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Evaluating the physical capture method of terminal restriction fragment length polymorphism for comparison of soil microbial communities

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Abstract

Terminal restriction fragment length polymorphism (T-RFLP) is a popular method of comparative microbial community analysis which is normally accomplished by tagging terminal restriction fragments (T-RFs) with a fluorescent primer. Here, we evaluate an alternative method of T-RFLP where T-RFs are physically captured using a biotinylated primer and streptavidin-coated beads. This eliminates one of the primary criticisms of T-RFLP, namely that T-RFs cannot be identified by sequence analysis, and also represents an alternative method for collecting T-RFLP profiles. Microbial communities from forest, agricultural, and turf soils were investigated using several sets of primers specific for different microbial groups. The physical capture method of T-RFLP resulted in similar profiles to those generated by fluorescent T-RFLP. The relationships among ecosystem types captured by both methods and revealed by ordination were virtually identical. The total variance in the profiles that was attributed to ecosystem type was approximately equal, or greater, when generated by the physical capture method, depending on the primers used. However, physical capture T-RFLP resolved fewer T-RFs than fluorescent T-RFLP, and this may reduce the sensitivity to changes in non-dominant populations within the community. Direct cloning and sequencing of physical capture T-RFs revealed that most bands were not comprised of sequences related to those in the database that would generate T-RFs of similar size. T-RFs should therefore be identified by sequencing, rather than by comparing the sizes of T-RFs to computer digests of database sequences. Physical capture T-RFLP should be a useful tool to identify T-RFs by sequencing, and for laboratories without economical access to equipment required to perform fluorescent T-RFLP.
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1. Introduction

Terminal restriction fragment length polymorphism (T-RFLP) is a culture-independent molecular genetic method for generating profiles or "fingerprints" of environmental microbial communities (Kitts, 2001; Tiedje et al., 1999). It has become a common tool in the characterization of soil microbes (Boyle et al., 2006; Bankhead et al., 2004; Pérez-Piqueres et al., 2006; Rasche et al., 2006). The method is based on sequence heterogeneity within a PCR-amplified gene. The most frequently used T-RFLP assays target the gene encoding the ribosomal small subunit using domain-specific PCR primers. One PCR primer is tagged by a fluorescent molecule so that restriction of the PCR amplicons results in fluorescently labeled terminal restriction fragments (T-RFs). Restriction fragments are separated by size using electrophoresis with a sequencing apparatus. The T-RFs are then visualized by excitation of the fluorescent molecule. Relative to other molecular methods, T-RFLP is fairly rapid and technically straightforward, and has the purported advantage that T-RF sizes can be tentatively related to sequences in a database (Marsh et al., 2000).

Like all methods, however, T-RFLP is not without limitations. It has been frequently noted that disparate taxa commonly generate the same size T-RF, limiting the ability to make meaningful comparisons to database sequences (Blackwood and Paul, 2003; Dunbar et al., 2001; Engebretson and Moyer, 2003; Pérez-Piqueres et al., 2006;

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Rogers et al., 2004). Furthermore, using the standard T-RFLP protocol, T-RFs cannot be directly sequenced to resolve this issue because they are not separated from other (non-terminal) restriction fragments, but are only differentially visualized. Also, the cost of running fluorescently labeled samples at core sequencing laboratories varies widely between institutions, and for some labs cost can limit the practicality of T-RFLP.

Here we evaluate a method of T-RFLP involving physical capture of T-RFs which can help to overcome these disadvantages. In physical-capture T-RFLP, PCR primers are labeled with biotin rather than a fluorescent molecule, and a restriction site for a "release enzyme" is also added to the 5′ end of the primer. After restriction with a different "profiling" enzyme, T-RFs are bound to streptavidin-coated paramagnetic beads. Non-terminal restriction fragments are removed, and then the T-RFs are released from the beads by incubation with the release enzyme. T-RFs can then be electrophoresed and visualized on high-resolution agarose gels, and bands can be excised for sequencing. The method was originally published by Nagashima et al. (2003), where it was used to identify two T-RFs from fecal bacterial communities. We examine the utility of the method for identifying T-RFs from complex soil communities and from a variety of phylogenetic groups. This can be used to identify T-RFs, as well as test the basic assumption that T-RFs in the same position in different samples represent the same taxa. We also provide the first evaluation of physical capture T-RFLP as a method of collecting T-RFLP profiles and ordinating communities, in comparison to fluorescent T-RFLP.

2. Materials and methods

2.1. In silico T-RFLP of database sequences

Small subunit ribosomal PCR primers used in this study include a standard pair of domain-specific bacterial primers (Eub338F and 1392R) and group-specific primers described by Blackwood et al. (2005) (Table 1). For each set of group-specific primers, a macro in SAS (version 8.2, SAS Institute, Cary, NC) was used to choose the optimal restriction enzyme, and primer to label, for T-RFLP (Table 1). Sequences from the ARB 2003 database (Ludwig et al., 2004) matching a primer set were imported into SAS, and T-RF lengths were calculated for each restriction enzyme present in REBASE (Roberts et al., 2005). The macro then determined the number and dispersion of T-RFs generated from the database for each combination of restriction enzyme and labeled primer. The theoretical digests were also evaluated for their taxonomic discrimination within the targeted group, and distribution of T-RFs >200 bp in length where phylogenetic information obtained from sequencing will be greatest. The macro is available from the authors.

2.2. Soil samples

To demonstrate physical-capture T-RFLP, soils were sampled from three treatments at the Farming Systems Project, established in 1996 at the Beltsville Agricultural Research Center, MD (Green et al., 2005). Soils at this site are silt loam Ultisols with approximately 1.8% organic C. Treatments sampled include (1) soybean managed using conventional practices for nutrients, weeds, and tillage, (2) bromegrass-clover ("grass") strips adjacent to the soybean plots, which had been mowed regularly since the establishment of the experiment, but otherwise not managed, and (3) deciduous forest plots. The forest is approximately 60 years old, and the soil profile does not indicate a history of tillage (M. Cavigelli, personal communication). Four field plots were sampled per treatment. Ten soil cores were taken within the A horizon from each field plot to a depth of 10 cm, after removal of plant litter. Samples were sieved over a 4.5 mm sieve, coarse roots and organic particles were removed, and soil was frozen until subsamples were removed for DNA extraction.

2.3. Method-comparison experimental design and general molecular methods

T-RFLP profiles were generated using both fluorescent and physical-capture T-RFLP for all primer pairs. For the

Table 1
Ribosomal primers used in this study (see also Blackwood et al., 2005)

Forward primer[a]	Reverse primer	Phylogenetic target	Sequence added to 5′ end of labeled primer	Profiling enzyme	°C[b]
Eub338F-0-III*	1392R	*Bacteria*	CAACATTTAAAT	MspI	37
ADF681F	1392R*	*Alphaproteobacteria*, some *Deltaproteobacteria, Fusobacteria*	GAACATTTAAAT	BslI	55
Beta680F*	1392R	*Betaproteobacteria*	GGAGATTTAAAT	BsmAI/BsrI	55
BLS342F*	1392R	*Bacilli*	GGAGATTTAAAT	HaeIII	37
Eub338F*	Act1159R	*Actinobacteria*	CAACATTTAAAT	DpnII	37
Eub338F-0-III*	Pln930R	*Planctomycetes*	CCTTGGCACC	BstNI	60
Bas1005F	5.8SR*	*Basidiomycota*	TCACATTTAAAT	HaeIII	37

[a]Primer labeled for T-RFLP is marked with *.
[b]Incubation temperature used for profiling enzyme digest.

Eub338F-1392R (general bacterial) assay, three replicate T-RFLP profiles (analytical replicates) were generated for each sample. One profile was generated for each sample using group-specific assays.

Genomic DNA was extracted from 0.4 g soil of each field plot using the Ultraclean Soil DNA extraction kit (Mo Bio Laboratories, Solana Beach, CA). All PCRs were performed as described in Blackwood et al. (2005), except that one primer was 5′ labeled. For fluorescent T-RFLP, the label was either hexachlorofluorescein or 6-carboxyfluorescein. For physical-capture T-RFLP, the label was biotin-TEG separated from the primer by a four nucleotide spacer and a release enzyme restriction site (Table 1). For each primer combination, triplicate 25 μL PCRs were pooled for each sample. All restriction digests described below were performed with reagents purchased from New England Biolabs (Beverly, MA). Restriction digest conditions were previously shown to result in complete restriction, without evidence of non-specific activity, using PCR product obtained from pure culture organisms (personal observations).

2.4. Fluorescent T-RFLP

Fluorescent-labeled PCR product was purified using an UltraClean PCR Clean-up Kit (MoBio), eluting with 25 μL H_2O. Twenty microliters of purified PCR product was then mixed with 10 U of the appropriate profiling restriction enzyme (Table 1), 2.33 μL of the enzyme's accompanying 10X reaction buffer, and BSA if recommended by the manufacturer. Restriction digests were incubated overnight at the appropriate temperature (Table 1). Digests were then purified using a QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA), eluting with 30 μL H_2O. Samples were sent to Michigan State University's Genomics Technology Support Facility where they were mixed with MapMarker 1000 DNA size marker (BioVentures, Murfreesboro, TN), and underwent capillary electrophoresis in an ABI PRISM 3100 Genetic Analyzer using standard conditions for fragment analysis (Applied Biosystems, Foster City, CA). Chromatograms were analyzed in Genescan (version 3.7, Applied Biosystems) and tables of peaks with heights over 100 fluorescence units were exported. Peaks comprising >1% of cumulative peak height for a sample were retained for analysis.

2.5. Physical capture T-RFLP

Biotinylated PCR product for physical-capture T-RFLP was digested by adding 10 U of the appropriate profiling restriction enzyme (Table 1) to the pooled PCRs and incubating overnight. After digestion, 70 μL/sample of Streptavidin Magnesphere Paramagnetic particles (Promega, Madison, WI) was prepared by washing three times in 0.5X SSC buffer, and then suspending in 18.7 μL 0.5X SSC per sample. Each digest was then mixed with 18.7 μL bead suspension and 1.75 μL 20X SSC. T-RFs were bound to the beads by incubating at 25 °C on a tube rotator for 1 h. Beads were immobilized using a 96-well plate magnet (Dynal Biotech, Oslo, Norway), and the liquid containing non-terminal restriction fragments was removed. Beads were washed twice with 0.1X SSC and once with H_2O, and then suspended in 10 μL restriction solution containing 5 U of release restriction enzyme (SwaI, except in the *Planctomycetes* assay where BanI was used), 1 μL of the accompanying 10X restriction buffer, and 0.1 μL BSA (10 μg/μL). T-RFs were separated from beads by incubating 3 h on a tube rotator at 25 °C (37 °C used for BanI). Samples were then electrophoresed at 200 V for 2.5 h on a 2% NuSieve 3:1 agarose gel (BioWhittaker, Rockland, ME) in TBE buffer. The size standards Lambda PstI (Fermentas, Hanover, MD) and PCR 20 bp Low Ladder (Sigma-Aldrich, St. Louis, MO) were diluted in restriction master mix and run in at least every eighth lane. Gels were stained 4–5 h with Gelstar (BioWhittaker) and visualized with 400–500 nm illumination on a DarkReader transilluminator (Clare Chemical, Dolores, CO).

2.6. Sequencing T-RFLP fragments

Several bands were excised from physical-capture T-RFLP agarose gels described above using sterile razors. DNA was extracted from agarose and suspended in 5 μL H_2O using the UltraClean 15 DNA Purification Kit (MoBio). A-tailing was then performed on these DNA fragments by incubating at 72 °C for 30 min with PCR components excluding primers. A-tailed samples were purified using the UltraClean PCR Clean-up Kit, eluting with 25 μL H_2O. DNA fragments were then cloned into *E. coli* JM109 using the pGEM-T Vector System (Promega) following the manufacturer's instructions, with some modifications to accommodate dilute T-RF DNA samples. For example, in extreme cases where the A-tailed, purified T-RF was barely visible on an agarose gel stained with Gelstar, 25 μL ligations were conducted using 12.5 μL 2X ligation buffer, 1 μL pGEM-T vector (50 ng/μL), 1.5 μL T4 DNA ligase (3 U/μL), and 10 μL purified T-RF DNA. Ligations were incubated overnight at 4 °C, and all 25 μL was used to transform 50 μL of *E. coli* cells.

Cloned inserts were amplified with M13 primers and digested with the restriction enzymes MspI and RsaI to screen clone libraries for multiple operational taxonomic units (OTUs). Restricted PCR amplicons were electrophoresed on 4% NuSieve 3:1 agarose gels and visualized with Gelstar as described above. PCR product from a clone representing each restriction pattern was submitted for sequencing at the USDA-ARS Environmental Microbial Safety Laboratory (Beltsville, MD). Sequences were aligned to the ARB 2003 database using the integrated aligner. Alignments were manually adjusted if needed. Sequences were placed in the ARB phylogenetic tree using the parsimony insertion function. Nearest matches in GenBank were also found using BLASTn to confirm results from ARB analyses. Nucleotide sequences

determined in this study have been deposited in the GenBank database under accession numbers AY555656–AY555675 and DQ508304–DQ508373.

2.7. Image processing and band class assignment

T-RFLP profiles require image processing to detect bands, and then bands from different profiles must be clustered into band classes before statistical analysis. For physical-capture T-RFLP, these steps were performed in the software GelCompar II (Applied Maths, Austin, TX) using automated algorithms. Band fluorescence intensity (peak height) was exported in community matrices, with samples as rows and band classes as columns.

For fluorescent T-RFLP, band detection is normally performed in software accompanying the sequencing instrument (described above), but there is no standardized way to cluster bands into band classes. We considered two methods of clustering T-RFs into band classes: (1) by importing peak tables into GelCompar II and using the automated band classification algorithm (2) by importing peak tables into Excel and using the PivotTable function with a bin size of 3 bp. Both methods of clustering fluorescent T-RFs into band classes were used for Eub338F-1392R profiles. Only the GelCompar II method was used for other primer pairs. Community matrices as described above were exported for statistical analysis.

2.8. Statistical analysis of community profiles

T-RFLP methods were compared by their sensitivity to differences between soil communities and analytical noise, and by the similarity of the relationships between communities that were revealed by the different methods.

Sensitivity was examined by partitioning variance in the profiles into portions derived from different sources. This was performed using redundancy analysis after Hellinger transformation (Blackwood et al., 2003) in Canoco 4.0 (Microcomputer Power, Ithaca, NY). For Eub338F-1392R profiles, three replicate profiles were generated for each sample (analytical replicates), so profile variance could be partitioned into (1) variance due to treatment (i.e. ecosystem type) (V_t), (2) variance between individual plots after removing treatment differences (V_p), and (3) analytical variance introduced by the profiling methods (V_a). V_a was equivalent to residual variance after taking treatment and plot variability into account, and could be separated from V_p because of the analytical replication employed. Under ideal circumstances, V_a is minimized. Each variance was expressed as a proportion of the total variance in the set of profiles for that analysis. For group-specific profiles, variance was partitioned into V_t and $V_p + V_a$ pools.

Canonical ordinations were generated from each source of variance in the redundancy analysis. These ordinations captured the relationships between communities derived only from an individual source of variance (V_t, V_p, or V_a). Mantel tests (Legendre and Legendre, 1998) were used to determine the similarity of ordinations of fluorescent and physical-capture T-RFLP profiles, and how this similarity changed depending on source of the variance. Mantel statistics can be interpreted as a correlation coefficient, with identical ordinations yielding a value of 1. Mantel testing was implemented in SAS version 8 IML (SAS Institute, Cary, NC). All hypothesis tests were performed by permutation, with sample identities randomly permuted 9999 times.

3. Results

3.1. General bacterial T-RFLP profiles

Physical-capture T-RFLP profiles show very similar banding patterns compared to those obtained by fluorescent T-RFLP profiles (Fig. 1). As expected, fluorescent T-RFLP was able to resolve some bands which appeared as a single large band on agarose gels (24 T-RF band classes

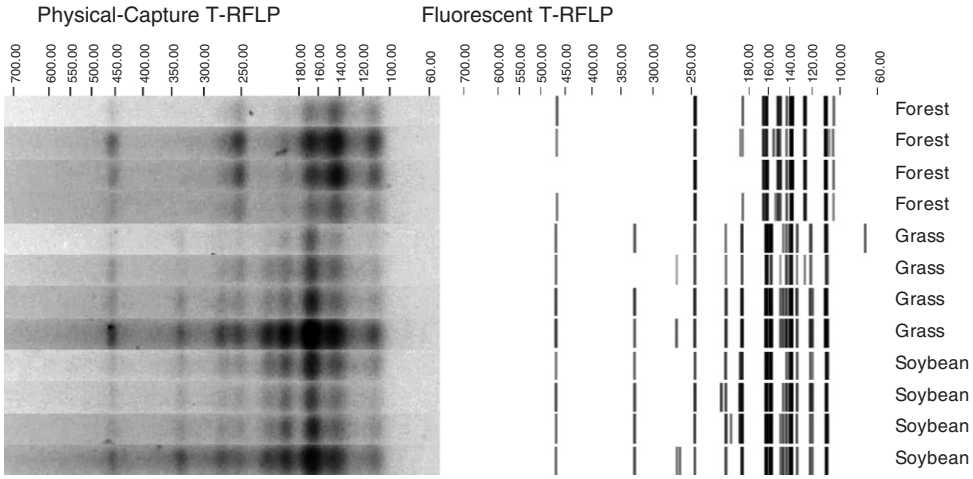

Fig. 1. T-RFLP profiles generated using primers Eub338F-1392R (general bacteria). Profiles are shown after processing in GelComparII. Each row shows T-RFLP profiles from one plot. Scale is in bp.

Table 2
Comparison of physical-capture and fluorescent T-RFLP using general bacterial primers

Factor	Variance explained (%)[a]			Mantel statistics[b]		
	PC	Fl GC	Fl Pivot	PC–Fl GC	PC–Fl Pivot	Fl GC–Fl Pivot
Treatment (V_t)	59**	63**	62**	0.99999	0.999	0.998
Plot (V_p)	19**	30**	24**	0.27*	0.34*	0.54**
Analytical (V_a)	22	7	14	−0.03	0.06	−0.007

*$p<0.1$.
**$p<0.01$.
[a]PC, physical-capture T-RFLP; Fl GC, fluorescent T-RFLP aligned in Gel Compar II; Fl Pivot, fluorescent T-RFLP aligned by PivotTable function in Excel.
[b]Correlations between canonical ordinations derived from each source of variance. Permutation tests of Mantel statistics for V_t ordinations had a minimum possible value of $p = 0.17$ because there were only three treatments being permuted; all comparisons of V_t ordinations were significant at this minimum p value.

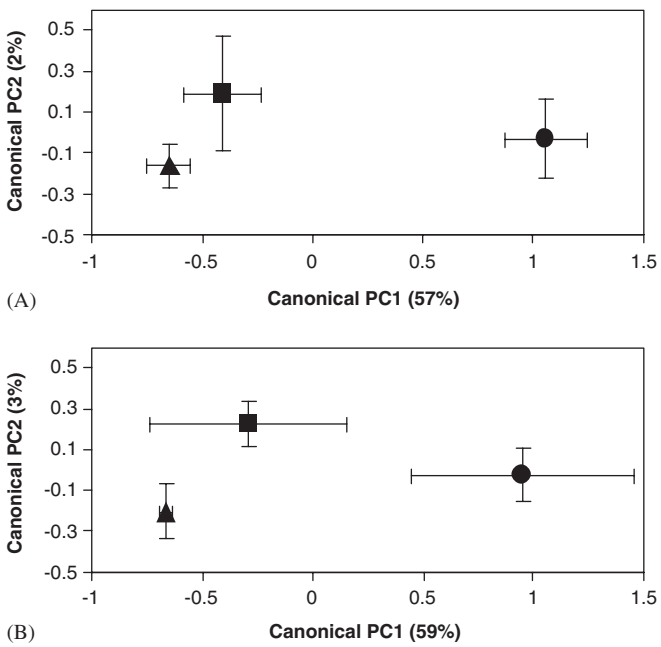

Fig. 2. Canonical ordination plots showing relationships between treatments for general bacterial T-RFLP profiles. (A) Physical capture T-RFLP ordination. (B) Fluorescent T-RFLP ordination. Squares = grass; triangles = soybean; circles = forest.

were present in physical capture T-RFLP profiles, and 38 were present in fluorescent T-RFLP profiles). The majority of the variance in the profiles was due to V_t, which was very similar in magnitude for physical-capture and fluorescent T-RFLP (Table 2). Canonical ordination plots (Fig. 2) and Mantel statistics (Table 2) indicate that ordinations capturing V_t were identical using the two methods. Error bars appear different between methods in Fig. 2, but these display extra information (from V_p) and are not a true difference between V_t ordinations. Examination of electrophoresis patterns (Fig. 1) indicates that bacterial communities from forest samples were quite different from grass or soybean samples, while grass and soybean communities were not distinguishable. This is also shown in ordinations (Fig. 2) by the very low proportion of the total variability accounted for by the second canonical axis which separates grass from soybean communities.

V_p and V_a were affected by the method of clustering fluorescent T-RFs into band classes (Table 2). Use of the fluorescent-PivotTable method resulted in a doubling of V_a relative to the fluorescent-GelCompar method (from 7% to 14%). The signal in the profiles that was obscured by the fluorescent-PivotTable method was due to V_p. V_a in physical-capture T-RFLP profiles was further increased to 22%, with a decrease in V_p. Note, however, that V_p was still found to be significant in physical-capture T-RFLP.

There is more complexity in V_p than V_t because there are 12 plots and only three treatments. At the same time, the signal within V_p is smaller than within V_t. It is therefore expected that the relationships captured by V_p will be more prone to noise than relationships between treatments. This noise is reflected in lower Mantel statistics between ordinations capturing V_p, although the ordinations are still somewhat similar (Table 2).

3.2. Group-specific T-RFLP profiles

Physical-capture and fluorescent T-RFLP profiles were also very similar for group-specific primers. Treatment significantly affected T-RFLP profiles in all cases (Table 3). For three groups (*Betaproteobacteria*, *Bacilli*, and *Actinobacteria*), the amount of variance attributed to treatment was higher in physical-capture T-RFLP profiles than in fluorescent T-RFLP profiles, while the reverse was true for only one group (*Alpha* and *Deltaproteobacteria*). Ordinations of treatment effects were very similar between fluorescent and physical-capture profiles, with Mantel statistics >0.98. An example comparison of *Actinobacteria* ordinations from physical capture and fluorescent T-RFLP profiles is shown in Fig. 3.

Forest profiles were clearly different from grass and soybean profiles for most groups (Fig. 4). Profiles from other plots appeared very similar to those shown in Fig. 4, except for *Basidiomycetes* profiles which were quite variable (data not shown). For each assay, forest communities were separated from grass and soybean communities

Table 3
Comparison of physical-capture and fluorescent T-RFLP using group-specific primers

Group[a]	Physical-capture T-RFLP		Fluorescent T-RFLP	
	Treatment effect variance (%)	# of T-RF categories	Treatment effect variance (%)	# of T-RF categories
Alpha and *Deltaproteobacteria*	52**	11	64**	35
Betaproteobacteria	85**	12	55**	22
Bacilli	47**	8	30*	24
Actinobacteria	80**	9	66**	30
Planctomycetes	51**	17	49**	46
Basidiomycetes	29*	17	29**	69

*$p<0.1$.
**$p<0.01$.
[a]Groups targeted in separate analyses using primers from Table 1.

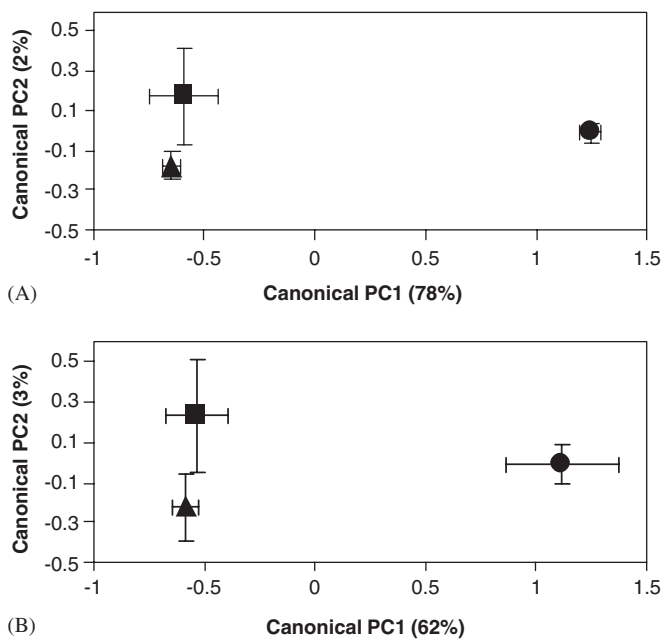

Fig. 3. Canonical ordination plots showing relationships between treatments for *Actinobacteria* T-RFLP profiles. (A) Physical capture T-RFLP ordination. (B) Fluorescent T-RFLP ordination. Squares = grass; triangles = soybean; circles = forest.

by the first ordination axis in ordinations reflecting V_t, accounting for 19% to 85% of the variance in the datasets. The soybean and grass treatments were separated on the second ordination axis in ordinations reflecting V_t, accounting for 0.3% to 10% of the variance.

3.3. Sequencing T-RFLP bands

DNA was isolated from the bands indicated in Fig. 4. Due to the small amount of DNA present in a T-RF band, in some cases relatively few clones were obtained (minimum of 10 for T-RF 14). Most of the inserts were within a few bases of the T-RF size predicted by image analysis. All sequences obtained were from the phylum or class targeted by the respective PCR assay except for sequences obtained from T-RFs 30 and 33 (Table 4). T-RFs 30 and 33 from the *Bacilli* assay were dominated by sequences which clustered with sequences from uncultivated organisms in the TM7 phylum. The cause for this anomaly is currently unknown since the primer BLS342F does not match known TM7 sequences. The two methods of identifying sequences, insertion into the ARB tree and BLASTn searching for matches within GenBank, generally yielded similar results (Table 4). BLASTn searching often could only match to sequences which were identified to the phylum level because of the many sequences from uncultivated organisms present in GenBank.

T-RFs 28 and 29 from the *Planctomycetes* assay were obtained from different treatments but were the same size, and would be expected to represent the same populations in a T-RFLP assay. We found that the same sequence OTU comprised a large proportion of both of these bands, accounting for 41% and 63% of clone libraries from T-RFs 28 and 29, respectively (Table 4). Note that T-RFs 28 and 29 are also approximately the same relative intensity (Fig. 4). In contrast, T-RFs 30 and 33 from the *Bacilli* assay are also the same size, but T-RF 30 represents 41% of the total signal of the forest profile, while T-RF 33 represents 8% of the soybean profile. T-RFs 30 and 33 have one dominant OTU in common, accounting for 98% and 36%, respectively, of the clone libraries. The difference in relative intensity between T-RFs 30 and 33 can therefore be attributed to the OTU in common between the two bands, as would be assumed in a comparative T-RFLP analysis. T-RF 33 would appear to include a greater diversity of sequences because of the reduced dominance of the shared OTU. Other sequences from T-RF 33 match various *Bacillus* and *Paenibacillus* sequences.

Table 5 shows the groups which include database sequences that could generate T-RFs of the size we investigated by sequence analysis. Some of the T-RFs could have been derived from database sequences from several genera, while others did not match any sequences.

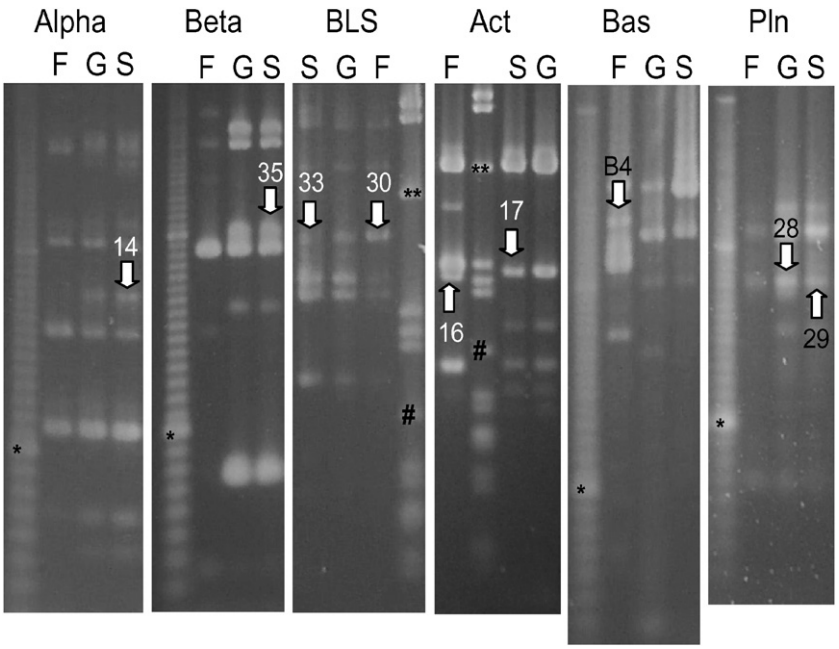

Fig. 4. Representative physical-capture T-RFLP profiles generated using group-specific primers, shown with DNA size-markers. Numbers indicate T-RF bands cut for sequencing. F = forest; S = soybean; G = grass; * = 200 bp band in 20 bp ladder; # = 339 bp band in λ PstI marker; ** = 805 bp band in λ PstI marker.

Table 4
Summary of phylogenetic characterization of dominant clones obtained from T-RFs

Band #	% of library	ARB categorization	BLASTn results		
			Summary of best matches	% Similarity	Length of match
14	40	*Sphingomonas*	*Sphingomonas*	94–96	361–363
14	60	*Myxococcus*	Uncultured *Deltaproteobacteria*	88–94	421–430
16	67	*Acidothermus*	Uncultured *Actinobacteria*	98–99	479–482
17	54	*Microthrix*	Uncultured *Actinobacteria*	97–99	445–492
28	45	*Gemmata*	Uncultured *Planctomycetes*	93–99	414–429
28	41	*Planctomyces*[a]	Uncultured *Planctomycetes*	88–97	401–438
29	63	*Planctomyces*[a]	Uncultured *Planctomycetes*	80–99	353–425
29	12	*Pirellula*	*Pirellula*	95–98	427–432
30	98	TM7[b]	Uncultured TM7	90–95	475–534
33	36	TM7[b]	Uncultured TM7	90–96	431–512
33	11	*Paenibacillus*	*Paenibacillus*	97–99	460–483
35	53	Uncultured *Betaproteobacteria*	Uncultured *Betaproteobacteria*	95–100	467–473
35	13	Uncultured *Betaproteobacteria*	*Oxalobacteraceae*	94–98	466–472
B4	55	*Homobasidiomycetes*	*Amanita bisporigera*	91	471
B4	16	*Ustilaginomycetes*	*Homobasidiomycetes*	96	300

[a] *Planctomyces* sequences which have matching phylogenetic results.
[b] TM7 sequences which have matching phylogenetic results.

Most of the sequences obtained from excised T-RFs did not match the groups predicted by in silico restriction of database sequences (compare Tables 4 and 5). T-RF 16 matched predictions from the database the best, with sequences affiliated with *Acidothermus* comprising 67% of the T-RF clone library, and being one of two groups that could generate a 477 bp band with the *Actinobacteria* assay.

4. Discussion

The physical capture method of T-RFLP resulted in quite similar profiles to those generated by fluorescent T-RFLP, although fluorescent T-RFLP exhibited enhanced resolving power of bands as expected. The differences between treatments captured by both methods and revealed by ordination were virtually identical. For

Table 5
Matches between sizes of sequenced T-RFs and predicted T-RF lengths of database sequences

Band #	Band size (bp)	Groups with predicted matches in target group
14	414	*Rickettsiales*, *Rhodospirillales*, *Desulfobacter*, *Methylobacterium*
16	477	*Streptosporangiaceae*, *Acidothermus*
17	490	*Mycobacterium*, *Rhodococcus*, *Nonomuraea*
28, 29	430	*Planctomyces limnophilus*
30, 33	679	*Paenibacillus*
35	511	None
B4	646	None

general bacterial T-RFLP profiles, where variance was fully partitioned, physical capture T-RFLP did result in an increase in V_a and a decrease in V_p, one of the biological sources of variability in the data. This was probably due to reduced accuracy in the electrophoresis and image analysis methods used. However, V_t was increased in physical capture T-RFLP for several group-specific assays (*Betaproteobacteria*, *Bacilli*, and *Actinobacteria*), with a concomitant decrease in the $V_a + V_p$ pool quantified for them. It is unlikely that one biological source of variability would increase at the expense of another one, so V_a was probably reduced in physical capture T-RFLP profiles for these assays. Based on these data, we suggest that the effects of physical capture T-RFLP on analytical variability will depend on the primers used and the unique suite of T-RF sizes that need to be discriminated. For example, in some cases where many peaks in several samples are present over a short window of T-RF length, there may be substantial noise in detection and clustering of these peaks due to variability in relative runtimes and the shapes of overlapping peaks. This noise would be eliminated by the method which pools the fluorescence of these peaks.

The weaker and more complex residual relationships between plots (V_p) were only partially similar between general bacterial physical capture and fluorescent T-RFLP profiles, but it must be noted that they were also only partially similar when two methods of clustering T-RFs into classes were used on the same fluorescent data. These results are similar to the sensitivity to binning method recently found by Hewson and Fuhrman (2006), who also found superior performance of a method that accommodated the distribution of bands to define centers of band classes. If the relationships captured by V_p can be altered by method of data processing, it can hardly be surprising that profiles involving different PCRs would also result in only partially similar relationships.

The number of T-RFs resolved was reduced by physical capture T-RFLP in all assays due to the differences in electrophoresis and image analysis mentioned above. This reduced sensitivity is one of the tradeoffs involved in performing physical capture T-RFLP as a community ordination method, and is likely to result in less sensitivity to changes in non-dominant populations within the community. However, as a method of comparing microbial communities, comparing the number of T-RFs present in profiles does not provide useful information about community diversity (Blackwood, 2006; Blackwood et al., in preparation; Loisel et al., 2006). Since the goal of performing T-RFLP is most often comparative community analysis, it is more relevant to compare methods by variance partitioning and sample ordination, which have already been discussed.

Physical capture T-RFLP allows one to obtain DNA sequences directly from T-RFs, which eliminates one of the primary criticisms of T-RFLP (Felske and Osborn, 2005). This is made possible because the non-terminal restriction fragments are removed, and individual T-RFs can be purified after being identified on a gel. We used this technique to evaluate one of the key assumptions in T-RFLP community analysis, that T-RFs of the same size from different environments represent the same (or similar) sequences. We confirmed that a *Planctomycetes* T-RF of a similar relative intensity in different samples represented largely the same taxon. In addition, a decrease in relative intensity of a *Bacilli* T-RF between samples could be attributed to a decrease in relative abundance of one of the taxa it represented (which actually turned out to be a sequence affiliated with the TM-7 group). Further tests of this assumption should be undertaken before it can be accepted as true in general. Previous attempts to relate T-RFs to specific sequences from the environment have involved evaluation of undigested PCRs by cloning and sequencing (Redfield et al., 2002; Sessitsch et al., 2001), or by screening clones with T-RFLP prior to sequencing (Grant and Ogilvie, 2004). However, T-RFs from complex soil communities can represent multiple taxa, and identification of T-RFs should take this into account by adequately sampling the sequences that make up a T-RF of interest. In a clone library of 100 clones, a typical T-RF comprising 2% of a pool of amplified ribosomal fragments has an 86% chance of being represented by 0 to 3 clones. This degree of sampling will be insufficient to reliably identify the sequences that make up many T-RFs in complex environments such as soil. Sequencing the same T-RF by physical capture T-RFLP can easily yield 20–50 clones, without wasted sequencing effort, and an identification that is made with much greater confidence. Ultimately, however, the goals of a study and the nature of the community should dictate the methods that are adopted. Another consideration is that phylogenetic reconstruction from short T-RFs is not recommended, although reliable identification may still be possible through classification algorithms such as those available on the RDP website (Cole et al., 2005).

It is clear from our data that identification of T-RFs only by comparison of T-RF sizes to computer digests of database sequences will often lead to incorrect conclusions. This is due in part to the unexplored diversity present in most environments, and the limited number of sequences

with phylogenetic affiliations present in the database. Variability in electrophoresis mobility of different sequences, which is inherent in the T-RFLP process (Kaplan and Kitts, 2003; Rasche et al., 2006), is also likely to contribute to misidentifications of T-RFs using comparison of T-RF size to the database.

Using group-specific T-RFLP assays instead of a general bacterial assay involves tradeoffs as well. The primary advantage is an increased ability to draw phylogenetic inferences from a group-specific assay, because of reduced complexity of the profiles and the increased likelihood that a T-RF band will represent a single taxon (Dunbar et al., 2001; Tom-Petersen et al., 2003; see also Gich et al., 2005). In addition, a group-specific assay is more sensitive to differences in composition of the particular group targeted. This is particularly important for complex communities such as soils. The disadvantage is that more assays are required to approach the phylogenetic breadth of a general assay. We used four group-specific assays within the bacteria, and many more would be necessary to cover the diversity of bacteria within soils (Janssen, 2006). Choice of restriction enzyme when performing T-RFLP has previously been pointed out to be an important factor in determining ability to discriminate taxa (Engebretson and Moyer, 2003), and is especially critical when using group-specific assays because of the lower diversity of target sequences. We found that systematic searches through a database of restriction enzymes resulted in optimal enzymes that were different for each group tested, and not necessarily among those typically used.

In conclusion, physical capture T-RFLP is a valuable tool which complements other methods of community analysis of soil microbes. Use of group-specific primers with optimal restriction enzymes can enhance the phylogenetic inferences that can be made from T-RFLP analyses. However, we found that sequencing of a T-RF must be performed if it is to be affiliated with specific taxa. As a community profiling method, physical capture T-RFLP profiles do not resolve as many bands as fluorescent T-RFLP, but performed approximately as well as fluorescent T-RFLP at discriminating our test communities. Relative performance of the methods should be evaluated on a case-by-case basis as there are idiosyncrasies associated with each set of primers and restriction enzymes, as well as with different sets of samples. Since the cost of equipment required to perform physical capture T-RFLP is a small fraction of the cost of equipment used in fluorescent T-RFLP, this method may also increase the opportunity for some labs to accomplish microbial community analysis.

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References

Bankhead, S.B., Landa, B.B., Lutton, E., Weller, D.M., McSpadden Gardener, B.B., 2004. Minimal changes in rhizobacterial population structure following root colonization by wild type and transgenic biocontrol strains. FEMS Microbiology Ecology 49, 307–318.

Blackwood, C.B., 2006. Analyzing microbial community structure by means of terminal restriction length polymorphism (T-RFLP). In: Cooper, J., Rao, J.R. (Eds.), Molecular Approaches to Soil, Rhizosphere and Plant Microorganism Analysis. CABI Publishing, Wallingford, Oxfordshire, UK (in press).

Blackwood, C.B., Paul, E.A., 2003. Eubacterial community structure and population size within the soil light fraction, rhizosphere, and heavy fraction of several agricultural systems. Soil Biology & Biochemistry 35, 1245–1255.

Blackwood, C.B., Marsh, T., Kim, S.-H., Paul, E.A., 2003. Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Applied & Environmental Microbiology 69, 926–932.

Blackwood, C.B., Oaks, A., Buyer, J.S., 2005. Phylum- and class-specific PCR primers for general microbial community analysis. Applied & Environmental Microbiology 71, 6193–6198.

Boyle, S.A., Rich, J.J., Bottomley, P.J., Cromack, K., Myrold, D.D., 2006. Reciprocal transfer effects on denitrifying community composition and activity at forest and meadow sites in the Cascade Mountains of Oregon. Soil Biology & Biochemistry 38, 870–878.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., Garrity, G.M., Tiedje, J.M., 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Research 33 (Database Issue), D294–D296.

Dunbar, J., Ticknor, L.O., Kuske, C.R., 2001. Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Applied & Environmental Microbiology 67, 190–197.

Engebretson, J.J., Moyer, C.L., 2003. Fidelity of select restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism. Applied & Environmental Microbiology 69, 4823–4829.

Felske, A., Osborn, A.M., 2005. DNA fingerprinting of microbial communities. In: Osborn, A.M., Smith, C.J. (Eds.), Molecular Microbial Ecology. Taylor & Francis Group, New York, USA, pp. 65–96.

Gich, F., Schubert, K., Bruns, A., Hoffelner, H., Overmann, J., 2005. Specific detection, isolation, and characterization of selected, previously uncultured members of the freshwater bacterioplankton community. Applied & Environmental Microbiology 71, 5908–5919.

Grant, A., Ogilvie, L.A., 2004. Name that microbe: rapid identification of taxa responsible for individual fragments in fingerprints of microbial community structure. Molecular Ecology Notes 4, 133–136.

Green, V.S., Cavigelli, M.A., Dao, T.H., Flanagan, D.C., 2005. Soil physical properties and aggregate-associated C, N, and P distributions in organic and conventional cropping systems. Soil Science 170, 1–10.

Hewson, I., Fuhrman, J.A., 2006. Improved strategy for comparing microbial assemblage fingerprints. Microbial Ecology 51, 147–153.

Janssen, P.H., 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Applied & Environmental Microbiology 72, 1719–1728.

Kaplan, C.W., Kitts, C.L., 2003. Variation between observed and true terminal restriction fragment length is dependent on true TRF length and purine content. Journal of Microbiological Methods 54, 121–125.

Kitts, C.L., 2001. Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. Current Issues in Intestinal Microbiology 2, 17–25.

Legendre, P., Legendre, L., 1998. Numerical ecology, second ed. Elsevier, Amsterdam, The Netherlands.

Loisel, P., Harmand, J., Zemb, O., Latrille, E., Lobry, C., Delgenès, J., Godon, J., 2006. Denaturing gradient electrophoresis (DGE) and

single-strand conformation polymorphism (SSCP) molecular fingerprints revisited by simulation and used as a tool to measure microbial diversity. Environmental Microbiology 8, 720–731.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H., 2004. ARB: a software environment for sequence data. Nucleic Acids Research 32, 1363–1371.

Marsh, T.L., Saxman, P., Cole, J., Tiedje, J., 2000. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Applied & Environmental Microbiology 66, 3616–3620.

Nagashima, K., Hisada, T., Sato, M., Mochizuki, J., 2003. Application of new primer-enzyme combinations to terminal restriction fragment length polymorphism profiling of bacterial populations in human feces. Applied & Environmental Microbiology 69, 1251–1262.

Pérez-Piqueres, A., Edel-Hermann, V., Alabouvette, C., Steinberg, C., 2006. Response of soil microbial communities to compost amendments. Soil Biology & Biochemistry 38, 460–470.

Rasche, F., Hödl, V., Poll, C., Kandeler, E., Gerzabek, M.H., Elsas, J.D., Sessitsch, A., 2006. Rhizosphere bacteria affected by transgenic potatoes with antibacterial activities compared with the effects of soil, wild-type potatoes, vegetation stage and pathogen exposure. FEMS Microbiology Ecology 56, 219–235.

Redfield, E., Barns, S.M., Belnap, J., Daane, L.L., Kuske, C.R., 2002. Comparative diversity and composition of cyanobacteria in three predominant soil crusts of the Colorado Plateau. FEMS Microbiology Ecology 40, 55–63.

Roberts, R.J., Vincze, T., Posfai, J., Macelis, D., 2005. REBASE—restriction enzyme and DNA methyltransferases. Nucleic Acids Research 33, D230–D232 (Database Issue).

Rogers, G.B., Carroll, M.P., Serisier, D.J., Hockey, P.M., Jones, G., Bruce, K.D., 2004. Characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16S ribosomal DNA terminal restriction fragment length polymorphism profiling. Journal of Clinical Microbiology 42, 5176–5183.

Sessitsch, A., Weilharter, A., Gerzabek, M.H., Kirchmann, H., Kandeler, E., 2001. Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. Applied & Environmental Microbiology 67, 4215–4224.

Tiedje, J.M., Asuming-Brempong, S., Nüsslein, K., Marsh, T.L., Flynn, S.J., 1999. Opening the black box of soil microbial diversity. Applied Soil Ecology 13, 109–122.

Tom-Petersen, A., Leser, T.D., Marsh, T.L., Nybroe, O., 2003. Effects of copper amendment on the bacterial community in agricultural soil analyzed by the T-RFLP technique. FEMS Microbiology Ecology 46, 53–62.