

Diversity of fungi in organic soils under a moorland – Scots pine (*Pinus sylvestris* L.) gradient

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Summary

The conservation and regeneration of native Scots pine (*Pinus sylvestris* L.) woodlands is being actively encouraged by conservation agencies in the UK because of their high biodiversity value. In the present study, the consequences of regeneration on terrestrial fungal communities was determined in three parallel transects running from open moorland, through an intermediate zone showing seedling colonization, into a mature Scots pine forest at Abernethy Forest, Cairngorm, Scotland. Soil cores were taken at 18 m intervals along each 180 m transect, and the diversity of the soil fungal community was investigated by DGGE and sequence analysis of ITS fragments PCR-amplified from DNA extracted from soil. Analysis of DGGE profiles generated for two of the three transects indicates a clear shift in the community from the moorland region of the transects to the forest region. Whereas a few bands were present at all sampling points across the transects, the majority of bands were unique to either the moorland or forest samples. FASTA database searches of ITS sequence data generated from excised DGGE bands revealed the closest species match for each band. In some cases, the similarity of ITS sequences to database sequences was poor, but the remaining sequences were most closely related to ITS sequences of both mycorrhizal and non-mycorrhizal fungi. The data are discussed in relation to the effect of native pine woodland expansion on the soil fungal community.

Introduction

Within the UK, native pine woodlands are an extremely

rare, indigenous ecosystem, with remnants located sparsely throughout the central and north-eastern Grampians and in the northern and western Highlands of Scotland, UK (Department of the Environment, 1994). Native pinewood forests are dominated by Scots pine (*Pinus sylvestris* L.) and are considered to be the western counterpart of the European boreal forests. They represent a precious natural resource of high biodiversity value and consequently current UK policy is to encourage expansion of remnant native pinewoods and to create new native pinewoods on appropriate sites within their natural geographical range (Department of the Environment, 1994). Whereas native woodland expansion is being encouraged, little is known about the potential effects of this process on the below-ground microbial diversity associated with bordering ecosystem and vegetation types. In Scotland, much of the native woodland expansion tends to be on highly organic soils supporting heather-moorland ecosystems which themselves have biodiversity value and where the soil contains a significant pool of sequestered C (Chapman *et al.*, 2001). Land use change on such soils as a result of afforestation may alter the structure and subsequent function of soil microbial communities. It is possible such changes may affect the functioning of key ecosystem processes, particularly the degradation of complex organic matter and the subsequent release and cycling of important nutrients including N, P, S and C, because of the importance of soil microbial communities in these processes. The proportion of fungal to bacterial biomass is higher in acid, organic soils than in mineral soils and fungi are the primary decomposers of structural plant litter. Nevertheless, little is known of the diversity of fungi in such soils. Whereas saprophytic fungi play a fundamental role in degradation processes, mycorrhizal fungi are important for the subsequent acquisition and translocation of nutrients to their plant hosts. The interaction between saprophytic and mycorrhizal fungi in the field is poorly understood, but microcosm studies suggest that the role of the interaction in controlling nutrient cycling may be more important than previously thought (Lindahl *et al.*, 1999; Setälä, 2000; Leake *et al.*, 2001). Consequently, competition between invading fungal species associated with colonizing Scots pine seedlings and the indigenous fungal population in bordering regions, may alter the overall structure of the fungal community and influence nutrient cycling processes.

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Our current understanding of the biodiversity and ecology of fungi in terrestrial ecosystems has been limited by the reliance on culture-based techniques for assessing fungal diversity. It has become increasingly clear that such techniques only detect a small fraction of the community, and it has been suggested that of an estimated 1.5 million fungal species in existence, only 5–10% have been formally described (Hawksworth, 1991; 2001; Hawksworth and Rossman, 1997). Recently, substantial advances have been made in fungal ecology through the development and application of molecular techniques to detect the presence of fungal taxa directly in environmental samples. In particular, the ability to characterize soil fungal communities by analysis of whole soil DNA extracts is seen as one of the major steps forward in fungal ecology (Horton and Bruns, 2001). In a number of recent studies, molecular techniques including cloning (Borneman and Hartin, 2000; Viaud *et al.*, 2000; Chen and Cairney, 2002; Vandenkoornhuysen *et al.*, 2002; Anderson *et al.*, 2003; Jumpponen, 2003; Landeweert *et al.*, 2003; Smit *et al.*, 2003), automated rRNA intergenic spacer analysis (ARISA) (Ranjard *et al.*, 2001), terminal restriction fragment length polymorphism (T-RFLP) (Dickie *et al.*, 2002; Lord *et al.*, 2002), denaturing gradient gel electrophoresis (DGGE) (van Elsas *et al.*, 2000; Vainio and Hantula, 2000; Möhlenhoff *et al.*, 2001; Penanen *et al.*, 2001; Schabereiter-Gurtner *et al.*, 2001; Kowalchuk *et al.*, 2003), temperature gradient gel electrophoresis (TGGE) (Smit *et al.*, 1999), and oligonucleotide fingerprinting of rRNA genes (OFRG) (Valinsky *et al.*, 2002) have been used to investigate fungal diversity. These approaches have been facilitated by the design of fungal-specific 18S rDNA (Smit *et al.*, 1999; Borneman and Hartin, 2000) and internal transcribed spacer (ITS) (White *et al.*, 1990; Gardes and Bruns, 1993) primers for the direct amplification of fungal DNA from environmental DNA extracts and subsequent identification in some cases by DNA sequencing. However, some concerns have been raised regarding potential bias of some primers to preferentially amplify certain fungal taxonomic groups (Smit *et al.*, 1999; Anderson *et al.*, 2003) and the potential lack of specificity for target fungal DNA in some instances (Borneman and Hartin, 2000; Anderson *et al.*, 2003). Such limitations should be taken into consideration when formulating conclusions on data generated using these techniques.

In the current work, DGGE was chosen as the fingerprinting method because of the ability of the technique to provide a rapid, visual indication of changes in microbial community structure and so that individual bands can be excised and sequenced in an attempt to identify their origin. Therefore, to test the hypothesis that forest expansion would lead to a change in the soil fungal community, fungal ITS regions amplified from total soil DNA were

analysed by DGGE to characterize fungal community structure across an environmental gradient in which colonization of moorland by pine had begun.

Results

DGGE profiles of fungal ITS fragments

Polymerase chain reaction amplification with the primers ITS1-F (containing a GC clamp) and ITS2 resulted in an intense PCR product approximately 300 bp in size for each sample. Secondary bands were also observed in the range 400–600 bp which varied in size and intensity depending on the sample (data not shown). Denaturing gradient gel electrophoresis analysis of the fungal ITS PCR products generated a complex banding pattern for each of the points along the three transects at the Abernethy field site (Figs 1–3). Denaturing gradient gel electrophoresis profiles were reproducible for each of the sampled transect points between different PCR reactions and different DGGE runs (data not shown). Visual analysis of the DGGE profiles generated for each of the three transects indicates a clear shift in the DGGE profiles from the moorland end of transects A and B to the pine forest end (Figs 1 and 2). Lanes 1–6 for transect A (Fig. 1) have several bands in common and the relative intensities of most of these bands were similar between lanes. For example, bands at positions marked M2 and M3 (Fig. 1) were only detected in the first six lanes. Similarly, bands at positions marked F1 and F2 were detected only in samples at the forest end of transect A (Fig. 1). Whereas the majority of bands in the DGGE profiles are unique to either the moorland or forest end of transect A, a small number of bands are common to both moorland and forest ends of the transect. For example, although bands at positions marked F3 and F4 are more dominant in lanes 7–11, a band at the same position is present in the majority of the samples from the moorland end of the transect (Fig. 1).

In some instances, bands at the same position from different transect points were excised and sequenced to determine whether bands running at the same position are in fact the same sequence type. For example, ITS sequence data for two excised bands for position M2 (A2a and A5a) and two excised bands for position M3 (A1a and A6a) from different lanes confirm that they are the same sequence type (see Figs 4 and 5).

Profiles for transects A and B were similar, in that there was a shift in DGGE banding patterns from the moorland to the forest ends of the transect. However, in transect B the shift did not occur until further along the transect (i.e. transect point 8; lane 8) (Fig. 2). There also appeared to be more bands in common between the moorland and forest ends of transect B than for transect A (Fig. 2). In

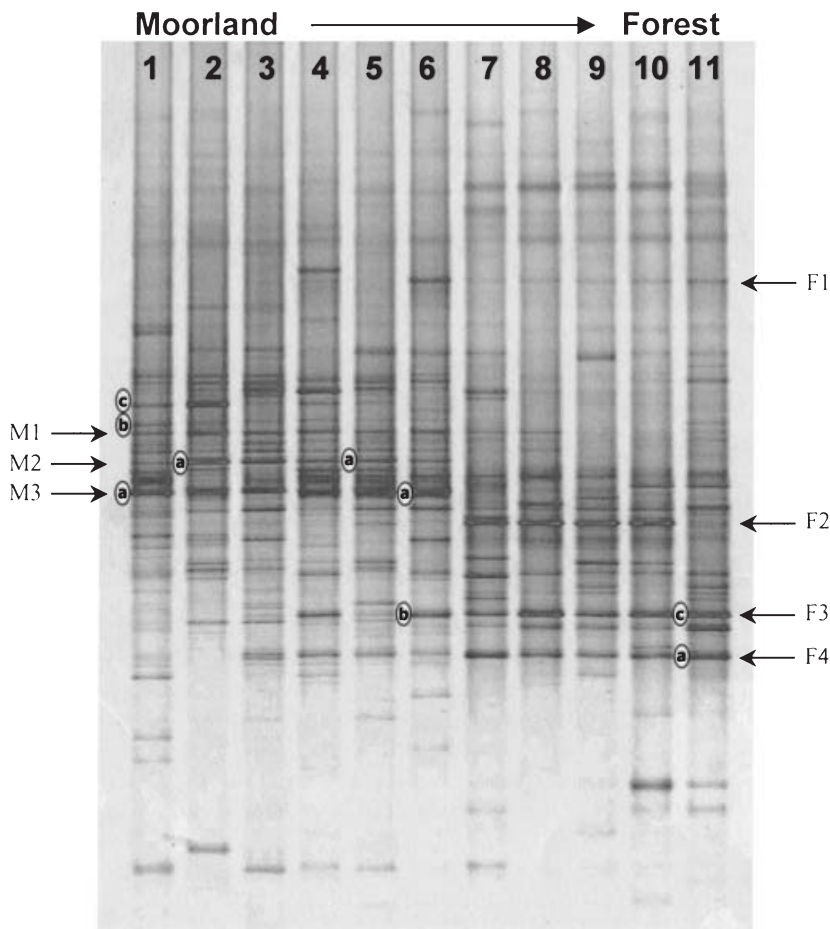


Fig. 1. DGGE analysis of partial fungal ITS sequences amplified from pooled DNA samples from transect A at the Abernethy field site. Lane numbers correspond to sampling points along the transect. Bands marked with letters to the left of the lane were excised from SYBR Gold stained gels and sequenced. Arrows indicate band positions highlighted in the text.

marked contrast to transects A and B, no clear trend was obvious in the DGGE profiles for transect C (Fig. 3).

Sequence and phylogenetic analysis of ITS-DGGE bands

Denaturing gradient gel electrophoresis profiles for the three transect samples revealed a clear shift between the moorland and forest ends of transects A and B whereas no clear trend was observed for transect C (Figs 1–3). Bands were therefore only excised from transects A and B for further sequence analysis (see Figs 1 and 2). In total, 19 DGGE bands from transects A and B were excised and sequenced. FASTA searches of sequenced bands revealed database sequences that had highest similarity with the unknown DGGE sequences and closest species matches are presented in Table 1. Although the majority of sequences had a high (>85%) similarity with fungal ITS sequences in the databases, sequence similarities (<80%) of five sequences (B1d, B1f, B7c, B8b and B9d) were low (Table 1), hampering their identification to the species level. To provide more information and accuracy to the identification and phylogenetic placement of

these ITS sequences, the two closest matches were reported for each sequence (Table 1) and were used in the phylogenetic analysis (Figs 4 and 5). Twenty-one per cent of sequenced DGGE bands were most closely related to fungi belonging to the basidiomycetes. Three out of the four sequences most closely related to basidiomycetes were isolated from the forest end of transect B. FASTA and phylogenetic analysis revealed band B9a to be most closely related to the ectomycorrhizal taxon *Piloderma* sp. whereas bands B8b and B9d were most closely related to *Clavulina cinerea* and *Armillaria novae-zelandiae* (Table 1, Fig. 4). The fourth basidiomycete sequence, A1c, was isolated from the moorland end of transect A and was most closely related to *Trichosporon* sp. (Table 1, Fig. 4).

The largest proportion (79%) of sequenced DGGE bands from both transects A and B had the highest sequence similarity to ascomycete taxa (Table 1). Sequenced bands B1d and B1f were most closely related to *Verticillium* sp. whereas B7c was most closely related to *Sporopachydermia* sp. (Table 1, Fig. 4). These three sequences clustered in the phylogenetic analysis with the

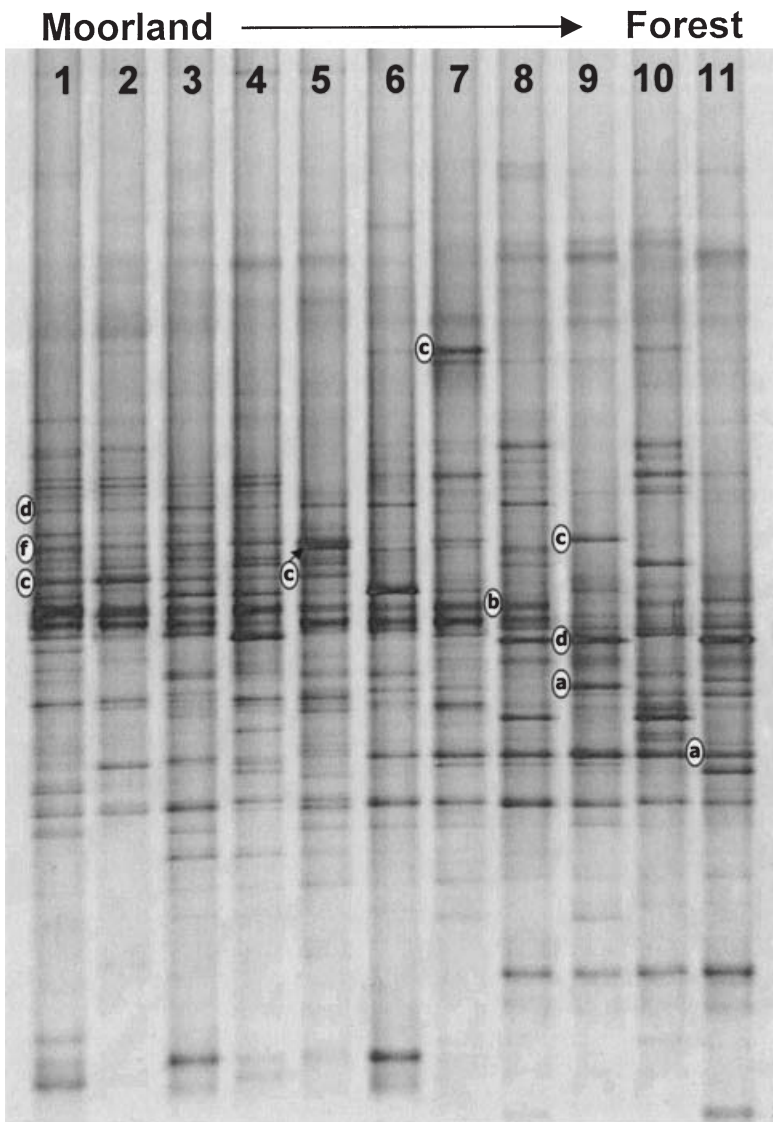


Fig. 2. DGGE analysis of partial fungal ITS sequences amplified from pooled DNA samples from transect B at the Abernethy field site. Lane numbers correspond to sampling point along the transect. Bands marked with letters to the left of the lane correspond to bands that were excised from SYBR Gold stained gels and sequenced.

bands most similar to basidiomycete taxa in group II (Fig. 4). The remaining sequences clustered tightly in the phylogenetic analysis as indicated by the short branch lengths in group I (Fig. 4). Band A11a was most closely related to *Penicillium* sp. whereas A1b and A11c were most closely related to *Guignardia philoprina* and *Neofabraea* sp. respectively. The remainder of the sequences clustering within this group are most closely related to a complex group of soil ascomycetes, some of which have been shown to form either ecto- or ericoid mycorrhizas (Table 1, Fig. 4). As the sequences falling within group I are so closely related to each other and so distantly related to the other sequences in the phylogenetic analysis, a second analysis was performed with these sequences alone to improve their placement within this group (Fig. 5).

In some cases, bands from the same position in a

DGGE gel from different points in the transects were sequenced to determine whether bands running to the same position were the same sequence type. Sequences of bands A1a and A6a (band position M3, Fig. 1) were identical (Fig. 5), whereas sequences from bands A2a, A5a and B1c were identical, although from two different transects, indicating the presence of the sequence type across both transect A and B (Figs 1, 2 and 5).

Discussion

A clear shift in fungal ITS-DGGE profiles was found along a moorland-pine forest environmental gradient suggesting a change in the soil fungal community structure across the gradient. Trends in a range of physical, chemical and biological properties have been demonstrated in previous research at the Abernethy forest field site, distinguishing

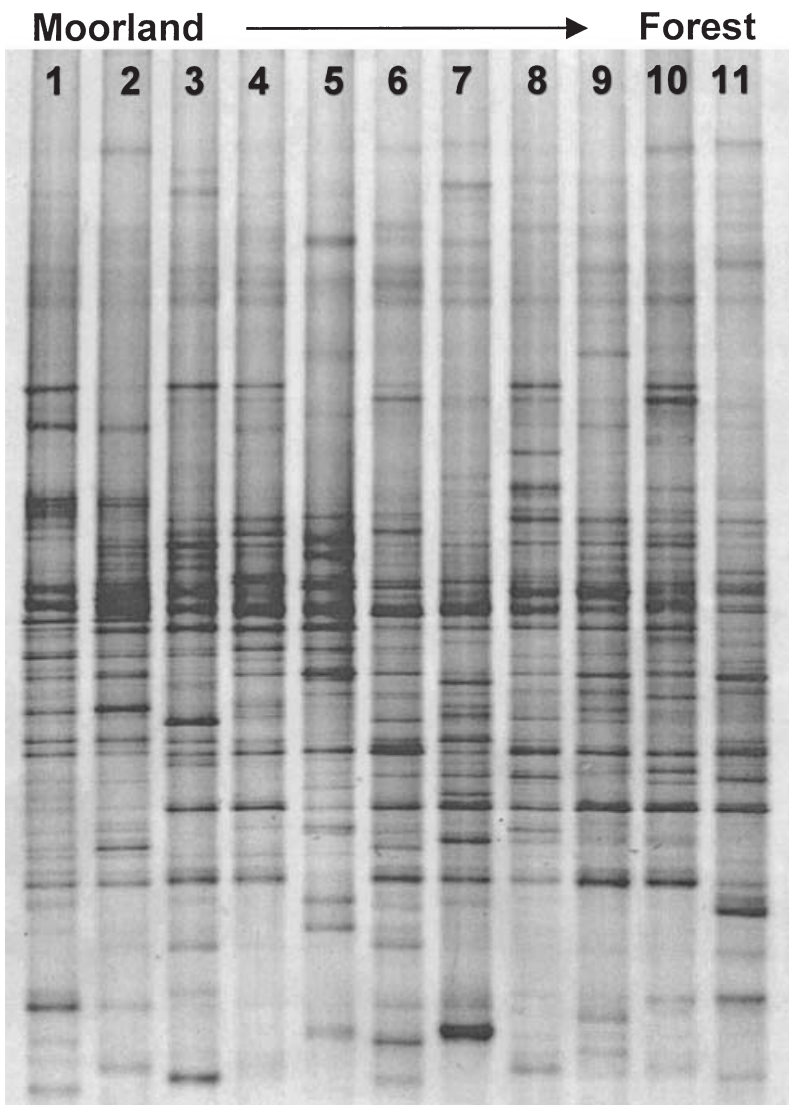


Fig. 3. DGGE analysis of partial fungal ITS sequences amplified from pooled DNA samples from transect C at the Abernethy field site. Lane numbers correspond to sampling points along the transect.

the moorland and forest ends of the transects (Chapman *et al.*, 2001; 2003). Tree colonization will alter the types and amounts of C inputs in litter and may increase soil drainage, both factors potentially altering fungal community structure. Interestingly, although a trend was observed for both transects A and B, no obvious shift was observed in ITS-DGGE profiles for transect C. Although this could be due to a variety of factors, transect C was distinguished by higher soil moisture content and more points along transect C were classified as 'bog' than transects A or B (Chapman *et al.*, 2003). Laboratory studies of fungi in pure culture have demonstrated differences between species in response to water stress (Mexal and Reid, 1973; Boddy, 1983; Coleman *et al.*, 1989). In addition, it has been shown that increasing the soil water content, and hence water potential, may inhibit fungal growth by uncontrolled influx of water resulting in cell lysis or by limitation of free oxygen (Cook and Papendick,

1970). Culture based studies of fungal communities in Swedish mires (moorlands) have been shown to differ between sites of different wetness (Nilsson *et al.*, 1992). Consequently, the development of fungal communities in transect C may therefore have been influenced directly by soil moisture content and by associated effects on tree growth and litter and exudate inputs.

Whereas the extremes of the transects represent mature Scots pine forest and moorland ecosystems, intermediate regions are characterized by the presence of colonizing Scots pine seedlings, representing a zone of transition between the two ecosystem types. This transition zone occurred over sampling points 2–6 for the three transects (Chapman *et al.*, 2003). ITS-DGGE data indicate that the presence of young colonizing seedlings in this 'transition zone' had little effect on the ITS-DGGE profiles and, by implication, on soil fungal community structure. A gradual change in fungal com-

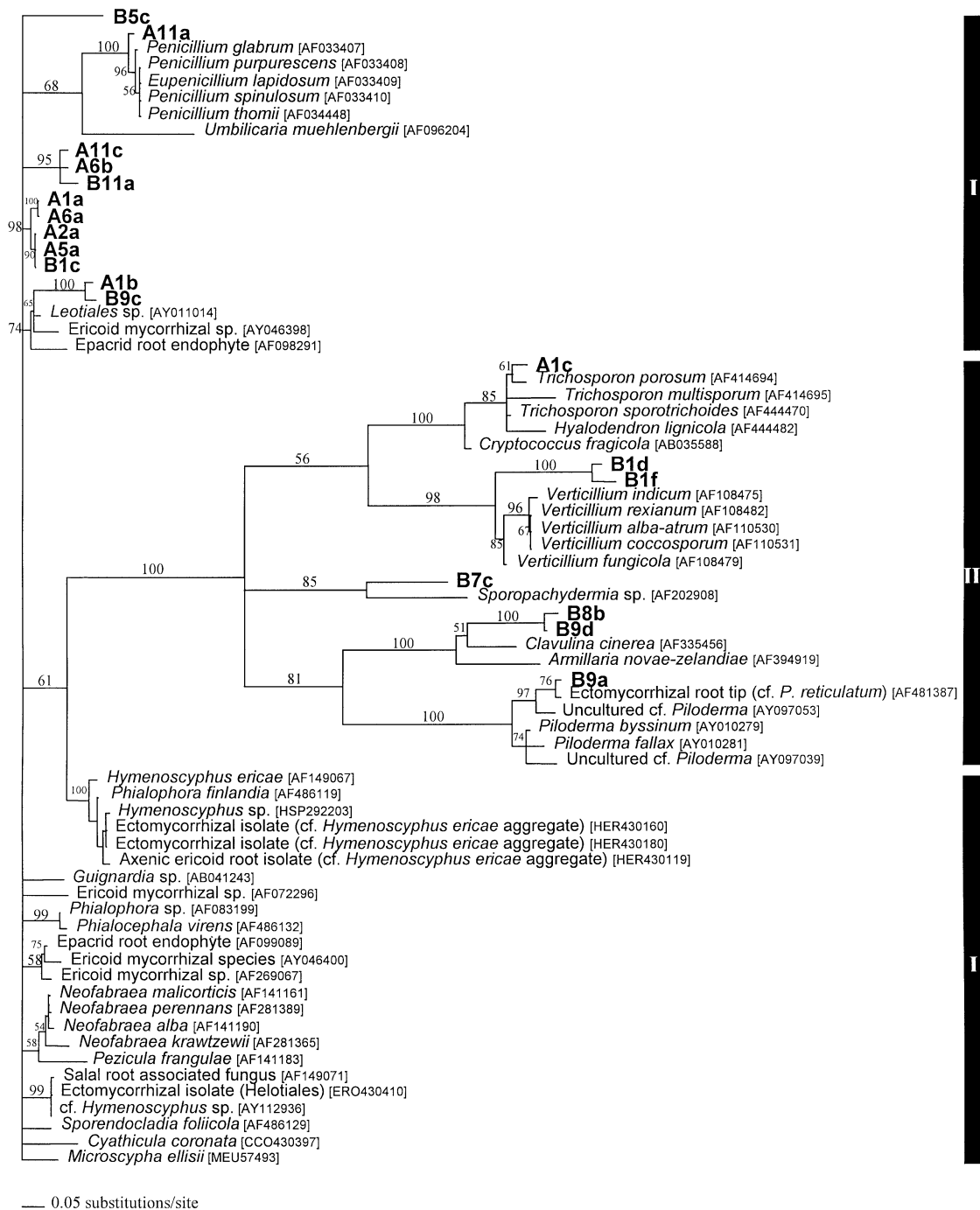


Fig. 4. Neighbour-joining tree showing the relationship between the 19 sequenced ITS DGGE bands from transects A and B (indicated in bold) with representative sequences from the GenBank/EMBL/DBJ databases. Band numbers correspond to those presented in Figs 1 and 2. The scale bar represents the number of base substitutions per site. Bootstrap values are shown for the major branches which had >50% support in a bootstrap analysis of 1000 replicates.

munity might have been expected over this zone, as a result of colonization of the seedlings in conjunction with their associated ectomycorrhiza. In contrast, ITS-DGGE profiles indicate a sharp boundary between established moorland and forest at the spatial scale investigated,

with few bands common to both extremes and considerable variation in relative intensity of common bands between extremes. A similar sharp boundary effect was observed in physical, chemical and biological properties at this site (Chapman *et al.*, 2003). Our data therefore

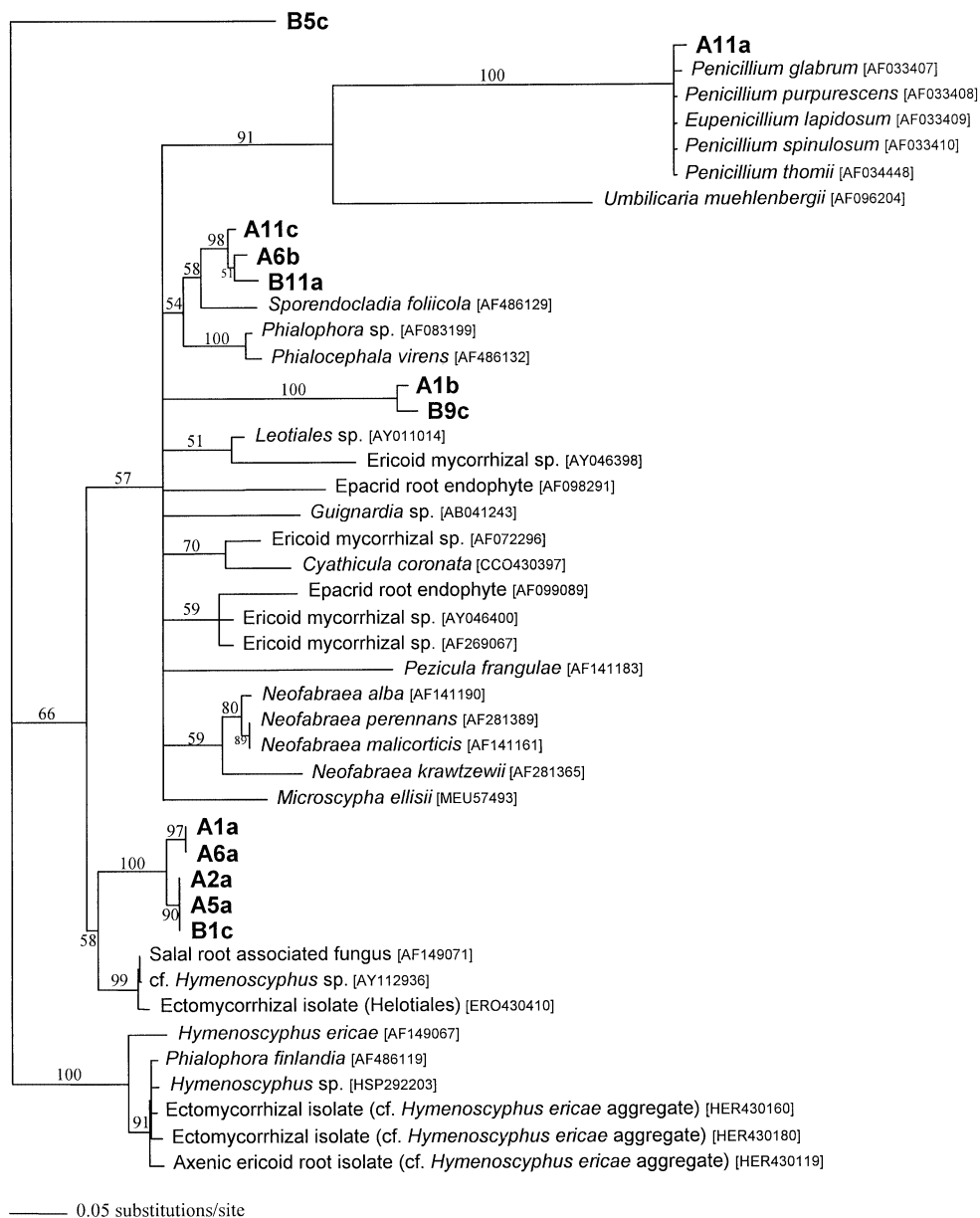


Fig. 5. Neighbour-joining tree showing the relationship between the 45 sequences which fell into group I in the phylogenetic analysis presented in Fig. 4. Transect sequences are indicated in bold. Remaining details as for Fig. 4.

suggest that *P. sylvestris* seedling expansion has little effect on the soil fungal community at an early stage of development but the clear differences in fungal communities in established moorland and mature forest samples indicate long-term changes associated with further development, expansion and maturation of the native pinewood forest.

Nineteen ITS-DGGE bands were excised and sequenced from transects A and B and the sequenced DGGE bands with similar electrophoretic mobilities in different samples were identical. Obtaining a clean, single ITS-DGGE band of sufficient quality for sequencing

required at least three rounds of excision and PCR re-amplification and in several cases was unsuccessful. Bands which could not be purified after three rounds of excision and PCR re-amplification were excluded from further analysis. These difficulties have been reported previously (e.g. Kowalchuk *et al.*, 2003) with the success rate dependent upon the complexity and resolution of the original profile and whether the single band actually represents a single sequence type. Previous research has shown that microbial community profiles can often consist of more sequence types than are detectable by even the most sensitive staining techniques, and that PCR prod-

Table 1. Two closest species matches obtained from FASTA searches between DNA of excised ITS-DGGE bands and sequences from the GenBank/EMBL/DDBJ databases.

Sequenced band ^a	Sequence length (bp)	Accession code	Closest species match	% Similarity	Overlap (bp)
A1a	238	AY324151	cf. <i>Hymenoscyphus</i> sp. [AY112936] ^b	91.9	212
			<i>Phialophora finlandia</i> [AF486119] ^b	87.1	233
A1b	246	AY324152	<i>Guignardia philoprina</i> [AB041243]	86.2	245
			<i>Phialocephala virens</i> [AF486132]	84.2	253
A1c	178	AY324153	<i>Trichosporon porosum</i> [AF414694]	99.4	177
			<i>Trichosporon sporotrichoides</i> [AF444470]	95.5	177
A2a	229	AY324154	cf. <i>Hymenoscyphus</i> sp. [AY112936] ^b	92.8	221
			<i>Phialophora finlandia</i> [AF486119] ^b	84.8	237
A5a	223	AY324155	cf. <i>Hymenoscyphus</i> sp. [AY112936] ^b	92.6	215
			<i>Phialophora finlandia</i> [AF486119] ^b	84.4	231
A6a	233	AY324156	cf. <i>Hymenoscyphus</i> sp. [AY112936] ^b	91.2	215
			Ericoid mycorrhizal sp. [AF269067] ^b	87.6	233
A6b	238	AY324157	<i>Neofabraea malicorticis</i> [AF141161]	92.9	238
			Ericoid mycorrhizal sp. [AF269067] ^b	92.2	231
A11a	253	AY324149	<i>Penicillium purpurescens</i> [AF033408]	94.0	234
			<i>Penicillium glabrum</i> [AF033407]	93.6	234
A11c	236	AY324150	<i>Neofabraea malicorticis</i> [AF141161]	93.2	235
			<i>Neofabraea alba</i> [AF141190]	92.8	235
B1c	241	AY324159	cf. <i>Hymenoscyphus</i> sp. [AY112936] ^b	92.8	222
			Ericoid mycorrhizal sp. [AF269067] ^b	88.8	240
B1d	228	AY324160	<i>Verticillium rexianum</i> [AF108482]	79.0	205
			<i>Verticillium coccosporum</i> [AF110531]	79.0	205
B1f	227	AY324161	<i>Verticillium coccosporum</i> [AF110531]	78.7	207
			<i>Verticillium rexianum</i> [AF108482]	78.7	207
B5c	264	AY324162	<i>Phialophora finlandia</i> [AF486119] ^b	86.0	215
			Ectomycorrhizal isolate (<i>H. ericae</i> aggregate) [AJ430180] ^b	86.5	215
B7c	182	AY324163	<i>Schizosaccharomyces pombe</i> [SPCP20C8]	66.5	167
			<i>Sporopachydermia</i> sp. [AF202908]	70.7	184
B8b	243	AY324164	<i>Clavulina cinerea</i> [AF335456] ^b	79.5	171
			<i>Armillaria novae-zelandiae</i> [AF394919]	69.1	256
B9a	221	AY324165	Ectomycorrhizal root tip (cf. <i>Piloderma reticulatum</i>) [AF481387] ^b	98.1	215
			Uncultured cf. <i>Piloderma</i> [AY097053] ^b	97.7	216
B9c	227	AY324166	<i>Leotiales</i> sp. [AY011014]	86.3	212
			Ericoid mycorrhizal sp. [AY046398] ^b	83.0	212
B9d	263	AY324167	<i>Clavulina cinerea</i> [AF335456] ^b	79.6	172
			<i>Armillaria novae-zelandiae</i> [AF394919]	67.4	261
B11a	219	AY324158	<i>Neofabraea malicorticis</i> [AF141161]	91.8	219
			Ericoid mycorrhizal sp. [AF269067] ^b	91.8	219

a. Band numbers correspond to those presented in Figs 1 and 2 for transects A and B.

b. Species known to form mycorrhizas.

ucts appearing as single bands on gels often consist of more than one sequence type (e.g. Schmalenberger and Tebbe, 2003).

FASTA analysis of sequenced ITS-DGGE bands from transects A and B demonstrated that the majority of sequenced bands had high (>85%) similarity with sequences in the databases with all sequenced bands being most closely related to either ascomycete (79%) or basidiomycete taxa (21%). This compares with cultivation-based estimates by Dickinson and Dooley (1967) for uncut bogs in which 77% of isolated fungi were identified as ascomycetes. Although ITS-DGGE data may actually reflect the relative abundance of ascomycete and basidiomycete taxa present in the transect soil samples, the possibility that DNA extraction and PCR conditions might introduce bias cannot be ignored (Smit *et al.*, 1999). The fungal ITS region was targeted

because it is taxonomically more informative than other genomic regions (e.g. 18S rDNA) (Bruns *et al.*, 1991). Nevertheless, five ITS sequences obtained in the current work had low similarity with fungal ITS sequences in the databases and on two occasions (B8b and B9d) the closest sequence match was with uncultured fungal ITS sequences generated from soil cloning studies (e.g. Chen and Cairney, 2002). These sequences had high similarity with the clone ITS sequences in the database but were not included in the phylogenetic analysis as they do not aid in the identification of unknown ITS sequences. Whereas this highlights the limitations imposed by existing fungal sequence databases when characterizing natural communities, it also suggests the presence of ecologically important, and perhaps unculturable, fungal taxa that are not currently represented in the databases.

The largest proportion of ITS sequences generated represent ascomycete taxa with the majority being most closely related to species in the fungal order Helotiales. Whereas the Helotiales is a complex group of fungi whose ecological roles are generally poorly understood, a large proportion of ITS sequences were phylogenetically closely related to species that are known to form ericoid mycorrhizas (Vrålstad *et al.*, 2002). The vegetation composition at the field site is heavily dominated by the ericaceous plant species *Calluna vulgaris*, *Vaccinium vitis-idaea* and *Vaccinium myrtillus* which produce very fine hair roots (approx. 0.1 mm) (Gimingham, 1960) that are difficult to remove from soil samples. Therefore, although it is highly likely that most of the detected ITS sequences originated from free-living soil fungi, it remains a possibility that some of the sequences originated from ericoid mycorrhizal fungal species present within fine hair roots. Previous vegetation surveys at Abernethy found *V. vitis-idaea* and *V. myrtillus* exclusively in the forest end of each transect while *C. vulgaris* was dominant at the moorland end, although its presence extended into the forest in transects B and C (Chapman *et al.*, 2003). Therefore, changes in the ericaceous vegetation structure between the forest and moorland ends of the transects may play a significant role in influencing the soil fungal community, particularly if there is a degree of host specificity in the selection and recruitment of specific ericoid mycorrhizal symbionts. Although numerous ascomycete fungi belonging to the Helotiales form ericoid mycorrhizal associations, several species within this broad group also form ectomycorrhizal associations (Vrålstad *et al.*, 2000). For example, *Piceirhiza bicolorata* is an ectomycorrhizal ascomycete belonging to the Helotiales group and is commonly found in association with *Pinus sylvestris* root systems in the field (Vrålstad *et al.*, 2002). Therefore, it is also possible that at least some of the variation observed within the ascomycete sequences represents the presence of ectomycorrhizal members of the Helotiales in the samples from the forest end of the transects.

The data presented show distinct differences between fungal communities associated with the forest and the moorland ecosystems, whereas colonizing Scots pine seedlings in the 'intermediate zone' appear to have little effect on the soil fungal community. Nonetheless, it is relatively clear that with the continued maturation of colonizing seedlings and subsequent expansion of native Scots pine woodlands, a change in the composition of the soil fungal community is a likely consequence. This change may, at least partially, be a result of changes in the understorey vegetation structure associated with canopy development of the Scots pine trees as they mature.

Experimental procedures

Field site description

Field samples were obtained from an area of mature Scots pine (*Pinus sylvestris*) at the southern edge of Abernethy Forest, Cairngorm, Scotland (National Grid Reference NJ027122), extending in a south-easterly direction into open moorland. Within the intermediate zone there was active seedling colonization by Scots pine that, at the time of sampling, were 10–15 years old, although growth was slow and few seedlings exceeded 1–1.3 m in height. Soil at the forest end consisted of a humus iron podzol (orthic podzol) changing to a peaty podzol (placic podzol) at the moorland end. Most of the intermediate zone was wetter than either end of the transects.

Soil sampling procedure

Samples were taken on August 25th 1998 along three parallel transects (A, B and C), each 180 m long and 50 m apart, running in an approximately south-east-north-west direction from the open moorland into the forest. Each transect was divided into 10 sections with 11 points in total, 18 m apart, starting from transect point No.1 in the moorland to transect point no. 11 in the forest. The boundary between the open moorland and the forest was found to be slightly different between transects such that transects A and B extended further into the forest than transect C. At each transect point a soil core was taken using a 35 cm long, square sectioned (5 × 5 cm) stainless-steel corer (Cuttle and Malcolm, 1979). Four further cores were taken perpendicularly, 1 and 2 m on either side of the transect point. The cores were taken to a depth of 30 cm or to the mineral horizon if the organic horizon was <30 cm. Where trees or substantial root systems were encountered, cores were taken from the nearest free area. After transport to the laboratory, the forest litter layer, or in the case of moorland samples, the undecomposed *Sphagnum* layer was removed and the top 15 cm of F_m (fermentation) and H_f (humification) horizons were separated from underlying mineral matter. At the forest end, the H_f horizon generally extended to a depth of 15 cm, but was shallower in a few samples. Where horizons were not clearly defined, samples were taken to 15 cm below the undecomposed *Sphagnum* layer. The five cores for each transect point were then bulked and sieved to 5 mm. All remaining root material was removed from the soil samples but, due to their small size (~0.1 mm) (Gimingham, 1960), it is possible that some fine hair root material from the ericaceous vegetation may have remained in the samples.

DNA extraction

DNA was extracted from duplicate 0.5 g soil subsamples using the method previously described by Griffiths *et al.* (2000). Briefly, this involved the addition of 0.5 ml of hexadecyltrimethylammonium bromide (CTAB) extraction buffer and 0.5 ml of phenol/chloroform/isoamyl alcohol (25 : 24 : 1; pH 8.0) to 0.5 g of soil in a Bio-101 Multimix matrix tube. The sample was then lysed for 30 s at a speed of 5.5 m s⁻¹ in a FastPrep bead beating system (Bio-101, Vista, California,

USA) and the aqueous phase was separated by centrifugation at 13500 r.p.m. for 10 min. The sample was extracted further with an equal volume of chloroform/isoamyl alcohol (24 : 1) before precipitating the nucleic acids with two volumes of 30% (w/v) polyethylene glycol 6000 in 1.6 M NaCl for 2 h at room temperature followed by centrifugation at 13500 r.p.m. for 30 min. Pelleted nucleic acids were then washed with cold 70% (v/v) ethanol and air-dried before resuspension in 50 μ l of Tris-EDTA buffer (pH 7.4). DNA from duplicate extractions was then pooled.

PCR amplification of fungal-specific genes

Fungal 18S rDNA and ITS regions were PCR-amplified using the primers EF4 (Smit *et al.*, 1999) and ITS4 (White *et al.*, 1990) in an attempt to amplify as broad a range of fungal taxa from the soil samples as possible (see Anderson *et al.*, 2003). Polymerase chain reactions were carried out on a Dyad DNA Engine thermal cycler (MJ Research, Waltham, USA) using 50 μ l reaction volumes containing approximately 50 ng of template DNA, 20 pmol of each primer, 2 mM MgCl₂, 250 μ M of each of dATP, dCTP, dGTP and dTTP, 10 \times buffer [20 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Tween 20 (v/v), 0.5% Nonidet P40 (v/v), 50% glycerol (v/v)], 1 μ l of a bovine serum albumin (BSA) solution (20 mg ml⁻¹) and 2.5 U of Expand High Fidelity DNA polymerase (Roche, Lewes, UK). Cycling parameters were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 5 min. Reactions were performed in duplicate and negative controls (containing no DNA) were included in each PCR. All amplification products were electrophoresed in 1.5% (w/v) agarose gels, stained with ethidium bromide and visualized under UV light. These products were then used as template in a nested PCR to generate products for DGGE analysis. Whereas several combinations of both 18S rDNA and ITS primers were tested for their suitability in DGGE analyses (data not shown), the sharpest and most consistent DGGE profiles were obtained with the primers ITS1-F (Gardes and Bruns, 1993) and ITS2 (White *et al.*, 1990) generating partial ITS products. A GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GCG GGG GCA CGG GGG G-3') (Muyzer *et al.*, 1993) was added to the 5' end of the ITS1-F primer to stabilise the melting behaviour of the DNA fragments. PCR and cycling conditions were as described above, except that BSA was omitted from the reactions.

Denaturing gradient gel electrophoresis (DGGE) analysis

Denaturing gradient gel electrophoresis analyses were carried out using the DCode universal mutation detection system (Bio-Rad, Hemel Hempstead, United Kingdom). Polyacrylamide gels (8% Acrylogel 2.6 solution; BDH, Poole, UK) were prepared with a 10% [0.7 M urea – 4% (v/v) formamide] to 50% [3.5 M urea – 20% (v/v) formamide] vertical gradient using a gradient former (Fisher Scientific, Loughborough, UK) and a peristaltic pump with a flow rate of 5 ml min⁻¹. Gels that were silver stained were poured onto the hydrophilic side of GelBond PAG film (BioWhittaker, Wokingham, UK) to facilitate handling during the staining process. Approximately

500 ng of each PCR product was loaded onto the gels and electrophoresis was performed in 1 \times TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 75 V and 60°C for 16 h. Gels were silver stained as previously described by McCaig *et al.* (2001) and were scanned using an Epson GT-9600 scanner. Bands of interest were identified for further analysis and were excised from re-run DGGE gels stained with SYBR Gold nucleic acid stain (Molecular Probes, Leiden, The Netherlands). Excised bands were reamplified as described above and re-run against the initial sample to ensure that the target band had been amplified. In most instances, a single band was obtained after 2–4 cycles of excision and PCR reamplification. Sequenced excised bands are referred to throughout in the format transect letter (A, B or C), transect point number (1–11), band reference [e.g. A1a].

DNA sequencing and data analysis

Polymerase chain reaction products generated from excised DGGE bands were purified using the Qiaquick PCR purification kit (Qiagen, Crawley, UK). Purified DNA was then sequenced using the BigDye Terminator Cycle Sequencing Kit on an automated DNA sequencer (ABI model 310, Applied Biosystems, Warrington, UK) using the primers ITS1-F and ITS2. DNA sequences were manually checked and edited where necessary using the Sequencher software package (version 3.0; Gene Codes Corporation, Michigan, USA). All sequences were analysed using the FASTA 3.0 program (Pearson and Lipman, 1998) to determine the closest matches in the GenBank/EMBL/DDBJ nucleotide databases. Final sequences were then aligned with sequences downloaded from GenBank using CLUSTALW Ver. 1.8.2 (Thompson *et al.*, 1994) and manual adjustments were made to the alignment where necessary. The proportion of invariable sites and the γ -distribution parameter were estimated by performing a Parsimony analysis using the F84 model and the heuristic search option in PAUP Ver. 4.0b10 (Swofford, 2002) and the tree scores were then calculated using the maximum likelihood option. Final trees were generated by performing a neighbour-joining analysis with 1000 bootstrap replicates using the parameters previously calculated from the parsimony analysis. All nucleotide sequences were submitted to the DDBJ/EMBL/GenBank databases under the accession numbers presented in Table 1.

Acknowledgements

This work was funded by The Macaulay Development Trust as part of The Macaulay Institute/University of Aberdeen Soil Health Initiative. C.D.C. is funded by the Scottish Executive Environment and Rural Affairs Department. The authors gratefully acknowledge the scientific cooperation of SNH and RSPB at Abernethy Nature Reserve and I. J. Alexander and S. J. Chapman for useful discussion.

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