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MINI-REVIEW

Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities

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Abstract Terminal restriction fragment length polymorphism (T-RFLP) analysis is a popular high-throughput fingerprinting technique used to monitor changes in the structure and composition of microbial communities. This approach is widely used because it offers a compromise between the information gained and labor intensity. In this review, we discuss the progress made in T-RFLP analysis of 16S rRNA genes and functional genes over the last 10 years and evaluate the performance of this technique when used in conjunction with different statistical methods. Web-based tools designed to perform virtual polymerase chain reaction and restriction enzyme digests greatly facilitate the choice of primers and restriction enzymes for T-RFLP analysis. Significant improvements have also been made in the statistical analysis of T-RFLP profiles such as the introduction of objective procedures to distinguish between signal and noise, the alignment of T-RFLP peaks between profiles, and the use of multivariate statistical methods to detect changes in the structure and composition of microbial communities due to spatial and temporal variation or treatment effects. The progress made in T-RFLP analysis of 16S rRNA and genes allows researchers to make methodological and statistical choices appropriate for the hypotheses of their studies.

Keywords T-RFLP · Microbial communities · 16S rRNA genes · Multivariate statistics

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Introduction

Several cultivation-independent methods lend themselves to the analysis of large numbers of samples and offer facile means to detect differences in the composition and structure of microbial communities. These methods include fingerprinting techniques based on 16S rRNA genes such as denaturing gradient gel electrophoresis (Muyzer et al. 1993), automated ribosomal intergenic spacer analysis (Fisher and Triplett 1999), and terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997). These fingerprinting techniques have been successfully used in numerous studies to explore microbial diversity of the predominant populations in various habitats and offer the advantage that they are more amenable to high throughput and more comprehensive than cultivation-dependent methods (Rappe and Giovannoni 2003; Torsvik et al. 2002; Weng et al. 2006).

T-RFLP analysis is one of the most frequently used high-throughput fingerprinting methods. Because of its relative simplicity, T-RFLP analysis has been applied to the analysis of fungal ribosomal genes (Genney et al. 2006; Johnson et al. 2004; Kennedy et al. 2005; Lord et al. 2002), bacterial 16S rRNA genes (Hullar et al. 2006; Katsivela et al. 2005; Noll et al. 2005; Pérez-Piqueres et al. 2006; Rasche et al. 2006; Schmidt et al. 2006; Sessitsch et al. 2001; Thies et al. 2007), and archaeal 16S rRNA genes (Kotsyurbenko et al. 2004; Leybo et al. 2006; Lu et al. 2005; Moeseneder et al. 2001; Ramakrishnan et al. 2000; Weber et al. 2001; Wu et al. 2006). In addition, T-RFLP has been used for the analysis of functional genes (Horz et al. 2000; Lueders and Friedrich 2003; Mintie et al. 2003; Pérez-Jiménez and Kerkhof 2005; Rich et al. 2003) such as those encoding for nitrogen fixation (Rösch and Bothe 2004; Tan et al. 2003) and methane oxidation (Horz et al. 2001; Mohanty et al. 2006). However, most frequently, the technique is used to amplify small subunit (16S or 18S) rRNA genes from total community DNA using polymerase chain reaction (PCR) wherein one or both of the primers used are labeled with a fluorescent dye. The resulting mixture of rRNA gene amplicons is then digested with one or more restriction enzymes that have four base-pair recognition sites, and the sizes and relative abundances of the fluorescently labeled T-RFs are determined using an automated DNA sequencer. Since differences in the sizes of T-RFs reflect differences in the sequences of 16S rRNA genes (i.e., sequence polymorphisms), phylogenetically distinct populations of organisms can be resolved. Thus, the pattern of T-RFs is a composite of DNA fragments with unique lengths that reflects the composition of the numerically dominant populations in the community. While T-RFLP shares problems inherent to any PCR-based method (Acinas et al. 2005; Becker et al. 2000; Crosby and Criddle 2003; Kanagawa 2003; Kurata et al. 2004; Lueders and Friedrich 2003; Polz and Cavanaugh 1998; Qiu et al. 2001; Reysenbach et al. 1992; Suzuki and Giovannoni 1996; Terahara et al. 2004; von Wintzingerode et al. 1997; Wang and Wang 1997), it has been shown to provide a facile means to assess changes in microbial community structure on temporal and spatial scales by monitoring the gain or loss of specific fragments from the profiles (Franklin and Mills 2003; Lukow et al. 2000; Mummey and Stahl 2003). When coupled with 16S rRNA clone library construction and clone sequencing, additional specific information on the composition of microbial communities can be obtained.

In this review, we evaluate the progress made in T-RFLP analysis and the associated statistical methods over the last 10 years since the method was introduced (Liu et al. 1997). We examine methods used to choose primers and restriction enzymes, distinguish between signal and noise, align T-RFLP profiles, identify plausible members, and monitor changes in microbial communities (Fig. 1). Other reviews have compared T-RFLP analysis to other fingerprinting techniques, evaluated the reproducibility of T-RFLP profiles, discussed some limitations and biases of the method (Anderson and Cairney 2004; Avis et al. 2006; Blackwood et al. 2007; Bent and Forney 2008; Clement et al. 1998; Dorigo et al. 2005; Egert and Friedrich 2003, 2005; Hartmann et al. 2007; Kitts 2001; Leckie 2005; Marsh 1999, 2005; Osborn et al. 2000; Rösch et al. 2006; Singh et al. 2006a; Smalla et al. 2007; Thies et al. 2001; Thies 2007), and reviewed multivariate methods to analyze molecular fingerprints (Ramette 2007).

Advances in T-RFLP methodology

Choice of primers

Ideally, primers chosen for T-RFLP analysis should be specific to the targeted taxonomic group yet sufficiently general so that they can amplify all bacterial populations that are of interest. There are no known primers that satisfy both of these criteria. For example, an *in silico* analysis of sequences done using the Probe Match tool of the Ribosomal Database Project (RDP) shows that the bacterial primer 8fm potentially amplifies only 76–98% of the bacterial 16S rRNA gene sequences in the RDP database (Table 1; Marsh et al. 2000). Worse still, this analysis does not take into account that sequence databases only contain a fraction of the extant bacterial diversity, which suggests that commonly used primers such as 8fm are far from universal. In addition, the 8fm primer is not 100% specific to bacteria because the primer also matches 19 16S rRNA archaeal genes of the 19,300 archaeal 16S rRNA gene sequences that were in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) as of June 2007. Although there is no perfect primer, tools such as the primer-prevalence tool (Shyu et al. 2007) on the Microbial Community Analysis (MiCA) website (http://mica.ibest.uidaho.edu/) and the Probe Match tool (Marsh et al. 2000) found on the Ribosomal Database Project website (http://rdp.cme.msu.edu/) facilitate the choosing of primers because they allow researchers to compare the specificity and selectivity of different primer sets based on sequences in the database. A limitation is that neither tool is equipped for the analysis of archaeal sequences.

The use of only one fluorescently labeled primer may result in underestimating the microbial diversity in a sample because different bacterial populations can share the same terminal restriction fragment length for a particular primer–enzyme combination (Marsh et al. 2000). This problem is reduced if two labeled primers are used; the premise being that some populations that cannot be resolved with one

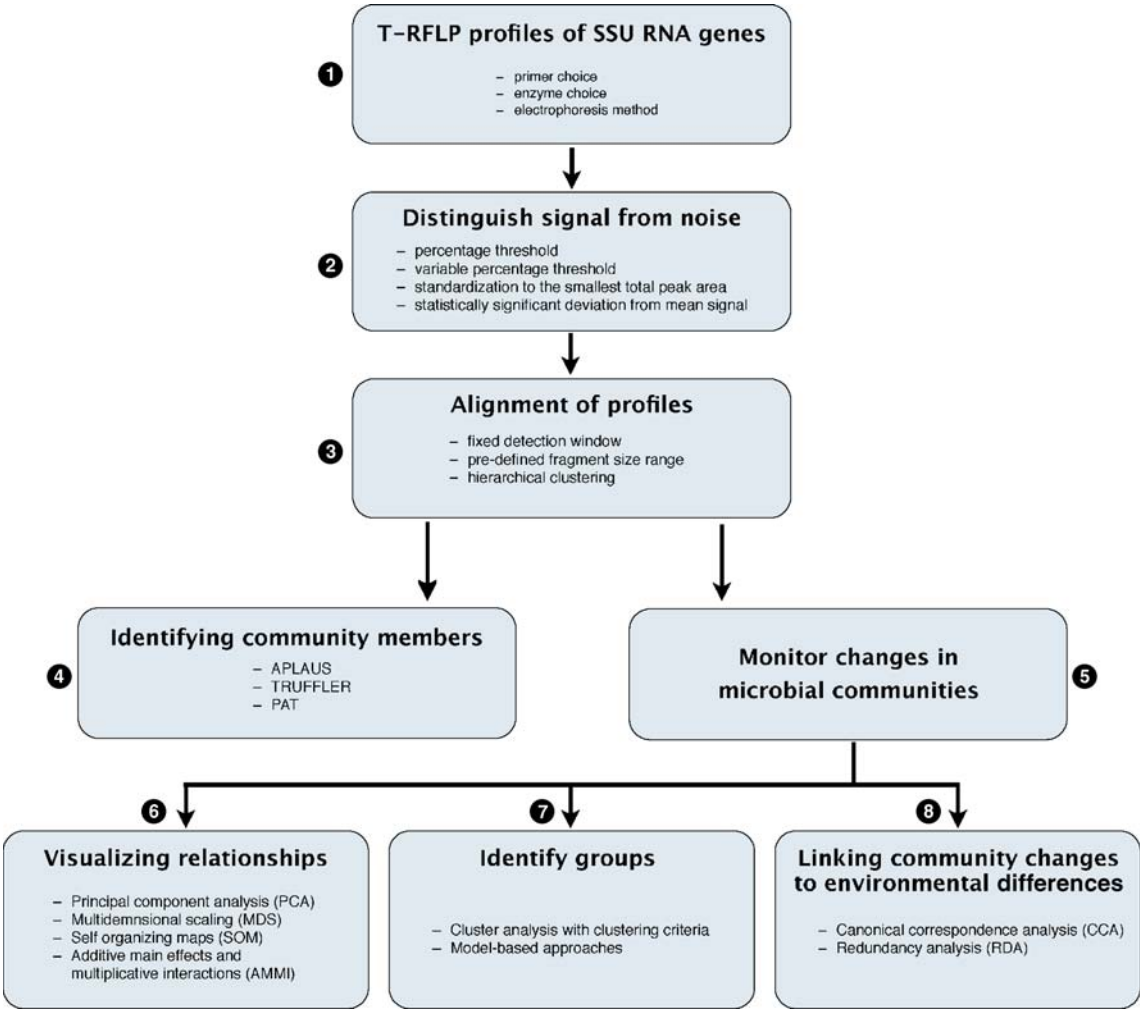

Fig. 1 Steps in the analysis of microbial community composition based on terminal restriction site length polymorphism analysis of 16S and 18S rRNA genes. Choose the primers, restriction enzymes, and electrophoresis method to be used to resolve fluorescently labeled DNA fragments (Step 1). Once the electropherograms have been obtained, the signals (*peaks*) are distinguished from baseline noise using one of four methods: (a) fixed percentage threshold, (b) variable percentage threshold, (c) iterative process of standardizing the profiles in respect to the smallest total peak height, or (d) an iterative process that defines signals as being three standard deviations from a mean of zero (Step 2). Finally, the fragment profiles are aligned by: (a) using a fixed detection window, (b) a defined fragment size range, or (c) a hierarchical clustering procedure (Step 3). Which steps are followed next depends on the objectives of the study. If the objective is to identify and classify microbial community members in individual samples, then (Step 4) web-based tools such as PAT, TRUFFLER, and APLAUS can be used. If the objective, however, is to assess differences in microbial communities (Step 5), PCA, MDS, SOM, AMMI, which are described in the text, can be used to visualize these differences (Step 6), while significant groups can be identified through cluster analysis using clustering criteria such as cubic clustering criterion, pseudo *F*-test, and model-based procedures (Step 7), or the observed differences can be linked to variation in the environments using CCA and RDA (Step 8)

primer might be distinguished on the basis of additional information provided from the terminal fragment generated by a second labeled primer (Liu et al. 1997). The resolution of populations can be enhanced even further through the use of three or more labeled primers. Zhou et al. (2007), for example, amplified 16S rRNA genes of human vaginal communities by amplifying 16S rRNA genes in two separate PCR reactions that employed two differentially labeled forward primers in combination with the same reverse primer. After digestion, the restriction products were combined prior to analysis.

Multiple primers can also be used in the same PCR reaction to study communities that contain different taxa, and this is referred to as multiplexing. For example, Singh et al. (2006b) analyzed soil microbial communities using three different primer sets that targeted bacteria, archaea, and fungi (Fig. 2). The authors evaluated the performance of this multiplexing by comparing T-RFLP profiles of

Table 1 Prevalence of primer target sequences in the Ribosomal Database Project II release 9 database

Primer[a]	Primer sequence (5′-3′)	No. sequences in database with target sequence	No. sequences (0 mismatches)	Fraction of total sequences	No. sequences (2 mismatches)	Fraction of total sequences
8F-Eub	AGAGTTTGATCCTGGCTCAG	74495	46389	0.62	71996	0.97
8fm-Eub	AGAGTTTGATCMTGGCTCAG	74495	56498	0.76	72634	0.98
49F-Eub	TNANACATGCAAGTCGRRCG	192557	136297	0.71	188399	0.98
334F-Eub	CCAGACTCCTACGGGAGGCAGGC	268238	19	7.08 E-05	246469	0.92
341F-Eub	CCTACGGGAGGCAGCAG	272435	244267	0.9	266479	0.98
519F-Univ	CAGCAGCCGCGGTAATAC	264039	222076	0.84	254083	0.96
786F-Eub	GATTAGATACCCTGGTAG	238996	188904	0.79	227388	0.95
536R-Eub	GWATTACCGCGGCKGCTG	268202	222455	0.83	252867	0.94
926R-Eub	CCGTCAATTCCTTTRAGTTT	199516	147271	0.74	190894	0.96
1113R-Eub	GGGTTGCGCTCGTTG	179777	137433	0.76	174470	0.97
1404R-Eub	GGGCGGWGTGTACAAGGC	127580	76505	0.6	121241	0.95
1406R-Univ	ACGGGCGGTGTGTRC	125548	108930	0.87	124112	0.99
1511R-Eub	GYTACCTTGTTACGACTT	44577	39399	0.88	43970	0.99

[a] Primer numbering is based on *Escherichia coli* 16S rRNA gene.

separate PCR reactions that used taxon-specific sets of primers to profiles based on multiplex PCR containing all three primer sets (Fig. 3). In addition, they investigated the effect of pooling individual PCR products before digestion with restriction enzymes. The results showed that profiles resulting from individual reactions, multiplexing, and pooled PCR products were consistent with one another in respect to the number, position, and relative intensity of peaks that represent the different microbes in samples.

Choice of restriction enzymes

The discrimination of bacterial populations by T-RFLP analysis relies on detecting 16S rRNA gene sequence polymorphisms using restriction enzymes (Liu et al. 1997). Typically, enzymes that have four base-pair recognition sites are used due to the higher frequency of these recognition sites. It has been shown by several groups that the use of more than one restriction enzyme facilitates the resolution of bacterial populations (Liu et al. 1997; Marsh 1999). This is due to the fact that different bacterial populations can share the same terminal restriction fragment length for a particular primer–enzyme combination but not others (Marsh et al. 2000).

The ability of different restriction enzymes to resolve unique sequences has been examined in studies of gene sequence databases, communities with different richness, and iterative random sampling from a derived database of T-RFs (Engebretson and Moyer 2003; Moyer et al. 1996). Engebretson and Moyer (2003) evaluated 18 restriction enzymes and found that *Bst*UI, *Dde*I, *Sau*961, and *Msp*I most often resolved individual populations in their model communities. For communities with more than 50 operational taxonomic units (OTUs), none of the restriction enzymes resolved more than 70% of the total OTUs. The authors concluded that T-RFLP can most efficiently be used for communities with low or intermediate richness.

In silico digestion to evaluate the ability of restriction enzymes to discriminate between sequences can be done using tools such as the T-RFLP analysis program (TAP) T-RFLP (http://rdp8.cme.msu.edu/html/TAP-trflp.html) and MiCA (http://mica.ibest.uidaho.edu/) for 16S rRNA genes and the ARB implemented tool TRF-CUT for functional genes (http://www.mpi-marburg.mpg.de/downloads/, Ricke et al. 2005). TAP T-RFLP is located on the RDP website, and it facilitates the choice of restriction enzymes by an *in silico* digest of all 16S rRNA genes in the database while using different primer–enzyme combinations (Marsh et al. 2000). TAP T-RFLP matches a chosen forward or reverse primer to every sequence of the database, and all the sequences that match the primer are digested *in silico* by the chosen restriction enzyme(s). The analysis provides answers to the following questions: (i) what enzyme(s) best discriminate phylotypes for estimates of population diversity, (ii) what enzyme(s) provides the best resolution of the targeted phylogenetic groups, and (iii) what primer–enzyme (s) combinations are best for a particular data set? The default output shows the results within RDP's phylogenetic hierarchy. In addition, the results can be ordered by sequence name or terminal fragment size. While TAP T-RFLP is a powerful tool to acquire a first impression of how well different restriction enzymes can resolve phylotypes, it also has certain limitations. It only allows one primer–enzyme combination to be specified, the data cannot be sorted, and the output cannot be printed or exported to other programs (Shyu et al. 2007). In contrast, MiCA allows the user to specify both forward and reverse primers, the number of mismatches between a primer and

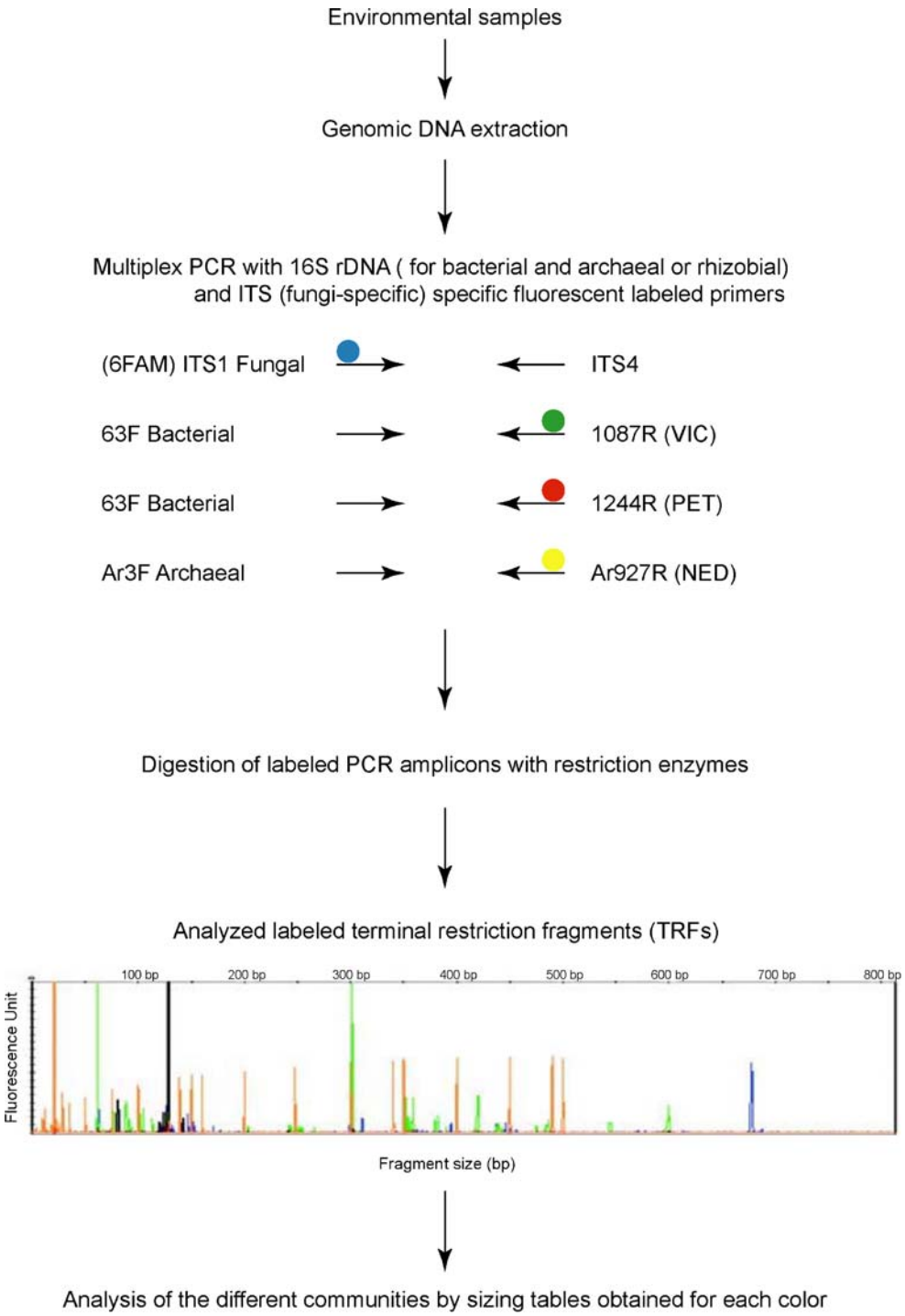

Fig. 2 Outline of the procedure for multiplex TRFLP (M-TRFLP) analysis. Primers for different taxa were labeled with different fluorophores which are depicted using *dots of different colors*. The fluorophores used in this study were 6-carboxyfluorescein (FAM; *blue*), VIC (*green*), NED (*yellow*), and PET (*red*; reprinted with permission from the American Society of Microbiology and based on Singh et al. 2006b)

the target sequences, choice of up to three restriction enzymes, and choice of the database to be used. The tool uses a query program that connects to the database, and it analyzes the data based on the parameters specified. The output is written in PHP, plain text, or comma separated value format. The drawback of these outputs is that they can be very large and difficult to interpret. An alternative is Restriction Endonuclease Picker (REPK; http://rocaplab.ocean.washington.edu/tools/repk), a program that automatically determines sets of restriction enzymes that differentiate user-designated sequences (Collins and Rocap 2007). The user input is a trimmed FASTA-formatted file that contains the sequences to be used for *in silico* analysis. After uploading the FASTA file, restriction enzymes can be selected from a list based on the latest REBASE database, or they can be defined by the user. Settings such as the minimum and maximum terminal fragment length allowed and the minimum threshold for the number of groups each enzyme must be able to distinguish

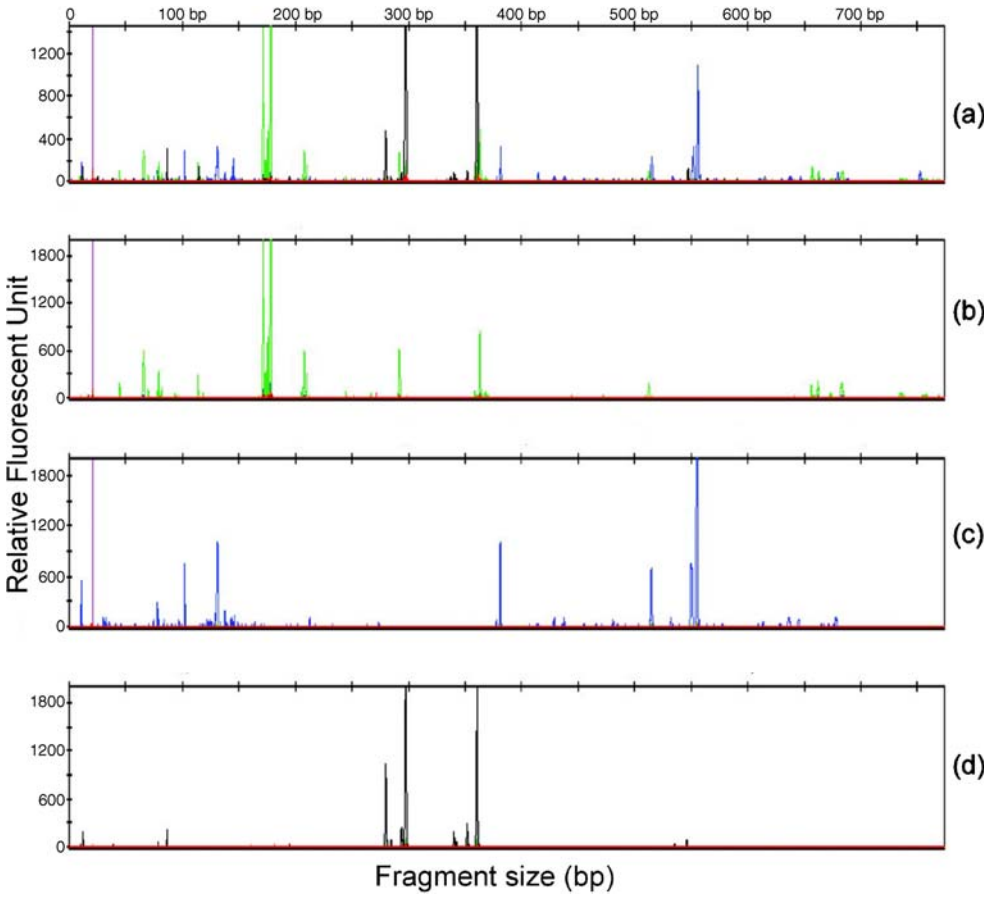

Fig. 3 Profiles obtained for archaeal, bacterial, and fungal communities by M-TRFLP and individual TRFLP analyses for a single sample. **a** Profile generated by M-TRFLP analysis for bacterial (*green*), fungal (*blue*), and archaeal (*yellow but appears black* on GeneMapper) communities together using multiplex PCR. TRFLP profiles generated for the same sample from PCR products obtained using only **b** bacterium-specific, **c** fungus-specific, and **d** archaeon-specific primers in separate PCR reactions (reprinted with permission from the American Society of Microbiology from Singh et al. 2006b)

(referred as the enzyme stringency filter) can be specified. The selected sequences are digested in both directions using all the restriction enzymes chosen. The sizes of the terminal fragments are determined and a matrix is generated. Terminal fragment sizes that are within a specified cutoff are 'binned' and considered the same size. Next, the program determines whether bins contain only sequences from a single bacterial group or sequences from different groups, meaning the enzymes chosen failed to discriminate between bacterial groups. The enzymes that do a sufficiently good job of differentiating among bacterial groups are identified using the enzyme stringency filter and saved in a final output. Collins and Rocap (2007) state that REPK is particularly useful if members of a certain taxonomic group must be distinguished or if populations found in a previously characterized habitat must be differentiated.

Although web-based tools and studies based on *in silico* experiments provide insight into the ability of restriction enzymes to resolve bacterial groups, the output from these tools should be used with caution. Investigators must remember that only a small fraction of the total bacterial diversity has been described and sequence databases are incomplete. Consequently, it is likely and even probable that samples will contain phylotypes that are not represented in any database. We recommend that primer–enzyme combinations chosen based on *in silico* analyses should be empirically evaluated to confirm that they best resolve the constituent populations in the samples to be analyzed.

Resolution of terminal fragments

Differences in the length and abundance of fluorescently labeled T-RFs in a sample are usually determined by capillary or polyacrylamide gel electrophoresis wherein the electrophoretic mobility of the T-RFs are compared to those of known size in an internal standard. The actual sizes of T-RFs are estimated by interpolation using algorithms such as the Local Southern algorithm that are available in software packages such as GeneScan and GeneMapper. The abundance of each T-RF is determined based on fluorescence intensity and expressed as either peak height or peak area. Generally speaking, T-RFLP analysis using capillary gel electrophoresis is more precise and reproducible than analyses done using polyacrylamide gels (Behr et al. 1999). Even so, run-to-run variability (generally ±1 bp) results in small size discrepancies even among terminal fragments of the same bacterial populations and therefore fingerprints need to be aligned. Fragment sizes are generally assigned to categories of operational taxonomic units or "bins". Each bin may actually include more than one phylotype depend-

ing on the species complexity of the community being analyzed, the phylogenetic relatedness of the populations present, and the resolving 'power' of the primers and enzymes used. A disadvantage of capillary systems is that they employ electrokinetic sample injection, in which charged molecules are injected into capillaries by applying an electric field (Behr et al. 1999). This can result in the preferential injection of smaller molecules, so salts and primers from the PCR reactions (Osborne et al. 2005; Tiquia et al. 2005) or the digestion reactions (Berg et al. 2005; Hoshino et al. 2006) should be removed prior to sample analysis.

Accurate fragment size determination is important, especially if the goal is to infer a plausible community composition from T-RFLP profiles. Plausible community compositions are determined using web-based tools, in which the sizes of T-RFs in a profile are matched with T-RF sizes derived *in silico* from the 16S rRNA gene sequences of phylotypes in a database. Sometimes, however, this is not straightforward because pseudo T-RFs (partially single-stranded amplicons) may be formed during PCR (Egert and Friedrich 2003, 2005) and different fluorophores can affect the electrophoretic mobility of fragments in different ways causing errors in determining fragment sizes (Tu et al. 1998). Although DNA fragment analysis by capillary electrophoresis is very precise (±1 bp), it is not necessarily accurate, a fact that is not widely known. In our experience, DNA fragments labeled with a fluorescein dye, such as 6FAM and HEX, migrate faster than DNA fragments labeled with a rhodamine dye such as ROX. The latter one is often used to label internal size standards. As a result, the sizes of terminal fragments labeled with HEX or 6FAM can be underestimated. Unfortunately, it is not easy to adjust for differences in migration behavior because the magnitude of the discrepancy is not constant across fragment sizes. For fragment sizes smaller than 100 bp, it is up to 11 bp (Hahn et al. 2001), it decreases to 2–3 bp for fragment sizes of about 500 bp, and then increases again for fragment sizes larger than about 700 bp (Shyu et al. unpublished data). Discrepancies between true and observed T-RF sizes can also be caused by the purine content (Kaplan and Kitts 2003). Furthermore, the performance of the algorithms used to size call the T-RFs deteriorates as the DNA fragment size increases. The commonly used Local Southern algorithm assumes that the migration time of fragments increases linearly with fragment size, but this is not true, and so the size calling of larger DNA fragments is more likely to be erroneous (Shyu et al. unpublished data). Currently, there is no solution to correct for these migration discrepancies that arise due to the use of different fluorophores, and users should take this into account when using T-RFLP data to determine the community composition.

Distinguishing signal from noise

As a first step in the analysis of T-RFLP profiles, the signal has to be distinguished from electronic noise. Differently said, the baseline has to be determined. Programs such as GeneScan and GeneMapper determine where a peak starts and ends, its height, and area, but the true baseline must be determined by the researcher. Ideally, the procedure used to distinguish signal from noise would be an automated objective approach that determines the true signal in each profile since the electronic noise varies from run to run. Either peak height or area can be used to distinguish signal from noise, and both have advantages and disadvantages (Kitts 2001; Lueders and Friedrich 2003). Several approaches to define baselines have been developed including fixed threshold (Lueders and Friedrich 2003; Osborn et al. 2000), proportional threshold (Dunbar et al. 2001; Osborne et al. 2006; Sait et al. 2003), and statistical determination of the threshold (Abdo et al. 2006).

The simplest approach to distinguish signal from noise is to impose a fixed detection threshold that is some arbitrarily chosen value, e.g., 50 or 100 fluorescence units (FU). Employing a high detection threshold such as 100 FU (Osborn et al. 2000) insures that the number of peaks attributable to noise is very low, but risks excluding small reproducible peaks (Dunbar et al. 2001). In addition, setting a fixed detection threshold assumes that profiles of samples are not subject to experimental variation that results from loading and detection efficiency. Dunbar et al. (2001) have shown that threshold cannot be arbitrarily set beforehand because the optimum threshold varies from sample to sample. Because the assumptions inherent in using a fixed detection threshold do not hold and because better approaches are available, we do not consider it to be a valid approach.

A more sophisticated approach is to use a constant *percentage* threshold (Sait et al. 2003). To do this, a matrix of all T-RFs and their peak areas that are present in sample profiles from a given study is generated. If a T-RF is not present in a particular profile, an area of zero is assigned. The dataset is then standardized by computing the proportion of the total area for each peak in a profile. To determine the baseline, a percentage threshold is chosen in such a way that the correlation between total peak area and number of peaks is minimized. The reasoning behind trying to minimize the correlation between number of peaks and total peak area is to control for differences due to variation in the amount of DNA injected. A strong correlation between the total peak area and number of peaks suggests that the larger number of peaks above the threshold results from a higher amount of DNA injected and not because of an increased richness of numerically abundant populations in a sample.

Dunbar et al. (2001) introduced a method that also addresses the problem that injecting different amounts of DNA may affect the apparent number and relative abundance of phylotypes represented in a T-RFLP profile. The method uses the profile with the smallest total peak height as a basis for normalizing the total peak heights of all other profiles. To accomplish this, the total peak height of each profile is calculated including only peaks with a height larger than 25 fluorescent units. The smallest total peak height for any sample in the dataset is divided by the total peak height for each remaining sample to produce a correction factor for each profile. This correction factor is used to adjust the peak height of the profile, and by doing so, adjust for differences on the amount of DNA injected. For example, assuming that 20,000 FU is the smallest total peak height, a profile with a total peak height of 40,000 FU would have a correction factor of 0.5. Accordingly, each peak height of the latter profile would be multiplied by 0.5. After this correction, some peaks will fall below the threshold of 25 FU and are eliminated. A new total peak height is then calculated for each profile and the process is repeated until each of them equals the smallest total peak height. The authors indicate that using a threshold of 25 FU fails to eliminate all noise from the data, and this affects further statistical comparisons. Therefore, only peaks that are present in all replicates of a sample after standardization were included in further comparisons. This approach is well thought through although the constant baseline value (25 FU) is subjectively chosen.

Electronic noise varies among profiles, so using the same percentage threshold to eliminate noise in all profiles is not appropriate. Therefore, Osborne et al. (2006) proposed the use of a variable percentage threshold to address this issue. As in Sait et al. (2003), the method of Osborne et al. (2006) attempts to minimize the correlation between total peak area and the number of peaks. In this method, a percentage threshold is individually determined for each profile so that the weakest relationship between the number of peaks and the total peak area results. Osborne et al. (2006) compared their method to the two procedures developed by Sait et al. (2003) and Dunbar et al. (2001) and concluded that their method was better able to distinguish signal and noise because replicate profiles were grouped most precisely.

A new statistical method has been developed to provide signal/noise discrimination based on statistical theory (Abdo et al. 2006). Using this procedure, the data are standardized by dividing the area of each peak by the total peak area of that particular sample. The standard deviation of the dataset is then computed assuming that the true mean is zero. Peaks with a relative area larger than three standard deviations from the mean are identified as true signal and are removed. This process is reiterated until no more 'true' peaks are identified. This method indirectly accounts for the variation in the injected DNA through profile standardization, which results in reducing some of the smaller peaks such that they are indistinguishable from noise. Still, this method can be more sensitive to identifying smaller peaks than the other described methods, which may result in slight differences in the observed richness of phylotypes in profiles. Although these differences have minimal impact on analyses using Euclidian distances based on peak area or height, analyses based on presence/absence matrices can amplify the effect of such richness differences and might produce inaccurate results.

Alignment of profiles

Run-to-run variability in T-RFLP analysis causes slight differences in the estimated sizes of T-RF fragments from the same bacterial phylotype. Because of this run-to-run variability, fingerprints need to be aligned before further statistical analyses can be done. The methods used to assign fragment sizes to length categories (bins) include nearest integer rounding, manual binning (Blackwood et al. 2003), and clustering-based statistical approaches (Abdo et al. 2006; Dunbar et al. 2001; Hewson and Fuhrman 2006; Ruan et al. 2006). The use of statistical approaches is far superior to rounding to the nearest integer or manual binning because so long as parameter choices are made based on empirical data and applied consistently, the automated procedures allow an objective analysis of large data sets with statistical justification.

The rounding method is the simplest method. The estimated fragment size is rounded to the nearest integer and each integer is treated as a bin. This method suffers from the limitation that the reproducibility of DNA fragment analysis by capillary electrophoresis is ±0.5 bp (Dunbar et al. 2001) so identical fragments in separate runs could be placed in different bins. For example, a fragment of 100.4 bp in one profile would be placed into a 100 bp bin, while the same fragment in a different profile could be measured to be of 100.6 bp, which would be rounded to the 101 bp and placed in a different bin. Since the difference between the two fragment sizes is just 0.2 bp, they probably should be grouped into a single category. Because of this problem, the rounding method is not an appropriate way to align T-RFLP profiles.

In contrast to rounding to the nearest integer, manual binning allows an experienced analyst to make intelligent choices in ambiguous cases. Manual binning introduces the possibility of subjective bias, and it has no high throughput capability; therefore, it is not recommended.

Hewson and Fuhrman (2006) describe a binning technique wherein profiles are aligned based on multiple bin windows of a fixed size. In their example, Hewson and Fuhrman used a bin window of 10 bp. If one excludes

fragment sizes smaller than 50 bp due to primer dimer peaks, then the first bin would contain all fragments of 50–59 bp, the second would contain all fragments with a size of 60–69 bp, and so on. The alignment is done several times, and each time the starting point of the window is shifted by 1 bp. In the second round of alignment, all fragment lengths from 51–60 bp, 61–70 bp, etc., are considered identical. The number of times the alignment is done equals to the size of the bin window, and in this example, it would be ten times (It should be noted that this method was developed for the analysis of data from fragment analysis using polyacrylamide gels and not capillary gel electrophoresis. This results in a larger run-to-run variability of fragment sizes; thus, large bin windows were proposed). For each frame, all pairwise similarities among the profiles are calculated, an unweighted pair-group method with arithmetic mean (UPGMA) tree is generated based on the maximum pairwise similarities among profiles, and this tree is used to draw conclusions about differences in microbial community. The performance of the binning method has only been evaluated based on samples with unknown community structure, and therefore, the ability of this approach to determine true differences in microbial communities remains unknown. An extension of this binning method has been developed by Ruan et al. (2006) to allow different bin window sizes depending on the fragment size because the reproducibility of fragment length measurements varies with fragment size (Brown et al. 2005).

The alignment of profiles based on a defined fragment size range, for example ±0.5 bp, was introduced by Dunbar et al. (2001). Because this analysis was done using capillary gel electrophoresis, all fragment lengths that differed less than 0.5 bp were considered identical and binned. However, as discussed above, a potential drawback to this approach is that in some cases, peaks with a size difference less than 0.5 bp can reproducibly occur in replicate profiles of the same sample. To address this problem, Dunbar et al. (2001) limited the maximum number of peaks that can be assigned to one bin to the number of profiles being aligned. This method is preferable to the method developed by Hewson and Fuhrman (2006) because by Dunbar's method, the decision as to whether two fragments are binned is based on the differences in the sizes of two fragments and not whether they fall within a preset fixed bin window.

Abdo et al. (2006) implemented hierarchical clustering to align T-RFLP profiles. This method is similar to the one described by Dunbar et al. (2001) though there is no limit to the number of fragments that can be in a bin. First, all fragment lengths from all fingerprints of interest are pooled, sorted, and duplicate fragment sizes are removed. Then hierarchical clustering using average linkage (UPGMA) is performed to identify fragments with lengths close enough to being binned (e.g., within a radius of ±1 bp). The clustering procedure starts by selecting the two fragments that have the smallest difference in size. These two fragments are grouped and form a bin that is represented by their average fragment size. This procedure continues to group fragments into bins until no more fragments or bins that have a size difference less than the defined radius. If fragments within a sample are binned together, their areas are summed and treated as a single peak. A benefit of this procedure is that the validity of this method was tested based on defined simulated data that were generated to mimic real data.

Identifying populations in microbial communities

There are several web-based tools available that allow users to identify plausible members of microbial communities based on T-RFLP data (Kent et al. 2003; Shyu et al. 2007; Wise and Osborn 2001). The tools differ as to whether they automatically combine the results of profiles from several restriction digests, account for both forward and reverse primer, account for the relative abundances of fragments, and allow for mismatches between primer sequence and templates. Allowing for mismatches is important because sequences that have a small number of mismatches with the primer sequence may still amplify in PCR reactions. What follows are short descriptions of some web-based tools that can be used to identify plausible members of a microbial community based on T-RFLP data.

A phylogenetic assignment tool (PAT) developed by Kent et al. (2003) uses a default database produced by using MiCA that contains the T-RFs predicted from an *in silico* analysis of 16S rRNA sequences using the bacterial 16S rRNA gene primer 8F and one of several restriction enzymes with tetrameric recognition sites. Custom-generated databases can be used as well. The T-RFs predicted from when a given primer–enzyme combination is used to match the empirically determined fragment lengths to those predicted from various phylotypes in the database. The ability of PAT to correctly determine community composition is increased by the analysis of multiple digests because species that are not resolved by a given restriction enzyme may be resolved by a different enzyme. The resolution of PAT could be further enhanced if T-RF sizes could be predicted from both the 5′ and 3′ ends of amplified fragments, but this is not a feature of the program. The method was validated by using T-RFLP in conjunction with sequencing cloned 16S rRNA genes to assess the variability of bacterial community structure throughout the water column of a humic lake. The taxonomic classes identified by T-RFLP and clone library analysis matched well. TRUFFLER is a program similar to PAT except sequences are retrieved from the European Molecular Biology Laboratory DNA database and mis-

matches between primer sequences and templates are allowed. The authors reported that there were discrepancies between predicted and actual fragment sizes (1–2 bp) but that overall, there was a good level of agreement (Wise and Osborn 2001). APLAUS is an algorithm that allows the definition of a bin size to address the problem of size calling errors caused by factors such as differences in migration behavior of the different fluorophores (Shyu et al. 2007). As with PAT, it allows the comparison of one or more T-RFLP profiles to the outcome of an *in silico* analysis of the database with the same primer–enzyme combination as used in the experiment (Shyu et al. 2007). An important difference is that data from the analysis of samples with multiple primer–enzyme combinations can be evaluated simultaneously, which narrows the possibilities for members of the community.

Although these tools can be used to gain insight to the possible members of microbial communities, there are several caveats one should be cognizant of when implementing them. First, DNA fragments labeled with different fluorophores may differ in electrophoretic mobility (Tu et al. 1998). Second, electrophoretic mobility can be influenced by sequence composition (Kaplan and Kitts 2003). Both of these will introduce discrepancies between the empirically determined and actual fragment sizes. Third, only a small percentage of 16S rRNA gene sequences are now archived in databases. Therefore, misidentification may result from the fact that known sequences in the database have the same sequence polymorphisms as novel and unknown sequences in the sample (Blackwood and Buyer, 2007). Even with these limitations, there are certain cases in which data from T-RFLP analysis of microbial communities can be used in conjunction with web-based tools to an advantage. For example, it is possible to presumptively identify and monitor specific bacterial populations within a microbial community so long as the sizes of the corresponding T-RFs are verified a priori. Nilsson and Strom (2002) developed a database that contains 16S rRNA gene sequences of fish pathogens. This database can be searched to presumptively identify common fish pathogens based on the T-RF lengths obtained with a defined primer pair and six restriction enzymes.

Monitoring changes in microbial communities

Advances in T-RFLP analysis make it possible to process many samples using multiple primer–enzyme combinations. The extensive datasets that result are too complex and noisy to be analyzed subjectively so statistical methods must be used.

We divide this discussion into three sections because in general, the objectives of studies that use T-RFLP can be divided into three categories: (a) visualizing relationships among fingerprints using principal component analysis (PCA; Clement et al. 1998; LaMontagne et al. 2002; Pereira et al. 2006; Pesaro et al. 2004; Pett-Ridge and Firestone 2005; Schwartz et al. 2007; Wang et al. 2004), multi-dimensional scaling (MDS; Denaro et al. 2005; Pett-Ridge and Firestone 2005; Terahara et al. 2004), self-organizing maps (SOM; Dollhopf et al. 2001), and additive main effects and multiplicative interactions (AMMI; Culman et al. 2006); (b) identifying significant groups using cluster analysis (Blackwood et al. 2003; Dickie et al. 2002; Fedi et al. 2005; Magalhaes et al. 2008; Moeseneder et al. 1999; Pesaro et al. 2004; Polymenakou et al. 2005; Schwartz et al. 2007; Smalla et al. 2007; Zhou et al. 2007) and model-based approaches (Tang et al. 2007); and (c) linking differences among the microbial communities to variation observed in the environments sampled using canonical correspondence analysis (CCA; Cao et al. 2006; Grüter et al. 2006; Magalhaes et al. 2008) and redundancy analysis (RDA; Blackwood and Paul 2003).

Visualizing relationships among microbial communities

PCA, MDS, SOM, and AMMI are useful methods to visualize similarities or dissimilarities among microbial communities. All four methods reduce the dimensionality of the data, which are then plotted in two–three dimensions. PCA uses a set of new variables (linear combinations of the original variables) to describe as much of the variance in the data as possible with as few variables as possible (Johnson 1998). The so-called first principal component—the first new variable—embodies the largest amount of variation in the data. The second principal component, which is orthogonal to the first principal component, takes into consideration the second largest amount of variation and so on (see Johnson 1998, for a more complete description). MDS can be divided into metric and nonmetric procedures. Metric MDS is also known as principal coordinate analysis. To construct a plot for metric MDS, all pairwise distances of all profiles are first computed. Then, the multidimensional distances are plotted in two–three dimensions in such a way that the original distances among the profiles are reflected as accurately as possible. In contrast to metric MDS, nonmetric MDS is based not on the metric distances between profiles but on the ranks of the distances between profiles. Importantly, MDS has a goodness of fit test that indicates how well the generated plot reflects the relations among the original data (Young 1987). Rees et al. (2004) extended the use of MDS to test for significant differences among groups through the analysis of similarity and they implemented similarity percentage analysis to calculate the contribution of individ-

ual T-RFs to the dissimilarity between samples. SOMs, which have been used in a few studies to analyze T-RFLP data (Dollhopf et al. 2001), organize data onto a two-dimensional grid. Each node on the grid is described by a model, and the models of neighboring nodes are more similar to each other than those located further away. Each T-RFLP profile is associated with a model on the grid that explains the structure of the profile, and accordingly, similar profiles will be organized more closely together on the grid than less similar profiles. For a detailed description, see Kohonen (2000). In comparison to PCA, SOM seems better able to detect differences between profiles that contain a large number of peaks (Dollhopf et al. 2001). Depending on the objective of the study, AMMI (Gauch 1992) can be more useful than PCA, MDS, and SOMs because it focuses on the differences in the response of bacterial populations to treatments (Culman et al. 2006). AMMI is a combination of analysis of variance and PCA.

There are limitations that should be considered when using these methods. PCA and MDS have the disadvantage that two–three dimensions may not be sufficient to reflect differences among the profiles accurately if the data structure is too complex. A limitation of PCA, MDS, and SOM is that none of them provide statistical support for the differences observed among microbial communities. Nonetheless, these methods can be seen as useful exploratory methods that give an impression on similarities and dissimilarities of microbial communities.

Identifying groups of microbial communities

Cluster analysis and Bayesian model-based analysis can be useful if the purpose of the analysis is to identify groups of similar profiles and offers the advantage of providing a measure of statistical support for each inferred cluster. Cluster analysis is based on computing pairwise distances between all profiles using a distance measure such as Euclidean and Bray–Curtis distance. Profiles are then clustered using a clustering algorithm such as single, complete, or average linkage to group profiles so that profiles within a group are more similar than profiles of distinct groups (Johnson 1998). To determine whether groups (clusters) are statistically significant, methods such as cubic clustering criterion (CCC) and pseudo *F*-test can be applied (Abdo et al. 2006). An extension of simple cluster analysis has been described by Abdo et al. (2006). It utilizes the outcome of cluster analysis to determine how many samples and which samples within each cluster should be used for the construction of clone libraries. This allows an investigator to describe a known proportion of total diversity present in samples of the cluster (Abdo et al. 2006). This is a useful objective procedure to minimize the number of samples that must be analyzed in detail by sequencing cloned genes.

A model-based Bayesian statistical tool (T-BAPS) was developed by Tang et al. (2007) to cluster microbial communities. T-BAPS operates by implying a priori that a certain number of groups exist based on a model that describes the probability that any of the observed profiles belongs to a group. Using a Markov chain Monte Carlo method, the parameters of the model for that particular number of groups are determined. T-BAPS is run multiple times assuming a different number of groups each time. Bayesian information criterion Monte Carlo approximations are then used to compare the models of the different runs to determine the model that describes the data best, and this model reflects the optimal number of groups (Raftery et al. 2006). Using both simulated and real data, it has been shown that T-BAPS performs better than standard cluster analysis. Unfortunately, Tang et al. (2007) do not state what was done to distinguish between signal and noise, or how profiles were aligned to account for run-to-run variation, and the authors assumed the data were normally distributed, which is not always the case for T-RFLP data. The advantage of model-based methods such as T-BAPS over many distance-based clustering techniques is that model-based procedures provide a measure of statistical support for each inferred cluster.

Linking changes among microbial communities to observed changes in the environment

CCA (Cao et al. 2006; Grüter et al. 2006) and distance-based RDA (Blackwood and Paul 2003) have been used to link the observed changes in microbial community structure to differences in environmental conditions. These methods differ in that RDA uses linear combinations of environmental variables to describe observed changes in community profiles, whereas CCA uses a bell-shaped relationship between microbial communities and environmental variables. The details of CCA and RDA are described in Ter Braak (1986) and Legendre and Anderson (1999), respectively. In their study, Grant and Ogilvie (2003) stated that redundancy analysis may not be appropriate if no strong environmental gradient is expected, in which case, exploratory ordination techniques are more applicable. We believe that this is not necessarily true and that it depends on the aim of the study. If the objective is to test if there is a correlation between changes in communities and variation in the environment, then RDA and CCA are both appropriate. If changes in microbial communities and changes in the environment are correlated, further studies can be done to determine which environmental factors are most strongly correlated with changes in the microbial

communities. Although methods such as RDA and CCA give some statistical support to determine whether samples from different treatments or localities have different community structures, there is a need for methods that allow better inference from T-RFLP data. Model-based methods are promising because they can deal with common characteristics of T-RFLP data such as nonnormal distribution and sparseness of data.

Distance measures

Many of the statistical approaches discussed above are based on measures that determine the similarity among profiles (distance measures). There are many different distance measures to choose from (Johnson 1998; Legendre and Gallagher 2001; Tullos 1997) and the choice of the distance measure used may greatly influence the results of the analysis. In the analysis of T-RFLP data using distance measures, there are two things to consider: (a) should the abundances of individual phylotypes be included as an important variable or should only the presence or absence of fragments be used and (b) should the absence of a peak in two samples be regarded as a similarity between them or have no impact on the distance between two profiles. Euclidean distance, the Morisita–Horn index, and Hellinger distance incorporate abundance, whereas measures such as Jaccard and Dice's coefficient only take into account presence/absence of T-RF sizes. Whether abundance is included in the analysis depends on whether the objective of the study is of a purely qualitative or a quantitative nature. If changes in abundance are included in the analysis, only standardized abundances should be used due to the run-to-run variation (Liu et al. 1997; Osborn et al. 2000). The simple matching coefficient incorporates the absence of a T-RF in two profiles as a similarity, whereas indices such as Jaccard and Bray–Curtis only account for T-RFs that are present in two profiles as a similarity between the two profiles. We suggest that similarity indices should be used in which the absence of a bacterial population in one of two profiles does not impact the distance between the profiles. This is because the failure to detect a population in a profile may not mean that it is absent from a sample but rather below the detection threshold.

Conclusion

Great progress has been made in T-RFLP analysis of 16S rRNA and functional genes. Technical developments such as implementing capillary gel electrophoresis and the use of multiple labeled primers and restriction enzymes resulted in an improved reproducibility and resolution of T-RFLP profiles, while web-based tools facilitate the choice of primer and enzymes. Various statistical methods for data analysis are being developed and used for the analysis of T-RFLP and this permits more expansive data sets to be objectively analyzed. These advances have greatly increased the utility of T-RFLP in studies of microbial community ecology.

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