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DETECTION OF BACTERIAL DIVERSITY OF VARIOUS HABITATS IN ÇORUM PROVINCE AND ITS CRIMINALISTICS CONTRIBUTION TO POSSIBLE CRIME SCENE STUDIES

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Abstract: Microorganisms are not homogeneously distributed in environments, soil systems are heterogeneous. Soil can be an important evidence value in forensic investigations. It is among the important evidences that contribute to the solution of forensic events in forensic sciences. *Bacteria* contained in the soil are microbiological evidences. Not all bacteria can be cultured by conventional methods and the amount of cultured bacteria remains limited. Metagenomic studies have been carried out for non-culturable *Bacteria*. The aim of this study is to perform DNA isolation from soil samples taken from Yeşil Lake (swamp), Faculty of Arts and Sciences garden, agricultural land, Sıklık (forest area) regions of Çorum Province in Türkiye and to determine bacterial diversity by metagenomic analysis of DNA isolated from soil samples. Density and differences of isolates according to habitats were determined. It is thought that the result of this study can shed light on previous crime scene studies in the determined habitats and will contribute to possible future crime scene studies and forensic science that may occur later.

Keywords: Habitat, DNA isolation, Bacterial diversity, Criminalistics

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1. Introduction

The most important time to find the evidence that guides the forensic events is when the crime scene investigation is done. Crime scene investigation and the evidence to be obtained are very important in terms of starting and ending the investigation in the best and correct way. All kinds of material are evidence. Evidence can be found at the macro or micro level, depending on the way the event occurred. There is evidence that can be obtained at the micro level in cases where the DNA is degraded and not sufficient at the crime scene. Soil is physical evidence that can be distinguished at a crime scene thanks to the variability of the microorganisms it contains. Since factors such as temperature, humidity, pH, climate, and topography affect the soil structure differently in each area, a result can be obtained by finding differences with comparison. Because of the complex structure of soils, the analysis of inorganic and organic components provides complementary independent information about the soil's geological origin, dominant vegetation, management and environment (Dawson and Hillier, 2010; Efeoğlu et al., 2022).

In forensic microbiology, it is important that many bacteria can be used as evidence. Bioterrorism makes a great contribution to forensic sciences to find the time and cause of death and to establish the plot in crime scene investigation studies. 16S rRNA gene sequence analyzes are performed to find the taxonomic connection of prokaryotes. The presence of 16S rRNA in all bacteria makes 16S rRNA gene sequencing a universal method. However, this method is not a perfect measure of gene sequence variation. 16S rRNA gene analysis is widely used to identify bacteria found in soil. Analysis is difficult due to the large number of bacterial species found in the soil. Scientists prefer to use 16S rRNA gene analysis to study bacteria (Carter et al., 2017).

In a study conducted by Efeoğlu et al., 2022 they aimed to evaluate the importance of identifying microorganisms in soil in terms of forensic science. The study covered 20 regions identified and marked outside the residential areas within the borders of Istanbul. 83% bacteria and 17% fungi were detected in soil samples and physical evidence samples (fabric, rubber, metal, and wood)

collected.

Demanèche et al. (2017) conducted a blind test to determine the origine of two samples (one from the mock crime scene and the other from a 50:50 mixture of the crime scene and the alibi area) compared to three control samples (soil samples from the crime scene, from a context site 25 m away from the crime scene and from the alibi site which was the suspect's home). Two biological methods, Ribosomal Intergenic Spacer Analysis (RISA) and 16S rRNA gene sequencing with Illumina Miseq, were used to evaluate the discriminatory power of soil bacterial communities. Promising results were already observed with molecular methods applied to soil extracted DNA under specific conditions, including T-RFLP, ARDRA, RISA and HTS (High-throughput sequencing) methods such as metagenomic and amplicon sequencing. As a results of this study, RISA or High-throughput sequencing (HTS) in soil DNA was able to identify single-origin soil samples, a combination of methods was required to accurately identify samples of mixed origin and soil DNA can be a useful tool for forensic science (Demanèche et al., 2017).

Although numerical estimation of prokaryotic cells was made, this result was reached as a result of culturing. However, contrary to what is known, the rate of non-culturable bacteria is much higher than that of bacteria that can be cultured and identified in this way. Metagenomic studies promise to reveal the gene sequence of the majority of microorganisms, which cannot be easily obtained by pure culture, by providing the confidence to clarify this situation. Thus, it is possible to reveal and identify the presence of unknown microorganisms (Kunin et al., 2008).

The aim of this study was to perform metagenomic analysis and genomic marking on soil samples collected from Yeşil Lake (swamp), Sıklık Nature Park (forest), agricultural land and the garden of the Faculty of Arts and Sciences of Çorum Province in Türkiye. As a result of the determination of the microbiological ratio by DNA analysis in the soil samples obtained, it is thought that it will guide the forensic sciences and investigation in terms of determining whether the incident took place in the visible place or in a different place.

2. Materials and Methods

2.1. Collection and Storage of Soil Samples

Soil samples were collected from Yeşil Lake (Latitude: 40.763084, Longitude: 34.863324), Faculty of Arts and Sciences' garden (Latitude: 40.566835, Longitude: 34.934049), Sıklık Nature Park (Latitude: 40.351975, Longitude: 35.023487) and an active cultivated agricultural land in Çorum Province (Latitude: 40.416385, Longitude: 35.034248). The distance between the fields is one of the reasons for their selection. The reason for choosing these areas is differences between forested area, inner city, aquatic environment and agricultural land also there are north-south differences in distance from each other.

While collecting the soil samples, attention was paid to the small number of people and acted quickly. Considering the hot weather, immediately after sampling, the soil was placed in a hot and cold protected aluminum-coated bag with ice cubes inside and was stored in a refrigerator at -20 degrees Celsius without wasting time. The soil sample was collected from a depth of 15-20 cm. This 0-15 cm depth was chosen because microbial activity has been reported to occur in the upper 15 cm (Castañeda and Barbosa, 2017). The samples taken were labeled and stored individually in ziplock bags. Samples taken during the daytime were packed in heat insulated bags containing ice cubes without waiting. In order to minimize heat loss, it was packed in bags, styrofoam boxes and delivered to the company to be analyzed within 18 hours.

2.2. DNA Extraction and Sequencing

DNA extraction and purification was performed using the Qiagen DNeasy PowerSoil Pro Kit. After the isolation, DNA concentrations and purity values of the samples were measured with a spectrophotometer device (Table 1). In DNA quantification with this device, measurements were made according to the absorbance values measured at 260 nm wavelength. The ratio of absorption values of DNA at 260 and 280 nm wavelengths (A260/A280) was used for purity control. If this ratio is in the range of 1.8-2.0, the isolated DNA is considered pure, and if it is outside the value range, the DNA is considered impure (Matlock, 2015). The obtained DNA samples were used in metagenomic analysis.

Table.1. Concentration values of isolated DNAs

Sample	Concentration (ng/ µL)
Sıklık Natural Park (SP)	33.516
Agricultural Land (AL)	23.955
Faculty of Art and Sciences (FAS)	19.409
Yesil Lake (YL)	25.816

Qiagen brand DNeasy PowerSoil Pro Kit (Figure 1) was used as the kit of choice, as it is effective and sensitive in the purification of proteins and nucleic acids in molecular biology applications and in the isolation of DNA from difficult samples such as compost, soil fertilizer and similar. QIAGEN's the second generation Inhibitor Removal Technology allows us to obtain high quality DNA that can be used immediately in difficult soil types such as environmental compost. With this kit, cell lysis is carried out by mechanical and chemical methods. Total genomic DNA is obtained by trapping on a silica membrane in spin column format and then eluted from the membrane by washing. The working protocol steps of the kit are as follows;

250 mg of soil, 800 µl of CD1 solution included in the kit are added to the PowerBead Pro Tube and vortexed. The tube used contains a buffer that will help disperse the soil particles, begin to dissolve the humic acids, and also

protect the nucleic acids from degradation. With a short vortexing process, the components in the tube are mixed and the buffer begins to disperse into the sample. After vortexing for a minimum of 10 minutes and ensuring that the samples are thoroughly homogenized, the tube is centrifuged at 15,000 rpm for 1 minute. The supernatant is transferred to a new 2 ml microcentrifuge tube. Add 200 µl of CD2 solution and vortex for 5 seconds. CD2 solution contains a reagent that can precipitate non-DNA organic and inorganic material including cell debris, humic substances, and proteins. It is centrifuged at 15,000 rpm for 1 minute and 700 µl of supernatant is taken into a new 2 ml microcentrifuge tube, avoiding the pellet. At this point, the pellet contains non-DNA humic acids, cell debris and proteins. Therefore, for the best DNA yield and quality, there should be no contamination from the pellet while taking the supernatant. 600 µl of CD3 solution, a highly concentrated salt solution, is added and vortexed for 5 seconds. Since DNA binds tightly to silica at high salt concentrations, the content of this solution is adjusted to allow DNA to bind. DNA binds selectively to the silica membrane in the MB spin column in the presence of high salt solution. Contaminants pass through the filter membrane and only DNA remains attached to the membrane. Here, 650 µl of lysate is loaded and centrifuged at 15,000 rpm for 1 minute. Transfer the MB spin column to a new 2 ml collection tube. 500 µl of EA solution, which is a wash bag that removes proteins and other non-aqueous substances, is added and centrifuged at 15,000 rpm for 1 minute. The bottom is discarded and the MB spin column is placed in the same 2 ml collection tube. 500 µl of C5 solution, an ethanol-based wash solution used to further clean the DNA attached to the silica filter membrane in the MB spin column, is added and centrifuged for 1 minute at 15,000 rpm. This wash solution removes residual salt, humic acid and other contaminants while allowing the DNA to remain attached to the silica membrane. The bottom is discarded and the MB spin column is placed in a new 2 ml collection tube. It is centrifuged at 16,000 rpm for 2 minutes. This rotation is important to remove all traces of the C5 solution. The MB spin column is placed in a new 1.5 ml Elution Tube. Add 50-100 µl of C6 solution to the center of the white filter membrane. Placing this solution in the middle of the membrane ensures that the membrane is wet so that the DNA is released from the membrane more efficiently and completely. The C6 solution is a salt-free (10 nM Tris) solution. The DNA bound in the presence of high salt is selectively liberated with the C6 solution. Centrifuge at 15,000 rpm for 1 minute and discard the MB spin column. As a result of these processes, DNA is isolated for other applications. The isolated DNA is amplified by PCR and sent for analysis for sequencing (URL, 2022).

After DNA isolation, NGS (Next Generation Sequencing) library was prepared and the sequencing process was started. Bioinformatics analysis was performed after sequencing. According to the absorbance values measured at 260 and 280 nm wavelengths of the initially isolated DNA, it was assumed to be pure between 1.8-2.0 values (Matlock, 2015), and the purity level was checked by reading with a spectrophotometer (Topal Sarıkaya, 2008).

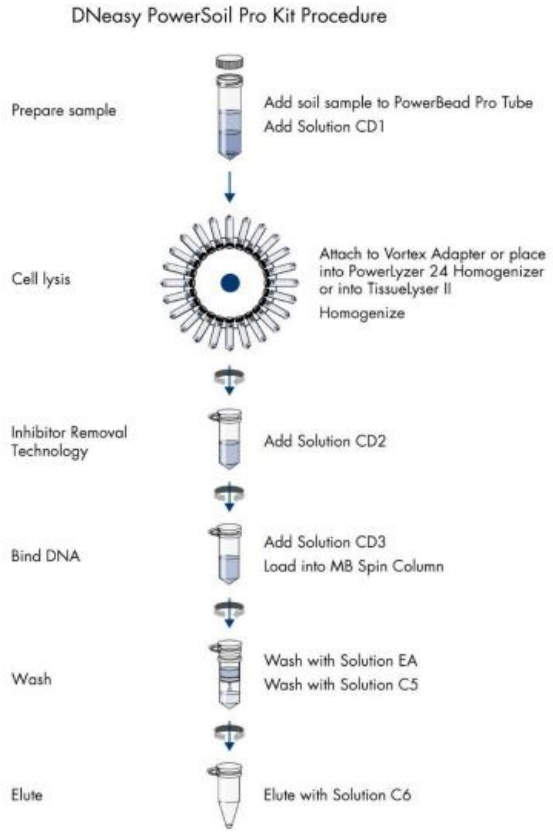

Figure 1. DNeasy powersoil pro kit procedure.

2.3. DNA Analyzing

When performing 16S amplicon sequencing and bioinformatics analysis, extracted DNA samples were subjected to amplicon sequencing library preparation using bacteria specific 341F (5'-CCTACGGGNGGCWGCAG-3) and 805R (5'- GACTACHVGGGTATCTAATCC-3') primers targeting V3-V4 region of the 16S rRNA gene. Prepared libraries were purified, quantified and further sequenced on MiSeq instrument (Illumina, USA) using 300bp paired-end chemistry. Demultiplexing and clipping of sequence adapters from raw sequences was performed by CASAVA data analysis software (Illumina, USA). The fragments with any mismatches to the barcodes or primers were excluded. PCR primers were removed from sequences using cutadapt (10.14806/ej.17.1.200) plugin within QIIME2 v2021.2 (10.1038/s41587-019-0209-9) as all amplicon sequencing workflow. Paired-end reads were joined (vsearch join-pairs) and quality filtered (quality-filter q-score-joined). Then, sequences were denoised using deblur (deblur denoise-16S) (10.1128/mSystems.00191-16). Taxonomy was assigned to each amplicon sequence variant (ASV) using 'feature-classifier classify-sklearn' plugin against the SILVA v138 database. Final ASV table was used to calculate alpha diversity metrics.

In the study, V3-V4 region was amplified based on Klindworth et al., 2013. The collected 4 metagenomic sequencing samples were sent to the USA through Artı Biotechnology. Since pure DNA was obtained, it was sent at room temperature. Sequencing of pure DNAs was performed using CASAVA software. As a result of the data obtained from the sequencing, alpha diversity metrics, taxonomic bar graphs and Krona pie chart graphs were created using DNA sequences, cleaned raw sequences, ASV (amplicon sequence variants) table.

3. Results

3.1. Phylum Level Distribution in the Soil

According to the results of metagenomic analysis, 1 kingdom, 26 phyla, 69 classes, 142 orders, 221 families, 382 genera and 658 species were identified in all samples. Bacterial branches in habitats are shown by a histogram graph (Figure 2).

In our study, the results of the analysis of the soil sample taken from the forest area (Sıklık Nature Park) showed that the bacterial distribution of this area is as follows: *Actinobacteriota* 64%, *Proteobacteria* 10%, *Chloroflexi* 10%, *Gemmatimonadota* 5%, *Acidobacteriota* 5%, *Planctomycetota* 5%, *Myxococcota* 0.8 %, *Firmicutes* 0.3% and *Methylomimirabilota* 0.2% (Figure 3). In a study by Lladó et al., 2017, it was determined that the acidic soils of coniferous forests mainly contain *Actinobacteriota*, *Proteobacteria* and *Acidobacteria*. Similarly, the presence of coniferous trees in the Sıklık Nature Park and the presence of more species of *Actinobacteriota* according to the analysis results of other soil samples support this information. At the same time, when all the analysis results are considered, the highest bacterial density was observed with 64% *Actinobacteriota* in the Sıklık Nature Park.

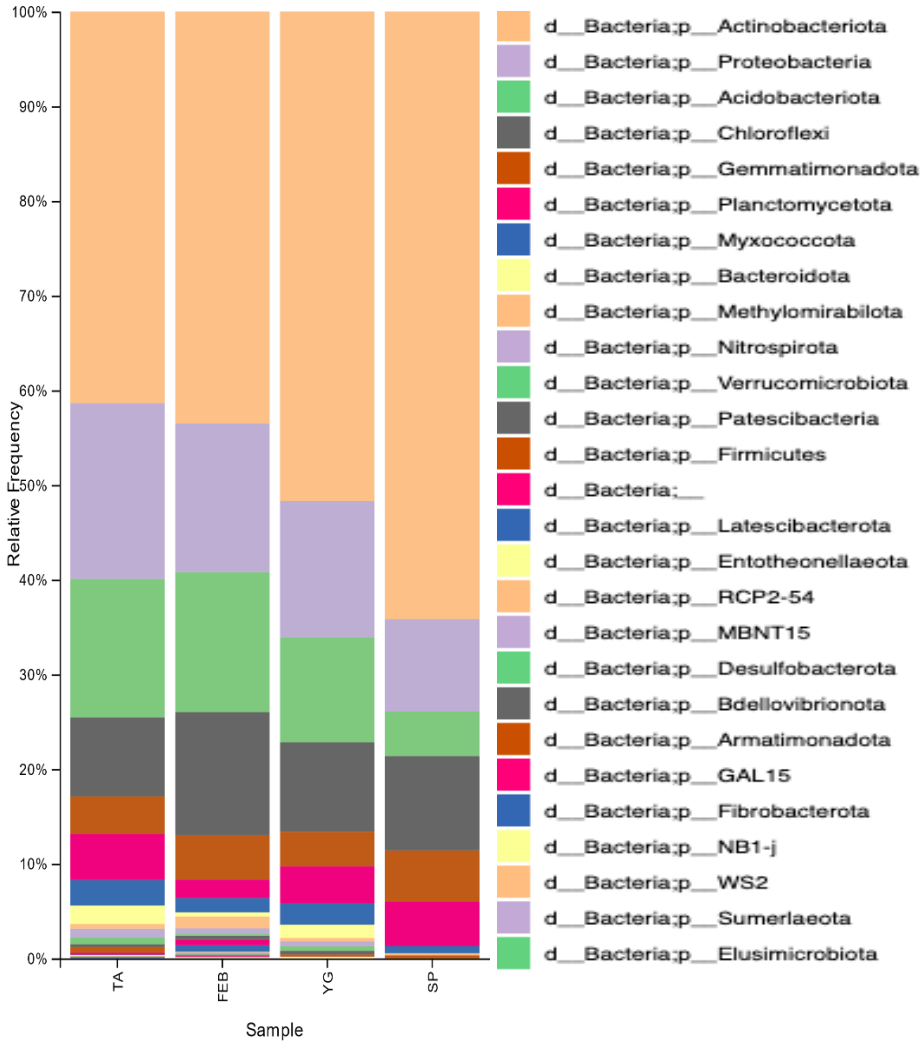

Figure 2. Representation of bacterial branches in habitats with histogram graph

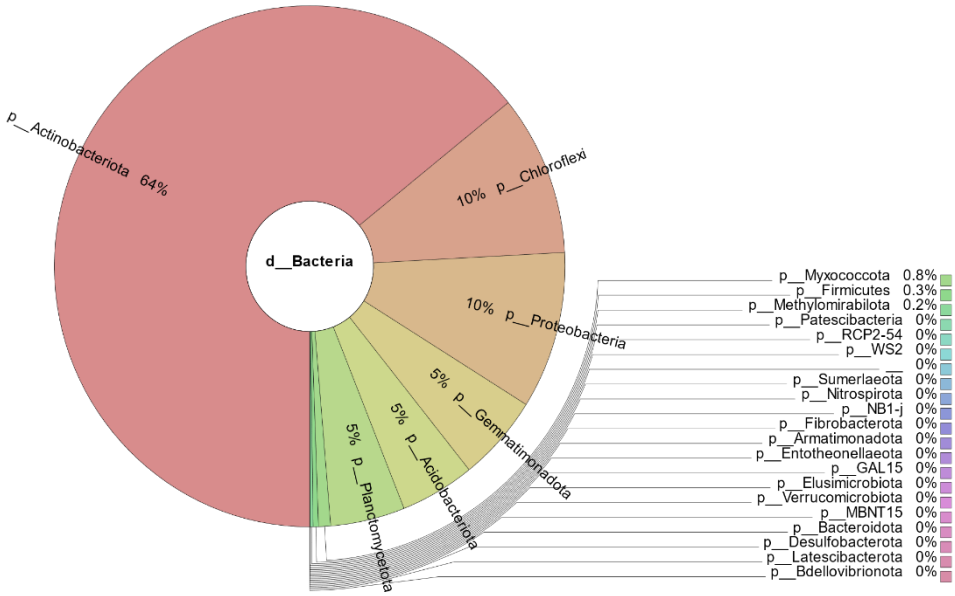

Figure 3. Representation of bacterial distribution in the soil sample taken from the Sıklık Nature Park on the Krona pie chart.

As a result of the analysis of the soil sample taken from the agricultural land, it was determined that while *Actinobacteriota* had the highest density with 41% followed by *Proteobacteria* 19%, *Acidobacteriota* 15%, *Chloroflexi* 8%, *Gemmatimonadota* 4%, *Planctomycetota* 5%, *Myxococcota* 3%, *Bacteroidota* 2%. 0.6% of *Firmicutes*, 0.5% of *Methylomirabilota*, 0.3% of *Patescibacteria*, but also the presence of 11 phyla less than 1% were determined (Figure 4).

As a result of the analysis of soil samples taken from the garden of Hitit University Faculty of Arts and Sciences, *Actinobacteriota* 44%, *Proteobacteria* 16%, *Acidobacteriota* 15%, *Chloroflexi* 13%, *Gemmatimonadota* 5%, *Planctomycetota* 2%, *Myxococcota* 2%, *Firmicutes* 0.03% and *Methylomirabilota* 1%, *Patescibacteria* was determined at a rate of 0.4% (Figure 5). At the same time, 14 different phyla were identified with percentages less than 1%. In the soil sample at the bottom of water we took from the Yeşil Lake as a swamp area, 52% *Actinobacteriota*, 14% *Proteobacteria*, 11% *Acidobacteriota* 9% *Chloroflexi*, 4% *Gemmatimonadota*, 4% *Planctomycetota*, 2% *Myxococcota*, 1% *Bacteroidota*, 0.2% *Firmicutes* and the presence of phyla such as 0.4% *Methylomirabilota* and 0.4% *Patescibacteria* were obtained (Figure 6).

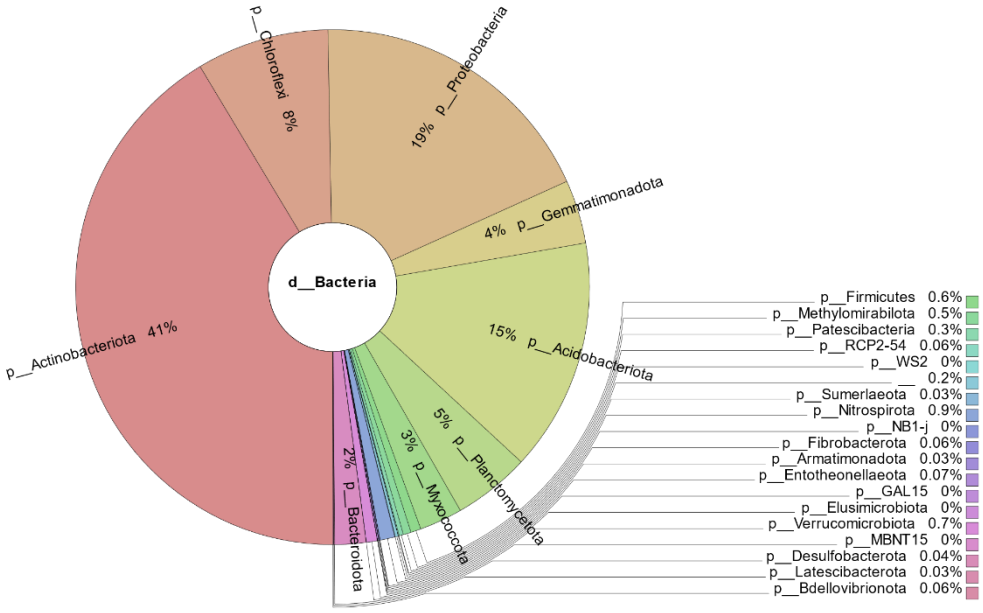

Figure 4. Demonstration of the bacterial distributions in the soil sample taken from the agricultural land on the Krona pie chart.

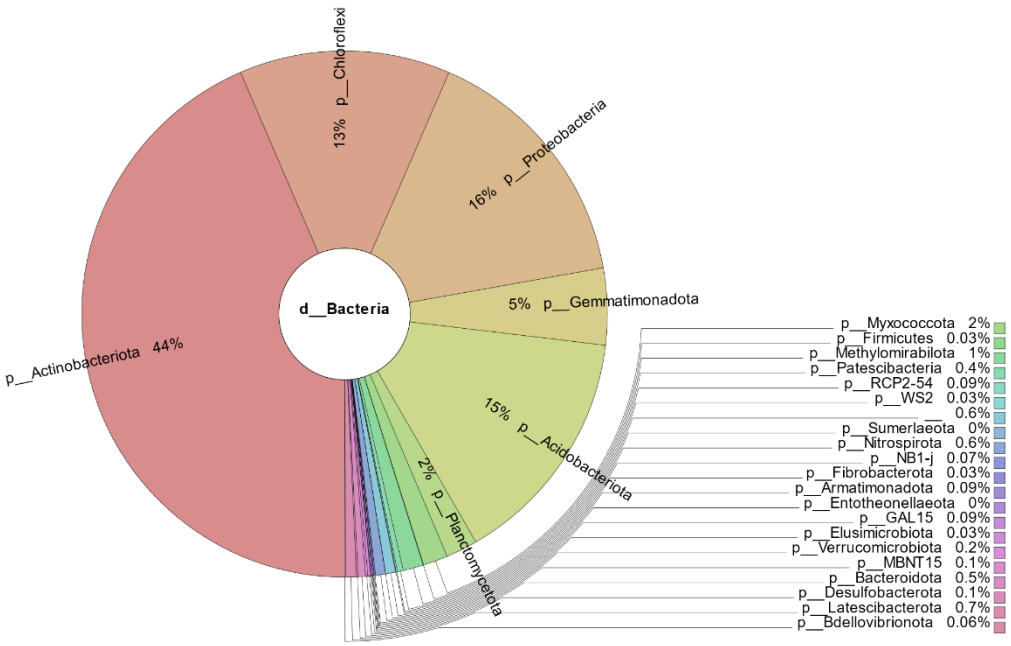

Figure 5. Demonstration of the bacterial distribution in the soil sample taken from the garden of the Faculty of Arts and Sciences on the Krona pie chart.

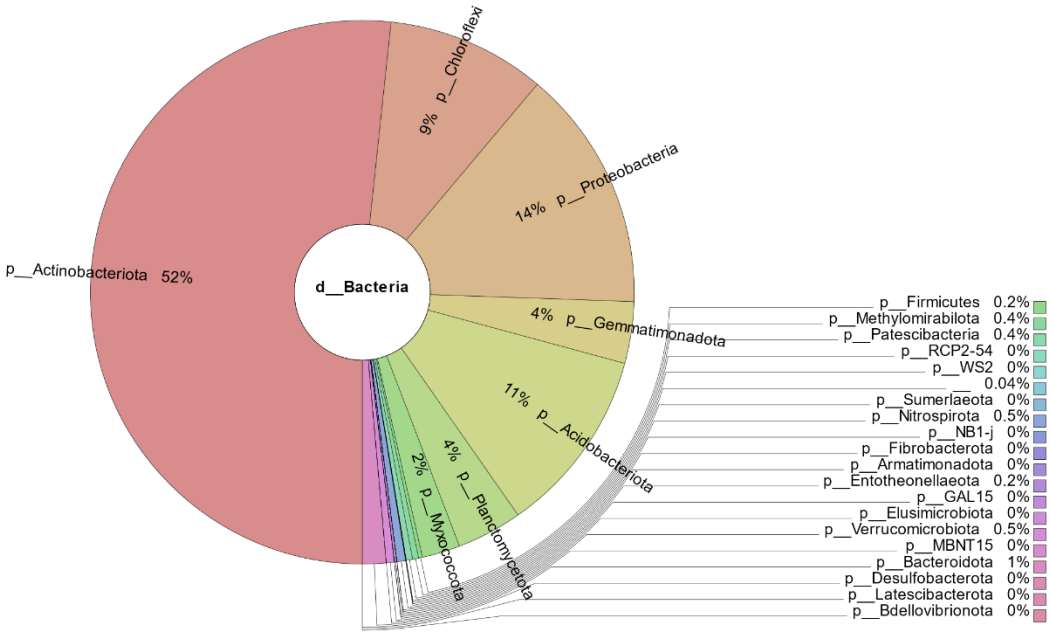

Figure 6. Demonstration of the bacterial distribution in the soil sample taken from the Green Lake on the Krona pie chart.

4. Discussion

It is present based on structures that geographical location is a more important factor than soil type in determining the microbial density in the soil (Habtom et al., 2019). In our study, the effective reason for determining the habitats was to take into account the areas with high crime rate and the potential to be a crime scene while selecting these areas, soil type was not taken into account and it was preferred that they were far from each other. It is also known that soil pH and C and N ratios in soil have an effect on the microbial community (Lauber et al., 2009; Rasche et al., 2011). At the same time, it has been determined in some studies that the soil microbial activity decreases compared to the summer season, especially in the winter months when the temperature factor decreases (Guoju et al., 2012). Based on these researches, in our study, considering the temperature factor, sample collection was carried out in summer when the microbial activity was the highest, cold storage was carried out and the current soil microbial community was frozen.

The aim of this study is to isolate DNA from soil samples

collected from Yeşil Lake, the garden of the Faculty of Arts and Sciences, an agricultural land and the Sıklık Nature Park regions of Çorum Province in Türkiye and to perform metagenomic analysis with the isolated DNA. It is thought that the results obtained will reveal the bacterial diversity in the soil samples and the diversity of soil bacteria in the determined habitats will shed light on the events that have occurred before and may occur in the future.

Within the scope of this study, DNA analysis was performed on soil samples taken from four different areas (the garden of the Faculty of Arts and Sciences, the Sıklık Nature Park, the Green Lake and an agricultural land) considering the temperature factor. Then the bacterial rates were determined by metagenomic analysis through service procurement from Artı Biotechnology Company has been done. The 16S bacterial rRNA gene was amplified by polymerase chain reaction (PCR) and sequenced. The taxonomic classification of the product amplicon sequence variants obtained as a result of PCR was performed in the SILVA v138 database. Percentages of reads at each taxonomic level between samples were analyzed and data are at the species level. All of the obtained data could not be included in our study due to the large file size.

Lladó et al. (2017) determined that five phyla (*Acidobacteriota*, *Actinobacteriota*, *Proteobacteria*, *Bacteroidota* and *Firmicutes*) were abundant in most soils. Similarly, the graphs in this study show that all five phyla are proportionally higher. Recent studies have shown that surface and buried soil communities exhibit different behavior during the weathering process. While microbial communities in surface soil tend to decrease in taxon richness, diversity and equality; Microbial communities in close contact with buried cadavers show contrasting characteristics (e.g., increased taxon richness, consistent diversity, and decreased evenness). Furthermore, while *Proteobacteria* was cited as the most abundant phylum in burial soil samples, the relative abundance of *Acidobacteriota* decreased and *Firmicutes* increased in surface cadaver-soil communities, while microbial community composition remained fairly constant in buried soil communities (Finley et al. 2015, 2016; Oliveira and Amorim, 2018).

In the samples we took, while the density of *Actinobacteriota* was 64% in the Sıklık Nature Park, it was 52% in the Green Lake, 44% in the garden of the Faculty of Arts and Sciences, and 41% in the agricultural land. It is thought that the density difference determined in the Sıklık Nature Park and the agricultural land is due to the acidic feature of the coniferous trees mentioned in the study by Lladó et al. (2017). The fact that the samples taken from the Sıklık Nature Park were taken the lower part of the trees, the presence of pine forests around the Yeşil Lake, the growth of similar trees in the garden of the Faculty of Arts and Sciences, and the absence of similar trees near the agricultural land indicate that the rate decreases when the tree density decreases.

For the *Proteobacteria* group, results were close to each other with 19% in the agricultural land, 16% in the Faculty of Arts and Sciences, 14% in the Yeşil Lake and 10% in the Sıklık Nature Park. This situation supports the information that *Proteobacteria* are involved in the global carbon, nitrogen and sulphur cycle (Kersters et al., 2006). In a study by Spain et al. (2009), it was stated that *Alphaproteobacteria* were the most abundant and diverse class in their study. Similarly, *Alphaproteobacteria* were found 10% in the Faculty of Science and Arts sample, 13% in the Agricultural Land, 11% in the Yeşil Lake and 6% in the Sıklık Nature Park, while *Gammaproteobacteria* was found 5% in the Faculty of Science and Arts and Agricultural Land samples, and 3% in Yeşil Lake and the Sıklık Nature Park. No data were available for the *Betaproteobacteria* class.

The *Acidobacteriota* group, which has the third largest ratio in terms of density, Kalam et al. (2020) stated that it is involved in the regulation of carbon, nitrogen and sulphur cycles. It is available in the literature that *Acidobacteriota* are dominant in microbial communities and also abundant in arable land (Kielak et al., 2009). According to the data obtained in our study, similarly, the presence of the *Acidobacterota* group was determined as 15% in the agricultural land and the garden of the Faculty of Arts and Sciences, 11% in the Yeşil Lake and 5% in the Sıklık Nature Park.

Even if our study was carried out to contribute to forensic science and to make microbial marking, the error rates of microorganism typing methods should be taken into account in a crime scene study, considering that there is a large amount of microorganisms in 1 gram of soil and the data we obtained belong to the sample area. Although we have come to a conclusion, it will be difficult to obtain data when there are incomparable samples due to the lack of a database with information on microorganisms found in Türkiye. Our study is a master's thesis, and with the increasing opportunities and technology in the future, another dimension can be added to metagenomic studies with more different examples.

5. Conclusion

Within the scope of this study, answers are sought to questions such as the relationship between the victim, the suspect and the crime scene at the crime scene, whether the soil samples found on the victim's soil or the suspect's belongings and the soil at the crime scene are similar and the extent of the microbial community. At the same time, our study can be considered as an important study in that it shows that phenotypic descriptions are not reliable and definitive results and that more reliable, inexpensive and short-term results can be obtained through metagenomic analysis.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	E.B.	D.T.	A.V.	A.T.
C	25	25	25	25
D	25	25	25	25
S		75	25	
DCP	25	25	25	25
DAI	25	25	25	25
L	25	25	25	25
W	75	25		
CR	25	25	25	25
SR	25	25	25	25
PM	25	25	25	25
FA	25	25	25	25

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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