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REVIEW ARTICLE

Technicalities and Glitches of Terminal Restriction Fragment Length Polymorphism (T-RFLP)

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Abstract Terminal restriction fragment length polymorphism (T-RFLP) is a rapid, robust, inexpensive and simple tool for microbial community profiling. Methods used for DNA extraction, PCR amplification and digestion of amplified products have a considerable impact on the results of T-RFLP. Pitfalls of the method skew the similarity analysis and compromise its high throughput ability. Despite a high throughput method of data generation, data analysis is still in its infancy and needs more attention. Current article highlights the limitations of the methods used for data generation and analysis. It also provides an overview of the recent methodological developments in T-RFLP which will assist the readers in obtaining real and authentic profiles of the microbial communities under consideration while eluding the inherent biases and technical difficulties.

Keywords Impreciseness · Microbial ecology · NGS, next generation sequencing · Reproducibility · Resolution

Introduction

Due to the limitations of culture-based methods towards the understanding of microbial communities, a number of culture-independent methods of community analysis have been developed [1–4]. T-RFLP is one such method which investigates variations in the lengths of terminal restriction fragments (T-RFs) of conserved molecular markers such as the 16S rRNA gene. It is an inexpensive, robust, reproducible, and rapid method for the study of microbial community structure. The potential of T-RFLP method for the study of community dynamics and structure is extensively studied for diverse habitats [5–14]. In addition to 16S rRNA gene, T-RFLP analysis also included genes representing a variety of functional groups [15–17].

A decade ago, T-RFLP was in an emerging stage but currently the number of T-RFLP related publications has exceeded more than a thousand, reporting application of the method, developments in methodology and data analysis. However, the popularity of T-RFLP seems to be decreasing as a choice of technique, primarily due to several inherent problems and secondly, due to the availability of other sophisticated, but expensive, methods such as next generation sequencing (NGS) and microarrays. Nevertheless, it is still remain as a method of choice for study of community dynamics (like 454 pyrosequencing, Illumina and Ion Torrent) due to its low cost, less intensive computation and massive parallel sample handling capacity.

Numerous methodological advancements have been made since the most recent extensive review [18] and it is timely to review the subject matter again in the light of new developments (Table 1). In this review, we shed light upon the limitations of T-RFLP pertaining to biases that create problems during data generation and analysis, and provide possible solutions to overcome them. In addition to highlighting points such as the effect of DNA extraction methods on the resolution of T-RFLP, we have incorporated a number of recent developments since last four years, e.g., the combination of denaturing high-pressure liquid chromatography (D-HPLC) with T-RFLP, effect of different dyes (used for labeling of primers) on the results; and focused on the applicability of T-RFLP in the era of NGS.

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Table 1 Steps of T-RFLP analysis, related technical challenges and proposed solutions

Steps of T-RFLP analysis	Technical challenges	Solutions	References
Extraction of community DNA	Partial lysis of cells and sample to sample variation in the amount of DNA	Optimize extraction procedure; use more than one method in combination	[21, 26, 56]
PCR amplification	PCR bias, multi template method of amplification and uneven distribution of marker genes among the community members	No solution for systematic bias; random bias can be minimized by using replicates and group specific primers; optimize amplification.	[28–30]
Digestion	Excess base line noise, poor resolution and low peak height	Ensure complete digestion; purify amplified product; purify digested DNA	[8, 24, 31, 44]
Data analysis	Poor resolution	Use group specific primers and optimize amplification; use single digestions with multiple enzymes; use 16S rRNA gene clone library	[8]
	Reproducibility	Run samples in replicates; standardize base line noise; standardize quantity of DNA loaded on the gel	[8, 13, 37, 47]
	Impreciseness in theoretical and practical T-RFs length	Use of multiple bin windows frames; use of clone library and molecular weight instead of bp length for T-RFs	[32, 34]
	Reduction of peak height with increase in retention time	Use peak area as a measure of similarity instead of peak height	[24, 39]

Analysis by T-RFLP is based on the differences in the restriction patterns of molecular markers like rRNA gene which generates a group-specific characteristic pattern of T-RFs after the restriction digestion and capillary electrophoresis. Methods described for obtaining the T-RF patterns are more or less similar with slight modifications [7, 8, 16]. It constitutes extraction of total community DNA, PCR amplification of molecular marker with labeled primer(s), purification of amplified products, restriction digestion of the purified amplicon with one or more restriction endonucleases (notably the 4-base cutters) and finally separation of T-RFs by capillary electrophoresis using an automated DNA sequencer equipped with data collection and analysis software [8]. Generally four base cutters restriction endonucleases are considered ideal for restriction fragment length polymorphism (RFLP) analysis as they cut the DNA strand more frequently and generate large number of smaller fragments (T-RFs here) consequently give better resolution.

Technical Advancements in Data Generation

Due to automation of method, generation of data has become simpler, robust and straightforward. However, some phases of the method like DNA extraction, PCR amplification, digestion and generation of replicates still require consideration. Points to be pondered over, while performing T-RFLP include: (1) DNA extraction and PCR amplification; (2) multiple single digestion of amplified products using one or more restriction endonucleases to enhance the resolution and specificity of T-RFs; (3) generation of data in replicates, at least at the electrophoresis level, to assess the reproducibility of the method and for statistical analysis.

Differential lysis of microbial cells, especially spore formers, during extraction of community DNA may lead to a bias in the relative amount of DNA in different samples [19–25]. Past data on community profiling by T-RFLP and other methods revealed that use of a single method for DNA extraction can significantly underestimate the total number of bacterial ribotypes [23, 25, 26]. Several DNA extraction methods for diverse communities and habitats are available [19, 20, 23, 25]. Therefore, a combination of different DNA extraction methods and the use of more than one genetic marker are recommended to obtain a more accurate evaluation of the diversity of the target organisms or functional groups [23, 25, 27, 28]. Multi-component amplification of the community DNA [28] and uneven distribution of copy number of the marker gene in community populations have their own issues [29]. The aforementioned factors provide an estimate only of species richness, but not of the relative abundance of species in a community due to the potential presence of multiple copies of a marker gene in the same cell.

Multiple single digestions with more than one restriction enzyme coupled with the use of group-specific primers enhance the resolution of peaks [8, 24] while incomplete digestion leads to artefactual peaks in the T-RFs [30]. A judicious choice of restriction enzymes for digestion of

amplified products [8, 24, 31] use of molecular weight instead of length of T-RFs for comparison [32], and confirmation of results by sequencing and other DNA fingerprinting methods may be used to enhance the resolution of the method [8, 24].

Variations in sample size (amount of DNA) loaded on capillary lead variability in data. Even with utmost care, some amount of sample to sample variability is the inherent part of the technique. Therefore, estimation and standardization of the amount of DNA is necessary for better resolution. To overcome the variability and improve the reproducibility of the data, apart from promoting the running of replicates Kaplan et al. [12] and Dunbar et al. [8] respectively proposed the methods for post-run data normalization based on the relative peak area and peak height of the generated TRFs.

Furthermore, it has been observed that DNA fragments labeled with different dyes show variations in mobility during capillary electrophoresis. According to Schütte et al. [18], fluorescein (6FAM and HEX) labeled DNA fragments move faster than rhodamine labeled DNA. Pandey et al. [33] have also reported variations in the T-RFLP profiles of the same sample due to the variation in the dyes used for labeling the primers.

The challenges of fragment size impreciseness and of baseline noise can be resolved to a certain extent by the use of multiple binning (sorting criteria for T-RFs) approach [34, 35] while the issue of unavailability of T-RFs in the databases can be overcome by the generation of clone library from a sample that is assumed to contain most, if not all, of the target genotypes of interest [4]. To overcome the current limitations of T-RFs size impreciseness and construction of clone libraries, Penny et al. [3] proposed an optimized protocol for T-RFLP that yields reliable T-RF sizes and uses denaturing high performance liquid chromatography (D-HPLC) as an alternative to cloning in order to gain direct access to the DNA sequence.

Developments in Data Analysis

Despite a high-throughput method of data generation, analysis of the data is still in its infancy. Past study showed that researchers often do not have a very good idea about data normalization, data transformation and statistical methods to be applied for the data generated [36]. Several points must be reckoned before analyzing the data of community analysis and some of them being: (1) setting of accurate fluorescence threshold to discriminate true peaks from baseline noise; (2) alignment of replicate profiles to a single consensus profile; (3) normalization of data for clustering and statistical analysis [13, 34, 36–39]; and (4) matching against databases for confirmation of specificity of T-RFs from clone libraries [24].

In the earlier days of T-RFLP, phylogenetic assignment was performed by a simple visual inspection and comparison of T-RFs. Now the method of data analysis is not limited only to visual inspection of peaks and comparison of profiles, but also includes cluster analysis, Bayesian analysis and methods of ordination like principal component analysis (PCA), redundancy analysis (RDA), correspondence analysis (CA), canonical correspondence analysis (CCA), multi-dimensional scaling (MDS), Additive Main Effects and Multiplicative Interaction (AMMI), self-organizing maps (SOM) etc. [18, 40–42].

Initially, selection of a minimum fluorescence threshold to eliminate the baseline noise was based on visual inspection and the researcher's judgment. Abdo et al. [37] reported that injudicious choice of minimum peak height may lead to difficulties in data analysis. Initially, a method for selection of minimum peak height based on variability and % similarity of T-RFs in replicated profiles was also proposed [13, 34]. Discarding of the peaks with a relative peak area (calculated by dividing the area of individual peak by the cumulative area of all peaks in the sample) of less than 1 % also helps in eliminating the background noise [35]. In addition to selection of the right peaks, alignment of peaks ('binning') also needs attention. In order to minimize the manual error of binning, Smith et al. has developed a free web-based program, T-Align (http://inismor.ucd.ie/~talign/) with an automated fragment analysis system [43]. The output generated by T-Align can be readily used for further statistical analysis of the peak alignments.

Similarities among T-RFLP patterns were initially calculated using similarity coefficients and used for the construction of dendrograms. Subsequently, similarity calculations using peak area instead of peak height were introduced [39, 44]. A recent review by Culman et al. [40] recommends the relativization of peak height and peak area to make up for the differences in the quantity of DNA extracted from different samples. They also observe that the usage of binary data (presence/absence of peaks) is less prone to variations in results than relativized peak height or area, and advocate the use of binary data or normalized peak heights for multivariate analysis over relativized peak area.

Ecological data also demand normalization and relevant transformations before statistical analysis. In brief, data normalization is performed for efficient organization of data to reduce the redundancy and increase interdependency or coherence in data; while data transformations increase interpretability and graphical appearance. Different ways of data transformations are available and Hellinger transformation is one that is highly recommended [40, 41]. Furthermore, appropriate association coefficients need to be chosen to calculate the dis-similarity among the

T-RFLP datasets [42]. Statistical methods used in microbial ecology (especially in community ecology) have been discussed comprehensively by various research groups [18, 40–42]. Generally, PCA, MDS, SOM and AMMI are recommended for visualizing the similarity and differences among the community members; cluster and Bayesian analysis for group identification; and CCA and RDA for linking the changes in microbial community in response to changes in the environment [18, 40]. Furthermore, Culman et al. [40] also suggested that method selection for data analysis should be based on complexity of T-RFLP dataset and outlined theoretical criteria of researchers.

Tools for Data Analysis

A number of tools have been developed for the handling of raw T-RFLP data [32, 45, 46]. Currently, a number of online tools like TAP-T-RFLP (http://rdp.cme.msu.edu), torast (http://www.torast.de), MiCA (http://mica.ibest.uidaho.edu/) and T-RFPred (http://nodens.ceab.csic.es/t-rfpred/) are available for the in silico digestion of 16S rRNA (database) sequences with a number of restriction enzymes and prediction of their relative T-RFs' patterns. Recently, a number of applications like phylogenetic assignment tool (PAT) [47], T-Align (http://inismor.ucd.ie/~talign/ [43]), ARB software integrated tool, TRF-CUT (http://www.mpi-marburg.mpg.de/braker/trfcut.zip [47]) TRiFLe (http://cegg.unige.ch/trifle/trifle.jnlp [48]) and T-RFLP statistical data analysis software (http://www.ibest.uidaho.edu/tool/T-RFLP_stats/index.php) [1] are available to perform tasks like profile comparison, statistical analysis of data and representation of similarity in the form of a dendrogram. T-Align generate a consensus profile from replicate data and compare generated consensus profile to get the result about community structure and composition, while PAT provide phylogenetic assignment in terms of species diversity and composition from user supplied data. TRF-CUT and TRiFLe introduced by Ricke et al. [45] and Junier et al. [48] respectively generate theoretical T-RFs by in silico digestion of user supplied database of small subunit rRNA gene sequences or any other functional gene of interest. In addition Stres et al. [49] developed BEsTRF to get the optimal resolution of T-RFLP data by using the user defined primer-enzyme-sequence database. The application by Abdo et al. [37] includes an algorithm for detection of base line noise, setting the fluorescence, alignment and binning of profiles, cluster analysis and finally selection of representative samples for construction of library. Calculation of interaction effects and beta diversity, relevant to ecology studies, can be calculated with T-REX (http://trex.biohpc.org/ [38]) free online software.

Comparison of T-RFLP to Other Community Profiling Methods

In addition to T-RFLP other culture independent methods available for community analysis are denaturation gradient gel electrophoresis/ temperature gradient gel electrophoresis (DGGE/TGGE) [50], length heterogeneity PCR (LH-PCR), amplified rDNA restriction analysis (ARDRA), phospholipid fatty acid analysis (PLFA), ribosomal intergenic spacer analysis (RISA), fluorescence in situ hybridization (FISH), single strand conformational polymorphism (SSCP) [4], construction of clone library and use of NGS. T-RFLP seems to have more advantages than DDGE, TGGE and SSCP techniques, because it can be standardized between different runs and different laboratories. T-RFs obtained during different studies can be organized in the form of a database for cross referencing by other workers [34]. Availability of huge 16S rRNA gene sequence database is also another favorable factor for this technique [5, 24]. Furthermore, phylogenetic inference obtained using T-RFLP has a greater resolution due to the use of superior capillary electrophoresis of automated DNA sequencer than other fingerprinting methods such as DGGE and SSCP. The reproducibility, robustness, and rapidness of T-RFLP enable researchers to generate huge amount of data (in replicates) in a short period of time to check the reproducibility and perform statistical analysis [24]. As compared to T-RFLP with the destructive nature of DNA sampling, DGGE stands at an advantage by enabling direct cloning of the profile element from the gel. As a solution, Penny et al. [3] introduced D-HPLC as an alternative to cloning in order to gain direct access to the DNA sequence information and circumvent the limitation of T-RFLP. Clone library based study of microbial community gives more resolution but it is costly, labor intensive and time consuming. In addition, NGS is an emerging alternative of T-RFLP for community profiling and provide more in-depth information but currently facing some critics. For example, rate of sequencing error in next generation sequencing is high than Sanger sequencing [51]. In addition, due to short read length it gives overestimate about microbial diversity and cannot use for species level resolution. Use of decades old primer and lack of systematic approach in primer designing without inclusion of new sequences from database underestimate the diversity. Kalia et al. [52], Porwal et al. [53] and Bhushan et al. [54] used extensive database of 16S rRNA gene sequences and developed species specific framework, signature nucleotide and restriction digestion pattern to increase the taxonomic resolution in genus *Clostridium, Bacillus*, and *Pseudomonas* respectively. Different approaches used by Kalia et al. [51] and Porwal et al. [52] can be used to get more taxonomic resolution by designing the species specific

framework for community analysis or for the study of effect of environmental perturbation on a particular species by T-RFLP or NGS. Although next generation sequencing is more attractive but at the same time it is cumbersome and costly as compared to T-RFLP. With the refined methods of data generation and analysis, it is now evident that the outcome of T-RFLP is satisfactory, at least for the study of trends in community dynamics as a consequence of change in environmental factors and external stimuli. It is also advisable to use T-RFLP for screening and sorting out the relevant samples for NGS. Finally NGS could be taken up on representative samples to get in-depth information about the structure and functions of the communities. Use of NGS and T-RFLP in combination is logical in order to save the cost and labor; and to conduct simultaneous studies on a large number of samples using different variables [55].

Conclusion and Future Prospects

T-RFLP is a high throughput and a rapid method for study and understanding the community structure, function and dynamics. The application of T-RFLP is not limited to a particular group of organisms or a specific kind of habitat. It is equally applicable to assess the diversity and dynamics of bacteria, archaea and eukaryotes from diverse habitats. However, due to some inherent technical biases and instrumental artifacts associated with the method, a meticulous care is a must, at least during the stages of data generation, DNA extraction, PCR amplification, digestion, use of replicates, setting of baseline noise and in the standardization of amount of DNA loaded on the gel. In conclusion, logical selection of primers and restriction enzyme(s), use of replicates and appropriate methods of data analysis will eventually improve the resolution of the method and phylogenetic assignment.

In brief, T-RFLP is a handy tool for a microbial ecologist in exploring the community structure, function and dynamics in a high throughput manner with low cost and labor. Even today, in the current era of next generation sequencing the applicability and importance of T-RFLP is not diminishing, particularly for the study of community dynamics and effect of external factors on community structure and functions. It seems that, in future, with improved 16S rRNA gene sequence database and primers, use of refined protocols and statistical tools for data generation and analysis, will improve the resolution and depth of the technique and T-RFLP will be able to provide quick and accurate information about community structure and dynamics in low cost and labor. In a nutshell T-RFLP is not going to be completely obsolete and will remain valuable in the field of microbial ecology.

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