Chapter 1
Molecular Approaches of Microbial Diversity in Agricultural Soil

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Abstract Soil presents a highly heterogeneous medium, and the different components of the soil (sand, silt, clay, and organic matter) enable various habitats for microbial communities which are great potential tools for elucidating community interactions in microbial ecology. These communities are made up of a diversity of organisms from bacteria, archaea, and eukarya domains. Microbial diversity in soil has vital importance in understanding the function of natural and agriculture soils.

Soil bacteria and fungi play pivotal roles in sustainable agriculture for removal of toxins and in various biogeochemical cycles consisting of carbon, nitrogen, phosphorus, sulfur important for agricultural soils. Soil microorganisms also promote plant growth, increase resistance against stress, etc.

However, when researching the soil for identification and discovery, problems with pure cultures and enrichment are often encountered. These limitations could be overcome by methodological strategies including molecular techniques such as

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indirect DNA techniques (cultivation of microorganisms and molecular identification), direct DNA techniques (polymerase chain reaction (PCR)-dependent methodologies such as Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Terminal Restriction Fragment Length Polymorphism (T-RFLP), 16S–18S Clone Library, Amplified Ribosomal DNA Restriction Analysis (ARDRA), real-time PCR), fluorescence in situ hybridization, microarray, metagenomics and transcriptomics which can be used in the determination of diversity of soil bacteria.

Recently, current developments in next-generation DNA sequencing methods, such as pyrosequencing and shotgun metagenome using bioinformatics tools, have contributed to increasing scientific attention and understanding of the complexity of microbial communities, functional traits and the relationship between communities and external drivers including environmental factors in soil. This chapter will discuss molecular approaches used for determination of microbiota, challenges encountered, and also future trends in the application of molecular tools to study soil microbial diversity.

Keywords Microbial diversity · Molecular methods · Agriculture · Microbial communities · Soil microbes · Metagenome

1.1 Introduction

Environmental microbiology research is often needed to evaluate the composition and diversity of microbial populations. Cultivation-dependent techniques are important in many ways but are limited for use in this task because of the bias forced by laboratory medium conditions. A diversity of culture-independent techniques targeting ribosomal RNA (rRNA) that solve culture bias by analyzing the structures of microbial communities and diversity based on their phylogenies have been developed. PCR-dependent or -independent microbial population analysis methods offer worthful, cost-effective, and high-throughput measurement of community composition.

The soil has a heterogeneous structure which consists of different solid fraction components that include sand, silt, clay, and organic matter (van Elsas and Trevors 1997; Garbeva et al. 2004; McCauley et al. 2005; Al-Kaisi et al. 2017). Soil is often defined with respect to the area of interest, and the best definition is considered to be a medium which is composed of minerals, organic matter, countless organisms, liquid, and gases. It supports life by acting as food source and habitat, etc. (Al-Kaisi et al. 2017). Therefore, the soil system must be dynamic, stable, and composite to serve these purposes (Garbeva et al. 2004; Al-Kaisi et al. 2017). The progenitor materials and originating factors affect the soil environment and functions and they promote the physical, chemical, and biological characteristics of soils whose characteristic influenced primarily the parent materials, and secondarily on vegetation, topography, and time (Jenny 1941; McCauley et al. 2005; Al-Kaisi et al. 2017).

Physically, soil can be considered to have three phases which consist of the solid, liquid, and gaseous phase. The solid phase shapes the soil matrix, the liquid phase is described as soil solution consisting of water in the soil system, and gaseous phase is defined as the soil atmosphere (Al-Kaisi et al. 2017). The soil matrix comprises particles varying in size, shape, chemical orientation, and number (McCauley et al. 2005; Al-Kaisi et al. 2017). Amorphous substances, particularly organic matter, generate the chemical and mineralogical composition of the soil matrix. They attack the mineral gains and can bind each other. The originated structure is called soil aggregates (Hillel 2003). The three phases of soil are continuously dynamic with constantly changing proportions influenced by the weather, human management, and vegetation. The stability of the soil and aggregates formed within can be deeply affected by tilling and cropping (Al-Kaisi et al. 2017).

Agriculture that has vital importance to ensure food safety, decrease poverty, and protect natural resources is the foundation of human existence. As the world population continues to grow, the need to provide food for agriculture will become one of the biggest challenges facing the agricultural society. To meet this challenge, it is necessary to focus on studying the soil biological system and the entire agricultural ecosystem. Soil is an important natural resource that contributes to the success of sustainable agriculture and interacts with the flora, microbiota, and fauna in the ground. Soil quality can be defined as the soil's ability to fulfill the necessary functions, such as producing healthy crops, resisting erosion, and minimizing its impact on the environment (Sharma 2015).

Faced with climate change, agriculture faces enormous challenges in using limited natural resources to supply food to the growing population. This great challenge cannot be met without sustainable development that meets today's needs and without compromising the ability of future generations to meet their own needs. Sustainable agriculture is a set of strategies, especially management, that can improve or maintain the quality and quantity of food supply without harming the environment or crop productivity in the long run. Sustainable agriculture is very important as it tries to meet our long-term agricultural needs by using special breeding techniques that try to make full use of natural resources that traditional agriculture cannot achieve. The principle is environmentally friendly and provides safe and healthy agricultural products. Microorganisms can promote plant growth and stress resistance, improve soil contaminated with heavy metal, restore nutrients, long-term soil fertility management, and reduce rock and fertilizer mineralization, so they have potential roles in sustainable agriculture (Rashid et al. 2019).

Productive and potential soil microbiota is only suitable for sustainable farming methods and may not be suitable for other alternative methods. Crop rotation is an additional dimension to optimize our soil and crop management practices such as organic change, conservation tillage, crop residue recycling, soil fertility improvement, soil quality preservation, and biological control of plant diseases. If used correctly, microbial communities can greatly benefit from agricultural practices (Singh et al. 2011).

Sustainable agriculture is not a specific set of methods but a broad concept. It includes advances in agricultural management practices and technology and is

increasingly recognized, indicating that traditional agriculture developed after the Second World War could not meet the needs of the growing population in the twenty-first century. Traditional agriculture is faced with reduced production or increased costs, or both. In agriculture, monoculture can cause topsoil depletion, affect soil viability, groundwater purity, beneficial microorganisms, insect life, and make crops vulnerable to parasites and pathogens (Singh et al. 2011).

Fundamental changes have occurred in global agricultural practices and food production. In the past, the main driving force was to increase the yield potential and productivity of food crops. Nowadays, the drive for productivity is increasingly coupled with the desire and even the need for sustainability. Sustainable agriculture involves the successful management of agricultural resources to meet human needs while preserving environmental quality and future natural resources. Improving agricultural sustainability requires the best use and management of soil fertility and its physical and chemical properties. Both depend on soil biological processes and soil microbial diversity. This increases the biological activity of the soil, increasing long-term soil fertility and crop health. This approach is of great concern to avoid degradation in marginal soils and restoration in degraded soils and areas where agriculture is not possible with high external inputs (Singh et al. 2011).

1.2 Microbial Diversity in Soil

Soil is a complex habitat for microorganisms in terms of typical qualities (Nannipieri and Badalucco 2003; Nannipieri et al. 2003; Pisa et al. 2011). The characteristics are grouped into four main headlines:

1. The microbial community of soil is highly variable owing to the rich environment (Nannipieri et al. 2003; Pisa et al. 2011). Soil microorganisms are made up of members of three domains: Eukarya, Bacteria, and Archaea (Fierer and Jackson 2006; Pisa et al. 2011). Microorganisms have easily colonized every area of the world because their genetics enable them to easily adapt. The genetic heterogeneity of microorganism communities causes widespread distribution in the world (Bouchez et al. 2016). So, there are fewer than one million bacteria species and 100,000 fungi species per gram of soil. However, there are a hundred thousand bacterial species in 1 ml of water and per 1 m^3 of air. These microbial communities also symbolize the large ratio of biomass in ecosystems (Bouchez et al. 2016). The bacterial diversity examinations are the most important methods to determine soil conditions according to nutrient cycle and productivity. The soil bacteria have a vital role in many processes consisting of decomposition, mineralization, biological nitrogen fixation, and denitrification (Boyle et al. 2008). Furthermore, some bacteria related to plants support their growth (Gray and Smith 2005; Pisa et al. 2011).
2. Soil is a poor system in the way of nutrient and energy source, compared to the appropriate nutrient medium in other habitats. However, the soil is a system that

consists of dissimilar elements and has no continuity (Stotzky 1997; Nannipieri et al. 2003).

3. The other unique property of soil as a microhabitat is the capability of adsorption of vital molecules such as proteins and nucleic acids by the solid phase (Nannipieri et al. 2003). Enzymes are absorbed by clay minerals or humic molecules, which protect nucleic acids against temperature, pH denaturation (Nannipieri et al. 1990, 2003).
4. The last but not least property is the avoidance of DNA denaturation. Clay, sand particles, and humic molecules are bound to DNA and protect it against the effect of nucleases degradation. The surface of soil mineral compounds has utmost important roles in reaction. However, electron transfer reactions are catalyzed by clay minerals, Mn (III and IV) and Fe (III) oxides. Also, abiotic reactions are catalyzed by clay minerals. These reactions are deamination, polymerization, polycondensation, and ring cleavage (Nannipieri et al. 2003).

The number of archaea and bacteria on the earth are 1.2×10^{30} cells and are spread out in five big habitats including deep oceanic subsurface (4×10^{29}), upper oceanic sediment (5×10^{28}), deep continental subsurface (3×10^{29}), soil (3×10^{29}), and oceans (1×10^{29}) (Flemming and Wuertz 2019). Microbial habitat is affected by the soil's physical and chemical environment, including water and gaseous behavior (Al-Kaisi et al. 2017; Flemming and Wuertz 2019). Therefore, the soil system impacts microbial diversity, efficiency, and performance (Nannipieri et al. 2003; Al-Kaisi et al. 2017). Soil bacteria have vital roles in the ecological and bioprocesses in contaminated and clean soils, including decomposition and transformation of soil substances, the cycles of carbon, nitrogen, and phosphorus (Su et al. 2012; Nema 2019). On the other hand, the contaminated soils have shown more microbial diversity compared to clean soils (Nema 2019). Microbial diversity is used often for expressing the distribution of bacteria, archaea, and fungi in different habitats. The term refers to genetic diversity which is related to the amount and distribution of genetic information among the microbial species. The microbial diversity is affected by various ecological and geographical factors (Nannipieri et al. 2003; Garbeva et al. 2004).

The aggregate arrangement of soil in different sizes allows shaping of diverse microbial communities in soil (Flemming and Wuertz 2019; Al-Kaisi et al. 2017). Macroaggregates behave as a defense for microaggregates which are opposite to activities of microorganisms in the soil. This situation is explained by the hierarchy theory of soil aggregate functions (Tisdall and Oades 1982; Al-Kaisi et al. 2017). Soil aggregates have pores of 50% total volume, which is an ideal condition for microorganisms to survive in soil systems. The pores are natural habitats for microbes occupying their walls. Water present in the soil allows microbes to move freely. Water movements in the soil perform an important function that promotes microbial life by also moving nutrients, gases, microbes, and their precursors (Al-Kaisi et al. 2017). To know the relationship between the soil, water, and microbial communities, the four critical elements need to be considered. These elements are (1) pH, (2) nutrient diffusion and flow rates, (3) mobility, and

(4) temperature (Standing and Killham 2007). Soil organic matter is found in different types, which affect the diffusion of food and energy for continuous microbial activities. Temperature is also an important element for the distribution of microorganisms in soil and is also related to the interaction of plant, animal, and microbes. The carbon sources of the rhizosphere depend heavily on temperature (Al-Kaisi et al. 2017). The pH of the soil is an indicative element for the generation and survival of various microbial types. The acidophiles grow best at low pH, and another group alkaliphiles prefer higher pH conditions (Staley et al. 2011).

1.3 Molecular Approach for Determination of Soil Diversity

Taxonomy is mostly used as an equivalent term of systematics or biosystematics. This has been divided into three parts: (a) classification, arrangement of microorganisms according to taxonomic groups, (b) naming of classified microorganisms, and (c) identification of undefined microorganisms (Agrawal et al. 2015). Two approaches of taxonomic classifications of microbes have been used. The culture-dependent technique is related to the phenotypic approach (Nural Yaman et al. 2019). The culture-independent techniques are informed on microbial diversity by using the phylogenetic markers (Agrawal et al. 2015; Panigrahi et al. 2019).

Culture-dependent techniques have been frequently used to do microbial diversity studies in natural and contaminated environments. However, these techniques are biased in evaluations of all microorganisms in the environment. The determinations of microorganisms by culture-dependent techniques have made known only about 1% of microorganisms. That is to say, there are no data on about 99% of the total number of microbes (Panigrahi et al. 2019). In microbial ecological studies, commercial media such as Nutrient Agar, Tryptic Soy Agar, Malt Extract Agar have been used to practice the traditional culture techniques. The media helped reveal a small part of microbial diversity. In any case, some culture medium conditions can be changed to optimize the growth conditions for the cultivation of different microorganisms. Despite the improvements of media, all microorganisms have not been successfully cultivated in the laboratory (Panigrahi et al. 2019).

Culture-independent techniques known as modern molecular approaches have been used to discover most of the unculturable microorganisms in laboratory conditions (Agrawal et al. 2015; Panigrahi et al. 2019). The primitive source of knowledge on culturable microorganisms consists of their biomolecules like nucleic acids, proteins, and lipids. The approaches related to nucleic acids have been performed using marker genes such as 16S and 18S rRNA (ribosomal RNA) for prokaryote and eukaryote microorganisms, respectively (Srivastava et al. 2019; Panigrahi et al. 2019). These biomolecules are phylogenetic markers used as a gold standard for the identification of taxonomic groups of microbial communities (Srivastava et al. 2019).

We will discuss different molecular determination techniques which are based on 16S/18S rRNA gene region amplifications as from the following headline.

1.3.1 DNA Extraction of Soil Microorganisms

DNA extraction from soil samples is difficult because of high clay and humic material concentrations. Furthermore, DNA binds strongly to clay particles which block the isolation of DNA into the extraction supernatant (Frostegard et al. 1999; Cai et al. 2006). Humic material has also the same size as DNA; therefore, this material can bind to DNA and it may be brown-colored same as DNA extracts. The presence of humic material in DNA extracts blocks the activity of some enzymes including DNA polymerases (Dong et al. 2006). In addition, humic material affects the DNA quantification determined by spectrophotometric methods. Both DNA and humic material absorbance values are measured at 260 nm and 230 nm. Alternative fluorometric methods (Qubit, Thermo Fisher Scientific) are less effective to measure humic materials. Therefore, the concentration of DNA is guessed more accurately compared to other measurement methods (Lear et al. 2018).

Commercial kits, especially PowerSoil® DNA Isolation kit, can remove the PCR inhibitors from soil DNA such as humic acid, clay, etc. (Lear et al. 2018). Except for commercial kits, some precautions are applied to decrease the contaminants from extracted DNA. Firstly, the DNA may be diluted before the PCR amplification. This enables the PCR to be successful. Secondly, DNA is precipitated by PEG (polyethylene glycol) to reduce humic acids (Griffiths et al. 2000). Another precipitator may be glycogen which is effective for DNA precipitation. It can be combined with PEG or ethanol.

1.3.2 PCR-Dependent Methods

Carl Woese reported the 16S rRNA gene region as a marker molecule for taxonomic studies towards the end of the 1970s. Then, life had been divided into three domains: Bacteria, Archaea, and Eukarya (Woese and Fox 1977; Woese et al. 1990). Just as 16S rRNA is a significant molecule for prokaryotic microorganisms, eukaryotic microorganisms also have the homolog molecules named as 18S rRNA gene region (Hughes et al. 2009).

Being responsible for the synthesis of proteins, ribosomes are found in every cell of organisms belonging to all three domains and they are considerably conserved. The ribosome comprises two main subunits which are small subunit (30S;16S rRNA) and large subunit (50S; 23S rRNA and 5S rRNA). These ribosomal RNA sequences have been used to identify microorganisms at the molecular level and are used to set up phylogenetic relationships (Aytar et al. 2015). Archaeal and bacterial systematics can use them because of their functional and structural stability. The genes are amplified from genomic DNAs of microorganisms by polymerase chain reaction (PCR). The universal primer pairs (Lane et al. 1985; Marchesi et al. 1998) are used in amplification. 16S rRNA has been often preferred instead of 23S rRNA due to full length and less favored region of 23S rRNA. *The 16S rRNA-based

techniques are preferable and reliable to illuminate microbial diversity studies in culture-independent techniques (Tripathi et al. 2019).

The microbial phylogenetic identification of fungal species (eukaryote) is performed by 18S rRNA-based techniques or ITS region-based techniques. The 18S rRNA region is a variable sequence being in small subunits in fungal genomes. Primer designing is feasible because of the iterative sequences in this region. Therefore, the identification according to 18S rRNA has become ideal. On the other hand, because the ITS region is less conserved, it is a better biomarker than others for identification at the fungal species level. The gene regions are amplified by using a polymerase chain reaction. The universal primer pairs (Borneman and Hartin 2000; Martin and Rygiewicz 2005) are required for successful amplification.

The ITS region has been sequenced and used for microbial identification as it is being done for other biomarkers. A necessity for these methodologies is the use of universal primers for 18S rRNA region (Borneman and Hartin 2000) and ITS region (Martin and Rygiewicz 2005; Aytar et al. 2014a, b). This method identifies eukaryotes with high sensitivity and specificity in a short time. Whole genome sequencing studies are increasing due to the continuous decrease in sequencing costs over time.

16S rRNA- and/or 18S rRNA-based PCR techniques including DGGE, TGGE, SSCP, ARDRA, T-RFLP, etc. can reveal details on microbial population structure in ecological niches.

1.3.2.1 Denatured Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE)

The differences in the 16S/18S rRNA gene regions of microbial communities in various environmental samples have been separated by DNA fingerprinting approach. This approach allows high-throughput sample and can be used for the marker sequences being phylogenetically or functionally important. DGGE or TGGE are DNA fingerprinting techniques that are most often used. The techniques are successfully performed to determine all microbial diversities (Zhao et al. 2011). Amongst often used techniques for microbial community study in environmental samples is PCR-DGGE that produces complex profiles of microbial communities in soil and rhizosphere (Fig. 1.1).

DGGE and TGGE are used to distinguish PCR-amplified ribosomal RNA fragments of microbial genomic DNA. The rRNA amplicons are the same length; however, variation in nucleotide compositions enables the distribution of microbial genetics fingerprinting on the gel (Rincon-Florez et al. 2013; Agrawal et al. 2015). Formamide and urea (in DGGE) or temperature (TGGE) has been used to melt the double-stranded DNAs, and melted DNAs have migrated partially on polyacrylamide gels by the electrophoretic mobility (Rincon-Florez et al. 2013). DNAs are extracted from samples and used as a template to amplify the amplicon with universal primer pairs targeted 16S or 18S rRNA regions. The forward primer has the GC clamps which are 30 bp lengths. The amplicons are loaded onto a polyacrylamide gel. The separation onto gel with different concentrations of denaturant agent

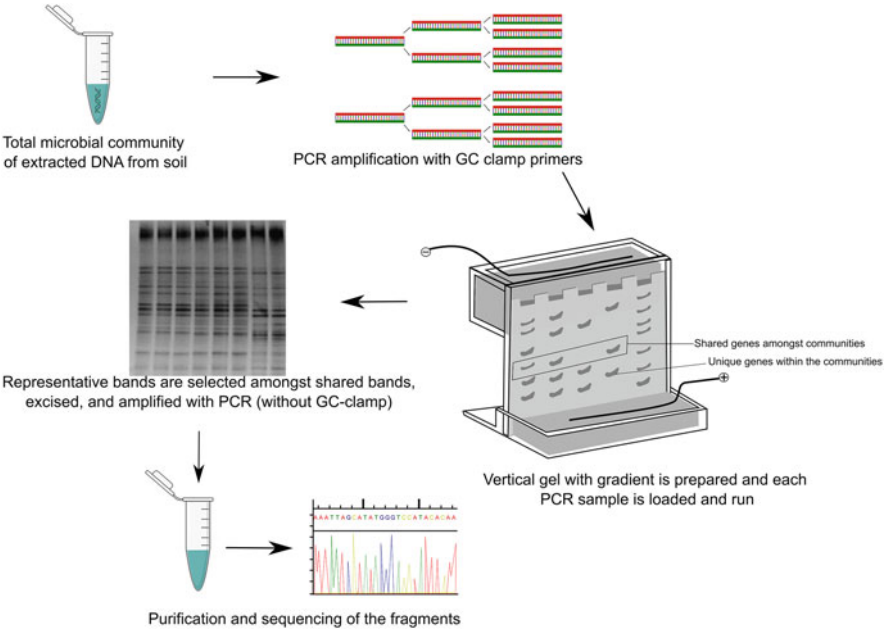

Fig. 1.1 DGGE diagram for determining microbial diversity in soil

(formamide and urea) happens according to melting points of double-strand DNA. After denaturation, DNA fragments can migrate differentially from beginning to end of the polyacrylamide gel, and they stop at the different points on the gel (Muyzer et al. 1993). At the end of the running gel, DNA can be visualized by staining (Agrawal et al. 2015). DGGE and TGGE only reveal microbial abundance within a community. On the other hand, the results can be misleading because single bands could mean multiple species while multiple bands may represent a specie (Agrawal et al. 2015).

Fungal profile from different environmental samples such as soil has been determined by using DGGE or TGGE. DGGE or TGGE fingerprints of environmental DNA from the rhizosphere have discovered the relationship between fungal profile and its habitat (Zhao et al. 2011). TGGE approach uses increasing temperature and uniform denaturant inside of denaturant gradient in DGGE gel. Therefore, bacteria and fungi are detected by TGGE compared to other molecular techniques (Bruns et al. 1999; Felske et al. 1998; Takaku et al. 2006; Ishii et al. 2000; Agrawal et al. 2015).

1.3.2.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP is another fingerprinting technique in which the forward or both forward and reverse primers are fluorescent-labeled (Fig. 1.2). Primers enable tagging targets

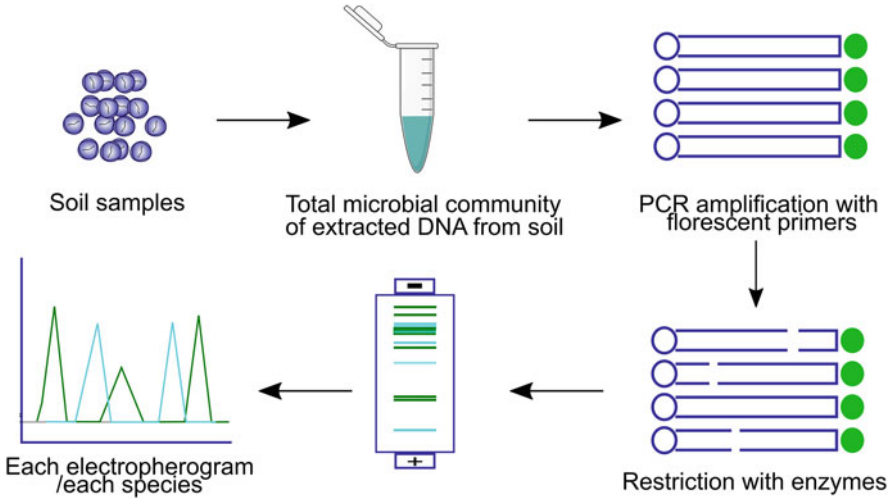

Fig. 1.2 T-RFLP diagram for microbial diversity in soil

to be amplified, then digestion is followed by a restriction enzyme. The sample can be run on a sequencing gel electrophoresis to know the sizes of the labeled terminal restriction fragments. Diverse combinations of restriction analyses of different soil microbial communities will display due to the changes in the gene sequencing. The genes are specific regions for organisms (Rincon-Florez et al. 2013).

This terminal-restriction fragment length analysis has several benefits, hence its rapid popularity: it is compatible in the laboratory, and in the end, electrophoresis is easily practicable (Zhao et al. 2011). The easy practicality originates from physical capture, fluorescence scanning, and primer with 32 labels. Therefore, 16S rRNA gene for bacteria and archaea and ribosomal genes for fungi have been used to exhibit soil microbial community (Zhao et al. 2011).

T-RFLP has been derived from RFLP and ARDRA. Their principles are so similar to each other. The most important difference is using fluorescence-labeled primers in this technique.

The microbial diversity of different environments has been revealed by this technique (Srivastava et al. 2019). Castaneda and coworkers have performed to compare community diversity of microorganisms between forest and vineyards (Castañeda et al. 2015). Similarly, the fungal community has been reported by Kasel and coworkers using this technique (Kasel et al. 2008).

1.3.2.3 Clone Libraries

Clone libraries have benefits to identify and characterize the dominant bacterial or fungal types in soil and thereby provide a picture of diversity and this pioneers microbial diversity studies (Fig. 1.3). This method depends on cloning PCR amplified biomarker genes of prokaryotes and eukaryotes and then their gene fragments

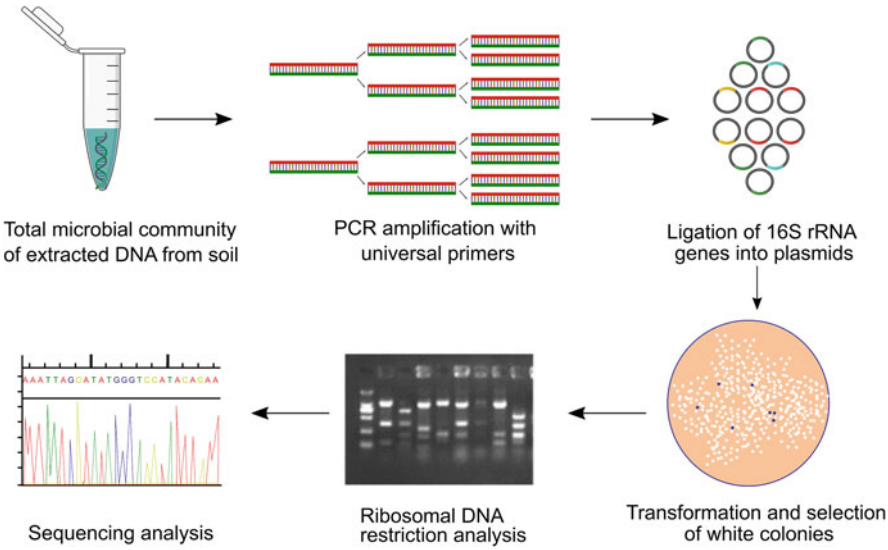

Fig. 1.3 16S–18S cloning diagram for microbial diversity in soil

sequences (Pal et al. 2019). The libraries should be large enough to describe the soil microbial community. There are a few studies about clone library constructions of soil environmental samples including hydrocarbon-contaminated soil (Dojka et al. 1998) because of some limitations and problems about a representative of soil microbiota (Garbeva et al. 2004; Sierra-Garcia et al. 2017).

1.3.2.4 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Amplified ribosomal DNA restriction analysis (ARDRA) has informed about the microbial diversity according to DNA polymorphism (Agrawal et al. 2015). ARDRA originates restriction fragments from the gene amplicons of 16S rRNA (Smit et al. 1997) and 18S rRNA (White et al. 1990), respectively, of bacterial-archaeal and fungal microbial populations in soil environments. Universal primers are not used to enable the knowledge about the specific organisms but are used to construct a pool of all microorganisms in soil environments (Rincon-Florez et al. 2013). The universal primers such as ITS-1 and ITS-4 are used in ARDRA-ITS (also termed ITS-RFLP). These primer pairs are specific for the evolutionary stable 18S and 28S rRNA genes region belonged to fungal ribosomes. 16S rRNA gene region is methodically used for bacterial and archaeal microorganisms, with appropriate primers (Choudhary et al. 2009).

Amplified marker genes were used in digestion reactions using restriction enzymes (Nocker et al. 2007; Rincon-Florez et al. 2013). The restriction enzymes (AluI, MspI, HaeIII, HinfI) recognize the region with four nucleotides and cut this

region (Rincon-Florez et al. 2013; Agrawal et al. 2015; Srivastava et al. 2019). These fragments of digested amplicons are loaded on agarose gel and separated according to their sizes. The dendrograms are obtained after running the digestion fragments.

The ARDRA is a sensitive molecular technique to inform the pattern of phylogenetic groups (Srivastava et al. 2019), but it does not give enough information about the types of microorganisms present in the soil environmental samples (Liu et al. 1997; Heyndrickx et al. 1996; Sklarz et al. 2009). ARDRA is also used to screen rapidly both colonies of clone libraries and isolates obtained from culture-dependent techniques.

1.3.2.5 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

One other approach which is used in the study of the diversity of microbial communities from environmental samples is Automated Ribosomal Intergenic Spacer Analysis. This method is based on the differentiation of the phylogenetic markers like the16S and 23S rRNA (Popa et al. 2009). An automated capillary laser detection system is used to determine the variation in the markers. The obtained peaks of the analysis are generated with universal primers (Nadarajah and Kumar 2019).

1.3.2.6 Random Amplification of Polymorphic DNA (RAPD)

Random Amplification of Polymorphic DNA is used to evaluate the difference and diversity in microbial habitat (Nadarajah and Kumar 2019). The method is applied with random primer and generated varied lengths of products. These DNA fragments are distinguished on the gel by bands representing different polymorphisms of different organisms. Visualization and comparisons can be done at the level of bands. The bands indicate the polymorphisms of different organisms. They can be visualized and compared in the form of bands (Nadarajah and Kumar 2019; Gohil et al. 2019).

1.3.2.7 Q- PCR

The Quantitative Polymerase Chain Reaction (Q-PCR) is generally used to determine the expression and abundance of marker gene regions. Marker gene used for this method might also be related to phylogenetic systematics in microbial communities. If the fluorescent stain (SYBR GREEN) or fluorescent probes (Taqman) are combined with conventional PCR conditions, this technique is called quantitative PCR (Rincon-Florez et al. 2013; Srivastava et al. 2019), and the amplicons can be measured in every cycle in real time (Smith and Osborn 2009). Many laboratory researchers start to use more frequently the Q-PCR because it is specific, sensitive, successful, reliable, and cost-effective. It can also be applied to detect the microbial

composition even at RNA (Bustin et al. 2005). On the other hand, evaluation of soil communities such as acidobacterial population in rhizospheres can be performed by real-time PCR. At the same time, real-time PCR primers being specific to taxonomic groups are used to discover bacterial and fungal microorganisms from the soil in advance. However, it does not require post-PCR procedures to avoid contamination. Therefore, this is different from other PCR techniques.

1.3.2.8 Single-Strand Cell Polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) analysis is a technique that is applied to detect differences in the sequence of single-stranded DNA as shown in Fig. 1.4 (Agrawal et al. 2015). The amplified fingerprint amplicons are loaded into a gel and separated by non-denaturating polyacrylamide gel electrophoresis (PAGE) (Srivastava et al. 2019). This approach has been performed to determine the

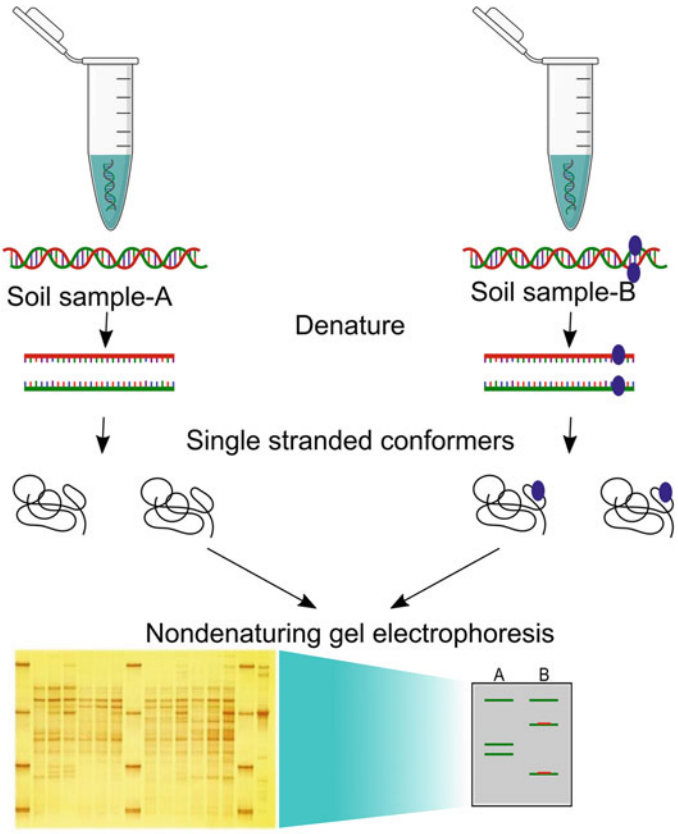

Fig. 1.4 SSCP diagram for microbial diversity in soil

differentiation among the pure culture isolated from the rhizosphere, the investigation of microbial diversity and the functional gene in contaminated environmental samples (Schwieger and Tebbe 1998; Peters et al. 2000; Junca and Pieper 2004). The general procedure of SSCP consists of PCR amplification from the template DNA, amplified product denaturation with heat and denaturants, and sample separation by non-denaturing polyacrylamide gel electrophoresis (Orita et al. 1989). SSCP separates DNA molecules of the same size whose sequences have different nucleotide. These molecules are distinguished according to their mobility on the gel (Rawat et al. 2005).

Bacterial and fungal diversity in communities has been investigated via single-strand conformation polymorphisms (SSCPs). The PCR products have been amplified with universal primers for 16S rRNA (bacteria) and 18S rRNA (fungi), from the template environmental DNA (Peters et al. 2000). This approach may be a substitute for DGGE and TGGE. SSCP does not need gradient gels prepared with denaturants (Agrawal et al. 2015). For TGGE, there is usually a need for specific equipment like the temperature gradient incubation system for gels through trivial electrophoretic chambers with SSCP temperature controls that can be used for the same purpose.

While TGGE-specific equipment such as a temperature gradient incubation system for electrophoretic gels is also needed, regular electrophoretic chambers with temperature control for SSCP can be used. An additional positive side of SSCP over DGGE/TGGE is that useful SSCP primers do not require GC clamp when running the PCR (Droffner and Brinton 1995).

1.3.2.9 Stable Isotope Probing (SIP)

Stable Isotope Probing (SIP) is a nucleic acid-based method used to identify bacterial communities in the environmental sample (McDonald et al. 2005; Schutte et al. 2008) (Fig.1.5). Either soil or plant is labeled with ^{13}C, a ^{13}C-labeled substrate is

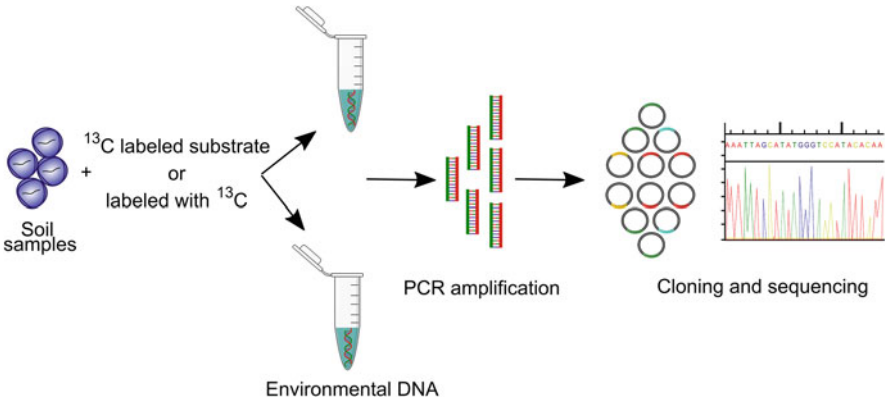

Fig. 1.5 Isotope array

added to soil or plant is marked with ^{13}C-CO_2. DNA in soil is extracted and a density gradient centrifuge is used to separate the ^{13}C marked DNA. Labeled DNA is the template to amplify PCR product which is cloned into a vector and this product is sequenced. Thus, the microbes that absorbed the marked substrates are identified. SIP approach has a big potential to identify microbes with functional activity. For this, the labeling degree should be very sensitive (Zhao et al. 2011).

This approach led to the understanding of how microorganisms vary in space in relation to carbon flow within the rhizosphere. The roles of fungal and bacteria interactions within communities have been investigated with SIP in the context of soil litter degradation. Different processes are followed by the method which also allows like matter fluxes and biochemical reactions in soil microbial samples. SIP may provide information related to carbon fluxes of soil microbial systems (Rincon-Florez et al. 2013).

1.3.2.10 DNA Microarray

Microarrays are classified into three main headlines combined by the different probe types used to study microbial populations. These are community genome arrays, rRNA-based oligonucleotide microarrays, and functional gene arrays (Zhao et al. 2011).

The results of microbial communities of environmental samples obtained by DNA microarrays are high throughput and comprehensive when compared with other techniques. Total DNA extracted from the sample is used as a template for amplification. They are hybridized to molecular probes which are added to the microarray surface (Gentry et al. 2006). Positive signals are numbered by confocal laser scanning microscopy, after hybridization. This method is rapidly evaluated by the microbial population analyses. The cause of rapid completion is related to the analysis of thousands of DNA sequences in a single array (Agrawal et al. 2015).

It might be said that using microarrays to investigate microbial populations in the soil is limited in microorganisms due to available probes. Microarray data might be confirmed by other methods such as nucleic acid blot hybridization and/or Q-PCR (Rincon-Florez et al. 2013).

1.3.3 PCR-Independent Methods

1.3.3.1 DNA-DNA Hybridization

Hybridization of nucleic acids including DNA or RNA extracted from different biological sources is based upon sequence homology between DNA and/or RNA (Agrawal et al. 2015). Specific probes are used in hybridization, which provides useful qualitative and quantitative molecular data for bacterial ecological studies (Clegg et al. 2000; Theron and Cloete 2000). This hybridization approach can lead to

the design of probes for extracted DNA or RNA but the mentioned design needs to use known model sequences consisted of a studied environmental sample. The oligo probes can be marked by fluorescent tags (Theron and Cloete 2000). On the other hand, the abundance of a specific group of microorganisms is determined by dot blot hybridization. This is a significant method to get information about the microbial community in environmental samples compared to similar ones (Agrawal et al. 2015). Large-scale study of DNA from the microbial community is performed by DNA reassociation kinetics to evaluate the diversity. In the case of DNA reassociation kinetics, the more complex the denatured DNA the slower the reassociation. This approach may be the only developed method that determines the total number of bacterial microorganisms in compost sample. This technique requires a good quantity of DNA which is always a challenge obtaining from soil constituting a major limitation (Torsvik et al. 2002).

1.3.3.2 Fluorescent iIn Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic technique for in situ detection of a specific gene, which has been used since the 1990s (Amann et al. 1995; Rincon-Florez et al. 2013; Srivastava et al. 2019). FISH is a generally implemented method for localizing, identifying, and isolating desired microbial taxa in environmental microbial ecology. Single-cell methods are committed to studying microbial population composition, and the efficiency of the method can be further improved through FISH technology (Amann and Fuchs 2008). Fluorescent stain or fluorochrome-labeled probes is preferred to detect the gene region of microorganisms in environmental soil samples. The complementary sequence and the fluorescent probe hybridize each other, and this group can be detected using fluorescence microscopy or confocal laser scanning microscopy. This technique helps to detect and visualize bacteria in the environment; at the same time, it is able to discover live cells by targeting the rRNA of microorganisms (Zhao et al. 2011). The results provide phylogenetical identification and counting in every cell. Diverse molecular probes (probes targeted Euc502, Eub338, and Arc915) have been directed towards the 16S rDNA genes of various taxa (Amann et al. 1995).

FISH technique is applied to study the cells of microorganisms with culture-independent techniques in laboratory conditions. FISH can reveal the taxonomic composition of a microbial population in contaminated soils (Ishii et al. 2004). This approach has been used to analyze the microbial diversity of agricultural soils with diverse pesticides and herbicides (Caracciolo et al. 2010). The FISH analysis is performed without cultivation of microorganisms, which has been reported firstly in 1989 (DeLong et al. 1989). These techniques are used often being reliable and rapid for soil samples (Sekar et al. 2003).

Studied soil microbial communities with fluorophore signal intensity is limited. To get over fluorescence problems in FISH technique, new methods use a single oligonucleotide combinatorial probe labeling, which is named multi-labeled FISH (MiL-FISH). In this approach, the technique will able to improve the signal intensity

and visualize the quality of every microbe in environmental samples (Schimak et al. 2016).

1.3.4 Next-Generation Sequencing Approach

Next-Generation Sequencing also called high-throughput sequencing is one of the culture-independent approaches and has been performed for determining of microbial diversity of complex environments such as soils. New technologies relating to DNA and/or RNA sequencing have been improved by advances in bioinformatics and other biotechnological methods. Metagenomics comprises DNA-based methods while metatranscriptomics comprises RNA-based methods. These methods play a major role in studying the microbial population in soil samples. Parallel sequencing platforms are performed most generally (Rincon-Florez et al. 2013). Metatranscriptome analysis reveals the enrichment and expression of genes in the soil environment (Pal et al. 2019) belonging to microorganisms. Metaproteome analysis has informed about protein complement of the microbial community in specific environmental conditions at a time point.

1.3.4.1 Metagenomics

Metagenomic is a culture-independent method that finds out the microbial community using only environmental DNAs (Srivastava et al. 2019; Demir et al. 2020; Nural Yaman et al. 2020). It can be called "environmental genomics" or "community genomics" according to Handelsman and coworkers (Handelsman et al. 2002). This technique does not require cultivation procedures. This term has been used firstly by Handelsman et al. (Handelsman et al. 1998) to explain the soil microbiota by using the concept of cloning of environmental DNA (Srivastava et al. 2019). It relies on shotgun sequencing and target gene sequencing, and their results generate two profiles of microbial community which are taxonomic profiling and functional profiling. This approach focused on the generation of taxonomic classification connecting to functional profiles of unculturable microorganisms (Rondon et al. 2000). Shotgun sequencing starts at the extraction of environmental DNA and continues to the cloning of environmental DNA to show the microbial habitat of environments. Then the constructed libraries are screened and can provide information about the microbial population at the taxonomic level (Srivastava et al. 2019; Nural Yaman et al. 2021; Aytar Çelik et al. 2021).

In targeted gene sequencing, the first step is the extraction of DNA from the soil environment. Then the 16S and/or 18S rRNA genes are amplified from soil DNA by using domain-specific primer, 341F/805R and 340F 915R (for prokaryotes, Herlemann et al. 2011), F1380/R1520 (for eukaryotes, Amaral-Zettler et al. 2009), and ITS3/ ITS4 (for fungi, White et al. 1990); then the products are purified and the adapters are added to amplicon. The fragments are both amplified and sequenced

(Sabale et al. 2019). The readings are blasted against the SILVA, Green Genes NCBI, and OTT (Balvočiūtė and Huson 2017). Identification of microbial communities living in environmental sites is completed.

Roche 454 Genome Sequencer (Roche Diagnostics Corp., Branford, CT, USA), HiSeq 2000 (Illumina Inc., San Diego, CA, USA), and AB SOLID™ System (Life Technologies Corp., Carlsbad, CA, USA) are used in metagenomic studies of soil samples. Other high throughput platforms are Ion Personal Genome Machine (Life Technologies, South San Francisco, CA, USA), Heliscope (Helicos Bioscience Corp., Cambridge, MA, USA), and PacBio RS SMRT system (Pacific Bioscience, Menlo Park, CA, USA) which are applied for metatranscriptomics (Rincon-Florez et al. 2013).

1.3.4.1.1 Application of Soil Metagenomics

Soil habitats are the richest of all the other environments on earth with regard to microbial diversity. Soil is the most microbially diverse habitat and is considered the unlimited resource for finding out novel genes, enzymes, biomolecules, bioactive compounds, and bioprocesses (Sabale et al. 2019). Soil metagenomics approaches led to the understanding of microbial communities and their functional interactions. Therefore, this method can be preferred in the determination of microbial community and discovery of new functional genes that code for biocatalysts with industrial potential. Sustainable industry and bioeconomy have often needed candidate enzymes, biomolecules, and processes to modernize the industrial process. Soil metagenomics approach helps the researcher to identify candidate unculturable microorganisms having huge potential instead of culturable classic microorganisms. The next-generation sequencing methods are applied to figure out the problems of identifying diversity on the soil microbiota caused by the complex structure of the soil. The results of the two approaches provide the advance for soil health, industrial applications, antibiotic studies, agriculture and bioremediation topics, and so forth (Sabale et al. 2019).

1.3.4.2 Metatranscriptomics

Soil metagenomics provides both taxonomic and functional information about the microbial population in soils. However, it can inform the interaction community and functional activity in soil (Srivastava et al. 2019). The exact functional roles of microbial communities are given insight by studying the mRNA transcriptional profiles of microorganisms (Pal et al. 2019). Metatranscriptomic approach also reveal transcribed genes of active microbes by using the complement of RNA obtained from entire microbial communities (Zarraonaindia et al. 2013).

Total mRNA directly extracted from a single cell or the environment, such as soil sample, is called the transcriptome and metatranscriptome, respectively (Mason et al. 2012; Li et al. 2014; He et al. 2015; Bashiardes et al. 2016). Studies enable profiling

the transcriptome of either the individual cell or the entire microbial community. The analysis in these approaches produces the information about the gene and microbes under specific environmental conditions such as soil and/or contaminated soil (Chistoserdova 2009; Bashiardes et al. 2016; Martinez et al. 2016). At the same time, active metabolic pathway(s) are found under studying environmental conditions (Srivastava et al. 2019).

In metatranscriptome analysis, total RNA is extracted firstly from the environmental sample. Complementary DNA (cDNA) is synthesized by using total RNA (Sahoo et al. 2019). The functional profile is constructed to use the map which generates RNA reads of functional gene sequences. Also, mRNA and rRNA are analyzed. mRNA is related to gene expression and rRNA is related to functional genes (Tveit et al. 2014). Environmental metatranscriptomics is studied on only mRNA that has been isolated from the environment and sequenced to show gene expression in the microbial community (Gosalbes et al. 2011).

1.3.4.3 Metaproteomics

The direct determination of protein expression from mixed communities of microorganisms from environmental samples can be possible by developing the traditional proteomic techniques, and the mentioned technique is called metaproteomics (Chakraborty et al. 2014; Pal et al. 2019; Sahoo et al. 2019). Proteogenomics can also be known for this method (Armengaud et al. 2013). Metaproteomics also provides information about proteins related to the microbial community at a certain time point and particular environmental conditions (one example for contaminated soil; Guazzaroni et al. 2013) in microbial ecology studies (Pal et al. 2019).

The metaproteomic analyses have been applied in four significant steps: (1) extraction, purification, and concentration of proteins; (2) denaturation of protein and reduction; (3) separation of protein separation, digestion, and analysis; and (4) spectroscopic identification of proteins (Schneider and Riedel 2010).

The biochemical techniques are applied to determine the stability level of protein. The extracted protein is analyzed by SDS-PAGE gel electrophoresis (one or two dimensional). In this way, the proteofingerprint analysis of microbial population is generated. Then, mass spectroscopy (MaLDI-TOF MS) can be used combined with gel electrophoresis (Maron et al. 2007; Srivastava et al. 2019; Pal et al. 2019).

1.3.4.4 Metabolomics

The profiles of whole metabolites in a single cell in a certain time and condition are studied in metabolomics. The next-generation technologies have widened to metabolomics technology, after the metagenomics, metatranscriptomics, and metaproteomics. The other -omics techniques, especially metagenomics, have demonstrated the power to determine the taxonomic and functional diversity of microbial communities of environmental samples in specific conditions (Malla et al. 2018).

The metabolome-based studies for environmental samples have shown microbial activities under the conditions where they live. In addition to this approach, other studies can be improved to profile the metabolic activity of communities according to changeable environmental factors. In general, secondary metabolites have been released under stress conditions. The metabolomics approach explains the functional roles of these metabolites (Malla et al. 2018).

1.3.4.5 Functional Diversity

The role of a microorganism in the ecosystem can be described as its functional diversity. Some of the mentioned roles are competition, synergy in the microbial community, forming of species together, and communication in the ecosystem. The functional diversity is interested in the interaction between microbes indifferent conditions (Laureto et al. 2015; Petchey and Gaston 2006) and can be predicted rightly by selecting functional and important properties that affect and change the ecosystem's balance. To evaluate this, functional diversity uses some biochemical and traditional methods. Besides, molecular techniques can be also used. Extracted environmental and/or genomic DNA and amplified PCR products are evaluated in this approach (Srivastava et al. 2019).

Studies on functional diversity may also investigate the significance of the individual characteristics. It looks for the answers to two questions. (a) How do species influence the ecosystem? (b) How do species respond to environmental differences? (Laureto et al. 2015).

1.3.5 Microfluidic Chips

The soil is a very diverse environment with so many different structural compositions harboring a large diversity of microorganisms. The study of these organisms has been very challenging because a large fraction of soil microbes are unculturable while others are found in very little amounts (Aleklett et al. 2018). Metagenomics has revealed a large functional diversity of soil microbial communities, but they do not replace culture techniques. Due to recent advances in microfluidics, high-throughput technologies, 3D bioprinting and single-cell analytics culture techniques have evolved from axenic to mixed cultures enabling the study of microbial communities and their underlying interactions. By creating microenvironments that mimic the natural environments, the behaviors of microorganisms can be studied in real time as in their natural environments (Nai and Meyer 2018). It is hoped that the exploration of the microbial dark matter will bring forth new antibiotics and beneficial metabolic pathways (Stanley et al. 2016).

Microfluidics has also improved cell sorting by producing far less damaged cells and higher precision. The possibility to customize the sorting process permits individual cells to be sorted and their roles as community members identified within large populations (Leung et al. 2012). Strategies to increase the precision of single-

cell sorting from culture-independent techniques include PCR-activated cell sorting and digital PCRs based on genetic sequences rather than cell size and labeling. Cells can be sorted based on particular genetic traits for metabolism or antibiotic production which enables studying the transcriptome of individual cells within soil microbial communities (Lim et al. 2015; Ottesen et al. 2006). Furthermore, using microfluidic-based quantitative real-time PCR, it is also possible to quantify species within microbial communities making feasible the monitoring of species dynamics over time (Kleyer et al. 2017). With microfluidic droplet barcoding, sequencing of up to >50,000 cells per run has been demonstrated (Lan et al. 2017). With this, researchers are therefore able to identify unculturable microbes carrying particular traits that can lead to numerous research opportunities within the environment (Ottesen et al. 2006).

Besides, the applications of microfluidics in the understanding of plant microbiome interactions may also permit us to characterize various important microbial consortia contributing to plant nutrient uptake (Stanley and van der Heijden 2017).

1.3.6 Combined Methods for Microbial Diversity

Some techniques can be combined to investigate microbial diversity in soil. For instance, FISH combined with microautoradiography is called FISH-MAR. This approach provides in situ identifications of microbial communities (Ouverney and Fuhrman 1999; Meyer et al. 2005). FISH-MAR detects the microbes, their activities, and specific substrate uptake profiles in the environmental microbial community (Lee et al. 1999). The sample obtained from the environment is incubated with compounds that have been tagged like 3H-acetate, 14C-pyruvate, 14C-butyrate, or 14C-bicarbonate after which it is fixed on a slide. Selected fluorescently labeled probes complementary to different 16S rRNA enable FISH analysis. Slide treatment with autoradiographic emulsion and silver particles allows for visualization with confocal scanning laser microscopy. Detecting radioactivity in combination with FISH allows for the detection of the metabolizers of the substrate of interest.

Furthermore, catalyzed reporter deposition (CARD) FISH is known as tyramide signal amplification, which also allows detection of microbes in the soil. CARD-FISH includes tyramide-labeled fluorochromes to amplify rRNA hybridization signals. Tyramide prevents the FISH staining, and many fluorescent probes come together at the target site (Pernthaler et al. 2002).

Another combined method is Chip-SIP, which contains stable isotope probing (SIP) and microarray approach. This technique utilizes the marker genes 16S and/or 18S rRNA genes and ion mass spectrometer which analyzes the relative isotope incorporation of the rRNA. This Chip-SIP approach helps in illuminating complex microbial diversity of environmental samples. Chip-SIP method is applied by comparing the different communities and/or different conditions. The researcher can make an analysis of these combinations: (a) the same community in different

substrates/nutrition, (b) different communities in the same substrate(s)/nutrition, (c) response of microbial community against temperature or nutrient concentrations (Mayali et al. 2019).

DNA-SIP probing employing 15N and 14N isotopes can also be combined with density gradient centrifugation to detect different DNA. It is also possible to use other isotopes such as ^{2}H and ^{13}C. For example, for the investigation of methylotrophs, substrates such as $^{13}CH_2OH$ and $^{13}CH_4$ have been included in soil samples to be investigated. Buoyant density gradient centrifugation showed good resolutions enabling effective separation of DNA that incorporated the labeled ^{13}C substrates. Using general PCR primers, the DNA can be amplified for further identification of the species by sRNA analyses. DNA-SIP can be further extended for use with multicarbon compounds which can allow for investigations of biodegradation rates (Dumont and Murrell 2005).

RNA-SIP has also been developed producing results even faster because lesser time is required in cells to synthesize RNA. Separation of RNA types can be achieved using cesium trifluoroacetate density gradient centrifugation. RT-PCR amplification can then be applied to obtain the corresponding DNA.

The investigation of microorganisms that are affected by root exudation for studies on rhizosphere-microorganism interactions can also be carried out using SIP techniques. Plants can be incubated with the stable isotope-containing substrates after which nucleic acids can be isolated from the rhizosphere. The DNA-containing isotopes can be obtained as mentioned above for further 16S rRNA analysis, development of metagenomic libraries to investigate functional genes, or other DGGE or microarray analysis (Dumont and Murrell 2005).

1.4 Challenges of Using Molecular Approaches for Analysis of Soil Environmental Samples

There are many types of techniques to determine the soil microbial diversity while they have numerous applications and importance in various analyses; their uses are also limited in many ways. Both culture-dependent and culture-independent approaches are very important separately. The two approaches do not replace each other and are more useful when applied together (Nai and Meyer 2018).

In amplification of marker genes, challenges or limitations affect the polymerase chain reaction in the soil system. DNA is used in a polymerase chain reaction as a template. Therefore, the inhibitor can bind to DNA during extraction. These inhibitors can also attack during the PCR leading to the generation of false-negative results. Nevertheless, wrong targets have also been amplified and false-positive PCR products have been produced. Another limitation is not being quantitative of the traditional form of PCR (Luby et al. 2016). However, RT-PCR method giving quantitative results has a limitation; this technique only allows a few gene(s) to be monitored per PCR reaction. As a solution to this problem, qPCR arrays can be used to allow the simultaneous quantification of hundreds of genes (Sen and Sarkar 2019).

DGGE, 16S rRNA-dependent technique has limitations related to artifacts of PCR (Dubey et al. 2020). PCR bias can be subjective and nondominant species can poorly resolute (Edet et al. 2017). Another limitation is that it is not possible to load all the samples on a single gel. Therefore, the gel variation can cause reproducibility (Dubey et al. 2020). PCR products from different organisms, despite differing nucleotide sequences, may also have the same melting point. This causes the generation of missing bands on the gel. To avoid the nonspecific binding, therefore, touchdown PCR is applied, and in this manner, specific binding might be increased (Gałązka and Grządziel 2016).

Another technique, SSCP works well for small fragments preferably 150–400 bp and is very simple and reliable. However, it is subject to PCR biases as well as DGGE. Besides, a major limitation of this technique appears to be the ability of some DNA strands to form multiple stable conformations. SIP methodology has also some problems such as the high cost of labeled substrates, labor-intensive, and low throughput.

Clone library, considered as a reliable technique, has also some limitations such as consuming time, labor-intensive, and cost (Sierra-Garcia et al. 2017). There are some restrictions in FISH technique, which is preferred for providing preliminary information. The signal intensity of the fluorophore used is the key limitation in FISH use. A multi-labeled FISH approach (MiL-FISH) employing combinatorial probe labeling is being proposed as a method to solve this fluorescence problem. The multi-labeled probe amplifies the signal from cells within the samples (Schimak et al. 2016).

Metagenomics approach can reveal the diversity of microbial communities from environmental samples. Recently developed tools which consist of microfluidics, bioprinting, high-throughput screening have been utilized to scrutinize microorganism identification and diversity. They should be applied with other culture-dependent methods to investigate and illuminate the diversity of microbial communities (Table 1.1) (Nai and Meyer 2018).

In metatranscriptome analysis, firstly, the RNA obtained directly from soil can restrict the process and its concentration might be often low. Therefore, additional amplification steps might be used to increase transcript concentrations (Frias-Lopez et al. 2008; Gilbert et al. 2011). Secondly, mRNA separation and the transcriptome of the sample can constrain. As a result, it may be possible to not obtain a statistically significant transcription pattern that represents most genes within a complex community. This therefore restricted earlier works to the more domineering species of the communities.

Metaproteomics has limitations: (1) the protein's source can be bad (e.g., soil sample); (2) molecules replicating proteins (unlike in DNA or RNA) may not have the ability; and (3) possibility for tedious protein identification and isolation (Sahoo et al. 2019).

Stable isotope probing techniques are faced with several limitations such as knowledge of the precise amounts of isotopes to be used to achieve effective resolution of DNAs on gels from complex communities, limited use to only 13C

Table 1.1 Advantages and disadvantages of molecular approaches used for determination diversity of microorganisms

Methods	Disadvantages	Advantages	References
DGGE/TGGE	PCR bias Single band could represent multiple species Same species could be represented by multiple bands Time-consuming Limited sequence information Only detects dominant species Dependent on DNA extraction efficiency	Rapid Reliable Reproducible Large number of samples can be analyzed simultaneously Bands can be excised, cloned, and sequenced for identification High resolution for dominant taxa presents in the sample	Rincon-Florez et al. (2013); Agrawal et al. (2015); Pal et al. (2019); Srivastava et al. (2019)
ARDRA	PCR bias More applicable to environments with low complexity Unknown sequences often limit the optimization of restriction enzymes Different bands can belong to the same group Labor- and time-intensive Several restrictions are needed for adequate resolution	Good comparison of microbial diversity in response to changing environmental conditions No special equipment required Highly reproducible microbial community Profiles Rapid monitoring of microbial communities over time	Rincon-Florez et al. (2013); Agrawal et al. (2015); Pal et al. (2019); Srivastava et al. (2019)
Methods	Disadvantages	Advantages	References
TRFLP	PCR biases Type of DNA polymerase can increase variability Underestimates community diversity because only a limited number of bands per gel can be resolved Especially of spore formers during the extraction of community DNA leading to biasness in DNA amount Choice of universal primers/restriction enzymes influences fingerprint data Lower discriminatory power Dependent on DNA extraction efficiency Artefacts might appear as false peaks Distinct sequences sharing a	Highly reproducible Convenient way to store data and compare between different samples Can be automated Large number of samples can be analyzed simultaneously Rapid, robust, inexpensive, less time-consuming	Rincon-Florez et al. (2013); Agrawal et al. (2015); Pal et al. (2019); Srivastava et al. (2019)

(continued)

Table 1.1 (continued)

Methods	Disadvantages	Advantages	References
	restriction site will result in one peak		
SSCP	PCR bias Lack of reproducibility Short fragments More than one stable conformation possible for some ssDNA Several factors like mutation and size of fragments can affect the sensitivity of the method Several factors like mutation and size of fragments can affect the sensitivity of the method	Rapid, reliable, and Reproducible Simultaneous analysis of a large number of samples No gradient required Possible to identify community members Screening of potential variations in sequences Helps to identify new mutations	Rincon-Florez et al. (2013); Agrawal et al. (2015); Srivastava et al. (2019)
Methods	Disadvantages	Advantages	References
Q-PCR	PCR bias Can only be used for targeting of known genes All inherent shortcomings of PCR	Speed, sensitivity, accuracy Discrimination of gene numbers across a wider dynamic range than is found with end-point PCR	Pal et al. (2019); Srivastava et al. (2019)
Nucleic acid Reassociation	Dependent on DNA extraction efficiency – Low sensitivity – Requires high copy number sequences for detection	Total DNA extracted – In situ study of DNA or RNA – Not influenced by PCR biases.	Agrawal et al. 2015
FISH	Autofluorescence of microorganisms Sequence information is required for probe design Limited number of probes could be used in a single hybridization experiment, low signal intensity, background fluorescence Specific detection FISH alone cannot provide any insight into the metabolic activities of microorganisms Difficult to differentiate between live and dead cells Difficult accessibility of target gene	DNA isolation and PCR bias independent Highly sensitive and quantitative Can use multiple fluorescent dyes to simultaneously detect different microorganisms Taxonomic and phylogenetic identification Visualization of uncultivable microorganisms Highly sensitive and quantitative	Rincon-Florez et al. (2013); Pal et al. (2019); Srivastava et al. (2019)

(continued)

Table 1.1 (continued)

Methods	Disadvantages	Advantages	References
Methods	Disadvantages	Advantages	References
SIP	Incubation and cycling of the stable isotope might cause biases within the microbial communities Prerequisite DNA synthesis and cell division to incorporate sufficient label into DNA for gradient separation	High sensitivity Provides evidence on the function of microorganisms in a controlled experimental setup Less labor-intensive and minimal instruments requires Phylogenetic resolution, provides ever-increasing resource for robust taxonomic and functional assignments	Rincon-Florez et al. (2013); Pal et al. (2019)
NGS	Massive data amount, a challenge for data analysis Overestimation of taxonomic classification with short read lengths High error rate	Provides more in-depth information about the composition and function of a whole microbial community	Pal et al. (2019)
DNA array	Culturing of organisms required Only detect the most abundant species – Culturing of the organisms required Variation in major ecosystem type can cause variability in the detection of targeted bacterial cells Difficulty in obtaining high-quality rRNA	Analyses a vast amount of genetic information simultaneously Not influenced by PCR biases Total DNA extracted One protocol can be utilized to identify different targeted bacteria simultaneously on a single array In situ study of DNA or RNA Use of DNA fragments increases specificity Large number of genes can be analyzed	Rincon-Florez et al. (2013); Agrawal et al. (2015); Pal et al. (2019); Srivastava et al. (2019)
Methods	Disadvantages	Advantages	References
Clone library	Labor intensive, Time-consuming, Expensive	More resolution	Sierra-Garcia et al. (2017); Pal et al. (2019)
RAPD	Low reproducibility	Rapid, inexpensive, and effortless, prior knowledge of sequence not needed	Gohil et al. (2019)
ARISA	PCR bias Economic and relevant for microbial community structure	Better resolution	Kovacs et al. (2010); Likar et al. (2017)

substrates, experiments are still carried out only in laboratory microcosms with limited applications in actual environments (Dumont and Murrell 2005).

1.5 Future Trends

Microbial diversity of environmental samples is investigated by culture-dependent and independent techniques. Culture-independent techniques can be divided into PCR-dependent and PCR-independent techniques. Culture-dependent techniques also use culture media and mimic the environmental conditions in the laboratory to isolate the microorganisms. However, this technique reveals only 1% of microorganisms from environments. On the other hand, molecular approaches investigate and determine more microorganisms than culture-dependent techniques. These approaches combined with bioinformatics tools analyze microbial communities. Nevertheless, they both have advantages and disadvantages.

In considering the future sustainability of agriculture, it is fundamental to evaluate and understand the roles that microbial communities play in the processes that govern ecological change in these ecosystems. Knowing soil microbiota and their applications in agriculture will promote sustainable agriculture, sustainable bioeconomy, enhancing product yield, providing healthy soil (Otwell et al. 2018). Techniques to be applied for figuring out interactions in microbial community and the ecological system should answer some questions:

- Do we investigate microorganisms that are used in the following processes (bioremediation, biosorption)?
- What is the role of microorganisms affecting product efficiency in the ecological system?
- When do we want to obtain the product, under which conditions, and which microorganisms will increase productivity?

New strategies can be followed to reveal the microbiota according to the changing environment selection. New methods to evaluate diversity may be in silico applications and systems biology approaches. Genome-scale metabolic models (GEMs) from in silico approaches are powerful tools to model an organism's/community's metabolic capabilities. The GEMs can be increased according to conditions and then can be used for comparisons. Before using the target-specific simulations to predict, these models should be repeatedly constructed and tried. Theories derived from lab-based studies can then be tested back in the field. The knowledge obtained from repetitive activities will allow for the computational understanding of field processes. This approach enables the understanding of interactions from microbial diversity to ecological and biogeochemical functions (Biggs et al. 2015; Oberhardt et al. 2011).

1.6 Conclusion

Soil structure is accepted as a very complex and composite environment. The determination of the interactions between microbial population and soil environment conditions is required because of improving new strategies about sustainable bioeconomy and industrial use, agriculture, bioremediation, and soil health.

Soil microbial population can be identified with culture-dependent techniques but it cannot mimic the environmental conditions in the laboratory conditions. Therefore, culture-independent techniques are applied to turn the disadvantages of cultivation. PCR-dependent or -independent techniques, next-generation sequencing technologies can exhibit the microbial community and relation between gene, protein, and this population. The target is microorganisms not arrived by cultivation in soil environmental samples.

The strategies discussed in this chapter have advantages and disadvantages that are related to one another. On the other hand, they have all been used to depict the microbial community in soil samples from the past to the present.

If we can get to know the oldest owners of the Earth's ecosystem better, we can make more use of them. For this, almost all of the soil microorganisms must be discovered. So, we will get to know the natives of the world and discover all the components in nature's toolbox. Thus, we will be able to make more use of nature to solve problems. According to industrial microbiologists, microorganisms are the first to come to mind when underground wealth is mentioned. Discovering and uncovering them is essential for a more livable world.

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