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Molecular and anatomical evidence for a three-way association between *Pinus sylvestris* and the ectomycorrhizal fungi *Suillus bovinus* and *Gomphidius roseus*

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Many intimate associations between different species of ectomycorrhizal fungi are inferred on the basis of the consistent co-occurrence of their fruit bodies. *Suillus bovinus* and *Gomphidius roseus*, where the latter never occurs without the former, is one example. This association was examined with PCR identification and light microscopy. *S. bovinus* and *G. roseus* were unambiguously separated on the basis of RFLPs of the PCR-amplified ITS region of ribosomal DNA. Tuberculate mycorrhizas of *Pinus sylvestris* sampled under fruit bodies of *G. roseus* and *S. bovinus* were investigated and the majority were identified as mixed associations involving both *G. roseus* and *S. bovinus*. Tuberculate mycorrhizas, which macroscopically resemble the ones of *Suillus* species, contained typical chlamydospores of *G. roseus* and they had haustoria where *G. roseus* hyphae penetrated the cortical root cells. Pine seedlings collected near the fruit bodies of the two species were mainly colonised by *S. bovinus*. Mycelial rhizomorphs collected under the fruit bodies of *G. roseus* were identified as *S. bovinus*, while both fungal species were present at the base of *G. roseus* fruit bodies. The significance of these observations and the possibility that *G. roseus* acts as a parasite are discussed.

INTRODUCTION

Some ectomycorrhizal fungi show consistent associations with each other as revealed by co-occurrence of fruit bodies and in mycorrhizal root tips, demonstrated by morphological observations (Brand 1992, Agerer 1992). An association between *Suillus* species and members of the *Gomphidiaceae* has for long been suspected because several *Gomphidiaceae* species only occur where their *Suillus* ‘partner’ is present. Recently studied examples involve *Gomphidius roseus* with *Suillus bovinus*, *Chroogomphus rutilus* with *S. granulatus*, and *C. helveticus* with different suilloid fungi (Agerer 1991). The ‘mixed’ mycorrhizas of these fungi show the typical appearance of mycorrhizas formed by *Suillus* species, but those of *Gomphidiaceae* may form haustoria in the cortical cells of the root (Brand 1992, Agerer 1990, 1991). The genera *Suillus* and *Gomphidius* are now considered to be closely related, both belonging to the group of suilloid fungi (Bruns *et al.* 1998).

Suillus bovinus and *G. roseus* both have narrow host ranges among ectomycorrhizal plants and typically form mycorrhizas with members of the *Pinaceae* (Molina *et al.* 1992). Agerer (1990, 1991) described the morphology of the *S. bovinus*–*G. roseus*–*P. sylvestris* mycorrhiza. PCR-amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA and subsequent digestion with endonucleases is a method

commonly used for the identification of mycobionts in single mycorrhizas (Erland 1995, Gardes & Bruns 1996, Dahlberg *et al.* 1997, Erland *et al.* 1999, Jonsson *et al.* 1999, Mahmood, Finlay & Erland 1999). The ITS-region has proved variable enough to separate most ectomycorrhizal species investigated, but conserved enough not to show much intraspecific variation, at least on a regional level (Kårén *et al.* 1997).

The aims of this study were to: (1) apply PCR-identification to study the interaction between *S. bovinus* and *G. roseus* in field-collected mycorrhizas and fungal rhizomorphs; (2) establish the frequency of detection of a three-way association between *S. bovinus*, *G. roseus* and *P. sylvestris* in mycorrhizas under fruitbodies of the two fungal species; (3) determine the identity of fungal rhizomorphs connected to fruitbodies and mycorrhizas of *S. bovinus* and *G. roseus*; and (4) investigate the anatomy of the three-way association between *S. bovinus*, *G. roseus* and *P. sylvestris*.

MATERIALS AND METHODS

Sampling sites and sampling technique

Roots and fungal material were sampled at three sites for molecular identification (site 1–3) and at one site for microscopic studies (site 4). Sampling was performed on 1–4

different points at each site where *G. roseus* fruit bodies appeared within 10 cm of *S. bovinus* fruit bodies.

Site 1: A moraine-filled path in a *Picea abies* forest with few *Pinus sylvestris* trees in southern Sweden (56° 23' N, 14° 20' E, September 1997).

Site 2: A *P. abies* forest with few *P. sylvestris* in southern Sweden (56° 29' N, 14° 13' E, October 1997) with a ground vegetation of *Vaccinium myrtillus* and *Deschampsia flexuosa*.

Site 3: A moraine-filled path in a mixed *P. abies* and *P. sylvestris* forest in southern Sweden (56° 15' N, 14° 04' E, August 1998).

Site 4: A pure stand of *P. sylvestris* on a dystric cambisol (derived from glacial outwash sediments) in northern Germany with a ground vegetation of *Vaccinium myrtillus* and *Deschampsia flexuosa* (52° 49' N, 13° 50' E, October 1993).

Sample preparation for PCR identification

Soil cores of 10 × 20 × 7.5 cm (width × length × depth), which at the surface contained fruit bodies of *G. roseus* and *S. bovinus*, were collected at sites 1–3 (4 cores at site 1; 4 cores at site 2; 1 core at site 3). The cores were transferred to the laboratory in plastic bags for sampling of mycorrhizal roots, fungal rhizomorphs and fruit bodies under dissecting microscope within 24 h. The soil cores from sites 1 and 3 had a poorly developed organic soil layer and therefore mycorrhizal root tips and rhizomorphs occurred mainly in the mineral soil. In the soil cores from site 2, mycorrhizal root tips and rhizomorphs occurred at the interface between the organic and the mineral soil layers. Samples were rinsed in water and stored frozen (−80 °C) until DNA extraction. Tuberculate mycorrhizas consisting of 5–20 root tips (see Treu 1990) were divided into fragments containing 3–5 root tips that were used for PCR identification.

DNA extraction

DNA was extracted from thawed mycorrhizas, rhizomorphs and fruitbody tissue following the procedures described by Erland *et al.* (1994) with slight modifications. Six hundred µl of extraction buffer (200 mM Tris–HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.2% mercaptoethanol) and 10 µl of proteinase K (10 mg ml^{−1}) were added to samples and incubated for 15 min at 65 ° for cellular lysis. The tissues were crushed with pestles and sand in Eppendorf tubes. The tubes were frozen in liquid nitrogen and thawed at 65 ° three times and then incubated at 65 ° for one hour. The samples were centrifuged for 10 min at 16 000 *g* to remove cell debris. The aqueous phase was extracted first with 500 µl of phenol/chloroform/isoamylalcohol (25/24/1, v/v/v), then with 500 µl of chloroform/isoamylalcohol (24/1, v/v) to remove the residual phenol after centrifugation at 16 000 *g* for 10 min for each extraction. The DNA was precipitated at −20 ° for 1 h in cold ethanol and pelleted by centrifugation at 16 000 *g* for 30 min. The pellets were washed again with 500 µl of 70% ethanol and centrifuged at 16 000 *g* for 10 min. The pellets were left to air dry at room temperature, dissolved in 50 µl Tris-EDTA buffer (1 mM EDTA, 10 mM Tris–HCl; pH 8.0),

and stored at −20 °. The DNA concentration of the extracts was determined spectrophotometrically at 260 nm.

PCR identification

The primers used for PCR were: ITS1 and ITS4 (White *et al.* 1990) to amplify the ITS region of the rDNA. The 50 µl reaction mixture for PCR contained the following final concentrations or total amounts: 500 pmol of each primer, 20 mM Tris (pH 8.3), 200 µM dNTP, 1.5 mM MgCl₂, 50 mM KCl and 2.5 units of Taq polymerase (Perkin–Elmer). To this mixture about 10 ng of DNA from fruitbodies or mycelial rhizomorphs or between 10 and 60 ng of DNA from mycorrhizas was added. PCR-amplification was performed with one cycle of 10 min at 94 °, followed by 35 cycles of 30 s at 94 °, 30 s at 53 °, 1.5 min at 72 ° and one cycle of 10 min at 72 °.

RFLP analysis of the PCR products was carried out with the restriction endonucleases *HinfI* and *TaqI* (Boehringer–Mannheim) by digesting 18 µl of PCR product with 2 µl of enzyme cocktail for 4 h, according to the manufacturers recommendations. The PCR products and their restriction fragments were visualised using 1% agarose + 1% Nusieve agarose gels stained with ethidium bromide.

Microscopical investigation

Tuberculate mycorrhizas from site 4 were fixed for microscopy and then divided into smaller pieces, which were embedded according to Münzenberger, Kottke & Oberwinkler (1992). Semi-thin sections (0.5 µm) of the mycorrhizal root tips were cut on an Ultracut Reichert ultramicrotome. The sections were stained with New Fuchsin–Crystal Violet (solution with 0.3 g New Fuchsin, 0.3 Crystal Violet, 40 ml glycerol and 60 ml sodium tetraborate; Merck) for light microscopy.

RESULTS

PCR amplification and identification of DNA from *S. bovinus* and *G. roseus*

PCR amplification of the ITS-region from fruit bodies of *S. bovinus* and *G. roseus* gave products of 700 bp and 780 bp respectively (Fig. 1). Cleaving with the restriction enzymes *HinfI* and *TaqI*, both gave distinctive RFLP patterns by which the two species could be distinguished (Fig. 1).

When between 5 and 70 ng total genomic DNA from mycorrhizal roots were used for PCR amplification, mixed *G. roseus* and *S. bovinus* DNA was detected over the whole range. DNVA-extracts from fruit bodies of *S. bovinus* and *G. roseus* were mixed in different ratios to get information about the proportions needed for amplification of the ITS region of both species simultaneously. When 50 ng of mixed DNA was used for PCR amplification, DNA of each species could be detected if constituting 1% of the total DNA. When the total amount of DNA was reduced to 5 ng, DNA of *G. roseus* was usually amplified if constituting at least 7% of the mixture and DNA from *S. bovinus* was amplified if constituting at least 4%. This

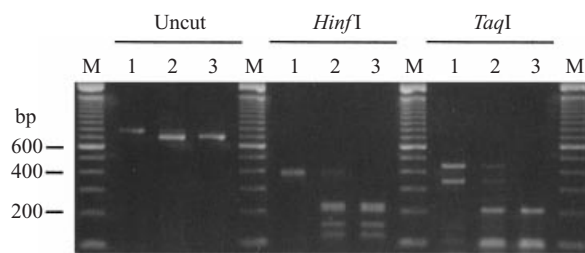


Fig. 1. Agarose gel electrophoresis of PCR amplified ribosomal DNA (ITS region) from 1. fruitbody of *G. roseus*, 2. mycorrhizal root tip containing both *G. roseus* and *S. bovinus* and 3. fruitbody of *S. bovinus*. Bands either show the uncut PCR product or the RFLP pattern after digestion with *HinfI* or *TaqI*.

shows that in a mixture of *G. roseus* and *S. bovinus*, less *S. bovinus* total genomic DNA is needed to amplify the ITS-region of that fungus.

PCR identification of tuberculate mycorrhizas

Pinus sylvestris tuberculate mycorrhizas, which resembled *Suillus* mycorrhizas, were found under the fruitbodies of *G. roseus*. In total, 10 tuberculate mycorrhizas originating from sites 1–3 were used for PCR identification (Table 1). Six of these were identified as three-way associations between *P. sylvestris*, *G. roseus*, and *S. bovinus* (Fig. 1). Three-way associations were found at all three sites investigated. The tubercles consisted of 5–20 Y-shaped mycorrhizal root tips. Three of the tubercles were identified as *S. bovinus* and from one tubercle no DNA could be amplified. From some of the tuberculate mycorrhizal systems with both *S. bovinus* and *G. roseus*, several subsamples from the same tubercle were taken and these consistently showed PCR-products of both fungi.

Microscopical investigation of tuberculate mycorrhizas

Tuberculate mycorrhizas are formed by many root tips and enveloped by a common hyphal mantle. Fungal rhizomorphs originated from between the root tips in the tuberculate

mycorrhizas (Fig. 2). The rhizomorphs had enlarged vessel hyphae surrounded by smaller hyphae. The individual root tips within the tubercles were also enveloped by hyphal mantles, which connected them (Fig. 3).

The hyphae of *G. roseus* within the tuberculate mycorrhizas penetrated the root cell wall and formed haustoria in cortical cells of the outer cortex (Figs 4–7). Several sections indicate that only a few cells of the cortex are infected at the same time. The presence of *G. roseus* was also evident from the occurrence of its characteristic chlamydospores (Agerer 1990). These were present at various depths in the hyphal mantle (Fig. 8).

Pine seedlings

Three one year-old pine seedlings were removed from the soil cores sampled at site 1. The mycorrhizal root tips of these seedlings were subjected to PCR-based identification (Table 1). Two of the seedlings were mainly colonized by *S. bovinus* according to PCR identification. One of these seedlings had small tuberculate mycorrhizas consisting of 2 to 6 root tips. DNA was successfully amplified from 7 out of 12 of these tubercles of which five were *S. bovinus*, one a mixture of *S. bovinus* and *G. roseus* and one was unidentified. Seven root tips with *S. bovinus* were identified in the second seedling out of 15 single mycorrhizas. DNA amplification from the third seedling was only successful for one out of 17 root tips, which showed the RFLP pattern of *S. bovinus*. Rhizomorphs connected to the seedlings were always identified as *S. bovinus* when DNA amplification was successful (Table 1).

Mycelial rhizomorphs in soil

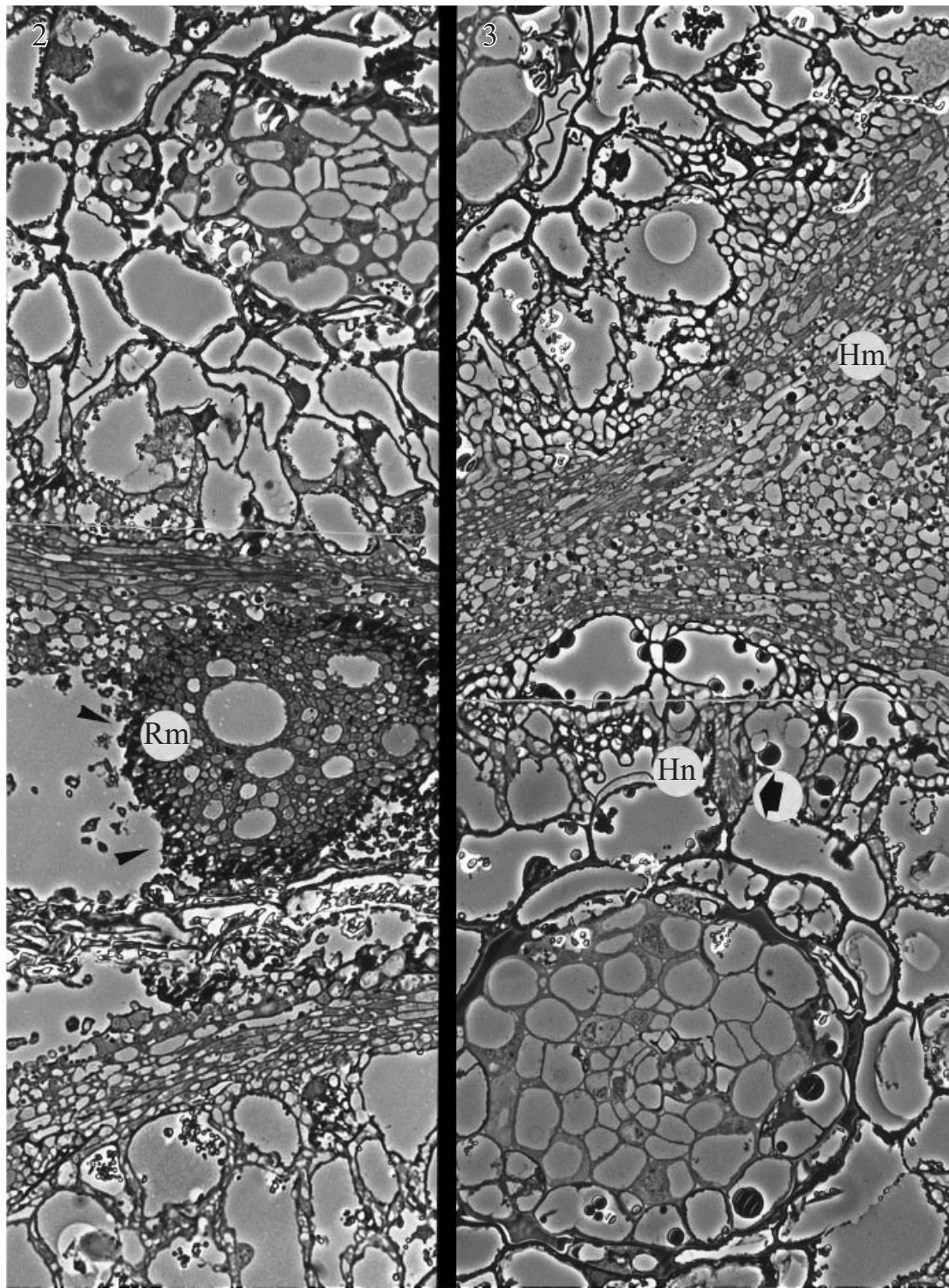
White rhizomorphs were commonly found in close proximity to the fruit bodies of *S. bovinus* and *G. roseus*. All rhizomorphs from which DNA was successfully amplified were identified as *S. bovinus* (Table 1). *S. bovinus* rhizomorphs were found under *G. roseus* at all three sites. One rhizomorph also contained DNA from an unidentified fungal species. DNA of *G. roseus* was not detected in any of the rhizomorphs.

Mycelium attached to the base of *G. roseus* fruit bodies

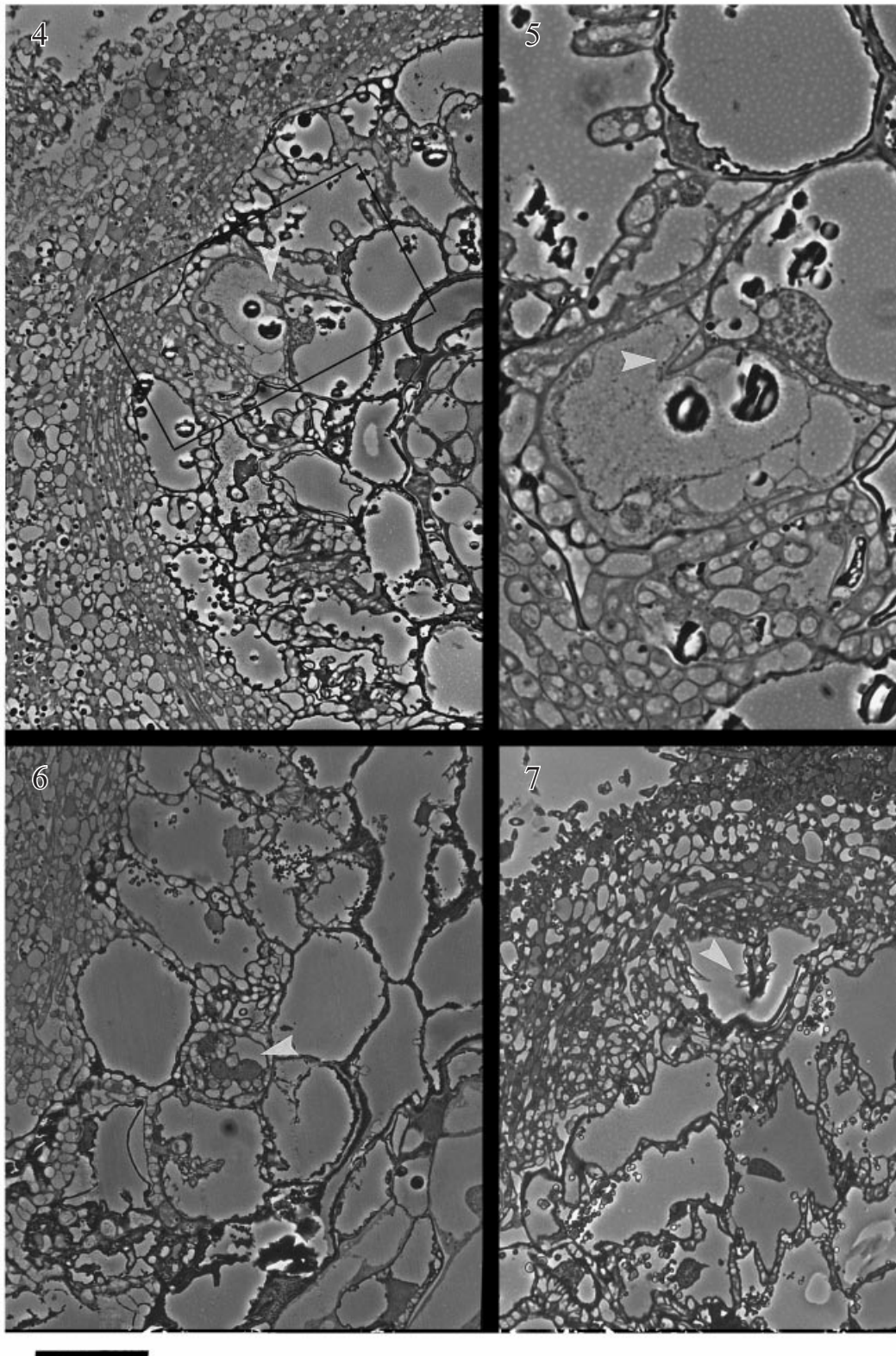
Table 1. PCR-identification of samples of root and mycelium collected under fruit bodies of *S. bovinus* and *G. roseus* at three sites in southern Sweden. The total number of samples analyzed is given and the identity of material where PCR amplification was positive is indicated. Mixed associations refer to samples where both *S. bovinus* and *G. roseus* were identified.

Type of sample	Total number	Mixed association	<i>Suillus bovinus</i>	Unidentified
Mature pine trees				
Tuberculate mycorrhizas	10	6	3	—
Rhizomorphs connected to tuberculate mycorrhizas	2	—	1	—
One-year old pine seedlings				
Mycorrhizal root tips	44	1	13	1
Rhizomorphs	12	—	3	—
Rhizomorphs under fruit bodies				
At the base of <i>G. roseus</i> fruit bodies	4	4	—	—
Under <i>G. roseus</i>	20	—	6	1*
Under <i>S. bovinus</i>	6	—	2	—

* Detected on the same root tip as *S. bovinus*.



Figs 2–3. Section through a *Pinus sylvestris*–*Suillus bovinus*–*Gomphidius roseus* tuberculate mycorrhiza. **Fig. 2.** A differentiated rhizomorph can be seen (Rm, arrows) between two mycorrhizal tips; note the randomly distributed enlarged hyphae. **Fig. 3.** Two mycorrhizal root tips connected by a thick hyphal mantle (Hm) containing numerous vacuolar bodies. The Hartig net (Hn) is well established showing finger-like structures (arrow). Bar = 50 µm.



Figs 4–7. Section through a tuberculate mycorrhizal system of *Pinus sylvestris*–*Suillus bovinus*–*Gomphidius roseus*. **Fig. 4.** A penetrating intracellular hypha of *Gomphidius roseus* in an outer cortical cell (arrow). Bar = 50 μ m. **Fig. 5.** Enlarged section of the penetrated cortical cell (represented as a rectangle in Fig. 4); the invading hypha (arrow) surrounded by the tonoplast of the vacuole is visible. Bar = 20 μ m. **Fig. 6.** A cortical cell filled with intracellular hyphae (arrow); note the irregularly shaped nucleus. Bar = 50 μ m. **Fig. 7.** Another cortical cell showing a haustorium of *Gomphidius roseus*. Bar = 50 μ m.

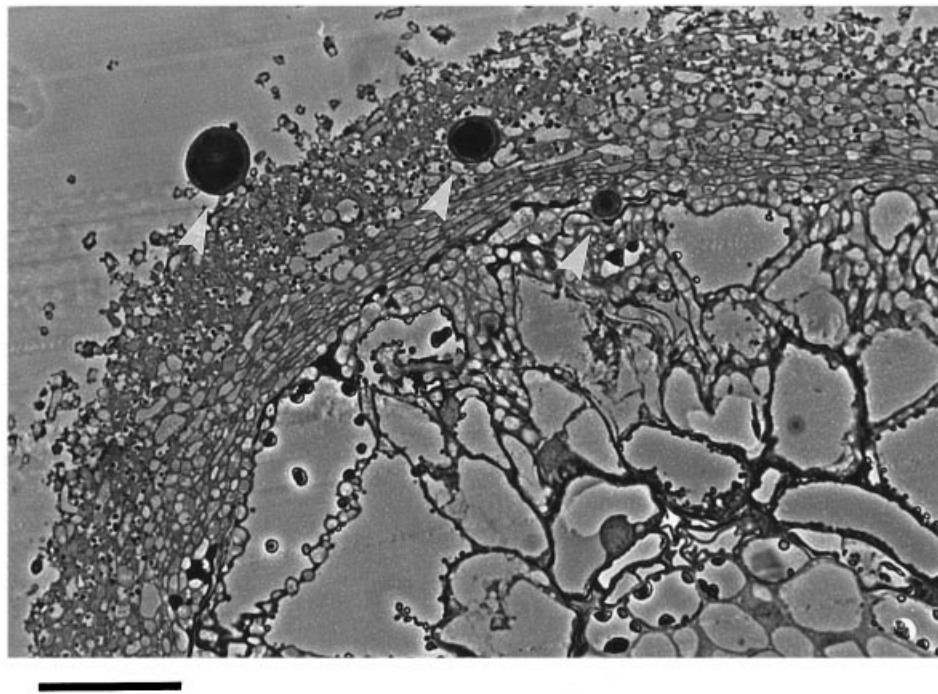


Fig. 8. Section through a mycorrhizal root tip of a tuberculate mycorrhizal system of *Pinus sylvestris*–*Suillus bovinus*–*Gomphidius roseus* revealing chlamydospores of *Gomphidius roseus* of different age (arrows) embedded in the hyphal mantle. Bar = 50 µm.

produced both *G. roseus* and *S. bovinus* PCR products (Table 1).

DISCUSSION

Here we show that the tuberculate mycorrhizal system is an important structure in the interaction between the ectomycorrhizal fungi *S. bovinus* and *G. roseus*. Tuberculate *P. sylvestris* mycorrhizas were analyzed from four different sites and interactions were detected at all sites. Chlamydospores and haustoria were identified as structures of *G. roseus* inside tuberculate mycorrhizas of *S. bovinus* (see also Agerer 1990, 1991), which otherwise show the morphological structure of *Suillus* mycorrhizas. *S. bovinus* colonization was also present on *P. sylvestris* seedlings near the fruit bodies sampled, but here, a mixed association was only observed once.

The distribution of *S. bovinus* was successfully tracked by PCR identification and the fungus was present in the mycorrhizas, in the rhizomorphs originating from the mycorrhizas, and rhizomorphs under fruit bodies of both fungal species. The presence of *G. roseus* was only detected in the *S. bovinus* mycorrhizas and as fruit bodies. Similarly, Agerer (1990) could not identify *G. roseus* in rhizomorphs connected to mycorrhizas with both species. However, since *S. bovinus* was present on the stipe of *G. roseus* fruit bodies, it is likely that *G. roseus* hyphae are present in, or connected to, *S. bovinus* rhizomorphs and initiate fruit body formation from these. If present in an amount lower than 7% of the total fungal DNA, *G. roseus* DNA would not be detected by our method, as indicated from the results where the proportions needed for amplification of both species simultaneously were tested. In an interaction that may be similar to the one described here, Agerer (1991) found rhizomorphs of an

unidentified *Suillus* species attached to the fruit bodies of *Gomphidius glutinosus*. Agerer (1991) also found *Rhizopogon* hyphae in the stipe of *G. glutinosus* and concluded that *Rhizopogon* can grow into the stipe of this fungus. Another explanation could be that the *G. glutinosus* fruit bodies are formed from hyphae associated with *Rhizopogon* rhizomorphs and that these are included in the fruit body by a purely physical interaction.

The test of PCR amplification of *in vitro* mixed DNA from *S. bovinus* and *G. roseus* indicates that the amount of *G. roseus* DNA in the tuberculate mycorrhizas may have been considerable. According to our results, over 7% of the DNA should have been of *G. roseus* origin for amplification to occur. In the mycorrhizas, plant DNA as well as substances inhibiting the PCR reaction may also have influenced the results, making the detection of both species at low concentration more difficult. In some cases the PCR products of *G. roseus* were of equal brightness to the PCR products of *S. bovinus*, whilst in other cases those of *S. bovinus* were brighter (see Fig. 1). Chlamydospores may make a major contribution to the DNA of *G. roseus* in the mycorrhizas. The fact that *G. roseus* was not detected in rhizomorphs except at the base of its fruitbodies in this study, indicates that if present in the rhizomorphs they have a low biomass. Hyphae of *G. roseus* (which are amyloid in contrast to *Suillus* hyphae) were not found in the rhizomorphs of *S. bovinus* in microscopical investigations (Agerer 1990).

Our finding that in soil *G. roseus* never occurred without *S. bovinus*, while *S. bovinus* often occurred alone, suggests that *S. bovinus* is a host for *G. roseus*. Agerer (1991) found that *G. roseus* could not be isolated unless co-cultivated with *S. bovinus*, but once established on the growth medium was it possible to grow pure cultures of the fungus. Mycorrhizas of

G. roseus have been observed but they seem to be very infrequent (Agerer 1994). The close association of *G. roseus* with *S. bovinus*, the formation of haustoria in the plant root cells and the observation that *G. roseus* does not seem to form an extensive soil mycelium, suggests that *G. roseus* acts as a parasite on either the fungal host, the plant host, or both. Further studies of these species in experimental systems may reveal their true functional relationships.

In studies where PCR identification of mycorrhizas is applied to study community structure of ectomycorrhizal fungi, it is relatively common that two PCR products are yielded from the same mycorrhiza. In some studies up to 20% of root tips are colonized by two fungi (Erland 1995, Erland *et al.* 1999). This may be due to a succession of fungal species or to competition between different fungi to colonize the root. It may also be due to close associations between species such as described here for *S. bovinus* and *G. roseus*. Suilloid fungi are very rarely detected on mycorrhizal roots in community structure studies even when their fruit bodies are abundantly present (Gardes & Bruns 1996). It is therefore difficult to obtain information from the literature on how often mycorrhizas formed by *Suillus* species are associated with other fungi. Future studies focusing on mycorrhizas connected to fruit bodies may reveal new types of close interactions between species of ectomycorrhizal fungi.

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