Mycorrhiza (2007) 17:259–270
DOI 10.1007/s00572-007-0129-2

REVIEW

# Using terminal restriction fragment length polymorphism (T-RFLP) to identify mycorrhizal fungi: a methods review

I. A. Dickie · R. G. FitzJohn

Received: 7 January 2007 / Accepted: 23 March 2007 / Published online: 12 April 2007
© Springer-Verlag 2007

**Abstract** Terminal restriction fragment length polymorphism (T-RFLP) is an increasingly widely used technique in mycorrhizal ecology. In this paper, we review the technique as it is used to identify species of mycorrhizal fungi and distinguish two different versions of the technique: peak-profile T-RFLP (the original version) and database T-RFLP. We define database T-RFLP as the use of T-RFLP to identify individual species within samples by comparison of unknown data with a database of known T-RFLP patterns. This application of T-RFLP avoids some of the pitfalls of peak-profile T-RFLP and allows T-RFLP to be applied to polyphyletic functional groups such as ectomycorrhizal fungi. The identification of species using database T-RFLP is subject to several sources of potential error, including (1) random erroneous matches of peaks to species, (2) shared T-RFLP profiles across species, and (3) multiple T-RFLP profiles within a species. A mathematical approximation of the risk of the first type of error as a function of experimental parameters is discussed. Although potentially less accurate than some other methods such as clone libraries, the high throughput of database T-RFLP permits much greater replication and may, therefore, be preferable for many ecological questions, particularly when combined with other techniques such as cloning.

**Keywords** Arbuscular mycorrhiza · Ectomycorrhiza · Molecular identification · Terminal restriction fragment length polymorphism (T-RFLP)

I. A. Dickie (✉) · R. G. FitzJohn
Landcare Research,
P.O. Box 40, Lincoln 7640, New Zealand
e-mail: dickiei@landcareresearch.co.nz

## Introduction

Mycorrhizal fungi occur in highly diverse communities (Bruns 1995) with fine-scale spatial partitioning (Dickie et al. 2002; Dickie and Reich 2005; Genney et al. 2006). Sporocarp production is only loosely related to belowground community patterns (Gardes and Bruns 1996), and many fungi produce cryptic and/or hypogeous sporocarps. Because of these difficulties, there has been an increasing reliance on molecular methods for identifying species from belowground structures (Horton and Bruns 2001), initially with restriction fragment length polymorphism analysis (RFLP, also known as ARDRA: amplified ribosomal DNA, rDNA, restriction analysis), and then increasingly with other techniques including sequencing, denaturing gradient gel electrophoresis (DGGE; Kowalchuk et al. 2002; Opik et al. 2003; Bougoure and Cairney 2005; Landeweert et al. 2005; Ma et al. 2005; Pennanen et al. 2005), and clone libraries (Landeweert et al. 2003; Renker et al. 2006), as well as terminal restriction fragment length polymorphism (T-RFLP, sometimes without hyphen).

Over the last several years, there has been an increasing interest in the use of T-RFLP for the identification of mycorrhizal fungi (citations in Table 1). It has been suggested that T-RFLP is more sensitive than DGGE for fungi (Brodie et al. 2003; Singh et al. 2006), although obtaining sequences directly from samples may be more easily performed with DGGE (Ma et al. 2005). T-RFLP also has significant advantages in cost over clone libraries, although clone libraries are likely the most accurate method of identifying species. Using clone libraries together with T-RFLP may permit both techniques to be used to their full potential: using T-RFLP to process large numbers of samples and clone libraries on selected samples to obtain identities of key species (Lindahl et al. 2006; Widmer et al. 2006).

**Table 1** Summary of papers that apply T-RFLP specifically to mycorrhizal fungi

| Citation(s)[a] | Analysis method | Primers | Matching error ($\delta$), in bp | Number of simultaneously labeled primers (/total labeled primers) | RFLP enzymes | PCR product length | Total profiles used ($n$) | Peak height used | Notes |
|---|---|---|---|---|---|---|---|---|---|
| *AMF* | | | | | | | | | |
| Lekberg et al. (2007) | Database | SSU–Glom1+ LSU–Glom1, ITS4+ITS5 | 2 | 2 | *Hin*fI | Yes | 3 | No | Nested PCR, ITS4+ITS5 primers labeled |
| Aldrich-Wolfe (2007) | Database | SSU–Glom1+ LSU–Glom1, ITS4+ITS5 | 3 | 2 | *Hin*fI, *Dpn*II | Yes | 5 | No | Nested PCR, ITS4+ITS5 primers labeled |
| Tonin et al. (2001) | Peak profile | MH2+MhPh1 | ?[b] | 1 | *Taq*I (*Bse*DI attempted but not used in analysis) | No | 2 | No | Used non-AMF-specific primers to investigate fungal diversity; also identified some AMF species based on predicted peak lengths from sequence data |
| Johnson et al. (2003); Vandenkoornhuyse et al. (2003) | Peak profile | NS32+AM1 | ?[b] | 2 | *Hin*fI, *Hsp*92II | No | 4 | No | Used a sophisticated approach to peak profiles, incorporating data from multiple enzymes |
| Mummey et al. (2005) | Peak profile | AM1+NS31 | 0.5 | 1 | *Dpn*II | No | 1 | Yes | Used 3 replicate T-RFLP profiles from each sample to reduce error in height quantification |
| Mummey and Rillig (2006) | Peak profile | LR1+FLR2, FLR3+FLR4 | ?[b] | 2 | *Taq*1 | No | 2 | Yes? | Nested PCR, FLR3+FLR4 primers labeled |
| *ECM* | | | | | | | | | |
| Dickie et al. (2002); Koide et al. (2005a, b) | Database | ITS1F+ITS4 | 3 | 2 | *Hin*fI, *Hae*III, (*Dpn*II) | Yes | 5 | No | *Dpn*II gave poor results and was abandoned |
| Zhou and Hogetsu (2002) | Database | ITS3+ITS4 | ? | 1/2 | *Hin*fI, *Alu*I | Yes | | | |
| Nara et al. (2003); Nara (2005) | Database | ITS3+ITS4, ITS1F+ITS4 | ?[b] | 1/2 | *Hin*fI | Yes | 2 | No | |
| Edwards et al. (2004) | Database | ITS1F+ITS4B | 2 | 1 | *Hin*fI, *Taq*I+ *Hae*III | No | 2–3 | Yes | *Hae*III used only where needed after *Hin*fI and *Taq*I |
| Burke et al. (2005); Burke et al. (2006b) | Database | NS1+58A2R; 58A2F+NLB4 | 10 | 2/4 | *Alu*I, *Hae*III | No? | 8 | Yes | Amplified ITS1 and ITS2 regions separately |
| Genney et al. (2006) | Database | ITS1F+ITS4 | 1.5 | 2 | *Hin*fI, *Taq*I | No | 4 | No | Used nested PCR with ITS4B in some cases |
| Lindahl et al. (2006) | Database | ITS1F+ITS4 | 2 | 2 | *Taq*I, *Cfo*I | No | 4 | No | Other restriction enzymes used when base 2 failed to distinguish species |
| Dickie, unpublished data | Database | ITS1F+ITS4 | 1.5 | 2 | *Hae*III, *Hpy*CH4IV | No | 4 | No | These are the methods presently used by the authors |

[a] Where more than one citation is listed, the papers use essentially identical methods.
[b] Where "?" is noted, the authors did not report the measure or it is unclear from the text.

T-RFLP can mean two different things: under one application, analysis focuses on peak profiles without identification of species, while in the other application, individual species are identified and analysis focuses on these identified species. While the chemistry behind the two techniques is similar, the distinction is important, as the two methods have fundamentally different outputs and are subject to different types of errors. Our goals in this review are to clarify the second of these two methods, which we term "database T-RFLP," and to discuss errors and methodological issues in database T-RFLP. Our focus is specifically on mycorrhizal research, although most of the review will be relevant to other fields as well. Other recent reviews cover T-RFLP in a more general sense (Thies 2007), as well as other molecular approaches to molecular analysis of mycorrhizal communities (Horton and Bruns 2001; Anderson and Cairney 2004; Martin 2007).

## Background

In a generic sense, T-RFLP refers to the use of fluorescently labeled primers combined with restriction digests to visualize sequence variation in either single- or mixed-species DNA samples. The T-RFLP technique was first developed in Liu et al. (1997) as a tool for assessing bacterial diversity and comparing the community structure of bacteria in environmental samples (Marsh 1999; Lukow et al. 2000; Kitts 2001). The data obtained are the sizes of the fragments of polymerase chain reaction (PCR) amplicons that contain the labeled primer (the terminal fragment lengths), observed as electropherogram "peaks." Variation in the presence and location of cutting sites results in different species having terminal fragments of different lengths.

In T-RFLP, as used by Liu and colleagues, a single fluorescent label and a single restriction digest are used. The data are then analyzed based on the number of peaks and the similarity of peak profiles across samples (Dollhopf et al. 2001; Edel-Hermann et al. 2004; Mummey et al. 2005). Because the original version of T-RFLP focuses on the peak profiles of communities and to distinguish it from other uses of the technique, we refer to this application as "peak-profile T-RFLP."

There are a number of important limitations of peak-profile T-RFLP. The technique relies on having primers specific to the functional group of interest. This prevents application to ectomycorrhizal fungi, a polyphyletic group with close affinities with saprophytic fungi (Hibbett et al. 2000), as there are no primers that can separate ectomycorrhizal DNA from other fungal DNA. Peak-profile T-RFLP also works on the assumption that a single peak represents a single species and that a single species results in a single peak. Based on these assumptions, diversity in peak-profile T-RFLP is calculated as the number of peaks (Klamer et al. 2002; Blackwood et al. 2003; Johnson et al. 2003; Vandenkoornhuyse et al. 2003), yet both of these assumptions have been shown to be false (Dunbar et al. 2000; Douhan et al. 2005; Avis et al. 2006). This can lead to a bias in the analysis of community similarity and estimates of diversity (Avis et al. 2006).

In 2002, two papers were published that applied T-RFLP to ectomycorrhizal fungal communities (Dickie et al. 2002; Zhou and Hogetsu 2002), both of which made significant and similar changes to the T-RFLP technique. In this application, which we refer to as "database T-RFLP," the analysis of communities is based on the presence or absence of species, with species presence being inferred by matching peaks from community T-RFLP profiles to a database of known T-RFLP patterns derived from sporocarps or other sources. Diversity is measured as the number of species identified, rather than being based on the number of peaks. This adaptation of T-RFLP is similar to RFLP analysis, widely used in prior studies of ectomycorrhizal fungi, with the important advantage that T-RFLP permits the identification of fungi in mixed-species samples (Dickie et al. 2002). As database T-RFLP relies on species identities rather than peak profiles, it largely avoids the potential problems of peak-profile T-RFLP: multiple T-RFLP profiles from single species can be grouped together, and multiple species that share a single T-RFLP can be at least recognized, if not fully corrected for. Since the initial description of the technique, database T-RFLP has been used by a number of different research groups to describe ectomycorrhizal fungal communities (Table 1).

The use of database T-RFLP, rather than peak-profile T-RFLP, for ectomycorrhizal fungi is necessary due to the polyphyletic nature of ectomycorrhizal fungi. In contrast, arbuscular mycorrhizal fungi (AMF) belong to a monophyletic phylum, the *Glomeromycota* (Schüßler et al. 2001), and there are putatively specific primers for this group. This makes it possible to use either peak-profile T-RFLP or database T-RFLP for AMF. In the earliest application of T-RFLP to AMF, some AMF species were identified based on matching a single terminal fragment to predicted terminal restriction fragment lengths from sequences (Tonin et al. 2001), a technique somewhere intermediate between peak-profile T-RFLP and database T-RFLP. While most other published work using T-RFLP on AMF has used peak-profile T-RFLP, there are at least two publications using database T-RFLP to identify AMF species (Aldrich-Wolfe 2007; Lekberg et al. 2007). Peak-profile T-RFLP rests entirely on the assumption that primers are specific for the group of interest and that all members of that group are amplified. The observation that putatively "AMF-specific" primers can amplify non-AMF species (Douhan et al. 2005; Renker et al. 2006; Aldrich-Wolfe 2007) and fail to amplify

some AMF groups (Renker et al. 2003) suggests that database T-RFLP may be more appropriate than peak-profile T-RFLP for AMF, as well as ectomycorrhizal fungi.

Outside mycorrhizal research, most other mycological studies have tended to use peak-profile T-RFLP (Klamer et al. 2002; Brodie et al. 2003; Edel-Hermann et al. 2004; Klamer and Hedlund 2004), but database T-RFLP has been applied to wood decay fungi (Råberg et al. 2005; Råberg et al. 2007; Allmer et al. 2006). Peak-profile T-RFLP has also been used to examine bacterial community responses to mycorrhizal fungi (Artursson et al. 2005; Burke et al. 2006a).

## Methodological considerations in using T-RFLP to identify species

### Method summary

The basic steps of database T-RFLP, compared with RFLP (or ARDRA) and peak-profile T-RFLP (Fig. 1), are (1) the extraction of DNA from either a single root, bulked roots, or soil; (2) PCR amplification of DNA with fluorescently labeled fungus-specific primers, typically followed by verification of amplification in agarose gels; (3) cleaning of PCR products to remove salts and primers; (4) digestion of PCR products with restriction enzymes; (5) electrophoresis in a capillary or slab sequencer; and (6) analysis of results.

Database T-RFLP is essentially a hybrid technique between RFLP and peak-profile T-RFLP, combining data from multiple restriction digests and comparing results with a database of knowns (as in RFLP), but running samples in a sequencer and only detecting terminal fragments (as in peak-profile T-RFLP). Compared with RFLP analysis, the main differences of database T-RFLP are:

1. Only terminal PCR fragments are detected, and the position (i.e., forward and reverse terminus) of those fragments is known.
2. The precision of fragment length estimation is greatly increased.
3. The detection threshold is greatly decreased.
4. Multiple species can be easily identified, such that samples with multiple PCR products do not need to be discarded.

The main differences of database T-RFLP compared with peak-profile T-RFLP are:

1. Multiple-labeled PCR primers and/or multiple RFLP digests result in multiple peak profiles for each sample, rather than a single profile.

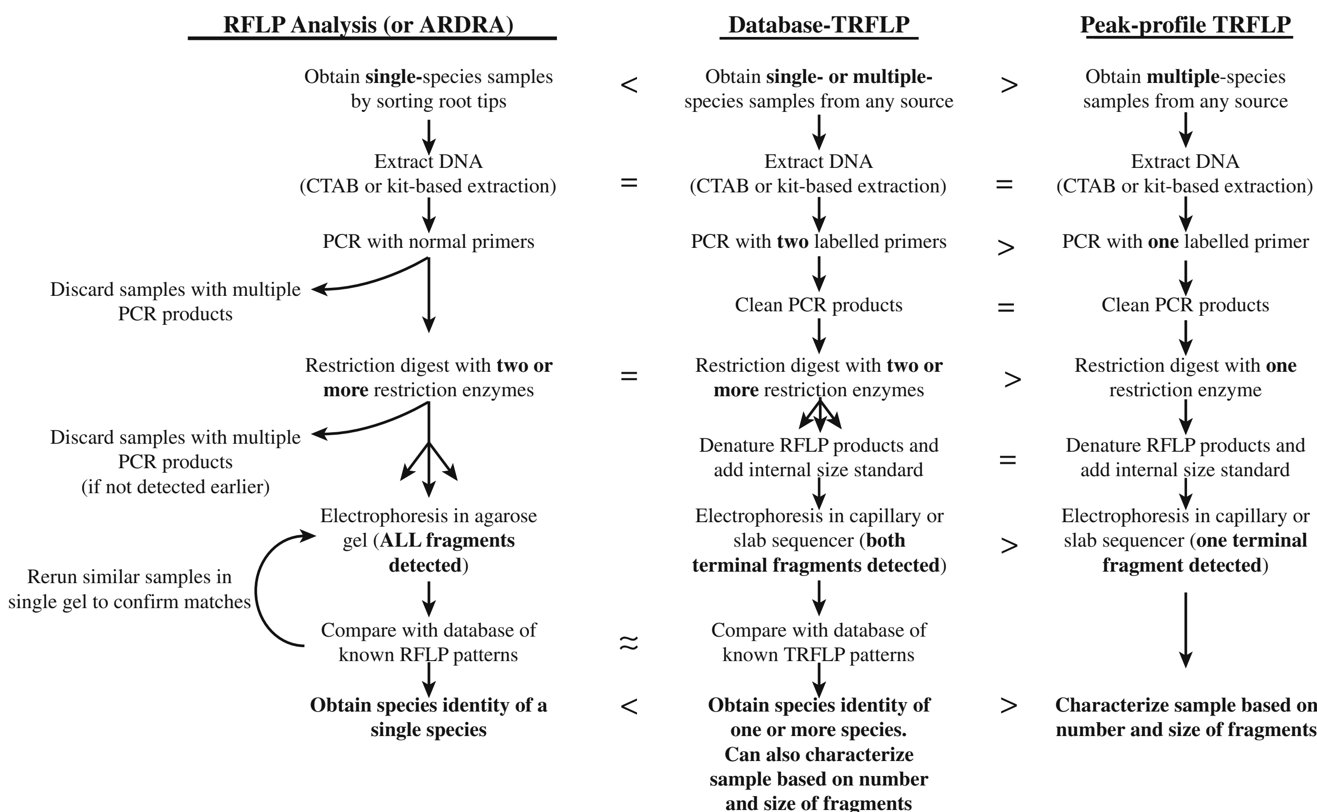


**Fig. 1** Schematic of method flow for RFLP, database T-RFLP, and peak profile T-RFLP. The *equal* and *approximate* signs indicate where two techniques are identical, or nearly identical, respectively. Where one technique is a subset or smaller version, or conversely a superset or larger version of the other, this is indicated by < and >, respectively

2. By using multiple peak profiles and comparing with a database of known species, individual species (or types) are identified.
3. Analysis of diversity and community structure is based on identified species.

The last point is probably the most critical. Because database T-RFLP focuses on identified species rather than peak profiles, it is much less sensitive than peak-profile T-RFLP to random noise in electropherograms or variation in peak profiles within species (Avis et al. 2006). Further, primer specificity to the group of interest is not required, hence, polyphyletic groups (e.g., ectomycorrhizal fungi) and groups where primers are not absolutely specific (e.g., AMF) can be analyzed. At the same time, species richness measured by database T-RFLP, which only detects species present in a database of knowns, is inevitably lower than richness quantified by peak-profile T-RFLP, which will detect all fragment lengths present in a sample. At times, it may be advantageous to use peak-profile analysis methods in conjunction with database T-RFLP as an approach to estimating unidentified diversity, particularly, if a well-developed database of T-RFLP profiles is not available.

As different research groups have concurrently developed T-RFLP, there have been some significant variations in the exact details of the methods including, for example, using only one labeled primer (Zhou and Hogetsu 2002; Edwards et al. 2004) or using multiple PCR regions rather than one (Burke et al. 2005). Some of these differences are summarized in Table 1.

## Sampling for T-RFLP

An important advantage of database T-RFLP over traditional RFLP techniques is the ability to distinguish multiple species in a sample. This allows the technique to be used on samples where multiple species are inevitably present, such as fungal hyphae in soils (Dickie et al. 2002). If database T-RFLP is applied to single ectomycorrhizal root tips, it also provides the opportunity to study ectomycorrhizal co-occurrence on single roots (Koide et al. 2005b). It has been suggested that the ability of T-RFLP to identify multiple species in a sample could permit bulked root samples or soil samples to be used to characterize fungal communities (Edwards et al. 2004; Burke et al. 2005) at a lower cost and effort than traditional methods requiring the sorting of individual root tips. A test with pooled PCR products found that T-RFLP could account for 93% of species on colonized root tips (Burke et al. 2005). It is not yet clear if this result can be extrapolated to bulked DNA extracts, as Burke and colleagues examined only pooled PCR products. A direct test of cloning on bulked PCR products versus cloning from a PCR on bulked roots found that non-specific amplification resulted in a failure to detect AMF fungi in bulked root samples (Renker et al. 2006), but only one bulked root sample was tested.

A concern in any application of T-RFLP to mixed species samples is that species present in low abundance may not be detected (Burke et al. 2005). Nonetheless, in one study, detection of added DNA comprising only 0.1–1% of the community suggests that T-RFLP is sufficiently sensitive to detect most species present in a mixed-species pool (Dunbar et al. 2000), although other studies have found that T-RFLP can overlook uncommon taxa (Allmer et al. 2006). Using soil DNA extracts is likely to produce a somewhat different picture of fungal communities than working from root-tip extracts (Koide et al. 2005a; Genney et al. 2006). This is an important area for further methodological development, including direct tests of different methods of profiling ectomycorrhizal fungal communities.

## Choice of primers and diagnostic gene regions

The choice of primers is critical in any molecular study. ITS1F and ITS4 are widely used for ectomycorrhizal ecology and have been adopted by several groups using T-RFLP (e.g., Dickie et al. 2002; Genney et al. 2006). Zhou and Hogetsu (2002) used the primers ITS3 and ITS4, which had low intraspecific variation in T-RFLP profiles within *Suillus grevillei*, yet retained sufficient interspecific polymorphism to allow species characterization; whether this can be extended outside the limited species list tested by Zhou and Hogetsu remains unclear. Edwards et al. (2004) use the basidiomycete-specific primers ITS1F and ITS4B; however, given the increasing recognition of ascomycetes as important components of ectomycorrhizal communities (Haskins and Gehring 2004; Trowbridge and Jumpponen 2004; Dickie and Reich 2005), this may result in a biased view of community composition. Burke et al. (2005) amplified the ITS1 and ITS2 regions separately in the hopes of increasing species discrimination. One concern in using shorter target regions is that the forward and reverse fragment sizes are increasingly likely to be correlated, reducing the potential information content. For AMF, the primers AM1-NS31 are widely used, but have also been criticized, as they amplify non-AMF fungi as well (Douhan et al. 2005; Aldrich-Wolfe 2007).

The exploration of further primers is well warranted. It would be valuable to find a reliable universal primer set that amplified DNA from outside of the internal transcribed spacer (ITS) region, preferably from non-ribosomal DNA, probably as an addition to the ITS1F/ITS4 primer pair rather than a replacement.

## Choice of restriction enzymes

The choice of restriction enzymes may affect the ability of T-RFLP to distinguish species. An optimal restriction enzyme would result in a wide range of fragment sizes, show relatively little variation within species, and have recognition sites in a high proportion of fungi. Enzymes with recognition sites in flanking regions (e.g., *Dpn*II and *Hpy*CH4III for ITS1F/ITS4 in ectomycorrhiza; see Table 2 for enzyme details) or, to a lesser degree, in conserved regions such as 5.8S (e.g., *Hin*fI) should be avoided, as they result in relatively poor species discrimination (Lord et al. 2002; Avis et al. 2006). The choice of whether to use *Hin*fI is problematic, as it is widely used (Table 1) and has a good track record of species discrimination, yet the conserved cutting site in 5.8S has been shown to reduce its efficiency (Avis et al. 2006).

Edwards and Turco (2005), using computer-simulated restriction digests of published sequences to evaluate six tetrameric (recognizing 4 bp) restriction enzymes in terms of 5′ fragment variation, found that *Hae*III had the highest number of different T-RFLP profiles of all six restriction enzymes tested. An evaluation of a wider range of 26 tetrameric RFLP enzymes found *Hae*III (again), and *Hpy*CH4IV had the highest number of usable fragments of all RFLP enzymes without recognition sites in the 5.8S region (Dickie, unpublished data). *Hae*III and *Hpy*CH4IV enzymes perform well in tests against a wide range of *Cortinarius* species, a group that can be problematic. Nonetheless, further optimization may be possible as neither enzyme has recognition sites in any of five collections of *Laccaria* sporocarps (Dickie, unpublished data). It is possible that the combination of multiple enzymes in a single digest would improve species resolution; this has not yet been tested. A particularly elegant approach is to use an extensive set of enzymes to develop a database of knowns and a smaller subset to analyze unknown samples, with additional digests selected based on the results from the initial analysis and known abilities of enzymes to distinguish species (Lindahl et al. 2006). For arbuscular mycorrhiza, *Dpn*II was suggested by Mummey et al. (2005), but a more recent publication by two of the same authors suggests *Alu*I and *Taq*1 (Mummey and Rillig 2006). Some authors also include undigested ITS length as an additional data-point in T-RFLP analysis (Table 1).

**Table 2** Summary of RFLP enzyme names used in the text[a]

| Name used in the text | Common isoschizimers | Recognition site |
|---|---|---|
| *Cfo*I | N/A | GCG^C |
| *Dpn*II | *Mbo*I | ^GATC |
| *Hae*III | *Bsu*RI | GG^CC |
| *Hpy*CH4III | *Taa*I | CAN^GT |
| *Hpy*CH4IV | *Mae*II | A^CTG |
| *Hin*fI | N/A | G^ANTC |
| *Taq*I | N/A | T^CGA |
| *Hsp*92II | *Hin*1II, *Nla*III | CATG^ |

[a] Where two or more citations use isoschizimers, we have used a single name—the earlier publication—for clarity.

## Running of T-RFLP in sequencer

There is some variation in the exact details of sequencer settings across different research groups. Settings can be found in published papers for ABI (Applied Biosystems) capillary sequencers with 35-cm capillaries (Koide et al. 2005b) and 50-cm capillaries (Avis and Feldheim 2005), ABI 337 slab sequencers (Dickie et al. 2002; Edwards et al. 2004), and Hitachi SQ-5500 and SQ-5500E slab sequencers (Zhou and Hogetsu 2002). Many of these details are probably relatively flexible, provided that the same settings are consistently used within any given experiment. Primers for T-RFLP are tagged with fluorescent labels. Because the choice of label changes the migration and, hence, apparent size of the fragment (Kaplan and Kitts 2003), the database and samples should use a consistent set of labels. Labels with strong differences in signal strength (e.g., 6FAM and NED) should be avoided.

Fragments larger than 500 bp can be difficult to size accurately, as extrapolation beyond the normal 500-bp size standard results in increased errors, and the 1,000-bp standard available from Applied Biosystems is not well optimized. Avis and Feldheim (2005) give one good method using the MapMarker1000 size standard, which has been used in a number of papers (Dickie et al. 2002; Koide et al. 2005b). If using GeneMapper software, the methods given by Avis and Feldheim can be improved by using the "advanced" size standard method, setting the lower limit of the analysis region to just below the 50-bp band and setting the peak half-width to 4 (Dickie and Park, personal observation).

## Developing a T-RFLP database

Database T-RFLP relies on having a database of known T-RFLP patterns. The most obvious source of known T-RFLP patterns is from sporocarp collections, either collected from a site of interest or obtained from herbarium material. Wherever possible, multiple independent collections should be used to encompass within-species variation and to guard against sporocarp contaminants (e.g., *Hypomyces*). Other options to build a database are via the characterization of mycorrhizal morphotypes and via sequencing from T-RFLP products (Dollhopf et al. 2001; Widmer et al.

2006; Lekberg et al. 2007). Unfortunately, obtaining T-RFLP patterns directly from sequence data is problematic, as the fluorescent label used and purine content of DNA influence migration rates (Kaplan and Kitts 2003). T-RFLP patterns that fail to match any known sample can sometimes be distinguished and added as "unknown knowns"—i.e., regularly encountered T-RFLP profiles of unknown identity—and added to the database of knowns on a provisional basis. In the case of ectomycorrhiza, however, it may be difficult to ensure that these "unknown knowns" are ectomycorrhizal, as dark-septate endophytes and saprotrophic or pathogenic fungi may also be amplified. Cloning and sequencing of PCR products from "unknown knowns" can resolve this issue (Lindahl et al. 2006).

## Analysis of data

Under database T-RFLP, the analysis is focused on identifying species by matching T-RFLP profiles to known patterns. There are a number of analytical programs for T-RFLP data that permit matching of profiles from multiple enzyme digests to individual species or phylogenetic groups (Kent et al. 2003). One Excel-based program, TRAMP, was developed specifically for ectomycorrhizal fungi (Dickie et al. 2002) and has been used by other researchers (Edwards et al. 2004; Allmer et al. 2006; Genney et al. 2006; Råberg et al. 2007). Two recently developed programs, FragMatch (Saari et al. 2007) and TRAMPR (FitzJohn and Dickie 2007), substantially improve on TRAMP. FragMatch, a Java-based program, permits greater flexibility in analysis parameters than TRAMP, including the ability to run multiple samples in a single run (Saari et al. 2007). We have also recently developed a comprehensive re-implementation of TRAMP, now named TRAMPR, in the statistical language R (R Development Core Team 2006). TRAMPR has significant advantages over TRAMP and FragMatch in providing complete control over matching parameters, permitting large data sets to be automatically analyzed; in automatically building a database of knowns from data (including clustering of knowns based on both species names and T-RFLP similarity); and in producing output (e.g., a species presence /absence matrix) that can be directly analyzed using standard community analysis methods (FitzJohn and Dickie 2007). Nonetheless, the R implementation may be challenging for casual users. In the TRAMP or TRAMPR approach, T-RFLP fragment lengths of each species in the database are compared to peaks in the unknown profile, and the minimum difference calculated. Matches are then determined based on whether the largest of these differences is less than an acceptable "matching error." FragMatch uses a slightly different calculation but the outcome remains essentially the same.

An important question is whether peak height should be considered in determining if a species match is valid. Some authors only consider a species to be present if the peak heights are relatively similar in magnitude (e.g., Edwards et al. 2004). The risk in taking this approach is that one species may mask the presence of another. This may occur where two or more species share a similar restriction fragment length, resulting in that peak having an intensity equal to the sum of the intensities of the matching species. Including heights will, therefore, result in a conservative matching of species, but may miss some species actually present. Neither TRAMPR nor FragMatch utilizes heights in matching peaks to species.

## The quantitative versus qualitative question

Perhaps, the largest question in T-RFLP methodology is whether the technique can be quantitative, i.e., used to estimate relative abundances based on peak heights. In the original development of the technique, Liu et al. (1997) stated that the technique was quantitative, but gave little direct evidence to support the claim. There are several reasons to doubt that T-RFLP has quantitative validity. First, the ratio of DNA to biomass is likely to be highly variable among different fungal species, due to variation both in the number of rDNA repeats per genome and of genomes per unit of biomass (Maleszka and Clark-Walker 1993). Second, the amplification of a species in PCR is known to be directly influenced by the presence of other species in the PCR mix. This could result in the apparent abundance of a species, varying only due to the presence of a second species in the PCR reaction. Third, the sequence composition of DNA can influence their amplification (Leckie 2005). Finally, in the analysis of replicate runs of the same sample, peak heights have been found to be variable (Vandenkoornhuyse et al. 2003). Nonetheless, recent empirical data suggest that peak heights are correlated with the abundance of fungi as measured by root-tip counts, although $r^2$ values of 0.28–0.56 suggest low predictive ability (Burke et al. 2006b).

## Data management

Perhaps, the most easily overlooked consideration in any attempt to use molecular techniques is the tracking of samples and storage of data. In T-RFLP, an individual run file from a 96-well plate (containing 48 samples with 2 digests per sample) may contain 4,000 or more rows of data. If all data are stored in a single Excel spreadsheet, less than 800 samples may exceed the limits of data storage. In addition, the identity of each sample must be tracked along

with all relevant data regarding collection history, possible clones, and sequences derived. Given this level of complexity and size of data, the development of a relational database is critical to ensure the integrity of data.

### Sources of error in T-RFLP

There are three major potential errors in T-RFLP: (1) erroneous combinations of either real peaks or “noise” in electropherograms, (2) shared T-RFLP profiles between two species, and (3) multiple T-RFLP profiles within a single species.

### Erroneous combinations of peaks

Particularly, in complex samples, there is a risk of erroneously identifying a T-RFLP profile as present when it is not. This could occur if all of the diagnostic peaks of a known species are randomly matched by peaks in an unknown sample despite species not actually being present. The probability of this type of error is a function of the acceptable matching error specified in the analysis ($\delta$), the length of the PCR target region ($r$), the number of peaks in a sample ($m$, where $m=r\times\theta$, and $\theta$ is the frequency of peaks in a sample, as peaks per base pair), the number of enzyme/primer pairs (or electropherograms) used in the analysis ($n$), and the number of T-RFLP profiles present in the database of knowns ($K$). The probability is approximated as

$$P = 1 - \left(1 - \left(1 - \left(1 - \frac{2\delta}{r}\right)^{m}\right)^{n}\right)^{K} . \quad (1)$$

This equation is not exact, as it assumes that fragment lengths are uniformly distributed across the region $r$ and that there is no correlation of fragment lengths in multiple primer enzyme pairs ($n$), which is demonstrably false. Nonetheless, the equation provides a useful first approximation in that it explicitly tests the effects of different parameters on the probability of erroneously combining peaks. A plot of the effect of varying $\delta$, $m$, $n$, and $K$ is shown in Fig. 2.

Given that the calculation is based on tenuous assumptions, the main value of Eq. 1 lies in permitting the effect of

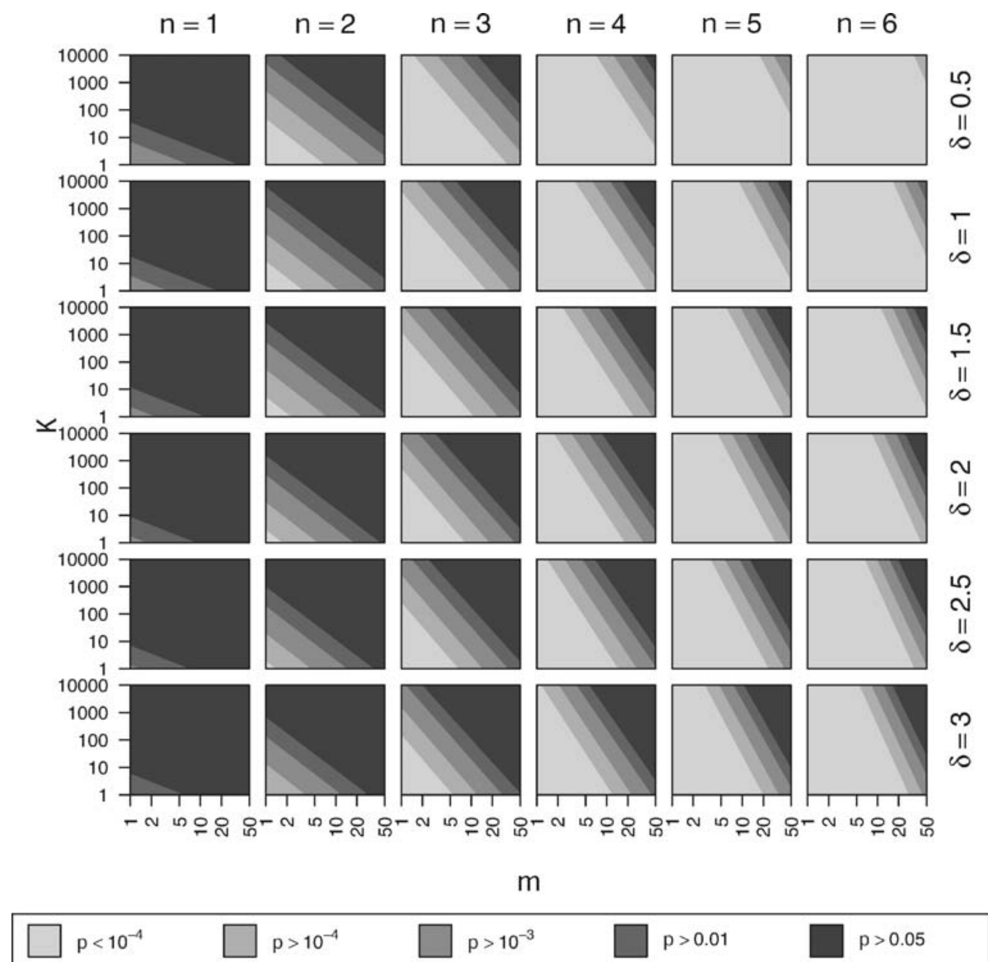


**Fig. 2** Graphical assessment of the effect of varying different parameters on the probability of erroneous peak combinations in T-RFLP as calculated by Eq. 1 as a function of $K$, the number of profiles in the knowns database; $m$ the number of peaks in the sample; $n$ the number of enzyme/primer pairs used in the analysis; and $\delta$ the acceptable matching error specified for an analysis region ($r$) of 700 bp

varying different aspects of experimental protocols to be evaluated. Using parameters taken from ectomycorrhizal research, the probability of making a mistaken match is $P=0.0016$ for parameters $r=700$ bp (the length of the ITS region), $\delta=1.5$ bp, $m=10$ peaks per profile, $n=4$ enzyme/primer pairs, and $K=500$ T-RFLP profiles in the database. Reducing $n$ from four to three enzyme/primer pairs significantly increases the risk of erroneous matches (e.g., $P=0.036$ for the parameters above). It has been suggested that locally derived databases are essential (Dickie et al. 2002), yet even increasing $K$ to 1,000 T-RFLP profiles results in $P=0.003$, implying that reasonably wide numbers of species can be included without increasing the risk of errors dramatically. The size of the target region ($r$) has only minimal direct effects on the outcome, provided that $\theta$ does not change ($P=0.00158$ for $r=300$ and $P=0.00156$ for $r=700$ with $\theta=10$ peaks/700 bp=4.3 peaks/300 bp=0.0143); however, if the same number of total peaks, $m$, are simply condensed into a smaller region, the probability of error is greatly increased for smaller target regions ($P=0.041$ for $r=300$ and $\theta=10$ peaks/300 bp=0.033). Where there is no recognition site for an enzyme, the error rate is significantly increased, as $r$ is reduced to the range of total PCR product lengths and the information from forward and reverse primers is redundant (potentially halving the effective value of $n$). An incidental by-product of the analysis is a demonstration of the weakness of peak-profile T-RFLP for species identification. With $n=1$, the risk of mistaken identifications rises well above $P=0.05$ for most parameter combinations (Fig. 2).

In some cases, $\delta$ has been elevated to as high as 3 bp to account for multiple peaks or profile variation within a species (Dickie et al. 2002; Koide et al. 2005b; Råberg et al. 2005). Eq. 1 suggests this may be disadvantageous, as $\delta=3$ raises $P$ to 0.023 for $n=4$. A better approach may be to include variant profiles as entries in the database and then aggregate database matches known to be the same species (see below).

## Shared T-RFLP profiles across two or more species

The second type of error is where T-RFLP correctly identifies a profile as being present, yet that profile is not unique to a single species. In such a case, species A may be treated as “present” due to the presence of species B, which happens to have the same T-RFLP profile. If species B is not in the database, this error may pass undetected. The first steps to minimizing this type of error are to maximize the number of species in the database so that shared patterns can be identified and to sequence a subset of identified samples to confirm identifications. Where multiple species are found to share a profile, these can either be analyzed as an artificial group (e.g., *Cortinarius* group 1), or sequences can be obtained and analyzed to find restriction enzymes that will differentiate the species. Additional digests can then be used as necessary to differentiate problematic groups (e.g., Edwards et al. 2004).

The frequency of shared T-RFLP profiles can be analyzed by examining databases of known species derived from sporocarps. In a collection of 93 known sporocarps analyzed using ITS1F and ITS4 primers with restriction digests using *Hae*III and *Hpy*CH4IV, we have found four cases of overlapping species: (1) a group of *Laccaria* species, all of which fail to digest with the two restriction enzymes chosen and, therefore, have minimal variation in fragment length; (2) a group that contains a *Cortinarius* sp. and a *Laccaria* sp., again driven by the failure of either collection to digest with the two enzymes; (3) a combination of *Macowanites carmineus* with an unknown *Russula* sp., both of which are in the Russulaceae; and (4) a combination of *Cortinarius meleagris* with an unknown *Descolea* sp., both of which are in the Cortinariaceae (Dickie, unpublished data). While limited, this suggests that (1) species that lack restriction sites (or equally species that restrict only at conserved restriction sites in 5.8S—see Avis et al. 2006) are more likely to be indistinguishable than species that have restriction sites, and (2) those that shared T-RFLP profiles are more common among related species than across disparate groups.

## Multiple T-RFLP profiles within a species

Ectomycorrhizal fungi can produce multiple terminal fragment lengths within a species and even within an individual (Kanchanaprayudh et al. 2003; Koide et al. 2005b; Avis et al. 2006). These are particularly a problem in peak-profile T-RFLP (as well as similar techniques such as DGGE) where they may bias estimates of diversity and measures of community profile shifts (Avis et al. 2006). Because database T-RFLP relies on species identifications rather than counting peaks, multiple fragments cause fewer problems.

Where multiple patterns are known to occur, they can be merged together before the analysis of community patterns (e.g., if *Suillus* pattern A or *Suillus* pattern B is present, record *Suillus* as present). This can be accomplished automatically using TRAMPR software (FitzJohn and Dickie 2007). Multiple T-RFLP profiles may cause errors in analysis where a species is present in two sites, with a different T-RFLP profile in each site. Unless both patterns are identified, this may result in the species being recorded as present in one site and absent in the other. Again, the risk of this type of error is decreased by having as many known species in the database as possible and sequencing and identifying as many unknown T-RFLP profiles as possible.

## Other problems in T-RFLP

The other potential problems of T-RFLP are not unique to the technique, but rather apply to any technique using PCR to study multiple-species communities (Anderson and Cairney 2004). In particular, relict DNA and DNA in non-target structures may bias the view of the community (Avis et al. 2006). The choice of primers may have very important effects as well. Aldrich-Wolfe (2007) found that 40% of T-RFLP types amplified by "AMF-specific" primers more closely matched non-AMF fungi than known AMF fungi, while the same methods exclude potentially important AMF *Paraglomus* and some *Archaeospora* (Renker et al. 2003). These generic problems of PCR are not easily corrected for; nonetheless, being aware of the potential problems may avoid over-interpretation of results.

## The statistical imperative: "Go forth and replicate!"

The choice of molecular methods for ecological research must be driven by the power of that method to detect ecologically meaningful patterns. At times, there is a trade-off between precision of species identifications and possible numbers of replicates. Fungal communities tend to have many species with low frequencies. Using data from one study, the most common 25% of species had frequencies as low as only 10% of the samples (Dickie and Reich 2005). As a hypothetical example, if a species were exclusively found in only one of the two treatments, but had a frequency of only 20% within that treatment (equivalent to 10% across the two treatments), the experiment would need to have 34 replicates of each treatment to achieve statistical significance of $\alpha$=0.05 and $\beta$=0.8 (calculated using the power.prop.test function of R, R Development Core Team 2006). This implies that an extremely high level of replication is required to be able to test for statistical patterns on even the most abundant 25% of species. Relatively few molecular methods can routinely achieve this level of replication at reasonable cost. The high throughput of T-RFLP has, however, permitted replication at quite high levels [e.g., 40 replicates in each of 4 treatments in Dickie et al. (2002)].

## Conclusions

T-RFLP analysis has already resulted in a heightened understanding of mycorrhizal ecology (Table 1) and is likely to become increasingly used. The technique has the significant advantage of low cost and relative simplicity, permitting sufficient replication to address important ecological questions. No technique is a panacea; while T-RFLP permits a high throughput of samples, it is subject to a number of different types of error. Whenever feasible, using multiple techniques may give the best view of community composition (Bougoure and Cairney 2005; Allmer et al. 2006). In particular, sequence analysis either directly from samples or from clone libraries will permit confirmation of a subset of T-RFLP identifications, greatly increasing confidence in results while still permitting the high throughput of T-RFLP.

There are promising technologies on the horizon that may well supercede T-RFLP. Microarray technologies have been proposed for mycorrhizal fungi (Anderson and Cairney 2004), but not yet widely applied. Another very recent development is methods based around 454 sequencing (Sogin et al. 2006; A. Jumponnen, personal communication), which show considerable promise for the analysis of mixed-species communities.

## Note on review methods

We have attempted to include all papers using T-RFLP for the study of the ecology of mycorrhizal fungi. These were found through a search on September 15, 2006 on the Web of Science (http://www.portal.isiknowledge.com, Thomson Scientific, Philadelphia, USA) using the search string "TS=(T-RFLP OR TRFLP OR "terminal restriction") AND TS=(mycorrhiz* OR ectomycorrhiz*)", and on Google scholar beta (http://www.scholar.google.com) using the string "(T-RFLP OR TRFLP OR "terminal restriction") AND (mycorrhizal OR mycorrhizae OR ectomycorrhizal OR ectomycorrhizae)", with the 100 highest-scoring hits (of 304) in Google Scholar examined in detail. We have not attempted to comprehensively review all literature on T-RFLP as applied to study organisms other than mycorrhiza. We give sincere apologies to the authors of any papers we have inadvertently omitted.

**Acknowledgment** Valuable discussions and sharing of data and concepts with P. Avis, S. Branco, I. Edwards, G. Forrester, D. Park, R. Smissen, and many others have contributed to this review. R. Molina and two reviewers provided helpful comments. The authors were supported by funds from the Foundation for Research, Science and Technology (FRST, NSOF) of New Zealand.

## References

Aldrich-Wolfe L (2007) Distinct mycorrhizal fungal communities on new and established host species in a transitional tropical plant community. Ecology 88:559–566

Allmer J, Vasiliauskas R, Ihrmark K, Stenlid J, Dahlberg A (2006) Wood-inhabiting fungal communities in woody debris of Norway spruce (*Picea abies* (L.) Karst.), as reflected by sporocarps, mycelial isolations, and T-RFLP analysis. FEMS Microbiol Ecol 55:57–67

Anderson IC, Cairney JWG (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ Microbiol 6:769–779

Artursson V, Finlay RD, Jansson JK (2005) Combined bromodeoxyuridine immunocapture and terminal-restriction fragment length polymorphism analysis highlights differences in the active soil bacterial metagenome due to *Glomus mosseae* inoculation or plant species. Environ Microbiol 7:1952–1966

Avis PG, Feldheim KA (2005) A method to size DNA fragments from 50 to 800 bp on a DNA analyzer. Mol Ecol Notes 5:969–970

Avis PG, Dickie IA, Mueller G (2006) A "dirty" business: testing the limitations of TRFLP analysis of soil fungi. Mol Ecol 15:873–882

Blackwood CB, Marsh T, Kim S-H, Paul EA (2003) Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Appl Environ Microbiol 69:926–932

Bougoure DS, Cairney JWG (2005) Fungi associated with hair roots of *Rhododendron lochiae* (Ericaceae) in an Australian tropical cloud forest revealed by culturing and culture-independent molecular methods. Environ Microbiol 7:1743–1754

Brodie E, Edwards S, Clipson N (2003) Soil fungal community structure in a temperate upland grassland soil. FEMS Microbiol Ecol 45:105–114

Bruns TD (1995) Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. Plant Soil 170:63–73

Burke DJ, Martin KJ, Rygiewicz PT, Topa MA (2005) Ectomycorrhizal fungi identification in single and pooled root samples: terminal restriction fragment length polymorphism (TRFLP) and morphotyping compared. Soil Biol Biochem 37:1683–1694

Burke DJ, Kretzer AM, Rygiewicz PT, Topa MA (2006a) Soil bacterial diversity in a loblolly pine plantation: influence of ectomycorrhizas and fertilization. FEMS Microbiol Ecol 57:409–419

Burke DJ, Martin KJ, Rygiewicz PT, Topa MA (2006b) Relative abundance of ectomycorrhizas in a managed loblolly pine (*Pinus taeda*) genetics plantation as determined through terminal restriction fragment length polymorphism profiles. Can J Bot 84:924–932

Dickie IA, Reich PB (2005) Ectomycorrhizal fungal communities at forest edges. J Ecol 93:244–255

Dickie IA, Xu W, Koide RT (2002) Vertical niche differentiation of ectomycorrhizal hyphae in soils as shown by T-RFLP analysis. New Phytol 156:527–535

Dollhopf SL, Hashsham SA, Tiedje JM (2001) Interpreting 16S rDNA T-RFLP data: application of self-organizing maps and principal component analysis to describe community dynamics and convergence. Microb Ecol 42:495–505

Douhan GW, Petersen C, Bledsoe CS, Rizzo DM (2005) Contrasting root associated fungi of three common oak-woodland plant species based on molecular identification: host specificity or non-specific amplification. Mycorrhiza 15:365–372

Dunbar J, Ticknor LO, Kuske CR (2000) Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl Environ Microbiol 66:2943–2950

Edel-Hermann W, Dreumont C, Perez-Piqueres A, Steinberg C (2004) Terminal restriction fragment length polymorphism analysis of ribosomal RNA genes to assess changes in fungal community structure in soils. FEMS Microbiol Ecol 47:397–404

Edwards IP, Turco RF (2005) Inter- and intraspecific resolution of nrDNA TRFLP assessed by computer-simulated restriction analysis of a diverse collection of ectomycorrhizal fungi. Mycol Res 109:212–226

Edwards IP, Cripliver JL, Gillespie AR, Johnsen KH, Scholler M, Turco RF (2004) Nitrogen availability alters macrofungal basidiomycete community structure in optimally fertilized loblolly pine forests. New Phytol 162:755–770

FitzJohn RG, Dickie IA (2007) TRAMPR: an R package for analysis and matching of terminal-restriction fragment length polymorphism (TRFLP) profiles. Mol Ecol Notes (in press). DOI 10.1111/j.1471-8286.2007.01744.x

Gardes M, Bruns TD (1996) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Can J Bot 74:1572–1583

Genney DR, Anderson IC, Alexander IJ (2006) Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium. New Phytol 170:381–390

Haskins KE, Gehring CA (2004) Interactions with juniper alter the abundance and composition of pinyon pine ectomycorrhizal fungal communities. Ecology 85:2687–2692

Hibbett DS, Gilbert L-B, Donoghue MJ (2000) Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. Nature 407:506–508

Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol Ecol 10:1855–1871

Johnson D, Vandenkoornhuyse PJ, Leake JR, Gilbert L, Booth RE, Grime JP, Young PW, Read DJ (2003) Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. New Phytol 161:503–515

Kanchanaprayudh J, Zhou Z, Yomyart S, Sihanonth P, Hogetsu T (2003) Molecular phylogeny of ectomycorrhizal *Pisolithus* fungi associated with pine, dipterocarp, and eucalyptus trees in Thailand. Mycoscience 44:287–294

Kaplan CW, Kitts CL (2003) Variation between observed and true terminal restriction fragment length is dependent on true TRF length and purine content. J Microbiol Methods 54:121–125

Kent AD, Smith DJ, Benson BJ, Triplett EW (2003) Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl Environ Microbiol 69:6768–6776

Kitts CL (2001) Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. Curr Issues Intest Microbiol 2:17–25

Klamer M, Hedlund K (2004) Fungal diversity in set-aide [sic] agricultural soil investigated using terminal-restriction fragment length polymorphism. Soil Biol Biochem 36:983–988

Klamer M, Roberts MS, Levine LH, Drake BG, Garland JL (2002) Influence of elevated CO2 on the fungal community in a coastal scrub oak forest soil investigated with terminal-restriction fragment length polymorphism analysis. Appl Environ Microbiol 68:4370–4376

Koide RT, Xu B, Sharda J (2005a) Contrasting below-ground views of an ectomycorrhizal fungal community. New Phytol 166:251–262

Koide RT, Xu B, Sharda J, Lekberg Y, Ostiguy N (2005b) Evidence of species interactions within an ectomycorrhizal fungal community. New Phytol 165:305–316

Kowalchuk GA, De Souza FA, Van Veen JA (2002) Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes. Mol Ecol 11:571–581

Landeweert R, Leeflang P, Kuyper TW, Hoffland E, Rosling A, Wernars K, Smit E (2003) Molecular identification of ectomycorrhizal mycelium in soil horizons. Appl Environ Microbiol 69:327–333

Landeweert R, Leeflang P, Smit E, Kuyper TW (2005) Diversity of an ectomycorrhizal fungal community studied by a root tip and total soil DNA approach. Mycorrhiza 15:1–6

Leckie SE (2005) Methods of microbial community profiling and their application to forest soils. For Ecol Manag 220:88–106

Lekberg Y, Koide RT, Rohr JR, Aldrich-Wolfe L, Morton JB (2007) Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. J Ecol 95:95–105

Lindahl BD, Ihrmark K, Boberg J, Trumbore SE, Högberg P, Stenlid J, Finlay RD (2006) Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytol 173:611–620

Liu WT, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment

length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

Lord NS, Kaplan CW, Shank P, Kitts CL, Elrod SL (2002) Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: comparison of 18S and ITS ribosomal regions. FEMS Microbiol Ecol 42:327–337

Lukow T, Dunfield PF, Liesack W (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiol Ecol 32:241–247

Ma WK, Siciliano SD, Germida JJ (2005) A PCR–DGGE method for detecting arbuscular mycorrhizal fungi in cultivated soils. Soil Biol Biochem 37:1589–1597

Maleszka R, Clark-Walker GD (1993) Yeasts have a four-fold variation in ribosomal DNA copy number. Yeast 9:53–58

Marsh TL (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr Opin Microbiol 2:323–327

Martin KJ (2007) Introduction to molecular analysis of ectomycorrhizal communities. Soil Sci Soc Am J 71:601–610

Mummey DL, Rillig MC (2006) The invasive plant species *Centaurea maculosa* alters arbuscular mycorrhizal fungal communities in the field. Plant Soil 288:81–90

Mummey DL, Rillig MC, Holben WE (2005) Neighboring plant influences on arbuscular mycorrhizal fungal community composition as assessed by T-RFLP analysis. Plant Soil 271:83–90

Nara K (2005) Ectomycorrhizal networks and seedling establishment during early primary succession. New Phytol 169:169–178

Nara K, Nakaya H, Wu B, Zhou Z, Hogetsu T (2003) Underground primary succession of ectomycorrhizal fungi in a volcanic desert on Mount Fuji. New Phytol 159:743–756

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

Pennanen T, Heiskanen J, Korkama U (2005) Dynamics of ectomycorrhizal fungi and growth of Norway spruce seedlings after planting on a mounded forest clearcut. For Ecol Manag 213:243–252

R Development Core Team (2006) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org

Råberg U, Högberg NOS, Land CJ (2005) Detection and species discrimination using rDNA T-RFLP for identification of wood decay fungi. Holzforschung 59:696–702

Råberg U, Brischke C, Rapp AO, Högberg NOS, Land CJ (2007) External and internal fungal flora of pine sapwood (*Pinus sylvestris* L.) specimens in above-ground field tests at six different sites in south-west Germany. Holzforschung 61:104–111

Renker C, Heinrichs J, Kaldorf M, Buscot F (2003) Combining nested PCR and restriction digest of the internal transcribed spacer region to characterize arbuscular mycorrhizal fungi on roots from the field. Mycorrhiza 13:191–198

Renker C, Weißhuhn K, Kellner H, Buscot F (2006) Rationalizing molecular analysis of field-collected roots for assessing diversity of arbuscular mycorrhizal fungi: to pool, or not to pool, that is the question. Mycorrhiza 16:525–531

Saari TA, Saari SK, Campbell CD, Alexander IJ, Anderson IC (2007) FragMatch—a program for the analysis of DNA fragment data. Mycorrhiza 17:133–136

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

Singh BK, Munro S, Reid E, Ord B, Potts JM, Paterson E, Millard P (2006) Investigating microbial community structure in soils by physiological, biochemical and molecular fingerprinting methods. Eur J Soil Sci 57:72–82

Sogin ML, Morrison HG, Huber JA, Welch DA, Huse SM, Neal PR, Arrieta JM, Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored “rare biosphere.” Proc Natl Acad Sci U S A 103:12115–12120

Thies JE (2007) Soil microbial community analysis using terminal restriction fragment length polymorphisms. Soil Sci Soc Am J 71:579–591

Tonin C, Vandenkoornhuyse P, Joner EJ, Straczek J, Leyval C (2001) Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of *Viola calaminaria* and effect of these fungi on heavy metal uptake by clover. Mycorrhiza 10:161–168

Trowbridge J, Jumpponen A (2004) Fungal colonization of shrub willow roots at the forefront of a receding glacier. Mycorrhiza 14:283–293

Vandenkoornhuyse P, Ridgway KP, Watson IJ, Fitter AH, Young JPW (2003) Co-existing grass species have distinctive arbuscular mycorrhizal communities. Mol Ecol 12:3085–3095

Widmer F, Hartmann M, Frey B, Kölliker R (2006) A novel strategy to extract specific phylogenetic sequence information from community T-RFLP. J Microbiol Methods 66:512–529

Zhou ZH, Hogetsu T (2002) Subterranean community structure of ectomycorrhizal fungi under *Suillus grevillei* sporocarps in a *Larix kaempferi* forest. New Phytol 154:529–539