Terminal Restriction Fragment Patterns: A Tool for Comparing Microbial Communities and Assessing Community Dynamics

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Abstract

Terminal Restriction Fragment (TRF) patterns, also known as Terminal Restriction Fragment Length Polymorphisms (T-RFLP), are a recently introduced PCR-based tool for studying microbial community structure and dynamics. Since the first review of TRF methodology (Marsh, 1999. Curr. Op. Microbiol. 2: 323-7), at least 35 new research articles were published that include this powerful tool in some part of their reports. This review covers some of the applications that TRF patterns were used for and provides a discussion of how to create and analyze TRF pattern data. This data has the advantage of being simply and rapidly produced using standard DNA sequencing equipment. The raw data are automatically converted to a digitized form that can be easily analyzed with a variety of multivariate statistical techniques. The identification of specific elements in a TRF pattern is possible by comparison to entries in a good sequence database or by comparison to a clone library. As an added advantage when investigating complex microbial communities such as those in soils and intestines, TRF patterns are recognized as having better resolution than other DNA-based methods for evaluating community structure.

Introduction

The Golden Age of Microbiology in the early 1900's was based on the isolation and characterization of pure cultures. However, the goal of a new cohort of modern microbiologists is the understanding of natural microbial community structures and dynamics. As the limitations of culture methods became clear many different techniques for evaluating microbial communities were developed. By far the majority of these use the Polymerase Chain Reaction (PCR) to amplify genes of interest directly from environmental samples without a culture bias (Kozdroj *et al.,* 2001; Ranjard *et al.,* 2000; Tiedje *et al.,* 1999). Existing PCR-based methods include Amplified Ribosomal DNA Restriction Analysis (ARDRA), Single Stranded Conformation Polymorphism analysis (SSCP), Thermal and Denaturing Gradient Gel Electrophoresis (TGGE and DGGE), Amplified Length Heterogeneity analysis (ALH) and Terminal Restriction Fragment (TRF) patterns or profiles (also known as T-RFLP analysis). All of these tools produce a pattern or profile of nucleic acids amplified from a sample and that pattern reflects the microbial community structure. This is a review of one of the newest tools for evaluating microbial communities, TRF patterns, a tool providing investigators with a large amount of easily analyzed data on microbial community structure. This review will cover the intricacies of production and analysis of TRF patterns, an overview of current applications for TRF patterns and a discussion of the advantages and disadvantages of this tool.

How TRF Patterns are Made

TRF patterns are generated and analyzed in a series of steps that combine PCR, restriction enzyme digestion and gel electrophoresis. DNA extracted from a sample is subjected to PCR using primers homologous to conserved regions in a target gene. A collection of sequences for the target gene from many different genetic backgrounds is necessary for the design of these primers. One primer is labeled on the 5'-end, usually with a fluorescent molecule. Analysis of the target sequences dictates which primer is most appropriate for labeling. The amplified DNA fragments (amplicons) are then digested with a restriction enzyme, usually one with a tetranucleotide recognition sequence. The digested amplicons are subjected to electrophoresis in either a polyacrylamide gel or a capillary gel electrophoresis apparatus, usually a DNA sequencer with a fluorescence detector so that only the fluorescently labeled terminal restriction fragments (TRFs) are visualized (Figure 1). Most investigators report using an automated fragment analysis program that calculates TRF length (bp) by comparing TRF peak retention time to a DNA size standard. These programs integrate the electropherograms and return TRF peak height and area. The patterns of TRF peaks can then be numerically compared between samples using a variety of mutivariate statistical methods. In addition, individual TRF peaks in a pattern can be identified by comparison to a clone library or by predictions from an existing database of sequences. The creation and analysis of TRF pattern data will be covered in detail later.

Current Reports with TRF Patterns

The history of TRF patterns and their adoption into mainstream microbial ecology was well covered in an excellent review of the method, referred to there as T-RFLP,

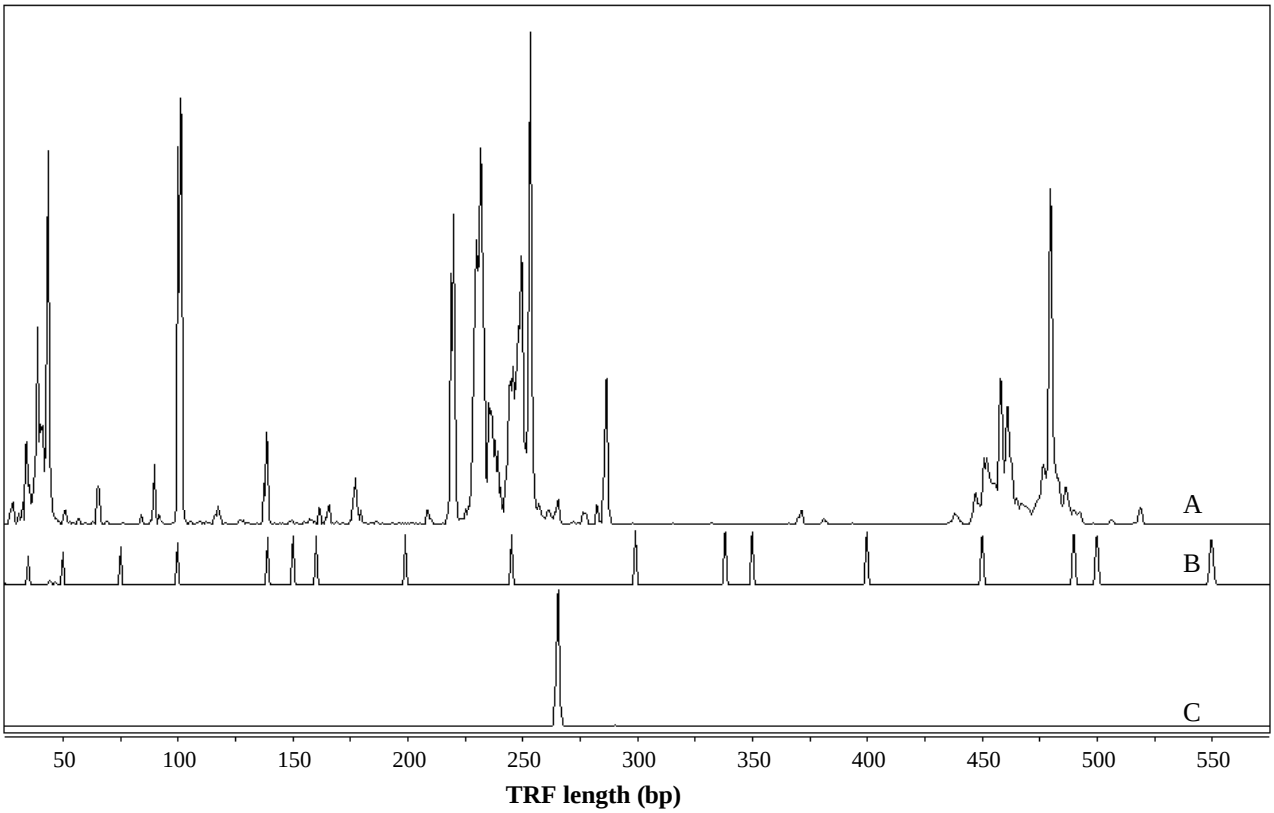

Figure 1. TRF pattern output from an ABI-310 genetic analyzer. Trace A is the actual TRF data, gathered in the blue channel (FAM dye). Trace B is the size standard ladder, in this case the ABI ROX500 ladder (red channel) with an additional standard fragment at 550 bp. Trace C is a digest standard, in this case the 16S-rDNA from *Pseudomonas oleovorans* labeled with the Hex dye (green channel). The traces represent data collected simultaneously during a single run but were separated in this figure for clarity.

by Terence Marsh (Marsh, 1999). Several advances have been made since that review was written and this paper will therefore focus on some of the emerging debate on how to obtain good TRF patterns and analyze them in meaningful ways. Marsh reported 8 papers on TRF patterns from 5 different groups of investigators, most of which discussed methods development. At the time the current review was written over 40 reports, from at least 16 laboratories, either used a TRF pattern as part of an investigation, advanced the method further or mentioned it in a review of methods (Table 1). While ten of these papers presented aspects of methods development, the majority reported investigations into microbial community analysis where TRF patterns were used to provide a broad view of the community.

Many studies of microbial communities focus on the *Eubacteriaceae* because several sets of 16S rRNA primers homologous to broadly conserved portions of the gene are well documented for eubacteria (Brunk, 1996). At this time, at least seven groups of investigators have produced 12 TRF papers on diverse eubacterial communities, ranging from bacteria in pig intestines to marine bacterioplankton. However, primers designed to observe taxonomic diversity in other groups of microbiota were also used to generate TRF patterns. At least two different groups using archaebacterial-specific 16S rRNA primers produced six papers investigating archaeal communities in soil and fish intestines (Table 1). Marsh *et al.* (1998) used 18S rRNA primers to describe fungal communities in sewage sludge. In the most focused taxonomic approaches, Bernhard and Field (2000a and 2000b) described TRF patterns created after PCR with 16S rRNA primers targeted to amplify only the *Bacteroides-Provotella* group of eubacteria and Derakshani *et al.* (2001) used 16S rRNA primers targeted to planctomycetes.

TRF patterns were also used to characterize functional diversity in bacterial communities. Primers with homology to broadly conserved sequences in functional genes were used to produce TRF patterns that served as a measure of diversity in functional genotypes from environmental samples. This category of investigation included reports on the functional diversity of N_2-fixation (nifH), nitrification (amoA), denitrification (nosZ) and mercury resistance (merR). The ecosystems investigated ranged from marine sediments to termite intestines (Table 1).

In addition to the investigative reports, three review papers (aside from Marsh, 1999) included TRF patterns in their discussions of microbial diversity measurement methods (Table 1). Notably, all these reviews were written in the last three years, an indication of the novelty of TRF patterns as a microbiological tool. The fact that the number of investigators using TRF patterns has tripled since Marsh wrote his review clearly shows the interest in this tool.

Table 1. Current literature involving TRF patterns

Author	Year	Primers	Subject
Avaniss-Aghajani	1994	16S-eubacterial	Method development (mycobacteria)
Avaniss-Aghajani	1996	16S-eubacterial	Method development (mycobacteria)
Brunk	1996	16S-eubacterial	Method development (database)
Liu	1997	16S-eubacterial	Method development (soils, intestines)
Clement	1997	16S-eubacterial	Method development (soils, feces)
Osborn	1999	16S-eubacterial	Method development (soils)
Moeseneder	1999	16S-eubacterial	Method development (marine)
Dunbar	2000	16S-eubacterial	Method development (soils)
Marsh	2000	16S-eubacterial	Method development (web tools)
Dunbar	2001	16S-eubacterial	Method development (database, soils)
Liu	1998	16S-eubacterial	Activated sludge
Phelps	1998	16S-eubacterial	Anaerobic biodegradation
Knight	1999	16S-eubacterial	Anaerobic biodegradation
Greenblatt	1999	16S-eubacterial	Amber
Lüdemann	2000	16S-eubacterial	Agricultural soil
Lukow	2000	16S-eubacterial	Agricultural soil
Flynn	2000	16S-eubacterial	Anaerobic biodegradation
Häggblom	2000	16S-eubacterial	Anaerobic biodegradation
Leser	2000	16S-eubacterial	Pig intestines
González	2000	16S-eubacterial	Marine algal blooms
Kerkhof	2000	16S-eubacterial	Soybean rhizosphere
Franklin	2001	16S-eubacterial	Dilution of sewage sludge
Moeseneder	2001	16S-eubacterial	Marine bacterioplankton
Kaplan	2001	16S-eubacterial	Rat feces
van der Maarel	1998	16S-archaeal	Fish intestines
Chin	1999a	16S-archaeal	Agricultural soil
Chin	1999b	16S-archaeal	Agricultural soil
Fey	2000	16S-archaeal	Agricultural soil
Lueders	2000	16S-archaeal	Agricultural soil
Ramakrishnan	2000	16S-archaeal	Agricultural soil
Marsh	1998	18S-fungal	Activated sludge
Bernhard	2000a	16S-group specific	*Bacteroides-Provotella* in the ocean
Bernhard	2000b	16S-group specific	*Bacteroides-Provotella* markers
Derakshani	2001	16S-group specific	Planctomycetes in soils
Bruce	1997	merR	Mercury resistance in sediments
Noda	1999	nifH	N_2 fixation in termite intestines
Ohkuma	1999	nifH	N_2 fixation in termite intestines
Horz	2000	amoA	Ammonia monooxidase genes
Scala	2000	nosZ	Denitrification in marine sediments
Marsh	1999		Review of T-RFLP method
Tiedje	1999		Review of community assessment methods
Bothe	2000		Review of nitrification and denitrification diversity
Ranjard	2000		Review of community assessment methods
Kozdrój	2001		Review of community assessment methods

Considerations for Gathering TRF Pattern Data

While it is simple to explain the basic method for obtaining TRF pattern data there are many pitfalls and tricks that are worth investigating before beginning a study (Table 2). Some of the comments in this section will apply to a wide variety of DNA and PCR based investigations of microbial communities but this is not meant to be a comprehensive list of the problems inherent in these approaches. Thus, most of the emphasis will be placed on those cautions or insights specifically applicable to the generation of TRF patterns.

DNA Extraction

DNA extraction techniques are as various as the habitats inhabited by microbial communities. Differential lysis of gram negative versus gram positive cells, especially spores, can bias the relative amounts of DNA present in an extract. A combination of physical (bead beating) and chemical/enzymatic cell lysis methods is most commonly touted as producing the best results (Frostegard *et al.,* 1999). Depending on the size of a sample, pooling of replicate extractions is recommended to limit random bias although systematic biases will persist.

PCR Bias

PCR has known biases when used in a multi-template manner such as is required for community analysis (Farrelly *et al.,* 1995; Polz *et al.,* 1998; Qiu *et al.,* 2001; Tanner *et al.,.,* 1998). Templates with good primer homologies will be preferentially amplified and some templates will not compete well for primers and be underrepresented or missing from the mixture of amplicons. In spite of these problems the abundance of a specific amplicon in a mixture (and thus of a TRF peak in a pattern) is reproducible and in direct proportion to the abundance of that template in the sample (Clement *et al.,* 2000; Dunbar *et al.,* 2000; Polz *et al.,* 1998). Given this fact it is clear that PCR amplification could bias estimates of organism abundance as a result of gene copy number. It is well known that rRNA genes vary significantly in copy number. Fogel *et al.* (1999) estimated that the range of rRNA gene copy number in eubacteria is from 1 to 13 with an average of 3.8 copies per genome. Thus, although amplicon abundance after PCR may be

Table 2. Steps in TRF production and analysis with suggestions and comments

Step	Comments and Suggestions
DNA extraction	Combining multiple extractions is recommended to limit biases
PCR	Keep amplicon size between 400 and 700 to ensure proper analysis
	10-100ng of template DNA per reaction 30 PCR cycles is average in the literature.
	Combining multiple reactions is recommended to limit biases
Digestion	Research best enzymes for each primer set.
	Use a digestion control for quality assurance.
	Make TRF patterns from more than one enzyme if you want better identification of community members and better resolution between communities
Electrophoresis	PAGE or CGE with appropriate fragment sizing software.
	Make sure DNA ladder covers appropriate range of lengths.
	Think about replicating electrophoresis runs to eliminate artifacts
Analysis	Bin fragments into single base pair bins, align as necessary.
	Standardize TRF patterns to account for differences in DNA loading
	Normalize TRF peak abundance relative to total DNA.
	Peak area is the preferred abundance measure
TRF Peak ID	Confirm with cloning if possible, otherwise use multiple enzyme digest patterns to direct database searching.
	Allow for a match window to cover discrepancies between predicted and observed TRF size based on electrophoretic properties.

proportional to cell abundance in the original sample the proportionality factor may vary significantly from one organism to another. In the final analysis, while TRF patterns may accurately describe the relative abundance of specific amplicons in a mixture, they cannot be used to estimate relative organism abundance without prior calibration.

Other PCR based artifacts such as the formation of chimeric amplicons are known to occur at frequencies up to several percent in controlled circumstances, but can also be minimized by decreasing the number of PCR cycles (Polz *et al.,* 1998; Qiu *et al.,* 2001). Although it is clear that systematic PCR bias cannot be controlled, many investigators in the TRF literature pooled multiple PCR reactions from a single sample to ensure random PCR artifacts were minimized. Between 20 and 35 PCR cycles were commonly reported although no obvious difference in TRF patterns was detected over this range of PCR cycles (Osborn *et al.,* 1999).

PCR Primer Choice

Selection of PCR primers is a key step in producing usable TRF pattern data. There must exist at least two regions of conserved sequence in the gene of interest to provide priming sites in genes from a broad range of organisms. In addition, the primers must be far enough apart for sufficient sequence divergence to exist between them since amplicons that are too short will result in patterns that do not reflect as much of the true diversity of the sample because the majority of amplicons will not contain a restriction site. Because tetranucleotide restriction enzymes are used to create TRF patterns, fragments longer than 1000 bp are statistically uncommon. However, if the primers are too far apart then the long amplicons will create some fragments that are too large for analysis since the accuracy of fragment sizing decreases with fragment length (see below). An optimal amplicon length is between 400 and 700 bp since this allows for the best possible estimation of diversity while avoiding the loss of data associated with long amplicons.

Digestion with Restriction Enzymes

After PCR the amplified DNA is digested to produce a pattern of different length fragments. The enzyme(s) chosen to digest amplicons is dictated by several parameters. If sufficient data exists, as is the case with 16s rRNA genes, it is possible to predict the fragment length expected and a digestion enzyme can be chosen that best reproduces the diversity expected in a sample. Brunk *et al.* (1996) looked at the distribution of predicted fragments from 16S rRNA genes in a sequence database (Maidak *et al.,* 2000) and recommended the use of *Hha*I, *Msp*I, *Rsa*I, and a combined digest of both *Rsa*I and *Hha*I. Dunbar *et al.* (2001) analyzed the phylogenetic resolution of TRF fragments from a range of enzymes and enzyme combinations. They found that 68% of the *Rsa*I generated TRF lengths were specific for less than four species of the same genus. They also concluded that the phylogenetic specificity of any TRF length would be greatly enhanced by the use of group specific primers.

Most of the investigators using 16S primers used the readily available enzymes *Hha*I and *Msp*I or their isoschizomers. A few labs report using *Rsa*I, *Hae*III, *Sau*3aI and *Alu*I, though these were used in conjunction with TRF patterns produced by other enzymes. One exception was publications from investigators at Rutgers (Haggblom *et al.,* 2000; Kerkof *et al.,* 2000; Knight *et al.,* 1999; Phelps *et al.,* 1998) that exclusively used *Mnl*I, an unusual enzyme that recognizes the non-palindromic sequence “CCTC”. With other more esoteric primer sets in the literature, the restriction enzymes used have varied considerably. Since other templates do not have the extensive database of sequences available for 16S rRNA genes a process of trial and error must be used to choose an appropriate enzyme.

Some researchers reported problems with incomplete digestion of amplicons creating artifactual peaks in TRF patterns (Clement *et al.,* 1997; Garcia *et al.,* 2000; Lukow *et al.,* 2000; Osborne *et al.,* 1999). Careful optimization of digestion protocols can relieve this problem and the inclusion of a digest control will help to track problems (Figure 1). Every batch of digestions should include one tube with end labeled DNA from a known organism for a control digestion. This sample is then run in a separate

lane on the sequencing gel to ascertain digestion completeness.

Electrophoresis of Digested Amplicons

Both polyacrylamide gel electrophoresis (PAGE) and capillary gel electrophoresis (CGE) were used to generate TRF patterns from digested PCR amplicons. In either case, determining the correct amount of DNA to load on a gel can be problematic. A TRF pattern containing a large number of similar size peaks will require more DNA loaded onto the gel since the signal will be diluted across all the peaks. However, patterns with very few peaks or an uneven distribution of peak sizes require less DNA to prevent saturating the fluorescence detector. Since the number and size distribution of individual peaks in a TRF pattern cannot be ascertained in advance some trial and error is required to discover the appropriate range for DNA loading. This datum is difficult to extract from the literature because it was not usually reported. Those reports that list amounts of DNA digested varied between 50 and 200 ng of DNA in a 20 to 50 ul reaction. The DNA is usually purified after digestion since the digestion buffer salts can interfere with electrophoresis. This means the amount of DNA actually loaded onto a gel is not known and it is often necessary to rerun samples that have been over- or under-loaded.

Both PAGE and CGE can produce detection artifacts that must be manually appraised. When using the Perkin-Elmer Applied Biosystems Inc. (PE-ABI) four color, automated DNA sequencers most artifacts can be recognized by the creation of a peak in all four color channels. These systems also allow for the inclusion of a DNA ladder labeled with a different dye to be run in the same lane as the sample DNA to ensure accurate TRF length determination.

Two groups reported that replication at each step in TRF pattern creation produced TRF patterns that did not vary significantly (Moeseneder *et al.,* 1999; Osborn *et al.,* 1999). However, Dunbar *et al*. (2001) reported an astounding 85% of all TRF peaks were irreproducible artifacts in a series of 9 replicate electrophoresis runs of the same DNA. In this experiment, the threshold for detection of peaks by the analysis software was set particularly low (half the level recommended by the software) and 90% of the irreproducible peaks were just above the detection threshold. In defense of this approach, Dunbar *et al*. suggest that repeating electrophoresis runs would be an excellent way to detect as many real TRF peaks as possible while still being able to exclude artifacts. Replication of electrophoresis runs would be especially useful in cases where presence/absence analysis was contemplated, as discussed below.

Considerations for the Analysis of TRF Pattern Data

As with the collection of TRF pattern data, there are many pitfalls to be taken into account when analyzing the data once it is collected (Table 2). Some of the comments in this section will apply to the analysis of any kind of data based on the differentiation of chemical species by chromatography, in this case an electrophoretic separation. As before, most of the emphasis will be placed on those aspects of analysis that pertain directly to TRF pattern data.

Approaches to TRF Pattern Analysis

Most TRF pattern reports presented at least one picture based analysis figure where differences in TRF patterns could be assessed by eye. Several reports went no further with their analysis than using the figure to show TRF peaks that were clearly present in TRF patterns from one sample and absent from another. Further effort in these reports was based on identifying organisms represented by these TRF peaks. Because of the problems discussed above, this can lead to some suspicion of the validity of these reports if no replication of TRF patterns was included.

Some investigators took the next step and used pairwise similarity coefficients to construct dendrograms showing how some microbial community structures clustered in similarity (Liu *et al.,* 1998; Leser *et al.,* 2000; Kerkof *et al.,* 2000; Dunbar *et al.,* 2000; Möeseneder *et al.,* 1999, Möeseneder *et al.,* 2001). Most of these comparisons were performed on a presence/absence basis where similarity is measured by the number of peaks two TRF patterns have in common. In this case there is no accounting for the relative size of a TRF peak. Several reports include the area of a peak into a similarity analysis either with a dendrogram output (Liu *et al.,* 1997; Liu *et al.,* 1998) or principle components analysis (Clement *et al.,* 1997, Franklin *et al.,* 2001; Kaplan *et al.,* 2001).

Another common method of analysis was to present the percent composition of a community by following the relative abundance of diagnostic TRF peaks through a series of samples. For example, Leuders *et al*. (2000) identified TRF peaks attributable to phylogenetic clusters of archaebacteria and presented percent contributions to the community for each group. The smaller or less diagnostic TRF peaks in a pattern were often ignored in these analyses.

Basic TRF Peak Calling

Most of the TRF pattern reports used the PE-ABI Genescan software to integrate peaks and place peak apices on a base pair scale relative to a DNA size ladder. Only one report (Möeseneder *et al.,* 1999) described a method of peak sizing based on different software. Using the Genescan software it is possible to dictate the peak detection threshold and most reports used the default of 50 fluorescence units. Some investigators reported raising the level to 100 units to ensure that noise peaks were not analyzed (Osborn *et al.,* 1999) while others recommended dropping the threshold to 25 units and used replicate electrophoresis runs to remove artifactual or noise peaks (Dunbar *et al.,* 2001).

Most investigators using PE-ABI DNA sequencers employed the standard PE-ABI DNA ladders (Rox500, Rox1000 or Rox2500) to size TRF peaks (Figure 1). However, DNA ladders from other sources were reported either as a supplement to the PE-ABI ladders (Kaplan *et al.,* 2001) or in a completely different system (Möeseneder *et al.,* 1999). No matter which ladder was used, most reports agreed that one base pair accuracy is only achievable up to about 700 bp.

The data produced by the Genescan package includes the peak height at apex, the apex position (in base-pairs with two decimals of accuracy, i.e. 145.34 bp) and the area under the peak. The peak recognition algorithms are not

complex and in many cases cannot correctly discriminate shoulders that result from small peaks on the edges of large peaks. In addition, background noise can result in a broad peak being called as several sub-peaks whose apices differ by less than one bp. The end result is that data must be quantized into one bp bins before further analysis. The quantized data can then be further manipulated depending on the preferred analysis method. A simple method for producing quantized data in one bp bins is to round TRF sizes to the nearest bp and then sum areas of peaks in the same bin (Clement *et al.,* 1997; Kaplan *et al.,* 2001). This method has some drawbacks that will be discussed below.

Peak Alignment Between Samples

The most difficult problem in analysis of TRF patterns stems from the accuracy of size determination for any given TRF peak. Genescan software returns a fractional peak apex position to the data table. The problem arises when attempting to align peaks from different patterns. Two peaks may be within 0.3 bp and yet fall into different 1 bp bins after rounding. For example, pattern A has a TRF peak apex at 134.42 bp and pattern B has one at 134.67 bp. Visual inspection shows that these TRF peaks should represent the same organism, yet a simple rounding routine will place these peaks into different bins. The result will change similarity profiles, skew presence/absence analysis and ruin analysis of individual TRF peak abundances between patterns.

One solution to this problem is an algorithm available on the Ribosomal Database Project (RDP) web site. The program returns a pair wise similarity coefficient based on a search for peaks within a defined distance of each other in the two patterns being compared (Marsh *et al.,* 2000). This is useful if a similarity coefficient is all that is required. Unfortunately, similarity coefficients do not determine the points of dissimilarity and so cannot point out TRF peaks that might be interesting. Another solution might be to export the raw electropherogram data to more sophisticated analysis programs that have more flexible integration algorithms. Unfortunately, export of the appropriate raw data is currently impossible with the existing software.

Dunbar *et al.* (2001) report the use of an alignment algorithm that gathers peaks within 0.5 bp and places only one peak from each sample into a one bp bin. However, this method was only used to align peaks from replicate TRF patterns, not between samples. In the end, manual analysis of eletropherograms may be the only way to resolve such problems. If the pattern of nearby peaks in the original electropherogram data shows a clear electrophoretic shift, then two TRF peaks can be confirmed as belonging in the same bin and the quantized data can be manually adjusted. Manual alignment of TRF peaks can be subjective and time consuming especially when analyzing large numbers of samples. However, this is true for the analysis of any other kind of electrophoretic data as well, where the resolution of electrophoresis is significantly lower. Fortunately, the process can be streamlined by careful examination of the quantized data. A quick scan of sample data in an array can sometimes show where a particular TRF peak in different samples may be running near a one-half bp mark and causing alignment problems.

Standardizing DNA Loading Between Samples

TRF pattern analysis can also be confounded by the fact that different amounts of DNA will be analyzed from each sample. The same DNA loaded at a higher concentration can result in the detection of more TRF peaks. Fortunately for most reports that ignored this problem, small differences in DNA loading between samples should not significantly perturb analysis of relative TRF peak abundance. However, detection discrepancies can be especially disturbing if a similarity analysis is being performed. As some investigators noted, very small peaks that result from either artifacts or differences in DNA loading can skew similarity profiles that are based on presence/absence data (Dunbar *et al.,* 2000; Kerkof *et al.,* 2000; Liu *et al.,* 1997). Because the amount of DNA loaded on a gel cannot be accurately controlled, an artificial detection threshold must be created for each sample so as to normalize peak detection thresholds.

Kaplan *et al.* (2001) presented a method for standardizing TRF patterns based on TRF peak area. The amount of DNA loaded onto a gel was estimated as the sum of all TRF peak areas in a pattern (total peak area). Multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total peak area in the sample with the smallest total peak area) by 580 area units (the smallest peak area detected by the Genescan software at the default 50 unit limit) created the new threshold value (Table 3). TRF peaks with area less than the new threshold value for a sample were removed from the data set.

Dunbar *et al.* (2001) presented a method for standardizing TRF patterns based on the peak height. The method follows the same steps outlined above except total DNA is estimated by a sum of peak heights. This makes sense on the surface since the software peak detection system has a detection threshold that is also based on peak height. However, peak area, not height is the most accurate measure of DNA abundance in an electropherogram because peak width increases as a function of retention time. Thus, a standard amount of a short DNA fragment will result in a peak with the same area but a larger height than the peak from a long fragment. Using a threshold based on peak height skews the relative abundance of TRF peaks from longer DNA fragments. Unsurprisingly, most investigators reporting analysis of relative abundance did so based on TRF peak area.

The last step before comparing TRF patterns is to normalize TRF peaks so that comparisons are performed on a relative abundance scale. This must be performed after normalizing detection thresholds because some TRF peaks may be removed after the new thresholds are applied. Most investigators reported relative abundance

Table 3. Example of procedure used to determine a threshold for the smallest observable peak for each sample in a dataset.

Sample	Total area	Ratio of total peak area	Threshold value
Smallest	200,000	1:1	580*
Big	400,000	2:1	1160
Bigger	2,000,000	10:1	5800

*Minimum detectable peak area with Genescan™ software detection threshold at 50 units.

on a percentage scale though some opted for parts per million.

Database Matching

Nearly every report using 16S rDNA TRF patterns took advantage of the extensive rRNA sequence database to attempt an identification of interesting TRF peaks. The majority of the reports used a clone library to back up their identification of a specific TRF peak. Two reports on soil archaebacteria (Fey *et al.,* 2000; Ramakrisna *et al.,* 2000) even used clones from previous reports to classify TRF peaks. However, several reports also used database comparisons to suggest possible taxonomic sources for TRF peaks of interest. When a single enzyme digest was used this resulted in very poor species resolution for a particular TRF size. In many cases a single TRF can represent several genera (Brunk *et al.,* 1996; Dunbar *et al.,* 2001; Marsh, 1999). Multiple enzyme digests should be able to better resolve TRF identity but the problem of correlating peaks from different enzyme derived TRF patterns remains thorny.

One difficulty with using a database predicted TRF size to identify a TRF peak from an environmental sample is that observed length and predicted length do not always match. Investigators reported that predicted TRF lengths were anywhere from 0 to 4 bp larger than the observed TRF length (Bernhard *et al.,* 2000a; Clement *et al.,* 1997; Gonzalez *et al.,* 2000; Kaplan *et al.,* 2001). Further work in our laboratory revealed an even larger range of discrepancy (Table 4). This discrepancy between predicted and observed TRF length appeared to be sequence dependant since organisms with similar predicted TRF lengths had different discrepancies. The discrepancies also increased as a function of TRF length.

Database matching is still possible, if a window is created for matching observed TRF lengths to database predicted TRF lengths. For example, a TRF peak observed at 345 bp could be produced from an organism with a predicted TRF size range of 344 to 349, a window of +1 to -4. This makes the database match significantly less specific. In many cases a single TRF length is predicted for several different species of bacteria (Brunk *et al.,* 1996; Dunbar *et al.,* 2001). Once a matching window is included in the analysis many more species could possibly produce the observed TRF peak. To overcome this obstacle TRF pattern data from several enzyme digests must be utilized in concert.

Kaplan *et al*. (2001) used a database to identify changing populations of bacteria in rat feces and included a +1 to -4 matching window. TRF pattern data from a combination of three enzyme digests was analyzed to identify TRF peaks whose abundance changed in concert. For example, in each of the three enzyme digests some TRF peaks were consistently small in old rats and large in young rats. Because these peaks decreased in concert with the rat's age the same organism(s) could have produced them. Those peaks were used to search a database and a set of three TRF peaks (one from each digest) produced a match to *Lactobacillus johnsonii*. Strains of this organism, isolated from several fecal samples, produced the predicted set of TRF peaks.

While database matching is possible, there is a clear advantage to confirming TRF identity by using a clone library, even if the process of cloning and sequencing is expensive and time consuming. A cloned fragment of DNA can be put through the TRF process and matched directly to TRF peaks in a pattern. This works well if the TRF peak of interest is of high relative abundance and a large number of clones can be inspected. Several investigators reported TRF peaks not represented in clone libraries and clones producing TRF fragments not seen in the community TRF pattern (Dunbar *et al.,* 2000; Phelps *et al.,* 1998). In addition, the frequency of clone abundance did not match the relative abundance seen in TRF patterns, perhaps the result of a cloning bias (Lueders *et al.,* 2000). This can result in a long search through a clone library for a low abundance TRF. Finally, RDP database analyses predict that several unrelated organisms can produce the same TRF size (Brunk *et al.,* 1996, Dunbar *et al.,* 2001). Thus, a single TRF peak in a pattern may represent more than one organism in the sample, even if only one clone in the library was found to match that TRF peak.

Table 4. Discrepancies between observed TRF sizes and those predicted by DNA sequence analysis.

Enzyme Digest		*Escherichia coli*	*Lactobacillus murinus*	*Bacillus licheniformis*
	Predicted TRF	168	209	272
*Hae*III	Observed TRF	167	207	269
	discrepancy	1	2	3
	Predicted TRF	235	281	266
*Sau*3aI	Observed TRF	234	278	262
	discrepancy	1	3	4
	Predicted TRF	335	218	203
*Hha*I	Observed TRF	334	215	200
	discrepancy	1	3	3
	Predicted TRF	389	513	419
*Rsa*I	Observed TRF	385	507	414
	discrepancy	4	6	5
	Predicted TRF	458	513	107
MspII	Observed TRF	451	507	102
	discrepancy	7	6	5

Advantages of the TRF Method

Even with the cautions and advisories covered above, the ease of obtaining robust TRF data is a significant advantage over other community profiling methods. With the automated systems available in many laboratories, hundreds of reproducible TRF patterns can be generated in under a week. This means it is possible to determine spatial and temporal shifts in community structures with some confidence. High through put allows a researcher to replicate samples and allows statistical precision that might otherwise not be possible.

In addition to the rapid production of data, the output form of the data allows for rapid analysis of large amounts of information. Fragment analysis software exists for most DNA sequencing machinery and this software will automatically digitize the electrophoresis output and export a tab delimited text file that can be loaded into standard statistical or spreadsheet packages. This sidesteps the time consuming step of manually digitizing the electrophoresis images generated with other methodologies. The combination of rapid data collection, instant digitization and simplified statistical analysis means that TRF patterns can be used to monitor microbial community dynamics on a scale and with a resolution that has never been achievable before.

The data produced by TRF patterns from 16S rRNA amplicons can also be used to search databases for matching sequences that might identify individual organisms in the community profile. Creating more than one enzyme-derived TRF pattern with the same samples can enhance resolution of the database match.

Last and perhaps most important, the electrophoresis systems used to generate TRF patterns are used for DNA sequencing and are therefore of much greater precision and resolution than what is used for almost any other community profiling method (Lüdeman *et al.,* 2000; Marsh *et al.,* 1999). This high definition system allows for comparisons of TRF patterns between gels while comparisons between profiles generated with DGGE for example are best made on the same gel because of the lower resolution inherent to the electrophoresis system.

Disadvantages of the TRF Method

The use and interpretation of TRF patterns is limited by the biases inherent to any DNA and PCR based investigation of environmental samples. DNA extraction, PCR and electrophoresis all have the ability to introduce artifacts and bias into TRF patterns. However this holds true for almost all the other community analysis methods in current use and so cannot be considered a disadvantage specific to TRF patterns.

Identifying the organisms responsible for a particular element in a profile is not simple for TRF patterns as compared to the possibilities offered by DGGE. While DGGE allows for southern blotting or direct cloning of profile elements, TRF patterns are destructively sampled and the DNA cannot be reclaimed. Database matching of TRF sizes is imprecise and may not produce species or even genera specific identification. Multiple enzyme digests can be correlated to produce more accurate identification from database matches though this requires collecting more data and employing more extensive analyses. In addition, if an existing sequence database is not complete some TRF peaks in a pattern might not be represented in the database. This is particularly true for target sequences other than the 16S rRNA gene. Most investigators have circumvented these shortcomings by simultaneously creating clone libraries that can be analyzed by TRF analysis and then sequenced for identification. This has the added advantage that larger DNA fragments than are commonly used in DGGE can be sequenced but it can also create difficult searches for low abundance TRF peaks.

Conclusions

Analysis of TRF patterns provides a rapid and reproducible way to compare microbial communities and assess community dynamics. Some care must be taken when preparing the data for analysis to minimize artifacts. The identification of specific elements in a TRF pattern is more difficult than with DGGE, although several methods exist for resolving this problem. TRF pattern data has the advantage of being simply and rapidly produced on existing and increasingly standard DNA sequencing equipment. TRF pattern data is automatically digitized and lends itself to easy analysis with a variety of multivariate statistical techniques. As the literature is showing, TRF pattern data allow for unprecedented opportunities to correlate the structure and diversity of microbial communities to the physico-chemical parameters of their surroundings.

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