Morphology and molecular diversity of arbuscular mycorrhizal fungi in wild and cultivated yew (*Taxus baccata*)

Tesfaye Wubet, Michael Weiß, Ingrid Kottke, and Franz Oberwinkler

Abstract: *Taxus baccata* L. roots collected from two sites in southern Germany were heavily colonized by arbuscular mycorrhizal fungi (AMF). The colonization pattern was of the *Paris* type. The diversity of the colonizing AMF species was investigated using polymerase chain reaction based molecular techniques. The internal transcribed spacer (ITS) region of the DNA from AMF within the roots was amplified using Glomeromycota-specific primers and then cloned and sequenced. Phylogenetic analysis using a data set of 5.8S rDNA sequences from a wide range of glomeralean taxa as well as data sets of partial ITS2 sequences from glomeralean subgroups indicated root colonization by four sequence types of *Glomus* and one sequence type of *Archaeospora*. These sequence types are distinct from any previously published sequences and differed between the two study sites.

Key words: arbuscular mycorrhiza, *Paris*-type AM, molecular diversity, ribosomal internal transcribed spacers, *Taxus baccata*.

Résumé : Des racines de *Taxus baccata* L. récoltées sur deux sites du sud de l'Allemagne, montrent une forte colonisation par des champignons arbusculaires mycorhiziens (AMF), formant une association de type *Paris*. Les auteurs ont examiné la diversité des espèces fongiques (AMF) colonisant ces racines, en utilisant des techniques moléculaires basées sur le PCR. Ils ont amplifié la région de l'espaceur interne transcrit (ITS) de l'ADN des AMF dans les racines, en utilisant des amorces spécifiques de Glomeromycota, avant de les cloner et de les séquencer. L'analyse phylogénétique, à l'aide d'un ensemble de données sur les séquences du rADN 5.8S provenant d'un large ensemble de taxons glomératéens, ainsi que d'un ensemble de données sur les séquences partielles de l'ITS2 provenant de sous groupes glomératéens, indiquent que la colonisation racinaire provient de quatre types de séquences de *Glomus,* et une séquence d'*Archaespora*. Ces types de séquences se distinguent de toutes les séquences déjà publiées, et diffèrent entre les deux sites d'étude.

Mots clés : mycorhize arbusculaire, AM de type *Paris*, diversité moléculaire, espaceurs ribosomiques internes transcrits, *Taxus baccata*.

[Traduit par la Rédaction]

Introduction

Arbuscular mycorrhizal fungi (AMF) form intimate symbiotic associations with most land plants (Smith and Read 1997). Recently, they have been separated from the polyphyletic Zygomycota and placed in a new monophyletic phylum, Glomeromycota (Schüßler et al. 2001). Identification of AMF is usually based on the morphology of multinucleate spores and related structures formed in the soil. However, because spores are usually produced on external hyphae that are easily detached from the root, it may be difficult to associate spores from a given taxon with the host plant under study. Spore production, which may be seasonal, is also highly dependent on soil physiological parameters and as a result, certain taxa, which may be functionally important, may be missed. Furthermore, the morphology and wall structures of spores vary at different stages of development (Clapp et al. 1995; Merryweather and Fitter 1998; Morton 1985; Walker and Trappe 1993). These restrictions make the identification of AMF colonizing certain plant hosts difficult to achieve with field-collected material.

There is increasing evidence that plant biodiversity and productivity in ecosystems are significantly influenced by AMF diversity (van der Heijden et al. 1998). It is also known that different AMF can simultaneously colonize a single root segment (Merryweather and Fitter 1998). Since AMF are obligate and functionally diverse symbionts, the fungi colonizing roots of a specific host are likely to have the most relevance to function (Helgason et al. 2002). In an attempt to determine the identity and diversity of AMF within roots, there is increasing interest in the use of molecular-based tools (Gadkar et al. 1997; Hahn et al. 1993; Lanfranco et al. 1995; Rosendahl 1989; Zeze et al. 1997).

Received 3 July 2002. Published on the NRC Research Press Web site at http://canjbot.nrc.ca on 7 April 2003.

T. Wubet,[1] M. Weiß, I. Kottke, and F. Oberwinkler. Universität Tübingen, Botanisches Institut, Spezielle Botanik and Mykologie, Auf der Morgenstelle 1, D-72076, Tübingen, Germany.

[1]Corresponding author (e-mail: wubet_mitiku@hotmail.com).

 doi: 10.1139/B03-020

Recent studies have used polymerase chain reaction (PCR) techniques coupled with isolate- or group-specific primers (Abbas et al. 1996; Clapp et al. 1995, 1999; Helgason et al. 1999; Millner et al. 2001; Redecker 2000; van Tuinen et al. 1998).

Nested PCR procedures with species-specific primers have been successfully used on material from pot cultures of known fungal species (Abbas et al. 1996; Chelius and Triplett 1999; Jacquot et al. 2000; Millner et al. 2001; van Tuinen et al. 1998) and also on field-collected material (Clapp et al. 1999; Daniell et al. 2001; Helgason et al. 1999; Kjøller and Rosendahl 2001; Turnau et al. 2001). However, most of the specific primers suggested and used in the study of AMF diversity in plant roots suffered from amplification of one glomeralean subgroup only and are therefore of restricted applicability (Clapp et al. 1999; Daniell et al. 2001; Millner et al. 2001; van Tuinen et al. 1998). The primer VANS1 (Simon et al. 1992) and the family-specific primers VAGIGA, VAGLO, and VAACAU (Simon et al. 1993) are apparently only applicable to a subgroup of AMF (Schüßler et al. 2001). The primer AM1 that was designed to be specific to Glomeromycota (Helgason et al. 1999) apparently excludes a number of fungal types from the recently recognized ancestral groups of the Archaeosporaceae and Paraglomeraceae (Daniell et al. 2001; Morton and Redecker 2001; Redecker et al. 2000*a*; Schwarzott and Schüßler 2001). Certain regions of the nuclear rDNA coding for the large ribosomal subunit proved to be useful in a single-strand conformation polymorphism protocol (Kjøller and Rosendahl 2000), but only one glomeralean clade comprising *Glomus caledonium*, *Glomus coronatum*, *Glomus geosporum*, and *Glomus mosseae* can be analyzed at present. Redecker (2000) has reported specific primers to amplify the internal transcribed spacer (ITS) region of six clades (Redecker et al. 2000*a*, 2000*b*). The fact that only the *Glomus versiforme* clade is neglected and that the origin of a given sequence can be verified using the 5.8S rDNA data set (Redecker et al. 1999) renders these an advantage over other currently available primers that have been suggested for the amplification of specific regions of AMF rDNA from colonized roots to study AMF molecular diversity.

The presence of taxol, an anticancer drug, in the bark and the leaves of *Taxus baccata* L. has generated recent interest in this and other species in the genus *Taxus* (Joyce 1993). Large-scale propagation of *Taxus* spp. is usually by cuttings, as germination is slow. The relatively few studies of the mycorrhizal status of *T. baccata* roots indicate that the species is arbuscular mycorrhizal (Bakashi and Thapar 1960; Khan 1971; Prat 1926; Strullu 1978; von Tubeuf 1896). However, there is no detailed description of the morphological features of the symbiosis. Enhanced growth and survival of rooted *T. baccata* cuttings following inoculation with AMF has been reported (Sainz et al. 2000). Inoculation programs may be further improved by using fungal species occurring in the roots of wild *Taxus* plants, but information about these species is lacking. Therfore, the aim of this study was to illustrate morphological characteristics of *T. baccata* mycorrhizas and to investigate the molecular diversity of AMF species in roots of *T. baccata* growing in southern Germany.

Materials and methods

Root sampling

Between October and November 2000, roots of *T. baccata* were sampled from two sites in southern Germany: a cultivated slightly acidic loam soil (pH = 6.0 and soil available P range of 16–30 ppm) at Tübingen (48°32′N, 9°03′E) and a mixed *Picea abies* – *T. baccata* forest growing on limestone soil (pH = 7.2 and soil available P range of 9–15.6 ppm) at Balingen (48°17′N, 8°49′E). At both study sites, five single standing plants were randomly selected and fine roots were excavated starting from the trunk and working out towards the fine roots. Roots were then washed under running tap water to remove attached soil and debris. Subsamples were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 1 h under vacuum on ice and alternatively in 50% ethanol and kept at 4°C for subsequent microscopical studies. Additional subsamples were kept at –20°C in 1.5-mL reaction cups for molecular studies.

Root preparation and microscopy

Ethanol-fixed roots were cut into short segments (approximately 1 cm) and stained following the modified procedure of Grace and Stribley (1991). Briefly, roots were treated with 10% KOH (2 h, 60°C), rinsed with distilled water, and treated with 10% HCl (v/v, 2 min, 25°C) before staining with 0.05% aniline blue in lactic acid (3 h, 60°C). Excess stain was removed during storage of the roots in lactic acid prior to light microscopic analysis. From every individual sample, five slides, each with five to six randomly selected stained roots (approximately 25–30 1-cm-long roots), were examined with a compound microscope for the presence of arbuscular mycorrhizal structures. Root samples fixed in 2% glutaraldehyde in 0.2 M phosphate buffer were postfixed with 1% OsO_4 in 0.2 M phosphate buffer (pH 7.2) for 1 h in the dark. The root tissues were then dehydrated for 10 min in each of a 10, 25, 50, 70, 85, 95, and 100% acetone solution followed by three 10-min treatments in 100% acetone. Samples were then embedded in Spurr's (1969) ERL using four infiltration steps done at 25°C: ERL–acetone in a ratio of 1:2 (overnight), 1:1 (30 min), 2:1 (30 min), and 1:0 (overnight). Semithin sections (70 µm) were stained with neofuchsin-crystal violet. Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate (Schmid and Oberwinkler 1996).

DNA isolation from AMF-colonized roots

Root samples (1–2 cm) were air dried and placed in 1.5-mL reaction tubes together with a tungsten carbide ball (3 mm) and ground (3 min, 13 000 rpm) using a mixer mill (MM 300; Retsch, Haan, Germany). DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol for the isolation of DNA from plant tissues. However, only one elution step with 100 µL of the elution buffer was used.

Nested PCR amplification

The target region for the PCR experiments was the ITS region of the nuclear rDNA including the pseudogene coding for the 5.8S ribosomal subunit. The first fungal DNA ampli-

fication was performed using the universal primer pair NS5 and ITS4 (White et al. 1990). DNA was amplified in a 50-μL reaction volume containing 5 μL of 10× PCR reaction buffer, concentrations of 0.2 mM of each dNTP and 0.5 μM of each primer, 2.5 units of *Taq* polymerase, and 5 μL of DNA template. The PCRs were done using Gene-Amp PCR System 2400 (Perkin-Elmer Corporation, Boston, Mass.) as described by Redecker (2000): after a hot start at 60°C, an initial denaturation of 3 min at 95°C was followed by five cycles of 30 s at 95°C, 30 s at 52°C, and 1.5 min at 72°C. Thereafter, 30 cycles were performed with an annealing temperature of 51°C followed by a final extension period of 7 min at 72°C. A 5-μL aliquot of each PCR product was analyzed on a 1.5% agarose gel followed by ethidium bromide (0.5 μg/mL) staining and photographed under UV light. A 1- to 3-μL aliquot was then used as template for the nested PCR amplification using the specific primers ACAU1660, ARCH1311, GIGA5.8R, GLOM1310, and LETC1670 and PCR conditions reported by Redecker (2000). The nested PCR products were then analyzed as outlined above.

Cloning and sequencing

Nested PCR products were purified following the QIAquick protocol (QIAGEN). Cloning was done using the pCR 2.1-TOPO vector system (Invitrogen, Breda, Netherlands) according to the manufacturer's instruction. White colonies (10–20) were then transferred to 2.2-mL reaction tubes with 500 μL of Lauria-Bertani (LB) selective medium containing kanamycin (50 μg/mL)and incubated overnight on a rotary shaker (150 rpm) at 37°C. Finally, 5 μL of the solution containing the transformed cells was used in a PCR with the primers and conditions of the nested PCR described above. Amplified PCR products were then purified and used in cycle sequencing with the PCR primers as sequencing primers. Cycle sequencing was conducted using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, Great Britain) according to the manufacturer's protocol except that reaction volumes were reduced by half and the kit was diluted 1:1 (v/v) with double-distilled water. Electrophoresis and data sampling was performed on an automated sequencer (ABI 373A Stretch; Applied Biosystems, Foster City, Calif.). Sequence editing was done using the program Sequencher™ version 3.1.1 (Gene Codes Corporation, Ann Arbor, Mich.). The sequences have been deposited at the National Center for Biotechnology Information (NCBI), GenBank (http://www. ncbi.nlm.nih.gov) under accession Nos. AY174685-AY174716.

Phylogenetic analysis

Sequence similarities were determined using the BLAST sequence similarity search tool (Altschul et al. 1997) provided by GenBank. Sequences were aligned with other published glomeralean sequences using the program ClustalX (Thompson et al. 1997) and further visual alignment was done in Se-Al version 2.03*a* (Rambaut 1996). GenBank accession numbers of sequences included in the phylogenetic analysis are given in Table 1.

Phylogenetic relationships were estimated with a Bayesian approach using Monte Carlo Markov chains (MCMC) as implemented in the computer program MrBayes (Huelsenbeck and Ronquist 2001). MCMC of trees are constructed that, after stationarity is reached, can be used to approximate the a posteriori probability that groups of taxa are monophyletic given the DNA alignment (i.e., the probability that corresponding bipartitions of the species set are present in the true unrooted tree including the given species). The power of this method to efficiently reconstruct phylogenetic relationships has been recently demonstrated (e.g., Murphy et al. (2001) for mammalian phylogeny; Maier et al. (2003) and Urban et al. (2003) for basidiomycetes subgroups). Four incrementally heated simultaneous MCMC were run over 1 000 000 generations using the general time-reversible model of DNA substitution with gamma distributed substitution rates (see Swofford et al. 1996), random starting trees, and default starting parameters of the DNA substitution model. Trees were sampled every 100 generations giving a total of 10 000 trees. From those trees that were sampled after the process had reached stationarity, a 50% majority rule consensus tree (including also compatible groups of lower frequencies) was computed to obtain estimates for the a posteriori probabilities. Branch lengths represent average values obtained from the sampled trees. To test the reproducibility of the results, computations were repeated five times using random starting trees and default starting values for the model parameters.

Results

Microscopic analysis

Initial AMF colonization of *T. baccata* roots was via a simple appressorium (Fig. 1*a*). Once within the roots, the hyphae passed through the epidermis and formed hyphal coils in the outer cortical cells (Fig. 1*b*). The fungus then advanced longitudinally and laterally within the cortex via intracellular hyphae. Hyphae either became noticeably narrower or remained unchanged (Fig. 1*c*) as they passed through the walls of contiguous cortical cells. Two major types of arbuscules were observed: either developing from hyphal coils (Fig. 1*d*) or arising from extensions of linear intracellular hyphae (Fig. 1*c*). Arbuscules were distributed in both the inner and outer cortical cells (Figs. 1*e* and 1*f*). Intercellular hyphae were completely absent. Intracellular spores (Fig. 1*g*) were frequently observed, while vesicles were rare or absent. Although variations in the level of colonization of examined root pieces were observed, the degree of colonization was above 76% at both studied sites.

Molecular analysis

A total of 32 sequences from the ITS region were obtained and analyzed using BLAST searches of the NCBI to reveal similarities with published sequences. Although the closest matches belonged to the Glomeromycota, in most cases they were only distantly related to the sequences obtained during this study.

A phylogenetic tree was constructed to illustrate the overall molecular diversity of AMF associated with *T. baccata* and to estimate the phylogenetic relationship between the sequences obtained in this study and those of named AMF. We restricted this analysis to 5.8S rDNA sequences from

Table 1. GenBank accession numbers for sequences included in the phylogenetic analysis.

Species	Accession No.	Species	Accession No.
Acaulospora denticulata	AJ239115	*Glomus etunicatum* 1	AF004683
Acaulospora laevis	AJ242499	*Glomus etunicatum* 2	GEU94712
Acaulospora mellea	AJ239116	*Glomus etunicatum* 3	GEU94711
Acaulospora morrowiae	AJ242500	*Glomus fasciculatum* 1	GFASMAB
Archeaospora trappei 1	AJ243419	*Glomus fasciculatum* 2	GFASMC
Archaeospora trappei 2	AJ243420	*Glomus geosporum*	AF413088
Archaeospora leptoticha	AB048681	*Glomus luteum* 1	AY035655
Endogone pisiformis	AF006509	*Glomus luteum* 2	AY035653
Entrophospora infrequens 1	U94714	*Glomus monosporum*	AF125195
Entrophospora infrequens 2	U94713	*Glomus mosseae* 1	GMFIN1A
Gigaspora albida	AF004707	*Glomus mosseae* 2	GMFIN2A
Gigaspora decipiens	AJ239119	*Glomus mosseae* 3	GMFIN2B
Gigaspora gigantea	AJ410752	*Glomus intraradices* 1	AF394777
Gigaspora rosea	AJ410749	*Glomus intraradices* 2	AF394754
Glomus clarum 1	GCL239123B	*Glomus intraradices* 3	AF394757
Glomus clarum 2	GCL243275	*Glomus intraradices* 4	AF394780
Glomus caledonium 1	AY035647	*Glomus coronatum* 1	GCOR1A
Glomus caledonium 2	AY035642	*Glomus coronatum* 2	GCOR1C
Glomus caledonium 3	AY035651	*Paraglomus brasilianum*	AF165920
Glomus claroideum 1	AY035649	*Paraglomus occultum*	AF004675
Glomus claroideum 2	AY035656	*Scutellospora castanea*	AJ00287
Glomus dimorphicum 1	GMDIM2B	*Scutellospora heterogama*	AF004692
Glomus dimorphicum 2	GMDIM1B	*Scutellospora pellucida*	AY035663
Glomus dimorphicum 3	GMDIM1A	*Scutellospora persica*	AJ410740

representative sequences obtained during this study, the corresponding closest matches from GenBank, and sequences representing the seven AMF genera (i.e., *Acaulospora*, *Archaeospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Paraglomus*, and *Scutellospora*).

Repeated Bayesian phylogenetic analysis yielded consistent tree topologies and a posteriori probabilities. Stationarity of the Markov chains was reached before 1000 trees had been sampled. Thus, the first 1000 trees were discarded and the remaining 9000 trees were included in the 50% majority rule consensus tree of each run. One of these trees is presented in Fig. 2. The AMF sequences from *T. baccata* represented five different 5.8S rDNA sequence groups of glomeralean fungi, four belonging to *Glomus* (grouped into *Glomus* I, *Glomus* II, and *Glomus* III) and one belonging to *Archaeospora*.

To get a more detailed view of the phylogenetic position of these sequences within the glomeralean subgroups supported by the 5.8S rDNA tree, phylogenetic analysis based on the 5.8S and parts of the ITS2 regions of the groups representing *Glomus* I, *Glomus* II, and *Glomus* III (supported by 95, 62, and 100% a posteriori probabilities, respectively (Fig. 2)) was carried out after removal of ambiguous positions in the alignment. In addition to those GenBank sequences that had been used in the generation of the 5.8S rDNA tree, further representatives of homogenous groups were included. The 5.8S/ITS2 tree (Fig. 3) demonstrated that the *Glomus* sequence types Taxusb-I1 (represented by clones 0502, 0504, and 0508) and Taxusb-I2 (represented by clones 0161 and 0171) obtained from mycorrhizal *T. baccata* roots are distinctly different from those of related AMF sequences of *Glomus intraradices* and *Glomus clarum* in the database. The *Glomus* sequence type Taxusb-II was also found to be different from the related *Glomus* sequences from the database (Fig. 4). The 5.8S/ITS2 analysis also showed that the *Glomus* sequence type Taxusb-III (represented by clones 021, 023, 024, and 028) is markedly different from the closely related *Glomus luteum*, *Glomus etunicatum*, and *Glomus claroideum* sequences from the database. Three of the *Glomus* sequence types (Taxusb-I1, Taxusb-I2, and Taxusb-II) were amplified from roots collected from the cultivated site (Tübingen), and one *Glomus* sequence type (Taxusb-III) and the *Archaeospora* sequence type (Taxusb-IV) were amplified from roots collected from the forest site (Balingen) (Table 2).

Discussion

Morphological data

The microscopic analysis revealed a high level of *Paris*-type (Gallaud 1905) AMF colonization of roots of *T. baccata* from both study sites, which supports a number of previous reports (Bakashi and Thapar 1960; Khan 1971; Prat 1926; Strullu 1978; von Tubeuf 1896). The recent finding that AMF species affect the morphology of arbuscular mycorrhizas (Cavagnaro et al. 2001) suggested that the observed *Paris*-type AMF colonization is due to the AMF species amplified from the colonized roots of *T. baccata*. The constriction of hyphae upon passage through the cell walls has also been documented (Cooke et al. 1993; Yawney and Schultz 1990; Whitbread et al. 1996). Yawney and Schultz (1990) also observed large intracellular hyphae passing through cortical cells unchanged. Cooke et al. (1993) suggested that this variation in morphology might be AMF species dependent. Following this hypothesis, the observation of unchanged and constricted traversing hyphae would indicate

Fig. 1. Light and transition electron micrographs of morphological characteristics of arbuscular mycorrhizal *Taxus baccata* roots. AP, appressorium; HC, hyphal coil; AC, arbuscular coil; AR, branched arbuscules; SP, spore; H, hypha. (*a*) Light micrograph of simple appressorium (AP) formed on the root epidermis. Scale bar = 10 µm. (*b*) Light micrograph of intracellular hyphal coil (HC) formed in cortical cell. Scale bar = 10 µm. (*c*) Light micrograph of arbuscules (AR) on a linear intracellular hypha. Note the constriction of the hypha traversing the host cell wall (arrowheads). Scale bar = 25 µm. (*d*) Light micrograph of an arbusculate coil (AC). Scale bar = 10 µm. (*e*) Light micrograph showing a longitudinal semithin section through an arbuscular mycorrhizal *Taxus baccata* root. Note the high level of colonization in the inner cortical region (arrowheads). Scale bar = 50 µm. (*f*) Transmission electron micrograph showing arbuscules and hyphae (H) in cells of mycorrhizal *Taxus baccata* roots. Scale bar = 9 µm. (*g*) Light micrograph of intracellular spores (SP). Scale bar = 25 µm.

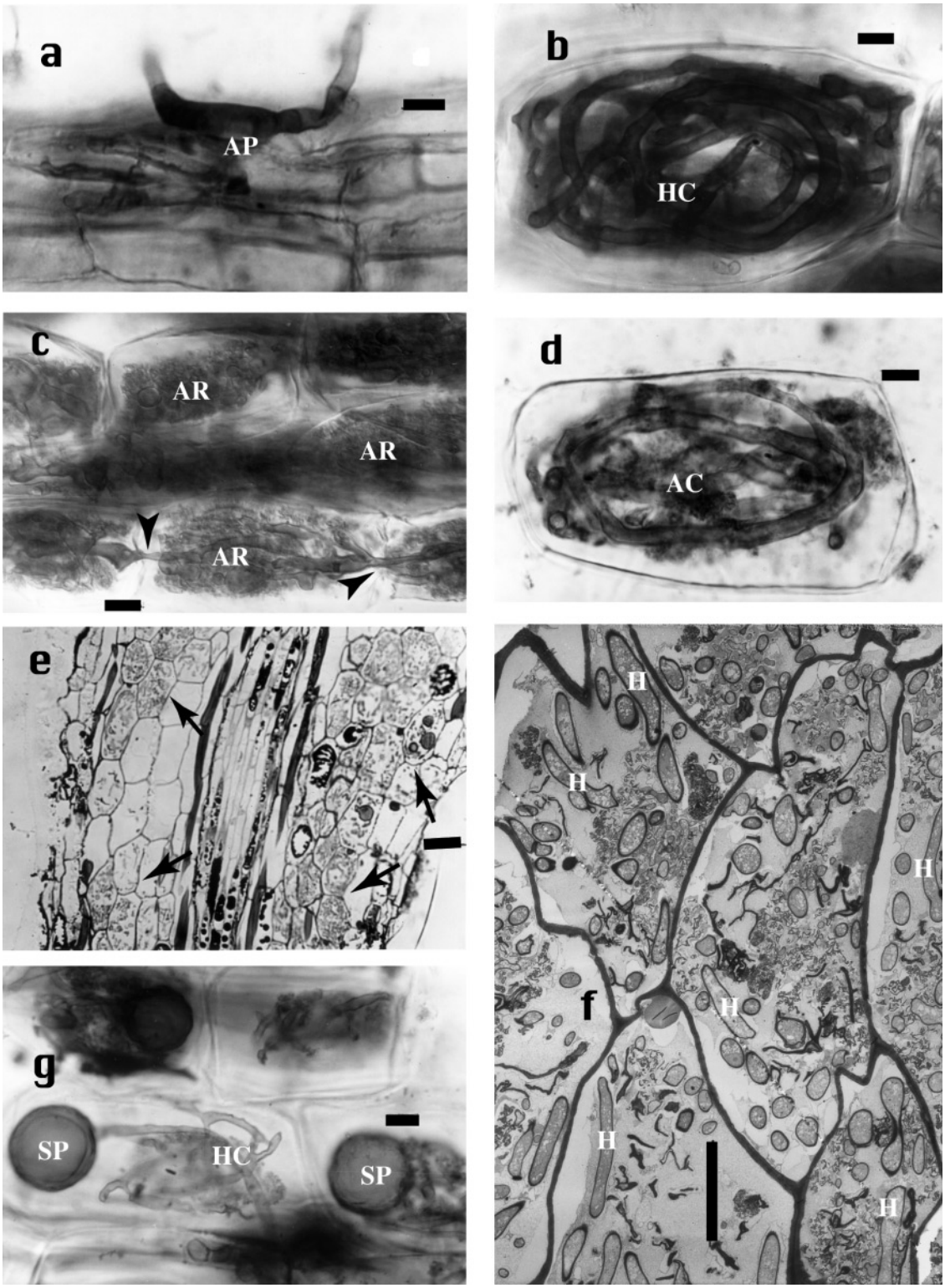


Fig. 2. Phylogenetic relationships of AMF of *Taxus baccata* and related glomeralean fungi: Bayesian Monte Carlo Markov chain analysis of an alignment of 5.8S rDNA sequences assuming the general time-reversible model of DNA substitution and gamma-distributed substitution rates. Numbers on branches are estimates of a posteriori probabilities that the respective groups are monophyletic given the alignment. Branch lengths represent average values over the sampled trees and are scaled in terms of expected numbers of nucleotide substitutions per site. The topology was rooted with *Endogone pisiformis*. Bolded clones in the frames are representatives of AMF sequences from this study.

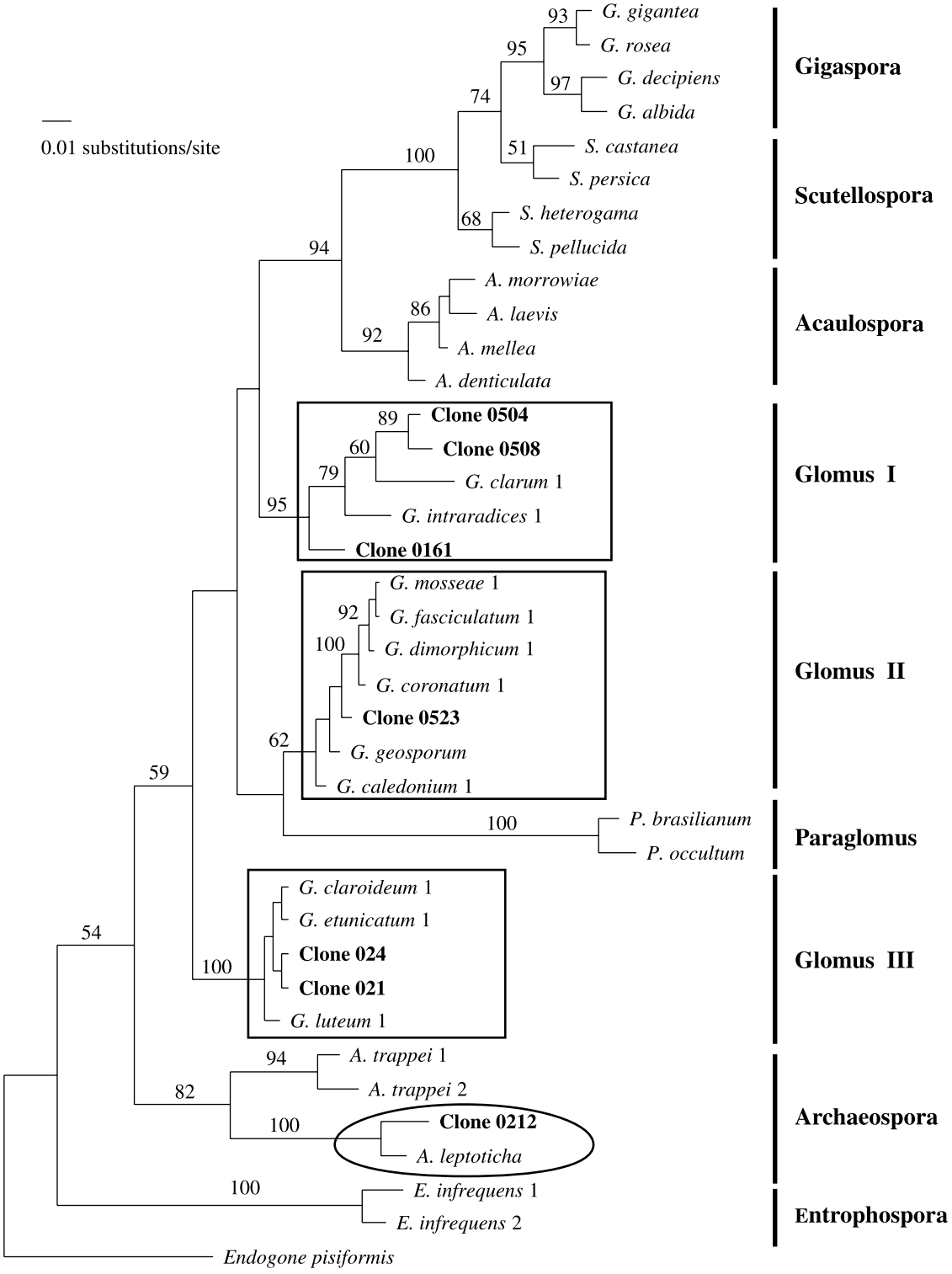


Fig. 3. Phylogenetic relationships of AMF of *Taxus baccata* and related glomeralean fungi restricted to the *Glomus clarum* cluster: Bayesian Monte Carlo Markov chain analysis of an alignment of 294 bp spanning the 5.8S rDNA and a portion of the ITS2 region, assuming the general time-reversible model of DNA substitution and gamma-distributed substitution rates. Numbers on branches are estimates of a posteriori probabilities. Branch lengths represent average values over the sampled trees and are scaled in terms of expected numbers of nucleotide substitutions per site.

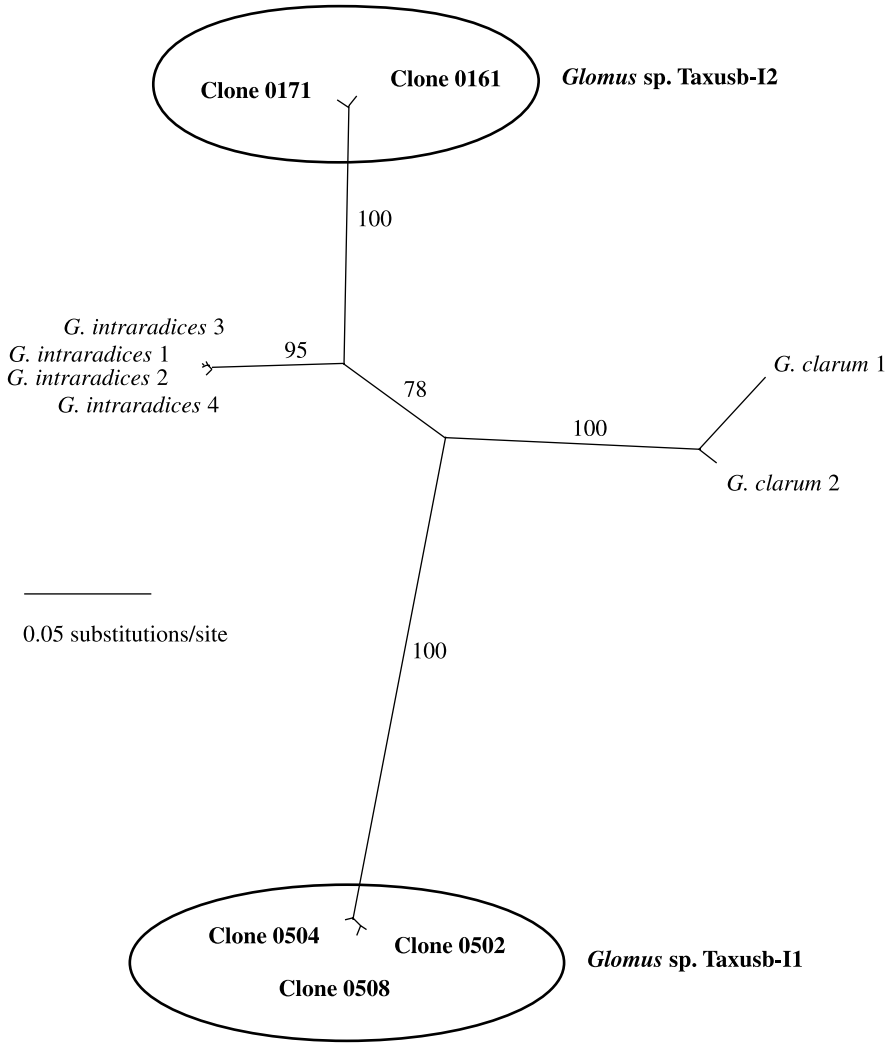


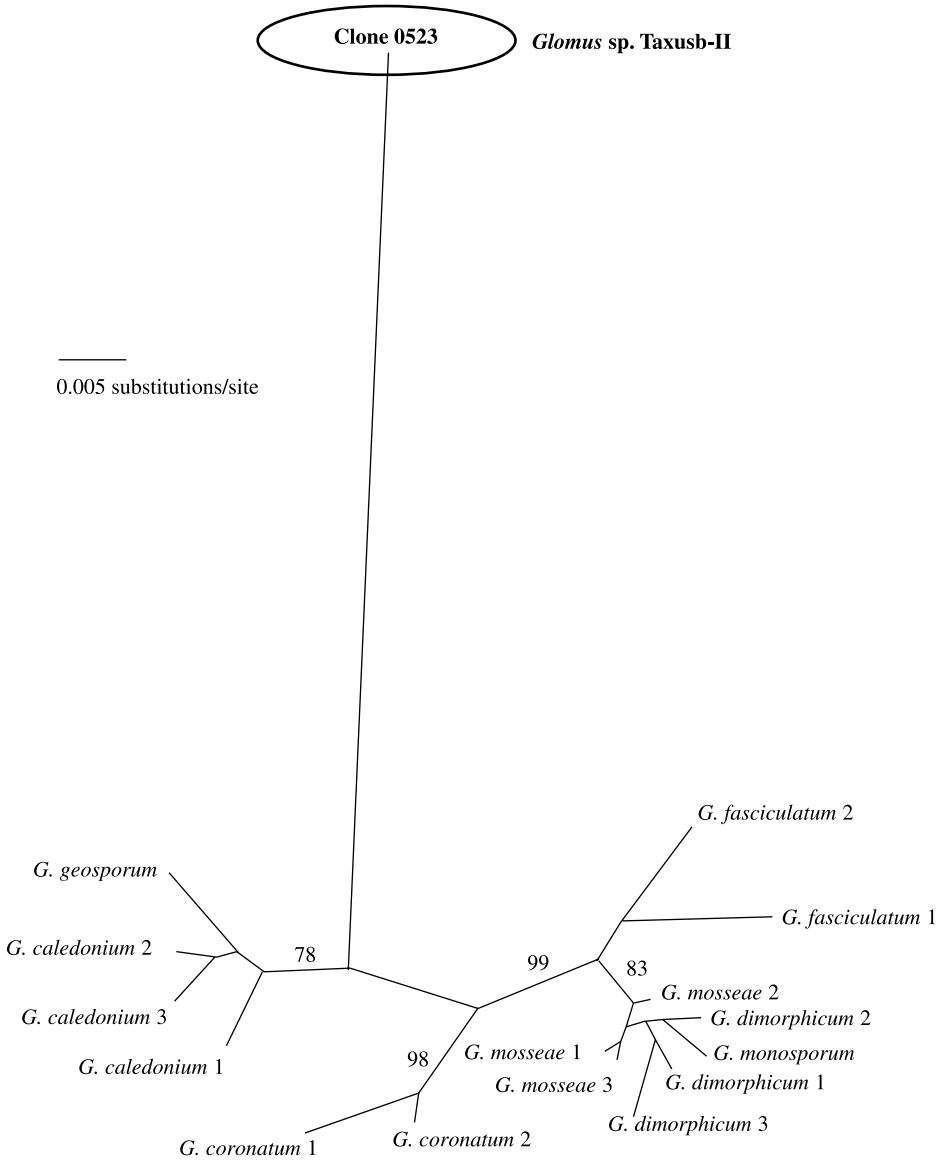
Table 2. AMF sequence types, number of clones, accession numbers, specific primers used for the nested PCR amplification, and the study sites where the sequence types were obtained.

Sequence type	No. of clones	GenBank accession No.	Primer for nested PCR	Study site
Glomus sp. Taxusb-I1	6	AY174685–AY174693	GLOM1310	Tübingen
Glomus sp. Taxusb-I2	9	AY174694–AY174699	ACAU1660	Tübingen
Glomus sp. Taxusb-II	1	AY174700	ACAU1660	Tübingen
Glomus sp. Taxusb-III	6	AY174701–AY174710	ACAU1660	Balingen
Archaeospora sp. Taxusb-IV	10	AY174711–AY174716	LETC1670	Balingen

colonization by potentially functionally different AMF species.

We observed the highest percentage of hyphal coils in the outer and central cortical cells of newly colonized roots. By contrast, the inner cortex and most of the central and outer cortical cells contained arbusculate coils. Similar spatial differences in the production of hyphal coils and arbusculate coils have also been observed in other plants forming *Paris*-type arbuscular mycorrhizas (Cavagnaro et al. 2001; Cooke et al. 1993; Kinden and Brown 1975; Whitbread et al. 1996). Although some level of organization in hyphal coil and arbuscular colonization was observed in newly colonized roots, hyphal coils were generally not restricted to outer cortical cells, and arbuscules and intracellular spores

Fig. 4. Phylogenetic relationships of AMF of *Taxus baccata* and related glomeralean fungi restricted to the *Glomus coronatum* cluster: Bayesian Monte Carlo Markov chain analysis of an alignment of 314 bp spanning the 5.8S rDNA and a portion of the ITS2 region, assuming the general time-reversible model of DNA substitution and gamma-distributed substitution rates. Numbers on branches are estimates of a posteriori probabilities. Branch lengths represent average values over the sampled trees and are scaled in terms of expected numbers of nucleotide substitutions per site.



were not limited to inner cortical cells. The dominance of arbusculate coil structures with a relatively low number of hyphal coils observed in this study suggests that hyphal coils in *Paris*-type arbuscular mycorrhizas develop into arbusculate coils and that the transition is rapid (Cavagnaro et al. 2001; Whitbread et al. 1996). Intracellular spores were observed in regions of extensive arbuscular development, while vesicles were rare.

Whitbread et al. (1996) reported that the frequency of vesicles in American ginseng (*Panax quinquefolius*) collected from a maple–beech woodlot was low. Brundrett and Kendrick (1990) also noted low levels of vesicles in woodland plants. The low frequency of vesicles in mycorrhizal roots of *T. baccata* might be due to the absence of vesicle-producing AMF species or the host physiology may be unfavorable for vesicle development. However, the molecular data, which revealed the dominance of *Glomus* spp., imply the latter to be the case. The frequent occurrence of intracellular spores in regions of extensive arbuscular development also indicates the possibility for the presence of AMF species that invest in intracellular spore formation rather than vesicle formation.

Reliability of the molecular data

The combination of BLAST searches with subsequent thorough phylogenetic analyses ensured that both the sequences obtained in this study and the reference sequences obtained from NCBI are of glomeralean origin. Thus, our analyses should not suffer from distortions caused by sequences from other fungal groups that were mislabelled as

glomeralean sequences in public databases, a problem affecting various molecular studies of the Glomeromycota (Clapp et al. 2002).

Phylogenetic tree inferred from 5.8S rDNA data

The relatively short 5.8S rDNA is known to provide information to estimate higher-level phylogenetic relationships rather than to resolve fungal phylogeny in detail (Cullings and Vogler 1998; Redecker et al. 1999). The phylogenetic hypotheses inferred from the 5.8S rDNA data (Fig. 2) are congruent with the results of published molecular phylogenetic analyses of the Glomeromycota. The monophyly of the Gigasporaceae (*Gigaspora*, *Scutellospora*) and of *Acaulospora* is supported by a 100 and 92% a posteriori probability, respectively. Both groups form sister taxa, which is supported by a 94% a posteriori probability. These results are consistent with phylogenetic hypotheses estimated from 5.8S (Redecker et al. 1999) and also from 18S rDNA data (Redecker et al. 2000*a*; Schüßler et al. 2001). The three subgroups of *Glomus* detected in this study were also found by Redecker et al. (1999) in their phylogenetic analysis based on 5.8S rDNA data from a wide spectrum of species, which also included basidiomycetes and ascomycetes material. The genus *Paraglomus* (*Paraglomus occultum*, *Paraglomus brasilianum*), which was obviously misplaced with ascomycetes and basidiomycetes in the 5.8S rDNA analysis by Redecker et al. (1999), clusters with the subgroups of *Glomus* in the present analysis, although this position is not significantly supported. The long genetic distance that separates the *Paraglomus* sequences from the other sequences included in this study is supported by the results of Schüßler et al. (2001) and Redecker et al. (2000*a*), who demonstrated that this genus represents one of the ancestral lineages of glomeromycetes.

The basal position in our 5.8S rDNA tree is obtained by *Entrophospora infrequens*, a species that has not yet been included in comprehensive molecular phylogenetic studies based on 18S rDNA data but also appears at a basal position in the 5.8S rDNA analysis of Clapp et al. (2002).

The MCMC-based Bayesian approach of phylogenetic analysis used in this study (Huelsenbeck and Ronquist 2001) was superior to the bootstrapped parsimony and the bootstrapped neighbor-joining analyses done using the same data set (data not shown; for a review of methods of molecular phylogenetic analyses, see Swofford et al. (1996)). Concerning significantly supported branches, results obtained by parsimony and neighbor-joining analyses were not in conflict with those inferred from MCMC analysis, but the latter method yielded a much higher resolution of the tree topology.

Phylogenetic position of the AMF sequences obtained

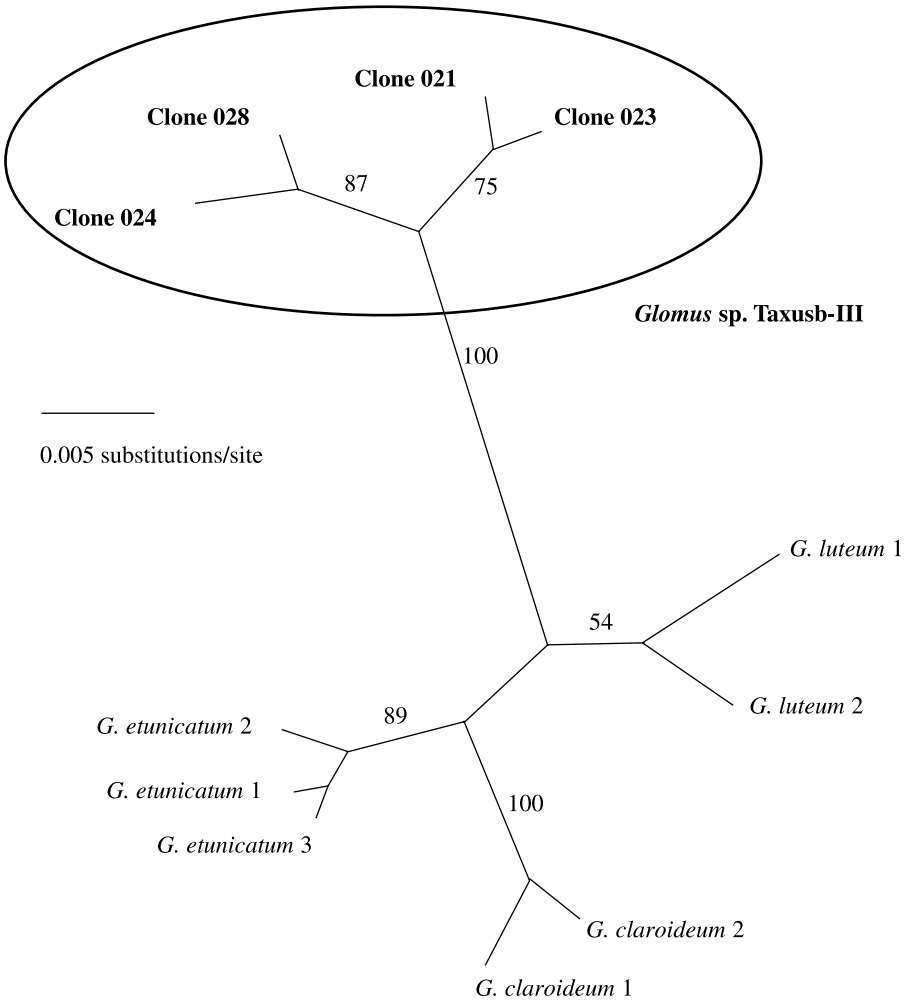
From the phylogenetic analysis of the 5.8S rDNA data set, most of the AMF sequence types found in *T. baccata* roots were significantly assigned to glomeralean subgroups (Fig. 2). However, the inclusion of clone 0523 in the *Glomus* II group lacked a high a posteriori probability. The phylogenetic position of clone 0212 in the *Archaeospora* group was completely resolved in the 5.8S rDNA analysis due to the low number of published sequences and their high degree of divergence. For each of the other glomeralean groups for which sequences were detected, phylogenetic analyses using all of the respective clones, together with an additional range of reference sequences, were performed to provide a better resolution of the phylogenetic position of the new sequences (Figs. 3–5). These analyses strongly support the view that the new sequences do not belong to any AMF species types with published 5.8S rDNA sequences. Considering the high level of ITS sequence diversity that have been documented from single spores and among spores of the same species of AMF (Hijri et al. 1999; Lanfranco et al. 1999; Lloyd-Macgilp et al. 1996; Pringle et al. 2000; Sanders et al. 1995), we assume that *Glomus* sp. Taxusb-I1 and *Glomus* sp. Taxusb-I2 form two distinct species types (Fig. 3). It is also likely that clones 024 and 028 in the *Glomus* III group and clones 021 and 023 represent two other species types (Fig. 5), but there is also the possibility that these sequences are variants of a single AMF species.

Although the assignment of the new sequences to subgroups of Glomeromycota was possible, the new sequences could not be assigned to particular species (Figs. 2–5). These sequences represent either species new to science or species with yet unpublished ITS sequences. The GenBank AMF database is presently too small to allow inter- and intraspecific polymorphisms of these fungi to be efficiently clarified. Species designation will only be possible after sequencing of spores of known, or new, species isolated from the study sites or from spore traps established using soil and roots collected at the sites.

Recently, Helgason et al. (2002) reported physical and functional selectivity in arbuscular mycorrhizal symbiosis in field soils and roots where a diverse community of AMF plays particular roles in association with individual hosts. The variation in the sequence types in colonized roots of *T. baccata* from the cultivated and forest sites strengthens the report of Helgason et al. (1999) who showed that the frequency of AMF sequence types in *Hyacinthoides nonscripta* differ with site. Smith et al. (2000) demonstrated the existence of significant functional diversity among AMF species in the soil from which they absorb phosphate. Thus, the difference in the sequence types in the study sites might be due to selectivity of the host based on functional complementarity at the respective sites. The result also indicates the necessity to isolate indigenous AMF spores and screen for the combination of functionally complementary species to be used in nursery propagation.

The primer pairs used for the nested PCR work were reported to specifically amplify the ITS regions of six clades of AMF (Redecker et al. 2000*a*, 2000*b*) with the exception of the *G. versiforme* clade (Redecker 2000). Therefore, our results should be considered as a survey of these clades with the possibility that there were additional AMF in the roots examined. The present study is the first to use these primers to study the diversity of AMF in field-collected material. However, we observed problems regarding primer specificity: the primer pair ACAU1660/ITS4 that was designed specifically for the Acaulosporaceae (Redecker 2000) also amplified an *Archaeospora* sequence (Fig. 2, clone 0212). By contrast, the latter sequence was not detected using the primer pair ARCH1311/ITS4 that should be specific for the Archaeosporaceae (Redecker 2000), although PCR products were obtained using the latter primer pair on other material (T. Wubet, data not included).

Fig. 5. Phylogenetic relationships of AMF of *Taxus baccata* and related glomeralean fungi restricted to the *Glomus claroideum* cluster: Bayesian Monte Carlo Markov chain analysis of an alignment of 295 bp spanning the 5.8S rDNA and a portion of the ITS2 region, assuming the general time-reversible model of DNA substitution and gamma-distributed substitution rates. Numbers on branches are estimates of a posteriori probabilities. Branch lengths represent average values over the sampled trees and are scaled in terms of expected numbers of nucleotide substitutions per site.



To obtain phylogenetic trees of higher resolution, which cover the wide genetic diversity within the Glomeromycota, future studies should investigate the use of other DNA regions and develop primers for these regions that are specific for the different subgroups. Potential candidates are the 18S rDNA that has been useful in resolving the phylogenetic relationships between higher fungi (e.g., Schüßler et al. 2001) and partial 28S rDNA, which has been successfully used in phylogenetic work on the basidiomycetes at different taxonomic levels (Berres et al. 1995; Begerow et al. 1997; Moncalvo et al. 2002; Weiß et al. 1998; Weiß and Oberwinkler 2001).

Acknowledgements

This study was supported by a grant from the Deutscher Akademischer Austauschdienst whose financial assistance is acknowledged. We would like to thank Dr. Ingeborg Haug for her comments on an earlier version of the manuscript. We also acknowledge the valuable suggestions made by the two anonymous referees of this paper.

References

Abbas, J.D., Hetric, B.A.D., and Jurgenson, J.E. 1996. Isolate specific detection of mycorrhizal fungi using genome specific primer pairs. Mycologia, **88**: 939–946.

Altschul, S.F., Thomas, L.M., Alejandro, A.S., Jinghui, Z., Webb, M., and David, J.L. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**: 3389–3402.

Bakashi, B.K., and Thapar, H.S. 1960. Mycorrhizae in *Taxus baccata* and *Pinus wallichiana*. Indian For. **86**: 16–17 plus plates.

Begerow, D., Bauer, R., and Oberwinkler, F. 1997. Phylogenetic studies on nuclear large subunit ribosomal DNA sequences of smut fungi and related taxa. Can. J. Bot. **75**: 2045–2056.

Berres, M.E, Szabo, L.J., and McLaughlin, D.J. 1995. Phylogenetic relationships in auriculariaceous basidiomycetes based on 25S ribosomal DNA sequences. Mycologia, **87**: 821–840.

Brundrett, M., and Kendrick, B. 1990. The roots and mycorrhizas of herbaceous woodland plants. II. Structural aspects of morphology. New Phytol. **114**: 469–479.

Cavagnaro, T.R., Smith, F.A., Lorimer, M.F., Haskard, K.A.,

Ayling, S.M., and Smith, S.E. 2001. Quantitative development of *Paris*-type arbuscular mycorrhizas formed between *Asphodelus fistulosus* and *Glomus coronatum*. New Phytol. **149**: 105–113.

Chelius, M.K., and Triplett, E.W. 1999. Rapid detection of arbuscular mycorrhizae in roots and soils of an intensively managed turfgrass system by PCR amplification of small subunit rDNA. Mycorrhiza, **9**: 61–64.

Clapp, J.P., Young, J.P.W., Merryweather, J.W., and Fitter, A.H. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol. **130**: 259–265.

Clapp, J.P., Fitter, A.H., and Young, J.P.W. 1999. Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizal fungus, *Scutellospora* sp. Mol. Ecol. **8**: 915–921.

Clapp, J.P., Rodriguez, A., and Dodd, J.C. 2002. Glomales rRNA gene diversity: all that glistens is not necessarily glomalean? Mycorrhiza, Online Edition. http://dx.doi.org/10.1007/s00572-002-0175-8.

Cooke, M.A., Widden, P., and O'Halloran, I. 1993. Development of vesicular arbuscular mycorrhizae in sugar maple (*Acer saccharum*) and effects of base–cation amendment on vesicle and arbuscule formation. Can. J. Bot. **71**: 1421–1426.

Cullings, K.W., and Vogler, D.R. 1998. A 5.8S nuclear ribosomal RNA gene sequence database: applications to ecology and evolution. Mol. Ecol. **7**: 919–923.

Daniell, T.J., Husband, R., Fitter, A.H., and Young, J.P.W. 2001. Molecular diversity of arbuscular mycorrhizal fungi colonizing arable crops. FEMS Microbiol. Ecol. **36**: 203–209.

Gadkar, V., Adholeya, A., and Satyanarayana, T. 1997. Randomly amplified polymorphic DNA using the M13 core sequence of the vesicular–arbuscular mycorrhizal fungi *Gigaspora margarita* and *Gigaspora gigantea*. Can. J. Microbiol. **43**: 795–798.

Gallaud, I. 1905. Études sur les mycorrhizes endotrophs. Rev. Gen. Bot. **17**: 66–83, 123–135, 223–239, 313–325, 425–433, 479–500.

Grace, C., and Stribley, D.P. 1991. A safer procedure for root staining of vesicular arbuscular mycorrhizal fungi. Mycol. Res. **95**: 1160–1162.

Hahn, A., Bonfante, P., Horn, K., Pausch, F., and Hock, B. 1993. Production of monoclonal antibodies against surface antigens of spores from arbuscular mycorrhizal fungi by an improved immunization and screening procedure. Mycorrhiza, **4**: 69–78.

Helgason, T., Fitter, A.W., and Young, J.P.W. 1999. Molecular diversity of arbuscular mycorrhizal fungi colonizing *Hyacinthoides nonscripta* (bluebell) in a seminatural woodland. Mol. Ecol. **8**: 659–666.

Helgason, T., Merryweather, J.W., Denison, J., Wilson, P., Young, J.P.W., and Fitter, A.H. 2002. Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. J. Ecol. **90**: 371–384.

Hijri, M., Hosny, M., van Tuinen, D., and Dulieu, H. 1999. Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zygomycota) is structured within multinucleate spores. Fungal Genet. Biol. **26**: 141–151.

Huelsenbeck, J.P., and Ronquist, F.R. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics, **17**: 754–755.

Jacquot, E., van Tuinen, D., Gianinazzi, S., and Gianinazzi-Pearson, V. 2000. Monitoring species of arbuscular mycorrhizal fungi in planta and soil by nested PCR: application to the study of the impact of sewage slugde. Plant Soil, **226**: 179–188.

Joyce, C. 1993. Taxol: search for a cancer drug. Bioscience, **43**: 133–136.

Khan, A.G. 1971. Mycorrhizal associations in gymnosperms of West Pakistan. Pak. J. Bot. **2**: 9–18.

Kinden, D.A., and Brown, M.F. 1975. Electron microscopy of vesicular–arbuscular mycorrhizae of yellow poplar. I. Characterization of endophytic structures by scanning electron stereoscopy. Can. J. Microbiol. **21**: 989–993.

Kjøller, R., and Rosendahl, S. 2000. Detection of glomalean fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). Plant Soil, **226**: 189–196.

Kjøller, R., and Rosendahl, S. 2001. Molecular diversity of glomalean (arbuscular mycorrhizal) fungi determined as distinct *Glomus* specific DNA sequences from roots of field grown peas. Mycol. Res. **105**: 1027–1032.

Lanfranco, L., Wyss, P., Marzachi, C., and Bonfante, P. 1995. Generation of RAPD–PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. Mol. Ecol. **4**: 61–68.

Lanfranco, L., Delpero, M., and Bonfante, P. 1999. Intrasporal variability of ribosomal sequences in the endomycorrhizal fungus *Gigaspora margarita*. Mol. Ecol. **8**: 37–46.

Lloyd-Macgilp, S.A., Chambers, S.M., Dodd, J.C., Fitter, A.H., Walker, C., and Young, J.P.W. 1996. Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol. **133**: 103–111.

Maier, W., Begerow, D., Weiß, M., and Oberwinkler, F. 2003. Phylogeny of the rust fungi: an approach using nuclear large subunit ribosomal DNA sequences. Can. J. Bot. **81**: 12–23.

Merryweather, J.W., and Fitter, A.H. 1998. The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta*. I. Diversity of fungal taxa. New Phytol. **138**: 117–129.

Millner, P.D., Mulbry, W.W., and Reynolds, S.L. 2001. Taxon-specific oligonucleotide primers for detection of *Glomus etunicatum*. Mycorrhiza, **10**: 259–265.

Moncalvo, J.M., Vilgalys, R., Redhead, S.A., Johnson, J.E., James, T.Y., Aime, M.C., Hofstetter, V., Verduin, S.J.W., Larsson, E., Baroni, T.J., Thorn, R.G., Jacobsson, S., Clémençon, H., and Miller, O.K., Jr. 2002. One hundred and seventeen clades of euagarics. Mol. Phylogenet. Evol. **23**: 357–400.

Morton, J.B. 1985. Variation in mycorrhizal and spore morphology of *Glomus occultum* and *Glomus diaphanum* as influenced by plant host and soil environment. Mycologia, **77**: 192–204.

Morton, J.B., and Redecker, D. 2001. Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera, *Archeaospora* and *Paraglomus*, based on concordant molecular and morphological characters. Mycologia, **93**: 181–195.

Murphy, W.J., Eizirik, E., O'Brien, S.J., Madsen, O., Scally, M., Douady, C.J., Teeling, E., Ryder, O.A., Stanhope, M.J., de Jong, W.W., and Springer, M.S. 2001. Resolution of the early placental mammal radiation using Bayesian phylogenetics. Science (Washington, D.C.), **294**: 2348–2351.

Prat, H. 1926. Étude des mycorhizes du *Taxus baccata*. Ann. Sci. Nat. Ser. 10. Bot. **8**: 141–164.

Pringle, A., Moncalvo, J.M., and Vilgalys, R. 2000. High levels of variation in ribosomal DNA sequences within and among spores of a natural population of the arbuscular mycorrhizal fungus *Acaulospora colossica*. Mycologia, **92**: 259–268.

Rambaut, A. 1996. Se-Al. Sequence Alignment Editor. Department of Zoology, University of Oxford, Oxford, U.K.

Redecker, D. 2000. Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza, **10**: 73–80.

Redecker, D., Hijri, M., Dulieu, H., and Sanders, I.R. 1999. Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of ascomycete origin. Fungal Genet. Biol. **28**: 238–244.

Redecker, D., Morton, J.M., and Bruns, T.D. 2000*a*. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). Mol. Phylogenet. Evol. **14**: 276–284.

Redecker, D., Morton, J.B., and Bruns, T.D. 2000*b*. Molecular phylogeny of the arbuscular mycorrhizal fungi *Glomus sinuosum* and *Sclerocystis coremioides*. Mycologia, **92**: 282–285.

Rosendahl, S. 1989. Comparisons of spore-cluster forming *Glomus* species (Endogonaceae) based on morphological characters and isozyme banding pattern. Opera Bot. **100**: 215–223.

Sainz, M.J., Iglesias, I., Pintos, C., Mansilla, J.P., and Vilarino, A. 2000. Improved production of nursery stock of *Taxus baccata* L. through management of the arbuscular mycorrhizal symbiosis. ISHS Acta Hortic. **536**: 379–384.

Sanders, I.R., Alt, M., Groppe, K., Boller, T., and Wiemken, A. 1995. Identification of ribosomal DNA polymorphism among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol. **130**: 419–427.

Schmid, E., and Oberwinkler, F. 1996. Light and electron microscopy of a distinctive VA mycorrhiza in mature sporophytes of *Ophioglossum reticulatum*. Mycol. Res. **100**: 843–849.

Schüßler, A., Schwarzott, D., and Walker, C. 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol. Res. **105**: 1413–1421.

Schwarzott, D., and Schüßler, A. 2001. A simple and reliable method for SSU rRNA gene DNA-extraction, amplification, and cloning from single AM fungal spores. Mycorrhiza, **10**: 203–207.

Simon, L., Lalonde, M., and Bruns, T.D. 1992. Specific amplification of 18S fungal ribosomal genes from vesicular–arbuscular endomycorrhizal fungi colonizing roots. Appl. Environ. Microbiol. **58**: 291–295.

Simon, L., Levesque, R.C., and Lalonde, M. 1993. Identification of endomycorrhizal fungi colonizing roots by fluorescent single-strand conformation polymorphism polymerase chain reaction. Appl. Environ. Microbiol. **59**: 4211–4215.

Smith, S.E., and Read, D.J. 1997. Mycorrhizal symbiosis. 2nd ed. Academic Press, London, U.K.

Smith, F.A., Jakobsen, I., and Smith, S.E. 2000. Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol. **147**: 357–366.

Spurr, A.R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**: 31–43.

Strullu, D.G. 1978. Histologie et cytologie des endomycorrhizes. Physiol. Veg. **16**: 657–669.

Swofford, D.L., Olsen, G., Waddell, P.J., and Hillis, D.M. 1996. Phylogenetic inference. *In* Molecular systematics. 2nd ed. *Edited by* D.M. Hillis, C. Moritz, and B.K. Mable. Sinauer Associates, Inc., Sunderland, Mass. pp. 407–514.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25**: 4876–4882.

Turnau, K., Ryszka, P., Gianinazzi-Pearson, V., and van Tuinen, D. 2001. Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in southern Poland. Mycorrhiza, **10**: 169–174.

Urban, A., Weiß, M., and Bauer, R. 2003. Ectomycorrhizae involving sebacinoid mycobionts. Mycol. Res. **107**: 3–14.

van der Heijden, M.A.G., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., and Sanders, I.R. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature (Lond.), **396**: 69–72.

van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., and Gianinazzi-Pearson, V. 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. **7**: 879–887.

von Tubeuf, C. 1896. Die Haarbildungen der Coniferen. Forstl. Naturwiss. Z. **5**: 173–193.

Walker, C., and Trappe, J.M. 1993. Names and epithets in the Glomales and Endogonales. Mycol. Res. **97**: 339–344.

Weiß, M., and Oberwinkler, F. 2001. Phylogenetic relationships in Auriculariales and related groups: hypotheses derived from nuclear ribosomal DNA sequences. Mycol. Res. **105**: 403–415.

Weiß, M., Yang, Z.-L., and Oberwinkler, F. 1998. Molecular phylogenetic studies in the genus *Amanita*. Can. J. Bot. **76**: 1170–1179.

Whitbread, F., McGonigle, T.P., and Peterson, R.L. 1996. Vesicular–arbuscular mycorrhizal associations of American ginseng (*Panax quinquefolius*) in commercial production. Can. J. Bot. **74**: 1104–1112.

White, T.J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols: a guide to methods and applications. *Edited by* M.A. Innis, D.H. Gelfand, and J.J. Sninsky. Academic Press, San Diego, Calif. pp. 315–322.

Yawney, W.J., and Schultz, R.C. 1990. Anatomy of a vesicular–arbuscular endomycorrhizal symbiosis between sugar maple (*Acer saccharum* Marsh.) and *Glomus etunicatum* Becker and Gerdemann. New Phytol. **114**: 47–57.

Zeze, A., Sulistyowati, E., Ophelkeller, K., Barker, S., and Smith, S. 1997. Intersporal genetic variation of *Gigaspora margarita*, a vesicular arbuscular mycorrhizal fungus revealed by M13 minisatellite-primed PCR. Appl. Environ. Microbiol. **63**: 676–678.