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DETECTING USEFUL RAPD MARKERS FOR DNA VIRUS DIVERSITY ANALYSIS IN SOIL SAMPLES

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Abstract

Viruses, including bacteriophages, influence bacterial life and contribute to soil structure modification. Their genomes primarily exist in DNA form. Therefore, it is essential to understand the diversity of DNA viruses in agricultural soils, which serve as habitats for beneficial bacteria such as *Rhizobium* and *Azotobacter* species. These viruses play a significant role in microbial dynamics by regulating bacterial populations, facilitating gene transfer, and impacting nutrient cycling within the soil ecosystem. This study aims to identify useful random amplified polymorphic DNA (RAPD) markers for detecting DNA virus diversity in soil. Twenty-five primers were tested using the PCR method with 50 soil samples collected from 44 locations where bean cultivation is prevalent in six geographical regions of Turkey. The soil samples were meticulously filtered using a 0.22-µm filter and the filtered samples were checked for eukaryotic and prokaryotic contamination markers. All primers' PCR amplification efficiency and the useful primers' polymorphism information content values were calculated. Binary data were obtained using Phoretix1D software and a dendrogram illustrating the DNA virus diversity of the soil samples was created. Principal coordinates analysis of the samples was performed using GenAlEx software. PCR and dendrogram analysis revealed the primers that effectively distinguished and compared DNA virus diversity in soils.

Key words: soil, DNA viruses, diversity, RAPD markers, PCR

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Introduction. The ecological functions of soil viruses are complex [1]. Many of the microorganisms living in soil are critical for maintaining soil fertility in sustainable agriculture [2]. Microbial ecologists now recognize that soil viruses are critical to global biogeochemical cycles [3]. Nitrogen fixation and the release of biologically active compounds that ensure the productivity of the soil microflora both occur with the help of bacterial groups such as *Rhizobacteria* and *Azotobacter* that promote plant growth [2]. It is known that some bacteriophage viruses negatively control the fixed nitrogen amount in the soil by infecting populations of the nitrogen-fixing symbiont *Mesorhizobium* isolated from chickpea nodules [4].

Viruses also serve as genomic reservoirs in ecological systems due to their effects on beneficial bacteria and soil-borne plant pathogens and their contributions to horizontal gene transfer [5, 6]. It was reported that bacteriophages that negatively affect bacterial communities change the organic and mineral structure of the soil [1]. In recent years, it has been hypothesized that T4-like phages increase bacterial mortality in soils where they are abundant, thereby suppressing soil organic carbon mineralization [1]. It is estimated that approximately 25% of the fixed carbon in the soil enters the microbial cycle via virus-induced lysis [5].

Viral diversity in agricultural soils affects product quality and yield depending on the soil's structure [7]. Although viral community structures in complex agricultural soils are largely unknown, a study conducted in China suggested that tailed bacteriophages and single-stranded DNA viruses represent the largest viral components in most soil habitats based on metagenomic analysis [8]. Bacteriophages, which have relatively smaller genomes than other viruses, represent an absolute majority of all organisms in the world. When the genome diversity of approximately 750 bacteriophage populations was examined, most were tailed phages (*Caudovirales*) with double-stranded DNA [9].

Different methods are used to determine viral diversity in soil and water samples. Pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR) are the preferred methods for generating genetic fingerprints of soil and aquatic viral communities [10, 11]. RAPD-PCR markers have been successfully used in many studies to characterize soil virus communities [11, 12]. In this study, 25 RAPD primers were tested using the PCR method to detect DNA virus diversity in soil samples collected from different regions of Turkey. Among these primers, the useful ones with high amplification efficiency were determined.

Materials and methods. ***Soil sample preparation.*** Pesticide-free fields were chosen from among 44 bean-growing locations across six geographical regions of Turkey (Marmara Region, Aegean Region, Mediterranean Region, Central Anatolia Region, Black Sea Region, Southeastern Anatolia Region). Fifty different soil samples were collected from these locations: T1 (Samsun-Cayir), T2 (Samsun-Cayir), T3 (Samsun-Mermer), T4 (Samsun-Mermer), T5 (Samsun-Yagbasan), T6 (Samsun-Burunalan), T7 (Ankara-Kacarli), T8 (Bilecik-Kepirler), T9 (Bilecik-Kizildamlar), T10 (Kutahya-Yenice), T11 (Kutahya-Tavsanli), T12 (Kutahya-Boztepe), T13 (Erzurum-Ispir), T14 (Diyarbakir-Silvan), T15 (Afyon-Cobansargi), T16 (Afyon-Saldikbey), T17 (Antalya-Fatih), T18 (Isparta-Savkoy), T19 (Antalya-Side), T20 (Bilecik-Elmali), T21 (Artvin-Akpinar), T22 (Artvin-Ibrikli), T23 (Kastamonu-Daday), T24 (Karabuk-Eflani), T25 (Sivas-Centrum), T26 (Mardin-Midyat), T27 (Düzce-Gölyaka), T28 (Erzurum-Devedagi), T29 (Erzurum-Devedagi), T30 (Erzurum-Devedagi), T31 (Erzurum-Oztoprak), T32 (Erzurum-Numanpasa), T33 (Tokat-Niksar), T34 (Giresun-Görele), T35 (Ordu-Akkus-1), T36 (Balikesir-Edremit), T37 (Rize-Kaplicalar), T38 (K. Maras-Dulkadiroplu), T39 (Ordu-Akkus-2), T40 (Eskisehir-Cukurhisar), T41 (Artvin-Arhavi), T42 (Hatay-Reyhanli), T43 (Trabzon-Karatepe), T44 (Balikesir-Burhaniye), T45 (Trabzon-Semizoglu), T46 (Ordu-Akkus-2), T47 (Eskisehir-Karacasehir), T48 (Kutahya-Akpinar), T49 (Amasya-Merzifon), T50 (Bilecik-Kepirler).

Samples taken from 30 cm below the soil's surface were stored using black nylon bags in a dry and dark environment at room temperature. Soil materials were added to half of the volumes of 50-mL Falcon tubes and the volumes were completed to 45 mL with Luria broth. The tubes were then incubated overnight at 4 °C. The aqueous phases of the precipitated samples were filtered using 0.22-µm polyether sulfone (PES) filters.

Contamination control PCR. Possible prokaryotic and eukaryotic contamination in the filtered soil samples was checked according to Segobola et al. [13] using the following discriminant markers: E9F "GAGTTTGATCCTGGCTCAG", U1510R "GGTTACCTTGTTACGACTT", ITS1 "TCCGTAGGT-GAACCTGCG G" and ITS4 "TCCTCCGCTTATTGATATGC".

RAPD-PCR. RAPD primers used in viral diversity and standard analyses [14] were selected for PCR (Table 1). The final concentrations for the reaction volume of 25 µL were 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 µM primer, 1 U Taq polymerase, and 1X PCR buffer. Amplifications were carried out with an Arktik Thermal Cycler (Thermo Scientific, USA) programmed for an initial denaturation step at 95 °C for 5 min, followed by 45 cycles of 95 °C for 55 s, 32–51 °C for 1 min, and 72 °C for 2 min with a final elongation at 72 °C for 7 min.

All reactions were performed in duplicate. The PCR products were separated on 1.2% agarose gel containing EtBr (0.5 µg/mL) with a 100-bp plus DNA ladder (Thermo Scientific, USA) and photographed using a gel documentation system (Carestream, USA).

Data analysis. Band profiles, band counting, and size determination for each RAPD primer were obtained using Phoretix1D Pro software. A dendrogram was drawn using the UPGMA clustering analysis method and MEGA 11 software with binary data to evaluate the genetic distance between samples. One hundred bootstrap replicates were generated for dendrograms. The polymorphism information content (PIC) values for each primer were calculated using the following

Table 1

RAPD primer names, sequences, efficiencies, and amplification properties

Primer names	**Sequence (5'-3')**	**Tm (°C)**	**Primer efficiency (%)***	**Total bands**	**Polymorphic bands**	**Polymorphism rate (%)**	**PIC value**
AAWS-1	CACCACCTGC	35	0	–	–	–	–
CRA-22	CCGCAGCCAA	39	100	21	21	100	0.565
CRA23	GCGATCCCCA	36	50	–	–	–	–
HCB-1	CCAGCAGCAG	36	50	–	–	–	–
HDC-1	CGCCGCCGCC	51	0	–	–	–	–
LWHS-1	GTTCGGGTCG	35	0	–	–	–	–
OPA6	GGTCCCTGAC	32	0	–	–	–	–
OPA9	GGGTAACGCC	35	10	–	–	–	–
OPA-13	CAGCACCCAC	35	100	33	33	100	0.543
OPB-17	AGGGAACGAG	32	50	–	–	–	–
OPL-5	ACGCAGGCAC	39	25	–	–	–	–
OPU-13	GGCTGGTTCC	34	0	–	–	–	–
OPU-16	CTGCGCTGGA	34	0	–	–	–	–
P2	AACGGGCAGA	35	0	–	–	–	–
P5	AACGCGCAAC	32	100	41	41	100	0.409
P11	GGCCGATGAT	32	100	39	39	100	0.448
P13	ACCGCCTTGT	32	100	34	34	100	0.533
P14	CAGCACTGAC	32	12	–	–	–	–
P16	TGGTGGCCTT	32	0	–	–	–	–
P17	GTAGCACTCC	32	10	–	–	–	–
P21	ACGGTGCCTG	34	100	28	28	100	0.587
P23	CGCCCAAGCC	32	10	–	–	–	–
P24	CGCCCTGGTC	34	0	–	–	–	–
RAPD-5	AACGCGCAAC	38	0	–	–	–	–
RWLS-1	GCGATCCACG	36	0	–	–	–	–

*: Ratio of amplified samples to the total number of samples

formula: PIC $= 2(1 - f)$, where f represents allele frequency [15]. Data for principal coordinates analysis (PCoA) were obtained using GenAlEx software.

Results. DNA sequences obtained from 50 soil samples from 44 bean-growing locations across six geographical regions of Turkey were amplified for the amplification performance testing of 25 RAPD primers. We also checked for eukaryotic and prokaryotic contamination using universal markers. Table 1 shows the primers' efficiency rates, PIC values, polymorphism rates, and total and polymorphic band numbers. The amplification results of six primers with 100% efficiency for all samples were visualized on agarose gel with EtBr (Fig. 1).

The dendrogram showing DNA virus diversity among the soil samples in Fig. 2, presenting colours to denote geographical regions and location numbers, was created according to the UPGMA clustering analysis method. PCoA was performed for 50 soil samples using GenAlEx software (Fig. 3).

Discussion. All organic and inorganic soil components play roles in determining soil productivity in important application areas such as agriculture [2, 3]. Bacteriophage viruses are particularly critical actors in the mechanisms that con-

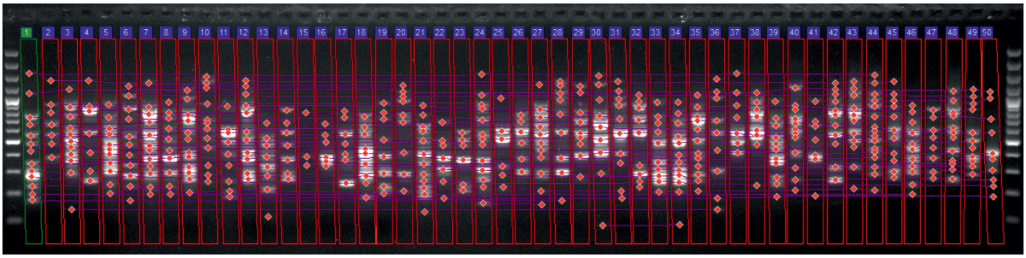

Fig. 1. Agarose gel image of amplification performed with the Opa-13 primer. M: 100-bp plus DNA ladder. 1–50: Soil samples

trol bacterial species playing an active role in soil productivity [6, 7]. Therefore, bacteriophages indirectly determine the structure and content of the soil. In addition, the negative control of plant-friendly bacteria negatively affects agricultural productivity. Lysis of beneficial nitrogen-fixing bacteria in chickpea and bean plant roots due to phage diversity is an example of these negative effects on agricultural production [4]. It is known that the vast majority of soil bacteriophages have DNA-based genomes [8]. Different DNA-based methods and markers for determining DNA virus diversity in agricultural soils exist [10] but RAPD-PCR markers are the most economical and widely preferred [12]. In 2020, the Hcb1 RAPD marker was effectively used to determine viral diversity in agricultural and early plant successional soil samples [11]. In the same year, Dias et al. [10] analyzed the DNA virus diversity of three soil samples collected from a pumpkin plantation in Brazil with three RAPD marker primers (RAPD5, OPL5, and P2). As an expected result, these two studies concluded that the number of amplified bands was relatively low. The first use of Hcb1 and several other RAPD markers in viral diversity analysis occurred in 2013. Srinivasiah et al. [12] performed DNA virus diversity analysis for three Antarctic soil samples. A very high amount of band amplification was obtained in that study, where four primers were effectively used.

In this study, we tested the usefulness of 25 RAPD primers in determining DNA virus diversity in soil samples. We used 50 soil samples from 44 locations across six geographical regions where bean cultivation is carried out in Turkey. In the previously mentioned studies, more limited numbers of soil samples and markers were used. This study aimed to find high-efficiency RAPD markers recognizing DNA virus genomes in all soil samples. We determined the primer products' band numbers, efficiency rates, and polymorphism statuses (Table 1) in the PCR amplification gels of the soil samples (Fig. 1). We observed that six RAPD primers gave high-efficiency results for all soil samples. Each primer had 21–41 different amplification DNA bands and 100% polymorphism. These results show that the primers have high discriminatory power in genetic diversity analyses. Using binary data obtained from gel band images with the Phoretix1D Pro program, we created a genetic diversity dendrogram with the UPGMA method (Fig. 2).

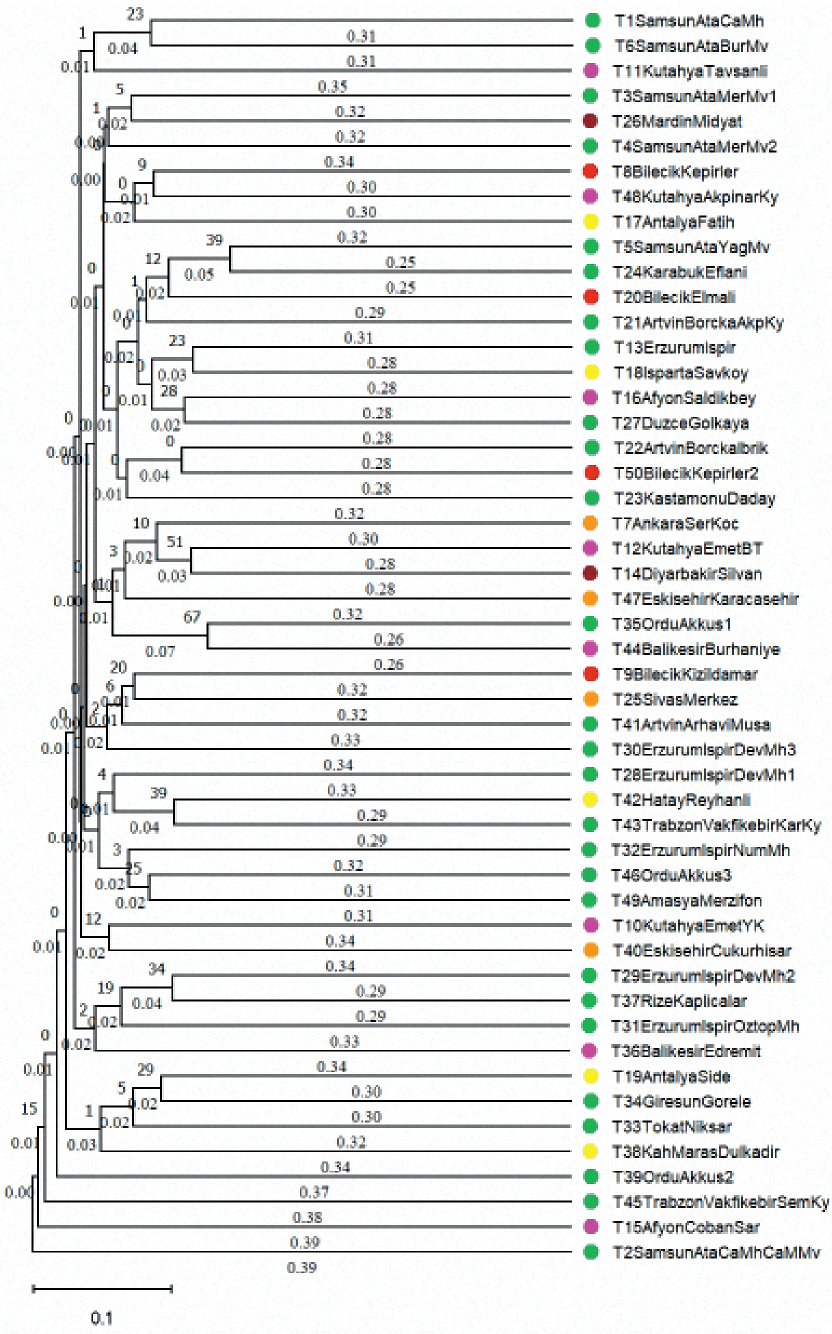

Fig. 2. Dendrogram showing DNA virus diversity among 50 soil samples. Geographical region colours: brick-colour (Marmara Region), pink (Aegean Region), yellow (Mediterranean Region), orange (Central Anatolia Region), green (Black Sea Region), brown (Southeastern Anatolia Region)

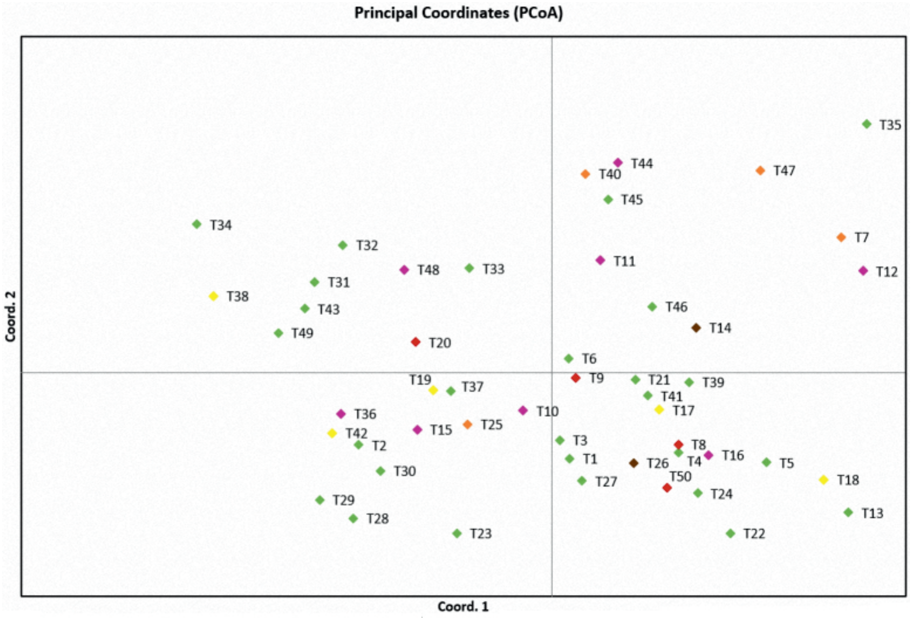

Fig. 3. Principal coordinates analysis (PCoA) of the genetic diversity of DNA viruses in 50 soil samples

In the dendrogram, we determined that some close or same-location samples branched side by side and that geographical regions were grouped separately according to colours. In the PCoA graph created with GenAlEx software, it was seen that the locations were separated more clearly according to the dendrogram result (Fig. 3). Neighbour locations in the dendrogram were also located close to each other in the PCoA graph. Our results confirmed that the six primers we selected from among 25 RAPD marker primers had high efficiency. These primers can be used in DNA viral diversity analyses for all soil samples. Three RAPD primers performed amplification for half of the soil samples. Six RAPD primers were efficient for fewer than 50% of all soil samples while 11 primers were not able to amplify at all. We recommend the six RAPD primer markers (CRA-22, OPA-13, P5, P11, P13, and P21) that were successful in PCR testing for DNA virus diversity analyses of soil samples collected from many different locations.

Previous studies have shown that the number of RAPD markers used to identify and compare soil viruses is limited. On the contrary, ten-nucleobase RAPD markers can easily match genome sequences of entire species and be used for identification [16]. They are effectively used in DNA analysis studies to compare different genomes, from base changes due to damage to diversity studies between species [17]. Determining suitable genome-matched markers among dozens of universal RAPD markers for genetic studies is critical. Sequence data obtained by RAPD marker amplification are used as references for genetic mapping, next-generation DNA sequencing, and metagenomic analyses [18]. RAPD markers such as CRA-22 can be used effectively in viral communities, especially to detect Baculoviruses [19]. Our study confirmed that this marker, which produces polymorphic amplification products, shows the presence of Baculovirus in all soil samples and is functional.

Beneficial or harmful viruses for plants may exist in thousands of different soils worldwide, yet to be discovered. Identifying markers that will identify undiscovered viral genomes and amplify them for DNA sequencing will increase the diversity of references required for large-scale analyses such as metagenomics. The six markers tested for bean cultivation soils in this study can be effectively used in all advanced analyses for the identification and diversity of viruses.

Conclusions. The high diversity in viral content of soil samples collected from 44 different locations in six geographical regions of Turkey, rich in microclimates, shows that this research is comprehensive. We identified six RAPD markers among 25 that provided viral genome amplification and high polymorphism in all 50 soil samples. These proposed markers will be helpful in future studies to discover new soil viruses and to identify viral components whose genome sequences are unknown. Amplification results obtained with useful RAPD markers will increase the reference diversity required for large-scale analyses such as metagenomics.

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