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Genotypic variations among isolates of *Marasmius quercophilus*, a white-rot fungus isolated from evergreen oak litter

A.M. Farnet, M. Roux, and J. Le Petit

Abstract: *Marasmius quercophilus* is a white-rot basidiomycete isolated from evergreen oak litter. This fungus plays an extensive role in litter degradation because of its cellulase, pectinase, and laccase production. Our objective was to investigate the potential genotypic polymorphism of this species using RAPD (random amplified polymorphic DNA) and the amplification and the sequencing of fragments ITS (internal transcribed spacer) from the rRNA repeat. An intraspecies polymorphism was observed for the *M. quercophilus* isolates studied as suggested by the tree found with RAPD data: seven subgroups were defined. The minimum similarity coefficient observed in these groups was 0.278. The sizes of fragments ITS 1 and ITS 2 were the same for each isolate (ITS 1: 320 bp and ITS 2: 450 bp), and the percentages of nucleotide variation were 1.25 and 1.11%, respectively. Few isolates from the studied population had the same RAPD pattern that would suggest a clonal development in some zones of the site of La Gardiole.

Key words: *Marasmius quercophilus*, intraspecific variability, ITS, RAPD.

Résumé : *Marasmius quercophilus* est un basidiomycète de la pourriture blanche isolé de la litière de chêne vert. Ce champignon joue un rôle important dans la dégradation de la litière de par sa production de cellulase, pectinase et laccase. Notre objectif consiste en la recherche d'un éventuel polymorphisme génotypique chez cette espèce en utilisant la RAPD (« random amplified polymorphic DNA ») et l'amplification suivie du séquençage des fragments ITS (« internal transcribed spacer ») à partir du cluster codant pour les ARNr. Un polymorphisme intraspécifique a été observé chez les isolats de *M. quercophilus* étudiés comme le suggère le dendrogramme trouvé à partir des données RAPD : sept sous-groupes ont été définis. Le coefficient de similarité minimum observé dans ces groupes était de 0,278. Les tailles des fragments ITS 1 et ITS 2 étaient identiques pour chaque isolat testé (ITS 1 : 320 pb et ITS 2 : 450 pb) et le pourcentage de variation des nucléotides étaient de 1,25 et 1,11%, respectivement. Quelques souches de la population étudiée présentaient le même profil RAPD ce qui suggère le développement de clones dans certaines zones du site de La Gardiole.

Mots clés : *Marasmius quercophilus*, variabilité intraspécifique, ITS, RAPD.

Introduction

Marasmius quercophilus is a white-rot fungus which colonizes evergreen oak litter, a typical Mediterranean ecosystem (Barbero and Loisel 1983). This fungus has not yet been the subject of investigations, although its enzymatic properties are of great interest. Tagger et al. (1998) have recently described the enzymatic properties of an isolate from the site of la Gardiole de Rians (Var, France), which is widely populated by evergreen oak, and thus the involvement of this fungus in litter degradation phenomena. Indeed, they have

showed the amylolytic, pectinolytic, and cellulolytic activities of this basidiomycete and also its extensive production of polyphenoloxidases such as laccases. Furthermore, the enzymatic systems involved in ligninolysis such as polyphenoloxidases have provoked considerable interest since they find applications in biotechnology (kraft pulp bleaching or aromatic pollutant degradation). In our study, 26 isolates were collected in the same site (La Gardiole de Rians). This site has been the subject of our research in many fields such as biological activity in the litter (Chabert et al. 1984), chemical analysis of tanins (Racon et al. 1987), and bacterial biodiversity (Périssol et al. 1993; Brunel et al. 1994).

The objective of this work was to study a potential genotypic polymorphism in *M. quercophilus* species since the genetics of this fungus has not yet been investigated. Genetic variations are mainly linked to mutations, genetic exchanges, spore spread, and adaptation to the environment. Thus, this study constituted a first attempt to investigate the biology and ecology of *M. quercophilus*, which seems to have an important role in the evergreen oak ecosystem. Furthermore, the genetic variability among the isolates studied may explain potential differences observed in their enzymatic properties. Molecular biology provides efficient tools such as RFLP (restriction fragment length polymorphism) after am-

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plification of the ITS (internal transcribed spacer) fragments from the rRNA repeat, or random amplified polymorphic DNA (RAPD) (Williams et al. 1990) to determine subspecies variability. The RAPD technique is not a time-consuming and expensive method. This may explain the rapid growth of interest for this technology in behavioural ecology and population biology in the past few years. It is based on the use of random oligonucleotides with priming sites in an inverted orientation, which are close enough to permit PCR (polymerase chain reaction) amplification. Both of these methods have been applied successfully for the detection of polymorphisms and the classification for various fungal species (Demeke et al. 1992; Fegan et al. 1993; Kumeda and Asao 1996; Overmeyer et al. 1996). Here, we report on the successful use of RAPD in the analysis of genetic diversity of the *M. quercophilus* species.

Materials and methods

Isolation of *Marasmius quercophilus* isolates

Carpophores of *M. quercophilus* were collected at the site of La Gardiole de Rians (Var, France), in diverse areas and on different substrata: white or evergreen oak leaves, acorns, or wood (Table 1). In this site, the four areas (about 20 m²) were all 100 m distant. Dikaryotic isolates were obtained by using the rhizomorphic form of the fungus: cultures from fungal cap were obtained on a malt-agar medium, 20 g·L⁻¹ (Bio Mérieux, Marcy l'Etoile, France) containing 50 mg·L⁻¹ chloramphenicol (Sigma). Then, pure mycelial cultures were grown on a special agar medium (whole wheat flour, 20 g·L⁻¹) that favours rhizomorph production. Rhizomorphs were cultured on a malt-agar medium (20 g·L⁻¹).

Enzymatic tests

Cultures were grown on mineral medium (Kirk et al. 1978) and 0.5 g·L⁻¹ cellulose. These cultures were homogenized with a sterile magnetic bar and were used to test for API Zym strips (Bio Mérieux, Marcy l'Etoile, France), which were incubated at 28°C for 24 h. This technique is a semiquantitative micromethod designed for the research of enzymatic activities. It allows a rapid study of 19 various enzymatic reactions. Assays for aromatic compound transformation were realized in Monod tubes as described by Tagger et al. (1998). The aromatic compounds tested included *p*-coumaric acid, ferulic acid, and tannic acid (Sigma).

DNA extraction

Still cultures were grown in a 250-mL Erlenmeyer flask containing 50 mL of malt extract (20 g·L⁻¹) at 30°C for 15 days. Cultures were filtered on microglass filters (Whatman, F/D, 2.7 µm), and the mycelia were frozen in liquid nitrogen and ground to a fine powder in a mortar. An Eppendorf tube was filled with about 0.1 g of the powdered mycelium, 0.5 mL of extraction buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS) was added, and the solution was well mixed and left to stand 10 min. The same volume of phenol and 0.3 mL of chloroform – isoamyl alcohol (2:1, v/v) were added. The mixture was shaken vigorously and left for 10 min. The suspension was centrifuged at 10 000 × *g* for 15 min, and the upper phase was removed carefully and transferred to a new Eppendorf tube. Ten microlitres of a 20% (w/v) solution of RNase A (Sigma) was added and the mixture was incubated for 10 min at 37°C. A volume of 0.4 mL of chloroform – isoamyl alcohol was added, and the solution was mixed and centrifuged at 10 000 × *g* for 10 min. The upper phase was transferred to a fresh Eppendorf tube, and one volume of isopropanol was added. The suspension was mixed by inversion and put at –20°C for 1 h. After

Table 1. Origin of the 26 *Marasmius quercophilus* isolates from the site of la Gardiole de Rians (Var, France).

Isolates	Natural substratum	Harvest date	Area
1a		Oct. 1990	3
2b		Oct. 1990	3
2c		Oct. 1990	3
3b		June 1994	1
4b		June 1994	2
5a	Wood	June 1994	1
5b	Wood	June 1994	1
6		Sept. 1996	2
8	Wood	Sept. 1996	3
9		Sept. 1996	1
10	Acorn	Sept. 1996	1
13		Sept. 1996	2
14		Sept. 1996	1
15		Sept. 1996	4
17		Sept. 1996	4
18		Sept. 1996	2
19	Acorn	Sept. 1996	2
21		Sept. 1996	3
22		Sept. 1996	1
23		Sept. 1996	1
24		Sept. 1996	2
25	Acorn	Sept. 1996	3
26		Sept. 1996	4
27		Sept. 1996	3
28	White oak leaves	Sept. 1996	3

Note: All the isolates were collected on evergreen oak leaves except for those specified. For isolate labelling, the number represented the carpophore, and the letter referred to the rhizomorph. When only one rhizomorph was isolated from the fungal cap culture, the isolate name included only a number.

centrifugation for 30 min, the DNA pellet was washed with 70% ethanol and centrifuged briefly. The supernatant was removed, and the pellet was dried under vacuum (10 min). The pellet was resuspended in 20 µL of TE buffer (0.01 M Tris-HCl, pH 8.0, 0.1 mM EDTA).

Amplification and sequencing of fragments ITS 1 and 2

Primers M1, M2, M3, and M4 were described by White et al. (1990). Primers M1 (5'-TCCGTAGGTGAACCTGCGG-3') and M2 (5'-GCTGCGTTCATCGATGC-3') were used for fragment ITS 1 amplification and primers M3 (5'-GCATCGATGAAGAACGCA GC-3') and M4 (5'-TCCTCCGCTTATTGATATGC-3') to amplify fragment ITS 2. The amplification conditions consisted of 75 mM Tris-HCl (pH 9), 20 mM (NH₄)₂SO₄, 0.01% Tween 20, 2.5 mM MgCl₂, 5 mM of each dNTP (Eurogentec, Seraing, Belgium), 100 pmol of each primer (synthesized by Eurogentec) using either primer M1 or M2, M3 or M4 with the appropriate primer, 5 ng genomic DNA, and 1 unit of *Taq* Polymerase (Goldstar, Eurogentec) in a final reaction volume of 100 µL. The reaction mixtures were prepared on ice and sealed with a drop of mineral oil before thermal cycling. The amplification program was the same for the two primer pairs: 30 cycles consisting of 30 s denaturation (93°C), 60 s primer annealing (55°C), and 45 s extension (72°C). This amplification was performed using the PTC 100 Thermal Controller (MJ Research, U.S.A.). The amplicons were separated on a 1.5% (w/v) agarose gel in TAE buffer with 0.1 µg·mL⁻¹ of ethidium bromide for 3 h at 4 V·cm⁻¹. The size marker was a 100-bp ladder (GenSura Laboratories, Inc.)

Fig. 1. The fragment ITS 1 and ITS 2 sequences of the four isolates specified: fragment ITS 1 (single strand) amplified with primer M1, fragment ITS 2 (single strand) amplified with primer M3. Different nucleotides are in boldface and underlined. The name of the isolate analysed is specified at the beginning of the sequence.

```

rRNA      24 5' TCCGTAGGTGAACCTGCGGTTCTTTGGAGATTAGTACTGTTGCTGGCCTTGTAAATGAGGCATGTCACGCTCT 3'
18S        17 5' TCCGTAGGTGAACCTGCGGTTCTTTGGAGATTAGTACTGTTGCTGGCCTTGTAAATGAGGCATGTCACGCTCT 3'
          18 5' TCCGTAGGTGAACCTGCGGTTCTTTGGAGATTAGTACTGTTGCTGGCCTTGTAAATGAGGCATGTCACGCTCT 3'
          2C 5' TCCGTAGGTGAACCTGCGGTTCTTTGGAGATTAGTACTGTTGCTGGCCTTGTAAATGAGGCATGTCACGCTCT 3'

24 5' ATCTCTAATCTATTATCCACCTGTGCACTTTTTTGTAGGAGTTCTTTAAAGGTTGGCTTGTGTAGGCTGAAAGGCTCGTGCAATG 3'
17 5' ATCTCTAATCTATTATCCACCTGTGCACTTTTTTGTAGGAGTTCTTTAAAGGTTGGCTTGTGTAGGCTGAAAGGCTCGTGCAATG 3'
18 5' ATCTCTAATCTATTATCCACCTGTGCACTTTTTTGTAGGAGTTCTTTAAAGGTTGGCTTGTGTAGGCTGAAAGGCTCGTGCAATG 3'
2C 5' ATCTCTAATCTATTATCCACCTGTGCACTTTTTTGTAGGAGTTCTTTAAAGGTTGGCTTGTGTAGGCTGAAAGGCTCGTGCAATG 3'

24 5' TTGACTAGAAGAGGCTTCTATGCTTACAATCCATTGAAGTATGTTGAGAATGCTTTTATTGGGACTTAAATGACCCCTTTAACT 3'
17 5' TTGACTAGAAGAGGCTTCTATGCTTACAATCCATTGAAGTATGTTGAGAATGCTTTTATTGGGACTTAAATGACCCCTTTAACT 3'
18 5' TTGACTAGAAGAGGCTTCTATGCTTACAATCCATTGAAGTATGTTGAGAATGCTTTTATTGGGACTTAAATGACCCCTTTAACT 3'
2C 5' TTGACTAGAAGAGGCTTCTATGCTTACAATCCATTGAAGTATGTTGAGAATGCTTTTATTGGGACTTAAATGACCCCTTTAACT 3'

24 5' TATACAACCTTTTAGCAACGGATCCCTTGGCTCTCGCATCGATGAAGAACGC 3'
17 5' TATACAACCTTTTAGCAACGGATCCCTTGGCTCTCGCATCGATGAAGAACGC 3'
18 5' TATACAACCTTTTAGCAACGGATCCCTTGGCTCTCGCATCGATGAAGAACGC 3'
2C 5' TATACAACCTTTTAGCAACGGATCCCTTGGCTCTCGCATCGATGAAGAACGC 3'

rRNA      24 5' GCATCGATGAAGAACGAGCCGAATGATCAATAACAAAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGC 3'
5.8S      17 5' GCATCGATGAAGAACGAGCCGAATGATCAATAACAAAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGC 3'
          18 5' GCATCGATGAAGAACGAGCCGAATGATCAATAACAAAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGC 3'
          2C 5' GCATCGATGAAGAACGAGCCGAATGATCAATAACAAAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGC 3'

24 5' GCCCTCTGGTATTCGGAGGGCATGCCTGTTGAGTGTCAATTAATCTCAACTCAAAAGTTTTTGTAACTTTTGAAGCTT 3'
17 5' GCCCTCTGGTATTCGGAGGGCATGCCTGTTGAGTGTCAATTAATCTCAACTCAAAAGTTTTTGTAACTTTTGAAGCTT 3'
18 5' GCCCTCTGGTATTCGGAGGGCATGCCTGTTGAGTGTCAATTAATCTCAACTCAAAAGTTTTTGTAACTTTTGAAGCTT 3'
2C 5' GCCCTCTGGTATTCGGAGGGCATGCCTGTTGAGTGTCAATTAATCTCAACTCAAAAGTTTTTGTAACTTTTGAAGCTT 3'

24 5' GGATGTGGGGTTTGCTAGCGTCTTTAGATGTTGCGCTCTCCTTAAAGTTATTAATGGGAATGCTTTTGTACACGATCCTGG 3'
17 5' GGATGTGGGGTTTGCTAGCGTCTTTAGATGTTGCGCTCTCCTTAAAGTTATTAATGGGAATGCTTTTGTACACGATCCTGG 3'
18 5' GGATGTGGGGTTTGCTAGCGTCTTTAGATGTTGCGCTCTCCTTAAAGTTATTAATGGGAATGCTTTTGTACACGATCCTGG 3'
2C 5' GGATGTGGGGTTTGCTAGCGTCTTTAGATGTTGCGCTCTCCTTAAAGTTATTAATGGGAATGCTTTTGTACACGATCCTGG 3'

24 5' GTGTGATAATTATCTACACTTTGGATAGTCTAATGGTCCATTGGGATTAATGGTATTGCGATAAACTTTGCTTTACTTGAGT 3'
17 5' GTGTGATAATTATCTACACTTTGGATAGTCTAATGGTCCATTGGGATTAATGGTATTGCGATAAACTTTGCTTTACTTGAGT 3'
18 5' GTGTGATAATTATCTACACTTTGGATAGTCTAATGGTCCATTGGGATTAATGGTATTGCGATAAACTTTGCTTTACTTGAGT 3'
2C 5' GTGTGATAATTATCTACACTTTGGATAGTCTAATGGTCCATTGGGATTAATGGTATTGCGATAAACTTTGCTTTACTTGAGT 3'

24 5' CTCAGACGAAAAGATAAGTTCTGCTTTCTAACCGTCTGTGTTACATGGACAATTTATTGACTATTGACCTCAAATCAGGTAGG 3'
17 5' CTCAGACGAAAAGATAAGTTCTGCTTTCTAACCGTCTGTGTTACATGGACAATTTATTGACTATTGACCTCAAATCAGGTAGG 3'
18 5' CTCAGACGAAAAGATAAGTTCTGCTTTCTAACCGTCTGTGTTACATGGACAATTTATTGACTATTGACCTCAAATCAGGTAGG 3'
2C 5' CTCAGACGAAAAGATAAGTTCTGCTTTCTAACCGTCTGTGTTACATGGACAATTTATTGACTATTGACCTCAAATCAGGTAGG 3'

24 5' ACTACCGCTGAACCTTAAGCA 3'
17 5' ACTACCGCTGAACCTTAAGCA 3'
18 5' ACTACCGCTGAACCTTAAGCA 3'
2C 5' ACTACCGCTGAACCTTAAGCA 3'

rRNA      28S

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Each fragment was sequenced in both directions using the appropriate primer with the T7 DNA polymerase sequencing kit (Pharmacia).

RAPD conditions

The reaction concentrations were identical to those described above. Among 20 primers tested (Operon Technologies Inc., Alameda, Calif., U.S.A.) the primer OPA 09 was chosen since every isolate showed a response. The OPA 09 sequence is 5'-GGGTAACGCC-3'. The reaction mixture was amplified through 30 cycles consisting of the following optimized program: 30 s denaturation (94°C), 30 s primer annealing (33°C), and 60 s extension (72°C). We used the optional step "increment" of the PTC 100 thermocycler to realize a progressive increase in temperature (0.5°C·s⁻¹) from annealing to extension. The amplified fragments were separated on a 1.5% agarose gel as described above. The band sizes were determined with the Image analysis software (Kodak Digital Science).

All amplification reactions were conducted at least three times in three separate experiments for each isolate.

Statistical analysis

Each of the RAPD bands was scored as discrete character in a binary matrix: 0 for absence and 1 for presence of reproducible

bands. Genetic similarity was estimated from this binary matrix with the RAPDistance program version 1.04 (Armstrong et al. 1997) using Nei's genetic distance coefficient (Nei and Li 1979):

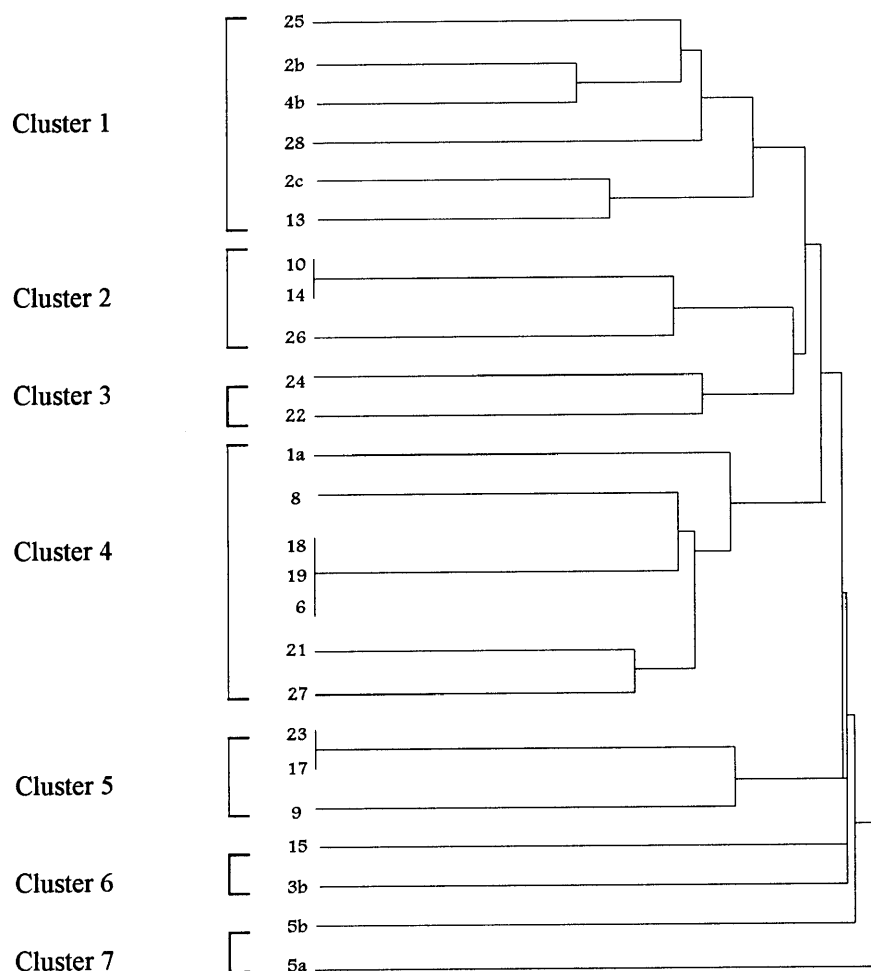
$$[1] \quad D_{xy} = \frac{1 - 2N_{xy}}{(N_x + N_y)}$$

where N_{xy} is the number of fragments shared by two isolates x and y , and N_x and N_y are the number of fragments for isolate x and y , respectively. Hierarchical cluster analysis was based on the neighbour-joining method (Saitou and Nei 1987).

Results

The use of primer pairs M1–M2 and M3–M4 led to the amplification of a single type of fragment in each case: about 320 bp for the ITS 1 fragment and about 450 bp for the ITS 2 fragment. Although the four *M. quercophilus* isolates sequenced did not show any differences in ITS 1 and ITS 2 sizes, the sequencing established nucleotide variability (Fig. 1): 1.25% for ITS 1 and 1.11% for ITS 2 (four nucleotides were different for fragment ITS 1, five nucleotides for fragment ITS 2).

Fig. 2. The tree of 26 *Marasmius quercophilus* isolates obtained from the RAPD results using the Nei and Li (1979) similarity coefficient and the neighbour-joining method (Saitou and Nei 1987). The tree was rooted using isolate 5a. The seven clusters observed are specified at the left of the tree.



We tested the 20 primers of kit A provided by Operon Technologies, and we obtained a RAPD profile for the 26 isolates tested with oligonucleotide OPA 09. With the other primers, a maximum of four isolates reacted to the amplification process. Each profile was reproducible from one amplification to another using newly extracted DNA from new cultures. The RAPD patterns obtained with primer OPA 09 showed 24 bands whose sizes varied from 80 to 6200 bp. Using statistical analysis by Nei and Li coefficient and the neighbour joining method as hierarchical clustering, we obtained a tree (Fig. 2). We observed that the branch lengths, which are proportional to genetic distance, were quite similar. This would suggest that the *M. quercophilus* isolates studied were fairly close genetically. Nevertheless, numerous deep branches in the tree demonstrated the presence of a polymorphism in this population. Indeed, the tree allowed us to group closely related isolates: cluster 1, which included isolates 13, 25, 28, 2b, 2c, and 4b; cluster 2 with isolates 10, 14, and 26; cluster 3 with isolates 22 and 24; cluster 4 with isolates 1a, 6, 8, 18, 19, 21, and 27; cluster 5 with isolates 9, 17, and 23. Four other isolates were separated from the rest of the population, and they were grouped in two other clusters:

cluster 6 with isolates 15 and 3b and cluster 7 with isolates 5a and 5b. The minimum similarity coefficient of Nei and Li (1979) observed in these different clusters was 0.278. The coefficient between isolates belonging to different clusters was greater, for example, the coefficient between isolates 10 and 1a was equal to 0.856 (when two isolates are different, this coefficient is equal to 1). When two isolates are identical the coefficient of Nei and Li (1979) is equal to 0: this was the case for some of the isolates studied, which were not differentiated (isolates 6, 18, and 19, and isolates 10 and 14). The tree also showed that isolates isolated from the same carpophore (carpophore 1, 2, 3, 4, or 5) were grouped in the same cluster. Some isolates collected from certain areas were grouped in the same clusters: clusters 1 and 4 in areas II and III and clusters 2, 5, 6, and 7 in areas I and IV.

Enzymatic tests such as acid phosphatase and β -galactosidase detection showed similar responses for all the isolates (data not shown). However, for other enzymes, we observed the same pattern in the different clusters described by the RAPD experiment. This was the case for the presence of ferulic acid (FA), tannic acid (TA), *p*-coumaric acid (*p*-CA),

Table 2. Ability to transform different substrates for the *Marasmius quercophilus* isolates in the different clusters determined by the RAPD study.

Cluster	Isolate	E	S	FA	<i>p</i> -CA	TA	EL	NAG	Area
Cluster 1	25	+	+	–	–	–	–	–	3
	2 <i>b</i>	+	+	–	–	–	–	–	3
	4 <i>b</i>	+	+	–	–	–	–	–	2
	28	+	+	–	–	–	–	–	3
	2 <i>c</i>	+	+	–	–	–	–	–	3
	13	+	+	–	–	–	–	–	2
Cluster 2	10	–	–	–	–	+	–	+	1
	14	–	–	–	–	+	–	+	1
	26	–	–	–	–	+	–	+	4
Cluster 3	24	–	–	–	+	–	–	+	2
	22	–	–	–	+	–	–	+	1
	1 <i>a</i>	–	–	–	–	–	+	–	3
Cluster 4	8	–	–	–	–	–	+	–	3
	18	–	–	–	–	–	+	–	2
	19	–	–	–	–	–	+	–	2
	6	–	–	–	–	–	+	–	2
	21	–	–	–	–	–	+	–	3
	27	–	–	–	–	–	–	+	3
Cluster 5	23	–	–	–	–	–	–	+	1
	17	–	–	–	–	–	–	+	4
	9	–	–	–	–	–	–	+	1
Cluster 6	15	–	+	+	–	–	–	–	4
	3 <i>b</i>	–	+	+	–	–	–	–	1
Cluster 7	5 <i>b</i>	–	+	–	–	–	+	–	1
	5 <i>a</i>	–	+	–	–	–	+	–	1

Note: Enzymatic tests were esculin (E), salicillin (S), ferulic acid (FA), *p*-coumaric acid (*p*-CA), and tannic acid (TA) transformation and esterase–lipase (EL) and *N*-acetyl β -glucosaminidase (NAG) presence.

esculin (E), and salicillin (S) transformation, esterase–lipase (EL), and *N*-acetyl β -glucosaminidase (NAG) (Table 2).

Discussion

ITS analysis did not distinguish between isolates; all the isolates were quite similar, and the sequences of fragments ITS 1 and 2 showed few differences for the four isolates tested (1.25 and 1.11%, respectively). Although both the ITS region and the intergenic spacer of the nuclear rRNA repeat unit evolve quickly and thus are useful in describing intra-specific genetic variations (White et al. 1990), intraspecies homology has already been reported using analysis of ITS fragments. For example, in the case of both *Fusarium oxysporum* Schlechtendahl isolates (Edel et al. 1996) and *Aspergillus* (Kumeda and Asao 1996) section *Flavi* (*A. flavus* Eries, *A. parasiticus* Speare, *A. oryzae* Ahlburg, *A. sojae* Sakagushi & Yamada, *A. tamarii* Kita), no differences were detected in the restriction patterns of fragments ITS 1 and 2 although isolates were collected from several different countries in both cases. Furthermore, Zimand et al. (1994) have observed great homology among *Trichoderma harzianum* Rifai isolates which were isolated from various sites (United States, Norway, Germany, Sweden). Extensive genetic stability has also been found in the plant pathogenic fungus *Calonectria morganii* Crous, Alfenas & Wingfield

(Overmeyer et al. 1996) and among *Metarhizium anisopliae* Metschnikoff isolates (Bidochka et al. 1994).

In this study, the RAPD data showed an overall genetic stability, since the branch lengths of the tree, which are proportional to genetic distance, were quite similar. This can be explained by the intraspecific nature of the study. Extensive genetic exchanges within a population lead to a decrease in genetic diversity, since this genetic flow occurs easily between isolates of the same species. But we also observed a greater degree of homology between some isolates forming subgroups, which demonstrated polymorphism in the population studied (the minimum coefficient of similarity was 0.278). This extensive polymorphism may be due to different environmental pressures, which vary with the area where the fungi were collected. Furthermore, a progressive separation of fungal hyphae even in a quite limited site may lead to genetic divergence. These interpretations are consistent with the fact that the isolates (except cluster 3) grouped in the same cluster were collected in the same areas (II and III; I and IV). These observations suggest that the environmental influences (temperature, moisture, pH) are the same in areas I and IV and in areas II and III. Thus, the isolates grouped in clusters 1 and 4 and clusters 2, 5, 6, and 7 seem to have adapted to the same environmental factors in areas II and III and in areas I and IV, respectively. However, this hypothesis has to be considered with caution, since the environmental factors of the different areas are not yet clearly defined, and

some of the isolates collected on various substrata (acorn, evergreen oak or white oak leaves) were grouped in the same cluster. Assigbetse et al. (1994) have also attributed the differentiation of *Fusarium oxysporum* f.sp. *vasinfectum* to the geographical location of the isolates. De la Bastide et al. (1994) have successfully distinguished *Laccaria bicolor* Maire isolates depending on the area where the isolates were collected. Rohel et al. (1997) have shown that some *Erynia neoaphidis* Remaudière & Hennebert isolates from different continents showed extensive homology, while other isolates of the same species presented differences even when they were collected in the same area in France. This demonstrated the wide variety of results possible.

The heterogeneity of this population was illustrated by the enzymatic data: isolates belonging to the same cluster had certain similar phenotypic properties. Phenotypic potential alone can not be used to estimate the variability of a population, but it can reflect heterogeneity previously detected by genome analyses. Wildman (1995) has also shown that the geographic location and the substrate of origin of *Fusarium acuminatum* Ellis & Everhart isolates influenced the morphological aspects of this fungus. Furthermore, toxin production was affected by the habitat of the different isolates of this fungal species. The *Fusarium compactum* Wollenweber isolates, which were also analysed in this study, differed in their carbon utilization patterns depending on the sites where they were collected.

In our RAPD study, several isolates from the same carpophore were grouped. It was expected that these isolates would be genetically close, since their genetic difference was due to meiosis alone, these dikaryons having been obtained from the recombination of the monokaryons produced by the same polysporic culture. The grouping of these isolates has thus showed the ability of the RAPD method to analyse genotype relationships at different taxonomic levels. The isolates that were considered as identical (6, 18, and 19; 10 and 14) were collected at the same time (1996). Isolates 6, 18, and 19 were collected in the same area (area II) and isolates 10 and 14 were collected in area I. The hypothesis of a clone dispersed in a site has already been described (Turner et al. 1997). Cooke and Rayner (1984) have also reported clone spread in ecosystems in the case of different basidiomycete populations. The somatic incompatibility loci are numerous and multiallelic. Both these situations minimize the chance of two isolates in a population having exactly the same somatic incompatibility alleles. This limits genetic exchanges and encourages the development of clonal populations.

This first attempt to analyse the *M. quercophilus* genome showed genetic polymorphism in this species. Further studies will help us to better describe this species, for example, using isolates from other sites or other geological substrata (such as siliceous soils). At the same time, this would allow us to investigate the potential influence of environmental factors on a fungal population.

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