ectomycorrhizal species have been described, with a whole range of life histories (Smith and Read, 1997). The majority of ECM fungi are basidiomycetes (e.g. in the genera *Tricholoma*, *Boletus*, *Amanita*), but there are also ascomycetes including the black truffle *Tuber melanosporum*. The root system of an individual host is usually colonized by numerous fungal species and genotypes (Horton and Bruns, 2001).

Most members of Ascomycota and Basidiomycota typically feature a vegetative thallus (termed mycelium, pl. mycelia) that is a branched network of microscopic filaments (= hyphae). Once a mycelium becomes established, it can grow indeterminately by apical extension of the hyphae, but segmentation of the mycelium can also occur resulting in separate physiological units or ramets. In a typical outcrossing basidiomycete, the life cycle includes a primary homocaryotic mycelium with a transient existence and a secondary mycelium, which results from hyphal anastomosis between two compatible primary mycelia (dikaryotization) or between a primary mycelium and a secondary mycelium (di-mon mating). At some stage during development the sexual process is initiated, and the secondary mycelium changes to a wholly or partly reproductive one, allowing channelling of resources to macroscopic structures (referred to as fruit bodies or sporophores). Sexual spores are produced by meiosis and mitosis after fusion of the two haploid nuclei in the basidia. Compatibility genetic systems maintain the individuality of a mycelium and regulate sexual exchange between

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mycelia of the same species (Moore and Novak Fraser, 2002).

Field studies of population structure provide a direct assessment of the potential for sexual or asexual reproduction, and are a prerequisite to understand genotype-by-genotype-by-environment interactions. Because ECM fungi are cryptic organisms and hyphal growth is indeterminate, establishing even the most basic population features (e.g. number and size of individuals) can be difficult. It often requires the development of efficient molecular markers with a high discriminatory power, particularly to identify the isolates from soil samples or mycorrhizae (Kretzer *et al*., 2003). There is also temporal and spatial heterogeneity in production and activity of the different stages of the fungal life cycle, including the mycelia in the soil. As a result, most conventional attempts to characterize populations of ECM fungi have used the occurrence of visible reproductive structures (i.e. fruit bodies) as an approximation for actual population size and how it varies in space and time (Dahlberg and Stenlid, 1990; 1994; 1995; Gryta *et al*., 2000; Redecker *et al*., 2001; Bergemann and Miller, 2002; Dunham *et al*., 2003; Hirose *et al*., 2004), but see, e.g. Guidot and colleagues (2001) or Kretzer and colleagues (2003) for the characterization of the below-ground populations.

Patterns of establishment and population history can be inferred from the diversity and size of the genetic individuals (= genets) identified at a site (Dahlberg and Stenlid, 1990). The balance between sexual and asexual reproduction has ecological and genetic consequences as it affects establishment dynamics, and results in markedly different populations. Where a high proportion of genets are present within a locality, the inference is that the population has arisen predominantly from meiotic spores produced by sexual recombination and random mating. There are examples of high levels of genotypic diversity and very small genets (a few cm^2) in populations of many ectomycorrhizal species, e.g. *Hebeloma cylindrosporum* (Gryta *et al*., 1997) and *Laccaria amethystina* (Gherbi *et al*., 1999; Fiore-Donno and Martin, 2001). In contrast to this diversity, the widespread occurrence of identical genets is indicative of clonality, i.e. establishment by mycelial growth. Such a situation has also been found in ectomycorrhizal populations, e.g. single genets of various species of *Suillus* often reach the size of tens to hundreds of metres (Dahlberg and Stenlid, 1990; 1994; Bonello *et al*., 1998; Hirose *et al*., 2004). The reason for maintenance of sexual recombination in clonal organisms is still an unresolved problem. If fungal genets successfully disperse spatially by clonal means, why maintain a process such as sexual reproduction, which involves fruit bodies production and the cost of meiosis, and how important is sexual reproduction for localized occupation of space?

In the present study, we report on the diversity patterns of *Tricholoma populinum* and *Tricholoma scalpturatum* in two habitats that were selected on the basis of host composition, age of the trees and environmental conditions. Literature surveys of plant–fruit bodies associations in ectomycorrhizal symbiosis suggest that *T. populinum* exclusively associates with poplars, whereas *T. scalpturatum* colonizes a wide range of conifers and deciduous trees in a diverse range of habitats (Bon, 1984; Courtecuisse and Duhem, 1994). Fruit bodies were collected and mapped over several years in two black poplar stands of similar age differing by their level of abiotic disturbance. Molecular fingerprinting methods are employed to delimit multilocus genotypes (= genets) and to assess genetic variation within and between populations. Polymorphic DNA markers are generated by the method of random amplified polymorphic DNA (RAPD; Welsh and McClelland, 1990; Williams *et al*., 1990), the method of inter-simple sequence repeat (ISSR; Gupta *et al*., 1994; Zietkiewicz *et al*., 1994) and the restriction of the amplified ribosomal intergenic spacer [polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the IGS2 region].

We asked whether there are differences in genetic diversity between the two species of *Tricholoma* that were fruiting side by side during the same period, and how patchy is the distribution of genets. Results from the two habitats are compared which address questions related to the local dynamics of fungal populations in response to habitat heterogeneity. We also discuss genet survival and try to evaluate the relative importance of clonal and sexual reproduction to population persistence in the two basidiomycete species.

Results

Fruiting patterns

The fruiting period of *T. populinum* and *T. scalpturatum* lasted approximately 1 week and took place in early November at both sites. Fruit bodies were unevenly distributed and formed dense patches separated by areas where they were rare or absent (Figs 1 and 2, and data not shown). The two species were rarely intermingled. *Tricholoma populinum* produced less fruit bodies than *T. scalpturatum*, but the biomass per individual fruit body was 5–10 times bigger (data not shown). Fruit bodies of *T. populinum* often occurred in clumps (Figs 1 and 2). At Monbéqui (disturbed MO site), fruit bodies of *T. populinum* were only observed in 1998 and 24 were scored (Table 1) whereas at Montaudran (undisturbed AF site), fruit bodies were recorded in 2000 and in 2002 (95 and 10 fruit bodies, respectively; Table 1). A total of 75 fruit bodies of this species were analysed (Table 1).

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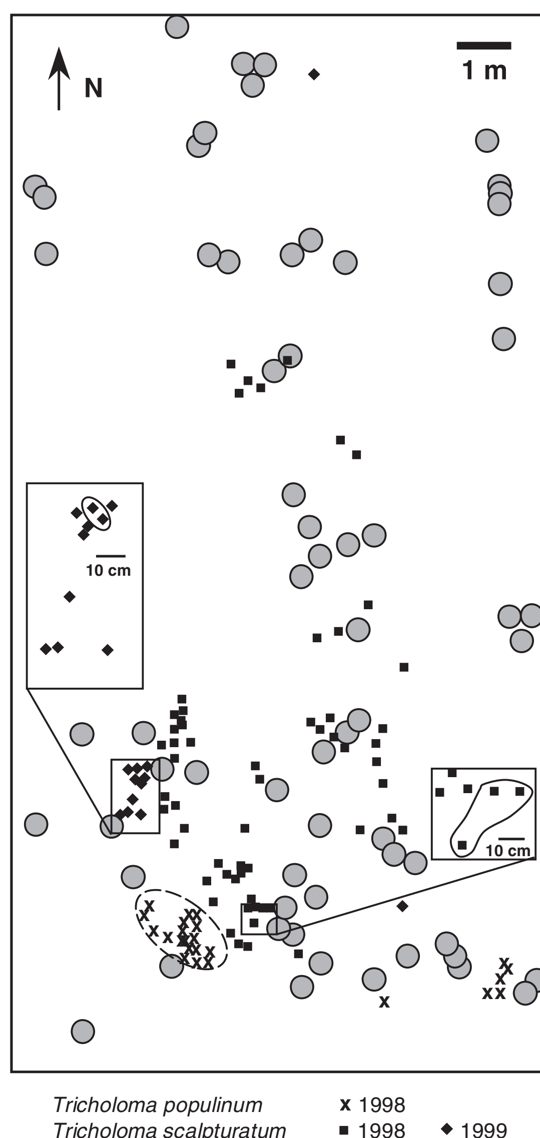

Fig. 1. Map of the disturbed MO site at Monbéqui, with positions of *Populus nigra* trees (large grey filled circles), *Tricholoma populinum* ('x') and *Tricholoma scalpturatum* analysed fruit bodies (squares in 1998 and diamonds in 1999). Genets that produced at least two fruit bodies are enclosed with dashed line and full line for *T. populinum* and *T. scalpturatum* respectively. Two enlarged views of dense patches of fruit bodies are presented. Fruit bodies that were collected and mapped but not submitted to a genetic analysis were omitted for clarity.

At the disturbed MO site, fruiting of *T. scalpturatum* was observed in 1998 and to a lesser extent in 1999, but not in the following years. Out of 76 observed and mapped fruit bodies, a total of 70 samples (92%) were genetically analysed (Table 1). Although the area where the fruit bodies emerged was of similar size, the undisturbed AF site was about five times more productive than the disturbed site. There were 296 and 91 fruit bodies in year 2000 and 2002 respectively (Table 1). Some of the areas where fruit bodies were observed in 2000 did not produce any in 2002 (Fig. 2 and data not shown). A total of 127 samples were selected for the molecular fingerprinting analysis, comprising 62 and 65 samples in year 2000 and 2002 respectively (Table 1). The selection of this subset of samples was based on the fruit body distribution in the field, with at least one analysed sample per square metre in year 2000.

Typing of fruit bodies using RAPDs, ISSRs and restriction analysis of the IGS2 region

Random amplified polymorphic DNA typing of *T. populinum* and *T. scalpturatum* was performed using six and seven primers respectively (Table 2). These primers produced reproducible and interpretable RAPD markers (Fig. 3). A clear polymorphism was observed within the populations, with a percentage value always higher in the *T. scalpturatum* population than in the *T. populinum* population, regardless of the primer used (Table 2). The RAPD analysis performed on 75 *T. populinum* fruit bodies gave 65 fragments of 242–1565 bp length, of which 45 (69%) were polymorphic (Table 2). Typing of 75 fruit bodies revealed 10 RAPD types on the two sites (Table 3). Among the 197 analysed *T. scalpturatum* fruit bodies, the seven primers yielded 102 reliable fragments ranging from 290 to 1885 bp (Table 2), of which 99 (97%) were polymorphic. Ninety-five types were identified (Table 3).

Inter-simple sequence repeat amplifications with R3 primer (Table 2) produced 14 and 30 fragments for *T. populinum* and *T. scalpturatum* respectively (Table 2). Fewer polymorphic fragments were detected in *T. populinum* than *T. scalpturatum* (64.3% versus 100%; Table 2). Of the 129 analysed *T. populinum* fruit bodies, seven ISSR types were identified, whereas 51 types were found among the 197 *T. scalpturatum* fruit bodies (Table 3).

Polymerase chain reaction amplification of the IGS2 region yielded a fragment of about 2.2 kb with *T. populinum* isolates and of 2.3 kb with *T. scalpturatum* isolates. Restriction analysis of the IGS2 region provided polymorphic band patterns (Table 3 and data not shown). This method shared the 129 *T. populinum* fruit bodies into seven groups (IGS2 types *PA* to *PG*; Fig. 4A), and the 196 analysed *T. scalpturatum* fruit bodies were separated into 11 groups of identical banding pattern (IGS2 types *SA* to *SK*; Fig. 4B).

Genet identification and congruence of molecular markers

Combined analysis using molecular fingerprinting methods grouped the analysed fruit bodies into 10 and 96 genets for *T. populinum* and *T. scalpturatum* (Fig. 4)

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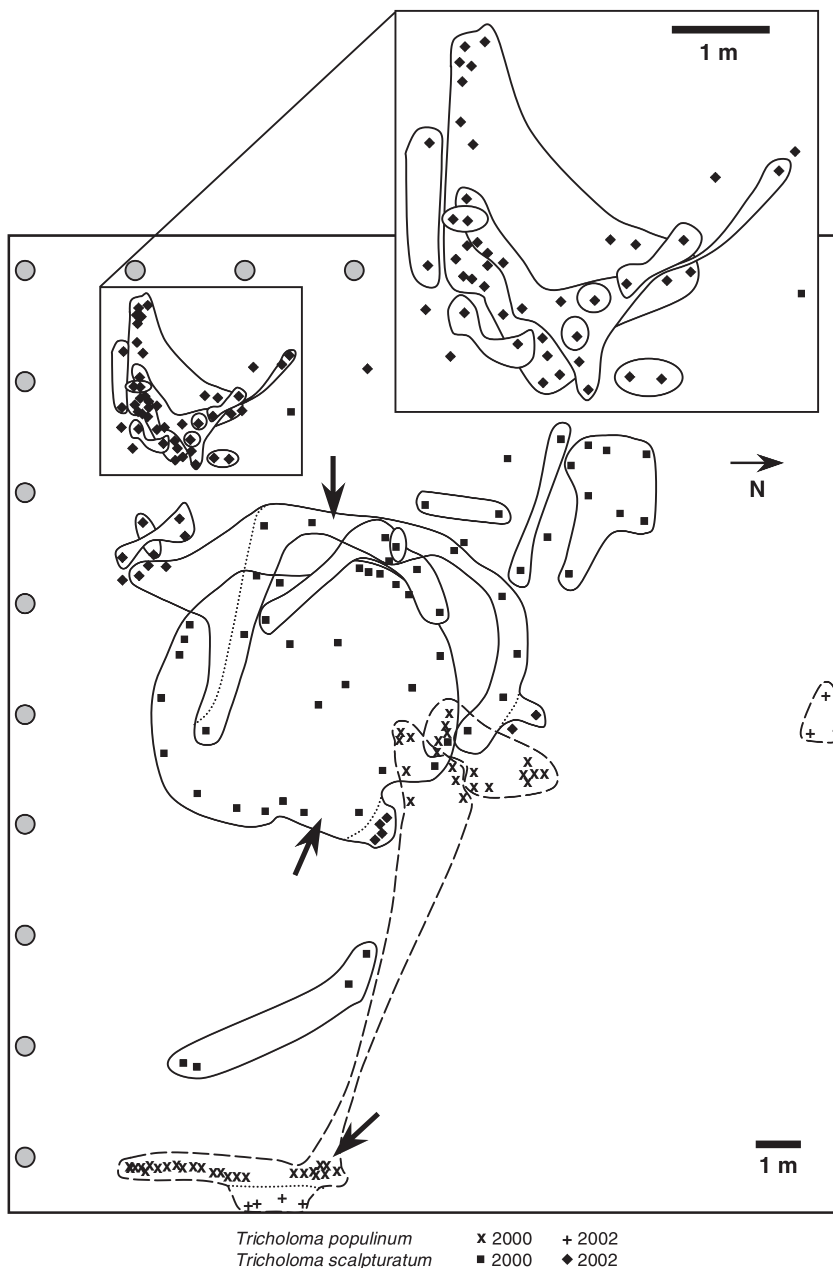

Fig. 2. Map of the undisturbed AF site at Montaudran with positions of *Populus nigra* trees (large grey filled circles), *Tricholoma populinum* ('x' in 2000 and '+' in 2002) and *Tricholoma scalpturatum* analysed fruit bodies (squares in 2000 and diamonds in 2002). Genets are represented as in Fig. 1. Two *T. scalpturatum* genets and one *T. populinum* genet (arrows) were detected over 2 sampling years; a dotted line represents the 2000's limits of these genets. An enlarged view of a dense patch of fruit bodies is presented. Fruit bodies that were collected and mapped but not submitted to a genetic analysis were omitted for clarity.

respectively. There was a significant correlation between the RAPD matrix of pairwise genetic distances and the ISSR matrix was significant for each of the two fungal species ($r = 0.89$ and $P < 0.001$ for *T. populinum*, $r = 0.88$ and $P < 0.001$ for *T. scalpturatum*), indicating congruence of the RAPD and ISSR data. If we assume random mating and taking into account the number of scored RAPD and ISSR fragments and their frequencies of presence/absence (Redecker *et al.*, 2001), the probability of obtaining two samples having the same multilocus pattern by chance was 4.1×10^{-9} and 3.3×10^{-10} for *T. populinum* and *T. scalpturatum* respectively.

Spatial and temporal distribution of genets

Distribution of fruit bodies on the maps revealed a spatial separation of the genets (Figs 1 and 2). *Tricholoma scalpturatum* genets were evenly distributed whereas *T. populinum* genets were generally restricted to specific areas.

Considering the maximum distance between the two furthest fruit bodies with identical patterns to estimate the size of the genets, there was evidence that populations had genets that were larger within the AF site than within the MO site (Table 1, Figs 1 and 2). For instance, the

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Table 1. Main characteristics of populations.

	Sites and sampling years						
	MO (disturbed site)			AF (undisturbed site)			
	1998	1999	1998/1999	2000	2002	2000/2002	All
Tricholoma populinum							
Scored fruit bodies	24	0	24	95	10	105	129
Analysed fruit bodies	24	–	24	44	7	51	75
No. of genets (refound[a])	7	–	7	2	2 (1)	3	10
Genotypic diversity (*PD*)	0.29	–	0.29	0.05	0.29	0.06	0.13
Fruit bodies per genet							
Range	1–18	–	1–18	13–31	3–4	3–31	1–31
Mean	3.4	–	3.4	20	3.5	17	7.5
Genets length range (m)[b]	0–1.8	–	0–1.8	2.6–11.5	0.9–1.3	0.9–11.5	0–11.5
Tricholoma scalpturatum							
Scored fruit bodies	63	13	76	296	91	387	463
Analysed fruit bodies	58	12	70	62	65	127	197
No. of genets (refound[a])	56	11 (0)	67	12	19 (2)	29	96
Genotypic diversity (*PD*)	0.97	0.92	0.96	0.20	0.29	0.23	0.49
Fruit bodies per genet							
Range	1–3	1–2	1–3	1–27	1–22	1–27	1–27
Mean	1.1	1.1	1.1	5.2	3.6	4.5	2.1
Genets length range (m)[b]	0–0.35	0–0.06	0–0.35	0–7.4	0–9.5	0–9.5	0–9.5

a. Between brackets, the number of genets identified the first year of sampling and found again the second year.
b. Range of maximum distance between two fruit bodies sharing the same pattern; the value is set to 0 m for single-fruit body genets.

maximum distance between the outermost *T. populinum* fruit bodies was 11.5 m and 1.8 m at AF site and MO site respectively. Furthermore, there was a positive relationship between the size of the genets and the number of fruit bodies. The largest genets at each site contained the highest number of fruit bodies (60% and 75% of the total number of analysed fruit bodies at the AF site and MO site respectively) (Table 1, Figs 1 and 2). A similar trend was detected for *T. scalpturatum* genets. The maximum width was 9.5 m at AF site and 0.35 m at MO site. The largest genets represented 21% and 4% of the total number of analysed fruit bodies at AF and MO site respectively (Table 1, Figs 1 and 2).

In order to ascertain genet persistence and clonal growth, fruit bodies were collected during several years. At the MO site, the analysis revealed 56 genets of *T. scalpturatum* out of 58 fruit bodies, in 1998. None of them was observed again in 1999 (Table 1). Thirteen sin-

Table 2. Designations of primers used for RAPD and ISSR amplifications, number of scored fragments and proportion of polymorphic fragments in each species.

		Tricholoma populinum		*Tricholoma scalpturatum*	
Primers	Sequences[a]	Scored fragments	Polymorphic fragments	Scored fragments	Polymorphic fragments
RAPD primers					
156	5′-GCCTGGTTGC-3′	10	80%	17	100%
152GC	5′-CGCACCCGCAG-3′	–	–	15	100%
CTB9	5′-CGGAGGATAGGATAAGTCG-3′	–	–	19	100%
ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	13	77%	–	–
ML5	5′-CTCGGCAAATTATCCTCATAAG-3′	13	69%	16	100%
NS1	5′-GTAGTCATATGCTTGTCTC-3′	–	–	7	86%
NS2	5′-GGCTGCTGGCACCAGACTTGC-3′	14	57%	10	100%
SS38	5′-GTCGACTCCTGCCAGTAGTCATATGCTT-3′	6	50%	17	88%
VANS32	5′-AAGCTCGTAGTTGAATTTCGG-3′	9	78%	–	–
All RAPD primers		65	69%	102	97%
ISSR primer					
R3[b]	5′-BDB(ACA)$_5$-3′	14	64%	30	100%
All primers		79	68%	132	98%

a. 156 and 152GC (Selosse *et al.*, 1998); CTB9 (http://plantbio.berkeley.edu/~bruns/primers.html); ITS1, ML5, NS1 and NS2 (White *et al.*, 1990); SS38 (Bousquet *et al.*, 1990); VANS32 (Simon *et al.*, 1992) and R3 (Hantula *et al.*, 1996).
b. With B = T, C or G and D = A, T or G.

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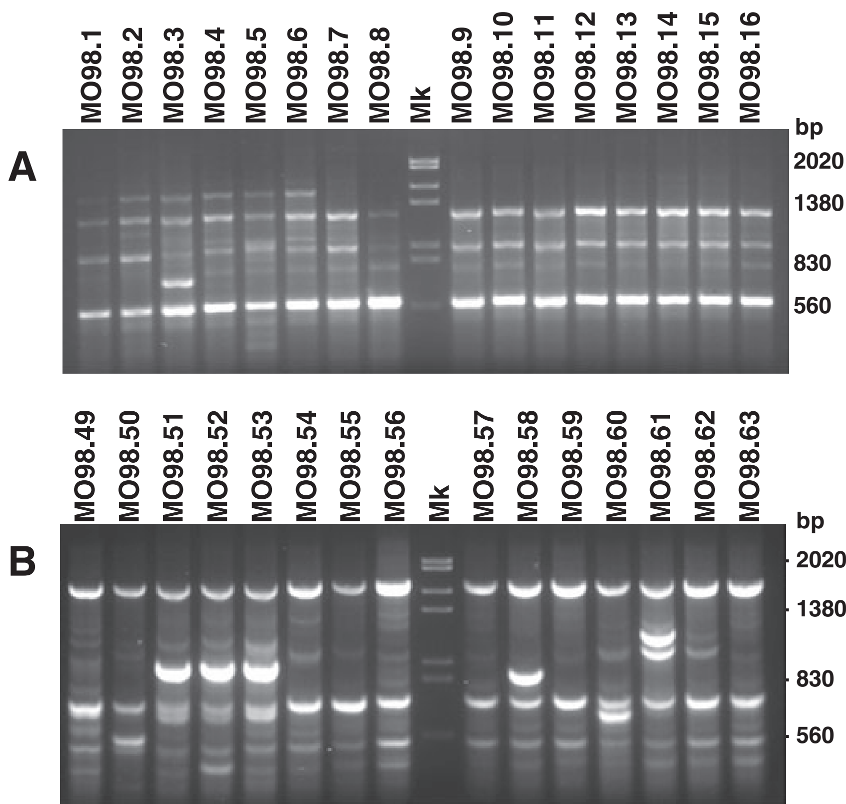

Fig. 3. Banding patterns of various fruit bodies produced by RAPD analysis using primer VANS32 for *Tricholoma populinum* (A) and primer ML5 for *Tricholoma scalpturatum* (B). Mk refers to HindIII–EcoRI double-digested λ DNA included as molecular weight marker.

gle-fruit body genets (out of 28) were observed at the AF site, but the population was also composed of genets that produced from 2 to 27 fruit bodies. Two large genets found in 2000 were also detected in 2002 (arrows, Fig. 2).

A similar trend in temporal pattern of genets was observed within the *T. populinum* population at the AF site. Two genets out of 44 fruit bodies were detected in 2000; the largest (represented by 27 fruit bodies) was still present 2 years later (four fruit bodies) (indicated by an

Table 3. Number of RAPD, ISSR and IGS2-RFLP types, and estimates of genetic diversity as revealed by calculation of Shannon index (*H*) and the percentage of putative polymorphic fragments (*P*).

	Number of types			*H*			*P*		
	RAPD[a]	ISSR	IGS2	RAPD	ISSR	RAPD/ISSR	RAPD	ISSR	RAPD/ISSR
Tricholoma populinum									
MO site									
1998	7	5	5	0.27 ± 0.30	0.15 ± 0.25	0.25 ± 0.30	48%	29%	44%
AF site									
2000	2	2	2	0.13 ± 0.25	0.04 ± 0.16	0.11 ± 0.24	22%	7%	19%
2002[b]	2 (1)	2 (2)	2 (2)	0.12 ± 0.24	0.04 ± 0.16	0.11 ± 0.23	20%	7%	18%
2000/2002	3	2	2	0.13 ± 0.25	0.04 ± 0.16	0.12 ± 0.24	22%	7%	19%
All	10	7	7	0.38 ± 0.28	0.36 ± 0.30	0.38 ± 0.28	69%	64%	68%
Tricholoma scalpturatum									
MO site									
1998	56	23	2	0.20 ± 0.25	0.19 ± 0.19	0.18 ± 0.24	50%	37%	47%
1999 [b]	11 (0)	10 (3)	1 (1)	0.19 ± 0.26	0.15 ± 0.23	0.18 ± 0.25	41%	30%	39%
1998/1999	67	30	2	0.21 ± 0.25	0.13 ± 0.20	0.19 ± 0.24	53%	37%	49%
AF site									
2000	12	8	7	0.38 ± 0.24	0.20 ± 0.23	0.34 ± 0.25	79%	50%	73%
2002[b]	19 (2)	15 (2)	4 (2)	0.37 ± 0.25	0.25 ± 0.21	0.33 ± 0.25	75%	70%	74%
2000/2002	29	21	9	0.39 ± 0.24	0.25 ± 0.20	0.35 ± 0.23	81%	73%	80%
All	96	51	11	0.38 ± 0.21	0.22 ± 0.15	0.35 ± 0.21	97%	100%	98%

a. The numbers of types identified with RAPD are equal to the numbers of different genets.
b. Between brackets, the number of types identified the first year of sampling and found again the second year.

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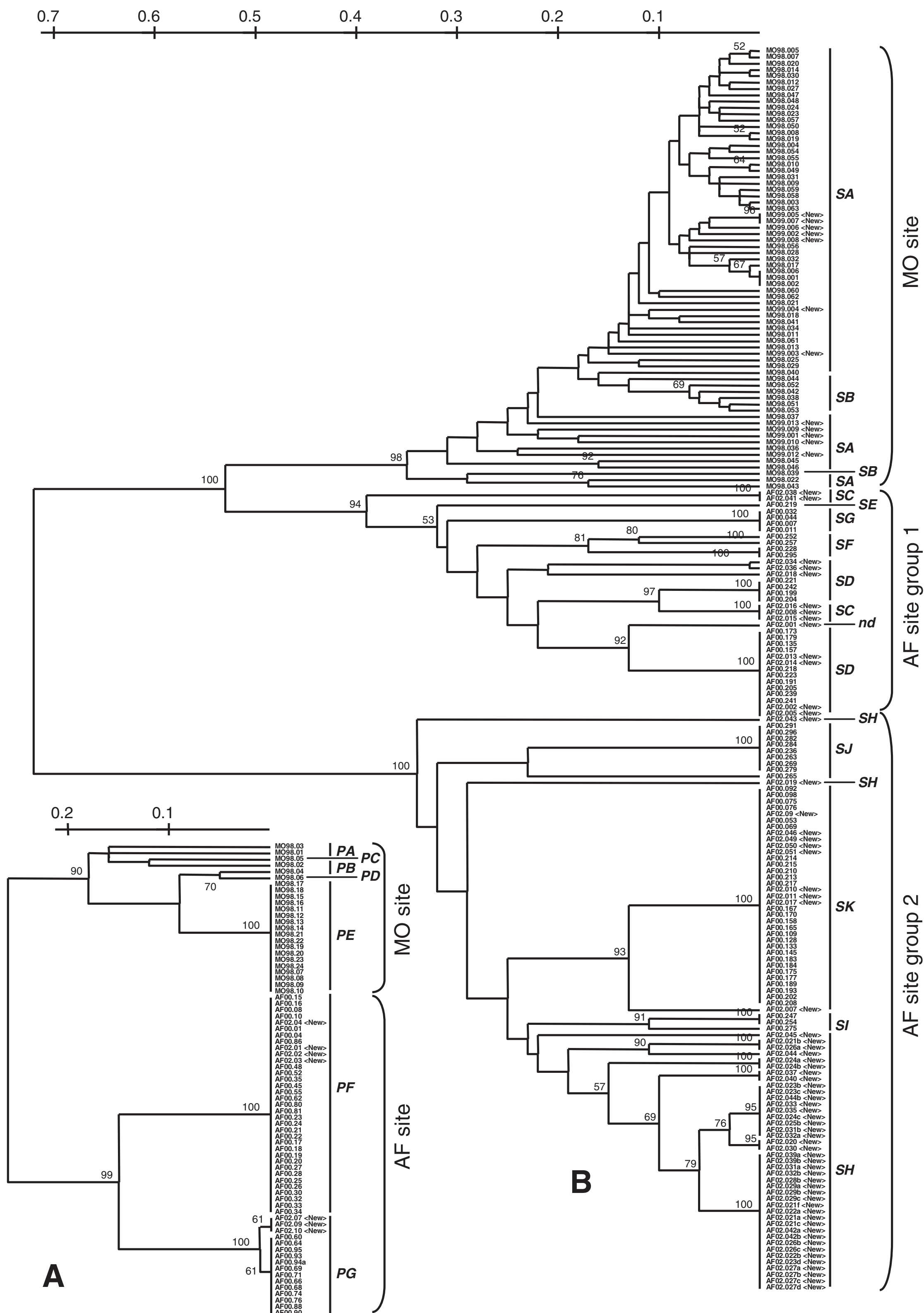

Fig. 4. UPGMA cluster analysis showing genetic relationship among the combined RAPD and ISSR patterns from *Tricholoma populinum* (A) and *Tricholoma scalpturatum* (B). The labels 'New' highlight the isolates collected during the second sampling year (1999 for isolates from MO site and 2002 for those from AF site). Italic letters indicates IGS2 types. The scale refers to genetic distance (Nei and Li, 1979). Bootstrap values over 50% are presented over concerned nodes.

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arrow on Fig. 1). The emerging positions of fruit bodies in 2000 and 2002 were <0.7 m apart. The temporal persistence of *T. populinum* genets could not be analysed at the MO site, because fruit bodies were only found in 1998. However, the size of the largest genet is indicative of temporal persistence.

Intra- and inter-species variation

The mean similarity index (SI) between pairs of genets was significantly higher for *T. populinum* (0.80, range from 0.69 to 0.99) than for *T. scalpturatum* (0.60, range from 0.17 to 0.99) (Mann–Whitney *U*-test, $P = 8.0 \times 10^{-6}$) (Fig. 5). Clustering of genets separated *T. populinum* genets into two large groups that correlated with the site population affiliation, indicating a higher degree of genetic similarity within than between populations (Fig. 4A). The combined RAPD and ISSR dendrogram generated for *T. scalpturatum* revealed three main clusters, each represented by 13–67 genets (Fig. 4B). One cluster included all the genets from the disturbed MO site and was closely linked to a cluster (AF site group 1), which consisted of genets from the undisturbed AF site. The third cluster (AF site group 2) also comprised genets from the AF site and was linked more distantly to the two previous groups (Fig. 4B). The genetic diversity (*H*) did not differ between the two species (Table 3). However, the proportion of distinguishable genets within populations was lower for *T. populinum* ($PD = 0.13$) than *T. scalpturatum* ($PD = 0.49$) (Table 1). Together, these results suggest that *T. populinum*, the poplar specialist, has a lower level of diversity than *T. scalpturatum*, the generalist species, which has a wider distribution.

Site differences in diversity

There was a threefold difference in the number of fruit bodies per multilocus genet (*PD*) between the two sites, with the population from the undisturbed site (AF) having the lowest number of genets in both species (Table 1, Figs 1 and 2). The molecular fingerprinting analysis revealed three *T. populinum* genets (out of 51 analysed fruit bodies) at the AF site, whereas the MO population had seven genets (out of 24 analysed fruit bodies). This pattern was even more pronounced in *T. scalpturatum*. Sixty-seven genets were found at the MO site, out of 70 analysed fruit bodies. Thus, 95% of *T. scalpturatum* fruit bodies had a unique pattern at this site. In contrast, only 29 genets were detected out of 127 analysed fruit bodies at the AF site.

The population of *T. populinum* within highly disturbed MO site also exhibited a greater level of genetic variation ($H = 0.25$ and $P = 44\%$; Table 3) than the population from the less disturbed AF site ($H = 0.12$ and $P = 19\%$; Table 3). This result is consistent with the number of observed genets (Table 1) and with the higher number of IGS2 types recorded within MO site (5; Table 3) than within AF site (2; Table 3). No significant difference was observed in mean SI value between MO genets and AF genets of *T. populinum* (Fig. 6A). In contrast, a negative relationship between the two estimators of diversity (*PD* versus *H*) was observed in *T. scalpturatum* (Tables 1 and 3). In this species, the AF population consisted of a few but highly dissimilar genets whereas the MO population is composed of numerous more closely related genets (Table 1, Fig. 4B). This trend was supported by the record of nine IGS2 types among the 29 genets from AF site whereas only two IGS2 types have been found among the 67 genets from MO site (Table 3, Fig. 4B). Thus, the similarity between genet pairs ranged from 0.17 to 0.99 for AF genets and from 0.50 to 0.99 for MO genets (Fig. 6B). The mean SI value was significantly higher for AF site population (0.50) than for MO site population (0.83) of *T. scalpturatum* (Mann–Whitney *U*-test, $P < 6.0 \times 10^{-6}$) (Fig. 6B).

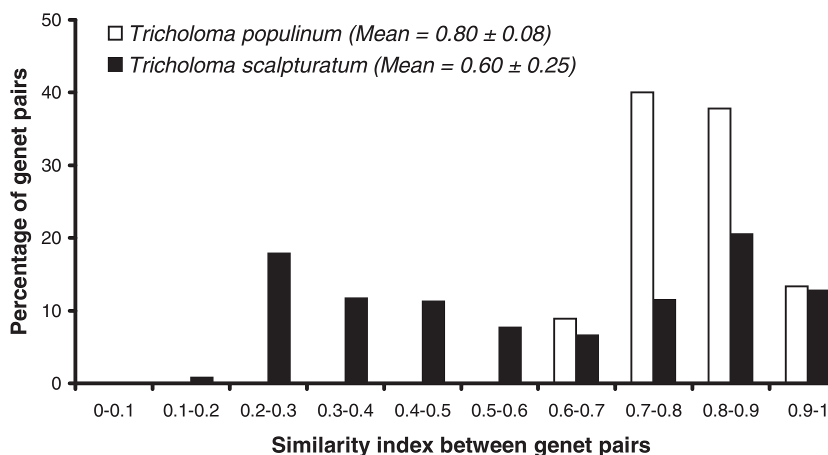

Fig. 5. Frequency distribution of similarity index calculated for genet pairs of *Tricholoma populinum* (in white) and for genet pairs of *Tricholoma scalpturatum* (in black). The two means of similarity index are significantly different (Mann–Whitney *U*-test, $P = 8.0 \times 10^{-6}$).

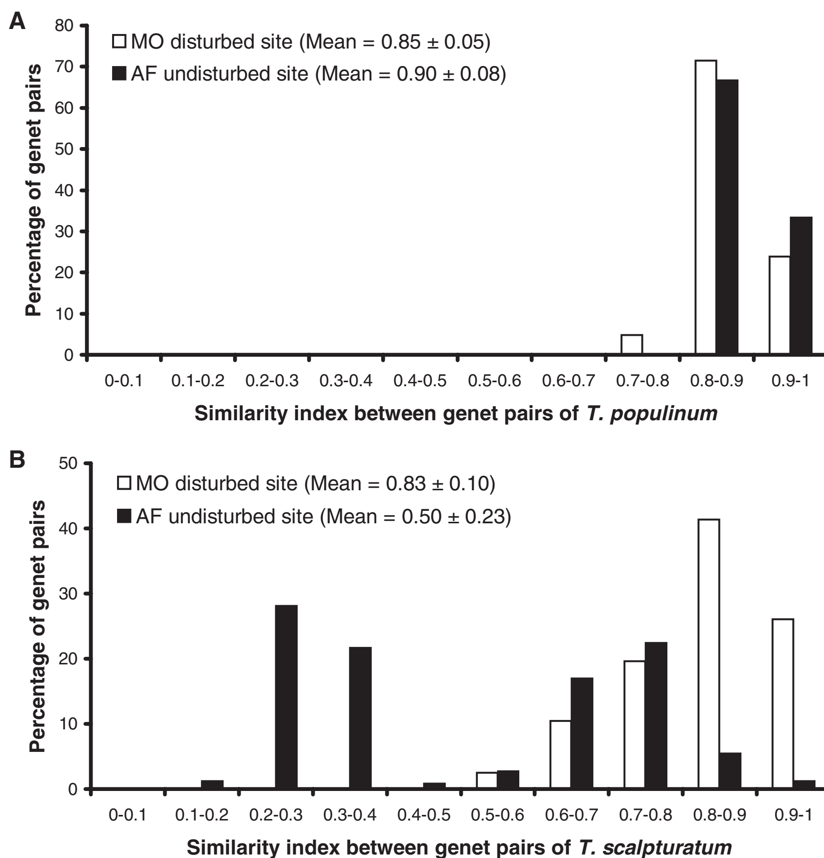

Fig. 6. Frequency distribution of similarity index calculated for genet pairs of *Tricholoma populinum* (A) and for genet pairs of *Tricholoma scalpturatum* (B) within MO disturbed site (in white) and within AF undisturbed site (in black). The two means of similarity index calculated for *T. populinum* (A) are not significantly different (Mann–Whitney *U*-test, $P = 0.19$) and those calculated for *T. scalpturatum* (B) are significantly different (Mann–Whitney *U*-test, $P < 6.0 \times 10^{-6}$).

Discussion

Our study aimed to compare two species of *Tricholoma* in their patterns of sexual reproduction and clonal growth under two different environmental conditions. Knowledge of the distribution of genets within populations provides insights into dispersal potential of the species and abilities of fungal individuals to interact with their environment. There are several ways in which characteristics of ECM fungal populations can be investigated but multilocus fingerprinting methods have been widely employed, e.g. RAPD (Jany *et al.*, 2002), ISSR (Zhou *et al.*, 1999; Sawyer *et al.*, 2001) and amplified fragment length polymorphism (AFLP) (Redecker *et al.*, 2001; Muller *et al.*, 2004). Despite their limitations, particularly for quantitative estimations (Brown, 1996; Sunnucks, 2000), these techniques detect variation at many loci and are relatively easy to assay (Moore and Novak Fraser, 2002; Burnett, 2003). The RAPD method revealed a high level of polymorphism within the *T. populinum* and *T. scalpturatum* populations, and enabled the successful identification of numerous genets (Tables 1 and 3). The ISSR method and RFLP in the PCR-amplified ribosomal intergenic spacer (IGS2 region) confirmed and extended the interpretation of the RAPD results (Table 3).

Assuming that the observed patterns are representative of the mycelium in the soil and roots, our results revealed clear differences in the colonization patterns of the two *Tricholoma* species. Within the undisturbed AF site, *T. populinum* population harboured three often large genets that consisted of multiple fruit bodies and the larger genet covered an area of at least 40–50 m^2 (Fig. 2). This size is compatible with the existence of perennial clones that extend in the soil by vegetative growth. The re-sampling of the largest genet 2 years later confirmed the long-term persistence of the clone. On the assumption that the distance between the outermost fruit bodies represents genet expansion (Dahlberg and Stenlid, 1994; Gryta *et al.*, 2000; Hirose *et al.*, 2004), we estimated the growth rate of this clone to be 0.35 m per year on average.

Despite the high number of fruit bodies and millions of sexual spores they produced, recruitment of new individuals of *T. populinum* was a rare event at the AF site. Under the disturbed environmental conditions of the MO site, there were also a few genets that produced several fruit bodies, with sizes prone for a minimal persistence of them over several years (Fig. 1). The observed patterns of diversity suggest that clones of *T. populinum* may have a competitive strategy, which promotes the local persistence of a clone. Examples of ECM fungal populations that

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comprised large (up to 300 m^2) and old genets have been reported in species from various genera such as *Suillus* (Dahlberg and Stenlid, 1990; Bonello *et al.*, 1998; Hirose *et al.*, 2004), *Cortinarius* (Sawyer *et al.*, 1999), *Xerocomus* (Fiore-Donno and Martin, 2001) and *Amanita* (Sawyer *et al.*, 2001).

In contrast, *T. scalpturatum* seems to invest more than *T. populinum* in sexual reproduction for localized occupation of space. In our short-term demographic study, *T. scalpturatum* produced five times more fruit bodies than *T. populinum*. A large proportion of the observed genets were represented by a unique fruit body, which suggests small mycelial patches in the soil or roots and colonization by meiospores. Most genets were ephemeral and did not persist over 1 year (Table 1). Rapid turnover of small (< 1 m^2) and short life-span genets are common population characteristics of ruderal and/or fugitive species. This pattern has been reported, e.g. for *H. cylindrosporum* (Gryta *et al.*, 1997; Guidot *et al.*, 2002) and for *L. amethystina* (Gherbi *et al.*, 1999; Fiore-Donno and Martin, 2001).

The RAPD and ISSR patterns revealed at least three large groups of isolates within *T. scalpturatum* (Fig. 4B). Two of these three groups coexisted at the same location (the AF site). At this point, it is unclear whether they represent morphologically cryptic species and whether they are reproductively fully isolated. However, the observed genetic differentiation and the maintenance over time of the two groups at the same site strongly suggest the existence of intersterility barriers and possibility of cryptic species. To support this hypothesis, we would need: (i) to determine the degree of isolation within and between populations of the two putative cryptic species, (ii) to test for the existence of morphological and/or biological cryptic species within *T. scalpturatum* and (iii) to obtain a phylogeography of *T. scalpturatum* and thus, clarify the conditions under which coexistence of distinct lineages (phylospecies) is expected to occur.

In addition to biological attributes of the species, local population dynamics depends on the environment. Under certain ecological conditions either mode of reproduction may be favoured. A study by Guidot and colleagues (2003) revealed that small-scale human activities (e.g. soil digging and soil compaction) promote the co-occurrence of small short life-span genets of *H. cylindrosporum* in old *Pinus pinaster* plantations. The pattern of genets also varies over time with expansion of existing genets and establishment of new genets into the population. Large individuals of *Suillus bovinus* and *Suillus variegatus* that grow vegetatively over long periods were observed in old forests but not in young stands (Dahlberg and Stenlid, 1990; 1994; Dahlberg, 1997). In our study, the two sites showed different patterns of population dynamics. Local populations of both species differ with respect to the number and size of genets and recruitment. The repeated process of disturbance in the riparian community provides the setting for new colonization patches and a mix of genotypes. Thus, genotypic diversity was 4.8 and 4.4 times higher in the disturbed MO site than in the undisturbed AF site for *T. populinum* and *T. scalpturatum* respectively. Much of the established genets within the MO population was of small size and did not persist over time. This trend was particularly pronounced in the generalist species. Thus, *T. scalpturatum* was constituted of numerous closely related genets (Figs 4B and 6B), suggesting inbreeding and local recruitment. The faster turnover of genets observed in *T. scalpturatum* may facilitate the maintenance of this species following disturbance. A contrasted pattern of population structure was observed at the undisturbed AF site. Populations of both species comprised a few long-lived clones that produced numerous fruit bodies. Selection of genotypes and differences in the success of remaining individuals may have led to a decrease in genet number and dominance of a few clones over time. Of specific interest is that a high genotypic diversity (Table 1, Fig. 4) was also observed within *T. scalpturatum* population, suggesting outbreeding and regular recruitment of new individuals by spores.

Reasons for the observed differences in patterns of diversity between the two species may be related to particular combination of traits associated with reproductive success and competitive abilities. The fitness of a genet is determined by traits associated with both successful production of spores and traits enhancing clonal propagation (e.g. cord-forming ability) (Pringle and Taylor, 2002). Territory size is important as it may lead to an increase in resources captured. The two species of *Tricholoma* may also differ in their ability to benefit black poplar as *T. populinum* has a much narrower host range than *T. scalpturatum*. One potential scenario is that the plant differentially rewards the two fungal species by controlling the amount of photosynthates allocated to the growth and reproduction of genets colonizing the roots. If so, one would predict that clones of *T. populinum* (the specialized species) would have performed better on black poplar than clones of *T. scalpturatum* (the generalist species), as a result of trade-offs in symbiotic performance. Obviously more work along those lines is needed to test the importance of natural selection and co-evolutionary processes in the evolution of the fungi. As a first step, we would suggest to perform controlled inoculations of different host plants and/or different developmental stages of the same host (e.g. poplar seedlings and cuttings) using different genotypes from the two species of *Tricholoma*.

In conclusion, there are differences between the two ECM fungal species with respect to sexual reproduction and clonal growth. *Tricholoma scalpturatum* populations have a large number of small and ephemeral genets prop-

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agated by spores, whereas *T. populinum* populations tend to contain large genets mainly propagated by mycelial extension. Our analyses also confirm that environmental discontinuities in space and time ('patchiness') and/or heterogeneity in resources have an effect on the local population structure of ECM fungi. We have documented patterns of diversity based on occurrence of fruit bodies. However, fruiting may vary unpredictably from year to year and independently of mycelial populations in the soil. As advanced PCR-based single-locus markers (e.g. microsatellites) become available for *T. populinum* and *T. scalpturatum*, it will be possible to amplify specific DNA sequences from mycorrhizae and from soil samples (Guidot *et al*., 2001; Kretzer *et al*., 2003). This will greatly enhance our understanding of cause and regulation of population changes.

Experimental procedures

Study sites

Our study was performed at two sites (MO and AF), which consisted of black poplar (*Populus nigra*) stands of similar age. The MO site is located in a riparian community at Monbéqui (43°53′45″N, 1°12′30″E), 50 km north of the city of Toulouse, in southwestern France. The sampling area (20 m by 10 m) consisted of a gravel bar naturally colonized by black poplars (Fig. 1) adjacent to the Garonne River. The age of the trees was estimated to be approximately 20 years old. The entire area was periodically subjected to floods (three to four times per year on average), resulting in heterogeneous soil topology and texture through erosion and deposition. Because environments under strong abiotic constraints are harsh environments for the establishment of living organisms (including fungi), we referred to this riparian site as the 'disturbed site'. The AF site is located in a grassland at Montaudran (43°33′36″N, 1°28′57″E) in the eastern suburb of Toulouse, about 50 km away from the MO site. The sampling area (22 m by 19 m) was bordered on its south and east sides with black poplars (Fig. 2). The trees were planted 25 years ago to create a hedge. This site is located far away from any potential source of major disturbance and consequently we referred to it as the 'undisturbed site'.

Collecting of fruit bodies and DNA extraction

Sampling sites were visited weekly during October and November, from 1998 to 2002 and from 2000 to 2002 at MO site and AF site respectively. All fruit bodies of *T. populinum* and of *T. scalpturatum* were collected except when they were densely grouped in a patch in which they were less than 2 cm apart from each other. In this case, only one fruit body per patch was sampled. Positions of collected fruit bodies were mapped with a precision of about 5 cm (Figs 1 and 2). After cleaning, fruit bodies sampled in 1998 were air-dried at 60°C for 1 day, and those sampled in 1999, 2000 and 2002 were freeze-dried overnight until further analyses. Genomic DNA was extracted from 50 to 100 mg of dried gill and inner tissue of fruit body cap according to the protocol described by Laitung and colleagues (2004).

Random amplified polymorphic DNA (RAPD) amplification and reproducibility

Initially, we tested 51 primers on a subset of samples from the two fungal species. Based on these preliminary results, we selected primers that (i) yielded polymorphisms and (ii) generate patterns, which included well-defined fragments.

Reactions were carried out in a 25 μl final volume containing 25 ng of template DNA, 200 μM of each dNTP, 0.4 μM of primer, 10 mM Tris-HCl (pH 9), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100 and 0.5 unit of *Taq* DNA polymerase (Promega Cat. # M1668). Amplifications were performed in a PTC-200 thermocycler (MJ Research) with an initial denaturation step of 2 min at 94°C, followed by five cycles of 1 min at 94°C, 1 min at 34°C, increments of 0.2°C s^{-1} from 34°C up to 45°C, 1 min at 72°C, 40 cycles of 30 s at 94°C, 30 s at 40°C and 1 min at 72°C, and a final extension of 5 min at 72°C. A negative control without template DNA was included in each run to test the absence of DNA contamination. Amplification products were separated by electrophoresis in 1× TAE buffer using a 1.4% agarose gel. Gels were ethidium bromide stained and numerical pictures of banding patterns were captured under UV light with a CDD camera supported by BIO-capt 97.03 (Vilber Lourmat, France) software for latter scoring. Fragments were automatically detected and sized using BIO1D++ 97.06 software (Vilbert Lourmat) from digitized gel images. Further visual comparisons of electrophoretograms were carried out to correct for any possible misinterpretations from the automated procedures.

For each primer, reproducibility of the patterns was checked using DNAs from six and eight fruit bodies of *T. populinum* and *T. scalpturatum* respectively. These samples were included in each amplification run and the amplified products were used as positive controls. Independent DNA extractions of these samples were also repeated three times.

Inter-simple sequence repeat (ISSR) amplification

Reactions were carried out using primer R3 (Table 2) as described for RAPD procedure except amplifications were performed in a Mastercycler thermocycler (Eppendorf) with an initial denaturation of 3 min at 94°C, followed by 36 repetitions of 30 s at 94°C, 1 min at 51°C and 3 min at 72°C, and ended by a terminal extension of 10 min at 72°C. The amplified products were separated and visualized as described above for the RAPD procedure.

Scoring of RAPD and ISSR fragments and data analysis

Only unambiguously clear fragments that were present in at least two samples were used in the analysis of RAPD and of ISSR patterns. Each variable fragment was treated as an independent locus with two alleles, coded as present (1) or absent (0) and a binary matrix of phenotypes was assembled. Samples, which had identical patterns for all RAPD and ISSR primers, were regarded as belonging to the same

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genet. The distribution of each genet was mapped according to the position of the collected fruit bodies at each site. Metric distance between the two outermost fruit bodies of the genets, therein refers to 'Dmax', was used to infer the size of the genet.

A matrix of pairwise genetic distances among the isolates was established for each species by calculating, for all pairs of isolates, the dissimilarity index $DI = 1 - 2n_{xy}/(n_x + n_y)$ where n_x and n_y refer to the number of present fragments in isolate X and in isolate Y, respectively, and $2n_{xy}$ refers to the number of bands shared by the two isolates (Nei and Li, 1979). To test for congruence between RAPD and ISSR data, the matrix of pairwise genetic distances calculated from RAPD data and the one calculated from ISSR data were compared. The level and significance of correlation between these two matrices were assessed for each species, using a Mantel test performed with the ADE-4 software package (Thioulouse *et al*., 1997) available at http://pbil.univ-lyon1.fr/ADE-4.

Cluster analysis based on the DI matrix (with combined RAPD and ISSR data) was calculated using the unweighted pair-group method with arithmetic mean (UPGMA) and a dendogram was constructed using TREECON 1.3b software (Van de Peer and De Wachter, 1994). Confidence of dendrogram branching was tested with 1000 bootstrap permutations of the data set. Genetic SI between genet pairs was derived from calculated DI values as $SI = 1 - DI$ (Nei and Li, 1979). The differences in SI between the two species and between populations of the two sites for each species were tested with the non-parametric Mann–Whitney *U*-test performed with PAST (software available at http://folk.uio.no/ohammer/past).

Genotypic diversity was estimated by the proportion of distinguishable genets (*PD*) as $PD = G/N$ where G is the number of genets and N is the number of isolates (Ellstrand and Rose, 1987).

Genetic diversity was estimated by the percentage of putative polymorphic loci (P) and by the average (H) of Shannon diversity indexes at each RAPD and each ISSR locus (H_a) calculated as: $H_a = -\Sigma p_i \log_2 p_i$ where p_i is the frequency of the ith allele at the considered RAPD locus (Lewontin, 1972). The allele frequencies at each RAPD and ISSR locus, P and H, were computed with POPGENE 1.31 software (Yeh *et al*., 1997) considering a dominant mode of inheritance in diploid organisms and assuming Hardy–Weinberg equilibrium.

Polymerase chain reaction-RFLPs of IGS2

The IGS2 region of the nuclear ribosomal intergenic spacer, which spans between the 5S gene and the 18S gene, was amplified using primers Rev5SA and RevNS1 (Selosse *et al*., 1996). Polymerase chain reactions were performed in a total volume of 50 μl including 10 ng of template DNA, 200 μM of each dNTP, 1 μM of each primer and 1 unit of GoTaq DNA polymerase (Promega Cat. # M3175) and appropriate buffer (Promega), using a PTC-200 thermal cycler (MJ Research). After an initial denaturation at 95°C for 3 min, the sequence was amplified by repeating 37 times the cycle: 1 min at 95°C, 1 min at 50°C and 3 min at 72°C. These 37 cycles were followed by a final extension at 72°C for 10 min. About 2 μg of the amplified product was digested by restriction endonucleases HaeIII and HinfI. Digestions were carried out using 2 units of enzyme in the conditions specified by the manufacturer (Promega). Restriction fragments were size fractionated in 2% NuSieve agarose (FMC Bioproduct) gel, and visualized as described above for patterns of RADP and ISSR. Restriction fragment length polymorphism patterns were compared side by side. Each isolate of *T. populinum* and of *T. scalpturatum* was then assigned to an IGS2 type therein designated by italic capital letters.

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