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Klara Hönig · Michael Riefler · Ingrid Kottke

Survey of *Paxillus involutus* (Batsch) Fr. inoculum and fruitbodies in a nursery by IGS-RFLPs and IGS sequences

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Abstract Maintenance and stress resistance of beech and oak plantlets is considerably improved when seedlings are inoculated in the nursery with *Paxillus involutus*, but success of mycorrhization is strongly related to the fungal isolate. To determine the success of individual *P. involutus* isolates used for inoculation, a molecular characterization was carried out and used to survey the mycorrhizas and the fruitbodies obtained in the nursery. Persistency of isolates was also surveyed on mycorrhizas 1 and 2 years after planting the seedlings to the field. Molecular characters for practical survey of the isolates were the length of the PCR product using primers CLN12/5SA for amplification and the restriction fragment length polymorphism of the intergenic spacer (IGS1) digested by *Hae*III. Sequencing of the IGS1 confirmed the length polymorphisms, but did not allow further discrimination of the isolates. Differences in IGS1-sequences between isolates increased with larger geographical distance of isolate origin, but genetically closely related isolates could not be separated. The molecular survey indicated that the *P. involutus* inoculants were not necessarily those on the mycorrhizas, but some were more competitive than others. Only the most efficient isolate was found to form mycorrhizas with a plant and within a nursery plot when several isolates were mixed for inoculation. An Australian *P. involutus* isolate (ANU) failed in mycorrhization of beech and oak in the nursery and the seedlings became mycorrhizal by another isolate. Fruitbody production in the nursery could be linked to the inoculated isolates. The isolates remained associated with the inoculated beech and oak for 2 years after outplanting to a forest area and arable land. The molecular survey, thus, helped to select efficient isolates of *P. involutus* for forestry application.

Key words *Paxillus involutus* · *Fagus sylvatica* · *Quercus robur* · IGS1 sequences · IGS1/RFLPs

K. Hönig · M. Riefler · I. Kottke (✉)
Eberhard-Karls-Universitity Tübingen, Botanical Institute, Systematic Botany and Mycology. Auf der Morgenstelle 1, D-72076 Tübingen, Germany
Fax: +49-7071-29-5344
e-mail: Ingrid.Kottke@uni-tuebingen.de

Introduction

Recent demand for beech (*Fagus sylvatica* L.) and oak (*Quercus robur* L.) seedlings for restauration of windcast areas was confronted with dieback of plantlets in bare-root nurseries in Germany. Maintenance and stress resistance of tree plantlets is considerably improved when seedlings become inoculated by ectomycorrhizal fungi in the forest nursery, but success of mycorrhization may be strongly related to the selected fungal species or isolate (Marx et al. 1991; Le Tacon et al. 1992; Rosado et al. 1994; Di Battista et al. 1996; Rudawska and Kieliszewska-Rokicka 1997). *Paxillus involutus* (Batsch) Fr. was found to be well suited for inoculation of beech and oak in bare-root conditions (Marx et al. 1991; Herrmann et al. 1992; Garbaye and Churin 1997; Kottke and Hönig 1998). Isolates of *P. involutus* obtained from fruitbodies of diverse forest stands were tested previously with respect to mycorrhization and growth-promoting effects (Kottke and Hönig 1998). While mycorrhizas formed by *P. involutus* can be easily identified by their outer appearance and sclerotia formation (Agerer 1997), no isolate-specific morphological differences are visible. It was, therefore, unclear whether the individual isolates were efficient in mycorrhization of seedlings, or whether more competitive isolates could replace others.

Molecular characters appeared to be an appropriate tool to survey the success of mycorrhization in the nursery and to follow the persistency of the individual isolates after outplanting. High intraspecific genetic variability of ectomycorrhizal fungi was found in the intergenic spacer (IGS1; Henrion et al. 1992; Gryta et

al. 1996). Henrion et al. (1994a) monitored the persistency of *Laccaria bicolor* (Maire) Orton on nursery-grown Douglas-fir *(Pseudostuga menziesii* Mirb.) on the basis of the length polymorphism of the PCR-amplified nuclear ribosomal IGS1 of single mycorrhizas. Additional discrimination of fungal isolates was gained from RFLP (restriction fragment length polymorphism) after digestion of the IGS product by *Hinf*I (Henrion et al. 1994a). Similar "fingerprinting" was less efficient for *Tuber melanosporum* Vitt. (Henrion et al. 1994b) and for *Tylospora fibrillosa* (Burt) Donk isolated from Sitka spruce mycorrhizas (Erland et al. 1994). Variability in the IGS region of *P. involutus* had not been previously studied and its suitability for isolate screening was not known. We, therefore, investigated the IGS1 region of 10 isolates of *P. involutus* obtained from fruitbody tissue. The DNA profiles were used to survey isolate-specific mycorrhization of beech and oak seedlings in the nursery and after planting to the field.

Materials and methods

Fungal isolates

Fungal isolates were obtained from tissue of *P. involutus* fruitbodies collected in two 80-year-old Norway spruce [*Picea abies* (L.) Karst.] stands, in two windcast areas close to one of the Norway spruce stands and in the nursery greenhouse where the inoculation experiment was carried out. One isolate was obtained from INRA, Nancy (Henrion et al. 1992). Details of origin and dates of sampling are shown in Table 1. Isolates are stored in the culture collection of the institute according to the isolate numbers presented in Table 1.

Media and culture conditions of isolates and inoculum

The mycelia were grown in Petri dishes on Hagem medium (1 l aqua dest., KH_2PO_4 0.5 g, NH_4Cl 0.5 g, $MgSO_4.7\,H_2O$ 0.5 g, $FeCl_3$ (1%) 0.5 ml, glucose 5 g, malt extract 5 g, 1.5% agar). The nursery inoculum was prepared on Perlite/Sphagnum/Hagem medium in 2-l Erlenmeyer flasks and grown for 2 months at 22 °C in the dark (Kottke and Hönig 1998). Only those flasks where the mycelium had spread through the whole substrate were used for inoculation.

Design of the forest nursery greenhouse experiment

The inoculation experiments were carried out in a state forest nursery (Schorndorf, Forest Administration Stuttgart, Germany). A folia-covered greenhouse (5×32 m) was designed according to Garbaye (1986) using peat moss as substrate and adding only low amounts of fertilizer (Osmocote, 15–30 g m^3 of N, P and K). Oak and beech were sown at spring time. The inoculum was mixed into the top layer of the peat at the time of sowing at approximately 1 l m^{-2}.

The inoculation experiments were carried out over 4 years. Although in this publication we mainly discuss the results obtained in the fourth year, information about years 1–3 inoculation in the nursery is necessary to understand the whole. During the first year (1990), *P. involutus* isolate 1315 was used for inoculation. A mixture of the isolates 1315, 1444 and ANU (1 : 1 : 1) was applied to the nursery beds in the second (1991) and third year (1992). In the fourth year, 10 isolates of *P. involutus* were introduced alone or mixed (1 : 1 : 1) as shown in Figure 1. The size of the individual plots in the fourth year was 2.5×4 m. There were no mechanical barriers between the individual plots.

In the second and third years, the nursery beds were steam fumigated before adding the inoculum. Part of the peat was taken out and stored in bags to be used as inoculum in the fourth year. This was to test how long the mycelium persisted in the peat without host plants.

During all 4 years of experiments, we obtained high rates of mycorrhizal seedlings (Herrmann et al. 1992; Kottke and Hönig 1998). Fruitbodies of *P. involutus* were produced in the nursery beds in the first and third year and were used for isolation of mycelia (Table 1).

Sampling of mycorrhizas

During the fourth year, seeds of both tree species germinated at high rates and the plots were densely covered with seedlings. Six months after sowing, 20 plantlets of each plot were taken at random and the percentage of mycorrhizal rootlets was estimated

Table 1 Origin of fruitbodies collected for isolation of *Paxillus involutus* mycelia from the cap tissue

Number of isolate (strain collection Tübingen)	Year of sampling	Site of sampling
1315	1988	80-year-old Norway spruce stand, Stadtwald Villingen, Black Forest, Germany
1444	1990	Folia-covered greenhouse, oak seedlings inoculated with *P. involutus* isolate 1315; forest nursery, Schorndorf, Baden-Württemberg, Germany
1506	1992	Folia-covered greenhouse, oak seedlings inoculated with *P. involutus* mixing isolates 1315+1444+ANU; forest nursery, Schorndorf, Baden-Württemberg, Germany
1523, 1524	1993	80-year-old Norway spruce stand, Bad-Waldsee, Southern Baden-Württemberg, Germany. Isolates of two fruitbodies growing a few centimetres apart
1525, 1526	1993	Regenerating Norway spruce on windcast area 100 m from the 80-year-old stand, Bad-Waldsee, Southern Baden-Württemberg, Germany. Isolates of two fruitbodies growing a few centimetres apart
1527, 1528	1993	Mixed, young regeneration on windcast area, 200 m from the 80-year-old Norway spruce stand, Bad Waldsee, Southern Baden-Württemberg, Germany. Isolates of two fruitbodies growing a few centimteres apart
ANU	1987	Poplar, campus of the Australian National University, Canberra, Australia (isolated by F. Lapeyrie, INRA, Nancy)

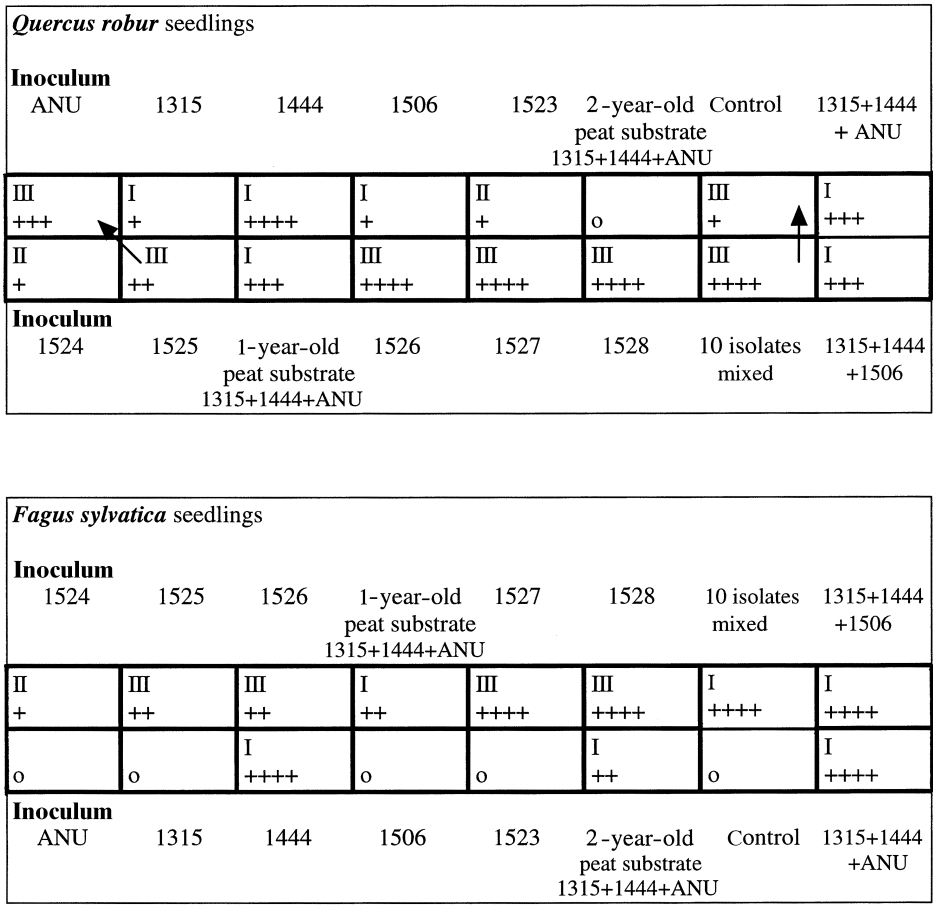

Fig. 1 Design of the experimental plots in the fourth year. Each square was 10 m^2 with no intervening mechanical barriers. Degree of mycorrhization: no mycorrhization (0), 1–30% (+), 30–50% (++), 50–70% (+++), >70% (++++). *Arabic numbers* indicate the isolates of *Paxillus involutus* used for inoculation within a plot. The 1- and 2-year-old peat substrate was used 1 or 2 years before in the nursery to inoculate oak and beech by mixing in isolates 1315, 1444 and ANU and was steam fumigated. *I–III* indicate the grouping of isolates arising from the molecular characterization. *Arrows* indicate spread of isolates. The control was without inoculum

using a dissecting microscope. To determine the success of the isolates, five plantlets per treatment were selected at random and 3–5 tips were mixed together and frozen in an Eppendorf tube for DNA extraction, taking at least five tubes per seedling. In total, 25 extractions per plot were examined.

Outplanting experiment

In the second year, 100 seedlings of oak and beech inoculated with the mixture of isolates 1315, 1444 and ANU and 100 control plants, were selected at random from the greenhouse material in November, kept cool between sawdust during the winter and planted in early May the next year in an arable plot and in a windcast area close to a Norway spruce stand. Twenty plants of each treatment were collected in October the first year and in June of the second year after planting. Persistency of *P. involutus* mycorrhizas was evaluated using a dissecting microscope. The mycorrhizas were collected and frozen in Eppendorf tubes as described above.

Molecular survey of fungal isolates

DNA extraction

DNA was extracted from mycelia and mycorrhizas according to the protocols of Edwards et al. (1991), and Henrion et al. (1992). Amplification of the IGS1 region was performed according to Henrion et al. (1994b) using the universal primer CNL12 in combination with primer 5SA or with primer PAXI (Fig. 2). The primer CNL12 was designed by Anderson and Stasovski (1992). The primer 5SA was designed by Duchesne and Anderson (1990) and later modified by T. Bruns (Henrion et al. 1992). We designed primer PAXI (5′-CAA TGC AAA GTG CCA TGG AAG-3′) according to sequences obtained using primer 5SA (see below). The locations of the primer sequences are given in Fig. 2. The thermal cycling parameters were an initial denaturation at 95 °C for 5 min, followed by 29 cycles of 93 °C for 2 min, 53 °C for 2 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. Controls without DNA were carried out to

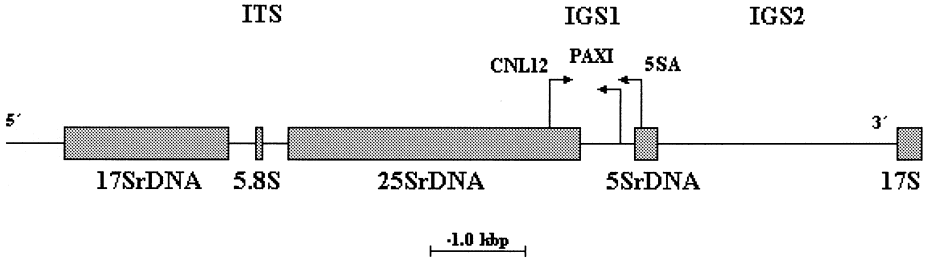

Fig. 2 Diagram displaying the position of the intergenic space (IGS1) between the nuclear large subunit (LSU or 25SrDNA) and the 5 S rDNA followed by the IGS2 and the nuclear small rDNA (17S) sequences. The primer annealing sites (CNL12, PAXI and 5SA) are indicated by *arrows*

check for the presence of contaminants in the reagents and reaction buffers.

RFLP and gel electrophoresis

The enzymes *Eco*RI, *Rsa*I, *Hinf*I, *Hae*III and *Mbo*I were used for RFLP analysis. Digestion was performed using 10 µl of the PCR product for 14–16 h with 3 units of enzyme according to the manufacturer's instructions. The digested PCR products were size-fractionated using agarose gels. Gel sizes were 9×6.5 cm (mini-gel, 1 or 2% agarose), 14×12 cm (midi-gel, 1.5% agarose) and 25×20 cm (maxi-gel, 1.7% agarose). Gels were stained with ethidium bromide (0.5 ml ml^{-1}) for 20 min and documented under UV light with a Polaroid camera.

Cycle sequencing

The PCR products of isolates 1315, 1444, 1523, 1526 and ANU were purified using the QIAquick Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The dsDNA was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an automated sequencer (ABI 373 A, Applied Biosystems). The primers CNL12, 5SA and PAXI were used to sequence both strands of the IGS1 region.

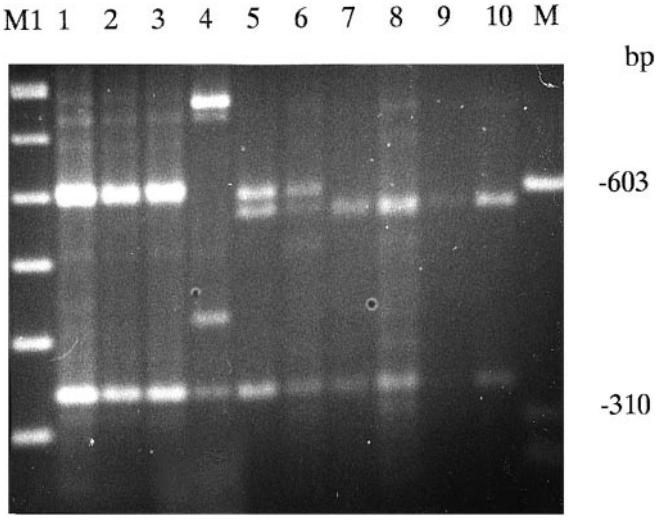

Fig. 3 RFLP profiles of the IGS1 digested by *Hae*III of the 10 *P. involutus* isolates used for inoculation of beech and oak in the nursery (see Tables 1 and 2). An agarose maxigel with molecular size markers M1 (100 bp) and M (Phi-X-174 digested by *Hae*III)

Results

Molecular characterization of the fungal isolates

The primers CNL12/5SA successfully amplified the IGS1 region of all the *P. involutus* isolates used for inoculation (Table 2). According to the total length of the IGS-PCR products, the fungal isolates could be separated into four groups. The isolates 1315, 1444 and 1506 showed one IGS1 fragment of 920 bp (group I). The isolates 1523 and 1524 both gave two IGS1 fragments of approximately 920 and 880 bp (group II). Isolates 1525, 1526, 1527 and 1528 gave one IGS band of approximately 880 bp (group III). The PCR of isolate ANU resulted in a significantly shorter product (740 bp, group IV).

*Mbo*I, *Rsa*I and *Eco*RI did not digest the PCR products properly. Digestion by *Hinf*I and *Hae*III resulted in distinct fragments and the RFLPs obtained could be used to characterize but not to separate further the four isolate groups. Only the results obtained by digestion with *Hae*III are presented (Table 2, Fig. 3) as the RFLPs were consistently more clearly separated than those obtained by digestion with *Hinf*I. All the isolates showed one band of approximately 320 bp. A second band of 600 bp appeared in isolates 1315, 1444 and 1506. The isolates 1525–1528 gave a second fragment of 560 bp, isolate ANU had a much shorter second band (420 bp). The isolates 1523 and 1524 showed two additional fragments (600 and 560 bp) which coincided with the second fragments of the two other isolate groups. The double banding could only be detected using maxigels (Fig. 3).

Cycle sequencing of about 360 bp of the IGS1 using primer CNL12 was successfully carried out with the selected isolates (Fig. 4). Sequencing behind bp 360 was only successful in the case of the Australian isolate, where about 500 bp were sequenced. Only

Table 2 Molecular characterization of the intergenic spacer (IGS1) of *P. involutus* isolates. For origin of the isolates see Table 1

Isolate number	Length of PCR product			RFLPs by digestion using *Hae*III				Grouping of isolates
	920 bp	880 bp	740 bp	600 bp	560 bp	420 bp	320 bp	
1315	+			+			+	I
1444	+			+			+	I
1506	+			+			+	I
1523	+	+		+	+		+	II
1524	+	+		+	+		+	II
1525		+			+		+	III
1526		+			+		+	III
1527		+			+		+	III
1528		+			+		+	III
ANU			+			+	+	IV

Fig. 4 Alignment of the sequences of the strains 1315, 1444, 1523, 1526 and ANU obtained by the primer CNL12: The *Hae*III restriction site is shown bold. Identical bases in the compared sequences are shown by * and blanks indicate differences

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                1
1315            ATGTGAATGGAGTTAAGATAGAGCTTGGCT-CGTGAACCAAATCAGGTGGGCTAGGCTAG
1444            ATGTGAATGGAGTTAAGATAGAGCTTTGCT-CGTGAACCAAATCAGGTGGGCTAGGCGAG
1523            ATGTGAATGGAGTTAAGATAGAGCTTTGCT-CGTGAACCAAATCAGGTGGGCTAGGCTAG
1526            ATGTGAATGGAGTTAAGATAGAGCTTTGCT-CGTGAACCAAATCAGGTGGGCTAGGCGAG
ANU             ATGGGAATGGAGTTAAGATAGAGCTTTGCTTCGTGAACCAAATCAGGTGGGGTAGGCTCG
                ****************************** ****************** *****  *
                61
1315            TCGGGCGGAAATGCCTGGCGCGCTCGTCTACGAATTGCAATCATGATATGCGCGGGGGTG
1444            TCGGGCGGAAATGCCTGGCGCGCTCGTCTACGAATTGCAATCATGATATGCGCGGGGGTG
1523            TCGGGCGGAAATGCCTGGCGGGCTCGTCTACGAATTGCAATCATGATATGCGCGGGGGTG
1526            TCGGGCGGAAATGCCTGGCGCGCTCGTCTACGAATTGCAATCATGATATGCGCGGGGGTG
ANU             CTGGGCGGAAATGCCTGGCGGGCTCGTCTACGAATTGCAATCATGATATGCGCGGGGGTG
                  ****************** ****************************************
                121
1315            AATCCTTTGCATACGACTTGAATGGGAACGGGGTACTGTAAGCGGTAGAGTAGCCTTGTT
1444            AATCCTTTGCAGACGACTTGAATGGGAACGGGGTACTGTAAGCGGTAGAGTAGCCTTGTT
1523            AATCCTTTGCAGACGACTTGAATGGGAACGGGGTACTGTAAGCGGTAGAGTAGCCTTGTT
1526            AATCCTTTGCAGACGACTTGAATGGGAACGGGGTACTGTAAGCGGTAGAGTAGCCTTGTT
ANU             AATCCTTTGCAGACGACTTGAATGGGAACGGGGTACTGTAAGCGGTAGAGTAGCCTTGTT
                *********** ********************************************
                181
1315            GCTACGATCCGCTGAGGTTAAGCCCTTGTGCTATANATTTGTTCGACCTTCGAAAGAAGT
1444            GCTACGATCCGCTGAGGTTAAGCCCTTGTTCTATAGATTTGTTCGACCTTCGAAAGAAGT
1523            GCTACGATCCGCTGAGGTTAAGCCCTTGTTCTATAGATTTGTTCGACCTTCGAAAGAAGT
1526            GCTACGATCCGCTGAGGTTAAGCCCTTGTTCTATAGATTTGTTCGACCTTCGAAAGAAGT
ANU             GCTACGATCCGCTGAGGTTAAGCCCTTGTTCTATAGATTTGTTCGACCTTCGAAAGAAGT
                ************************************************************
                241                                             285
1315            CGGGCTTTCTCTTTTCTTTTCTTTCGTCACGCTCTCTGCTTTAG***GGCC***GGTTCATTCGAA
1444            CGGGCTTTCTCTTTTCTTTTCTTTCGTCACGCTCTCTGCTTTAG***GGCC***GGTTCATTCGAA
1523            CGGGCTTTCTCTTTTCTTTTCTTTCGTCACGCTCTCTGCTTTAG***GGCC***GGTTCATTCGAA
1526            CGGGCTTTCTCTTTTCTTTTCTTTCGTCACGCTCTCTGCTTTAG***GGCC***GGTTCATTCGAA
ANU             CGGGCTTTCTCTTTTCTTTTCTTTTGTCACACTCTCTGCTTTAG***GGCC***GGTTCATTCGAA
                ************************ ***** ****************************
                301
1315            TCATTCGAACACGGCACTGCT
1444            TCATTCGAACACGGCACTGCT
1523            TCATTCGAACGCGGCACTGCT
1526            TCATTCGAACACGGCACTGCT
ANU             TCATTCGAACATGGCACTGCT
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very few differences appeared between the isolates. Three 1-bp exchanges were found among the German isolates, and seven 1- or 2-bp exchanges in the Australian isolate ANU. Of the 360-bp sequence, 276 bp were within the highly conserved 25SrRNA, and only the remaining sequence can be considered as specific for *P. involutus* (Fig. 4). Within this region, *Hae*III (GG↓CC; Fig. 4 in bold) produced the 320-bp fragment obtained with all isolates.

Cycle sequencing of the IGS1 using primers 5SA and PAXI yielded identical sequences of about 240 bp with all isolates (not shown). Sequences of isolate ANU obtained with PAXI were aligned to the final sequences obtained by primer CNL12. This means that the complete sequence of the PCR product was obtained, confirming the length of 740 bp for isolate ANU. The other isolates displayed ambiguities following the 240-bp region. Sequences, including the ambiguities, were exactly the same for isolates 1315 and 1444. Therefore, only part of the sequences of isolate 1444 are shown (Fig. 5). A section of 38 bp was lacking in isolate 1526 when aligned with isolate 1444 (Fig. 5). The result corresponds to the approximately 40-bp shorter PCR fragment of isolate 1526 (Table 2). Isolate 1523 could not be sequenced beyond 240 bp using primers 5SA and PAXI. All sequences obtained were transmitted to GenBank and were assigned the accession numbers AF204763–AF204775.

Isolates obtained from fruitbodies collected only a few centimetres apart in the field plots could not be separated on the basis of IGS1 characters. The molecular characters indicate that the fruitbody from which isolate 1444 was obtained was produced by isolate 1315, which was used as inoculum in the nursery in the first year. Likewise, isolate 1506 was obtained from a fruitbody of isolate 1315 or 1444, which were used in a mixed inoculum in the second year. It becomes clear from the IGS1 sequences why *Mbo*I, *Rsa*I and *EcoR*I did not digest the DNA and no RFLPs were obtained with these enzymes.

Fig. 5 Alignment of the sequences of the isolates 1444 and 1526 obtained by the primers 5SA and PAXI revealing the 38-bp gap in isolate 1526. The letters *K*(=G/T), *M*(=A/C), *R*(=A/G), *S*(=G/C), *W*(=A/T) and *Y*(=C/T) indicate two bases found at one position: Identical bases in the compared sequences are shown by * and blanks indicate differences

Survey of the *P. involutus* isolates in the nursery

Survey of mycorrhization of beech and oak plantlets in the nursery plots at the end of the season showed a percentage of mycorrhizal rootlets per plant between zero and more than 70% according to fungal isolate and plant species (Fig. 1). The length polymorphisms of the PCR product and the RFLP patterns obtained by digestion of the IGS1 region with *Hae*III were efficient for following the success or failure of the different isolate groups (I to IV), but not of all the individual isolates. Identification of fungal isolate groups resulted from distinct banding in the agarose gels. Consistently, only one group of isolates was detected in the mycorrhizas on one seedling. Only one group of isolates was found on the five plants collected within a plot. This was also true for mycorrhizas from plots where a mixed inoculum had been applied (Fig. 1).

In most plots, the inoculated isolate colonized the rootlets and persisted during the season (Fig. 1). However, isolate ANU was not detected either in plots where it had been applied alone or in plots of mixed inoculum (Fig. 1). Instead, isolate 1525 of the adjacent plot was found to have invaded the ANU-oak plot, as shown by the RFLP-pattern corresponding to isolate group III. When all 10 isolates were applied as a mixture, isolates of group III were the only colonizers of oak, whereas isolates of group I colonized beech. Isolate group III was also efficient in colonizing the oak control plot. Isolates of group I persisted in the substrate even after fumigation and produced mycorrhizas with both tree species (Fig. 1, 1-year-old peat), while mycorrhization occurred only in the beech plot when the substrate was already 2 years old (Fig. 1).

Although, the IGS1 fragment and RFLP allowed discrimination only of isolate groups, we can conclude from the plots where single isolates were used as inoculum that isolates 1444, 1527 and 1528 were the best colonizers of beech and oak seedlings, followed by isolates 1525 and 1526. Isolates 1523 and 1524 were less successful (Fig. 1). The isolate from Australia and isolate 1315 failed or gave very weak mycorrhization in the fourth year experiment, although isolate 1315 successfully colonized beech and oak and produced fruitbodies in the first year. Isolate 1315 had obviously lost its mycorrhizal potential.

Molecular monitoring of *P. involutus* isolates 1 and 2 years after outplanting of mycorrhizal seedlings to the field

Beech and oak seedlings successfully inoculated with the mixture of isolates 1315, 1444 and ANU during the second year of experiments were planted in an arable plot and a windcast area close to a Norway spruce stand. During two vegetation periods, *P. involutus* mycorrhizas persisted on 30–50% of the rootlets of beech and oak (not shown). Control plants did not become mycorrhizal with *P. involutus*. In the third year, the beech plants lacked *P. involutus* mycorrhizas, while the mycorrhizas were still found on the oak plants. Control plants, although mycorrhizal, still lacked *P. involutus* mycorrhizas. The PCR products of the IGS1 region and the RFLPs obtained with *Hae*III from all investigated *P. involutus* mycorrhizas corresponded to the banding pattern of isolate group I. No other banding patterns were detected (not shown).

Discussion

The molecular techniques used in this research provided an effective means of discriminating between phenotypically indistinguishable mycorrhizas formed by different groups of isolates of *P. involutus*. Persistence, failure and migration of the groups of isolates in the nursery plots could be monitored by the total length and by the RFLP patterns of the IGS1 after digestion with *Hae*III. Partial sequencing of this DNA fragment confirmed the length polymorphisms of the groups of isolates but did not yield further distinguish-

ing characters. Thus, genetically closely linked isolates, like those obtained from fruitbodies produced in the inoculated nursery plots or from neighbouring fruitbodies in the field, could not be discerned. Isolate 1315 and 1444, differing significantly in their mycorrhizal potential in the fourth year of experiments, could not be properly monitored when applied as a mixed inoculum. Probably more variable DNA regions such as IGS2 or microsatellites could be more discriminating. However, the loss of mycorrhizal potential of isolate 1315 might also be due to somatic changes. Isolates from different geographical sites were sufficiently heterogenous in the IGS1 to follow their success in mycorrhization of the beech and oak seedlings, the differences becoming larger with greater distance.

The isolates 1523 and 1524 displayed a double-banded PCR fragment (920/880 bp) and two RFLP fragments of similar length (600/560 bp; Table 2). The isolates may be heterokaryotic, with the length of the two IGS1 bands and the RFLPs corresponding to either isolate group I or III. To test this suggestion, investigation of monokaryotic isolates would be necessary (Selosse et al. 1996, 1998), but this was outside the scope ot this study. The ambiguities hampered sequencing beyond 240 bp of these isolates.

The length of the PCR product and the RFLPs indicated that the fruitbodies collected in the nursery were produced by the inoculum and most probably did not originate from wind-borne indigenous isolates. However, we cannot be totally sure as, so far, we did not investigate the fruitbodies in the nearby forest, which by chance may have the same IGS1 length and RFLPs. The same criticism may apply to the survey of the outplanted seedlings, as we did not investigate mycorrhizas of the surrounding trees. The conclusion that isolate 1315 persisted on the inoculated seedlings is, however, supported by the fact that no *P. involutus* mycorrhizas were found on the control plants. No other mycorrhizal types were found on seedlings in the greenhouse.

The detection of only one isolate group connected to a seedling and within a plot when mixed inocula were applied was rather unexpected. Although we screened only five plants per plot and may have overlooked exceptions, the consistency of this finding with both tree species and the high mycorrhizal rate support the following interpretation: the first isolate becoming symbiotic in a plot by chance is stimulated by the symbiotic state and rapidly spreads over the root system of the seedling and infects other seedlings successively. The most efficient isolate belonged to group III in the case of oak, but to group I in the case of beech. This difference may then be only arbitrary, as the two groups of isolates efficiently formed mycorrhizas with either the tree species.

The failure of mycorrhization by the isolate ANU was unexpected and was only detected by the molecular screening. The Australian isolate ANU was found to be most competitive and efficient in mycorrhization of *Quercus petraea* M. Liebl. and *Q. robur* in northeastern France (Garbaye and Churin 1997). The isolate was obtained from a fruitbody connected to poplar, which is an exotic tree in Australia and thus was most probably introducd by seedlings or cuttings from North America (F. Lapeyrie personal communication). It was recently shown from sequences of the LROR-LR5 region of 28S that the *P. involutus* complex consists of several distinct groups (Bresinsky et al. 1999; Jarosch and Bresinsky 1999 and personal communication). One group contains collections from gardens and parks linked to *Betula, Corylus, Populus, Quercus* and *Tilia.* This group has a close relationship to *P. vernalis* from North America and is well separated from forest samples. These authors also sequenced isolate ANU (LROR-LR5 region of 28S) and found a close relationship to the *P. vernalis* group (personal communication). The result may explain why ANU failed to inoculate our forest beech and oak proveniences.

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