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THE FIRST DECADE OF TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) IN MICROBIAL ECOLOGY

Blaž STRES [a)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Dept. of Food Science and Technology, Večna pot 111, SI-1000 Ljubljana, Slovenia, Ph.D., M.Sc., e-mail: blaz.stres@bf.uni-lj.si.

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ABSTRACT

Terminal restriction fragment length polymorphism (T-RFLP) was introduced to environmental microbiology only a decade ago but it soon became a molecular tool of choice, due to its high throughput and phylogenetic resolution. Fierce discussions accompanied the new method leading to sophistication of the data preparation, acquisition, manipulation and standardization of analysis. Consequently, numerous approaches were proposed at various steps and also criticized. As a result, a combination of variable percentage threshold and Bray-Curtis index used in non-metric multidimensional scaling are now being accepted. Their combination offers a balance between noise elimination and information retention yielding a powerful and yet easily interpreted method to examine community patterns based on T-RFLP data. Its current state of the art and future developments highlight the potential of the method in the field of microbial ecology. However, a more standardized approach and a higher level of control at all stages of T-RFLP fingerprinting are needed.

Key words: microbiology / microbial ecology / molecular genetics / T-RFLP

PRVO DESETLETJE RESTRIKCIJSKEGA POLIMORFIZMA DOLŽINE KONČNIH FRAGMENTOV (T-RFLP) V MIKROBNI EKOLOGIJI

IZVLEČEK

Proučevanje restrikcijskega polimorfizma dolžine končnih fragmentov (T-RFLP) tarčnih genov se na področju mikrobne ekologije pričelo šele pred desetletjem. Metoda je hitro postala zelo priljubljena zaradi svoje filogenetske ločljivosti in enostavne analize velikega števila vzorcev. Razvoj priprave vzorcev, zajemanja podatkov, obdelave in standardizacije metod analize so v veliki meri obkrožale silovite razprave. Raziskovalci so tako na vsaki stopnji kritično preizkusili veliko število različnih pristopov. Danes kombinacija tehnik, kot so prag variabilnih deležev, koeficient Bray-Curtis v ne-merskem večdimenzionalnem umerjanju predstavlja ravnotežje med odstranjevanjem šuma in zadrževanjem informacij. Tako je nastalo zelo uporabno orodje z relativno enostavno interpretacijo za proučevanje tipizacijskih profilov T-RFLP mikrobnih združb. Njegova trenutna stopnja dovršenosti in predvidene razvojne izpopolnitve v prihodnosti kažejo na velik potencial tega orodja na področju mikrobne ekologije. Hkrati pa bo potrebno uveljaviti tudi bolj standardizirane in bolje kontrolirane izvedbe posameznih stopenj tipizacije mikrobnih združb s T-RFLP.

Ključne besede: mikrobiologija / mikrobna ekologija / molekularna genetika / T-RFLP

INTRODUCTION

The Golden Age of Microbiology in the early 1900's was shaped on extensive isolation and characterization of pure cultures. As the limitations of culture methods became clear many different techniques for evaluating microbial communities were developed in order to enable 'modern' microbiologists to understand natural microbial community structure and dynamics. The far majority of such studies use polymerase chain reaction (PCR) to amplify genes of interest directly from environmental samples without culture bias. However, the use of PCR introduced other sources of bias which were already addressed elsewhere (Kent and Triplett, 2002). In addition to Amplified Ribosomal DNA Restriction Analysis (ARDRA), Single Stranded Conformation Polymorphism analysis (SSCP), Thermal and Denaturing Gradient Gel Electrophoresis (TGGE and DGGE), Length Heterogeneity analysis (LH), Terminal Restriction Fragment Length Polymorphism (T-RFLP) was introduced in early 1997 (Liu *et al.*, 1997; Bruce, 1997). As all of the above mentioned approaches produce a pattern or profile of nucleic acids amplified from a sample, T-RFLP emerged as a tool providing researchers with a large amount of easily analyzed data on microbial community structure. As with any other method, certain aspects need to be highlighted from fundamentally technical perspective. After all, they influence substantially the final conclusions drawn from studies in microbial ecology.

HOW TERMINAL RESTRICTION FRAGMENTS (T-RFS) ARE MADE

T-RFLP patterns are generated and analyzed in a series of steps that combine PCR, restriction enzyme digestion and capillary or gel electrophoresis. From a collection of phylogenetically divergent sequences for the target gene, primers homologous to conserved regions in target gene are designed and used for amplification of a fragment. One primer, either forward or reverse, is labeled at 5'-end with a fluorochrome. The resulting fluorescently labeled amplicons are then digested with a number of restriction enzymes in separate reactions. Restriction enzymes are usually specific for tetranucleotide recognition sequence in amplified product. The digested amplicons are purified in order to remove excess salts resulting from PCR and restriction reactions. Subsequently, they are subjected to capillary electrophoresis using DNA sequencer with a fluorescence detector.

In this way only fluorescently labeled fragments are detected, their length automatically determined according to internal standards and their fluorescent signal integrated as peak height or area. The programs enable simple transfer of resulting sequencer data to text files that are easily transported. Alternatively, raw chromatographic files can also be analyzed with programs used for comparing spectral data (e.g. BioNumerics, Gelcompare (AppliedMath, Belgium)). The patterns of T-RF peaks can then be numerically compared between samples using a variety of multivariate statistical methods (Kitts, 2001; Osborn *et al.*, 2000; Dunbar *et al.*, 2001).

SECOND THOUGHTS ABOUT T-RFLP

As any other method, T-RFLP analysis also suffers from its own inherent pitfalls that need to be taken into account before, during and after data collection. In the next paragraphs the following crucial steps in T-RFLP analysis are highlighted: (i) sample preparation and DNA extraction; (ii) PCR specificity and bias; (iii) digestion with restriction endonucleases; (iv) pseudo-terminal restriction fragments; (v) incomplete digests; (vi) differential migration; (vii) aligning raw T-RFLP data sets.

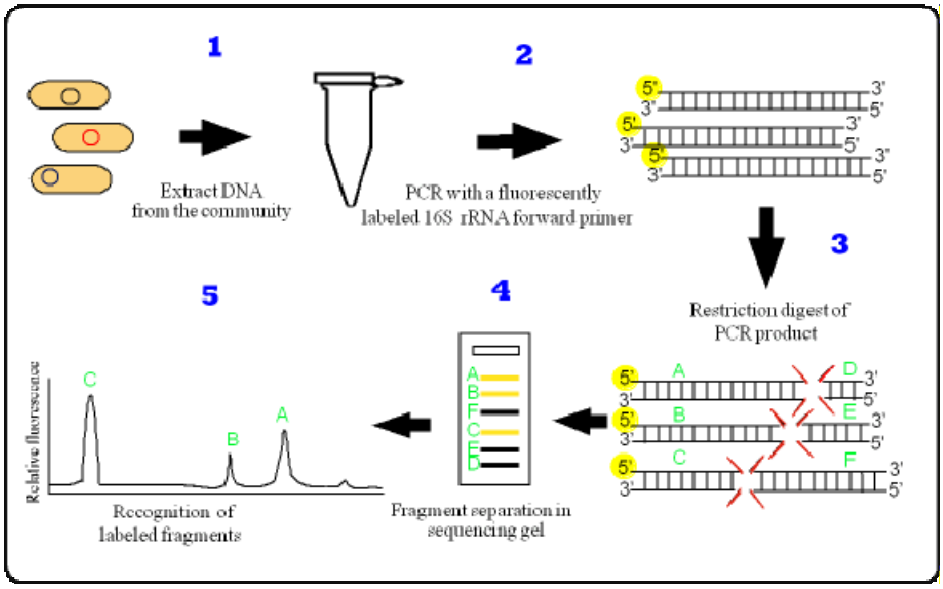

Figure 1. Schematic diagram of T-RFLP approach to community fingerprinting of genes coding for 16S rRNA (modified from Grüntzig *et al.*, 2002).
Slika 1. Shematičen prikaz tehnike T-RFLP pri tipizaciji genov za 16S rRNA združbe (povzeto po Grüntzig in sod., 2002).

(i) Sample preparation and DNA extraction

Fidelity of sample preparation and DNA extraction is often neglected in published literature although this part is possibly one of the most crucial steps in the analysis as representative samples, representative sample size and DNA extraction protocol should be selected and verified carefully to minimize the possibility of later misinterpretation of results (Morris *et al.*, 2002; Stres *et al.*, 2004; Stres and Tiedje, 2006). In order to minimize the inherent random bias present in soil, sediment or feces sample composition, numerous replicate samples should be analyzed or even replicate extractions pooled (Kitts, 2001). As subsequent steps in preparation of T-RFLP patterns may be highly dependent on starting DNA purity and extent of fragmentation, it is needless to say that those factors should be controlled and kept at minimum or at least constant among various samples and replicates. It would also be worthwhile to consider estimating the degree of cell lysis, DNA adhesion and presence of extracellular DNA during preparation of DNA extracts from complex environmental samples.

(ii) PCR specificity and bias

When amplifying complex mixtures of sequences PCR bias has been identified (Farelly *et al.*, 1995; Qiu *et al.*, 2001). It has been shown that to all odds the abundance of a specific amplicon in a mixture is reproducible and in direct proportion to the abundance of that template in a sample containing high diversity (Clement *et al.*, 2000; Dunbar *et al.*, 2000). However, primer selection and PCR conditions are those that can be constantly improved. The numbers of ribosomal, housekeeping and other functional genes sequences in databases increase exponentially thus enabling constant improvement in primer quality through their evaluation using freely available tools (Amplicon (http://sourceforge.net/projects/amplicon/), Blast at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), FunGene (http://flyingcloud.cme.msu.edu/fungene/)) (Lueders and Friedrich, 2003). In this respect also classical optimization of PCR composition and cycling parameters should not be neglected. However, one thing should constantly be kept in mind: it is in the nature of PCR to amplify dominant groups of target sequences with higher

chance. In a community comprised of 10^8 cells, only those populations representing up to 0.1% (10^5 cells) of microbial community have the chance to be detected at best. The appearance or loss of a fragment from a fingerprint is therefore interpreted as a result of changes in detectability, but not a complete loss of population (Kent and Triplett, 2002).

(iii) Digestion with restriction endonucleases

Amplicon digestion with restriction endonucleases should be explored to determine which enzyme results in the highest number of peaks detected therefore yielding 'best' enzyme for each primer and PCR conditions set. Although diversity of fragments is increased, such approaches using only one restriction enzyme, limit general comparability of the results. Therefore the use of multiple enzymes is suggested in order to obtain better resolution between different communities (Kitts, 2001; Engebretson and Moyer 2003; Rosch and Bothe, 2005). By choosing the appropriate number and types of restriction endonucleases, an investigator increases the probability that the resulting arrays of T-RFs size distributions more accurately reflect the natural diversity of microbial populations within a sampled community (Engebretson and Moyer, 2003; Lueders and Friedrich, 2003).

(iv) Pseudo-terminal restriction fragments

Formation of single-stranded pseudo-terminal restriction fragments (pseudo T-RFs) was reported (Egert and Friedrich, 2003) as a result of incomplete amplification during PCR. Their fraction is dependent on the number of cycles used in PCR. Digestion of amplicons with single-strand-specific nucleases prior to separation on sequencer completely eliminated pseudo T-RFs. However, the importance of formation of secondary structures during PCR amplification for pseudo T-RF formation should be less of a concern during amplification of functional genes as their secondary structure is usually less complex in comparison to genes for 16S rRNA (Egert and Friedrich, 2003).

(v) Incomplete digests

In some instances enzymatic digestion of amplicons yields incomplete digests due to various reasons, such as template purity, complexity, PCR salt interference or traces of PCR enhancers and additives. Therefore longer restriction times and higher enzyme concentration in restriction reactions of purified PCR products from ecological samples are suggested. Absence of incomplete digestion can be determined by simple incubation of restriction reaction mixtures for varying periods of time, verifying disappearance of uncleaved amplicons and plotting the number of fragments relative to duration of restriction reaction.

(vi) Differential migration

Since there is some experimental error in reading of the fragment lengths, the matching of them must be tolerant to minor differences. Furthermore, this error is highly dependent on the fragment length as shown in table 1. The major fraction of discrepancies in lengths of sequenced fragment and those observed at separation of T-RF on sequencer, arise from differential migration of internal ladder (usually ROX or TAMRA labeled) and the FAM or other labeled sample fragments. The reason is that for example ROX label has 12 more carbon atoms than 6-FAM label (Applied Biosystems). In addition, some marker ladders are double stranded yielding double peaks after separation thus confusing size calling software that then needs to be manually adjusted.

Table 1. Informative scale of difference – lengths of T-RFs and maximum length differences (Marsh *et al.*, 2000)
Preglednica 1. Informativni razpon ugotovljenih razlik – dolžine končnih restrikcijskih fragmentov (T-RF) in maksimalne razlike v dolžinah (Marsh in sod., 2000)

True length of T-RF, bp Prava dolžina T-RF, bp	1	200	400	600	800	1200
Difference, bp Razlika, bp	0.5	0.8	1.2	4	8	20

Purine content (A, G) of fragments was also identified as a source of size variation indicating that subtle differences in molecular weight can significantly affect the observed T-RF length. The remaining factor regularly observed is a variation between observed T-RF lengths in replicate runs of the same sample. The primary cause of this variation was attributed to fluctuations in ambient temperature and minute variations in gel composition and capillary quality after each use (Kaplan and Kitts, 2003). A way to mitigate the above mentioned discrepancies it to determine migration size of already sequenced fragments under strictly defined conditions.

(vii) Aligning raw T-RFLP data sets

The first step in analyzing T-RFLP data that is often overlooked in the literature is an appropriate method for aligning the resulting peaks of the raw T-RFLP data sets. Peaks reflecting the size of T-RFs present are measured in base pairs, and the area and height of each peak are determined in relative fluorescence units. The Gene-scan software (Applied Biosystems), most often used according to published literature, generally reports fragments sizes to 1/100 of a base pair. Aligning macros of resulting files in Excel (Microsoft) were developed and used successfully to round peak sizes to the nearest integer value and then align all the peaks against the rounded sizes of the fragments thus significantly reducing subjective biases and time during manual peak aligning (Rees *et al*., 2004; Hewson and Fuhrman 2006). However, the issue of the use of peak area versus peak height has not been resolved yet completely as the use of one over the other has its positive and negative aspects and the choice is more sample dependant (Dunbar *et al.*, 2000; Dunbar *et al.*, 2001).

NO MAN'S LAND

Getting to the point where T-RFLP data is collected from sequencer may appear complicated at first glance. This is, however, not the case as analyzing T-RFLP data is currently even more challenging, facing us with an immediate question: how should one reflect on the collected data so as not to overstretch the final conclusions?

It is very important to choose an appropriate community analysis method for use with T-RFLP. The most simplistic approach is to compare presence or absence of different peaks. Such approach is valid, however it lacks the benefits of a quantitative analysis. So far, principal component analysis (PCA) (Braker *et al.*, 2001), cluster analysis (Braker *et al.*, 2001; Kraigher *et al.*, 2006), self- organizing neural networks (Dollhopf *et al.*, 2001) and multidimensional scaling (Wolsing and Prieme, 2004) were explored. However, the T-RFLP analysis requires a more standardized approach, especially one that has statistical rigor and that is easy to carry out. However, before analyzing data sets and extracting information about similarity or changes in community structure, we have to focus on data preparation and standardization.

In this respect, the use of T-RFLP has been criticized by some to lack the degree of resolution required for analyzing complex microbial communities, such as those found in soil (Dunbar *et al.*, 2000; Dunbar *et al.*, 2001; Engebretson and Moyer, 2003) because of the difficulty in assigning accurate identity to each T-RF in complex profiles of genes. Individual soil samples contain a large diversity of microorganisms, estimating many thousand of species (Curtis and Sloan, 2004). Therefore each peak in a profile generated from DNA extracted from a complex community must represent multiple T-RFs of the same size originating from different species (Sessitsch *et al.*, 2001; Engebretson and Moyer, 2003). An *in-silico* analysis of a set of 4600 16S rRNA gene sequences suggested that each T-RF would represent a mean of 9.1 to 18.5 different sequences depending on restriction endonuclease selected (Engebretson and Moyer, 2003). While assignment of identities may be uncertain at present, it does not preclude the use of the fingerprinting technique to compare whole communities on relative basis.

There is, yet, no agreed-upon method for normalizing samples with different amounts of DNA, which would allow easy comparison of profiles with different total amounts of fluorescent label (Blackwood *et al.*, 2003). An appropriate method for calculating a threshold, baseline or minimum fluorescence cutoff needs to be determined as any similarity measures based on presence or absence of small noisy peaks may have profound impact on the conclusions drawn. There are three published threshold determination protocols: (i) constant percentage threshold (Sait *et al*., 2003), (ii) constant baseline threshold (Dunbar *et al.*, 2001) and (iii) variable percentage threshold (Osborne *et al.*, 2006).

(i) Constant percentage threshold (Sait *et al.*, 2003)

T-RFs that are present in at least one sample are added to the data set of all profiles that lack that T-RF, and an area or height of zero is assigned to that T-RF if it was not detected. A threshold area value is then used to remove small peaks that may be detected purely as a result of the amount of DNA applied before separation. The area that each T-RF contributed is calculated as a proportion of the total area for all T-RFs in that profile. These proportions are then assigned to the appropriate T-RFs as a relative area. T-RFs that contribute less than a designated threshold percentage are reassigned a value of zero.

(ii) Constant baseline threshold (Dunbar *et al.*, 2001)

The total areas in all of the profiles in a data set are normalized to the same value as that of the profile having the smallest total area. Then all of the peaks in each trace are reduced proportionally by the factor required to yield that normalized total area. This constant baseline threshold is usually set as the smallest peak area detected in the unmanipulated data sets (rounded up to 50 fluorescent units). Peaks with an area equal to or smaller than this threshold are then removed from profiles after normalization. Additional analyses can then be carried out reanalyzing the data using constant baseline threshold set to 100 fluorescent units or more.

(iii) Variable percentage threshold (Osborne *et al.*, 2006)

As in the previous approach, the total area of each profile is divided by different arbitrary values (divisors) to yield numbers that are used as percentage thresholds. For each divisor, all peaks that contribute less than the percentage threshold calculated for that profile are removed. Then, for each divisor, the remaining number of peaks is plotted against the total area, so that each profile contributes one point on that plot. Osborne *et al.* (2006) have found that in case there is very little variation in the total area of the profiles, there is no detectable relationship between the number of peaks and the total area, and no threshold needs to be applied. If standardization is required, a useful divisor to start with is 1,000 times the mean total area for the data set. Different

divisors are then tested, and the divisor that results in the weakest relationship between the number of peaks remaining and the initial total area can be considered to be the optimal divisor. The unique percentage threshold value for each profile is calculated by dividing the total area of that profile by the optimal divisor. Peaks that contribute less than that percentage threshold are then removed from that profile before analyzing relationships.

The comparison of all three approaches suggested that their use allows the subsequent analysis to detect real groupings by eliminating noise. However, the variable percentage method appears to be the most useful of the methods tested, providing a balance between noise elimination and information retention (Osborne *et al.*, 2006). The authors of this study also suggested once more that pooling PCRs (Hackl *et al.*, 2004), using a number of restriction enzymes and generating consensus profiles from multiple separations (Dunbar *et al.*, 2001) enable more confident interpretation of T-RFLP analyses to be made.

If there is no agreed upon and widely accepted method for standardization of T-RFLP raw data files there is even less congruency regarding the methods comparing the resulting standardized and manipulated T-RFLP data. So far, various similarity indices, grouping methods were proposed and criticized. Although often described in the literature, Euclidean coefficients are not an appropriate method to determine similarity within T-RFLP data sets, as they do not handle adequately data containing blocks of double zeros or joint absence of fragments (Rees *et al.*, 2004). Also the application of PCA has not been widely accepted as a good tool for T-RFLP data analysis as the data may not always be normally distributed. The most widely accepted are Bray-Curtis coefficient coupled with non-metric multidimensional scaling (NM-MDS) (Wolsing and Prieme, 2004), analysis of similarity (ANOSIM) and similarity percentage (SIMPER) (Rees *et al.*, 2004). The Bray-Curtis coefficient is suggested as an ideal coefficient to be used for the construction of similarity matrices. Its strengths include its ability to deal with data sets containing multiple blocks of zeros in a meaningful manner. In combination with NM-MDS this yields a powerful and yet easily interpreted method to examine community patterns based on T-RFLP data (Wolsing and Prieme, 2004).

FUTURE DIRECTIONS

Currently, efforts are directed towards coupling of quantitative PCR and T-RFLP assays into single molecular assay allowing quantification first and subsequent fingerprinting of detected microbial community members (Yu *et al.*, 2005). In addition, T-RFLP profiles of DNA and RNA extracted from complex microbial communities are now being explored, on ribosomal and functional level (Mengoni *et al.*, 2005) and linked to *in-silico* analyses of matching T-RFs with sequence database entries. Such approaches technically enable high-throughput identification of dominant groups of moderately complex microbial communities (Marsh *et al.*, 2000; Kent *et al.*, 2003). Further, multiplex-T-RFLP was just recently explored thus enabling rapid and simultaneous analysis of bacteria, archaea and funghi in the same PCR reaction (Singh *et al.*, 2006).

However, all technical issues have not been resolved yet and in this respect significant advances need to be made to enable sound use of all aspects of T-RFLP as a method. As data preparation and handling can now be more uniformly conducted also differences in microbial community structures can be more rigorously tested and determined. To identify the critical factors that influence population distribution and activity in complex environments more sophisticated statistical and mathematical techniques are needed to model the relationship between microbial community structure, function and environmental characteristics (Morris *et al.*, 2002; Stres and Tiedje, 2006). In this respect, reconsidering the above mentioned pitfalls, fundamental methodological aspects of T-RFLP and published evidence on their misuse all lead

to sobering conclusion: if more effort were put into standardizing the T-RFLP methodological approaches first, more reliable interpretations of ecologically relevant data would emerge from a decade of T-RFLP use in microbial ecology. As one would put it – a decade of many missed opportunities, but many hard lessons learned. I think it's time to put them to practice.

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