

Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities

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Abstract

Microbial populations in complex environmental samples are difficult to characterize; current techniques are incomplete and time consuming. We investigated a polymerase chain reaction (PCR)-based method for rapidly comparing bacterial communities independent of culture or cloning. Community 16S rRNA genes were amplified and fluorescently labeled by PCR. The labeled products were digested by a restriction enzyme and the labeled, terminal restriction fragments (TRFs) were separated by electrophoresis and detected by laser-induced fluorescence on an automated gene sequencer. PCR parameters were optimized using an in vitro model community of known organisms. Community comparisons were made between deer fecal pellets, petroleum hydrocarbon-contaminated sands and pristine sand. Principal components analysis (PCA) was used to compare the resulting TRF patterns (TRFPs). Patterns derived from a single enzyme digest did not result in accurate community characterizations. Accurate characterizations reflecting the expected bacterial community biology were only achieved by combining TRFP data derived from different enzyme digestions. Suggestions are offered for future use of this technique.

Keywords: 16S rRNA; Bacterial communities; PCR; Principal components analysis; Restriction analysis; TRFP

1. Introduction

Microbial ecology studies are limited by the complication of identifying and counting the microorganisms present in any given ecosystem. Visual observations are limited by the small number of possible morphologies. Biochemical tests, while more informative than morphological studies, are restricted to cultured species. Genetic techniques, particularly those that utilize the polymerase chain reaction (PCR) to amplify sample DNA, are more useful than culture or direct observation for assessing sample diversity [1–5]. While PCR-based methods can assess a wider range of organisms than culture techniques, they are limited by inherent biases. Sequence-based differential amplification and/or poor homology between templates and ‘consensus’ primers can result in unequal or no amplification of certain templates in a community [6,7].

To date, most PCR-based methods rely on amplifying, cloning and sequencing 16S rRNA genes (16S rDNA) from sample DNA. Cloning can detect both known and unknown sequences in a sample, but the process is time consuming. One must sequence several thousand clones from a single sample to

definitively characterize a soil community that may harbor as many as 4000 species per gram [8]. Identification of individual community members, however, is not required for substantive studies of microbial communities.

Patterns derived from the diversity of 16S rDNA sequences in a microbial community have been used to rapidly characterize environmental samples [2,3]. One example, amplified ribosomal DNA restriction analysis (ARDRA), creates patterns by digesting amplified community 16S rDNA with restriction enzymes [9,10]. While faster than cloning, ARDRA data relies on the visual resolution of all restriction fragments. Visual detection may not resolve a significant fraction of the fragments, thereby reducing the amount of data available for analysis. Additionally, the number of restriction fragments per organism presents a problem when community restriction fragments are visually resolved. Restriction fragment analysis effectively divides the visual signal of any one organism's DNA. Therefore, fragments from organisms with a large number of restriction sites may be eliminated from the resulting pattern while fragments from those organisms with few restriction sites are more likely to appear in the pattern. Ideally, a community restriction pattern technique would both resolve all available fragments and limit those fragments to one per organism.

Fluorescent-labeled PCR, in which one or both primers carry a 5' fluorescent molecule, can be followed by restriction enzyme digestion to produce fluorescent-labeled terminal restriction fragments. This technique was previously evaluated with in vitro cultures as a means of identifying individual bacteria [11,12], and its utility for complex samples was modeled using a sequence database [13]. Patterns reflecting genetic diversity are produced by electrophoretic separation and detection of only the labeled terminal restriction fragments (TRFs) on an automated gene sequencer. In these patterns, each TRF can be used as a data point for community comparison via statistical analyses.

Using 16S rDNA sequences for differentiating bacteria based on TRF length cannot produce the ideal of one data point per species. A TRF pattern (TRFP) produced from amplified 16S rDNA is a simplified representation of the community. Different species, regardless of taxon, may produce identical

length TRFs with a given restriction enzyme [13]. However, organisms that produce identical TRFs with one restriction enzyme may produce unique TRFs with a different restriction enzyme. Thus, a more accurate community representation may be achieved by combining multiple TRFPs derived from different restriction enzyme digests.

Here we report the use of TRFPs in the analysis of different microbial communities; in vitro communities, deer fecal pellets, and coastal dune sands. In vitro control communities were used to optimize methodology, while deer fecal pellets were examined as an example of complex sample communities where inter-sample similarity was expected. Pristine sands and petroleum hydrocarbon-contaminated sands were examined to investigate changes in TRFPs that might correspond to a defined environmental change.

2. Materials and methods

2.1. Organisms and samples

In vitro communities were created by combining DNAs extracted from a pure culture of each strain listed in Table 1. Deer (*Odocoileus hemionus*) fecal pellets were collected in sterile tubes on the campus of California Polytechnic State University, San Luis Obispo, and used for DNA isolations within 24 h. Pellets from two discrete piles were used and labeled Deer 1 and Deer 2. Pristine (Sand 1) and petroleum hydrocarbon-contaminated sand were collected from a coastal oil field at Guadalupe, CA, and stored at 4°C until used. The petroleum contaminants were either a mid-distillation cut (Sand 2), approximately C₈ to C₃₆, or crude and waste oil (Sand 3), approximately C₈ to C₄₂ [14].

2.2. DNA isolations

Both in vitro culture and sand DNA were isolated by a phenol:chloroform-isoamyl alcohol method modified from Sambrook et al. [15] by the addition of a lysis step. Cell lysis was accomplished by incubating (24 h for sand and 1 h for cultures at 28°C) 200 µl of pelleted cells in 200 µl of lysis

buffer consisting of 100 mM NaCl, 10 mM Tris HCl, 25 mM disodium EDTA and 700 U/l lysozyme.

DNA from deer fecal pellets was isolated using the FastDNA Medium-Hard Tissue kit with a FastPrep bead beater (all kit components from Bio 101, Vista, CA, USA). Prior to processing, 50 mg of fecal pellet was incubated in 250 μ l MT Buffer (Bio 101) and 750 μ l 200 mM sodium phosphate buffer (pH 8.0) for 24 h at 4°C in MH FastDNA tubes (Bio 101). The tubes were processed on the bead beater for 5.0 s at 5.0 m/s. The incubation and processing were repeated. DNA was then isolated according to the manufacturer's SpinFilter protocol (Bio 101).

DNA isolated from all sample types was visualized on a 1.5% agarose gel in pH 8.25 TBE buffer (89.2 mM Tris base, 88.9 mM boric acid, 2.47 mM disodium EDTA) stained with ethidium bromide and quantified by comparison with DNA standards (*MspI* digest of pUC18, Sigma, St. Louis, MO, USA).

2.3. PCR

Primers homologous to 16S rDNA conserved regions were used to amplify a fragment from bases 8–536 in *E. coli*. This region and the primer binding sites were identified as optimal for this application using the author's spreadsheet macro analysis of published 16S rRNA sequences [16]. Primers were adapted from Bruce et al. [17] by the addition of a G to the 3' end of the reverse primer, K2R. The forward primer, K1F, was 5' labeled with FAM®, a fluorescent sequencing dye (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA). The sequences of K1F and K2R were as follows: K1F, 5'-AGA GTT TGT TCC TGG CTC AG-3' (consensus for eubacterial SSU rDNA [17]); K2R, 5'-GTA TTA CCG CGG CTG CTG G-3' (universal to all SSU rDNA [17]). The underlined T in K1F replaces an A from the consensus primer to eliminate a *Sau3A* cut site. The underlined C is an additional one-base pair mismatch with K1F for the published *P. vulgaris* 16S rDNA sequence [16]. All other in vitro community organisms used had one or fewer mismatches.

Reactions were carried out in 50 μ l with 1 \times buffer (Perkin-Elmer), 0.6 mM dNTP, 8 μ g/ml bovine serum albumin, 2 mM MgCl₂, and 1 U of Taq DNA polymerase (Perkin-Elmer). To overcome

inhibitors, template DNA for fecal and sand samples was diluted to 0.5 ng per reaction, at which point all samples amplified equally. Two in vitro communities were constructed by combining either equal or random amounts of DNA extracted from pure cultures. Reaction temperatures and cycling for fecal and sand samples were: 94°C for 2 min, 35 cycles of 94°C for 1 min, 48.5°C for 1 min, 72°C for 2 min, followed by 72°C for 10 min. In vitro communities were subjected to the same PCR parameters with the addition of successive trials at annealing temperatures of 55, 51 and 45°C. Triplicate reactions were prepared for sand and fecal samples. Products were visualized on 2.0% agarose gels and quantified as above.

2.4. Primer removal and amplicon concentration

For sand and fecal pellet samples, replicate PCR reactions were combined in 30-kDa molecular weight cut-off filters and concentrated. The concentrate was washed twice and resuspended in sterile deionized water. Resuspension volumes were varied to equalize amplicon concentrations.

2.5. Amplicon digestion

Digestion reactions contained 0.1 μ g of labeled DNA and were incubated for 12 h at 37°C. The 15- μ l reactions contained 1.3 U/l of either *Sau3A*, *HaeIII*, *MspI* (New England Biolabs, Beverly, MA, USA) or *HhaI* (Promega, Madison, WI, USA) in the manufacturer's recommended reaction buffers.

2.6. Electrophoresis and fragment size determination

Electrophoresis samples containing 2 μ l of digestion reaction, 5 μ l formamide (BioRad, Benecia, CA, USA), 1 μ l load buffer and 1 μ l ROX500® size standard (Perkin-Elmer, Applied Biosystems Inc., Fremont, CA, USA) were denatured at 95°C for 5 min. Approximately 2 μ l of each sample was applied to a 6% polyacrylamide (19:1 bis from BioRad) gel and run at 30 W for 16 h on an ABI 373 Sequencer. TRF sizing was performed with the electropherogram output from GeneScan 2.0.2 software using a third-order least-squares size curve and heavy smoothing. Manual peak calling was made for peaks

with a minimum height of 20 fluorescence units above background. Fragments were resolved to one base pair by manual alignment of the size standard peaks from different electropherograms. Inconsistencies in the Genescan software peak calling routine prevented the use of peak area data.

2.7. Data analysis

TRFPs from all five environmental samples were compared using principal components analysis. Sample data consisted of Boolean character sets (1 or 0) corresponding to the absence or presence of a given TRF in a pattern. The total number of elements analyzed equaled the number of unique TRFs observed in all samples digested with the same enzyme. Analyses were performed for each set of samples digested with a given enzyme and for all three enzyme digestions combined.

3. Results and discussion

3.1. Single organism terminal restriction fragments

In order to identify individual organisms in a TRFP from the in vitro community, TRF lengths were determined for all organisms used. In cases

where database entries existed for the organism, TRF lengths were within 5 bp of the predicted length. An extra TRF was observed for some organisms. In most cases the shortest TRF was the most abundant fragment. Longer TRFs corresponded to either undigested amplicon or the predicted length of partial digestion products. *M. luteus*, *P. fluorescens*, *A. tumefaciens* and *M. phlei* consistently produced multiple TRFs, possibly indicating 16S rDNA sequence heterogeneity in these organisms (Table 1). Multiple heterogeneous 16S rDNAs in a single organism have been previously observed in *Mycobacterium terrae* [18] and some marine isolates [19]. Digestion with *MspI* resulted in similar or identical length TRFs for five different organisms (Table 1). Therefore, *MspI* was excluded from further testing with the in vitro community. *Sau3A* and *HhaI* resulted in unique TRF lengths for all organisms, although some TRFs differed in length by a single base pair (Table 1). *HaeIII* was not tested on the in vitro communities.

3.2. In vitro community TRFPs

The creation of TRFPs from the in vitro community was used to optimize PCR and enzyme digestion reactions. When the PCR annealing step was 48.5°C, expected TRFs corresponding to all in vitro com-

Table 1
Predicted and observed TRF lengths for in vitro community organisms

Organism	Amplified fragment length: predicted	TRF length after digestion with <i>Sau3A</i> :		TRF length after digestion with <i>MspI</i> :		TRF length after digestion with <i>HhaI</i> :	
		predicted	observed	predicted	observed	predicted	observed
<i>Proteus vulgaris</i>	529	121	125 , 278	496	497	213	214
<i>Mycobacterium phlei</i>	503	113	118 , ~500	160	163 , 284 ^a	367	368
<i>Enterobacter aerogenes</i>	NDE	NDE	278 , ~520	NDE	497	NDE	371
<i>Micrococcus luteus</i>	505	505	215 ^a , ~500	163	165	369	~ 510 , ~ 515 ^a
<i>Citrobacter freundii</i>	529	273	277 , ~520	496	494 , ~525	373	373
<i>Pseudomonas fluorescens</i>	NDE	NDE	194 , 271	NDE	493	NDE	155, ~ 530
<i>Streptococcus faecalis</i>	NDE	NDE	323 , 437	NDE	~ 545	NDE	218
<i>Alcaligenes faecalis</i>	529	196	200 , 277	496	497	67	67
<i>Agrobacterium tumefaciens</i>	471	240	244 , 471	402	128 ^a , 407	340	339 , 470
<i>Staphylococcus hominis</i>	537	305	310 , 426	156	156 , ~540	239	239
<i>Flavobacterium indolgenes</i>	518	66	69	202	206 , 488	225	227

NDE, no database entry for predictions [16]. Bold entries indicate a TRF whose peak area was greater than 40% of total peak areas.

Note: electropherogram peaks resulting from fragments longer than 500 base pairs were resolved to ± 5 bp.

^aPeak of unknown origin; not a predicted partial digestion product.

munity organisms except *P. vulgaris* were detected. Annealing temperatures greater than 49°C resulted in the loss of some fragments while temperatures less than 48.5°C did not increase fragment recovery (Fig. 1). TRFPs for the in vitro community were identical among replicate digestions for all enzymes tested (data not shown). Although some organisms in the community produced secondary TRFs (Table 1), the community TRFPs were reproducible and consistent (data not shown).

3.3. Bacterial community TRFPs from environmental samples

The optimum PCR parameters identified using the in vitro community were next applied to create TRFPs from environmental samples. The choice of

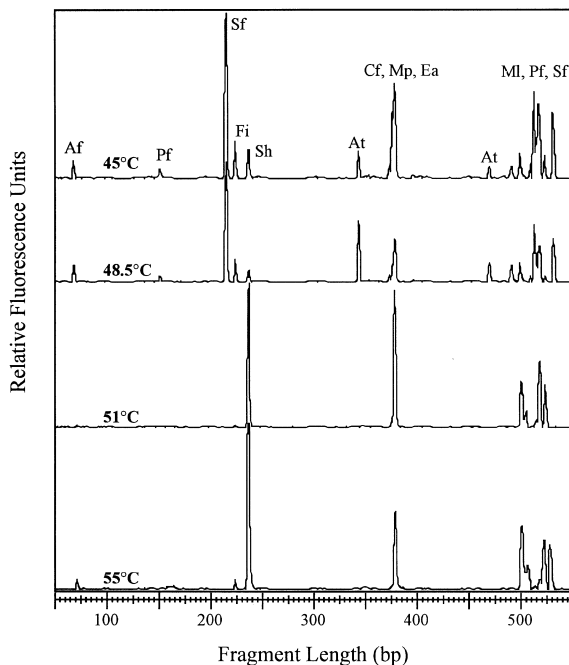


Fig. 1. Electropherograms of the in vitro community subjected to five different annealing temperatures during PCR and digested with *HhaI*. Labeled peaks correspond to the TRF for individual organisms (Table 1). Abbreviations of organisms: Af, *Alcaligenes faecalis*; At, *Agrobacterium tumefaciens*; Cf, *Citrobacter freundii*; Ea, *Enterobacter aerogenes*; Fi, *Flavobacterium indolgenes*; MI, *Micrococcus luteus*; Mp, *Mycobacterium phlei*; Pf, *Pseudomonas fluorescens*; Sf, *Streptococcus faecalis*; Sh, *Staphylococcus hominis*.

Table 2
Number of terminal restriction fragments per pattern

Sample	<i>MspI</i>	<i>HaeIII</i>	<i>HhaI</i>	Total number of TRFs
Deer 1	24	35	36	95
Deer 2	26	48	32	106
Sand 1	56	60	36	152
Sand 2	30	50	39	119
Sand 3	41	37	30	108

enzyme greatly affected the number of TRFs visible in any one pattern. The enzyme *HaeIII* gave the greatest number of TRFs in three out of five samples. The maximum number of distinguishable TRFs as postulated by Brunk et al. [13] was not reached with any of these samples. Each TRFP had 25–60 discernible TRFs that could be used to differentiate one bacterial community from another (Table 2). Fig. 2 shows complete *HaeIII* TRFPs as an example; Fig. 3 shows the one-base pair resolution of the technique. Visual inspection of the TRFPs in Fig. 2 provides intuitive confirmation of the differences between the bacterial communities sampled. The deer samples appear to be very similar and still quite different from all the sand samples. However, an objective analysis of community relatedness can be achieved

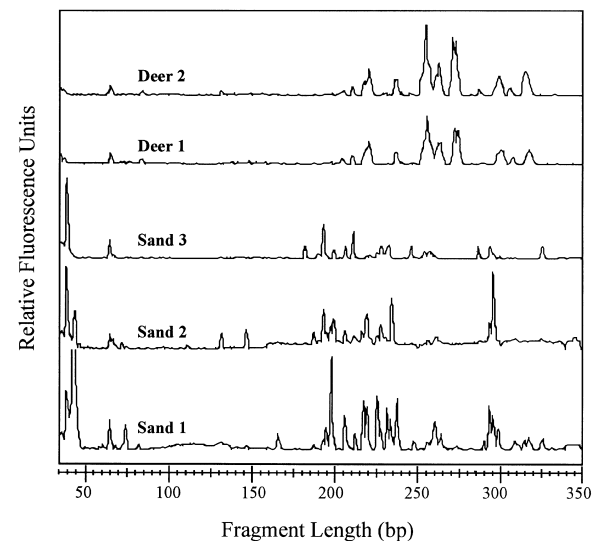


Fig. 2. Electropherograms of the TRFPs derived from *HaeIII* digestions of environmental bacterial community DNAs.

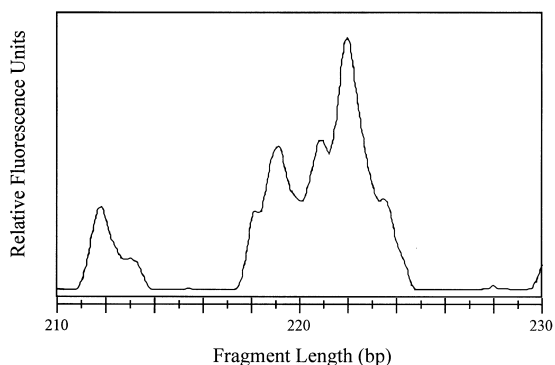


Fig. 3. Close-up of the *HaeIII* TRFP from Deer 2 showing 1 bp TRF length resolution.

only by assigning a defined length to each TRF in a pattern.

Several issues are important when calling peaks to one-base pair resolution. We found Genescan version 2.0.2 software inconsistent in its recognition of peaks. However, manual peak calling introduces a subjective element to data collection. To limit this effect we maintained a dual panel view with samples in one panel and the internal size standards in the

second. This allowed alignment of two different electropherograms and the comparison of sample peaks to standard peaks. Even with these procedures, wide peaks and those with ‘shoulders’ presented problems. Size standard peaks tend to widen and develop shoulders with an increase in fragment size. Therefore, resolution is lost with increasing fragment sizes. We found that consistent well-formed peaks were rare for TRFs greater than 350 base pairs, and therefore our analysis only included peaks smaller than 351 base pairs.

3.4. Multivariate analysis of TRFPs

Principal components analysis (PCA) of the TRFPs arranged the five samples differently depending on the enzyme used to create the pattern (Fig. 4a–c). Analysis of the *MspI* and *HaeIII* TRFPs alone failed to produce significant clusters. The *HhaI* TRFPs did place the deer fecal samples together, but also paired a petroleum hydrocarbon-contaminated sand (Sand 2) with the pristine sand (Sand 1). However, when the information from all three digests was combined, each community TRFP had

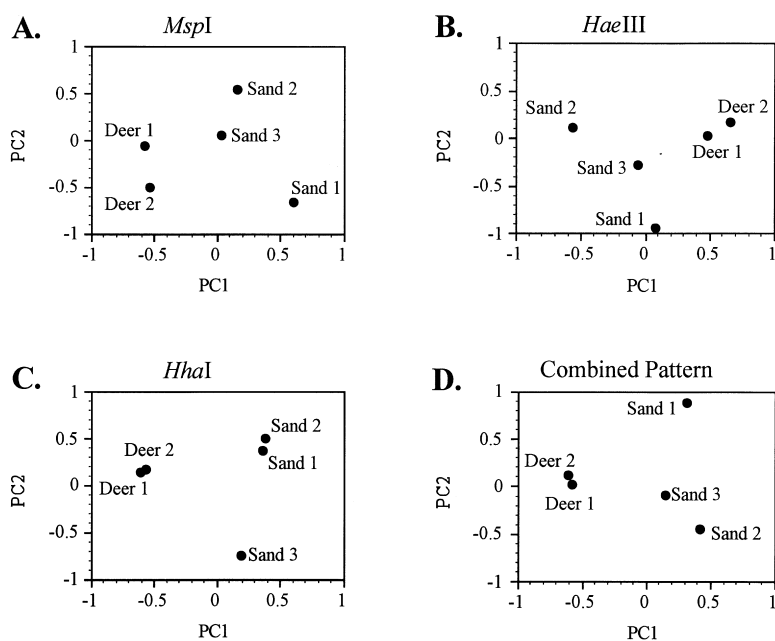


Fig. 4. Principal component analyses of TRFP data sets from environmental samples. Data sets derived from digestions with: (A) *MspI*; (B) *HaeIII*; (C) *HhaI*; and (D) all three digests combined.

from 98 to 152 elements for analysis (Table 2). PCA of the combined data resulted in a clear pairing of the two deer fecal samples (Fig. 4d). Principal component 1 (PC1) separated deer fecal samples from sand samples, and principal component 2 (PC2) separated the pristine sand from the two petroleum-contaminated sands. Thus the PCA results correlated with the expected bacterial community biology of each sample best when the data from multiple enzyme digestions was combined. Essentially, the complex pattern expected from the community is only reflected in the combination of several relatively simple TRFPs.

3.5. Limitations of the method

Our work has identified some limitations of the TRFP technique as described here. (i) The tendency of PCR to differentially amplify templates prevented quantification of relative abundance in the community [6]. The *in vitro* community DNA produced TRFPs of unequal relative abundance (electropherogram peak area) with different annealing temperatures (Fig. 1). Thus, TRF abundance does not reflect an organism's abundance within the sample community. Furthermore, differential amplification can result in the 'loss' of some community templates. For example, the TRF corresponding to *P. vulgaris* was not observed in the *in vitro* TRFP (Fig. 1, Table 1). (ii) Individual TRF detection was limited by electrophoresis technology. One-base pair resolution up to 600 bp would allow maximum information retrieval from PCR with the chosen primer set. Such resolution may be achieved by the new generation of automated capillary gel electrophoresis DNA sequencers. (iii) As expected, patterns derived from a single enzyme digest did not result in accurate community characterizations (Fig. 4). Accurate characterizations were only achieved by combining TRFP data derived from different enzyme digestions. (iv) Statistical analysis for community comparison was restricted to Boolean (1 or 0) data due to software shortcomings. Peak area could be included in TRFP analysis with the use of more capable integration software. We are investigating GelCompar® (Applied Math, Belgium) software for this purpose.

4. Conclusions and recommendations

Distinctive bacterial community patterns can be rapidly generated using fluorescently labeled, PCR-amplified 16S rDNA terminal restriction fragments using the method described here (TRFP). TRFPs will be useful for investigating complex communities where community dynamics are on a time scale that requires rapid analysis and the number of bacterial species in a sample may be quite large. Composed of *only* the terminal restriction fragment(s) from each organism, TRFPs represent a manageable data set. Although different organisms, even unrelated species, produced the same TRF with a given restriction enzyme digestion (Table 1, [13]), this information reduction was mitigated by the use of multiple enzymes (Fig. 4). Organisms that produce identical length TRFs with one restriction enzyme may produce different length TRFs when digested with another restriction enzyme (Table 1). Thus, the best community comparisons result from analysis of the combined data from different enzyme digests (Fig. 4).

This paper does not address our work-in-progress on the effects of DNA extraction, template concentration, and PCR bias on the reproducibility of TRFPs from environmental samples. However, the similarity in TRFPs from different deer fecal pellets (Fig. 2) indicates a high level of reproducibility may be possible. At present, to best describe a bacterial community, we make the following conservative recommendations: first, combine multiple DNA extractions from each well-homogenized sample to minimize extraction bias. Similarly, combine amplicons from multiple PCRs to reduce PCR bias. Load as much as DNA from each digestion as is practical onto the polyacrylamide gel to allow detection of less abundant fragments. Finally, combine TRFP data from three or more different restriction enzyme digests to increase the information derived from each community.

5. Notation

A	adenine
ABI	Applied Biosystems Incorporated (Per-

	kin-Elmer Applied Biosystems Division)
ARDRA	amplified ribosomal DNA restriction analysis
C	cytosine
DNA	deoxyribonucleic acid
G	guanine
PCA	principal components analysis
PCR	polymerase chain reaction
rDNA	ribosomal DNA
rRNA	ribosomal ribonucleic acid
T	thymine
TRF	terminal restriction fragment
TRFP	terminal restriction fragment pattern

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