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DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI IN THE ROOTS OF PERENNIAL PLANTS AND THEIR EFFECT ON PLANT PERFORMANCE

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following papers, which are referred to in the text by the relevant Roman numerals.

I. C. Renker, M. Zobel, M. Öpik, M.F. Allen, E.B. Allen, M. Vosátka, J. Rydlová and F. Buscot. 2004. Structure, dynamics and restoration of plant communities: does arbuscular mycorrhiza matter? In: V. Temperton, R. Hobbs (eds.), *Assembly rules in restoration ecology — bridging the gap between theory and practice*, pp. 189–229. Island Press, Washington.

II. M. Öpik, M. Moora, J. Liira, U. Kõljalg, R. Sen and M. Zobel. 2003. Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forest and grassland soils. *New Phytologist*, 160: 581–593.

III. M. Moora, M. Öpik, R. Sen and M. Zobel. 2004. Rare vs. common *Pulsatilla* spp. seedling performance with arbuscular mycorrhizal inoculum from contrasting native habitats. *Functional Ecology*, accepted for publication.

IV. M. Öpik, M. Moora, J. Liira, S. Rosendahl and M. Zobel. Comparison of communities of arbuscular mycorrhizal fungi in roots of two *Viola* species. Manuscript.

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The contribution of M. Öpik to the respective papers as follows: 10% (paper I), 80% (paper II), 40% (paper III), and 80% (paper IV).

ABBREVIATIONS

AM	arbuscular mycorrhiza
AMF	arbuscular mycorrhizal fungus/fungi
bp	base pair
DCA	detrended correspondence analysis
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
LSU rDNA	large subunit ribosomal RNA gene
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SSCP	single stranded conformation polymorphism
SSU rDNA	small subunit ribosomal RNA gene
T-RFLP	terminal-restriction fragment length polymorphism
TWINSPAN	two-way indicator species analysis

INTRODUCTION

The aim of the present thesis is to describe the communities of arbuscular mycorrhizal (AM) fungi in the roots of perennial herbaceous plants and to investigate their effects on plant performance. In particular, the focus is on pairs of congeneric plant species, similar in morphology and ecology, but showing different abundances in nature.

Arbuscular mycorrhiza. The term 'mycorrhiza' (Greek *mykes*, fungus + *rhiza*, root) implies an association of fungi and plant roots. However, the mycorrhizal associations, recognised now as the nutrient absorptive formations in soil, also include plants with no roots — bryophytes and pteridophytes (Smith & Read 1997, p. 2). The underground organs of gametophytes of bryophytes and gameto- and sporophytes of pteridophytes host the symbiotic fungi. 'Symbiosis' is here understood as the regular coexistence of dissimilar organisms, and covers associations from parasitism to mutualism (beneficial to both parts) (Smith & Read 1997). It is accepted widely that mycorrhiza, not plant root, is the principal organ of nutrient uptake from the soil (Pearson & Jakobsen 1993, Smith & Read 1997).

Arbuscular mycorrhiza is probably the most widespread terrestrial symbiosis. It is formed between obligate biotrophic fungi of the phylum Glomeromycota (Schüßler *et al.* 2001) and plants of more than 60% of families (Smith & Read 1997). The fungi comprise a monophyletic group of *c.* 150 described species (Walker & Trappe 1993), originally assigned to the order Glomales within Zygomycota (Morton & Benny 1990). Recent studies involving morphological and DNA sequence characters (ribosomal genes and single-copy protein-encoding genes), however, have shown that the order Glomales constitutes a distinct group outside the four previously recognised phyla of the fungal kingdom (Morton and Redecker 2001, Schüßler *et al.* 2001, Helgason *et al.* 2003, Corradi *et al.* 2004).

Important features of the AM fungi are their presumed asexuality, production of large soil-borne spores harbouring hundreds or thousands of nuclei, and multinucleate mycelium without true septa (Smith & Read 1997). It is still a matter of debate as to whether the nuclei within a single spore are genetically different (Kuhn *et al.* 2001) or identical to each other but harbouring polymorphic gene copies (Pawlowska & Taylor 2004). The genome, recently shown in the case of *Glomus intraradices*, can be haploid and of small size compared to other eukaryotes (*c.* 14 Mb) (Hijri & Sanders 2004). Despite obvious asexuality, some cryptic recombination events may exist in AM fungi (Gandolfi *et al.* 2003).

The symbiotic association itself is ancient, the first spore fossils originating from the Ordovician (Redecker *et al.* 2000a). It has been hypothesised that the

first land plants, with no roots but with protostelic rhizomes, were arbuscular mycorrhizal, the fungus being essential for the plant in scavenging mineral nutrients from poor primary soils (Pirozynski & Malloch 1975).

Today four orders, seven families and eight genera of AM fungi are recognised (Schüßler *et al.* 2001). The number of AM fungal species is unknown, and has been suspected to be much larger than 150, based on selectivity between fungal and plant species and the high proportion of total AM fungal diversity commonly detected in natural communities, compared to the number of plant species (Helgason *et al.* 2002).

Functions of arbuscular mycorrhiza. Arbuscular mycorrhizal fungi give a major contribution to plant nutrition, promoting mostly the uptake of phosphorus, but also other immobile ions such as zinc and copper, and the uptake of mobile nitrogen (Gildon & Tinker 1983, Smith & Read 1997, Bago *et al.* 2001). As for the phosphorus uptake, the provision of the nutrient by AM fungi can reach 100% of the plant's P uptake (Smith *et al.* 2003). The fungi can also protect plants from infection by root pathogenic fungi and nematodes, alleviate drought stress, improve soil structure, and confer heavy metal resistance to plants (Newsham *et al.* 1995, Leyval *et al.* 1997, Smith & Read 1997, Gonzales-Chavez *et al.* 2002).

AM fungi can confer protection against root pathogens to plants (Azcón-Aguilar and Barea 1996). Decreased pathogen development has been observed in mycorrhizal and non-mycorrhizal parts of a root system, indicating that both localised and systemic resistance can be induced in response to AM fungal colonisation (e.g., Cordier *et al.* 1998, Pozo *et al.* 2002).

Mycelium of AM fungi has high metal sorption capacity compared to other microorganisms (Joner *et al.* 2000), which may result in accumulation of metals in the rhizosphere of mycorrhizal plants but not in shoots (Tonin *et al.* 2001). Species of AM fungi show different sensitivity to heavy metals, ranging from intolerance to tolerance to fairly high amounts of a pollutant (Jacquot *et al.* 2000, Del Val *et al.* 1999).

Better resistance to drought of mycorrhizal plants was first reported in 1970s (thoroughly reviewed by Smith & Read 1997). Whereas it is agreed that AM fungi affect plant water relations (Augé 2001), other important aspects related to the drought resistance are the nutrient uptake from dry soil via fungal hyphae (Smith & Read 1997), and improved water-stable soil aggregation caused by secretion of a glycoprotein glomalin by AM fungi (Rillig *et al.* 2002).

Plants provide fungi with photosynthetically fixed carbon, their only source of energy. Fungal spores may germinate without the presence of a plant partner, and the resulting mycelium has a limited ability for non-symbiotic growth using the nutrient reserves of the spore (Bago & Bécard 2002). The fungi are, otherwise, however, entirely dependent on the plant as energy source and cannot complete their life cycle without the plant partner. The basis of obligate symbiosis for the fungus may lie in morphological and functional bipolarity of

AM fungal mycelium — intraradical fungal structures (hyphae, arbuscules) acquire carbon from the plant, transform it into storage lipids that are transferred to other parts of the mycelium, whilst, in the opposite direction, transfer of mineral nutrients from fungus to the plant takes place. Extraradical fungal structures (mycelium in soil, branched absorbing structures) take up mineral nutrients from the soil and transfer them to the plant root; in the opposite direction, the carbon is exported from intraradical to extraradical mycelium to build spores and mycelium (Bago & Bécard 2002).

The above-described array of functions of the AM symbiosis means that the individual fungal species/isolates are also diverse in their implementation. Fungi from different genera have been shown to differ in the efficiency of uptake and transport of phosphorus (Jakobsen *et al.* 1992) and nitrogen to plants (Azcon *et al.* 2001), the ability to protect plants from pathogens (Azcon-Aguilar & Barea 1997), heavy metal stress (Joner *et al.* 2000, Tonin *et al.* 2001) and the ability to alleviate drought stress (Ruiz-Lozano *et al.* 1995). Specifically, differential up- and down-regulation of several plant functional genes involved in nutrient uptake and transport have been reported in symbiosis with different AM fungi (Burleigh *et al.* 2002, 2003, Ravnskov *et al.* 2003). From the point of view of the plant, the fungi differ in their carbon expenditure, whilst providing nutrients to the plant with differing efficiencies (Pearson & Jakobsen 1993, Dodd *et al.* 2000). Therefore, depending on the balance between provision of mineral nutrients and the amount of carbon used, different fungi may constitute different cost to a plant, which also varies with plant age and developmental stage (Wright *et al.* 1998).

Methods to study AM diversity. Conventional means of studying the natural populations and communities of AM fungi are based on identification of asexual soil-born spores. Taxonomy of AM fungi is still largely based on spore morphological characters, with a few exceptions (Sawaki *et al.* 1998, Declerck *et al.* 2000, Kramadibrata *et al.* 2000, Redecker *et al.* 2000b, Lanfranco *et al.* 2001, Morton & Redecker 2001, de Souza *et al.* 2004). Morphological character states change during the course of development of spores (Morton *et al.* 1995), commonly complicating the identification of field specimens. Further, the presence of spores in the soil does not always coincide with fungal colonisation in roots (Clapp *et al.* 1995), but the morphology of the fungal intraradical structures allows identification on family level at best (Merryweather & Fitter 1998). Such primary identification constraints have set the limits on studying AM fungi.

The development of molecular biology methods has allowed the circumvention of many of the above limits, first by applying isozyme (Rosendahl & Sen 1992) and immunology methods (Sanders *et al.* 1992), followed by nucleic acids-based approaches (reviewed by Clapp *et al.* 2002b, and **paper I**). Amplification of AM fungal genes from environmental samples with the aid of primers of different specificities now allows fairly accurate

detection and identification of AM fungi both in the soil and in plant roots. Starting with the work of Simon *et al.* (1992), different sets of primers, mostly amplifying nuclear ribosomal genes of AM fungi, have been developed. For research into the natural communities of AM fungi, primers amplifying all AM fungi, but excluding other fungi and plant hosts, are desirable. Best fitting these requirements is the primer pair NS31 (Simon *et al.* 1992) coupled with AM1 (Helgason *et al.* 1998), designed to amplify (AM) the fungal and exclude the plant small subunit nuclear ribosomal rRNA gene (SSU rDNA). The AM1 primer was later shown to exclude the deeply branching Archaeosporaceae and Paraglomaceae families (Daniell *et al.* 2001), but attempts to develop better AM fungi-specific primers have not been successful (J.P.W. Young & T.J. Daniell, *pers. comm*.). However, this primer pair and the SSU region are the most widely used targets in AM fungal community studies (results reviewed in the section 'Distribution patterns…', page 14). A range of isolate-, species- and group-specific primers is available (reviewed by Clapp *et al.* 2002b; later additions include Kjøller & Rosendahl 2000, Millner *et al.* 2001a,b, Turnau *et al.* 2001, Yokoyama *et al.* 2002, Geue & Hock 2004).

Other nuclear ribosomal DNA regions have been used in AM research, including the internal transcribed spacer (ITS) flanking the 5.8S gene, and the large ribosomal subunit (LSU) gene. The ITS region is widely used in mycological and ectomycorrhizal research, but has been found to be rather variable among AM fungi. Many AM fungi possess divergent copies of the ITS region within an individual spore (nucleus) (Lloyd-MacGilp *et al.* 1996, Redecker *et al.* 1997, Jansa *et al.* 2002a, Pawlowska & Taylor 2004), which complicates its use with environmental material. Despite the complications, the ITS and/or the 5.8S gene have been applied to answer specific questions (Antoniolli *et al.* 2000, Pringle *et al.* 2000, 2003, Hildebrandt *et al.* 2001, Bidartondo *et al.* 2002, Jansa *et al.* 2002a,b, Wubet *et al.* 2003, 2004, **paper I**).

The LSU rRNA gene, though less used in the case of AM fungi, may be desirable due to its higher variability, allowing distinction of taxonomical groupings difficult to separate on the basis of SSU rDNA sequences (Kjøller & Rosendahl 2000). Again, multiple divergent copies of the LSU gene may occur in a single spore (Clapp *et al.* 2001). LSU rDNA primers specific to the *Glomus mosseae-intraradices* species group were designed by Kjøller and Rosendahl (2000) and have been used to monitor this fungal group in an agricultural field (Kjøller & Rosendahl 2001) and natural forest/meadow ecosystems (**paper IV**). Species-specific primers of the LSU region have been useful for detection of AM fungi in microcosm experiments as well as in natural plant roots (van Tuinen *et al.* 1998b, Jacquot *et al.* 2000, Jacquot-Plumey *et al.* 2001, Turnau *et al.* 2001).

Amplification of AM fungal genes from environmental samples with the goal of detection or identification, if using general primers, needs to be followed by an amplicon separation and/or screening step, because the amplicon consists of amplifed gene fragments of multiple co-existing organisms. The separation

can be achieved via electrophoresis or cloning of PCR products. Cloning followed by clone screening with restriction enzymes and sequencing of representatives of RFLP groupings has been widely used in natural AM fungal community studies (Helgason *et al.* 1998, 1999, 2002; Daniell *et al.* 2001; Husband *et al.* 2002a,b; Vandenkoornhuyse *et al.* 2002). Alternatively, PCR products of the same length but different sequence can be separated by acrylamide gel electrophoresis in a gradient of denaturant (DGGE, denaturing gradient gel electrophoresis; Myers *et al.* 1987) or as single-stranded molecules moving at different speeds dependent on their conformation (SSCP, single strand conformation polymorphism, Orita *et al.* 1989). Separated bands can be identified either by comparison with band positions of known organisms, or by sequencing and sequence comparisons against databases. DGGE is widely used in environmental microbiology for microbial community monitoring and is useful for screening of numerous samples. The method was applied in this study (**paper II**) and by Kowalchuk *et al.* (2002) to describe natural root- inhabiting AM fungal communities of *Pulsatilla* spp. and *Ammophila arenaria*, respectively. The second, SSCP, is a sensitive and invaluable method for distinction of DNA fragments differing only by a few base pairs, and therefore has been applied as a pre-sequencing screen of samples in extensive population studies of AM fungi (Clapp *et al.* 2001, Rodriguez *et al.* 2001, Jansa *et al.* 2002b). The method was used in this study (**paper IV**), by Kjøller and Rosendahl (2000, 2001) and by Jansa *et al.* (2003) to monitor specific groups of AM fungi.

With the use of molecular detection/identification methods, the question of individual and species boundaries within Glomeromycota arises. There is scarce information about the intra-specific variability of AM fungi at the molecular level, complicating the interpretation of the results of phylogenetic analysis. One may ask, is the 97% similarity level sufficient as a species-delimiting criterion? The question is further complicated by the high degree of variation of rDNA regions demonstrated in some AM fungal species (Lanfranco *et al.* 1999, Clapp *et al.* 1999, 2001, Antoniolli *et al.* 2000, Rodriguez *et al.* 2001, Jansa *et al.* 2002, 2003). However, one should be careful to avoid contaminating sequence data (Schüßler 1999, Pringle *et al.* 2000, 2003, Clapp *et al.* 2002b, Schüßler *et al.* 2003). The problem may partly lie in the asexual nature of the Glomeromycota. However, the sequence grouping concept remains the most valid and applicable system for delimiting AM fungal taxa in the field, at least until a better understanding of the genetical organisation and taxonomy of AM fungi is achieved (Clapp *et al.* 2002a).

Dispersal and propagule bank of AM fungi. AM fungi propagate and disperse via soil-borne (asexual) spores, mycelial fragments and colonised root pieces (Smith & Read 1997). The large size of the spores (30 to 700 µm) and their formation in the soil are a reason for their poor dispersal ability (Molina *et al.* 1992). The means of dispersal include wind, water, and small animals

(earthworms, rodents, grasshoppers, etc; e.g. Warner *et al.* 1987, Reddell & Spain 1991, Allen *et al.* 1992, Gange 1993, McGee & Baczocha 1994, Janos *et al.* 1995, Mangan & Adler 2002). Wind was also found to disperse mainly small *Glomus* spores, up to a distance of *c.* 2 km (Warner *et al.* 1987). The importance of wind as a dispersal agent is probably higher in open, wind-eroded ecosystems. Rodents have been shown to disperse considerable quantities of sporocarp-forming *Glomus* species in their droppings in a tropical forest; importantly, the spores extracted from faeces had retained the ability to form mycorrhiza (Mangan & Adler 2002).

Sporulation of some AM fungi, especially those from the genera *Scutellospora* and *Gigaspora*, needs to be preceded by a prolonged phase of root colonisation (Dodd *et al.* 2000). Also, spores are the main source of inoculum for these genera (Jasper *et al.* 1993, Boddington & Dodd 2000a,b). On the contrary, for many *Glomus* species, sporulation is not required to colonise new roots (Klironomos & Hart 2002). Therefore, *Glomus* spores may be relatively infrequent in native soils, even if root colonisation by *Glomus* is abundant (Jasper *et al.* 1991, Clapp *et al.* 1995). Colonisation of new roots is preferably started from intact mycelium in the soil, or from hyphal or colonised root fragments (McGee 1989). Some, but not all, *Glomus* species (e.g., *G. invermaium*) are rather vulnerable to disturbance that disrupts the mycelial network; this is in contrast to *Gigaspora*, *Acaulospora* and some other *Glomus* species (e.g., *G. monosporum*, *G. manihotis*), which may show higher colonisation initiation upon disturbance (Braunberger *et al.* 1996, Boddington & Dodd 2000b). *Glomus*, but not *Scutellospora* and *Gigaspora* have been observed to form anastomoses between hyphae of the same isolate (Giovannetti *et al.* 1999, 2001). The anastomosis formation of *Acaulospora* mycelia has not been studied. The anastomoses create a mycelial network with a potential for multidirectional nutrient flow that is adjustable to the needs of different parts of the mycelium, possibly including regrowth in damaged mycelial regions.

Spores of AM fungi may maintain the ability to germinate over several years (McGee *et al*. 1997). Additionally, the spores can germinate for several times before a host plant is encountered; the presence of a plant host is not required for the germination to take place (reviewed by Bago *et al.* 1998). Spore dormancy is another mechanism that contributes to the survival of AM fungi and maintains a pool of spores in the soil. A dormant spore is defined as *one that fails to germinate although it is exposed to physical and chemical conditions that will support germination and hyphal growth of apparently identical, but non-dormant, spores of the same species* (Tommerup 1983). Obviously, this is a physiological condition that needs an activation in order to be terminated, e.g. by temperature or storage (Louis & Lim 1988, Juge *et al.* 2002). Dormancy may be an important mechanism for synchronising spore germination with rapid root growth and favourable conditions for colonisation in temperate regions (Tommerup 1985). There is experimental evidence that spore dormancy varies with AM fungal species (reviewed by Juge *et al.* 2002). However, *Glomus*

mosseae isolates from different geographic locations have shown either no dormancy (Douds & Schenck 1991), or they need storage at low positive (+6ºC, Hepper & Smith 1976) or at negative temperatures (–10ºC, Safir *et al.* 1990) in order to break dormancy, indicating adaptation to different climatic conditions.

The mycelium of AM fungi can survive a period of freezing (Kabir *et al.* 1997, Addy *et al.* 1998). More interestingly, root colonisation by *Glomus* species was shown to be little affected by simulated winter as compared to severe reduction in next-season colonisation of *Scutellospora* and *Acaulospora*; the combination of plant host and fungus species also affected the fungal response to freezing (Klironomos *et al.* 2001). Thus, freezing tolerance is another important characteristic of AM fungi inhabiting periodically frozen soils.

The persisting spores, and other propagules such as intact mycelium, hyphal and colonised root fragments, in the soil may therefore be called a 'propagule bank' that is 'waiting' for suitable conditions to germinate and/or grow and eventually colonise new plant roots.

Distribution patterns of AM fungi in natural communities. Traditional methods of studying natural AM fungal communities have for a long time included identification of spores extracted and/or trapped (via so-called 'trap-culturing' with plants, e.g. Stutz & Morton 1996) from the soil. However, it is still not known, what exactly determines the sporulation intensity of AM fungi in natural environments. Furthermore, the spore populations in the soil do not necessarily correlate with the fungi colonising plant roots (Clapp *et al.* 1995); neither is there a direct relationship between sporulation and root colonisation levels (see Dodd *et al.* 2000). Therefore, while spores identified from soils constitute important information regarding the species pool with potential to colonise plant roots in a given location, as well as revealing some biological and functional properties of morphospecies, they should not be equated with the AM fungal community at a site without further information. Thus, alternate means to detect and identify AM fungi in field samples (soil or plant roots) are needed in order to study AM fungal communities in nature.

Conventional spore-based investigations suggest that distribution of AM fungal spores is not random in natural communities. The presence and abundance of the spores of an AM fungal species can be affected by biotic and abiotic factors, such as host plant species, ecosystem type, soil pH, soil moisture, total soil C and N, temperature, season, disturbance regime, etc. (Schenck & Kinloch 1980, McGraw & Hendrix 1984, Koske 1987, Gibson & Hetrick 1988, Johnson *et al.* 1991, 1992, 2003, Boddington & Dodd 2000, Egerton-Warburton & Allen 2000, Eom *et al.* 2000, Carvalho *et al.* 2003, Lovelock *et al.* 2003).

The first step forwards in the molecular AM fungal community analysis is represented by investigations where AM fungi are isolated from the soil and cultured; specific primers are developed based on these taxa, and are used to

detect the presence of the fungi in roots (Jacquot-Plumey *et al.* 2001, Turnau *et al.* 2001, Ferrol *et al.* 2003, Calvente *et al.* 2004). The morphological identification of retrieved spores from the soil can also be confirmed by sequencing (e.g. Jansa *et al.* 2002a).

The increasing number of molecular surveys of root colonising AM fungal communities allow identification of some preliminary patterns of natural diversity of AM fungi. Different numbers of fungal species have been identified from a range of ecosystems (summarised in Table 1): 13 AM fungal sequence types from a temperate broad-leaved forest, 10–24 from different temperate grasslands, 19 from tropical rain forests, 2–7 from temperate arable fields, 22 from an afromontane forest, 14–20 from temperate wetlands (Helgason *et al.* 1998, 1999, 2002, Daniell *et al.* 2001, Husband *et al.* 2002a,b, Vandenkoornhuyse *et al.* 2002, Heinemeyer *et al.* 2004, Wirsel 2004, Wubet *et al.* 2004). However, probably the highest known AM fungal richness at a single site, 37 taxa in an old-field, was detected by extensive trap-culturing (Bever *et al.* 2001).

In addition to the absolute number of species, species distribution patterns within a community, expressed as diversity indices, vary remarkably between sites/ecosystems and across seasons. For example, the Shannon diversity index (*H*) of AM fungal communities may show remarkable variation: 0.98 to 1.19 in different arable fields, and 0.45 to 1.49 across one season in the same agricultural fields (Daniell *et al.* 2001), 1.36 to 1.62 in nearby seminatural woodland in July and December (Helgason *et al.* 1999), 1.71 in a temperate grassland (Vandenkoornhuyse *et al.* 2002), 2.33 in a tropical forest (Husband *et al.* 2002b), 2.58 in an Afromontane forest (Wubet *et al.* 2004), and 2.4 in temperate wetlands (Wirsel 2004).

In most of the published studies so far there is insufficient data to make firm conclusions about the structure of AM fungal communities. However, it is apparent that the dominant species or species groups may differ with season and site, host species, habitat and ecosystem type (Helgason *et al.* 1998, 1999, 2002, Daniell *et al.* 2001, Husband *et al.* 2002a, b, Heinemeyer *et al.* 2004, Wirsel 2004, Wubet *et al.* 2004). Strikingly, the AM fungal communities of co-occurring plant species, taxonomically related or not, may differ significantly (Vandenkoornhuyse *et al.* 2002, 2003), and the fungal communities inhabiting the same plant species at different sites may be divergent (Helgason *et al.* 1999, Wubet *et al.* 2003, 2004, Wirsel 2004). There are examples of host selectivity by AM fungi (Helgason *et al.* 2002), but extreme specificity has been shown only in the case of epiparasitic myco-heterotroph plants (Bidartondo *et al.* 2002).

Table 1. Summary of AM fungal community surveys in a range of ecosystems. Number of detected fungal 'species' (sequence types) in relation to sampling and sample screening effort is shown.

Ecosystem	No. of sites studied	No. of AMF types per site	No. of plant spp. studied per site	No. of root samples analysed	No. of clones screened per study	No. of clones sequenced per study	Ref.[1]
Temperate forest	2	5–8	1–3	33–37	66–154	22–62	1, 2
Temperate arable field	4	2–7	1–2	79	303	72	3
Temperate grassland	3	10–24	1–2, ?	24–47	43–2001	16–88	**II**, 4, 5
Temperate wetland	2	14–20	1	13	546	54	6
Boreal forest	1	10	2	26	83	83	**II**
Tropical forest	4	18–22	1–2	20–54	558–1536	10–90	7, 8
Afromontane forest	2	11–15	1	50[2]	?	109	9

[1] 1, Helgason *et al.* 1999; 2, Helgason *et al.* 2002; 3, Daniell *et al.* 2001; 4, Vandenkoornhuyse *et al.* 2002; 5, Heinemeyer *et al.* 2004; 6, Wirsel 2004; 7, Husband *et al.* 2002a; 8, Husband *et al.* 2002b; 9, Wubet *et al.* 2004. Papers that provide comparable data and had an intention to describe the entire community of AM fungi were included.

[2] number of samples collected; number of samples actually cloned is not given in the paper.

Role of AM in plant population dynamics. The full population growth of a plant can only be exhibited when the individual has its full complement of obligate mutualists (Crawley 1997). Thus, the role of symbiont limitation in the fate of plant populations can be a decisive one.

The role AM fungi play in plant population dynamics has not been heavily studied, partly due to ignorance of underground processes, partly due to methodological difficulties related to the manipulation of obligate symbiotic fungi for experimentation (reviewed by Read 2002). However, it is well known that AM fungi are intimately involved in plant life through roles in nutrient uptake, biotic and abiotic stress alleviation, and possibly other effects (Smith & Read 1997). Fungal effects may change plant fitness via improved sexual or clonal reproduction, and changes in competitive abilities of plant individuals, thus shaping density as well as size and reproductive hierarchy of plant populations (Streitwolf-Engel *et al.* 2001, Koide & Dickie 2002).

Mycorrhizal colonisation affects several components of plant reproductive behaviour. Mycorrhizal plants pollen can sire more seeds; mycorrhizal plants produce more flowers and more pollen per flower than nonmycorrhizal plants (Poulton *et al.* 2001a,b). These effects are largely due to improved P nutrition, which is demonstrated by the appearance of the same effects in plants grown in conditions of higher P availability (Poulton *et al.* 2002). Mycorrhizal plants can flower for a longer period of time, produce more seeds, and have reduced levels of seed and fruit abortion (Carey *et al.* 1992, Koide *et al.* 1994, Lu & Koide 1994). The seeds of mycorrhizal plants may have considerably higher phosphorus content, which was also the case for seedlings emerging from seeds of mycorrhizal parents (Koide *et al.* 1988, Lu & Koide 1991). Thus, AM appears to have a parental effect on plant growth. In conditions of intraspecific competition, offspring of mycorrhizal parents are larger and show higher survival; a larger proportion of plants are reproductive, and in turn produce more seeds (Heppell *et al.* 1998).

Vegetative reproduction can be influenced by AM fungal colonisation as well. AM fungal colonisation has a strong effect not only on overall biomass of clonal plants, but has also been shown to affect clonal growth traits such as ramet number and size in *Prunella vulgaris* and *P. grandiflora*, the effect being independent of AM fungal effect on biomass (Streitwolf-Engel *et al.* 1997, 2001). Furthermore, different fungal isolates show differential impact on the clonal reproduction of the two *Prunella* species.

AM fungi can influence the outcome of interspecific (Fitter 1977, Hartnett *et al.* 1993) and intraspecific competition of plants (Moora & Zobel 1996). Competition intensity is generally increased by mycorrhizal colonisation, manifested in increased size inequality, while the mycorrhizal growth response itself is smaller at high plant densities (Moora & Zobel 1998, Facelli & Facelli 2002). There have been a number of studies where the root AM fungal colonisation of naturally growing plants has been suppressed with fungicides. The results of these studies show that reduction of AM root colonisation may

result in changes in relative abundance of plant species in a particular community (Koide *et al.* 1988, Gange *et al.* 1990, 1993). However, removal of mycorrhizal colonisation can also have no apparent effects on plant performance or abundance, though the particular plant species is consistently forming mycorrhizal relationship. In such a case, the function of symbiosis can be the alleviation of pathogen stress (Newsham *et al.* 1995), which in non-mycorrhizal conditions would adversely affect plant competitive ability, since, when removing AM fungi, the pathogenic fungi that are suppressed, if present, in mycorrhizal root systems may also be removed (West *et al.* 1993, Newsham *et al.* 1994).

Seedlings may become colonised with AM by 'entering' into the common mycorrrhizal network (CMN) (Newman 1988), as the mycelium of an AM fungus may colonise multiple plant individuals at the same time (Heap & Newman 1980, Francis & Read 1984). It could be expected that a seedling in the common mycorrhizal network would gain growth benefit compared to nonmycorrhizal seedlings or those not in the network (Koide & Dickie 2002), through the supply of mineral nutrients and, to a lesser extent, of the photosynthate spent on the fungus, presuming that the fungus receives it from larger plants. However, in pot-experiment conditions, mycorrhizal colonisation has not been shown to improve seedling growth in the vicinity of an adult plant as compared to nonmycorrhizal seedlings, whilst seedlings growing alone may gain significant growth benefit from some mycorrhizal fungi (Moora & Zobel 1998, Kytöviita *et al.* 2003). On the other hand, in a microcosm experiment simulating native grassland with a pre-existing mycelial network, seedlings clearly benefited from AM (van der Heijden 2004). Also, in a gap simulation experiment with disrupted mycorrhizal network, seedlings of highly mycorrhizal responsive plant species could barely survive without mycorrhiza (Francis & Read 1995).

Being aware of all the above effects of AM on plants as compared to non-mycorrhizal plants, it should be kept in mind that in natural conditions there are hardly any plant communities without AM fungi. Rather, the important aspects are the composition of the AM fungal community and both relative and absolute frequencies of individual AM fungi (species/isolates) as well as their spatio-temporal distribution.

Identifying the composition of AM fungal communities is so important because AM fungi differ in the provision of functions to plants and in their efficiency in each of the functions. The most obvious effects are those on plant biomass. In two breakthrough papers, van der Heijden *et al*. (1998a,b) demonstrated that combinations of AM fungi isolated from calcareous grassland and used to inoculate plant species from the same community show strikingly different effects on plant performance (measured as biomass). However, in a similar experiment with abandoned-field plant species and three AM fungal species from Hawaii (but not isolated from the same fields), the growth effects were rather minor (Stampe & Daehler 2003). The fungal isolates from the above

mentioned calcareous grassland are also known to differ in their effects on plant clonal growth (Streitwolf-Engel *et al.* 1998, 2001). The functional background of the above effects may be due to the fact that coexisting plant species obtain differential amounts of soil nutrients when inoculated with different AM fungi (van der Heijden *et al.* 2003). Probably the most thorough study of a single ecosystem, involving 10 AM fungal species and 10 local plant species from an old-field, demonstrated a considerable complexity in the host-fungus relations (Klironomos 2003). In the set of species in the study, there was no single fungus that enhanced or suppressed the growth of all plant species, or any plant species that responded uniformly negatively or positively to mycorrhizal colonisation.

Consequently, impacts of AM fungal taxa on plant populations are most probably different(ial), and not uniform. Because of such effects on plant species, the species composition of AM fungal communities can affect the outcome of plant competition (Fitter 1977, Hartnett *et al.* 1993, Ronsheim & Anderson 2001, Bever 2003) and the performance of plant individuals and populations.

OUTLINE OF THE THESIS

The thesis aims to identify patterns of AM fungal communities associated with potentially co-occurring congeneric plant species that show different abundance, and to investigate the effect of AM fungi on plant performance. Two plant species pairs were chosen for the detailed study of AM fungal communities in plant roots: rare *Viola elatior* and common *V. mirabilis*; less abundant *Pulsatilla patens* and more abundant *P. pratensis*. In order to further specify the effects of the distinguished AM fungal communities on plant performance, the two *Pulsatilla* spp. were inoculated with soils containing AM fungal communities from two different sites in a pot experiment of factorial design.

Earlier studies by our working group (Zobel & Moora 1995, Moora & Zobel 1996, 1998) demonstrated differential plant performance in the presence of a natural AM fungal community as compared to non-mycorrhizal conditions. Such a comparison, however, does not improve the understanding of natural patterns of plant distribution, since a non-mycorrhizal situation occurs only in exceptional cases in nature, such as exposure of new substrates after severe disturbance, etc. (Allen *et al.* 1992). Thus, we were interested in the effect of natural variation of AM fungal community composition and on the effect of different AM fungal communities on plants.

First, we hypothesised that different communities of AM fungi have different effects on the performance of the same plant species. Second, it was hypothesised that the plant species with lower abundance may display more specificity towards symbiotic fungi. Thus, the AM fungal communities of a less abundant plant species, compared to a common one, were expected to include fungi that are either not present, or are less consistently found in the roots of the more abundant plant species. Consequently, AM fungal communities of a less abundant plant would appear less diverse compared to a common plant, in case of a more strict specificity between fungi and a rare plant; or, they would appear more diverse, if there are fungi common to both plants, added by rare-plant-specific fungi. If specific relations were true, specificity towards a fungal symbiont would limit the establishment of a less abundant plant species in the case the specificity is mutual and the fungus/fungi is therefore not commonly present.

Further, it was hypothesised that a less abundant plant species, having more specific associations with its symbiont(s), shows a more pronounced differential performance in the presence or absence of the required organism(s), whilst the more abundant plant species may not show different performance in relation to different AM fungal communities, being less sensitive to the identity of fungal symbionts.

Therefore, the following questions were posed:

1. Are the communities of AM fungi variable in different localities?
2. Do the communities of AM fungi differ between congeneric plant species with different abundances?
3. Do different AM fungal communities have differential effects on a particular plant species?
4. Do plant species of different abundances respond differentially to particular AM fungal communities?

MATERIALS AND METHODS

Plant species

***Pulsatilla patens* (L.) Mill., *P. pratensis* (L.) Mill. (Ranunculaceae) (papers II, III).** Both species are long-living perennials with an upright, branching rhizome (Klimeš *et al.* 1997) that results in a clump in older plants. Vegetative spreading occurs only by infrequent splitting of bigger clumps (Rysina 1981, Wildeman & Steeves 1982). The roots are thick and little branched, reaching a considerable depth (*c.* 50–100 cm, M. Öpik, *pers. obs*.). Both species flower in early spring and are pollinated by insects; the seeds mature in mid-summer and are wind-dispersed. Germination occurs in late summer or in the next spring. Seedlings of the species are slow growing and rarely observed in nature. The species inhabit dry forests with relatively open canopies, grasslands, road verges, old gravel pits and other open sites.

P. patens s.l. shows circumpolar distribution, growing in Eurasia and North America (Hultén & Fries 1986). *P. patens* subsp. *patens* is confined to Eastern Europe and reaches its northernmost limit of distribution close to the White Sea (Jalas & Suominen, 1989). *P. pratensis* is a European endemic species with northernmost localities in southern Karelia (Hultén & Fries 1986, Jalas & Suominen 1989). There are presently only a handful of local Estonian populations of *P. patens* (Fig. 1a), the sizes of which vary from a single plant to a few populations containing thousands of individuals. In contrast to *P. patens*, *P. pratensis* is relatively abundant in many parts of the country (Fig. 1b), though there are also some small local populations in fragmented agricultural landscapes.

***Viola elatior* Fr., *V. mirabilis* L. (Violaceae) (paper IV).** The two species are perennial forbs, *Viola elatior* has a plagiotrophic branching rhizome and may show extensive vegetative spread; *V. mirabilis* has an upright branching rhizome and limited vegetative spreading ability (Klimeš *et al.* 1997). *V. elatior* prefers somewhat more well-lit, warm and moist habitats than *V. mirabilis* (Ellenberg *et al.* 1991), growing in mesic calcareous soils in wooded meadows and sparse deciduous forests, *V. mirabilis* in mesic calcareous wooded meadows and deciduous and mixed forests (Kukk 1999). Both species produce chasmogamous seeds from open insect-pollinated violet flowers in spring, and cleistogamous seeds from closed obligately self-fertilised flowers during the whole summer.

V. elatior is distributed from central Europe to the mountains of central Asia, though the Asiatic distribution is not fully known, *V. mirabilis* in central and Eastern Europe and western Asia (Hultén & Fries 1986). Despite relatively similar ecology and morphology, these two species show contrasting abundances in Estonia (Kukk 1999) and elsewhere. In Nordic countries, *V. mirabilis* is widespread while *V. elatior* occurs only in some localities on the island of Öland (Mossberg *et al.* 1992, Gärdenfors 2000). In Estonia,

V. mirabilis is common (Fig. 1d) while *V. elatior* is recorded from 11 to 13 local populations (Estonian Flora, Talts (1973) and the database of Distribution Maps of Estonian Vascular Plants, Institute of Zoology and Botany, Estonian Agricultural University) (Fig. 1c).

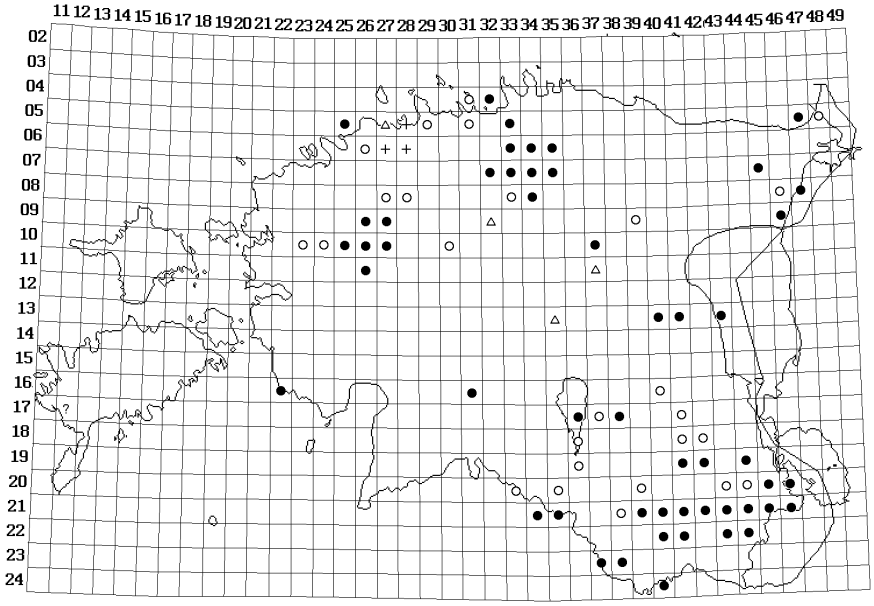

Fig. 1a.

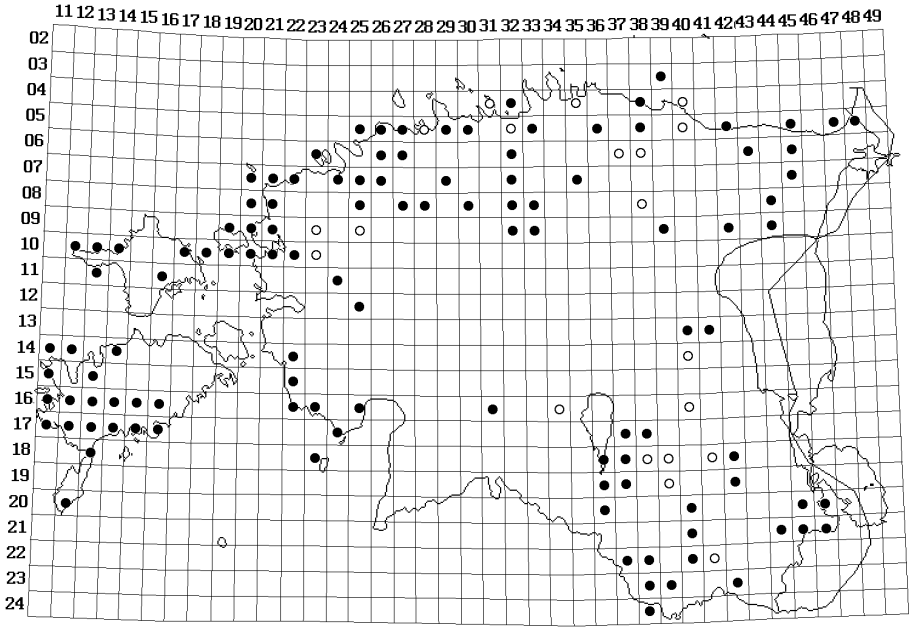

Fig. 1b.

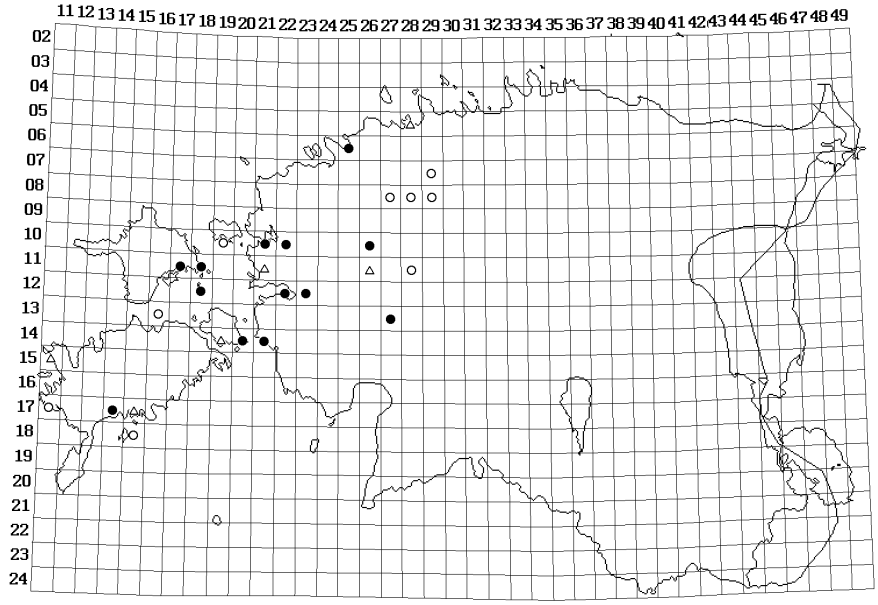

Fig. 1c.

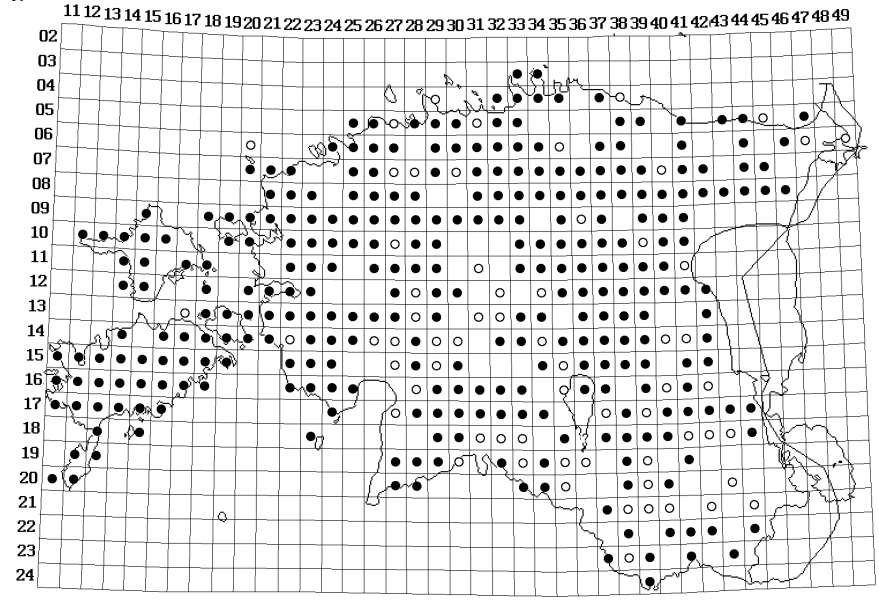

Fig. 1d.

Fig. 1. Distribution of (a) *Pulsatilla patens*, (b) *P. pratensis*, (c) *Viola elatior*, and (c) *V. mirabilis* in Estonia. Filled circles — recordings after 1970; open circles — 1921–1970; open triangles — before 1921; + – extinct habitat; ? — uncertain recording. All four distribution maps originate from the database of Distribution Maps of Estonian Vascular Plants, Institute of Zoology and Botany, Estonian Agricultural University (reproduced with permission).

Fungal reference species

The species and isolates of AM fungi used as reference taxa in molecular analyses are listed in Table 2.

Table 2. List of fungal isolates used as references in molecular analyses.

Fungal species	Isolate	Provided by	Paper
Acaulospora laevis GERD. & TRAPPE	BEG 13	M. Giovannetti	II
Glomus caledonium (T. H. NICHOLSON & GERD.) GERD. & TRAPPE	BEG 86	S. Rosendahl	IV
G. geosporum (T. H. NICHOLSON & GERD.) C. WALKER	BEG 11	V. Gianinazzi-Pearson	II
G. geosporum (T. H. NICHOLSON & GERD.) C. WALKER	BEG 90	S. Rosendahl	IV
G. intraradices N.C. SCHENCK & G.S. SM.	IMA 6	M. Giovannetti	II
G. mosseae (T. H. NICHOLSON & GERD.) GERD. & TRAPPE	BEG 84	S. Rosendahl	II, IV
Scutellospora castanea C. WALKER	BEG 1	V. Gianinazzi-Pearson	II

Field sites and sampling of natural plant roots

***Pulsatilla* spp. (papers II and III).** Roots of naturally growing adult *P. patens* and *P. pratensis* were collected in August 1999 from the sites listed in Table 3. More detailed descriptions of the two sites whose soil was used as inoculum in pot experiments is given in **Paper III**. The distance between sites was, in all cases, at least 60 km (Fig. 2). Roots of three plant individuals of the species present at each site (except Soomaa, where only one individual was sampled, due to the very small population size) were excavated from a depth of 15–45 cm and stored in 40% ethanol at 4 ºC until processing.

***Viola* spp. (paper IV).** Plant roots were sampled in September 1999 from five sites (Fig. 2), the details of which are given in Table 4. Sites 1, 2 and 3 are located within a circle of 10 km; the distance to other sites was over 100 km. The whole root system of a sample plant was excavated, washed, and stored in 40% ethanol at 4 ºC. Two individuals of each species were sampled per site. From each individual, six, 1 cm-long fragments of fine roots were randomly taken for molecular analysis. From some samples fungal DNA was not detected in nested PCR (Table 4).

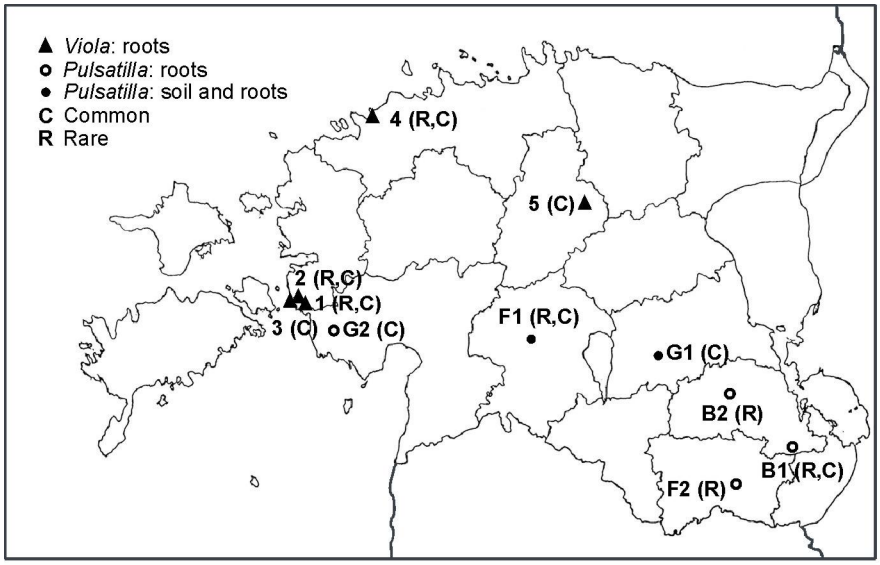

Fig. 2. Field sites of origin of plant root samples for AM fungal diversity description (*Pulsatilla* — **paper II,** *Viola* — **paper IV**) and soil samples for plant performance experiment (*Pulsatilla* — **paper III**).

Table 3. Field sites of origin of soil inocula and root samples (papers II and III).

Site code	Site	Location	Characteristics	Sampled		Study plants present	
				Soil	Roots	*P. patens*	*P. pratensis*
F1*	Soomaa	Central Estonia	Extensive boreal Scots pine forest	+	+	+	+
B1*	Piusa	SE Estonia	Dry open area alongside railway line, bordered by a boreal Scots pine forest		+	+	+
G1*	Pangodi	Central Estonia	Dry meadow within agricultural landscape	+	+		+
G2	Varbla	W Estonia	Dry meadow within forested landscape		+		+
F2	Vastseliina	S Estonia	Boreal Scots pine forest		+	+	
B2	Palo	Southern-Central Estonia	Roadside area borders by a boreal Scots pine forest		+	+	

*F – forest, B – 'borderland', G – grassland

Table 4. Field sites of origin of *Viola* spp. root samples. Two plant individuals were excavated from each site and six root fragments were subjected to molecular analysis. Number of root fragments successfully amplified by PCR is given in parentheses (**paper IV**).

Site code	Site	Location	Ecosystem	Characteristics	Soil	Management	Samples collected (amplified) per plant	
							V. elatior	*V. mirabilis*
1	Laelatu	W Estonia	wooded meadow	rich in vascular plant species	calcareous, 15–20 cm thick humus-rich layer, bedrock limestone rubble	mowing; has been carried out ±continuously	1: 6 (4) 2: 6 (3)	1: 6 (5) 2: 6 (4)
2	Virtsu	W Estonia	former wooded meadow	rich in vascular plant species		mowing; ceased ca 10–15 yr ago	1: 6 (2) 2: 6 (6)	1: 6 (6) 2: 6 (6)
3	Puhtu	W Estonia	coastal deciduous forest	rich in vascular plant species; dominants *Quercus robur* L., *Fraxinus excelsior* L.		old forest stand	Not present	1: 6 (5) 2: 6 (3)
4	Niitvälja	NW Estonia	former wooded meadow	within agricultural landscape; meadow is overgrown by shrubs (*Corylus avellana* L.) and deciduous trees (*F. excelsior*, *Populus tremula* L., *Betula* spp.)	calcareous, 25–30 cm thick humus layer, bedrock limestone-rich fine material	mowing; ceased ca 20–30 yr ago	1: 6 (6) 2: 6 (6)	1: 6 (5) 2: 6 (5)
5	Koeru	Central Estonia	coniferous forest	dominants *Picea abies* (L.) H. Karst., *C. avellana*	brown forest soil	old forest stand	Not present	1: 6 (6) 2: 6 (6)

Design of experimental studies and sampling (papers II and III)

Mature seeds of *Pulsatilla patens* and *P. pratensis* were collected at the end of June and beginning of July 1999 from three local populations of both species in Estonia and were then pooled. Seeds, visually examined and carefully selected to avoid those attacked by herbivores or pathogenic fungi, were sown on 16th July 1999 (hereafter called the summer experiment; **paper III**) and on 08th February 2000 (hereafter called the spring experiment; **paper II**). These dates approximately mimic the time of real establishment in nature — either immediately after seed set (June-July), or from the transient over-wintered seed bank in spring (March-April).

The natural soils used for the seedling establishment experiments originated from two of the sites — a grassland G1 and a Scots pine forest F1 — where adult plants had also been sampled (Fig. 2, Table 3). The soils, dry arenosols with weakly differentiated horizons, were collected from ten random locations in both target ecosystems in the first half of July 1999 and in the second half of August 1999, for the summer and spring experiment respectively. Topsoil samples (a grey mineral layer at a depth of 2–10 cm underlying the thin litter layer) from each site were pooled for use in the experiments.

In the summer experiment, a 1:1 mixture of natural soil and sterile sand was used as an establishment substrate; sterile sand served as non-mycorrhizal control. The experiment was conducted under natural conditions in the Botanical Garden of the University of Tartu. Seeds were sown at a constant density (1.2 seeds/cm^2) into pots (4 × 13 × 18 cm, depth × width × length). Pots were carefully watered with tap water as required. Treatments were replicated six times. 10 to 12 seedlings were sampled at the age of nine weeks (time after mass germination) and used for molecular identification of root AM fungi.

In the spring experiment, a 1:1 mixture of the two natural soils was used, where one of the soils had been autoclaved (40 min at 121 °C); a 1:1 mixture of two autoclaved soils served as non-mycorrhizal control. The soil parameters of different mixtures were fairly similar (Table 1 in **III**). The experiment was conducted in a greenhouse of the Viikki Biocentre of Helsinki University. Seeds were sown at the same density as above into pots (9 × 12 cm, depth × diameter), and later thinned to one individual per pot. Pots were carefully watered with tap water as required. Every treatment was replicated 10 times. Plants were grown in daylight (day length 16 h) for 14 weeks. Four to five plants per treatment were harvested five weeks (first harvest) and 14 weeks (second harvest) after germination for 1) biomass and nutrient (tissue N and P) analyses, 2) molecular analyses, and 3) root colonisation estimation.

Estimation of mycorrhizal colonised root length (paper III)

The percentage of AM root colonisation was estimated on the basis of full root system (first harvest) or 1–2 g (fresh weight) random root pieces (second harvest) of *Pulsatilla* seedlings. Root samples were stained with typan blue according to Koske and Gemma (1989) and the percentage colonisation was determined following Rajapakse and Miller (1992).

Molecular analysis

DNA extraction from roots and spores (papers II and III). DNA was extracted from the whole root system of an experimental seedling or several randomly sampled root segments from field plants (total length *c*. 5 cm). The DNA extraction procedure involving modified chloroform extraction and isopropanol precipitation method, was performed as in Heinonsalo *et al.* (2001). Shortly, root samples were ground in 750 µl CTAB buffer (2% cetylammoniumbromide, 20 mM EDTA, 100 mM Tris-HCl, 1.4 M NaCl) with help of fine quartz sand and micropestle, incubated at 65°C for 1 h, and centrifuged (14 000 g) for 5 min. Thereafter, equal volume of chloroform was added to the supernatant and samples were centrifuged 15 min (14 000 g). The upper phase was collected, precipitated with 750 µl of isopropanol at –20°C for at least 1 h and centrifuged for 30 min (14 000 g). The pellet was washed with 200 µl ice-cold (–20°C) 70% ethanol, centrifuged 5 min at 7000 g, dried and resuspended in 25 µl water or TE.

DNA was also extracted from batches of 5–20 spores of control strains pre-cleaned by sonication for 2–3 seconds, twice in sterile water and once in TE buffer. Spores were crushed in 50 µl TE and centrifuged five min at 13000 rpm to remove spore debris.

DNA extraction from roots and spores (paper IV). DNA was extracted from a total of 96, 1 cm-long root pieces in total by the Chelex extraction method (van Tuinen *et al.* 1998b). DNA was extracted from spores of reference fungi by washing the spores twice in sterile water and crushing them in 50 µl TE buffer. After centrifugation (5 min. 14 000 g) the supernatant was directly used in PCR.

PCR and Denaturing Gradient Gel Electrophoresis (papers II and III). An approximately 590 bp fragment of small subunit ribosomal RNA gene (SSU rDNA) was amplified by PCR using the universal eukaryotic primer NS31 (Simon *et al.*, 1992; Fig. 3), extended to include a GC-clamp (Table 5), paired with a more AM fungal specific fungal primer AM1 (Helgason *et al.* 1998, Fig. 3) designed to exclude plant DNA sequences. The PCR cocktail (total volume 50 µl) contained 1 unit DyNAzyme^{TM} II DNA Polymerase (Finnzymes

Table 5. List of primers used to amplify AM fungi from plant roots.

Primer	Sequence (5'–…–3')	Gene	Specificity	Paper	Reference
NS31f	TTG GAG GGC AAG TCT GGT GCC	SSU	Eukaryotes	II	Simon *et al.* 1992
AM1r	GTT TCC CGT AAG GCG CCG AA	SSU	(AM) fungi	II	Helgason *et al.* 1998
NS31-GCf[1]	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G-TTG GAG GGC AAG TCT GGT GCC	SSU	Eukaryotes	II	**Paper II**
LSU 0061f (=LR1)	AGC ATA TCA ATA AGC GGA GGA	LSU	Eukaryotes	IV	van Tuinen *et al.* 1998b
LSU 0599r[1] (=NDL22)	TGG TCC GTG TTT CAA GAC G	LSU	Eukaryotes	IV	van Tuinen *et al.* 1998b
LSURK 4f	GGG AGG TAA ATT TCT CCT AAG GC	LSU	*Glomus mosseae-intraradices* group	IV	Kjøller and Rosendahl 2000
LSURK 7r	ATC GAA GCT ACA TTC CTC C	LSU		IV	Kjøller and Rosendahl 2000

[1] f – forward primer, r – reverse primer.

OY, Espoo, Finland), 1 × DyNAzyme™ buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton® X-100), 25 pmol of each primer, 200 nmol of each dNTP, 0.1% dried non-fat milk (De Boer *et al.* 1995, Edwards *et al.* 1997), and 25 µl DNA template. Thermocycling was carried out as follows: 3 min at 95°C followed by 30 or 40 cycles of 45 sec at 94°C, 1 min at 60°C, 3 min at 72°C, and a final extension 7 min at 72°C in a PTC-100 thermocycler (MJ Research Inc., Waltham, MA, USA). Negative water or TE buffer controls were included to check for contamination of reagents. Amplified SSU rDNA fragments were identified in 1.6% agarose gels (Sambrook *et al.* 1989). Where required, two or three 50 µl PCR reactions were performed from a DNA template, the products pooled, and the DNA concentrated by isopropanol precipitation and ethanol washing as before.

DGGE was performed with the DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). Electrophoretic parameters were as follows: 6% (wt/vol) polyacrylamide gel (37.5:1 acrylamide:bis-acrylamide, 1× TAE buffer, 1 mm thick, 16 × 16 cm) containing a gradient of a denaturant from 22 to 35%, generated with urea-formamide (Muyzer *et al.* 1993). Electrophoresis was carried out for four hours at 150 V in 1× TAE buffer at a constant temperature of 60°C. Gels were stained with ethidium bromide and visualised under UV epifluorescence light using a Fluor-S Imager and Quantity One software (both Bio-Rad, Hercules, CA, USA).

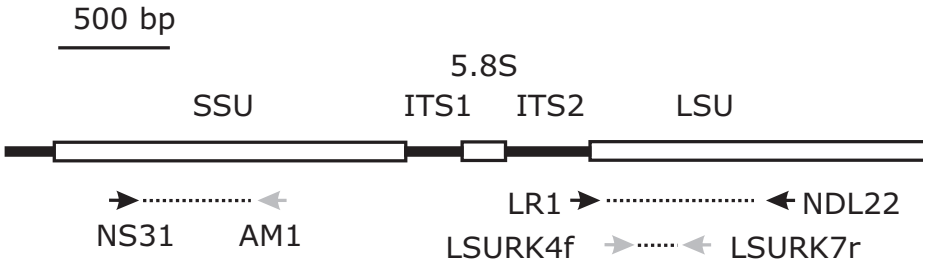

Fig. 3. Positioning of the PCR primers used in the study. Boxes, RNA genes; thick lines, transcribed spacers; black arrows, general primers; grey arrows, specific primers.

Cloning and sequencing (papers II and III). All DNA fragments clearly resolved by DGGE were excised from gels and stored in a 50 µl TE buffer at –20ºC until processed. DNA in TE buffer was reamplified with primers NS31/AM1 as before (no non-fat milk added), with a slightly modified thermocycling programme (3 min at 95°C followed by 40 cycles of 45 sec at 94°C, 45 sec at 60°C, 45 sec at 72°C, and a final extension of 10 min at 72°C), and the fragment of expected length (550 bp) was purified from low temperature gelling agarose gel by a modified glassmilk purification method

using silica instead of glassmilk (Boyle & Lew 1995). Purified PCR products were ligated into pGEM®-T Easy vector and cloned in *E. coli* JM109 (both Promega, Madison, WI, USA) according to the manufacturer's instructions. Four to six putative positive clones were screened for sequence differences by NS31/AM1 amplification and restriction analyses (*Alu*I, *Hin*fI and *Rsa*I). Representative sequences of all the different RFLP types detected from roots of single plants were sequenced. Inserts of clones to be sequenced were re-analyzed by DGGE under the described conditions. Following this quality check, re-confirmed sequences containing plasmids were isolated using the Wizard® *Plus* Minipreps DNA Purification System (Promega, Madison, WI, USA). Sequencing was carried out by cycle sequencing of both strands of the insert with primers T7 and SP6 (A. I. Virtanen Institute, University of Kuopio, Kuopio, Finland, and Institute of Biotechnology, University of Helsinki, Helsinki, Finland). Sequences are lodged in the EMBL database under the accession numbers AJ418855-AJ418900 and AJ496040-AJ496119.

PCR and SSCP (paper IV). Nested PCR with eukaryote-specific primers LSU 0061/LSU 0599 (LR1/NDL22, van Tuinen *et al.* 1998b; Table 5, Fig. 3) in the primary PCR reaction and specific primers LSURK4f/LSURK7r (Kjøller & Rosendahl 2000; Table 5, Fig. 3) in the nested reaction was performed as in Kjøller and Rosendahl (2000), except that the primary PCR product was diluted 1:100 prior to use in the nested reaction. The two primer combinations amplify 700 and 300 bp fragments of LSU, respectively. Products of positive nested PCR reactions (77 samples in total) were analyzed by SSCP electrophoresis (Kjøller & Rosendahl 2000). SSCP gels were run for 3 hours at 4ºC using Hoefer Mighty Small™ II SE 250 horizontal gel electrophoresis units (Hoefer Scientific Instruments, San Fransisco, CA). Gels were stained by silver staining using PlusOne DNA Silver Staining Kit (Amersham Pharmacia Biotech, UK).

The SSCP banding patterns obtained from different root samples were compared by eye and the bands grouped according to similar mobility. Usually, two bands occurring close to each other were recognised as a sequence group, presuming that two single strands of the same DNA fragment have folded in distinct conformations and have moved at slightly different speeds. If only a single band was present, it was interpreted as a separate group, presuming that two single strands have folded up in one conformation in the case of such a particular combination of nucleotides. In cases where gel-to-gel comparisons of band positions were hard to make, the samples from different gels were re-run together.

Analysis of molecular data (paper II)

Forward and reverse strands of sequenced products were assembled with Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were aligned manually using Se-Al Sequence Alignement Editor 2.0 (http://evolve.zoo.ox.ac.uk/software/Se-Al/main.html), taking SSU rRNA secondary structure information into account (Wuyts *et al.*, 2002). Similarity comparisons were performed with the BLAST 2.0 algorithm (Altschul *et al.* 1997). Sequences were screened for possible chimeric origin using the Chimera Check 2.7 algorithm of the Ribosomal Database Project II (RDP-II) internet site (http://rdp.cme.msu.edu). In addition, sequences of 58 glomalean and two outgroup taxa (*Endogone pisiformis* Link, *Mortierella polycephala* Coem.) were acquired from GenBank/EMBL databases. The sequence alignment is deposited in the EMBL database (accession number ALIGN_000585). The aligned data matrix included 529 characters. 25 ambiguously aligned nucleotide positions were excluded from further analysis. Phylogenetic analyses were performed with PAUP* version 4.0b10 for Macintosh (Swofford 2002) as follows: 1) maximum parsimony analyses: (a) heuristic search option, random addition of sequences, 100 replicates, tree bisection-reconnection (TBR) swapping, MulTrees on, all characters unordered and of equal weight, gaps treated as missing characters, no more than 50 trees saved in each replicate; (b) heuristic search option, TBR swapping of shortest trees found in previous analysis, all characters unordered and of equal weight, gaps treated as missing characters. 45100 trees were found that were of the same length as the shortest tree in the first analysis. Not all trees were found because of computer memory constraints; 2) parsimony bootstrap values were computed over 100 replicates, no more than 500 trees saved in each replicate because of computer time constraints; 3) distance analysis: neighbour joining analysis with Kimura 2-parameter substitution model.

Statistical data analysis

Paper II. AM fungal communities in *P. patens* and *P. pratensis* root samples were described on the basis of presence/absence of fungal sequence groups identified in the phylogenetic analysis. Compositional analysis of the fungal communities was performed using multivariate cluster analysis implemented in PC-ORD ver. 4.01 for Windows (McCune & Mefford 1999). Similarities in root colonising AM fungal community composition among samples were identified following the application of Ward's linkage method with the Euclidean distance measure.

Paper III. For analysis of plant establishment rate, repeated measures ANOVA was conducted with plant species (*P. patens, P. pratensis*) and soil inoculum (forest, grassland, sterile) as fixed factors, and time as the repeated measures factor. Biomass, percentage of root AM fungal colonisation, and tissue P and N concentration (percentage of plant dry biomass) data were subjected to ANOVA. Biomass and percentage AM fungal colonisation data were log and arcsin transformed, respectively, prior to statistical analysis. All analyses were conducted with the Windows version of STATISTICA (StatSoft, Inc., Tulsa, OK, USA).

Similarities of AM fungal communities were calculated on the basis of the fungal sequence groups presence/absence in a root system by multivariate cluster analysis (Ward's linkage method and Euclidean distance measure) implemented in PC-ORD ver. 4.01 (McCune & Mefford 1999).

Paper IV. The AM fungal communities in roots of plant individuals were classified and the distribution of the community types described by rearranging the sample (plant individual)-AMF species table, using two-way indicator species analysis (TWINSPAN). This method unites ordination and classification of samples, utilising presence/absence of pseudo-species defined by the logarithmic frequencies of AM fungal sequence types as computed in PC-ORD 4.01 (McCune & Mefford 1999). AM fungal sequence types were classified as constant, characteristic, or differentiating types according to a common routine in plant sociology (Westhoff & van der Maarel 1973). In addition, the dataset of 1 cm-long root samples (AM fungal species detected/not detected) and the data on the whole plant individuals (detection frequencies of AM fungal species) were ordinated separately using DCA, implemented in PC-ORD 4.01 (McCune & Mefford 1999). The effect of plant species and study site on AM fungal species richness per plant was estimated using two-way ANOVA with mixed factors and Tukey's HSD test in SAS 6.12 (Littell et al. 1996). In the mixed model of species richness in 1-cm-long root fragments, the plant individual was considered as a random factor.

RESULTS

Diversity of AM fungi in plant roots: number and identity of sequence groups/genotypes (papers II, IV)

Arbuscular mycorrhizal (AM) fungi were detected in roots of two plant species pairs: less abundant *Pulsatilla patens* and more abundant *P. pratensis* (**paper II**), rare *Viola elatior* and common *V. mirabilis* (**paper IV**).

Paper II. AM fungi in the roots of *Pulsatilla* spp., growing either in the pot experiment or in the field, were identified with the help of AM fungal-specific PCR of nSSU rDNA, DGGE (Fig. 2 in **II**), cloning, sequencing and phylogenetic analysis.

19 sequence groups of AM fungi were recognised in *Pulsatilla* spp. roots (Fig. 4): 14 putative *Glomus*, two *Acaulospora*, two *Scutellospora* and one *Gigaspora* grouping. Four sequence groups showed high similarity to database sequences of respective glomalean species or isolates: MO-G2 (*Glomus* sp. UY 1225), MO-G7 (*G. hoi*), MO-G11 (*G. mosseae*), MO-G10 (*G. caledonium*). Six other sequence groups were related to root-derived, but taxonomically unknown sequence types: MO-G3 (Glo8), MO-G5 (Glo2), MO-G6 (Glo7), MO-G1 (Glo21), MO-G4 (Glo18), MO-G12 (Glo13). The remaining ten sequence groups showed no closely related database sequences, nor relationships with known taxa was resolved by phylogenetic analyses.

The number of AM fungal sequence groups detected in *Pulsatilla* root samples ranged from zero to six, on average, 1.76 and 1.33 groups in the pot-experiment and field plant roots (samples with no amplification excluded), respectively. The statistical significance of the above difference was not estimated due to imbalance of sample sizes of experimental and field plants.

The total number of AM fungal sequence groups detected from a site, taking into account both experimental plants and naturally growing plants, was 14 in grassland (G1) and 10 in forest (F1) roots (Table 1 in **II**).

Paper IV. AM fungi in the roots of field collected *Viola* spp. plants were detected by SSCP fingerprinting, where presence/absence of bands at specific positions was interpreted as presence/absence of particular fungal genotypes. The fungal LSU rDNA was amplified from plant roots by nested PCR with primers specific for the *Glomus mosseae-intraradices* species group.

12 distinct AM fungal genotypes were detected altogether. Spores of three fungal isolates (*Glomus mosseae*, *G. geosporum*, *G. caledonium*; Table 2) were concurrently analysed by SSCP, but the banding patterns of these species did not match with any of the genotypes recognised from plant root samples.

The number of AM fungi detected from 1 cm root fragments (3.8 in average) and plant root systems as a whole (7.5 in average) did not differ significantly between plant species or sites. The number of AM fungi was smaller in root fragments of *V. elatior* from site 1 as compared to site 2 (Tukey's HSD test, $P < 0.05$; plant species and site interaction significant, $F = 5.19$, $P = 0.008$).

Communities of AM fungi: plant species related patterns (papers II, IV)

Paper II. Multivariate cluster analysis by Ward's linkage method (Fig. 6) was performed in order to identify patterns in AM fungal communities of experimental and field collected roots of *Pulsatilla* species. The AM fungal community composition did not show any clear host plant specific patterns (but see below). There were eight and two infrequent sequence groups, which were identified only from the roots of *P. pratensis* and *P. patens*, respectively.

Paper IV. The AM fungal communities of *Viola* spp. were analysed with the aid of TWINSPAN and DCA. In both analyses, fungal communities in the whole root systems of rare *V. elatior* appeared to be more variable in comparison with those of common *V. mirabilis* (Fig. 1 and Table 2 in **IV**).

TWINSPAN analysis distinguished constant fungal genotypes (Co), detected at all sites and in both plant species, characteristic genotypes for sample groups (ChA1, ChB), and sample group differentiating genotypes (D). The samples of *V. elatior* appeared in marginal positions in the TWINSPAN table (Table 2 in **IV**) and were more variable in genotype composition, compared to the centrally positioned and more uniform *V. mirabilis* samples. Samples of the same plant species from sites 3, 4, and 5 were located side-by-side in the TWINSPAN table, indicating high similarity. Samples of the two plant species from site 4 appeared to be clearly different in their fungal communities (Table 2 in **IV**).

Detrended correspondence analysis (DCA) ordination was applied to the dataset of AM fungal communities in whole plant root systems (frequencies of genotypes) and to the dataset of individual root fragments (presence/absence data of fungal genotypes). The first two axes of the DCA ordination of individual plant data described 45.3% of the total variation in fungal community composition. Again, the *V. mirabilis* samples showed higher similarity within and between sites; the *V. elatior* samples were scattered near the margins of the ordination plane (Fig. 1 in **IV**).

DCA of the root fragments dataset (presence/absence of genotypes) showed much greater variation among samples, and samples from individual plants were scattered over the ordination plane (Fig. 5). This indicates considerable

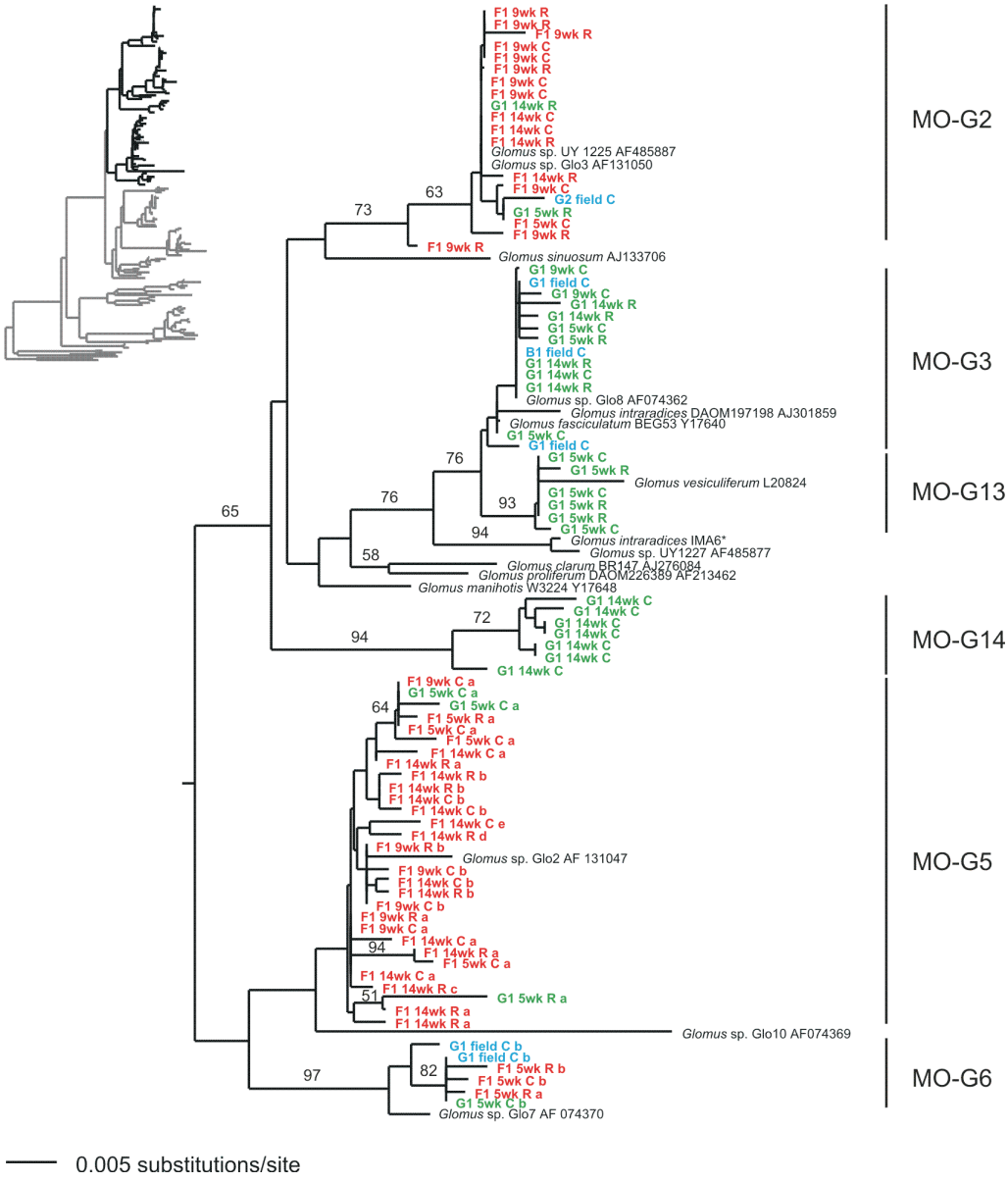

Fig. 4. Neighbour joining tree inferred from nSSU rDNA sequences of all identified AM fungi (Glomeromycota taxa) in *Pulsatilla* spp. roots and corresponding sequences of known and unknown (field samples) taxa, using *Endogone pisiformis* and *Mortierella polycephala* as outgroup species. The sequence groups (MO-G2 etc.) identify distinct clusters of sequences with similarity >97%. Branch lengths correspond to the expected nucleotide substitutions per site. Parsimony bootstrap frequencies higher than 50% (100 replicates) are shown. Note source colour coding: blue, field plants; red and green, experiment pot plants grown on forest F1 and grassland G1 soil inoculum, respectively. Individual sequences codes are identified as follows: (locality) (harvest) (host species). Locality: as in Fig. 5. Harvest: field — field plants; 9 wk — seedlings from summer experiment; 5 wand 14 wk — seedlings from spring experiment. Host species: R — rare *P. patens*; C — common *P. pratensis*. * — isolate sequenced in this study. a, b, c, d, e identify different RFLP types in groups MO-G5 and MO-G6.

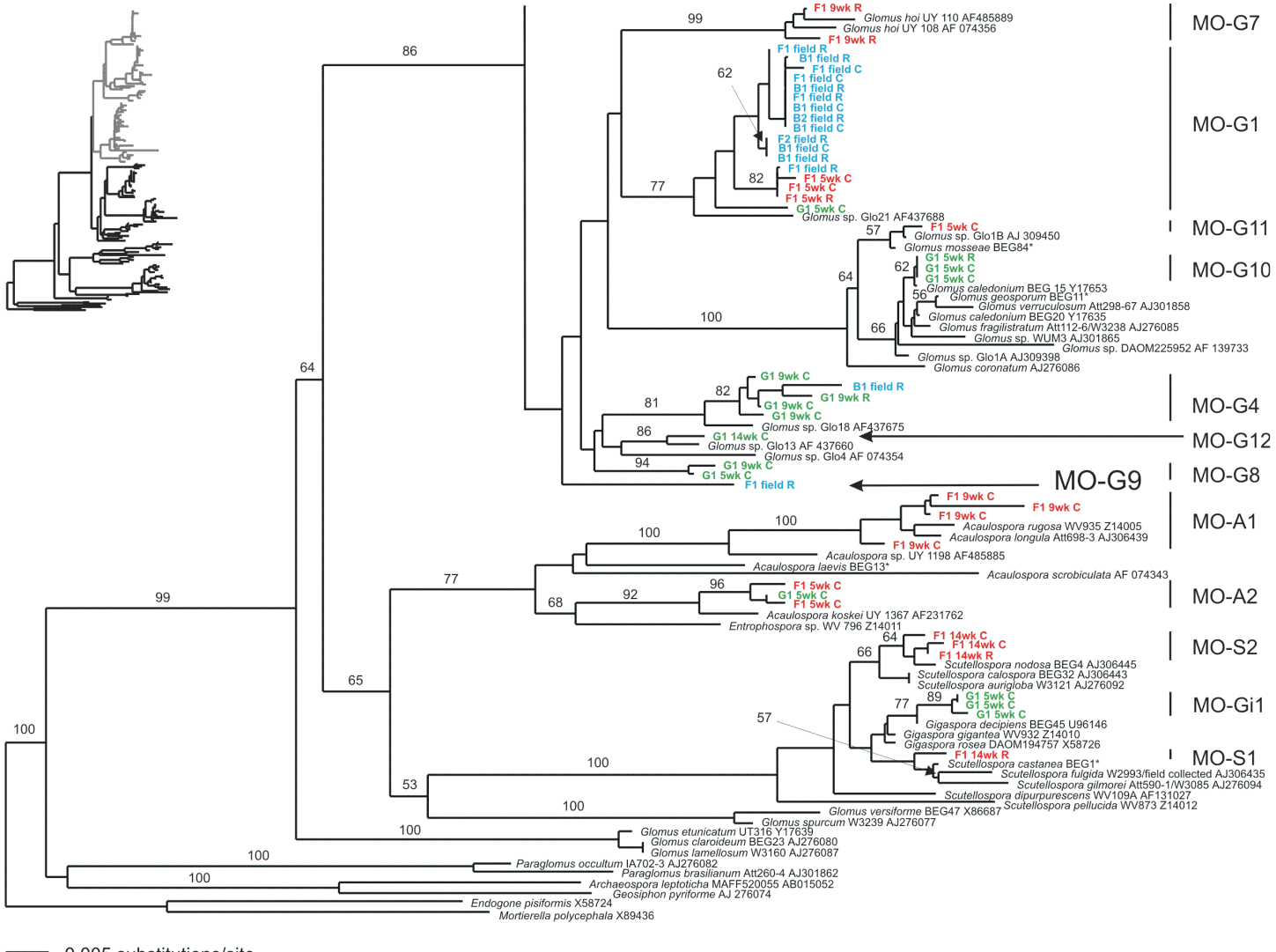

Fig. 4 *(continued).*

within-root-system variation of AM fungal communities, whereas total variation between plant individuals is much smaller, suggesting also different spatial niche occupancy of fungi.

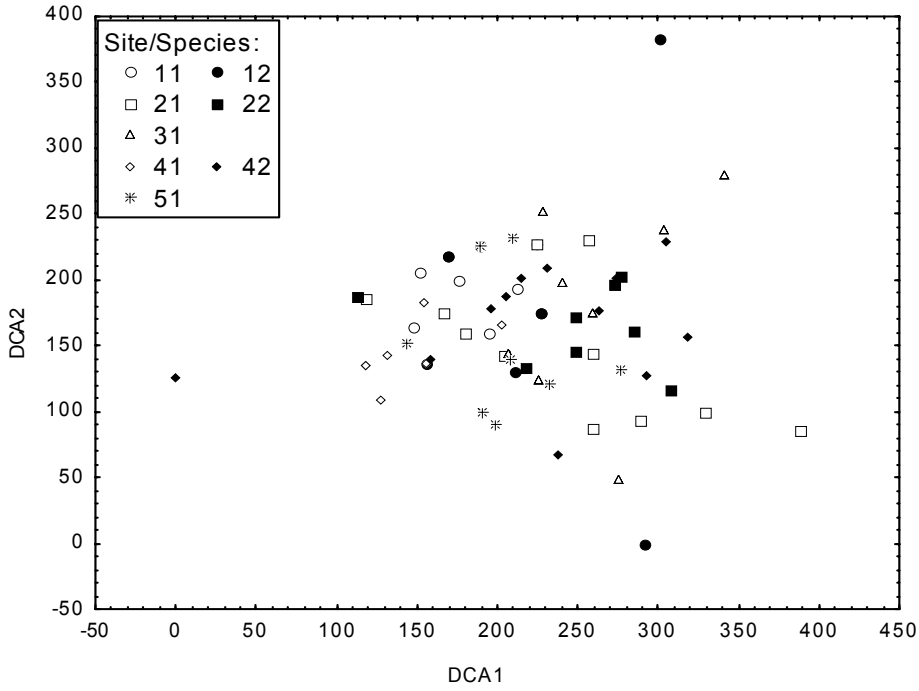

Fig. 5. DCA of AM fungal communities in 1-cm-long root fragments of *V. mirabilis* (1, open symbols) and *V. elatior* (2, filled symbols) from five and three sites, respectively. Site codes as in Table 4.

Communities of AM fungi: site-related patterns (papers II, III, IV)

Papers II, III. Multivariate analysis identified site-related differences in AM fungal community composition in the roots of *Pulsatilla* spp. When the dendrogram was pruned at the level 50% information retained, six major clusters were recognised. Most field plants appeared in a single well-defined cluster, whilst the plants from the pot-experiment were clearly grouped according to the origin of soil inoculum (Fig. 6).

The composition of AM fungal communities in the roots of experimental *Pulsatilla* spp. showed differences among field sites — the occurrence and abundance of fungal sequence groups in the forest site differed from that in the grassland site (Table 1 in **II**). Sequence group MO-G3 was exclusively characteristic to grassland-soil-treatment plants, occurring in 45.5% of success-

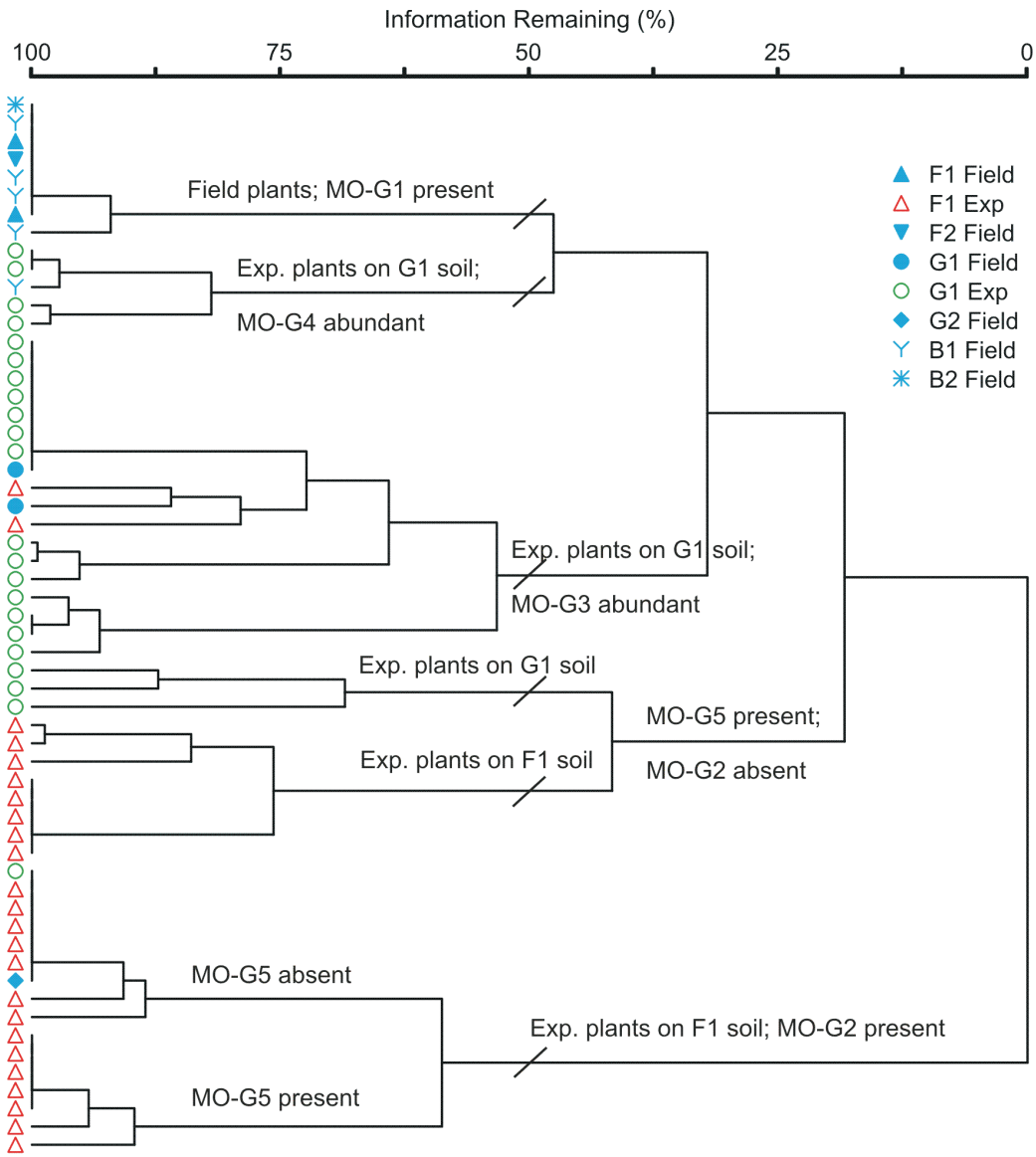

Fig. 6. Grouping of root AM fungal communities in relation to soil inoculum treatment of experimental plants; field plants from six sites are included. Hierarchical cluster analysis by Ward's linkage method with Euclidean distance was used. Community similarities were calculated on the basis of fungal sequence group presence/absence within root samples. Sample groups were defined at the level of 50% information remaining as indicated with '/' marks. G — dry meadow within fragmented agricultural landscape, F1 — extensive boreal pine forest area, B1 — open sandy area bordered by boreal pine forest, G2 — dry meadow, F2 — boreal pine forest, B2 — roadside bordered by boreal pine forest. Source colour coding: blue — field plants; red and green — experiment pot plants grown on forest F1 and grassland G1 soil inoculum, respectively.

fully amplified samples. Two other sequence groups, MO-G2 and MO-G5, were characteristic to forest-soil-treatment plants (in 58.3% and 62.5% of samples); these groups were also present in grassland-soil-treatment plants, but at lower frequencies.

Paper IV. In DCA ordination diagram, *Viola* spp. samples from the same site tended to be located close to each other, but no clear differentiation between AM fungal communities originating from each of the six sites was obtained (Fig. 1 in **IV**).

Communities of AM fungi: field vs. pot-experiment plants (paper II)

Pulsatilla spp. field plants were almost exclusively colonised by a single sequence type, MO-G1, which was rarely detected in pot-experiment plant roots. Five other sequence groups identified in field plant roots were more infrequent, including a sequence group specific to adult plant roots (MO-G9) (Table 1 in **II**).

Plant performance as related to AM fungal colonisers (paper III)

Performance of *Pulsatilla* spp. when colonised by different AM fungal communities was tested in a seedling establishment experiment using soil inocula from grassland G1 and forest F1 sites. The seedling establishment of *Pulsatilla* spp. was significantly influenced by the type of soil inoculum ($F = 14.19$, $P < 0.001$) and was lower on sterile control soil (28% *P. patens*, 21% *P. pratensis*), compared to natural soil treatments. In natural soil treatments, the final establishment rates of *P. patens* (51%) and *P. pratensis* (30%) were significantly different ($F = 10.58$, $P = 0.002$). The establishment rate was higher on forest F1 soil as compared to grassland G1 soil (45% and 36%, respectively). There was a significant interaction between plant species and time ($F = 18.03$, $P < 0.001$), indicating that the establishment rates of the two species were similar at first, but that later the establishment of *P. patens* was higher than that of *P. pratensis*.

Plant biomass, root AM fungal colonisation and tissue phosphorus concentration were significantly higher, but tissue nitrogen concentration lower, in plants grown with grassland soil inoculum, compared to plants grown with forest soil inoculum (Fig. 1 and Table 2 in **III**). The plant biomass did not differ between the two plant species, but soil inocula had differential effects on the growth of the plant species ($F = 3.24$, $P = 0.043$). Inoculated with grassland G1

soil, *P. pratensis* displayed more vigorous growth than *P. patens*; forest F1 soil inoculum did not induce differential growth of the two plant species. The grassland inoculum induced an order of magnitude higher rate of plant growth in both species than the other two soils did (Fig. 1B in **III**). The shoot biomass variation was apparently the main cause of the total biomass variation, since mostly the same significant main effects and interactions were found in the analyses of shoot and total biomass. Root biomass differed among soil treatments and in time. The grassland soil supported the highest root production; root biomass of plant species did not differ. Shoot to root ratio decreased with time on the grassland soil inoculum and sterile soil, but not on the forest soil inoculum; rapid shoot growth was favoured during the first five weeks, later switching to more intensive root growth (Table 2 in paper **III**).

Mycorrhizal root colonisation levels were about twice as high with grassland than forest soil inoculum (Fig. 1C in **III**), with no plant species-specific differences. Root colonisation was positively correlated with plant total biomass ($r = 0.630$, $P = 0.007$, Pearson correlation). Plant tissue P concentration was higher, and N concentration lower in plants grown with grassland inoculum compared to plants grown with forest soil inoculum. There were no differences detected among plant species (Fig. 1D,E and Table 2 in **III**). Plant total biomass and root colonisation levels were positively correlated with tissue P, and negatively correlated with tissue N concentration ($r = 0.384$, $P = 0.019$; $r = 0.669$, $P = 0.003$; $r = –0.587$, $P < 0.001$, $r = –0.750$, $P = 0.050$, Pearson correlation).

DISCUSSION

Natural AM fungal diversity: number and identity of species. Comparable data regarding the number of root colonising AM fungal sequence groupings per site vary from two sequence types detected in a temperate arable field (Daniell *et al.* 2001) to around 20 types in tropical forests (Husband *et al.* 2002), a temperate grassland (Vandenkoornhuyse *et al.* 2002) and a temperate wetland (Wirsel 2004; see also Table 1 in this study). Along with the apparently natural patterns of AM fungal sequence type richness over different ecosystems, within ecosystems AM fungal richness has been observed to rise with increasing sample size and number of clones screened (Table 1). The total number of AM fungal species in a given site has recently been estimated statistically on the basis of observed patterns of AM fungi associated with common reed in a wetland ecosystem, and the statistically estimated number of sequence types exceeded the detected number of types (14 to 20 types over sites and seasons) by 0 to 6 types (Wirsel 2004). Analysis of the relation between sample size and the detected number of ectomycorrhizal fungi demonstrated that greater sampling effort would have been required in most studies, whilst the required sample size needed in order to exhaust the fungal diversity differs between studies (Horton & Bruns 2001). The total number of AM fungal types at any given site has not yet been tested via exhaustive sampling and clone screening. In the present investigation, we were able to detect 10 and 14 AM fungal sequence groups in *Pulsatilla* species' roots inoculated with forest and grassland soil, respectively. In the context of the roughly similar sampling and screening efforts of previous studies, our data fitted well with the corresponding numbers of detected fungal types from different ecosystems (see Table 1).

The majority of AM fungal sequence types detected from natural ecosystems, both in the present study, and those of other authors, show no sequenced relatives among described and named fungi. However, different authors have independently detected several of these 'unknown' sequence types (Table 6). For example, one of the dominant fungal types of many natural communities, *Glomus* sp. MO-G5 of this study, corresponds to the *Glomus* sp. Glo2 detected in temperate broadleaved woodlands, temperate grasslands, boreal and tropical forests (Helgason *et al.* 1998, 1999, 2002, Husband *et al.* 2002a,b, Vandenkoornhuyse *et al.* 2002, **paper II**; see Table 6). It is reasonable to assume that most of the 'unknown' sequence types are AM fungi that have not yet entered the sequence databases, either because they have not been isolated into culture, or because the gene fragment analysed has not yet been sequenced from the particular fungus. However, even in the case of long-studied ecosystems, only a few of the new sequence types have been successfully cultured, e.g. an isolate *Glomus* sp. UY1225, corresponding to sequence types Glo3 (Helgason *et al.* 2002) and MO-G2 of this study, detected from temperate woodlands and grasslands and boreal and tropical forests (Helgason

et al. 1998, 1999, 2002, Husband *et al.* 2002a,b, Heinemeyer *et al.* 2004, **paper II**; Table 6). *Glomus* sp. UY1225, a culturable AM fungus that is frequently detected in many natural communities, is not taxonomically described. Thus, there is an obvious need for coordinated work by taxonomists and molecular ecologists of AM fungi.

A number of new AM fungal sequence groups were identified in this study that showed no close similarities to any database sequences, namely *Glomus* sp. MO-G8, MO-G9 and MO-G14. Later, sequence types closely related to MO-G8 have been identified from tropical forest (Glo14b, 97% similarity, Husband *et al.* 2002a) and a temperate wetland (group 11, 99% similarity, Wirsel 2004); and to MO–G14 (Glo 47 AY512355, 98% similarity, T.R. Scheublin *et al.* unpublished, NCBI database, http://www.ncbi.nlm.nih.gov/). Additionally, sequence groups that correspond to the sequence types of 'unknown' status of other authors were detected (Table 6). Obviously, there is a gap in our knowledge regarding a large part of AM fungal diversity in natural ecosystems, potentially including functionally important fungi.

Studies of AM fungal communities in temperate ecosystems have suggested a predominance of *Scutellospora* and *Acaulospora* sequence types in forest understorey plant roots (UK; Helgason *et al.* 1998, 1999, 2002) and *Glomus* types in grassland (UK; Vandenkoornhuyse *et al.* 2002, Heinemeyer *et al.* 2004). However, in our case, the large majority of sequence groups detected in the roots of *Pulsatilla* experimental plants, inoculated with either grassland or forest soil, belonged to the genus *Glomus* (14 groups); only three Gigasporaceae and two Acaulosporaceae groups were detected. The observed prevalence of *Glomus* sequence groups over the other families of AM fungi in the studied forest site may have several explanations.

First, it may well reflect the real composition of the given AM fungal communities. Other studied temperate systems include arable fields with prevalence of a *Glomus mosseae*-like sequence group (UK, Helgason *et al.* 1998, Daniell *et al.* 2001); sand dunes showing equal incidence of *Glomus* and *Scutellospora* types in the roots of marram grass (*Ammophila arenaria* in Netherlands, Kowalchuk *et al.* 2002); forest and cultivated habitats of yew (*Taxus baccata*), whose roots hosted *Glomus* and *Archaeospora* groups (Germany, Wubet *et al.* 2003); and wetland habitats of common reed hosting only *Glomus* sequence types (Wirsel 2004). Thus, there appears to be considerable variation in AM fungal dominance in different temperate ecosystems that are still far from well studied. Further, the understorey plant communities of the pine forest site from this study and of the broad-leaved woodland in the UK (above) are considerably different, and may be the reason for differences in AM fungal community composition.

Second, our experimental design, aiming to simulate a gap environment in a boreal forest or grassland, might have promoted more 'ruderal' AM fungi, i.e. fungi that are able to rapidly germinate and/or colonise plant roots. When setting up the experiment, the potting soil was mixed, thus damaging the hyphal

network and thus suppressing disturbance-sensitive fungi. *Pulsatilla* seedlings tend to establish in nature in disturbed microsites or gaps (Uotila 1996, Pilt & Kukk 2002), therefore the experimental design mimicked a natural situation faced by the germlings. Further, soil disruption can promote colonisation by *Acaulospora* and *Scutellospora* (Jasper *et al.* 1991), probably because hyphae of these genera tend to lose their infectivity rapidly (Jasper *et al.* 1993) and spores are the main source of inoculum for *Scutellospora* (Klironomos & Hart 2002). These groups, however, did not dominate in the *Pulsatilla* roots. Consequently, the experimental design should not have caused the lack of colonisation of Gigasporaceae and Acaulosporaceae groups in the *Pulsatilla* roots.

In addition, the AM1 primer used for amplification of AM fungi from plant roots is known to exclude the basal families Archaeosporaceae and Paraglomaceae (Redecker *et al.* 2000b, Daniell *et al.* 2001). Thus, even if present in roots, the current approach would not have revealed these fungi.

Intriguingly low numbers of AM fungal sequence groups were detected in the roots of *Pulsatilla* field plants from six sites, compared to pot-experiment plants. Because of limited sampling of native endangered plant species *P. patens*, for obvious reasons, sample size differences between field plants and experimental plants (12 vs. 46 samples) could have influenced the structure and composition of the AM fungal community detected. Amplification success rate was also quite low in the case of field plants, possibly due to lower root colonisation. A large majority of field plants of both *Pulsatilla* spp. yielded a single sequence group, *Glomus* sp. MO-G1, which was rarely detected in experimental plants. Admitting the above technical constraints, it is still tempting to make a few speculations on other possible explanations of the observed diversity difference: (1) 'natural' succession of AM fungal community in roots as a plant ages. As shown previously, two equally dominant AM fungal types (Glo1b and Glo8, representing 26% and 24% of the total number of clones) in roots of 3-month-old *Tetragastris panamensis*, a tropical forest tree, were replaced by an overwhelmingly dominant sequence type (Glo18, 82%) in the roots of the 5-year-old seedlings; the number of sequence types declined from 13 and 15 in 3-month-old and 1-year-old seedlings to eight and seven sequence types in the roots of 2 and 5-year-old seedlings (Husband *et al.* 2002b). It has been recently shown that the optimal AM fungal species may be different for seedlings and adults of some plant species, and that seedlings may benefit from AM even if adult plants do not (van der Heijden 2004). This observation may be an underlying reason for a succession of AM fungal communities in different-aged plants; (2) lower sporulation rate of MO-G1 and/or larger sensitivity to disturbance, which may result in lower colonisation rates of seedlings in experimental soils with disturbed AM fungal mycelial systems; and (3) vertical differences in root colonising fungal communities in upper and deeper soil layers, as has been shown for ectomycorrhizal fungi in symbiosis with Scots pine in similar boreal podzol horizons (Heinonsalo *et al.* 2001). The roots of the adult field plants reach considerable depth (commonly 50–100 cm), whereas the

soil inoculum was collected from upper soil layers, which, in field conditions would be encountered by the roots of seedlings. However, proper sample sizes and comparable plant growth conditions are required in order to test the regularity of these differences.

AM fungal community patterns: related to plant host and site. Both plant host and site related patterns of AM fungal communities have been described earlier. Coexisting plant species in a community may show divergent AM fungal communities, two grass species, or a grass and a legume in a temperate grassland for example (Vandenkoornhuyse *et al.* 2002, 2003). An extreme specificity was shown to exist between AM fungi and epiparasitic plant species, which were colonised by very few AM fungi from the surrounding AM fungal community (Bidartondo *et al.* 2002). The same plant host in different sites can host AM fungal communities with different dominants (Helgason *et al*. 1999, Wubet *et al.* 2004) or distinct composition (Wubet *et al.* 2003, Wirsel 2004). Apart from this, the fungal community structure can show considerable variation in time, either seasonally or from year to year (Helgason *et al.* 1999, Husband *et al.* 2002b, Heinemeyer *et al.* 2004).

In the present study, two pairs of congeneric plant species with different abundance show different patterns in regard to AM fungal communities in their roots. AM fungal communities of *Viola* spp. differed between plant species; in the case of *Pulsatilla* spp., root AM fungal communities of established field plants from six sites showed no significant differences, but AMF communities in the roots of experimental seedlings differed according to the site of origin of soil inoculum.

In the case of *Viola* spp., different abundances of the same AM fungi in the roots of the two plant species were detected. This may indicate that there is a preference between plant host species and some fungal genotypes over the others. The five study sites themselves did not vary markably in their fungal community composition. The more common *V. mirabilis* exhibited more selectivity towards its root-colonising fungi, which was apparent from the smaller variability of fungal communities in its roots compared to the AM fungal communities of *V. elatior*. Thus, our initial hypothesis was not supported; we did not find the less common plant species to have more specific AM relations.

The opposite was apparent in the case of *Pulsatilla* spp. The soil inocula, originating from two study sites, resulted in colonisation of plant roots with different characteristic AM fungal sequence groups, whilst the plant species, when grown on the same soil inoculum, hosted similar AM fungal communities. However, plants sampled directly from the field hosted almost uniformly just one AM fungal sequence group (see above).

The discrepancy between the results from *Viola* and *Pulsatilla* surveys may be explained in several ways. First, different methodological approaches were used to detect AM fungi in *Viola* and *Pulsatilla* roots. The LSU rDNA primers

used in the *Viola* survey may reveal more intra- and interspecific variation, but cover only a part of the total AM fungal diversity, namely the *Glomus mosseae-intraradices* group (Kjøller & Rosendahl 2000). The SSU rDNA primers used in the *Pulsatilla* survey cover nearly all AMF taxa (Helgason *et al.* 1998, Daniell *et al.* 2001). However, the main body of SSU sequence groups detected from *Pulsatilla* roots was within the same *Glomus* group, which is covered by the LSU primers used in the *Viola* survey. Further, quantitative data of root colonising AM fungi was obtained for *Viola* spp., because several samples from a root system were analysed; only one sample per root system was analysed from the roots of *Pulsatilla* spp., yielding the presence/absence data of AM fungi in a plant, and the number of plants from the sample set that host a particular fungal sequence group.

Second, the different patterns of associated AM fungal communities are perhaps related to plant life history. *Pulsatilla* species are rather long-lived; their critical life-stage from the survival point of view is the seedling phase. During that stage, *Pulsatilla* individuals are extremely vulnerable to environmental fluctuations, e.g. drought, and are expected to gain a lot from mycorrhizal symbiosis. The root fungal communities in seedlings were diverse, compared to mostly single-fungus communities, detected in established plants. As both *Pulsatilla* species are highly responsive to mycorrhiza in the seedling phase (**paper III**, and see below), they might appear to be less selective. The difference in AM fungal richness between adults and seedlings might indicate that the adults are either much less dependent on mycorrhiza than seedlings, or, that there is considerably higher degree of specificity between adult *Pulsatilla* plants and the fungal sequence group overwhelmingly colonising their roots. The two *Viola* spp. hosted different AM fungal communities that can be interpreted as differential selectivity between AM fungi and the two host plant species, but further investigations are required to find out why.

The site-specific variation in AM fungal communities depends evidently on the local environmental conditions and management history of the sites. The latter influences the plant community structure, which in turn will affect the associated organisms, including AM fungi. Changes in AM fungal communities may be observed both with plant community succession (Janos 1980, Johnson *et al.* 1991) and with changing land use intensity (Oehl *et al.* 2003). In the case of the pine forest and the grassland sites with *Pulsatilla* spp., studied by us, the development of a seminatural grassland in an area where the natural vegetation consists of forest has brought diversification of the plant community, and, most importantly, the inclusion of a considerably wider range of AM forming plant species. Though the more diverse assortment of host plants may support a more diverse community of AM fungi (Johnson *et al.* 2004), the number of AM fungal species detected in the plants growing in forest and grassland soils was approximately the same. The most obvious difference between the two AM communities lies in the single dominant fungal species of the grassland site, specific to the site, and the two dominant fungal species of the forest site, being

much less frequently detected in grassland-soil-inoculated plants. Consequently, one can say that the two investigated sites have different AM fungal communities, but extrapolation of the data to ecosystem types and explanation of the origin of differences obviously requires more descriptive and experimental investigations.

Roles of AM fungi in plant performance. Seedling recruitment of plant species that are highly responsive to mycorrhiza may be entirely dependent on the presence of mycorrhizal fungi in the system (Francis & Read 1995). In this study, the seedlings of two *Pulsatilla* spp. inoculated with native AM fungal communities showed better establishment and developed an order of magnitude higher biomass by the end of the experiment, compared to non-mycorrhizal seedlings. Based on this experiment, the mycorrhizal dependency (defined as the *extent to which a plant benefits from the presence of AMF compared to when it is absent*, van der Heijden (2002), ranges from –100 to +100%) is 97% and 98% for *P. patens* and *P. pratensis*, respectively. Consequently, these two plant species are very highly mycorrhizal dependent.

Mycorrhizal dependency has been expected to be positively related to mycorrhizal species sensitivity, i.e. the variation of a plant species' growth response to different AM fungal species (van der Heijden 2002). Thus, it would have been expected that the *Pulsatilla* species respond differentially to different AM fungi. Our data showed, first of all, different plant growth reaction to inoculation with grassland and forest soil inoculum, resulting also in different root-colonising AM fungal communities — both *Pulsatilla* species grew better when inoculated with the grassland soil AM fungal community. Earlier authors have also suggested that differences in soil AM fungal communities underlie divergent growth responses of *Andropogon gerardi* (Johnson 1993) and *Poa pratensis* (Frank *et al.* 2003) to inoculation with different soils, but the fungi were not identified in plant roots in either case.

Better performance of two *Pulsatilla* species when inoculated with grassland inoculum suggests that both plant species respond specifically to the particular fungus/fungi in that inoculum. The physicochemical soil parameters were not significantly different between the treatments. Differential growth response to certain fungal isolates has been observed in the case of *Prunella vulgaris* and *P. grandiflora*, both common in their native habitats, when inoculated with a set of native AM fungi and showing equal colonisation levels (Streitwolf-Engel *et al.* 1997). Further, cross-inoculation of ten plant and ten AM fungal species from an old-field demonstrated a diverse range of responses between plant-fungus pairs, with no fungus benefiting all plants (Klironomos 2003). In our case, one may hypothesise that the dominant AM fungus in grassland soil, *Glomus* sp. MO-G3, might have been responsible for the observed differential growth response of the two plant species on that soil mixture. In order to test this hypothesis, however, the fungus must be isolated into

culture. Finally, more complex plant-fungal relations, or significant effects of less abundant fungi, cannot be ruled out.

The successful establishment of seedlings in natural communities may be strongly influenced by the availability and colonisation ability of AM fungal propagules (Hart & Reader 2002b, Hart *et al.* 2003). We do not know whether the propagule densities in soils of the grassland and the forest site differed. However, the presence of a more diverse plant community consisting of 33 arbuscular mycorrhizal plant species in the grassland site, as opposed to sparse cover of five AM plant species in pine forest understorey, might support a more diverse (cf. Johnson *et al.* 2004) and dense AM fungal community. For example, in a heathland community dominated by *Calluna vulgaris* (as was the pine forest site understorey in our study) AM colonisation level was related to the AM host density (Genney *et al.* 2001). Data from the present study does not indicate significant differences in numbers of AM fungal sequence groups from between the grassland and the forest site, but the differences in composition of AM fungal communities were evident, and were accompanied by slower root colonisation development, lower final colonisation levels and smaller plant biomass in seedlings inoculated with forest soil. The mycorrhizal colonisation levels of 1-month-old *Pulsatilla* seedlings inoculated with grassland soil were 25–45%, as compared to less than 10% in forest-soil-inoculated seedlings. The fast colonisation accompanied by faster growth of plants, indicating the presence of efficient compatible symbionts, or high density of compatible symbionts, that might be of decisive importance for the *Pulsatilla* seedlings for survival. Additionally, their sensitivity to drought (Pilt & Kukk 2002) could result in reduced seedling survival in most years, whilst colonisation by drought stress alleviating AM fungi (Ruiz-Lozano *et al.* 1995) may give a considerable bonus to seedlings.

The dominant AM fungus in grassland soil in this study, *Glomus* sp. MO-G3, is related to *G. intraradices*, which is a frequently isolated species in trap-cultures. Some isolates of this species show ability for rapid root colonisation and an outstandingly large effect on plant biomass (Hart & Reader 2002a,b). Koch *et al*. (2004) demonstrated a considerable variation in mycelial growth and sporulation among ten *G. intraradices* isolates from a single arable field. A related sequence group (Glo8) has been detected from temperate woodland, grassland, wetland and arable fields, and from tropical forest (Helgason *et al.* 1998, 2002, Daniell *et al.* 2001, Husband *et al.* 2002a,b, Heinemeyer *et al.* 2004, Wirsel 2004); a fungus matching the sequence group has not yet been isolated and consequently its symbiotic performance is not known.

The two dominant fungi from the forest soil in this study, *Glomus* sp. MO-G2 and MO-G5, are related to an almost identical *Glomus* sp. isolate UY1225 (= Glo3) and sequence group Glo2, respectively. The first is known to confer some benefit to a relatively broad range of plant hosts (Helgason *et al.* 2002). Both fungi have been detected in roots of a range of plant species in most of the undisturbed ecosystems for which comparable data exists (Helgason *et al.* 1998,

1999, 2002; Husband *et al.* 2002a,b, Heinemeyer *et al.* 2004), suggesting low specificity of these taxa and possibly little contribution to plant growth. Lack of these taxa in disturbed environments like arable fields (Daniell *et al.* 2001) and periodically flooded wetlands (Wirsel 2004) suggests sensitivity of the two fungi to disturbance.

Coupling of plant and AM fungal communities. The last decade has brought new information on natural AM fungal communities in plant roots in an array of ecosystems worldwide. Several fungal species or sequence groups have been detected in various ecosystems, whilst others appear to be less common or seem to be characteristic to specific ecosystems (Table 6). A level of selectivity exists between AM fungi and their plant hosts (van der Heijden *et al.* 1998a,b, Helgason *et al.* 2002, Vandenkoornhuyse *et al.* 2002). Consequently, plant communities of different composition may promote diversification of the associated AM fungal community (Johnson *et al.* 2004). Different AM fungal communities have been observed in the roots of coexisting plant species, in the roots of the same plant species in different stands of the same plant community type, and in the roots of the same or different plant species in different types of plant communities (Helgason *et al.* 1998, 1999, Vandenkoornhuyse *et al.* 2002, 2003, Wubet *et al.* 2003, 2004, Wirsel 2004, **paper II**, **paper IV**). The above evidence of plant species related patterns suggests a degree of specificity in plant-fungal association, but the plant community related patterns suggest a larger role of edaphic factors in determining the composition of AM fungal communities. Clearly the data is too fragmentary and the number of plant species studied too small to draw any firm conclusions. However, considering the degree of specificity between plant and fungal species varies from low to high, i.e. there are generalist and specialist AM fungal types, both patterns may be observed depending on which plant species are studied. Thus, there is still no clear answer to the essential question: is there a relationship between plant and AM fungal species distributions?

The need for 'fungal sociology' (or geobotany), i.e. establishment of the distribution of AM fungal communities in nature, was articulated by Renker *et al.* (2004 — **paper I**). Given the above information, it is highly probable that specific coupling of AM fungal communities with plant communities occurs in natural ecosystems. In order to verify this hypothesis on an ecosystem scale, studies and experiments need to be performed, in which considerably larger sample sizes are processed in order to allow statistical analysis. The number of plant species studied from any given ecosystem has been small in all the investigations so far conducted and published (Table 1). The number of plants sampled, spatial and time-scale covered, as well as clones screened per sample has to increase considerably to allow community-ecological questions to be answered. This goal can be reached using high-throughput molecular methods, e.g. terminal-restriction fragment length polymorphism (T-RFLP) and

Table 6. Detection of most common AM fungal sequence types in natural plant roots from different ecosystems.

	Ref.[1]	Glo1a[2]	Glo1b = MO-G11	Glo2 = MO-G5	Glo3a = MO-G2	Glo4 = W18	Glo7 = W4= MO-G6	Glo8 = W1= MO-G3	Glo9 = MO-G7	Glo10 = W2	Glo12 = W19	Glo 14a	Glo 14b= W11= MO-G8	Glo17 = W8	Glo 18= W8	Glo 19= W12	Glo 20	Glo 21= MO-G1
Tropical forest	7,8		+	+	+			+	+	+		+	+	+	+		+	+
Temperate broadleaved forest	1,2,10			+	+		+	+	+									
Temperate arable field	3,10	+	+			+		+		+								
Temperate grassland	4,5	+	+	+	+	+		+	+		+	+		+	+	+	+	+
Temperate wetland	9					+	+	+		+	+		+	+	+	+		
Temperate grassland	**Paper II**			+	+		+	+					+					+
Boreal forest	**Paper II**		+	+	+		+		+									+

[1] 1, Helgason *et al.* 1999; 2, Helgason *et al.* 2002; 3, Daniell *et al.* 2001; 4, Vandenkoornhuyse *et al.* 2002; 5, Heinemeyer *et al.* 2004; 6, Wirsel 2004; 7, Husband *et al.* 2002a; 8, Husband *et al.* 2002b; 9, Wubet *et al.* 2004; 10, Helgason *et al.* 1998. Papers that provide comparable data and had an intention to describe the entire community of AM fungi were included. Sequence types that were comparable and were detected in at least two studies were included.

[2] GloXXX/AcauXXX/ScutXXX, sequence type designations from references 1–8, 10; MO-XXX, designations from this study (paper II); WXXX, designations from reference 9.

Table 6 (*continued*). Sequence types specific to particular ecosystems are separated with vertical line.

	Ref.[1]	Glo23= W1= MO-G13	Glo24 = W2	W8= MO-G4	Acau1	Acau3	Scut1	Scut2	Scut3	Acau9	Scut4	Glo5	Acau2	Acau4	Acau5	Glo6	Glo 13= MO-G12
Tropical forest	7,8						+	+	+	+	+						
Temperate broadleaved forest	1,2,10				+	+	+					+	+	+	+		
Temperate arable field	3,10	+	+		+		+									+	
Temperate grassland	4,5	+	+			+		+	+								+
Temperate wetland	9	+	+	+													
Temperate grassland	**Paper II**	+		+													+
Boreal forest	**Paper II**																

[1] 1, Helgason *et al.* 1999; 2, Helgason *et al.* 2002; 3, Daniell *et al.* 2001; 4, Vandenkoornhuyse *et al.* 2002; 5, Heinemeyer *et al.* 2004; 6, Wirsel 2004; 7, Husband *et al.* 2002a; 8, Husband *et al.* 2002b; 9, Wubet *et al.* 2004; 10, Helgason *et al.* 1998. Papers that provide comparable data and had an intention to describe the entire community of AM fungi were included. Sequence types that were comparable and were detected in at least two studies were included.

[2] GloXXX/AcauXXX/ScutXXX, sequence type designations from references 1–8, 10; MO-XXX, designations from this study (paper II); WXXX, designations from reference 9.

high-throughput sequencing. T-RFLP was first applied to AM by Tonin *et al.* (2001), and was recently used to unveil plant species and plant community related AM fungal community patterns (Vandenkoornhuyse *et al.* 2003, Johnson *et al.* 2004). High-throughput approaches will be particularly valuable if the capacity of the methods is coupled with the extant knowledge on AM fungal RFLP types, thus adding to the body of information on AM fungal biogeography.

Further, there is clearly a lack of quantitative data on individual AM fungal colonisation in natural plant roots. The data of this study (**paper IV**) suggests both spatial separation within a root system and host-related differential colonisation levels of specific AM fungi. This quantitative aspect of AM fungal community composition is highly likely to affect our understanding of AM fungal distribution patterns in nature (cf. Sanders 2002). The first attempts to apply quantitative molecular methods (real-time PCR) to AM fungi have enabled the measurement of the DNA amount of two AM fungal species in plant roots (Böhm *et al.* 1999, Alkan *et al.* 2004) or in soil (Filion *et al.* 2003) in experimental conditions. Development of methods that allow quantification of AM fungi in natural communities, within plant roots and rhizosphere soil, coupled with molecular identification of fungi in the very same samples, will provide the essential evidence needed to uncover the AM fungal community patterns and dynamics in natural ecosystems that are desperately needed in order to advance the understanding of interrelated functioning of arbuscular mycorrhizal fungi and plants at the scale of genes to the scale of ecosystems.

CONCLUSIONS

- Sequence groups of AM fungi in the roots of experimental *Pulsatilla* seedlings that were more abundant either in seedlings inoculated with grassland soil (*Glomus* sp. MO-G3) or in seedlings inoculated with forest soil (*Glomus* spp. MO-G2 and MO-G5) were identified. There were obvious site-dependent differences in AM fungal community compositions in the roots of *Pulsatilla* spp. seedlings.
- 10 and 14 AM fungal sequence groups were detected in *Pulsatilla* spp. roots in a pine forest and grassland site, respectively. 12 groups were detected from the roots of *P. patens* and 15 groups from the roots of *P. pratensis*. Consequently, we did not identify plant host species- or site- related variation in AM fungal sequence group richness.
- The genotypic composition of AM fungal communities in *Viola* spp. roots differed with host plant species, though there was very little variation in fungal genotype richness. The roots of the two plant species were colonised by fungal communities that differed in the abundance of fungal types obtained from similar pools of fungal genotypes.
- We failed to identify AM fungal sequence groups specifically or preferentially colonising less abundant plant species as compared to more abundant congeneric species. In the case of *Pulsatilla* spp., the composition of root colonising AM fungal communities was similar in both species. In the case of *Viola* spp., the composition of root colonising AM fungal communities was more variable in the roots of the rare species, but there were no fungal genotypes specifically colonising the majority of individuals of the rare species.
- Establishment and growth of *Pulsatilla* spp. in the field was influenced by the composition of native AM fungal communities — the fungal community of grassland origin had a stronger positive effect on the performance of *Pulsatilla* species than the fungal community of forest origin. At the same time, the positive effect of the grassland AM fungal community was stronger in the case of the more common *Pulsatilla pratensis*. Thus, the congeneric plant species exhibited differential performance as a response to specific AM fungal communities.

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SUMMARY IN ESTONIAN

Arbuskulaar-mükoriissete seente mitmekesisus mitmeaastaste taimede juurtes ning nende mõju taimede kasvule

KOKKUVÕTE

Käesoleva doktoriväitekirja eesmärgiks on kirjeldada arbuskulaarset mükoriisat (AM) moodustavate seente kooslusi mitmeaastaste rohttaimede juurtes, ning selgitada nende seente mõju taimede kasvule.

Arbuskulaar-mükoriissed seened on taimejuurte obligatoorsed sümbiondid ning looduslike maismaakoosluste loomulik komponent. Olles nende juurestiku jätkuks mullas, varustavad AM seened taimi mineraalainetega, kaitsevad patogeenide eest ning leevendavad põua ja raskemetallide poolt põhjustatud stressi. Erinevad AM seeneliigid mõjutavad erinevalt taimeliikide kasvu, vegetatiivset ja sugulist paljunemist, konkurentsivõimet jm. ning on seeläbi taimeindiviidide ja -populatsioonide elukäigus tähtsateks mõjuriteks. Seetõttu on oluline teada, millised seensümbiondid asustavad looduslike taimede juuri ja kuidas need seened mõjutavad taimede kasvu.

Käesolevas töös uuriti kahte taimeliikide paari, mis on Eestis erineva arvukusega: palu-karukell (*Pulsatilla patens*) ja aas-karukell (*P. pratensis*); kõrge kannike (*Viola elatior*) ja imekannike (*V. mirabilis*). Neil liikidel määrati molekulaarsete meetoditega juuri asustavad AM seened erinevates looduslikes kasvukohtades ning looduslike AM seenekooslustega nakatatud katseidanditel (*Pulsatilla*). Kannikeste juurte AM seened määrati ribosoomi suure alaühiku geeni (LSU rDNA) amplifitseerimisel *Glomus mosseae-intraradices* liigirühma spetsiifiliste praimeritega, seenekoosluse sõrmejäljed saadi üheahelalise konformatsiooni polümorfismi (SSCP) meetodil. Karukellade AM seened määrati ribosoomi väikese alaühiku geeni (SSU rDNA) amplifitseerimisel AM seente spetsiifiliste praimeritega, produktide kloneerimisel, sekveneerimisel ja fülogeneesianalüüsil. Mõlema karukella liigi idandite kasvu hinnati faktoriaalses potikatses erinevate looduslike AM seenekoosluste manulusel, kus töötlustena kasutati kahte erinevat, metsa- ja niidumullast pärit AM seenekooslust.

Eestis ja mujal haruldase kõrge kannikese juuri asustavad AM seenekooslused olid tunduvalt varieeruvamad kui tavalise imekannikese juurte seenekooslused. Mõlema liigi kasvukohtade, sh. selliste, kus esinevad mõlemad taimeliigid, seenekoosluste koosseisus suuri erinevusi ei leitud. Karukellade juuri asustavad metsa- ja niidumullast pärit AM seenekooslused erinesid karakterliikide ning liigirikkuse poolest. Samas ei erinenud taimeliigid märkimisväärselt oma seenekoosluste poolest, kui taimed olid kasvanud sama päritolu AM seenekooslusega. Kokku tehti kindlaks 19 AM seene sekventsirühma esinemine karukellade juurtes, neist 14 perekonnast *Glomus*, kaks

Acaulospora, kaks *Scutellospora* ja üks *Gigaspora* perekonnast. Neli sekventsirühma olid sarnased teadaolevate liikide järjestustega, kuus rühma aga juurtest pärinevate 'tundmatute' järjestustega.

Niidu- ja metsa AM seenekooslustega muldadel oli palu- ja aas-karukella kasv oluliselt erinev. Niidult pärinevad AM seened soodustasid suurusjärgu võrra suurema biomassi arengut mõlemal taimeliigil, samuti kasvasid aas-karukella idandid sel mullal oluliselt suuremaks kui palu-karukella idandid. Seega mõjutasid niidumullas esinenud seened kahe taimeliigi kasvu erinevalt. Selliste AM seente olemasolu, mis on tavalisele karukellaliigile kasulikumad, võib olla üheks põhjuseks, miks palu- ja aas-karukell on erineva leviku ja ohtrusega.

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