Mycorrhiza (2002) 12:19–27
DOI 10.1007/s00572-001-0142-9

ORIGINAL PAPER

Jean Weber · Jesús Díez · Marc-André Selosse
Denis Tagu · François Le Tacon

SCAR markers to detect mycorrhizas of an American *Laccaria bicolor* strain inoculated in European Douglas-fir plantations

Accepted: 8 November 2001 / Published online: 21 December 2001

Abstract The American strain S238N of the ectomycorrhizal fungus *Laccaria bicolor* (Maire) Orton has been used to inoculate Douglas-fir [*Pseudotsuga menziesii* (Mir.) Franco] plantations in France over the last two decades. *Laccaria* fruit bodies are scarce in mature plantations, which precludes further assessment of its persistence by fruit body surveys. Our objective was to develop new markers to identify this strain and its eventual non-fruiting progeny on root tips. We converted nine random amplified polymorphic DNA markers into sequence characterized amplified region (SCAR) markers. Two of these SCAR markers enabled us to detect S238N on roots of seedlings and mature trees. No amplification of non-fungal (host plant, bacterial, etc.) DNA was observed. Moreover, both SCARs were amplified from *Laccaria*-like mycorrhizas in a Douglas-fir plantation inoculated 14 years ago, demonstrating the long-term persistence of the inoculant strain. We also obtained a SCAR marker to detect one strain of European origin (*L. bicolor* 81306), indicating that SCARs are potential markers to type the naturally occurring genets. Thus, SCAR markers are of great value in studying the persistence of inoculant strains and the effects on local populations of introducing foreign strains.

Keywords Douglas fir · Ectomycorrhizas · *Laccaria bicolor* · Random amplified polymorphic DNA · Sequence characterized amplified region

J. Weber and J. Díez contributed equally to this work

J. Díez (✉) · D. Tagu · F. Le Tacon
Unité Mixte de Recherche INRA-UHP,
Interactions Arbres/Micro-organismes,
INRA-Nancy, 54280 Champenoux, France
e-mail: diez_muriel@yahoo.com
Fax: +34-91-8855066

J. Weber
National Institute of Education,
Nanyang Technological University,
Natural Science Division,
Biology Section, 1 Nanyang walk,
Singapore 637616

M.-A. Selosse
Institut de Systématique (IFR CNRS 1541),
Muséum National d'Histoire Naturelle,
43 rue Cuvier, 75005 Paris, France

Present address:
J. Díez, Dpto. de Biología Vegetal,
Universidad de Alcalá, 28871 Alcalá de Henares,
Madrid, Spain

Introduction

Successful forest plantation sometimes requires deliberate introduction of ectomycorrhizal fungi (Smith and Read 1997). Hence, such fungi are used in tree nurseries as inoculants (Le Tacon et al. 1992). A research program was developed in France over the last 15 years to improve growth of Douglas fir [*Pseudotsuga menziesii* (Mir.) Franco] by inoculation with strains of *Laccaria bicolor* (Maire) Orton (Le Tacon et al. 1992). This fungus is distributed worldwide and is common in both forest nurseries (Henrion et al. 1994) and mature forest stands (Last et al. 1987). Soil-growing dikaryotic mycelia are regarded as the main vegetative stage. Soil mycelia form mycorrhizas with tree roots (Smith and Read 1997) and give rise to fruit bodies (Fries and Mueller 1984).

Strain S238N of *L. bicolor* is used commercially in France to inoculate Douglas fir in nurseries and forest plantations. This strain can increase the total volume of wood produced by 60% (Selosse et al. 2000). Unfortunately, morphological identification of fruit bodies and mycorrhizas is not sufficient to distinguish *Laccaria* genets (genetic individuals) and molecular tools are required. Survival of the S239N strain has been studied in nursery and in Douglas-fir plantations using PCR-based techniques (Henrion et al. 1994; Selosse et al. 1998a). Molecular typing of *Laccaria* sporophores with random amplified polymorphic DNA (RAPD) markers confirmed that this inoculant strain was still present and dominant (ca. 38% of fruit bodies) 14 years after inoculation (Selosse et al. 1998a, b).

The basidiomycete *L. bicolor* is heterothallic with a bifactorial mating-type system and multi-allelic mating-type factors (Kropp and Mueller 1999). North American strains are genetically divergent from European strains, yet they are sexually compatible (Mueller and Gardes 1991; De la Bastide et al. 1995). Furthermore, strain S238N is sexually compatible with a strain isolated from Saint-Brisson (France) (Selosse et al. 1998a). Hybrid spread may outcompete or displace indigenous genotypes. Despite the persistence and abundant fruiting of S238N, neither introgressed nor inbred progeny of this strain were detected among sporophores (Selosse et al. 1998a). However, hybrid and/or inbred genets could exist solely as vegetative mycelia and thus remain undetected by basidiocarp typing. Such genets may not fruit because of genetic sterility or low mycorrhizal colonization (Lamhamedi et al. 1994; Selosse et al. 1998b). The scarcity of *Laccaria* fruit bodies in mature plantations of Douglas fir, probably because of canopy closure (Jansen and de Nie 1988), is a further hindrance to *Laccaria* population assessment by basidiocarp surveys. Thus, molecular markers are necessary for detecting potential progeny of S238N on roots.

Genets can be identified with RAPD markers using DNA extracted from fruit bodies and mycelium pure cultures, but not with DNA extracted from mycorrhizal root tips. Primers used in RAPD amplification can amplify DNA from both symbionts (plant and fungus) and other microorganisms of the rhizosphere, e.g. bacteria (Garbaye and Duponnois 1992). This would give rise to complex DNA patterns. The only available marker to detect strain S238N on mycorrhizal root tips is the rDNA intergenic spacer (IGS) (Henrion et al. 1994; Martin et al. 1999). A single marker, however, is insufficient to distinguish a strain from its descendants. RAPD may be transformed to give fungal-specific markers. After cloning and sequencing RAPD fragments, longer primers can be designed to specifically amplify the so-called sequence characterized amplified region (SCAR) (Paran and Michelmore 1993). As longer primers, SCAR primers specifically amplify fungal DNA from the mycorrhizal complex-DNA matrix. A short set of SCAR markers thus produce multilocus fingerprints. Such fingerprints allow the identification of mycorrhizas of strain S238N and its progeny. SCAR markers have been of great value already for identifying organisms in complex samples, e.g. pathogenic microorganisms or fungi used for biocontrol in plants (Toth et al. 1998; Becker et al. 1999). In particular, SCAR markers enable the identification of truffle species in roots of inoculated seedlings (Gandeboeuf et al. 1997; Bertini et al. 1998). Encouraged by these advances, we sought to design SCAR markers for identifying mycorrhizas of *L. bicolor* S238N.

Materials and methods

Strains

The strains used in this study are listed in Table 1. The American strain S238N of *Laccaria bicolor* (Maire) Orton was isolated by J. Trappe (Oregon State University, Corvallis, Ore., USA) and R. Molina (USDA Forest Service, Corvallis, Ore., USA) from a fruit body collected under *Tsuga mertensiana* (Bong.) Carr. at Crater Lake, Ore., USA in 1976 (Di Battista et al. 1996). Strain S238N was then transferred to the Forest Microbiology Laboratory (INRA-Nancy Center, France). This strain has since been introduced as inoculant into tree nurseries and plantations in France, e.g. the experimental plantation of Saint-Brisson (Nièvre, France) in 1987 (Selosse et al. 2000).

A set of five monokaryons obtained from in vitro spore germination was also used. The spores were isolated from one basidiocarp of S238N (Selosse et al. 1996). In addition, we used a set of five field isolates of *L. bicolor* isolated from fruit bodies in association with Douglas fir [*Pseudotsuga menziesii* (Mir.) Franco] at Saint-Brisson (Selosse et al. 1998a, b; 1999). The field isolates consisted of local strains, among them N109 and N77. These two strains were identified as S238N using RAPD markers (Selosse et al. 1998a).

The study material also included isolates of *L. bicolor* from Douglas-fir and Norway spruce plantations in central France and isolates of *L. laccata* and *L. proxima* from European coniferous stands (Henrion et al. 1994). Additional isolates of fungi that may form mycorrhizas with Douglas fir were analyzed. These field isolates were from different regions of Europe (Henrion et al. 1994). All strains used in this study are preserved in the Collection of Ectomycorrhizal Fungi at the INRA-Nancy Center (France). They are subcultivated every 2–3 months on solid modified-Pachlweski's medium (Selosse et al. 2000) at 25°C in the dark.

Mycorrhiza sampling

From April to June 2000, Douglas-fir mycorrhizas were collected from a greenhouse, a tree nursery and a forest plantation. Mycorrhizas were examined under a dissecting microscope and classified as *Laccaria*-like, *Thelephora*-like, *Rhizopogon*-like or *Chalciporus*-like morphotypes (Selosse et al. 2000). Mycorrhizas and root tips were preserved at –20°C pending DNA extraction. Although our original sample was larger, we only used DNA extractions that tested positive for the amplification of the intergenic 28 S/5 S spacer (IGS1).

Mycorrhizas were sampled from 1-year-old seedlings growing in the greenhouse of the INRA-Nancy Center (France). Ten S238N-inoculated and 10 non-inoculated seedlings were used. Twenty-five *Laccaria*-type and 15 *Thelephora*-like mycorrhizas were analyzed. Greenhouse seedlings were inoculated with S238N as described by Villeneuve et al. (1991).

Mycorrhizas were collected also in the tree nursery of the Naudet Company (Autun, France). Inoculation for commercial purposes has been practiced in this nursery since 1992. Seedlings were inoculated with S238N as described by Villeneuve et al. (1991). Inoculated seedlings were transplanted to the upper 30 m (treatment I) of a 75-m-long nursery bed, and non-inoculated seedlings were transplanted to the lower 45 m (treatment II). Seedlings were planted 2–10 cm apart in four rows separated by 20 cm. In April 2000, we randomly sampled 10 seedlings per treatment with the constraint that samples were taken at least 2 m apart. Twenty-five *Laccaria*-like mycorrhizas and 15 *Rhizopogon*-like mycorrhizas were analyzed per treatment.

Mycorrhizas were sampled also in the experimental plantation of Saint-Brisson. In this plantation, inoculated seedlings were planted during spring 1987. Ten 225-ml soil cores were randomly collected from the upper 10 cm of soil within a 7.2×7.2-m^2 S238N-inoculated plot. The soil cores were spaced 1 m apart. Roots were separated from the soil and washed. Fifty *Laccaria*-

Table 1 List of strains used in this study. These strains are preserved in the Collection of Ectomycorrhizal Fungi of the INRA-Nancy Center (France)

Strain	Species	Host	Origin
S238N	*Laccaria bicolor* (Maire) P.D. Orton	*Tsuga mertensiana* (Bong.) Carr	Crater Lake, Ore., USA
Monokaryons obtained from *Laccaria bicolor* strain S238N			
H18[a]	*L. bicolor*	*Pseudotsuga menziesii* (Mir.) Franco	INRA-Nancy, France
H63[a]	*L. bicolor*	*P. menziesii*	INRA-Nancy
H82[a]	*L. bicolor*	*P. menziesii*	INRA-Nancy
H90[a]	*L. bicolor*	*P. menziesii*	INRA-Nancy
H97[a]	*L. bicolor*	*P. menziesii*	INRA-Nancy
Laccaria bicolor strains present at the Saint-Brisson plantation			
N77[b,c]	*L. bicolor*	*P. menziesii*	Saint-Brisson (Nièvre, France)
N109[b,c]	*L. bicolor*	*P. menziesii*	Saint-Brisson (Nièvre)
N18[c]	*L. bicolor*	*P. menziesii*	Saint-Brisson (Nièvre)
N93[c]	*L. bicolor*	*P. menziesii*	Saint-Brisson (Nièvre)
N246[c]	*L. bicolor*	*P. menziesii*	Saint-Brisson (Nièvre)
L. bicolor strain isolates from forest stands in Europe			
Cham3	*L. bicolor*	*P. menziesii*	Chammet (Limousin, France)
83216	*L. bicolor*	*Picea abies* Karst	Grand Grammont (Limousin)
81306	*L. bicolor*	*P. menziesii*	Barbaroux (Limousin)
Laccaria isolates from Europe			
CNRF	*Laccaria laccata* (Scot.) Fr.	*Fagus sylvatica* L.	INRA-Nancy
83.222	*L. laccata*	*P. abies*	Grand Grammont (Limousin)
Prag	*Laccaria proxima* (Bou.) Pat.	*P. abies*	Prague (1986), Czechoslovakia
LL004	*L. proxima*	Unknown	(Edinburgh, UK)
Potential ectomycorrhizal fungi of *P. menziesii* at Saint-Brisson			
Gram1	*Lactarius hepaticus* Plowr.	*Picea abies*	Grand Chammot (Limousin)
Nancy	*Cenococcum geophilum* Fr.	*Tilia* sp	Nancy
Gram	*Chalciporus piperatus* (Bull.: Fr.) Bat	*P. menziesii*	Grand Grammont, (Limousin)
Peyrat	*Rhizopogon subareolatus* Smith	*P. menziesii*	Peyrat, France
IK1444	*Paxillus involutus* (Bastch) Fr.	Unknown	Goldboden, Germany

[a] Monokaryon obtained from spore germination of a fruit body of the S238N strain
[b] Identified as S238N by RAPD typing (Selosse et al. 1998a)
[c] Strain isolated from fruiting genets collected at Saint-Brisson

like mycorrhizas (six soil cores), and 20 *Chalciporus*-like mycorrhizas (two soil cores) were analyzed.

DNA extraction

Total DNA was extracted from pure fungal cultures of monokaryotic or dikaryotic mycelia, non-mycorrhizal root tips and mycorrhizas using an hexadecyltrimethylammonium bromide (CTAB) proteinase-K protocol (Henrion et al. 1994).

PCR reactions

All PCR reactions were run in 0.2-ml tubes in a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, Conn., USA). Reaction tubes contained 1× reaction buffer (20 mM Tris HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X100), 0.2 mM dNTP, 0.02–2 ng/µl template DNA and 0.06 u/µl *Taq* DNA polymerase (Quantum-Appligène-Oncor, Illkirch, France).

The thermocycling program for all amplifications was 3 min denaturation at 94°C, 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min, and a final elongation at 72°C for 10 min. RAPD amplification was carried out with conditions designed to enhance reproducibility, i.e. high annealing temperature (50°C) and high primer concentration (3.5 µM) (Selosse et al. 1998a). All RAPD reactions were performed at least twice. We used two different dilutions from two different extractions.

RAPD primers were: 152GC, 5′-CGCACCCGCAG-3′; 152 C, 5′-CGCACCGCAC-3′; 152G, 5′-CGCACC GCAG-3′; 156: 5′-GCCTGGTTGC-3′; 157: 5′-CGTGGGCAGG-3′; 173: 5′-CAGGCG GCG T-3′; 174, 5′-AACGGGCAGC-3′. All primers were supplied by LifeTechnologies, Paisley, UK.

Election of RAPD markers

Amplification patterns were compared to identify bands present only in S238N (and its haploid progeny). Amplification products were separated by electrophoresis on 8% polyacrylamide or 2% agarose gels in a 1× Tris-borate-EDTA buffer (Sambrook et al. 1989). The gels were stained with 4 µg/ml ethidium bromide and photographed under UV light. A 100-bp DNA ladder (Gibco-BRL, Life Technologies, Paisley, UK) was included in the gels as a molecular size marker.

Recovery of RAPD markers from polyacrylamide gels

Nine RAPD bands present only in the American S238N strains were recovered from the gels (see Table 2). In addition, three RAPD bands present only in the European strain *L. bicolor* 81306 were extracted (see Table 2). These bands were excised with a clean, sharp scalpel and placed into a 1.5-ml microcentrifuge tube with 1 ml of diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate; 1 mM EDTA, pH 8, 0.1% SDS). After incuba-

Table 2 SCAR markers obtained in this study. The strain from which RAPD fragment was cloned is shown in parentheses

Strain	SCAR	GenBank accession no.
S238N	SCAR-$157_{400(H90)}$	AF306317
	SCAR-$157_{350(H90)}$	AF306318
	SCAR-$157_{350(H90)}$	AF306320
	SCAR-$157_{220(H90)}$	AF306324
	SCAR-$174_{240(H82)}$	AF306321
	SCAR-$174_{220(H82)}$	AF306322
81306	SCAR-$152C_{470(81306)}$	AF306316
	SCAR-$156_{290(81306)}$	AF306323
	SCAR-$157_{210(81306)}$	AF306319

tion at 50°C for 30 min, they were centrifuged 1 min at 10,000 *g* and then filtered through a paper filter (Prolabo, Fontenay-Sous-Bois, France) using a 1-ml hypodermic syringe. DNA was purified with a PCR QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The DNA fragment was re-amplified with the same primer and RAPD amplification conditions described above. The re-amplified DNA fragments were purified with a QIAquick PCR Purification Kit (Qiagen).

Cloning and sequencing

Each purified RAPD product was ligated into the pCR4-TOPO plasmid vector of a TOPO-TA Cloning Kit (Invitrogen, Groningen, The Netherlands) following the manufacturer's instructions. Recombinant plasmids were used to transform TOPO thermocompetent *Escherichia coli* cells provided with the kit. The size of cloned RAPD fragments was checked by PCR using the universal primers T3 and T7. Plasmid DNA was purified from bacterial cells (QIA prep Spin Miniprep Kit; Qiagen). Both strands of each insert were sequenced with a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer) using the T3 and T7 primers. The sequence products were analyzed with an ABI model 373 DNA fluorescent sequencer (Perkin Elmer Applied Biosystems). Sequences were assembled with Sequencher 3.0 for MacOS from Genes Codes (Ann Arbor, Mich., USA). The consensus sequences were deposited in the GenBank of the National Center for Biotechnology Information (NCBI); accession numbers AF306316–AF306324 (Table 2). A search for sequence identity was conducted at the NCBI website (http://www.ncbi.nih.gov) using Blastn and Blastx algorithms (Altschul et al. 1997).

Primers design and SCAR nomenclature

We designed SCAR primers of 18–20 bases (Table 3). They typically included the sequence of the corresponding RAPD primers, a high percentage of GC bases and ended by G or C. Suitable Tm was calculated using the web page of the Virtual Genomic Center (http://alces.med.umn.edu/rawtm.html) (Breslauer et al. 1986). Primer specificity was tested prior to synthesis by virtual PCR using Amplify 1.2 software (University of Wisconsin, Madison, Wis., USA). Care was taken to avoid secondary structures, cross hybridization and primer dimer generation. Marker nomenclature follows standard conventions for primer designation, i.e. the name of the primer followed by the molecular weight of the base-pair band. The nomenclature also includes in parentheses the name of the strain from which it originated and the primer pair used for the amplification, e.g. SCAR-$157_{400(H90)}$-1–4: RAPD primer 157, 400 bp band from the strain H90, using the primers 1 and 4 given in Table 3.

Table 3 SCAR primers designed in the present study. The monokaryon from which the SCAR was issued is shown in parentheses. Italics indicate the sequence from the original RAPD primer. SCAR markers were designed to optimize *Tm* and to avoid formation of dimers (*Tm* temperature °C)

SCAR	Primer sequences (5′–3′)	Position	Size	*Tm*	Type of primer
$157_{400(H90)}$	(1) *cgtgggcagg*aacgaatgag	1	20	66.2	Forward
	(2) ccaggatcgcgaggctttcc	41	20	68.6	Forward[a]
	(3) gccgaccttgaaatcacaac	390	20	60.3	Reverse[a]
	(4) *cgtgggcagg*tcgtgttcg	421	19	68.5	Reverse
$157_{220(H90)}$	(1) *cgtgggcagg*acgatttac	1	19	61.3	Forward
	(2) *cgtgggcagg*cgcagaattg	224	20	71.5	Reverse
$157_{430(H90)}$	(1) *cgtgggcagg*catcggag	1	18	68.7	Forward
	(2) ctggccccggttcttcacg	115	19	68.4	Forward[a]
	(3) gtcggaggtttcgaacatcg	423	20	62.8	Reverse[a]
	(4) *cgtgggcagg*agagagggac	466	20	66.8	Reverse
$157_{350(H90)}$	(1) *cgtgggcagg*gccattgggg	1	20	76.0	Forward
	(2) *cgtgggcagg*tggggacgc	353	19	74.0	Reverse
$174_{220(H82)}$	(1) *cgggcagc*cacagacctc	1	18	65.4	Forward
	(2) *cgggcagc*acgaagataag	118	19	61.7	Reverse
$174_{240(H82)}$	(1) *cgggcagc*aatgaccgctcc	1	20	72.7	Forward
	(2) *cgggcagc*acgttgtactcg	269	20	67.3	Reverse
$152C_{470(81306)}$	(1) *cgcaccgcac*caagacatg	1	19	67.3	Forward
	(2) ggcgtaccctggtgttcttc	54	20	61.7	Forward[a]
	(3) ccgcttgccccccatcctgg	482	19	72.5	Reverse[a]
	(4) *cgcaccgcac*ctggcggactg	516	21	77.7	Reverse
$156_{290(81306)}$	(1) *gcctggttgc*tcaggatcag	1	20	63.2	Forward
	(2) *gcctggttgc*cgggagggta	297	20	70.8	Reverse
$157_{210(81.306)}$	(1) *cgtgggcagg*caatgaaat	1	19	64.1	Forward
	(2) *cgtgggcagg*gcaccagtagc	204	21	71.5	Reverse

[a] Internal primer

SCAR amplification conditions

Twelve SCAR pairs were tested for strain specificity to S238N. Each primer pair was tested on strains, on non-mycorrhizal roots, and ectomycorrhizas. For IGS1 amplification, 200-fold DNA dilutions were used to counter effects of the high copy number of the intergenic spacer. The IGS1 was amplified using the primers 5SA (5′-cag agt cct atg gcc gtg gat-3′) and CLN12 (5′-ctg aac gcc tct aag tca g-3′) at 0.5 mM. The IGS1 and SCAR reactions were carried out under the same conditions as the RAPD reaction. The annealing temperature of the SCAR amplification was raised to achieve exclusive amplification for strain S238N (65°C). Controls with no DNA were included in each set of PCR amplifications to test for contamination of reagents.

Results

SCAR markers

Six RAPD markers, present only in S238N (and its haploid progeny), were cloned and sequenced (Table 2). These markers were recovered from monokaryons issued from strain S238N (H82 or H90). In addition, three RAPD markers were detected only in *Laccaria bicolor* 81306: $152C_{470}$, 156_{290} and 157_{210}. These three RAPD bands were also cloned and their sequences characterized (Table 2). All the amplified sequences included the corresponding primer sequences at both ends. Nucleotide homology searches found no similar sequences in the GenBank database.

Strain specificity of the SCAR markers

At an annealing temperature of 59°C, no SCAR primer pair (Table 3) amplified a band only present in S238N (including N109 and N77) or its haploid progeny. At 65°C, two primer pairs, SCAR-$157_{400(H90)}$-1–4 and SCAR-$174_{240(H82)}$-1–2, amplified one single DNA fragment only from S238N and part of its progeny. The SCAR-$157_{400(H90)}$-1–4 primer pair amplified a DNA fragment from three monokaryons (H90, H63 and H18) (Fig. 1), but not from monokaryons H82 and H97. Similarly, the primer pair SCAR-$174_{240(H82)}$-1–2 amplified DNA from monokaryons H82, H97, H63 and H18, but not from monokaryon H90 (Fig. 1). Therefore, S238N strain is heterozygous at both loci. The presence of SCAR-$174_{240(H82)}$ and SCAR-$157_{400(H90)}$ represents the

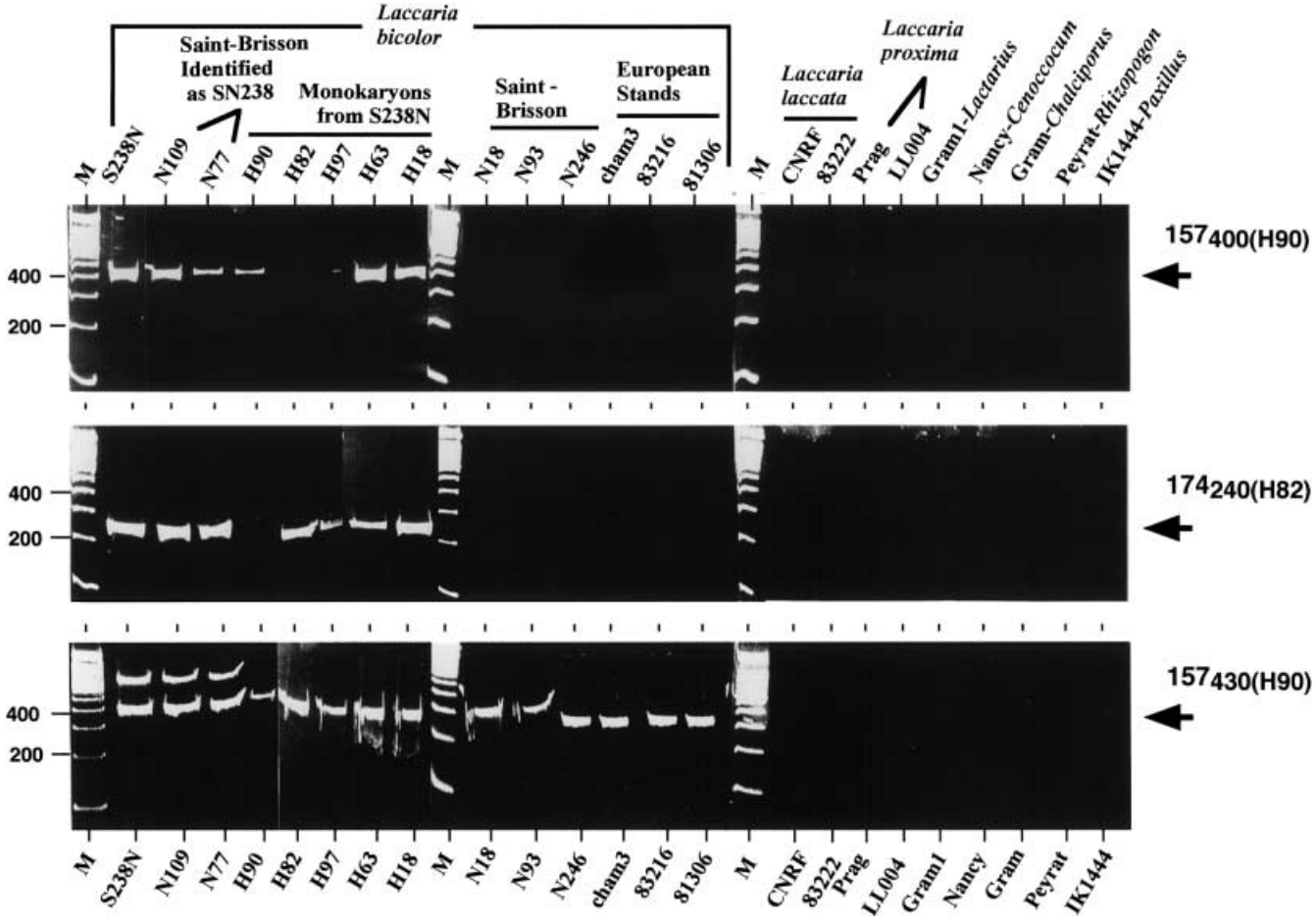

Fig. 1 SCAR markers for *Laccaria bicolor* S238N on acrylamide gels. Amplification was performed on DNA extracted from the mycelial pure cultures. Strain data are described in Table 1. The SCAR-$157_{400(H90)}$ was amplified with primer pair 1–4; the SCAR-$174_{240(H90)}$ with primer pair 1–2; and the SCAR-$157_{430(H90)}$ with primer pair 1–4 (Table 3). A band over the SCAR-$157_{430(H90)}$ was unique to the S238N strain (*M* 100-bp ladder)

Table 4 SCAR marker amplification for strains used in the present work. See Table 1 for the strain code (+/– weak amplification just observable on the UV transilluminator; – no information, *null* the primer pair did not amplify the corresponding SCAR marker)

Strain	SCAR							
	157_{430} (primer pair)		157_{400}		157_{350}	157_{220}	174_{240}	174_{220}
	(1–3) (1–4)	(2–3) (2–4)	(1–3) (1–4)	(2–3) (2–4)	(1–2)	(1–2)	(1–2)	(1–2)
L. bicolor S238N	430[a]	430[a]	400	400	350	220	240	220[c]
Obtained from the strain S238N of *Laccaria bicolor*								
L. bicolor H18	430	430	Null	–	350	–	Null	220[c]
H63	430	430	400	–	350	–	240	220[c]
H82	430	430	Null	–	350	–	240	220[c]
H90	430	430	400	–	350	–	Null	220[c]
H97	430	430	Null	–	350	–	240	–
Laccaria bicolor strains present at the Saint-Brisson plantation								
L. bicolor N77	430[a]	430[a]	400	–	350	–	240	220[c]
N109	430[a]	430[a]	400	400	350	220	240	220[c]
H18	430	430	400	(+/–)	350	220	240	220[c]
N93	430	430	Null	–	350	–	Null	220
N 246	430	430	Null	400b	350	220	–	220
Laccaria bicolor strain isolates from forest stands in Europe								
L. bicolor Cham3	Null	Null	Null	400	350	220	Null	220[c]
83216	430	430	Null	400	350	–	Null	220
81306	430	430	Null	400+	350	220	Null	220
Laccaria isolates from Europe								
L. laccata CNRF	430	(+/–)	Null	Null	Null	Null	Null	(+/–)
83222	Null	430	Null	400[b]	350	220	–	220
L. proxima Prag	Null	(+/–)	Null	Null	Null	–	–	(+/–)
LL004	Null	(+/–)	Null	Null	Null	Null	Null	220
Potential ectomycorrhizal fungi of *P. menziesii* at the Saint-Brisson plantation								
Lactarius hepaticus Gram1	Null	(+/–)	Null	–	Null	–	Null	(+/–)
Cenoccum geophilum Nancy	Null	(+/–)	Null	–	Null	–	Null	(+/–)
Chalciporus piperatus Gram	Null	Null	Null	–	Null	–	Null	(+/–)
Rhizopogon subareolatus Peyrat	Null	Null	Null	–	Null	–	Null	–
Paxillus involutus IK1444	Null	(+/–)	Null	–	Null	–	Null	(+/–)
Annealing temperature	65°C	65°C	65°C	59°C	65°C	59°C	65°C	65°C

[a] Additional band of 1000 bp; [b] Additional band of 450 bp; [c] Additional band of 240 bp

dominant allele and the lack of amplification indicates the recessive (null) allele. DNA extracts from monokaryon H90 amplified SCAR-157_{400} but not SCAR-174_{240}. DNA extracts from H82 and H97 amplified SCAR-174_{240} but not SCAR-157_{400}. Extracts from two further monokaryons (H18 and H63) amplified both markers. The two SCAR markers are, therefore, dominant and not tightly linked.

All the other SCAR markers were amplified from several *Laccaria* strains, e.g. SCAR-$157_{430(H90)}$ and SCAR-$157_{350(H90)}$ (Table 4). A retarded band of ca. 1,000 bp appeared for the dikaryotic strains S238N, N109 and N77 above the band of SCAR-$157_{430(H90)}$. This band did not appear in the monokaryons issued from S238N (Fig. 1). Additional DNA bands were also observed above other SCAR bands. We obtained three SCAR markers from *L. bicolor* 81306. Among them, at an annealing temperature of 65°C, SCAR-$156_{290(81306)}$ was found only in 8306.

Typing mycorrhizas with SCAR markers

Primers 1 and 4 of SCAR-157_{400} amplified one band for *Laccaria*-like mycorrhizas of nursery-inoculated seedlings (*n*=25 mycorrhizas, 10 seedlings). Similarly, only one other band was amplified with the primers 1 and 2 of SCAR-174_{240}. SCAR-157_{400} and SCAR-174_{240} were not found in *Laccaria*-like mycorrhizas of non-inoculated greenhouse seedlings (*n*=25 mycorrhizas, 6 seedlings). Positive amplification was also found in *Laccaria*-like mycorrhizas collected within the inoculated part of the nursery bed (*n*=25, 10 seedlings). In contrast, no amplification was observed in *Laccaria*-like mycorrhizas from the non-inoculated part (*n*=25, 6 seedlings). No amplification was obtained for *Thelephora*-like mycorrhizas of greenhouse seedlings (*n*=15, 5 seedlings) or *Rhizopogon*-like mycorrhizas of nursery seedlings (*n*=30, 6 seedlings) (Fig. 2).

SCAR-157_{400} and SCAR-174_{240} markers were amplified from 10% of *Laccaria*-like mycorrhizas collected in

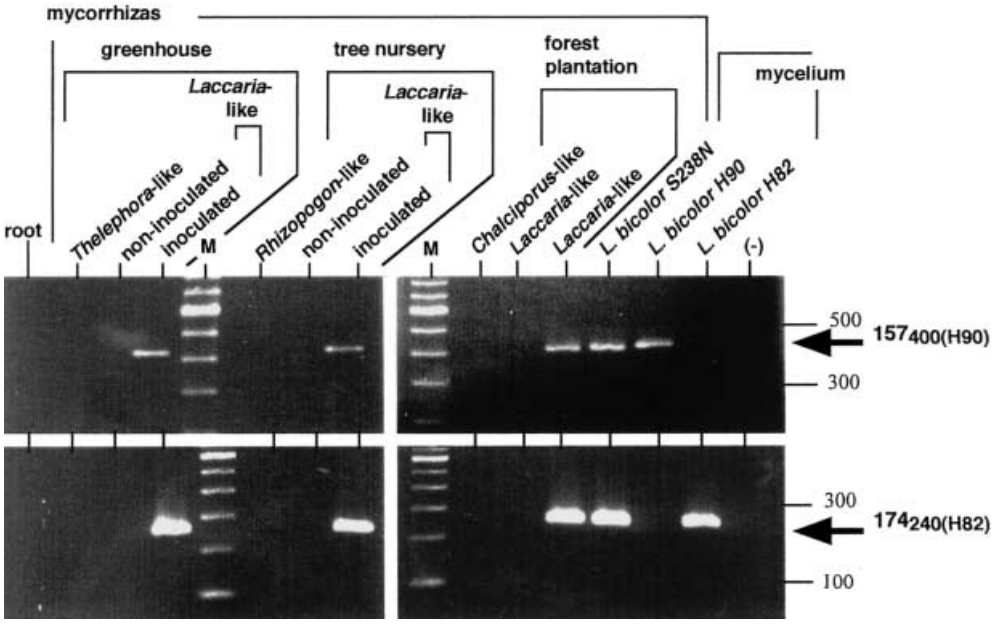

Fig. 2 Identification of *L. bicolor* S238N on ectomycorrhizas using the primers SCAR-$157_{400(H90)}$-1–4 and SCAR-$174_{240(H90)}$-1–2 (see Table 2 for sequences). Ectomycorrhizas were recovered from inoculated greenhouse and nursery seedlings and from soil cores in the Saint-Brisson plantation. Both markers distinguished naturally occurring *Laccaria* genets from the S238N strain. Agarose ethidium bromide-stained gel (*M* 100-bp ladder)

the forest plantation (*n*=50 mycorrhizas, 6 soil cores). In contrast, no amplification was observed for *Chalciporus*-like mycorrhizas (*n*=20, 2 soil cores) and 90% of *Laccaria*-like mycorrhizas. Local *Chalciporus* and *Laccaria* genets probably formed these mycorrhizas. Finally, no amplification was observed for non-mycorrhizal roots (*n*=8, 2 soil cores) (Fig. 2).

Discussion

SCAR markers to identify *Laccaria bicolor* S238N

In our study, the majority of the SCAR markers were found in most of the *Laccaria* strains assessed, suggesting that these SCAR primers would anneal also for other *Laccaria* strains at the same loci. Low specificity has been reported previously in other organisms (Hernández et al. 1999). Extending the primers from 10 to 20 nucleotides could reduce the effect of mismatching at the fixation site of the primer and prevent such polymorphism. However, we cannot exclude the possibility that we have cloned non-polymorphic bands undetectable by ethidium bromide staining (Hernández et al. 1999).

SCAR-157_{400} and SCAR-174_{240} markers were present only in part of the haploid progeny, revealing allelic polymorphism. This contradicts the previous observation of homozygosity (Selosse et al. 1998a) in the corresponding RAPD markers for S238N. Extending primer length from 10 to 20 bases probably induced more mismatching at primer fixation sites in one of the two alleles. If so, this would preclude amplification in monokaryons carrying this allele (Hernández et al. 1999). An alternative explanation is the presence of two bands of the same size in the RAPD profile. One of them may be homozygous, thereby preventing observation of the heterozygosity of the cloned band. In both scenarios, the level of heterozygosity of S238N might have been underestimated in previous work (Selosse et al. 1998a).

Both SCAR-157_{400} and SCAR-174_{240} represent allele-specific markers, as illustrated by the fact that both markers segregated within the haploid progeny. Our study was performed on a limited set of strains collected in France. Similar tests should be performed on a larger number of strains to determine whether both markers are strain specific. Such specificity must be evaluated before the markers are used in field studies. In particular, it will be necessary to verify whether these markers are absent from the fungal population of Saint-Brisson.

Identification of the mycorrhizas of *L. bicolor* S238N

False negatives are a common problem with SCAR markers. We chose the IGS1 of *L. bicolor* S238N as a control for failure in SCAR amplification resulting from low DNA quality. Under our amplification conditions, samples positive for both SCARs often did not amplify the IGS1 (data not shown). This might be because of the larger size of the IGS1 (800 bp), the higher SCAR-primer *Tm*, and/or the stringent temperature used in the SCAR amplification (65°C). Consequently, the intergenic spacer was a suitable control for DNA quality. We only used DNA samples that amplified the IGS1 after 200-fold dilutions. Hence, we prevented failures in amplification resulting from low-quality DNA. Moreover, these primers did not amplify DNA fragments from the host plant *Pseudotsuga menziesii*. Thus, they will allow us to investigate the persistence of *L. bicolor* S238N in large-scale mycorrhiza surveys. We also observed successful and specific amplification from *Laccaria*-like mycorrhi-

zas. Neither soil microorganisms nor root DNA prevented amplification of mycorrhiza samples.

SCAR markers were originally developed for marker-assisted selection in plant breeding programs (Paran and Michelmore 1993; Lahogue et al. 1998). They have proven to be of great value for identifying organisms in complex samples (Toth et al. 1998; Becker et al. 1999). SCAR markers were obtained to identify truffles of economic value at the species level. These markers allow verification of the reliability of seedlings intended for truffle plantations (Gandeboeuf et al. 1997; Bertini et al. 1998). SCAR markers have been used also in a few cases for typing mycorrhizas. To our knowledge, however, the present study is the first report of SCAR markers used for the identification of single genets in ectomycorrhizal root tips.

Detection of the S238N progeny on mycorrhizas

SCAR markers are used for the identification of hybrids and for the characterization of introgression (Mandolino et al. 1999; Hernández et al. 1999). In our case, the SCAR markers obtained will improve our capacity to detect strain S238N and its potential progeny on mycorrhizal roots. They will be particularly useful in assessing mature plantations where fruiting is currently scarce. A preliminary mycorrhiza assessment on the persistence of strain S238N at Saint-Brisson was carried out using the nuclear rDNA IGS1 locus (Selosse et al. 2000). The PCR-amplified IGS1 of strain S238N produced two characteristic bands when resolved in polyacrylamide gels. These bands correspond to two heteroduplexes formed after PCR denaturation and further cross-hybridization between the DNA strands of these alleles. The observation of heteroduplexes in mycorrhizas does not truly determine whether the inoculant strain or its progeny colonizes the roots. In the case of inbreeding, these heteroduplexes would prevent distinction of S238N from half of its F1 (also heterozygous for this locus). Potential hybrids would also present heteroduplexes. In addition, homozygous F1 of S238N genotypes could not be detected.

In conclusion, we obtained two SCAR markers to detect mycorrhizas of the inoculant strain *L. bicolor* S238N. Both markers are dominant and not tightly linked. Mycorrhizas displaying only one of the SCARs might be hybrids or S238N self-crossed descendants. The corresponding RAPD markers and the IGS1 locus are unlinked (Selosse et al. 1998a). Three heterozygous unlinked markers (IGS included) are sufficient to identify recombinants if they occur in silva at any significant frequency. However, they did not allow discrimination between S238N and its progeny. New unlinked SCAR markers would be required to identify genets as parental, intercontinental hybrid, inbreed or backcrossed genets. In addition, we have obtained a SCAR marker to detect a strain of *L. bicolor* of European origin. This demonstrates the ability of the SCAR markers to identify local genets.

Acknowledgements This work was supported by a postdoctoral Marie Curie Individual Fellowship of the European Community program Human Potential under the contract number HPMF-CT-1999-00174 and a "Ramón y Cajal" contract from the MCyT (Spain) to J.D., and funded in part by the CNRS program "Vie et Societé" managed by the Institut Français de Biodiversité. We appreciate suggestions by F. Martin on an earlier version of the manuscript and the comments of anonymous reviewers. We are indebted to D. Bouchard and P. Vion for their help during the mycorrhiza sampling. We thank the owners of Saint-Brisson plantation and the Naudet Company for access to experimental plots. The assistance of C. Delaruelle and A. Kohler with DNA sequencing is gratefully acknowledged.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Becker EM, Ball LA, Hintz WE (1999) PCR-based genetic markers for detection and infection frequency analysis of the biocontrol fungus *Chondrostereum purpureum* on Sitka alder and trembling aspen. Biol Control 15:71–80

Bertini L, Agostini D, Potenza L, Rossi I, Zeppa S, Zambonelli A, Stocchi V (1998) Molecular markers for the identification of the ectomycorrhizal fungus *Tuber borchii*. New Phytol 139: 565–570

Breslauer KJ, Frank R, Blocker H, Marky LA (1986) Predicting DNA duplex stability from the base sequence. Proc Natl Acad Sci USA 83:3746–3750

De la Bastide PY, Kropp BR, Piché Y (1995) Vegetative interactions among mycelia of *Laccaria bicolor* in pure culture and in symbiosis with *Pinus banksiana*. Can J Bot 73:1768–1779

Di Battista C, Selosse MA, Bouchard D, Stenström E, Le Tacon F (1996) Variations in symbiotic efficiency, phenotypic characters and ploidy level among different isolates of the ectomycorrhizal basidiomycete *Laccaria bicolor* strain S238. Mycol Res 100:1315–1324

Fries N, Mueller GM (1984) Incompatibility systems, cultural features and species circumscription in the ectomycorrhizal genus *Laccaria* (Agaricales). Mycologia 76:633–642

Gandeboeuf D, Dupré C, Roeckel-Drevet P, Nicolas P, Chevalier G (1997) Typing *Tuber* ectomycorrhizae by polymerase chain amplification of the internal transcribed spacer of rDNA and the sequence characterized amplified region markers. Can J Microbiol 43:723–728

Garbaye J, Duponnois R (1992) Specificity and function of Mycorrhization Helper Bacteria (MHB) associated with the *Pseudotsuga menziesii–Laccaria laccata* symbiosis. Symbiosis 14:335–344

Henrion B, Di Battista C, Bouchard D, Vairelles D, Thompson BD, Le Tacon F, Martin F (1994) Monitoring the persistence of *Laccaria bicolor* as an ectomycorrhizal symbiont of nursery-grown Douglas-fir by PCR of the rDNA intergenic spacer. Mol Ecol 3:571–580

Hernández P, Martin A, Dorado G (1999) Development of SCARs by direct sequencing of RAPD products: a practical tool for the introgression and marker-assisted selection of wheat. Mol Breeding 5:245–253

Jansen AE, de Nie HW (1988) Relations between mycorrhizas and fruitbodies of mycorrhizal fungi in Douglas-fir plantations in the Netherlands. Acta Bot Neerl 37:243–249

Kropp BR, Mueller GM (1999) *Laccaria*. In: Cairney JWG, Chambers SM (eds) Ectomycorrhizal fungi. Key genera in profile. Springer, Berlin Heidelberg New York, pp 65–88

Lahogue F, This P, Bouquet A (1998) Identification of a codominant SCAR marker linked to the seedlessness character in grapevine. Theor App Genet 97:950–959

Lamhamedi MS, Godbout C, Fortin JA (1994) Dependence of *Laccaria bicolor* basidiome development on current photosynthesis of *Pinus strobus* seedlings. Can J For Res 24:1797–1804

Last FT, Dighton J, Mason PA (1987) Successions of sheathing mycorrhizal fungi. Trends Ecol Evol 2:157–161

Le Tacon F, Alvarez IF, Bouchard D, Henrion B, Jackson RM, Luff S, Parlade JI, Pera J, Stenström E, Villeneuve N, Walker C (1992) Variations in field response of forest trees to nursery ectomycorrhizal inoculation in Europe. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CAB, Wallingford, pp 119–134

Mandolino G, Carboni A, Forapani S, Faeti V, Ranalli P (1999) Identification of DNA markers linked to the male sex in dioecious hemp (*Cannabis sativa* L.). Theor Appl Genet 98:86–92

Martin F, Selosse MA, Le Tacon F (1999) The nuclear rDNA intergenic spacer of the ectomycorrhizal basidiomycete *Laccaria bicolor*: structural analysis and allelic polymorphism. Microbiology 145:1605–1611

Mueller GM, Gardes M (1991) Intra- and interspecific relations within *Laccaria bicolor* sensu lato. Mycol Res 95:592–601

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistant genes in lettuce. Theor Appl Genet 85:985–993

Sambrook J, Fritsch EF, Maniatis T (eds) (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, New York

Selosse MA, Costa G, Di Battista C, Le Tacon F, Martin F (1996) Segregation and recombination of ribosomal haplotypes in the ectomycorrhizal basidiomycete *Laccaria bicolor* monitored by PCR and heteroduplex analysis. Curr Genet 3:332–337

Selosse MA, Jacquot D, Bouchard D, Martin F, Le Tacon F (1998a) Temporal persistence and spatial distribution of an American inoculant strain of the ectomycorrhizal basidiomycete *Laccaria bicolor* in a French forest plantation. Mol Ecol 7:561–573

Selosse MA, Martin F, Le Tacon F (1998b) Survival of an introduced ectomycorrhizal *Laccaria bicolor* strain in a European forest plantation monitored by mitochondrial ribosomal DNA analysis. New Phytol 140:753–761

Selosse MA, Martin F, Le Tacon F (1999) Structure and dynamic of artificially introduced and naturally occurring *Laccaria* spp. genets in a European Douglas-fir plantation. Appl Environ Microbiol 65:2006–2014

Selosse MA, Bouchard D, Martin F, Le Tacon F (2000) Survival after outplanting and effect of two *Laccaria bicolor* strains inoculated on Douglas-fir [*Pseudotsuga menziesii* (Mir.) Franco]. Can J For Res 30:360–371

Smith SE, Read DJ (eds) (1997) Mycorrhizal symbiosis, 2nd edn. Academic, New York

Toth IK, Hyman LJ, Taylor R, Birch PRJ (1998) PCR-based detection of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* in plant material and its differentiation from *X. c.* pv. *phaseoli*. J Appl Microbiol 85:327–336

Villeneuve N, Le Tacon F, Bouchard D (1991) Survival of inoculated *Laccaria bicolor* in competition with native ectomycorrhizal fungi and effects on the growth of outplanted Douglas-fir seedlings. Plant Soil 135:95–170