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Molecular Characterization of Phytoplasma-Associated Diseases in Squash (Cucurbita pepo L.) and Cucumber (Cucumis sativus L.) in Türkiye

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Research Article

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Abstract

Phytoplasmas are major pathogens responsible for severe losses in cucurbit production globally, yet their molecular characterization remains limited, particularly in Türkiye. During surveys conducted in Hatay, Türkiye, in 2024, phytoplasma-associated symptoms, including phyllody, virescence, sterility, general chlorosis, and stunting, were observed in squash (*Cucurbita pepo*) and cucumber (*Cucumis sativus*) plants. Total nucleic acid was extracted from 27 symptomatic plants using the CTAB method, and the presence of the pathogen was confirmed by PCR targeting the 16S rRNA gene using universal primers F1/R0 and F2n/R2. PCR analysis confirmed the presence of 16SrII-D subgroup phytoplasmas in symptomatic plants, with amplicon sizes of 1.4 kb and 1.2 kb, consistent with previous studies on phytoplasma diagnostics. BLAST analysis revealed that the sequences exhibited high similarity to Squash phyllody disease (SqPHY, PV056007) and Cucumber phyllody disease (CuPHY, PV056009) isolates, both belonging to the 16SrII-D subgroup. Furthermore, these isolates showed close genetic relationships with Yazd squash phyllody phytoplasma (KR822805) and Cucumber phyllody phytoplasma (MK453519) previously identified in Iran. Phylogenetic analysis confirmed the clustering of these isolates within the 16SrII-D subgroup, and these findings were further supported by virtual RFLP analyses. This study provides the first molecular evidence of 16SrII-D subgroup phytoplasmas infecting cucurbits in Türkiye, emphasizing their regional spread and the urgent need for vector control strategies.

Introduction

Cucumber (*Cucumis sativus* L.) and summer squash (*Cucurbita pepo* L.) are economically and agriculturally significant vegetable crops belonging to the Cucurbitaceae family, widely cultivated across various regions worldwide. Cucumber is extensively consumed due to its edible fruit, commonly used in salads, pickles, and cooked dishes (Navitha et al. 2019). Additionally, cucumber is a valuable dietary component rich in vitamins, amino acids, fiber and minerals, and is widely utilized in skincare and health products (Li et al. 2024). Similarly, squash (*C. pepo*) serves as a key vegetable crop, processed into various food products and used as animal feed. Its fruit is a rich source of vitamins, amino acids, polysaccharides, fiber, and essential minerals (Kumar et al. 2018). Moreover, pumpkin seeds contain high protein levels and low fat, making them a nutritious and health-promoting food source (Devi et al. 2018).

According to the Food and Agriculture Organization (FAO), Türkiye is a major cucumber producer, cultivating approximately 35,278 hectares with an annual production of 1,871,712 metric tons. Additionally, Türkiye plays a crucial role in global squash production, with 85,964 hectares under cultivation and an annual yield of 726,594 metric tons (FAOSTAT, 2023). However, both crops are susceptible to various pests and diseases from germination to harvest, leading to significant economic losses (Venkataravanappa et al. 2019). Cucurbits are economically significant crops cultivated worldwide, yet their production is increasingly threatened by emerging phytoplasma-related diseases.

Phytoplasmas are among the major pathogens responsible for severe losses in fruit, vegetable, cereal, and tree production worldwide (Lee et al. 2000). Cucurbit crops are susceptible not only to viral, fungal, and bacterial infections but also to phytoplasmas, which can severely impact yield and quality (Wang et al. 2021; Xu et al. 2021). Phytoplasmas are obligate intracellular plant pathogens belonging to the class Mollicutes and are transmitted by phloem-feeding insect vectors such as leafhoppers, psyllids, and other sap-sucking insects (Koinuma et al. 2020). Additionally, they can spread through parasitic plants like dodder (Cuscuta spp.) and plant propagation techniques (Chang and Chen, 2024).

Infected plants exhibit a wide range of symptoms, including witches' broom, phyllody, yellowing, virescence, dwarfism, and sterility, depending on the phytoplasma strain and host plant species (Salehi et al. 2015; Usta et al. 2017; Ghayeb Zamharir and Azimi 2018; Muttappagol et al. 2022). Phytoplasma-related diseases in cucurbits have been reported worldwide, including witches' broom in *Cucurbita pepo* (Rao and Kumar 2017), phyllody in cucumber (Hosseini et al. 2015), and yellowing in squash (McCoy et al. 1989).

Molecular approaches, particularly 16S rRNA gene analysis, are widely used for phytoplasma detection and classification (Bertaccini et al. 2014). To date, at least 16 ribosomal groups and multiple subgroups of phytoplasmas infecting vegetable crops have been identified (Kumari et al. 2019). In Türkiye, previous studies have identified *Candidatus* Phytoplasma trifolii (16SrVI-A subgroup) in cucurbits (Usta et al. 2017; Çarpar and Sertkaya 2022), but no molecular characterization of 16SrII-D subgroup phytoplasmas had been conducted prior to this study.

Given the increasing reports of phytoplasma-related diseases in cucurbits, this study aimed to identify and characterize phytoplasma strains associated with phyllody disease in cucumber and squash using PCR assays and 16S rRNA sequence analysis. The findings contribute to understanding the genetic diversity of phytoplasmas affecting cucurbits in Türkiye and highlight the need for effective disease management strategies.

Materials and methods

Sample Collection and DNA Extraction

In October 2024, cucumber and summer squash plants exhibiting symptoms of phyllody, floral virescence, sterility, yellowing, little leaf, and dwarfing were identified in agricultural fields located in Kırıkhan district, Hatay province of Türkiye (36°28'36.1"N 36°22'14.6"E). A total of 27 symptomatic and 3 asymptomatic cucumber and squash plant samples were collected and stored at -80°C for further analysis.

Total Nucleic Acid Isolation and Polymerase Chain Reaction (PCR) Studies

Total DNA was extracted from the collected plant samples using the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987). For phytoplasma detection, direct (Direct-PCR) and nested (Nested-PCR, nPCR) polymerase chain reactions were performed using universal primer sets targeting the 16S-23S ribosomal gene region. In Direct-PCR, the primer set R16F1/R16R0 was used, resulting in a 1.4 kb amplification product, while in Nested-PCR, the primer set R16F2n/R16R2 (Gundersen and Lee 1996) was used, generating a 1.2 kb amplification product. The thermal cycling conditions for Direct-PCR included an initial denaturation at 94°C for 3 minutes, followed by 35 cycles consisting of denaturation at 94°C for 1 minute, annealing at 57°C for 2 minutes, and extension at 72°C for 3 minutes, with a final extension step at 72°C for 10 minutes. For Nested-PCR, the conditions differed slightly, with denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute. DNA extracted from asymptomatic cucumber and squash plants was used as a negative control in PCR analysis.

Sequence Analysis

Nested-PCR (nPCR) products were separated by 1% agarose gel electrophoresis and visualized using a UV transilluminator (Bio-Rad, Hercules, CA, USA). In this study, 13 randomly selected squash and cucumber samples were chosen for sequencing. The nPCR products were bidirectionally sequenced by BM Labosis (Ankara, Türkiye). The obtained DNA sequences were aligned, assembled, and trimmed using MEGA-X software, and the final sequences were analyzed using BLAST (Basic Local Alignment Search Tool) to identify homologous sequences in the database. The BLAST results indicated that the sequenced samples were related to '*Candidatus* Phytoplasma aurantifolia', belonging to the peanut witches'-broom phytoplasma group (16SrII). Three of the 13 sequenced samples were submitted to the NCBI GenBank, and their accession numbers were obtained.

Phytoplasma Identification by RFLP Analyses and Phylogenetic Evaluation

A phylogenetic tree was constructed using the Neighbor-Joining method with the Maximum Composite Likelihood model in MEGA-X (Kumar et al. 2018). Bootstrap analysis was performed with 1000 replicates to assess branch support. The obtained sequences were analyzed using *i*PhyClassifier (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi, accessed on December 26, 2024) for phytoplasma classification and virtual restriction fragment length polymorphism (RFLP) analysis (Zhao et al. 2009).

Results

Field Survey and Molecular Detection

In October 2024, an unusual phenomenon was observed in cucumber and squash plants growing in an agricultural field in Kırıkhan district, Hatay province, Türkiye. Compared to symptomless squash plants with a creeping stem growth habit, the affected plants exhibited a single upright stem formation, along with virescence, characterized by the presence of green leaf-like structures resembling flowers, and phyllody, defined as the development of leaf-like structures emerging from floral organs. Additionally, a reduction in leaf size (Fig. 1) further increased the suspicion of phytoplasma infection.

Infected plants exhibited complete inhibition of fruit formation, resulting in a 100% yield loss compared to healthy plants. The incidence of this phenomenon was recorded as approximately 85% in both the squash field and the adjacent cucumber field (Fig. 1A).

Phylogenetic Analysis

The phytoplasmas responsible for squash and cucumber disease symptoms observed in Türkiye were identified as squash phyllody disease (SqPHY, PV056007) and cucumber phyllody disease (CuPHY, PV056009) isolates, respectively. The nucleotide sequences of the 16S rRNA gene were submitted to the National Center for Biotechnology Information (NCBI) and used in phylogenetic analyses. According to BLAST analysis, the 16S rRNA sequences of SqPHY and CuPHY isolates showed 99–100% similarity with the Yazd (Iran) squash phyllody phytoplasma (KR822805) and Cucumber phyllody phytoplasma (MK453519) reference isolates (Fig. 2). This high similarity strongly supports the classification of these isolates within the 16SrII-D subgroup. A phylogenetic tree, constructed using the Neighbor-Joining method, is presented in Fig. 2. SqPHY and CuPHY isolates formed a monophyletic cluster with 16SrII-D phytoplasmas from Iran and the Middle East, with a bootstrap support value of 98%, suggesting a common regional lineage (Fig. 2). These findings indicate that the studied phytoplasma isolates are genetically closely related to regionally distributed strains, suggesting their potential role in the spread and epidemiology of 16SrII-D group/subgroup phytoplasmas in Türkiye.

Additionally, digital RFLP profiles were generated as part of the *i*PhyClassifier analyses and are presented in Fig. 3. The results confirmed that SqPHY and CuPHY isolates belong to the 16SrII-D subgroup and are genetically closely related to regional phytoplasma strains. Overall, these findings, along with the analyses summarized in Fig. 2, Table 1, and Fig. 3, indicate that SqPHY and CuPHY are among the most dominant phytoplasma strains within the 16SrII-D subgroup and suggest their potential for regional spread as significant disease agents.

Table 1
Abbreviations, common name of the diseases, subgroups, and GenBank accession numbers of isolates used in phylogenetic analyses, including sequences from the 16S and 16S-23S rDNA regions registered in the GenBank database

Strain	Common Name of the Disease	16SrDNA Group-Subgroup	Genbank Accession Number
PHYPPI	Candidatus phytoplasma pini	16SrXXI-A	AJ632155
PHYPCA	Candidatus Phytoplasma castaneae	16SrXIX-B	AB054986
LYD	Coconut lethal yellowing phytoplasma	16SrIV-A	AF498307
PHYP35	Picris echioides yellows phytoplasma	16SrIX-C	Y16389
PHYPPH	Candidatus Phytoplasma phoenicium	16SrIX-D	AF515636
EY1	Elm yellows phytoplasma	16SrV-A	AY197655
CusPh	Cucumis sativus phytoplasma	16SrVI-A	MH004458
CuPh	Cucumber phyllody phytoplasma	16SrVI-A	JF508516
PHYPTR	Candidatus Phytoplasma trifolii	16SrVI-A	AY390261
PHYPUL	Elm phytoplasma	16SrVI-A	AF268895
GR1	Goldenrod yellows phytoplasma	16SrIII-D	FJ376627
CaWBYNO1	Cactus witches'-broom phytoplasma	16SrII-C	AJ293216
CaWBYN	Cactus witches'-broom phytoplasma	16SrII-H	EU099569
WBDL	Candidatus Phytoplasma aurantifolia	16SrII-B	U15442
EgPh	Eggplant phyllody phytoplasma	16SrII-A	MH667642
SqP	Yazd(Iran) squash phyllody phytoplasma	16SrII-D	KR822805
CuPh	Cucumber phyllody phytoplasma	16SrII-D	MK453519
SqPHY	**Squash phyllody disease**	**16SrII-D**	**PV056007**
CuPHY	**Cucumber phyllody disease**	**16SrII-D**	**PV056009**
CuP	Shahrekord cucumber phyllody phytoplasma	16SrXII-A	MK402983
PHYPSO	Stolbur phytoplasma	16SrXII-A	AF248959
MPV	Mexican periwinkle virescence phytoplasma	16SrXIII-A	AF248960
ChTY-Mo3	Candidatus Phytoplasma meliae	16SrXIII-G	KU850940

Strain	Common Name of the Disease	16SrDNA Group-Subgroup	Genbank Accession Number
PHYPMA	Candidatus Phytoplasma mali	16SrX-A	AJ542541
PHYPPR	Candidatus Phytoplasma prunorum	16SrX-F	AJ542544

Discussion

Phytoplasmas are obligate plant pathogens that cause significant economic losses in agriculture. Belonging to the Mollicutes class of bacteria, phytoplasmas localize in the phloem tissue of plants and induce a variety of symptoms, including general chlorosis, stunted growth, virescence (green petal formation), shortened internodes, enlarged buds, small leaves, witches' broom, phyllody, enlarged calyx, floral abnormalities, and vascular discoloration (Asudi et al. 2021; Wang et al. 2024). These infections pose a severe threat to global agricultural productivity. Phytoplasmas have been reported to cause yield losses ranging from 30–80% in potato (Ember et al. 2011; Castillo Carrillo et al. 2018), up to 60% in tomato (Navrátil et al., 2009), 40–100% in eggplant (Rao et al. 2011; Rao and Kumar 2017), 30–80% in sesame (Ghulam Sarwar and Akhtar 2009; Salehi et al. 2017), and potentially 100% in cucumber (Wang et al. 2022).

Although traditional methods such as electron microscopy, histochemical staining, and serological assays have been used for phytoplasma detection and classification, molecular techniques like the polymerase chain reaction (PCR) have revolutionized this field. PCR allows sensitive and specific detection of phytoplasmas by amplifying conserved gene regions using universal primers (Wang et al. 2024). Commonly used genetic markers for phytoplasma diagnosis include 16S rDNA, SecY, SecA, Tuf, and rp genes. In 2022, the International Research Program on Comparative Mycoplasmology (IRPCM) Phytoplasma/Spiroplasma Working Team updated the classification guidelines for phytoplasmas, recommending that the 16S rDNA sequence should be at least 1,200 base pairs (bp) in length for reliable identification (Bertaccini et al. 2022).

In this study, F1/R0 (Lee et al. 1994) and R16F2n/R2 (Gundersen and Lee 1996) primers were used to amplify the 16S rDNA gene, producing approximately 1.2 kb PCR products. After sequencing, 16S rDNA nucleotide sequences were analyzed, and phylogenetic analysis confirmed that the phytoplasma responsible for phyllody symptoms in cucumber and squash in Hatay belongs to the 16SrII-D subgroup. The RFLP pattern analysis performed using the *i*PhyClassifier software (Zhao et al. 2009) further supported this classification.

Globally, phytoplasmas infecting cucumber have been classified into different subgroups depending on geographical distribution. In Iran, cucumber phyllody phytoplasma has been identified as belonging to the 16SrII-M subgroup (Dehghan et al. 2015; Salehi et al. 2015), whereas in China, it falls within the 16SrII-A subgroup (Xi et al. 2024), and in India, it has been classified under 16SrI-X (Muttappagol et al.

2022). Similarly, in Iran, phytoplasmas affecting squash have been grouped into 16SrVI-A (Ghayeb Zamharir and Azimi, 2018). To date, over 100 phytoplasma-associated diseases have been identified worldwide, with the 16SrII group being the second most prevalent group in terms of plant hosts (Che et al. 2024). The ability of phytoplasmas to cause varied symptoms in different host plants presents a significant challenge in disease management and control.

In Türkiye, phytoplasma-related diseases have had a negative impact on agricultural production, affecting vegetables, fruits, ornamental plants, and field crops (Girsova et al., 2017; Salas-Muñoz et al. 2018; Oksal et al. 2017; Güller 2021; Usta et al. 2021). In cucumber, phytoplasmas have been linked to various disease groups (Montano et al. 2007; Xi et al. 2024). Previous studies identified *Candidatus* Phytoplasma trifolii in Turkish cucurbits (Usta et al. 2017; Çarpar and Sertkaya, 2022). Unlike previous reports of *Candidatus* Phytoplasma trifolii in Turkish cucurbits, our study confirms the presence of 16SrII-D subgroup phytoplasmas, revealing a broader distribution of phytoplasma lineages in the region.

The present study is the first report of cucumber and squash phyllody disease caused by a 16SrII-D subgroup phytoplasma in Türkiye. These findings contribute to a better understanding of phytoplasma diversity and their potential role in regional disease outbreaks. Further studies on phytoplasma epidemiology, insect vectors, and disease management strategies are needed to mitigate the spread of 16SrII-D phytoplasmas in Türkiye.

Conclusion

This study provides the first molecular evidence of 16SrII-D subgroup phytoplasma infecting cucumber and squash plants in Türkiye. The findings confirm the regional distribution of these pathogens and emphasize the necessity of comprehensive epidemiological studies to develop effective management strategies for phytoplasma-associated diseases. The results of this study underscore the urgent need for enhanced vector surveillance, integrated pest management strategies, and breeding programs for phytoplasma-resistant cultivars to ensure the sustainability of cucurbit production in Türkiye.

Declarations

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflicting of Interests: The author declare no potential conflicts of interest with respect to the research.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the author.

Consent for publication Not applicable.

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Figures



Figure 1

General chlorosis and dwarfism symptoms in cucumber and squash plants (A, B), along with phyllody and virescence symptoms (B, C, D and E), dwarfing and little leaf symptom (B), green leaf-like structures

resembling flowers and phyllody (B, C, D and E), Comparison of infected and healthy flower structure (D).

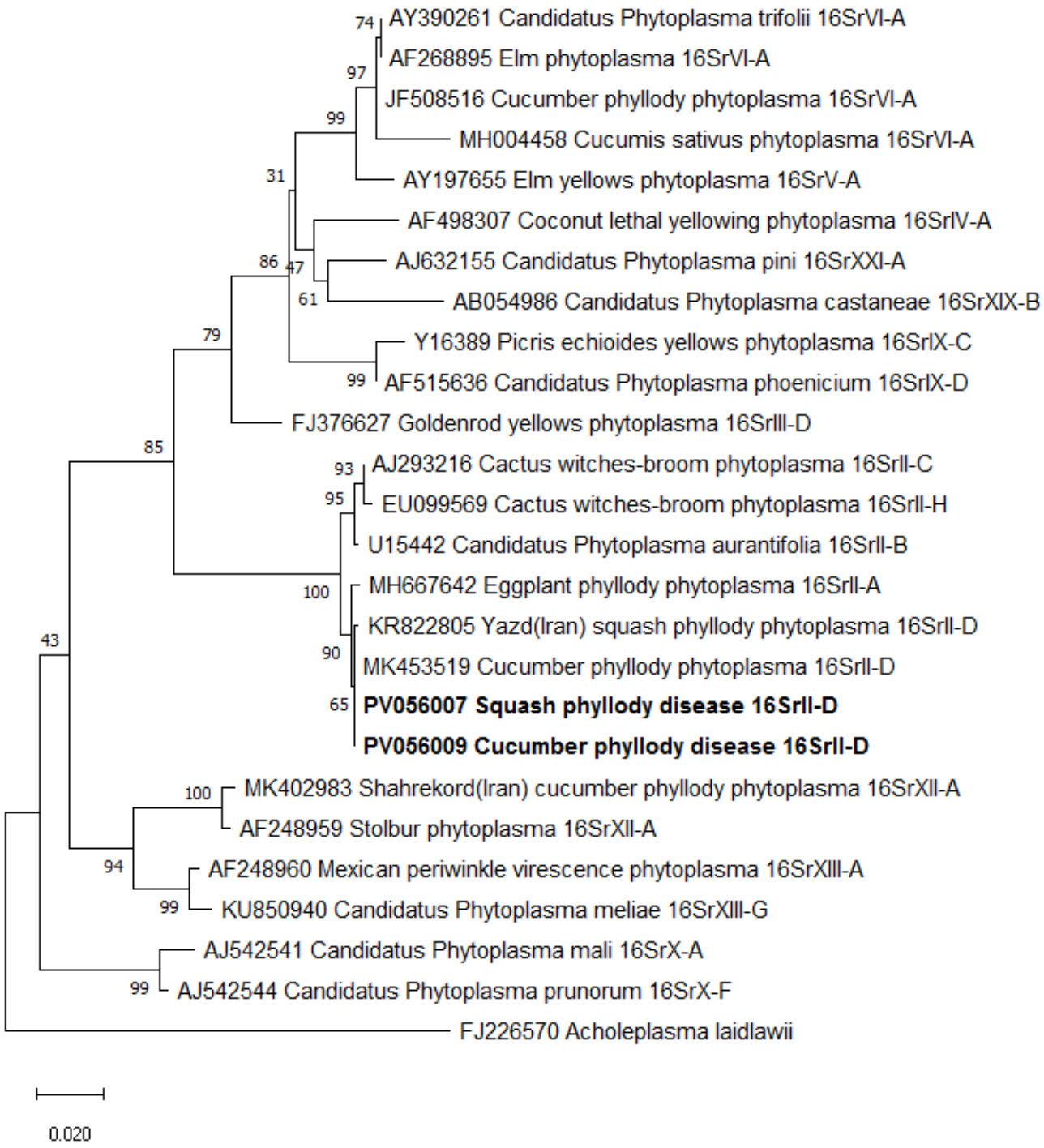


Figure 2

A phylogenetic tree constructed from full-length 16S rDNA nucleotide sequences of different groups registered in the GenBank database. The tree was generated using the MEGA-X program with 1000 bootstrap replications. The isolates from this study are highlighted in bold, and *Acholeplasma laidlawii* (GenBank accession number: FJ226570) was used as the outgroup

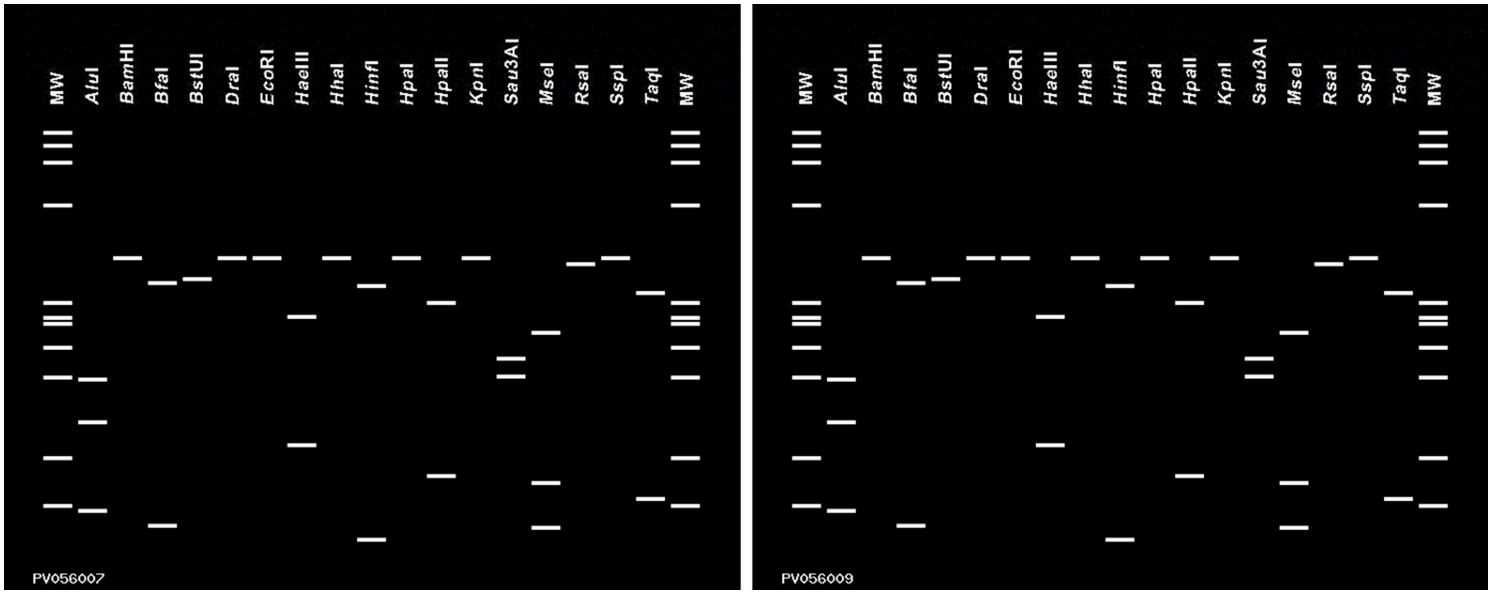


Figure 3

Digital RFLP patterns of 16S rDNA nucleotide sequences submitted to GenBank (GenBank accession numbers: PV056007 and PV056009), generated using the *i*PhyClassifier program. The sequences were digested with 17 restriction endonuclease enzymes, and the resulting virtual RFLP profiles were visualized as agarose gel-like patterns