Chapter 6
Emerging Methodologies for the Molecular Analysis of Soil Microbiota from Polluted Soil Sites

Ridhuvarshini, Pavethra, Sophia Reena, and Sivaranjani

Abstract The soil microbiome performs a wide range of crucial functions; however, we have a limited understanding of its biodiversity. Extracting microbes from polluted sites could reveal potential microbes that could be used to mitigate pollution better than conventional microbes. Soil DNA may be extracted directly, amplified using polymerase chain reaction, and profiled to reveal more about the soil microbiome's taxonomy and function than ever before. Current procedures frequently combine DNA sequencing with other methods like denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (TRFLP), amplified rDNA restriction analysis (ARDRA), amplified ribosomal intergenic spacer analysis (ARISA), and cloning. The advantages and disadvantages of these methods are discussed, and new developments that have relevance as an appliance shedding light on the soil microbial ecology are also included. Soil diversity cannot be assessed using just one approach; therefore, picking the right one and using newly discovered information can significantly improve our understanding of soil microbes for their specific applications in mitigating.

Keywords Soil microbiome · Bacterial ecology · Polluted soil · Emerging methodologies · Microbial profiling · Direct DNA sequencing

6.1 Introduction

The diversity of the soil microbiome is little understood, even though the soil microbiome performs a wide variety of essential tasks. Direct DNA extraction, amplification by polymerase chain reaction, and profiling of soil microbes have made hitherto undisclosed information on the taxonomy and function of the soil microbiome accessible. It is common practise to combine DNA sequencing with other

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techniques, including DGGE, TGGE, SSCP, TRFLP, cloning, and amplification of rDNA restriction and ribosomal intergenic spacer regions. Innovations that have significance as an appliance shining a light on the soil microbial ecology are also mentioned, along with the pros and cons of these approaches. We can learn much more about soil bacteria if we use the correct method for assessing soil diversity and incorporate newly found information.

Additional advancements are provided by more recent techniques like microarrays and high-throughput sequencing. In this review, various molecular methods used to study soil microbiota are reviewed, along with the pros and cons of each method.

6.2 Bioinformatic Analysis

The initial computational method was based on the sequencing platform, with distinct software modules envisioned for early analysis of diverse systems. However, the programs need to be linked to establish automated "channels" to identify the possible genes and taxonomic groups illustrated by the sequencing data and their relative frequency of occurrence. When one genome is sequenced in short reads, it creates overlapping expansions that allow the "assemblage" of shorter reads into longer ones. When the genomic sequence is presented as a "scaffold," the job becomes much less complicated. Extended sequences can be matched by searching the NCBI databases. Arranging the various sequences of the initiator in the best possible way is critical for further study. PHYLIP, the PHYLogeny Inference Package, can silently infer phylogenetic trees and sequence family relationships. Genome annotation is assigning biological meaning to DNA sequences and identifying genes. There is a need for software that can build de-novo genomes from short-reads (i.e., less than 50 b), with significant errors projected with extended-reads of 100 b; nevertheless, verifying restricted sequences is challenging. Therefore, it is highly improbable that microbial genomes from diverse and mixed populations can be reconstructed using high-throughput, short-read-length techniques. A mix of tactics catering to readers with varying attention spans (short, medium, and long) would be required. With the expanding breadth of metagenomic research and public databases comes the requirement for high-performance computing and automated applications. Independent and autonomous organizations are developing alternative public pathways to examine metagenomic data. Disparities in metabolic profiles among a few biomes have been uncovered using the mg-RAST server (Meyer et al. 2008; Dinsdale et al. 2008). The sequence was analyzed using BLASTX in addition to ribosomal (GREENGENES, RDP-II), chloroplast, and mitochondrial databases (via ACLAIME). MEGAN is a one-of-a-kind method that rapidly assesses the biodiversity of metagenomic samples using visual outputs, allowing researchers to compare and contrast many data sets, such as functional evaluations and metadata (Huson et al. 2007) (Fig. 6.1).

Understanding the subject's research hot spots and potential future research trajectories can be done using statistics and examining the literature's subject keywords (Shi et al. 1999). This study employed VOS viewer version 1.6.16 (van Eck and

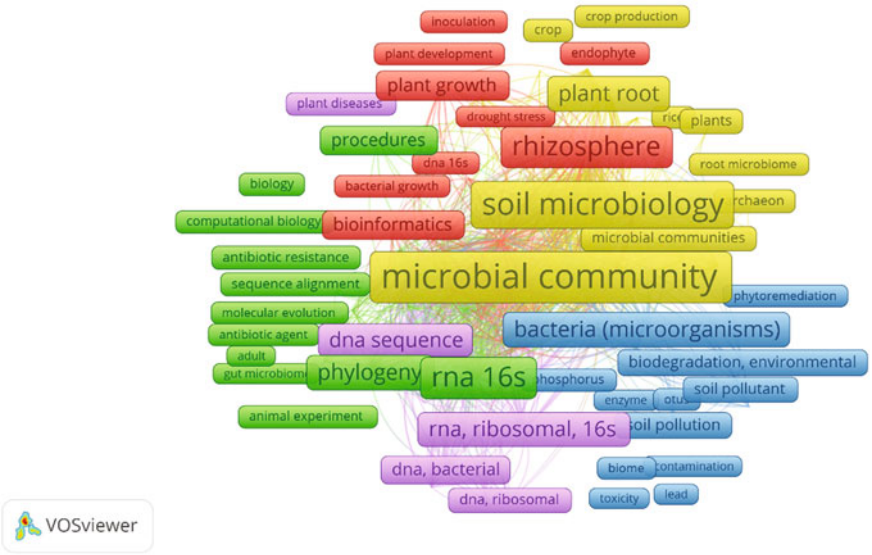

Fig. 6.1 VOSviewer mapping of keywords

Waltman 2010) to assess descriptive data and consider recent research and historical trends. The Scopus database output for the keyword "TITLE-ABS-KEY (Soil microbiota AND molecular analysis)" is also included for co-occurrence analysis in the VOS viewer. The nodes linked with the soil microbial community, soil bacteria, plant-microbe interactions, rDNA, and molecular analysis are deduced from the yellow, blue, red, violet, and green color clusters. The size of the nodes (frames) reveals how frequently they are used, and the separation between the frames and connecting lines reveals how interconnected and linked they are. All nodes are most connected to the cluster of adsorption keywords, the core content, and the most key phrase for all keywords. Figure 6.2 displays the global collaborations identified by the document search. The blue color of the map represents global research collaboration. The pink border that divides the states demonstrates the authors' level of involvement. It is incredible how the countries that have published the most articles on the soil microbiome have worked together. A generic function summary of bibliometrix analysis done by Bibliometrix R-Tool (Aria and Cuccurullo 2017) for the Scopus database search of a given keyword is provided in Table 6.1.

6.3 Community Profiling Techniques and Limitations

Community fingerprinting techniques have been widely employed in microbiota-ecology research, greatly enhancing our understanding of the variety of soil microbiota. For example, denaturing gradient gel electrophoresis (DGGE), temperature

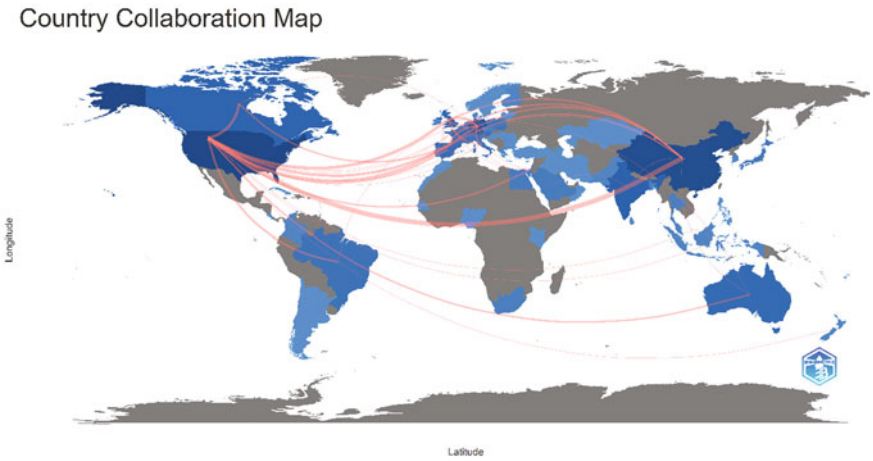

Fig. 6.2 Country collaboration map of research of the title

Table 6.1 Scopus database keyword search and paper hits

Description	Results
Main information about the data	
Timespan	2008:2022
Sources (Journals, Books, etc.)	139
Documents	277
Annual growth rate (%)	30.15
DOCUMENT AVERAGE AGE	3.6
Average citations per doc	52.39
References	20,472
Document contents	
Keywords plus (ID)	3319
Author's keywords (DE)	920
Authors	
Authors	1595
Authors of single-authored docs	6
Authors collaboration	
Single-authored docs	6
Co-authors per doc	6.49
International co-authorships (%)	35.02

(continued)

Table 6.1 (continued)

Description	Results
Document types	
Article	222
Book	2
Book chapter	8
Editorial	1
Erratum	1
Note	1
Review	41
Short survey	1

gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA). In addition, amplified tRNA-serine-leucine-nucleotide polymorphism (ART) is a method for identifying (van Elsas 2000).

While each community profiling approach is distinct, many share the same DNA features used in the separating process. Both denaturant gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) use the melting behavior of DNA to separate fragments of the same size but different sequences; this is because DNA with different sequences (i.e., different G + C contents) melts at different locations in a polyacrylamide gel (TGGE). Because of the potential for many bands and an exaggerated estimation of diversity, the use of degenerate primers is limited in DGGE/TGGE. Since the advent of DGGE, these methods have become the most widely used community fingerprinting techniques in bacterial ecology, making their advantages and disadvantages clear to anybody interested in studying soil microbial communities. Soil fungal communities may be analyzed rapidly and effectively with DGGE/TGGE, making it a helpful tool for studying changes in community composition (Anderson et al. 2003a). The techniques' benefits include the ability to study and compare several samples on a single gel and the speed and simplicity of such comparisons. Specialized software programs have substantially improved the study of community fingerprints by permitting comparison of the position and relative intensity of distinct bands within gels. This paves the way for further statistical analysis of the data and an appreciation of the ecological conclusions drawn from it. Accurate comparison, however, is highly dependent on suitable internal standards and the prior establishment of gel resolution and quality. The need to compare multiple gels owing to a large number of samples emphasizes the significance of this. One of the fundamental limitations of DGGE is its lack of repeatability across gels, even if the gel-making process may be improved by using, for example, the same flow rate of denaturant solutions and the same equipment for each gel (Fromin et al. 2002).

The methods used to create such profiles of a whole community are not without their flaws. Compared to more oversized products, smaller DNA fragments (500 bp)

provide better resolution across bands in a profile, limiting the taxonomic information that can be gleaned by sequencing excised bands (Landeweert et al. 2003). More importantly, even the most sensitive staining techniques sometimes fail to identify all the variability that occurs in a sample, especially for the less prominent members of the group. It has also been shown that a single band on a gel may contain many sequence types (Schmalenberger and Tebbe 2003). T-RFLP uses automated DNA sequencing technology to provide significantly better throughput than gel-based community profiling approaches (Marsh 1999). T-RFLP varies from conventional RFLP in that it employs fluorescently tagged PCR primers (forward, reverse, or both primers) before restriction digestion and size measurement of fluorescently labeled terminal restriction fragments using a DNA sequencer. T-RFLP may simplify the community profile without compromising the variety found by identifying the last segment of each 18S rDNA or ITS sequence in a sample. T-RFLP is an improvement on the ARDRA method of community profiling. There are methods like ARISA, which are similar, but they assess whole amplicons instead of simply the terminal fragments that result after restriction digestion (Leckie et al. 2004). Using an automated DNA sequencer for ARISA and T-RFLP increases throughput and improves accuracy in sizing the generated fragments by adding an internal standard in each sample and maintaining stable operating parameters. Although T-RFLP has been used to analyze the diversity of soil fungi and to detect ECM fungi in soil samples (Lord et al. 2002; Dickie et al. 2002), a robust T-RFLP database is necessary for identifying individual fungi species. The lack of ease with which sequence information may be extracted from T-RFLP peaks makes identifying previously unknown species in a sample a formidable task. Occasionally, artificial identification has been made using virtual restriction digests based on public database sequences; however, this is risky because it presumes that only one species or operational taxonomic unit (OTU) can have a peak of that size in a sample and that the database sequence is correctly identified and of high quality.

Cloning PCR amplicons from environmental DNA has also been used to evaluate soil fungal diversity (Jumpponen 2003). However, clones may be screened using RFLP to categorize clones into OTUs prior to DNA sequencing, reducing the number of clones required for sequencing and making it difficult to determine how many clones must be analyzed to sample the diversity contained in a single sample effectively. The development of collectors or species abundance curves shows that the number of 18S rDNA clones that need to be analyzed in agricultural soil is far smaller than the number of clones that would have been required for a library of bacterial 16S rDNA to have covered the diversity microbiota (Anderson et al. 2003). These researchers found that more ITS clones than 18S rDNA clones from the same sample needed to be screened to ensure coverage of the fungal diversity in the sample. Without analyzing many clones, it is difficult to discriminate between familiar and unusual sequence types in a sample.

DNA sequencing and phylogenetic analysis are commonly used in tandem with community profiling methods for the taxonomic identification of species present in a sample; however, it is crucial to remember that environmental DNA samples may also contain chimera DNA sequences. The techniques mentioned above provide an

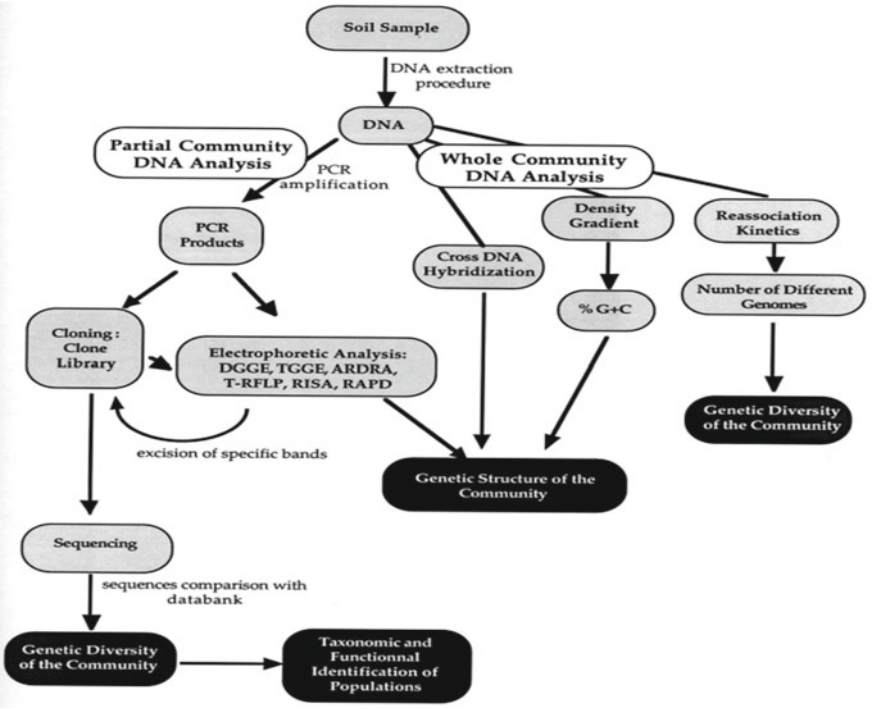

Fig. 6.3 Schematic representation of the different molecular approaches

array of adequate resources for assessing soil fungus populations. Although each has certain technical limitations that prevent it from being fully utilized, its widespread application in bacterial ecology over the past decade has led to steady development and refinement. Each community profiling method varies in its taxonomic resolution due to factors such as the PCR primers chosen for the first PCR amplification of the community DNA and the lack of extensive sequencing information in public databases. Fungus ecologists have been working on loosening these constraints for years, and their efforts will likely bear fruit soon (Figs. 6.3 and 6.4).

6.4 Current Developments in Molecular Ecology of Soil Fungi Profiling

The spatial and temporal dynamics of resident fungus, as well as the diversity of fungi in plant roots, the rhizosphere, and/or bulk soil, have been considerably improved by recent investigations of soil fungal ecology employing PCR amplification from total extracted DNA.

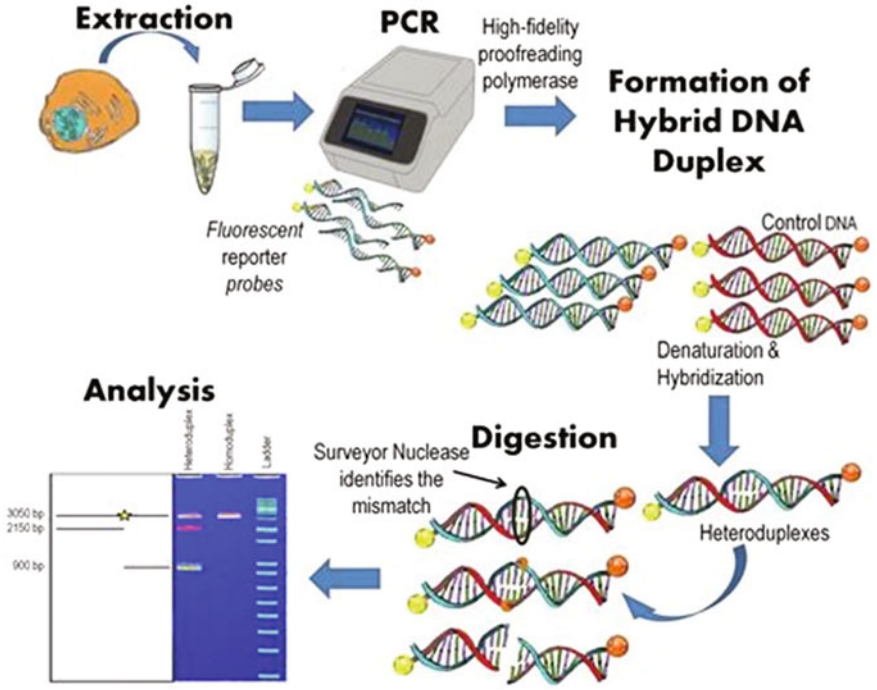

Fig. 6.4 Extraction and analysis of genetic information of soil microbes

Support for the long-held belief that growing fungi from the soil only allows for a limited perspective of variety was provided by the cloning and sequencing of 18S and ITS rDNA PCR products from total soil DNA (Hunt et al. 2004). Using 18S rDNA amplification in conjunction with TGGE and DGGE in both microcosms and field soil, we show that fungal diversity is lower in the rhizosphere compared to that in bulk soil, and that there is an influence of plant age. The bare soils around glacier ice edges and terminal moraines contain unique fungal communities, as shown by an analysis of 18S rDNA clones. Soil community composition has been shown to shift in response to various treatments, including petrochemical pollution, nitrogen addition, controlled vegetation burning, elevated atmospheric CO_2 concentration, and the addition of compost or manure, as determined by studies employing 18S or ITS rDNA amplification coupled with cloning, DGGE, SSCP, or T-RFLP analysis. The DGGE-detected communities lacked the taxonomic diversity of the T-RFLP-detected communities. However, because several DNA extraction methods and primers were employed, it is unclear to what extent these differences reflect variations in the relative resolution of the approaches.

However, the taxonomic richness of the DGGE profiles was similar to that of an isolated fungal assemblage from the same host, despite sequencing differences. While Phoma and Microdochium were predicted to be major players in the A. arenaria root fungal community based on isolated assemblages, they were not found using DGGE.

Additional research using 18S rDNA PCR and DGGE on A. arenaria endophytes demonstrated that the taxonomic structure of AM fungal assemblages in roots and that inferred from spore diversity are unrelated. It has been common practise to directly amplify AM fungus from whole root DNA for quite some time. Testing for AM infection in roots using competitive PCR has been shown to be doable. While it has been around for some time, it has just lately been put to use to resolve pressing ecological concerns. Helgason and coworkers (1998) used partial 18S rDNA PCR from total root DNA and cloning to compare AM fungal diversity in the roots of monoculture arable crop plant hosts and forest plant species. It was believed that the prevalence of AM fungus, which can infect a wide range of host plants, was not due to monoculture planting but rather an effect of agronomic management approaches on diversity. Different AM communities have been found in the roots of several plant species, however, according to a different study that used the same techniques in combination with T-RFLP (Vandenkoornhuyse et al. 2003). More evidence that floristic diversity may have a substantial effect on endophyte diversity was provided by Johnson and colleagues (2004), who compared the AM fungal assemblages in the roots of Plantago lanceolata bait plants in microcosms grown in monoculture and mixed plant communities. They were able to do so by employing a PCR and T-RFLP technique based on an 18S rDNA subset.

6.5 Popular Approaches

Community profiling techniques have recently made significant strides in soil mycology. These methods are already being used in labs all over the world, and it is expected that our knowledge of the composition and evolution of soil fungal communities will expand dramatically in the near future as a result. But researchers shouldn't just choose any old set of primers or community profiling technique to assess fungal diversity; they should weigh the pros and cons carefully in light of their goals, the fungi of interest, and the data they want to analyse. In addition, we have emphasised in this review the limits of the methodologies that must be taken into account in the context of individual studies and their respective hypotheses. Some of these restrictions apply across all fields of ecology, whereas others have been addressed in soil bacterial community investigations, leading to improved methods. Diversity analysis based on taxonomic richness and relative abundance, along with community dynamics, is far more difficult than analysing, say, the taxonomic richness of soil fungus. This is because fungi are mycelial in structure, and spores are likely to coexist in any given sample. The technique of stable isotope probing (SIP), which classifies organisms according to their nucleic acids' abilities to use certain stable isotope-marked substrates, has been successfully applied to the separation of soil bacteria into their respective functional groups (Morris et al. 2002). In particular, its benefits to soil fungus have just lately been recognised. These scientists present a convincing case for using this method to investigate microbial DNA in soil. Our findings show that SIP may be used to examine fungal RNA, which integrates 13C quicker

than DNA. This is in contrast to bacterial DNA, which incorporates carbon isotopes more slowly. In a practical setting, analysing fungal RNA obtained from soil enables the detection and identification of metabolically active community members rather than dormant individuals. Consequently, this is a major development in our understanding of fungus and the ecosystems in which they exist. Fungal DNA and RNA levels in soil have also been quantified using real-time PCR (Lueders et al. 2004). Opportunities for further development of metagenomics techniques and microarray technology to study the composition and activity of microbial communities seem promising. These methodologies, when used in tandem with community profiling techniques, allow us to get a more nuanced understanding of fungal communities and the roles they play in the ecological processes of soil.

6.6 Isotopic Labeling

This can be done with the use of circumstantial evidence, such as when scientists try to link a change in the abundance of a certain group or set of genes with the event in question. By using isotopes, substrates may be individually verified for membership in a certain category (Dumont and Murrell 2005). Micro-autoradiography (MAR) requires the introduction of radiolabeled substrates or nucleotides, such as 3H-labelled thymidine, into the soil. While FISH applied to soil may allow for the identification of live cells in their natural setting, it suffers from the same limitations as other direct imaging techniques (Wagner et al. 2006; Rogers et al. 2007). In order to better identify certain subpopulations, it is encouraged to label soil-extractable PLFAs or nucleic acids. When methane oxidizers in soil are exposed to 13CH4, stable isotope probing (SIP) is most often observed in the interaction of 13C with PLFAs or DNA (Bull et al. 2000; Radajewski et al. 2000). Growing cells take up 13C from certain substrates in their DNA and rRNA. In order to separate the 13C-enriched fraction from the unlabeled fraction of isolated soil nucleic acids, density gradient centrifugation can be used. Methods for SIP are outlined by Kreuzer-Martin (2007); these techniques are useful for examining separate groups that make use of specific substrates like methane, and they may also give more broad information on the members of actively forming soil communities. Many soil communities, for instance, have been studied using various 13C substrates in conjunction with DNA and RNA-SIP (Manefield et al. 2002; Lueders et al. 2004; Whiteley et al. 2006).

6.7 BrdU Labeling

The thymidine nucleotide analogue 5-bedroom-20-deoxyuridine (BrdU) can be used alone or in conjunction with an exogenous substrate to mark dividing soil cells. Using any of the current community DNA analysis techniques, DNA from the less active minority may be compared to DNA from cells containing BrdU. In soils from several

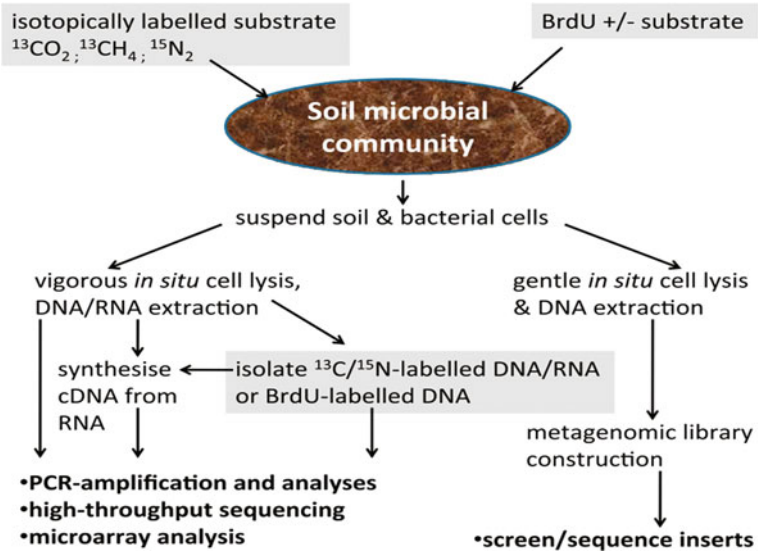

Fig. 6.5 Flow chart showing DNA and RNA extraction from soil. The soil microbial community will include bacteria, archaea, fungi, protists, and microfauna

sites that were incubated at either 4 or 25 °C, we found that BrdU was useful for assessing both the total and growing communities (Figs. 6.5 and 6.6). The data reveal that, in contrast to the profiles generated from actively reproducing bacteria that absorbed BrdU, whole-genome DNA profiles do not cluster according to soil type or incubation temperature. Other possible uses for BrdU include identifying members of disease-resistant plant-related active microbial communities in soil and identifying bacteria connected with arbuscular mycorrhizal fungus hyphae (Artursson and Jansson 2003). Researchers from many institutions collaborated on the project (Hjort et al. 2007).

6.8 The Potential of Microarrays in Soil

Microarrays have been constructed in recent years using sequence data from publically available bacterial genomes deposited in databases like GenBank at the NCBI. Recent literature on the use of microarrays in soil microbial ecology (Sessitsch et al. 2006). The PhyloChip, designed by Gary Andersen and his colleagues at the Lawrence Berkeley National Lab in the United States, is a phylogenetic microarray that uses databases of 16S rRNA gene sequences. With a total of 500,000 probes, this high-density array has the potential to identify w9000 unique species or taxa. To reflect the ever-evolving nature of DNA databases, these microarrays are routinely

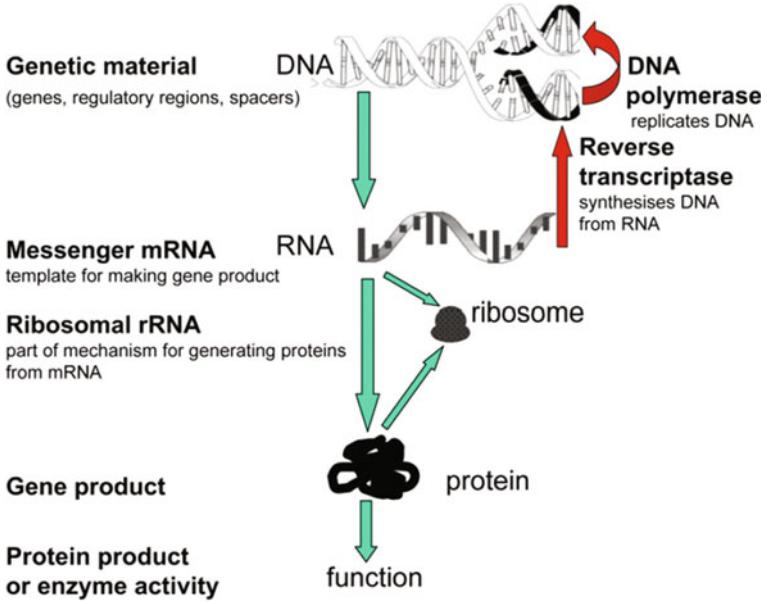

Fig. 6.6 The relationship between DNA, RNA forms, protein functions, and pathways

upgraded to include the most recent data. Some researchers believe that these techniques, as opposed to traditional cloning/sequencing procedures, may show a greater diversity of bacterial phyla and species in soil (DeSantis et al. 2007). In addition to showcasing the richness of ecosystems, these arrays can provide insight into how various taxa are represented within a given treatment, such as rhizosphere vs. bulk soil (DeAngelis et al. 2009). When it comes to identifying the people who make a community tick, rRNA may soon supplant genomic DNA as the gold standard. While it can provide some insight into the composition of known microbial communities, the array does not include all of the functional genes involved for the major activities in soil. The GeoChip, created by Jizhong Zhou at the University of Oklahoma in the United States, is based on functional gene sequences, whereas phylochips are based on non-functional gene sequences. By employing GeoChip 2, for instance, researchers have shown that the number and variety of genes involved in organic C degradation in soils is on the rise. GeoChip 2 has the potential to uncover over 10,000 genes in over 150 different functional areas. To wit: (Zhang et al. 2007). Functional genes in several species may be identified using probes, yielding information on biodiversity. The already outstanding identification rate of 47,000 genes across 292 gene families utilising the new and improved GeoChip 3 is expected to rise as additional sequencing data becomes available. The RNA that is collected from soil can provide information about which genes were active right before sampling, which is something that DNA cannot do. Microarrays, on the other hand, rely on already known DNA sequence information, therefore they are unable to identify new groups

that may become critical to particular functions. Because so little is known about the taxonomic diversity and functional gene sequences of soil fungi, microarrays for soil fungal communities are also now unavailable.

6.9 High-Throughput Sequencing

Traditional cloning and Sanger sequencing take a long time, thus only a small number of samples may be processed at once. Automatic diagnostic techniques (sometimes known as "lab-on-a-chip" technology) are now under development. Utilizing these techniques, researchers may now analyse hundreds of samples to study microbial functional diversity. Thanks to recent advancements in massively parallel high-throughput pyrosequencing techniques, hundreds of thousands of sequences may be processed in parallel. At now, the "GS FLX Titanium Series" machine can sequence one million fragments with an average read length of 400 bp; however, this will increase to 800 bp in the near future. Information on the most recent models may usually be found on company websites, and other commercial high-throughput sequencing equipment offer a more extensive variety of shorter sequences. In comparison to the Applied Biosystems Inc. SOLiD3 system, which promises to sequence 10–15 Gb every 6–7 day run with reads of up to 50b, the Illumina Inc. Genome AnalyserIIx, as of this writing, offers to sequence 20–25 Gb per 9–10 day run. With the fast development of new technologies, new approaches like nanopore sequencing are being created (Mardis 2008). In a 2009 study (Stoddart et al. However, weighing the benefits and drawbacks of each approach is outside the scope of this study and is being done on several online forums. In order to alter the DNA that has been collected from soil, PCR amplification with gene-specific primers can be performed. The relative abundance of bacterial and archaeal 16S rRNA genes (the mean from four soils was w140,000 bacterial; w9000 archaeal sequences) and the total taxonomic diversity of these species in soil were determined using universal 16S rRNA gene primers, for example (Roesch et al. 2007). This approach has the limitation that it assumes all prokaryotes have 16S rRNA gene sequences comparable to the primers used in the PCR amplification stage. However, many bacteria and archaea are able to be cultured despite deviating from the standard primer sequence for this gene. But the technology will develop into a more useful tool as more and more critical pieces may be scheduled with each iteration. Recent advances in high-throughput sequencing have rendered direct and unbiased sampling unproductive because of their inability to unearth uncommon but ecologically significant categories without prior selection. In the future, only the bioinformatic processing of sequence data is anticipated to limit the potential expansion of sequencing, which is now useful and will become much more so as more advanced methods are developed (Pop and Salzberg 2008).

6.10 Conclusion

There are obstacles to exploring the microbiota due to the large number and variety of soil microbiota and the varied nature of the soil, irrespective of the site being polluted or otherwise. Results from small samples that may be used for the study have made traditional protocols unappealing; nevertheless, cutting-edge methods increase the number of soil samples included. Understanding the fundamental mechanisms of each method for investigating microbiota diversity and activity is crucial for mitigating the demerits and capitalizing on the strengths of the various approaches.

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