

Gel Electrophoresis Based Genetic Fingerprinting Techniques on Environmental Ecology

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1. Introduction

Molecular tools in environmental microbiology have been applied extensively in last decades because of the limitations in culture-dependent methods (Amann *et al.*, 1995; Muyzer *et al.*, 1996; Head *et al.*, 1998). Despite isolation techniques are provided detailed knowledge about the single species in terms of morphology, biochemistry, and also genetic (Bitton, 2005), they have important drawbacks. The first one is to find the selective media favoring the desired microbial group. Additionally, isolated species cannot reflect their behaviors in the natural environment. Until today, 19.000 microbial species have been isolated (DSMZ, 2011; <http://www.dsmz.de>), however it is accepted that this number is only a small portion of real diversity (Amann *et al.*, 1995). Besides, using the molecular tools in natural and engineering systems, we can find the answer to the questions such as 'which species do exist?', 'which species are active?', 'how many microorganisms are there?', which species do utilize the specific compounds?'

Microbial ecology studies need identification of species based on a comprehensive classification system that perfectly reflects the evolutionary relations between the microorganisms (Pace, 1996). Zuckerkandl and Pauling (1965) indicated that nucleic acids could document evolutionary history. Due to the pioneering studies, nucleic acids, especially 16S rRNA, are the ultimate biomarkers and hereditary molecules probably because of their essential role in protein synthesis, making them one of the earliest evolutionary functions in all cellular life-forms (Olsen *et al.*, 1986; Pace *et al.*, 1986; Woese, 1987; Stahl *et al.*, 1988). In particular, 16S rRNA and 16S rDNA have been used in phylogenetic analysis and accepted as ideal evolutionary chronometer.

Genetic fingerprinting techniques are one of the most applied molecular tools based on 16S rRNA in microbial ecology studies. These techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), amplified ribosomal DNA restriction (ARDRA) or restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphism (T-RFLP), and single strand conformation polymorphism (SSCP), have been developed for estimation of diversity in ecosystems, screening clone libraries, following the diversity changes with respect to time

and location and also identification of species (Hofman-Bang, 2003). This approach comes into prominence because of fast, less labor-intensive features (Muyzer and Smalla, 1998).

These methods have been used to characterize the microbial diversity in different environments such as activated sludge (Liu *et al.*, 1997; Curtis and Craine, 1998), anaerobic reactors (Leclerc *et al.*, 2004), sediments (Muyzer and De Wall, 1993, Cetecioglu *et al.*, 2009), lake water (Ovreas *et al.*, 1997), hot springs (Santegoeds *et al.*, 1996), biofilm (Santegoeds *et al.*, 1998). The method can be used for as both qualitative and semi-quantitative approaches on biodiversity estimations.

In this chapter, these genetic fingerprinting techniques based on gel electrophoresis are discussed. Also exemplarily applications are presented.

2. Microbial ecology and characterization of microbial community via molecular tools

Biochemical conversions occurred in environment are determined by black box model because of limitations to identify microbial communities which are responsible of these (un)known processes (Amann *et al.*, 1995). Acquisition of pure cultures is necessary to obtain an insight into the physiology, biochemistry and genetics of isolated microorganisms. In spite of developments on cultivation methods everyday, still a small portion of the microbial species within the nature can be isolated by culture-dependent techniques (Giovannoni *et al.*, 1990). Another problem in microbial ecology is the complications on identification and classification of the species based on their morphological features. Since the morphological features of the microorganisms cannot give the detailed information about their evaluation relationships. In order to determine the role of microbial diversity in natural or engineered systems, the questions about microbial population including 'Who is there? How many microorganisms are there them? Where are they located? What are they doing? How do populations respond to changes in environmental conditions? What is the relationship between diversity and community stability?' have to be answered. Accordingly, culture independent methods, which give information about microbial ecosystem in terms of diversity, function, etc., are more reliable (Muyzer *et al.*, 1998; Head *et al.*, 1998).

To increase our knowledge about microbial communities and our understanding of their composition, dynamics and interactions within microbial ecosystems, nucleic acid analysis give a wide range opportunity nowadays. Molecular phylogeny not only employs nucleic acid documentation and evolutionary history but also provides a motivation for identification and quantification of microbial species (Olsen and Woese, 1993). The phylogenetic tree of all living organisms is represented in Figure 1. Ribosomal RNA and its gene are the main biomarkers and hereditary molecules for prokaryotes because of their essential role in protein synthesis making them one of the earliest evolutionary functions in all cellular life-forms (Woese, 1987). Therefore prokaryotes can be detected, identified and enumerated by the analysis of 16S rRNA and 16S rDNA.

16S rRNAs and 16S rDNAs, which encode them, are ideal biomarker because they exist in all prokaryotes, they have conserved and their variable regions give the opportunity to identify species even strains as seen in Figure 2. While the conserved regions of 16S rRNA make this molecule as an evolutionary clock instead of their selectively neutral mutational

changes (Woese, 1987, Amann *et al.*, 1995), their variable regions allow phylogenetic determination on different taxonomic level (Amann *et al.*, 1995; Head *et al.*, 1998).

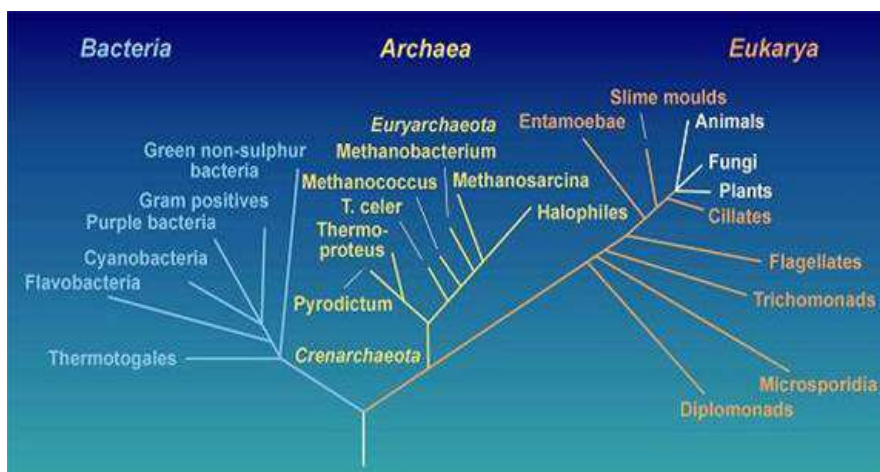


Fig. 1. The rRNA phylogenetic tree of life (Madigan *et al.*, 2009).

As a result, to design general or specific primers and probes for 16S rDNAs and 16S rRNAs provides study options about identification and evolution of microorganisms because this molecule is fairly large (≈ 1500 nucleotides) including sufficient sequence information. Also the abundance is high within most cells (10^3 to 10^5 copies) and they can be detected easily (Amann *et al.*, 1995). While even secondary structure of 16S rRNA molecule is highly conserved, many variable regions randomly change during evolution. This differential variation explains the relationship between microorganisms evolutionarily. Data obtained from this analysis are adequate to compare statistically significant phylogenetic relations (Olsen *et al.*, 1986). Therefore 16S rRNA and its encoding gene have been widely used to investigate community diversity. The rapidly growing 16S rDNA sequence data bank, accessible (<http://www.ebi.ac.uk/>) provides the opportunity to get information about 16S rDNA sequences of the determined cultured and uncultured species (Dahllöf, 2002).

In spite of the advantages of using 16S rRNA molecule for phylogenetic analysis, the main limitations are that the heterogeneity between multiple copies of this molecule in one species interferes pattern analysis, confuses the explanation of diversity obtained from clone libraries and sequences retrieved from banding patterns (Dahllöf, 2002).

3. Fingerprinting techniques and their application areas

Fingerprinting techniques provide a separation in microbial community according to their genetic pattern or profile (Muyzer, 1998). A variety of fingerprinting techniques such as *denaturing/temperature gradient gel electrophoresis*, *amplified ribosomal DNA restriction analysis*, *terminal restriction fragment length polymorphism*, and *single strand confirmation polymorphism* has been developed to assess diversity and dynamics in the ecosystem (Hofman-Bang, 2003). The first fingerprinting technique was used in 1980's, which based on the electrophoretic separation in high-resolution polyacrylamide gels of 5S rRNA and tRNA

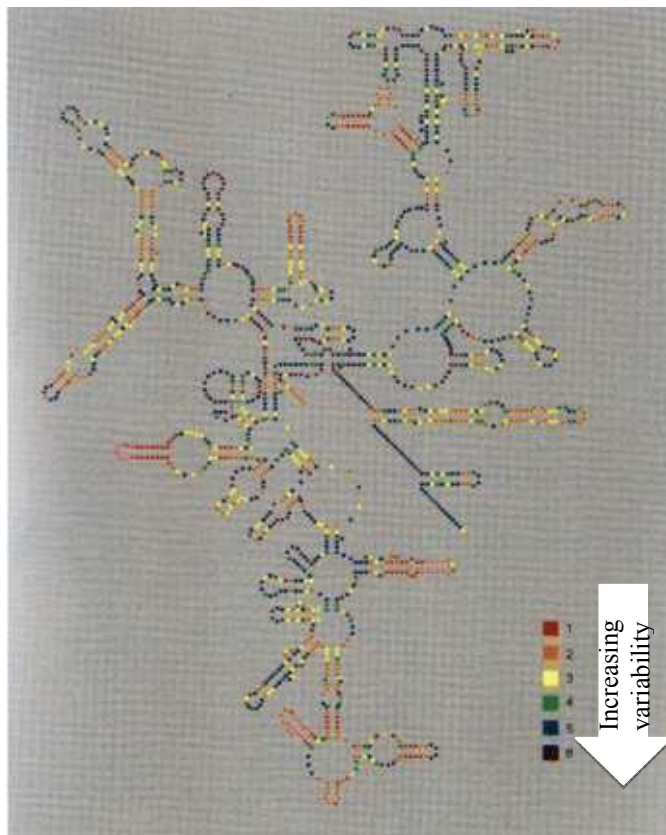


Fig. 2. Secondary structure of the 16S rRNA of *E. coli*, showing conserved and variable regions (Van de Peer *et al.*, 1996).

obtained from natural samples (Hofle, 1988 and 1990). In 1993, Muyzer *et al.* introduced a new fingerprinting technique to apply on microbial ecology, *denaturing gradient gel electrophoresis* (DGGE). In this method, PCR amplified DNA fragments can be separated according to their nucleic acid pattern. This method has become widespread in a short time. Then another similar technique has been developed, *temperature gradient gel electrophoresis* (TGGE). These methods provide not only analysis of the structure and species composition of microbial communities but also identification of several uncultured microorganisms (Heuer *et al.*, 1997 and Cetecioglu *et al.*, 2009).

3.1 Denaturing/Temperature Gradient Gel Electrophoresis (DGGE/TGGE)

DGGE is a gel electrophoresis technique to separate same length-DNA fragments based on their base sequence differences. In theory, it is sensitive to observe even one base difference on sequence because of melting patterns of the fragments (Muyzer *et al.*, 1993). This method provides a fast, and labor-intensive approach to determine the diversity and the microbial community within an ecosystem, to monitor the changes on dynamics and also to screen the

clone libraries (Muyzer and Smalla, 1998). Furthermore, DGGE can be used as qualitative and semi-quantitative approach for biodiversity estimations.

3.1.1 Principles of the experiment

The optimal gradient is the main concern for DGGE/TGGE experiments since the main purpose is separation of DNA fragments according to their melting behaviours. Perpendicular polyacrylamide gels are used according to incremental gradients of denaturants or temperature. The sample including same-length DNA fragment mixtures is loaded to gel for running by electrophoresis. After completing electrophoresis, the gel is stained by a dye such as ethidium bromide, SYBR gold, SYBR green, etc. for obtaining sample pattern. While linear gradient is created by chemical denaturants as urea and formamide for DGGE, temporal temperature gradient is used to separate the DNA fragments in TGGE. Melting pattern of double strand DNA fragments is based on their hydrogen bond content: GC rich DNA fragments melts at higher denaturant/temperature region of the gradient. Complete separation of the double strand DNA is prevented by using GC-clamp primer during the amplification of target DNA region (Dorigo *et al.*, 2005). The schematic explanation of DGGE is given in Figure 3.

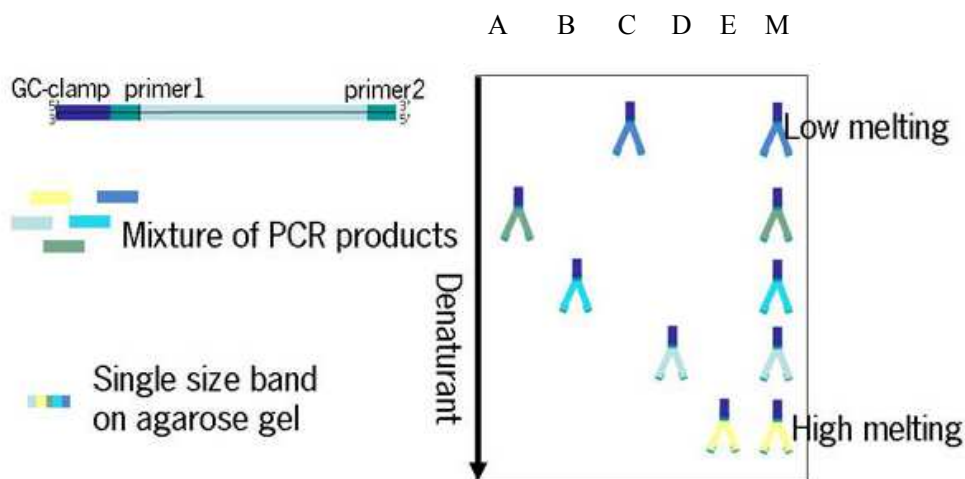


Fig. 3. Principle of DGGE (A: organism a, B: organism b, C: organism c, D: organism d, E: organism E, M: mix sample) (Plant Research International, 2011).

The main difficulties and limitations of the DGGE/TGGE can be listed as:

1. Proper primer selection to represent whole community
2. Optimization of electrophoresis conditions (Muyzer *et al.*, 1993)
3. Limitations on sensitivity for detection of rare community members (Vallaey *et al.*, 1997)
4. Separation of only small DNA fragments up to 500 bp (Muyzer and Smalla, 1998)
5. Biases coming from PCR amplification such as chimeric products or fidelity errors
6. Heteroduplex formations, multiple bands or due to resolution of the gel, or different fragments resulting from existence of several rRNA coding regions, (Curtis and Craine, 1998).

3.1.2 Application area

DGGE/TGGE is used for several purposes in microbial ecology. The first and the most common application is to reveal and to compare community complex of the microbial diversity within different environments. Curtis and Craine (1998) used this technique to show the bacterial complexity of different activated sludge samples. Connaughton *et al.* (2006) used PCR-DGGE method to find out bacterial and archaeal community structure in a high-rate anaerobic reactor operated at 18 °C. This technique was used to reveal the microbial community in a lab-scale thermophilic trickling biofilter producing hydrogen (Ahn *et al.*, 2005). Another biofilm study showed the bacterial diversity in a river by 16S rDNA PCR-DGGE method (Lyautey *et al.*, 2005). In another study, the authors showed that the different bacterial and archaeal profiles within the highly polluted anoxic marine sediments in the different locations from the Marmara Sea (Cetecioglu *et al.*, 2009). Ye *et al.* (2011) showed the temporal variability of cyanobacteria in the water and sediment of a lake.

Furthermore the scientists use these techniques, mostly DGGE, to analyse the community changes over time. Santagoeds *et al.* (1998) used PCR-DGGE method to monitor the changes in sulphate reducing bacteria in biofilm. Ferris and Ward (1997) also performed similar approach to reveal seasonal changes in bacterial community from hot spring microbial mat. Kolukirik *et al.* (2011) used 16S rDNA PCR-DGGE technique to represent the local and seasonal bacterial and archaeal shifts in hydrocarbon polluted anoxic marine sediments.

These fingerprinting techniques are widely used to monitor simple communities instead of complex environments. It is one of the detection methods to analyse the cultivation/isolation approaches and to determine the enrichment cultures (Santagoeds *et al.*, 1996; Ward *et al.*, 1996; Teske *et al.*, 1996; Muyzer, 1997; Bucholz-Cleven *et al.*, 1997).

Also DGGE/TGGE are commonly chosen for comparison of the efficiency of the DNA extraction protocols (Heuer and Smalla, 1997; Lieasack *et al.*, 1997) and the screening of the clone libraries (Heuer and Smalla, 1997; Lieasack *et al.*, 1997, Kolukirik *et al.*, 2011) because rapid and reliable results are caused to perform less time (Kowalchuk *et al.*, 1997).

3.2 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Recognition site of restriction enzymes are changed for different microbial species. The principle of amplified ribosomal DNA restriction analysis (ARDRA), also called as restriction fragment length polymorphism (RFLP), is based on this knowledge. The combination of PCR and restriction can, for example, be used for enhanced amplification of minor DNA templates (Green and Minz, 2005).

In the first step of this technique, ribosomal DNA is amplified by PCR to avoid undesired and/or dominant DNA templates. Then, the 16S rDNA PCR products are digested into specific DNA fragments by restriction enzymes. At the final step, the fragments are loaded to high-resolution gel for electrophoresis. The schematic representation of the principle of ARDRA is given in Figure 4. The main advantage of this technique is to provide rapid comparison of rRNA genes (Moyer *et al.*, 1994).

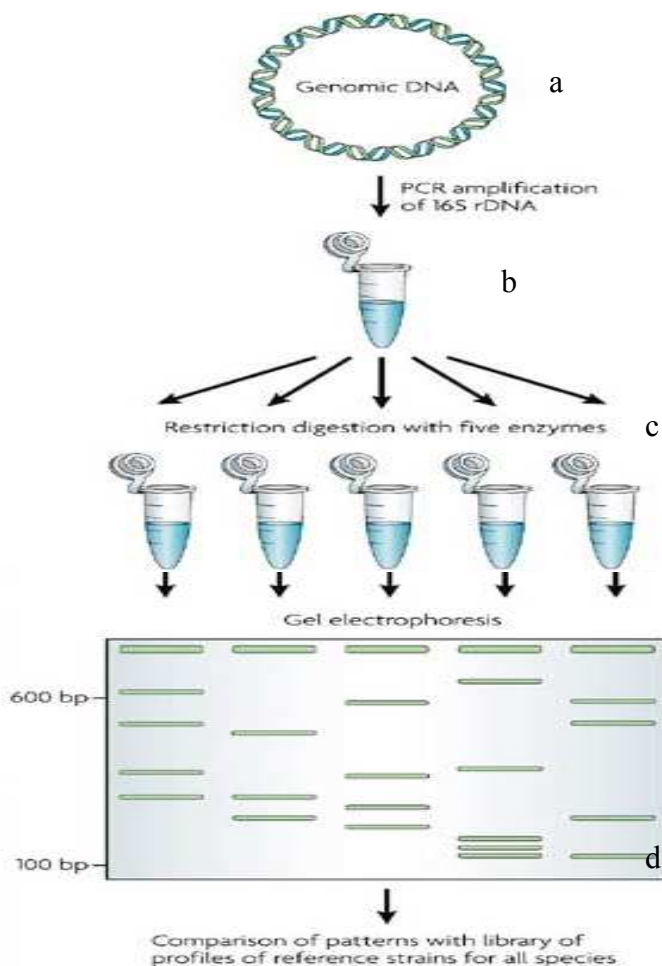


Fig. 4. Steps of ARDRA (a: Genomic DNA extraction, b: PCR reaction for specific region, c: restriction digestion, d: gel electrophoresis) (Dijkshoorn *et al.*, 2007).

The application areas of this technique are also similar to DGGE. It is varied from detection isolates or clones to determination of whole community in an environment. For these different purposes, different gel types can be used. While agarose gel is sufficient to detect isolates or clones, polyacrylamide gels are necessary for better resolution in the community analysis (Martinez-Murcia *et al.*, 1995).

In the literature, there are different studies performed by ARDRA. Lagace *et al.* (2004) identified the bacterial community of maple trees. A wide variety of the organisms were detected from different groups. Barbeiro and Fani used this technique to investigate more

specific bacterial group, Acinetobacteria, within 3 sewage treatment plants (1998). In 1995, Vaneechoutte and his colleagues performed similar study for Acinetobacter strains. They showed that this technique is less prone to contamination problems for detection. In another study, ARDRA was used to screen bacterial and archaeal clone libraries to detect the microbial community within an anaerobic reactor to treat fodder beta silage (Klocke *et al.*, 2007). Also there are some studies to investigate the microbial community in soil (Smith *et al.*, 1997; Viti and Giovannetti, 2005).

3.3 Terminal Restriction Length Polymorphism (T-RFLP)

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is another fingerprinting technique to obtain profiles of microbial communities. The principle of this method is to separate the genes according to position of their restriction site closest to a labelled end of an amplified gene (Figure 5). The main difference from ARDRA is that the restriction enzymes using in T-RFLP only detect terminal restriction fragments (T-RF). Also this method is used qualitative and quantitative analysis like DGGE (Liu *et al.*, 1997).

The method is carried out in a series of steps including PCR, restriction enzyme digestion, gel electrophoresis and recognition of labelled fragments. Like most other fingerprinting techniques, PCR amplification of a target gene is the first step of T-RFLP.

After DNA extraction, target gene amplification is carried out using one or both the primers having their 5' end labelled with a fluorescent molecule. Then amplicons are digested by restriction enzymes. Following the restriction reaction, the digested DNA fragments are separated using either capillary or polyacrylamide gel electrophoresis in a DNA sequencer with a fluorescence detector so that only the fluorescently labelled terminal restriction fragments (TRFs) are visualized. At the final step, electropherom is obtained as a result of T-RFLP profiling. Using this graph, electropherom, only target restricted DNA fragments are detected and also satisfactorily quantified by automated electrophoresis systems. Quantification analysis gives an opportunity to make various statistical methods, such as similarity indices, hierarchical clustering algorithms, ordination methods, and self-organizing maps (Liu *et al.*, 1997).

In the literature, T-RFLP was carried out for different purposes like other fingerprinting techniques. In 1997, while Liu *et al.* used this technique to characterize microbial diversity in different environments such as activated sludge, enriched sludge from lab-scale bioreactor, aquifer sand, termite, Moeseneder and his colleagues (1999) optimized T-RFLP to determine marine bacterioplankton communities and to compare this technique to DGGE. In 2000, Horz and his colleagues reported major sub-groups of ammonia oxidizing bacteria by using *amoA* functional gene. Methane-oxidizing bacteria from landfill site cover soil were detected by T-RFLP combined with RNA dot-blot hybridization (Stralis-Pavese *et al.*, 2006). Also in the same study, RFLP method is used to screen clone libraries. Lueders and Friedrich tried to determine PCR amplification bias by T-RFLP in 2003. Blackwood and his colleagues used T-RFLP for quantitative comparison of microbial communities from different environments such as soil and bioreactors (2003). Additionally this technique was used to screen clone libraries (Moeseneder *et al.*, 2001). Liu *et al.* (2011) performed T-RFLP to determine the microbial shift during bioremediation of petroleum hydrocarbon contaminated soil.

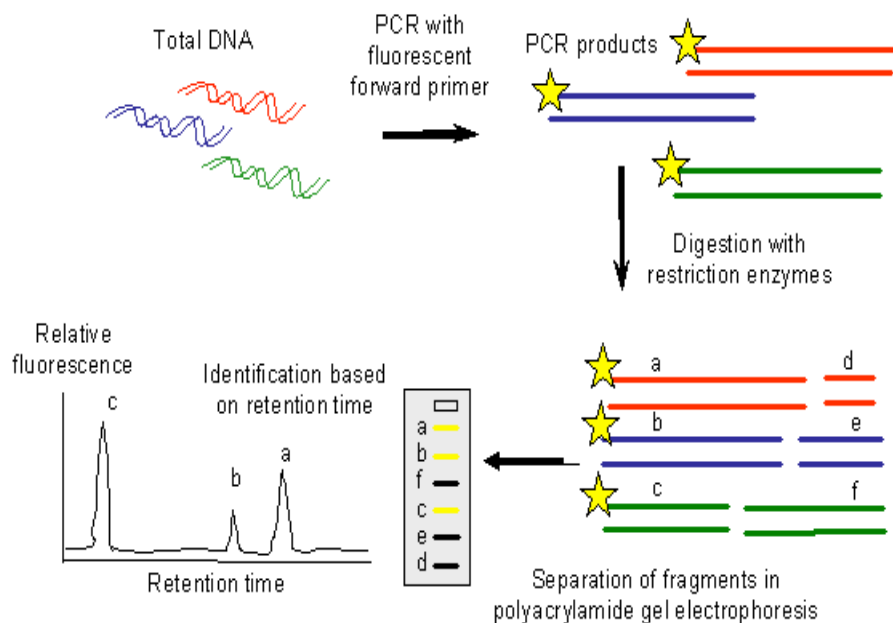


Fig. 5. Steps of T-RFLP (Kaksonen, 2011).

3.4 Single Strand Conformation Polymorphism (SSCP)

Single Strand Conformation Polymorphism (SSCP) is also a fingerprinting technique to separate same-length DNA fragments according to their differences in mobility caused by the secondary structure. The principle of this technique is represented in Figure 6. None of denaturant is used in this method to detect the mobility of the secondary structure of DNA fragments. Each band on SSCP gel corresponds to a distinct microbial sequence, indicating the presence of a microbial strain or species retrieved from the sample (Leclerc *et al.*, 2001; Lee *et al.*, 1996). The main limitation of SSCP, which is similar to DGGE/TGGE, is that one single strand DNA sequence can form more than one stable conformation and this fragment can be represented by multiple bands (Tiedje *et al.*, 1999). The advantage of this technique compared to other fingerprinting methods is that it does not require GC-clamp and gradient gel. SSCP is easier and more straightforward.

SSCP is mostly performed to determine the microbial community profile in different environments such as bioreactor and natural ecosystems. Firstly Lee *et al.* (1996) applied this method to obtain genetic profile of microbial communities. Then Schwieger and Tebbe (1998) used SSCP to determine the community profile including up to 10 bacterial strains. In another study, this method was combined with colony PCR to determine population levels of single and multiple species within plant and environmental samples (Kong *et al.*, 2005). Schmalenberger *et al.* (2008) investigated bacterial communities in an acidic fen by SSCP following by sequencing analysis. In this study, each representative

band was cut, then cloned and sequenced to identify species. Also SSCP was carried out to determine the bacterial profile in an aerobic continuous stirred tank reactor (CSTR) treating textile wastewater (Khelifi *et al.*, 2009). Also this technique was applied for determination of *Clostridium* sp. based on difference their [Fe-Fe]-hydrogenase gene (Quemeneur *et al.*, 2010).

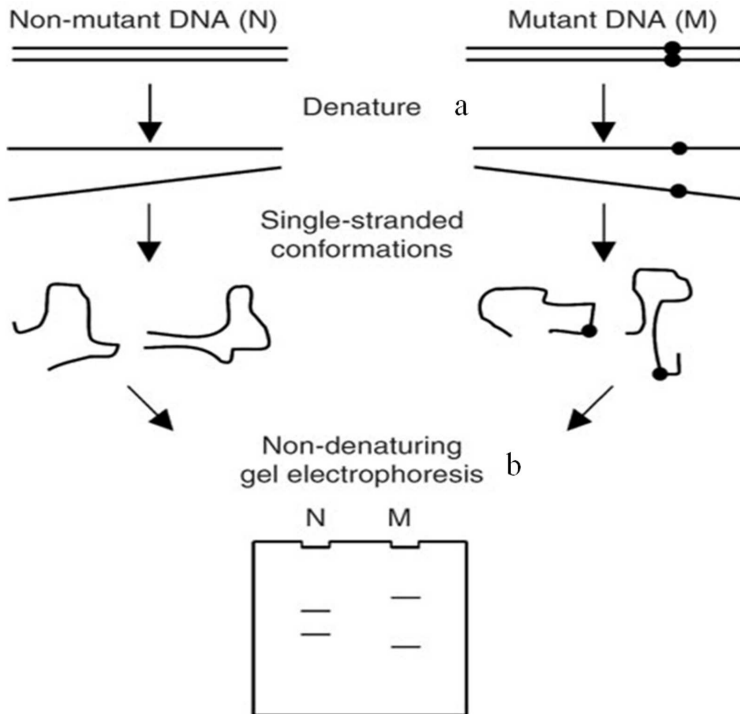


Fig. 6. Steps of SSCP (a: denaturation of ds DNA, b: electrophoresis) (Gasser *et al.*, 2007).

4. Conclusion

The principles of all fingerprinting techniques are similar. DGGE/TGGE, ARDRA, T-RFLP and (SSCP) have been developed to screen clone libraries, to estimate the level of diversity in environmental samples, to follow changes in community structure, to compare diversity and community characteristics in various samples and simply to identify differences between communities. While some of the scientists have showed that sensibilities and resolution of all these techniques are similar, DGGE is still more common application compared to other mentioned techniques. The main reasons of it are that the application of

DGGE is easier and more effective and also less equipment is necessary for it.

5. References

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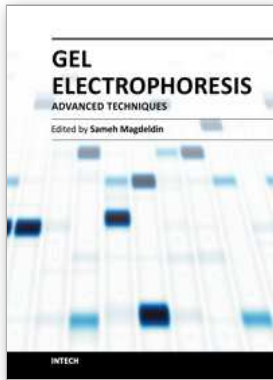
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As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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