

Modification of the terminal restriction fragment length polymorphism analysis for assessment of a specific taxonomic group within a soil microbial community

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ABSTRACT

A double color terminal restriction fragment length polymorphism analysis was applied for the diversity assessment of a complex bacterial community in a deciduous forest soil. Application of the second label was used to project a specific taxonomic group of bacteria, actinomycetes in our case, to the universal fingerprint. The diversity of actinomycetes was evaluated directly as a percentage of the whole bacterial community by comparing the corresponding terminal fragments of the same length.

Keywords: bacterial community; *Actinobacteria*; terminal restriction fragment length polymorphism

Rapid analysis of diversity in complex microbial communities remains an elusive but important goal in microbial ecology (Dunbar et al. 2000). The shortcomings of methods that rely on cultivation are well known. Direct amplification of bacterial 16S rRNA genes from extracted soil DNA provides the most comprehensive and flexible means of sampling bacterial communities, but the methods that are effective in community analysis are still quite limited in number and scope (Dunbar et al. 2001). Analysis of individual 16S rRNA gene clones in multiple libraries is an expensive and inefficient approach in the studies of bacterial communities. So methods such as thermal or denaturing gradient gel electrophoresis (TGGE, DGGE), heteroduplex analysis, or terminal restriction fragment length polymorphism (T-RFLP) analysis are employed in assessment of the 16S rRNA diversity because they are far more rapid and therefore more amenable to field-scale experiments, in which replication is important (Dunbar et al. 2000).

T-RFLP method relies on variation in the position of restriction sites among sequences and determination of the length of fluorescently labeled terminal restriction fragments (TRFs) by high-resolution gel electrophoresis on automated

DNA sequencers (Avaniss-Aghajani et al. 1994, Liu et al. 1997). The community profile arises from a selected gene, either encoding 16S (18S) rRNA for taxonomical profiling or a protein-coding gene to follow a specific function accomplished by the community, e.g. nitrogen assimilation, humification and mineralization, or secondary metabolite production and resistance (Čermák et al. 2008). The method has been successfully applied to characterization of complex bacterial communities e.g. in soil (Hackl et al. 2004), epiphytic (Becker et al. 2008) and endophytic communities (Conn and Franco 2004), or human intestine (Jernberg et al. 2005, Matsumoto et al. 2005).

The method has its drawbacks which must be considered in data interpretation. First, the method results are influenced by the initial amplification by PCR, which changes the original proportion of selected marker gene and may lead to the loss of gene types of low frequency. However, this is true for most methods but the metagenomic libraries and the Geochip (He et al. 2007) because only those methods use directly the whole community DNA in a real proportion. Yet, their protocol is very time consuming and expensive and more so, the detection limits are substantially higher. Second,

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TRF profiles cannot provide reliable information describing relative phylotype richness and evenness (Dunbar et al. 2000). Third, the measurement of TRF lengths by sequencer produces continuous data, while the DNA sequence length is a discrete variable, so the transformation leads to mistakes in some peak lengths identification. And finally, given a random nature of restriction site occurrence in the sequence, the data follow no hierarchy, i.e. the TRF size represents a single isolated feature and TRF size similarity does not implicate any degree of sequence homology.

However, T-RFLP analysis has also many advantages as compared to other DNA (PCR) based techniques (Marsh 1999): (i) T-RFLP technology with capillary or slab gel electrophoresis (using DNA sequencer) has considerably higher resolution than the commonly used gel electrophoretic systems; (ii) quantity and size of TRFs are detected by laser-induced fluorescence and the output is digital and readily converted into numeric data which enables subsequent statistic analyses; (iii) T-RFLP offers a possibility to compare the obtained data with data stored in sequence databases, e.g. the Ribosomal Database Project (RDP) or with data from other studies; (iv) also, a common drawback of hybridization and PCR detection is that only those genes, which are sought to be detected and the choice of their sequence is limited by a number of sequences currently available in public databases. The T-RFLP analysis overcomes this restriction. The amplification of 16S rDNA is accomplished from primers universal for eubacteria, i.e. unknown sequences within this taxon are not lost (with an exception of bias resulting from predominance of a few species) and may be revealed by appropriate restriction. T-RFLP is capable of simultaneous detection of more than 100 TRFs. Individual TRFs correspond to various taxonomic groups depending on endonuclease digestion, which may be optimized to focus on the taxa of interest. The detected TRF may be assigned to a taxonomic group or species provided its 16S rDNA sequence is available. However even for unknown species, the method yields an information on their presence and TRF sizes, which may be used to clone the respective fragments and thus to identify the new species. If one or a few species predominate in the sample, the whole pattern is affected resulting in undetectability of minor species. The problem can be solved by PCR amplification from sets of appropriate group-specific primers rather than from the universal ones.

It can be concluded that the method proved so far very effective in elucidating similarity relation-

ships between communities and has good detection sensitivity. The TRF method should be especially used for rapid analysis of replicate samples in field scale studies (Dunbar et al. 2000). The robust ability of the method to differentiate microbial communities has been validated. However, for highly complex soil bacterial communities, the method showed to be ineffective in assessing relative phylotype richness and structure (Dunbar et al. 2000). The method we propose represents an approach which can precisely assess the microbial community structure.

MATERIAL AND METHODS

Site. Soil was collected at a site of a deciduous forest composed of *Quercus robur* and *Carpinus betulus* near Srbsko in the Czech Karst area, the Czech Republic. The soil corresponds to a Cambisol and contains (%): clay – 26, silt – 57, and sand – 17.

Sample preparation. The essential step in sample preparation is DNA extraction. It has been shown that inadequate extraction procedure can change both the quantity and quality of environmental DNA and lead to non representative T-RFLP profiles. The recommended DNA extraction was described previously by Sagova-Mareckova et al. (2008).

Double color terminal restriction fragment length polymorphism for 16S rRNA gene. PCR amplified 16S rRNA genes or their fragments end labeled with a fluorescent dye (e.g. HEX, FAM) were cleaved with a site-specific restriction endonuclease to obtain genetic fingerprints of bacterial communities. In short for an example of eubacterial-actinomycete profile: purified PCR products from the 16S rDNA region using primers 16Seu27f-HEX and 16Seu783r (Sakai et al. 2004) for the eubacterial amplicon and purified PCR products of the 16S rDNA region amplified by the same universal forward primer 16Seu27f but labeled with FAM and actinomycete specific reverse primer 1114act (Kyselková et al. 2008) are mixed in a ratio 2:1. Always, the common primer must be labeled by a different color for each of the two or more specific taxonomic groups assessed. The total amount of PCR products in the mixture should be at least 200 µg and the same in all samples. Then, the PCR product mixture is cleaved by a frequently cutting restriction enzyme, e.g. AluI, HaeIII or MspI. Appropriate restriction endonucleases with high phylogenetic resolution are selected from TRF prediction computed by

ARB (Ludwig et al. 2004) and TRF-CUT (Ricke et al. 2005) software or using web-based tools MICA 3 (Shyu et al. 2007) with sequence data from Ribosomal Database Project. After inactivation of the restriction enzymes and purification, the arising fragments are separated on a capillary sequencer with internal Rox-labeled size standard and analysed in GeneScan (Applied Biosystems) software.

Crude data elaboration. Crude profiles are filtered, i.e. reduced to only the largest TRF peaks representing 95% of the total of peak heights (areas), to eliminate the noise intermittently exceeding the cut-off limit (Abdo et al. 2006). The filtered profiles are suitable for statistical evaluation using PCA or clustering analysis.

RESULTS AND DISCUSSION

The method of double color T-RFLP was tested in a simultaneous profiling of the whole bacterial (Figure 1A) and particular actinobacterial (Figure 1B) communities in a deciduous forest soil in Srbsko in the Czech Karst (Čermák et al. 2008, Kyselková et al. 2008). In bacterial and actinobacterial profiles, 39 and 31 peaks were detected, respectively. The actinobacterial profile was compared with a set of 72 16S rDNA sequences cloned from the site (Kyselková et al. 2008) providing direct identification for a part of the profile (Table 1). The taxa contributing TRF sizes not identified among the clones were estimated by *in silico* simulated amplification 16S rDNA sequences from the RDP

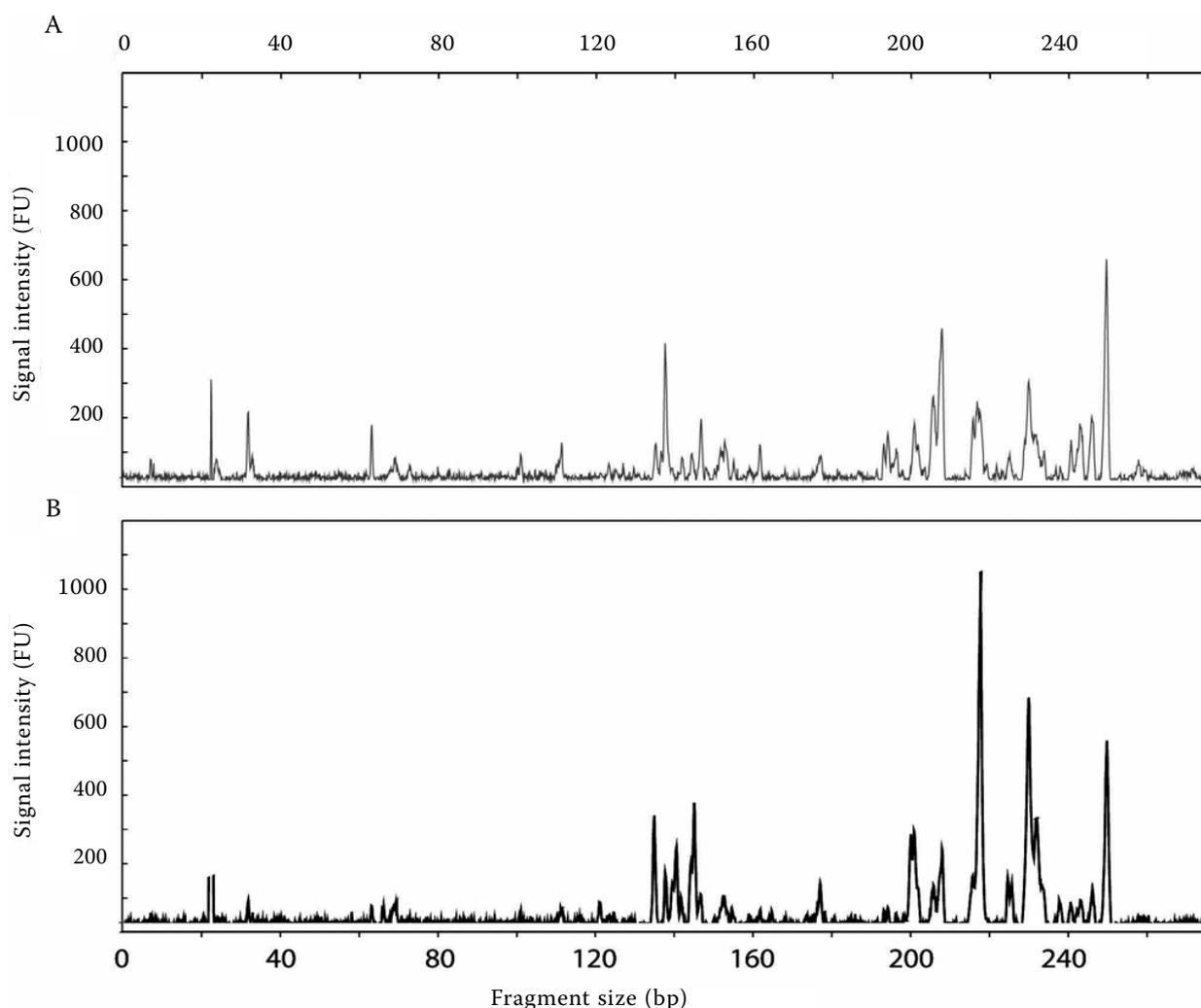


Figure 1. T-RFLP profiles of soil bacterial community generated by AluI cleavage of 16S rDNA amplicons prepared with primers (A) universal for *Bacteria* and (B) specific for *Actinobacteria* (FU, fluorescence unit)

Table 1. TRFs detected in bacterial and actinobacterial profiles with estimation of the corresponding taxonomic group based on cloned 16S rDNA sequences (in bold) or *in silico* amplification and cleavage of database sequences

Detected TRFs		No. of clones	Taxonomic group identification		
<i>Bacteria</i>	<i>Actino-bacteria</i>		suborder	family	other
63	63				uncultured
	66		Frankineae	Frankiaceae	
69	69		Micrococccineae	Promicromonosporaceae	(+ uncultured)
101					
111					
	121				uncultured
135	135		Corynebacterineae	Gordoniaceae	
137	137		Corynebacterineae	Nocardiaceae, Mycobacteriaceae	
	140	3	Propionibacterineae	Nocardiodaceae	
	141		Corynebacterineae	Corynebacteriaceae	(+ Coriobacteriales)
142					
144					
	145		Micrococccineae	Brevibacteriaceae, Cellulomonadaceae	
	146		Micrococccineae	Micrococccaceae	(+ uncultured)
147					
152	152	2	Propionibacterineae	Propionibacteriaceae	
153					
155					
162					
177	177		Streptosporangineae	Nocardiopsaceae	
193					
194	194		Corynebacterineae	Mycobacteriaceae	(+ uncultured)
196					
	200	1	Micromonosporineae	Micromonosporaceae	
201	201	1	Corynebacterineae	Mycobacteriaceae	
206	206		Streptomycineae	Streptomycetaceae	(+ uncultured)
208	208				uncultured
216	216				uncultured
217	217		Kineosporineae	Kineosporiaceae	uncultured
	224				uncultured
225	225		Propionibacterineae	Nocardiodaceae	(+ uncultured)
230	230	1	Micromonosporineae	Micromonosporaceae	
231					
	232	7	Micromonosporineae	Micromonosporaceae	
234					
	237	3	Propionibacterineae	Nocardiodaceae	
	240				uncultured
241					
243	243				uncultured
246	246	9	uncl. Actinobacteria		
249					
	250				uncultured
258					
315					

database with the same primers and subsequent calculation of theoretical AluI TRF sizes.

The method of multiple colors T-RFLP profiles can be mostly used to identify the percentage of a specific taxonomic group in the total diversity of a particular soil microbial community. In fact, the same approach can be used in the assessment of percentage of a particular gene type in any selected functional gene homologues. Above that, the method is also suitable in finding the diversity of the cultivable versus non cultivable community fraction by analyzing the community DNA extracted from a cultivation plate and comparing it to that of environmental DNA by using two different labels for the forward or reverse primers.

The method of multiple colors T-RFLP profiles can also help in diminishing some of the T-RFLP troubles and disadvantages. The deviation in peak retention times in capillary electrophoresis is considered one of the factors influencing the T-RFLP results significantly (Marsh 2005, Hartmann and Widmer 2008). So far, the best solution of this problem was, to our opinion, suggested by Nakano et al. (2008) who proposed to use both forward and reverse labeled primers and compare the movement of the fragment lengths from both sides. The method we propose can also adjust the deviation at least for the comparison between the two or multiple profiles.

The second most important influencing factor was found to be the differences in the amplification efficiency by PCR. This problem has not been overcome because also qPCR performed with universal primers enhances certain sequence amplification over others since the primers are degenerated. However, it was demonstrated that proportion of individual strains in an artificial community remained generally constant after quantity manipulation of individual strains (Hartmann and Widmer 2008). This finding is well in line with our observation that the clones we received from the same amplicon as the T-RFLP always reflected the major peaks in any T-RFLP profile. Consequently, it seems that the dominating taxa in a community may be determined with a reasonable precision in spite of the PCR's negative influence. Again, the multiple colors T-RFLP method comparing several different restrictions in one run can further improve the picture by direct assignment of some peaks to a particular taxonomic group.

The third issue often discussed in the association with T-RFLP application is the incomplete or nonspecific restriction which can increase the number of fragments. It was suggested that this

shortcoming can be treated by including an amplified product from a well-characterized individual sequence in the restriction step (Marsh et al. 2000). The method we proposed includes this step only in a modified version by comparing two or more profiles in a taxonomic hierarchy.

Further, the method we propose can also lead to a more detailed identification of sequences because two independent restrictions are performed on each sequence type.

It was shown that the analysis of independent digests using multiple restriction enzymes increases specificity because an individual TRF may correspond to 15 or more species (Nocker et al. 2007). The proposed method similarly as the digest by multiple enzymes offers an approach to the fragment lengths identification and leads to increased confidence of ecological interpretations.

Finally, it has been observed previously that the whole community assessment using universal eubacterial primers by T-RFLP is not as precise and reproducible as when the assessment is limited to a lower taxonomic unit, i.e. division (Dunbar et al. 2001). The proposed method answers the needs very well because in the multiple colors T-RFLP profile the specific profile(s) can show simultaneously the more precise specific profile on the background of the universal profile and can serve in both identification of peaks in the universal profile and also in the precision of the universal profile by the direct comparison of multiple profiles. In a direct comparison of two or several taxa, the mistake of fragment length assignment coming from the transposition of continuous to discrete data is overcome by the proposed method because of the direct comparison of two or more profiles due to the simultaneous cleavage, separation and fragment size assignment.

In conclusion, T-RFLP was described as a valuable method for comparison of complex communities when high throughput and high sensitivity are required without the need for direct sequence information (Nocker et al. 2007). The approach appeared to reliably detect the relative changes in microbial community structures with a high quantitative precision, which is a prerequisite for monitoring effects on microbial communities (Hartmann and Widmer 2008). However, Hartmann and Widmer (2008) concluded that owing to the above mentioned biases, representation of the real microbial community structure and diversity in a given sample will probably not be possible. The method we have described does not present a straight forward solution for the

problems described above but offers a considerable improvement in all assessments which include a community structure description.

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