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Detection of *Spiroplasma citri* from citrus trees in Turkey by molecular techniques

Türkiye'de turunçgil ağaçlarında moleküler tekniklerle *Spiroplasma citri*'nin belirlenmesi

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ABSTRACT

Spiroplasma citri is the causal agent of Citrus Stubborn Disease (CSD) on cultivated citrus in the arid and semi-arid climate. Detection and characterization of this agent at the early stage of infection in citrus orchard and young citrus saplings in the nursery is of utmost importance to prevent the introduction and spread of the disease in *Spiroplasma citri* free areas. The current study was conducted in order to investigate the possible existence of different *Spiroplasma citri* strains in Turkey. Leaf and fruit samples were collected from ten years old citrus trees showing mostly short internodes, upright and mottled leaves, off-season blossoming and typical small and acorn-shaped fruits symptoms associated with citrus stubborn diseases in different villages of Kozan town of Adana province in Turkey. Polymerase chain reaction (PCR) was performed using Spiralin-f/r, P89-r/f and P58-6f/4r primer couples for the detection and characterization of the Turkish isolates. No positive sample was detected with the primers Spiralin-f/r. On the contrary, all samples reacted positively to the P89-r/f and P58-6f/4r primer couples. The obtained amplicon from the P89-r/f primers pair was sequenced and the phylogenetic tree was constructed. Phylogenetic tree showed that the Turkish *Spiroplasma citri* isolates are closely related to the Mexican, Spanish, Algerian, and Italian than those isolated from Morocco, Egypt and USA.

MAKALE BİLGİSİ

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***Anahtar Kelimeler*:**

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ÖZ

Spiroplasma citri, kurak ve yarı kurak iklimde turunçgiller üzerindeki turunçgil palamutlaşma hastalığı (Citrus Stubborn Disease (CSD))'na neden olan bir etmendir. Bu etmenin turunçgil bahçeleri ve fidanlıklarda bulunan genç fidanlardaki enfeksiyonunun erken aşamada tespiti ve karakterizasyonu, hastalığın *Spiroplasma citri*'den ari bölgelere yayılmasını önlemek için büyük önem taşımaktadır. Bu çalışma, Türkiye'deki farklı *Spiroplasma citri* suşlarının muhtemel varlığını araştırmak amacıyla yapılmıştır. Eylül ayında Adana ilinin Kozan ilçesinde farklı köylerde bulunan turunçgil bahçelerindeki Stubborn hastalığına benzer çoğunlukla boğum arası kısalma, yapraklarda kaşıklanma ve leke, sezon dışı çiçeklenme ve tipik küçük palamut tipi meyve simptomları gösteren ağaçlardan yaprak ve meyve örneği toplanmıştır. Türk izolatlarının saptanması ve karakterizasyonu için, polimeraz zincir reaksiyonu (PZR) Spiralin-f/r, P89-r/f ve P58-6f/4r primer çiftleri kullanılarak gerçekleştirilmiştir. Spiralin-f/r primerlerinde pozitif sonuç saptanmamıştır. Buna karşın, testlenen tüm örnekler P89-r/f ve P58-6f/4r primer çiftlerine pozitif sonuç vermiştir. P89-r/f primer çiftinden elde edilen PZR ürünleri sekanslanmış ve filogenetik ağaç oluşturulmuştur. Elde edilen filogenetik ağaç, önemli bir turunçgil üretim merkezi olan Adana ilindeki turunçgil ağaçlarında saptanan *Spiroplasma citri* izolatlarının, Fas, Mısır ve Amerika Birleşik Devletleri (ABD)'nden izole olanlardan daha ziyade Meksika, İspanyol, Cezayir ve İtalyan izolatlarıyla yakından ilişkili olduğunu kanıtlamıştır.

1. Introduction

Spiroplasma citri a phloem-limited mollicute, is the causal agent of citrus stubborn disease on citrus species (Saglio et al. 1973). The pathogen also infects other plant species inducing many economically important plant diseases like brittle root disease in horseradish (Fletcher 1986) or purple leaf disease in carrot (Lee et al. 2006).

The *Spiroplasma citri* infected citrus trees, are mostly showing stunting of the tree, short internodes, upright and mottled leaves, off-season blossoming, and typical small and acorn-shaped fruit (Calavan 1979; Bové 1984; Bové1988; Calavan and Bové 1989; Bové and Garnier 2000). The causal agent of the stubborn can be transmitted in a circulative and propagative manner by several species of leafhoppers including *Circulifer tenellus* and *Neoalithurus haematoceps* (Oldfield et al. 1977; Liu et al. 1983).

Diagnosis of citrus stubborn disease in the field is often difficult, whereas, the detection of the causal agent by culturing on artificial media and biological indexing is laborious, expensive or time-consuming (Tully 1983; Yokomi et al. 2008). *Spiroplasma citri* can be also detected using antisera by ELISA (Saillard and Bové 1983; Clark et al. 1989). PCR detection targeting the spiralin gene is considered as the most efficient tool to detect *Spiroplasma citri*, even if the PCR assays have been developed recently (Lee et al. 2006; Yokomi et al. 2008; Yokomi and Sisterson 2011). These detection assays are still inconsistent and variable in their ability to detect some *Spiroplasma citri* isolates/strains, depending on the gene targeted.

The Stubborn disease was observed for the first time in İzmir province of Turkey by Azeri (1973). Kersting and Şengonca (1992) have found *Balclutha hebe* (Kirkaldy), *Cicadulina bipunctella* (Matsamura) and *Orosius orientalis* (Matsamura) leafhopper as new host of *Spiroplasma citri*. They reported that *Circulifero pacipennis* complex which come from sesame field (Lethierry) was able to transmit *Spiroplasma citri* to *Catharanthus roseus* L., while these complex identified as *Circulifer haematoceps* (Mulsan&Rey) (Hemiptera: Cicadellidae) (Kersting et al. 1993). Kersting and Başpınar (1997) detected *Spiroplasma citri* in sesame using ELISA assay, in Kahramanmaraş, Gaziantep-Kilis, and Şanlıurfa provinces. Moreover, the *Spiroplasma citri* was also detected by ELISA test on *Sorgum halepense* (L.) Pers.in the Eastern Mediterranean region of Turkey (Uygur et al. 1991; Sertkaya and Çınar 2002).

In this context, an investigation was carried out in order to detect and isolate different *Spiroplasma citri* from plant tissue in some Turkish citrus orchards and infected plants grown in the greenhouse, and then partially characterized by targeting the putative adhesin P89 gene and the P58 putative adhesin-like gene.

2. Materials and Methods

2.1. Survey and sample collection

During the survey, citrus leaves and fruits were collected from Navelina orange (*Citrus sinensis* (L.) Osbeck) trees (Fig. 1) evidencing putative symptoms associated with the citrus stubborn disease well as *Spiroplasma citri*-infected Riored grapefruit (*Citrus paradise* Macfadyen) plants. Petiole leaf and midribs were excised and codified as TR-26; midrib from Navelina, TR-26f; columella from Navelina fruit, TR-27; midrip from Navelina fruit, TR-28; midrib from Navelina trees, TR-28f; columella from Navelina fruit, Tr-29; midrib from Navelina trees in field, TR-31; midrib from Riored plant from orchard in different village of Kozan town of Adana province in Turkey. All the samples were tested by PCR using the primer pairs targeting spiral in gene and the most abundant membrane protein, the putative P89adhesingene and the P58 putative P58 adhesin-like gene (Yokomi et al. 2008).

2.2. DNA extraction and Polymerase Chain Reaction (PCR)

Spiroplasma genomic DNA was extracted from 1 g of fresh citrus leaves and fruits columella of diseased and healthy plants as described by Ahrens and Seemüller (1992). The leaves were homogenized in 4 ml of CTAB buffer (2% w/vc etyltrimethyl ammonium bromide, 1.4 M NaCl, 0.2 %2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, 2%polyvinylpyrrolydone, pH 8.0) and 1.5 ml aliquots of the extract were incubated at 65°C for 30 min. An equal volume of chloroform-isoamyl alcohol (24:1) was added to the lysis and mixed vigorously for 1 min and centrifuged at 12.000 rpm for 10 min. This step was repeated twice. The aqueous nucleic acid layer was precipitated overnight at -20°C with 0.6 volume of isopropanol. The pellet obtained following the centrifugation at 8.000 rpm for 10 min was washed with 70% ethanol, vacuum-dried and suspended in 50 µl sterile water. Then it was used as DNA template for direct PCR amplification with the previously specified primers (Tab. 1).

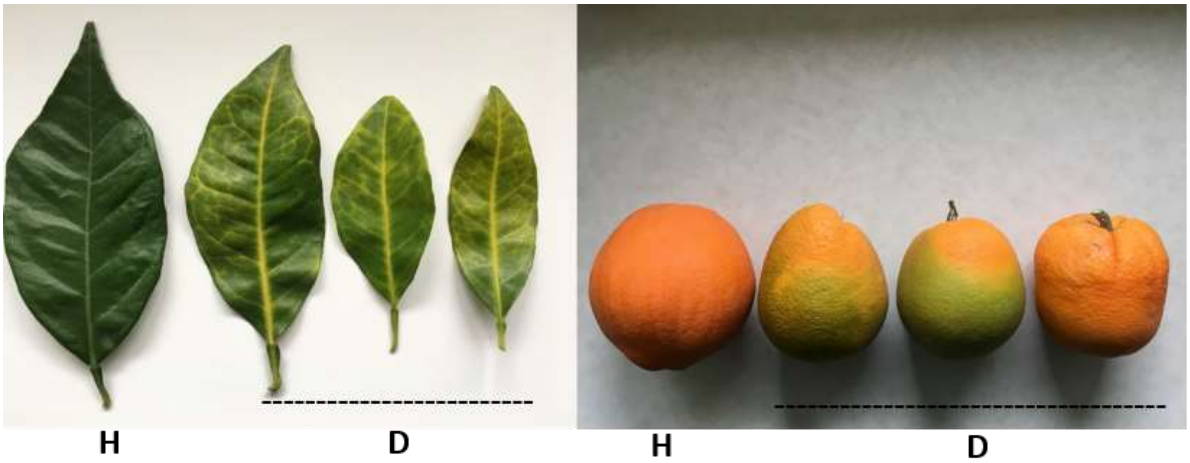

Figure 1. Citrus leaves and fruits from Navelina orange showing symptoms associated to the Citrus stubborn disease in filed. H; healty, D; diseased.

Table 1. Primers (Yokomi et al. 2008) used for polymerase chain reaction (PCR) for the detection of *Spiroplasma citri*.

Primer	Target gene	Primer sequence	Expected size
Spiralin-f	Spiralin	GTCGGAACAACATCAGTGGT	675 bp
Spiralin-r	Spiralin	TGCTTTTGGTGGTGCTAATG	
P89-f	Putative P89 adhesin gene	ATTGACTCAACAAACGGGATAA	707 bp
P89-r		CGGCGTTTGTTAATTTTTGGTA	
P58-6f	Putative P58 adhesin-like gene	GCGGACAAATTAAGTAATAAAAGAGC	450 bp
P58-4r		GCACAGCATTTGCCAACTACA	

PCR amplification was performed in 50 µl reaction mixtures containing 1 µl of extracted DNA, 5 µl of Dream Taq Green buffer (10X), 1 µl of dNTPs (10 mM), 1 µl of forward primer (10 pmol) and 1µl of reverse primers (10 pmol), 0.25 µl of Dream Taq DNA polymerase (5u / µl) (Thermo) and 40.75 µl sterile water. The PCR reactions were performed on Techne TC 4000 thermal cycler. The cycling parameters consisted of 3 min. of denaturation at 95°C, followed by 40 cycles of 30 sec. at 95°C, 50 sec. at 52°C for (Spiralin-f/r and P89-r/P89-f) at 56°C for (P58-6f and P58-4r) and 1 min at 72°C, one cycle of 10 min at 72°C. The PCR products were electrophoresed in 1% agarose gel in 1x TAE buffer (40 mM Tris acetate, 20 mM Acetic acid, 1 mM EDTA, pH 8.0) together with 100 bp DNA marker (Thermo), stained with ethidium bromide and observed on UV trans illuminator.

2.3. Sequencing and phylogenetic analysis

The PCR products obtained from seven positive samples to *Spiroplasma. citri* (six samples Navelina, one sample Riored) were excised from agarose gel, then washed and eluted by centrifugation through siliconized glass wool, as described by Gromadka (1995). The eluted DNAs were sequenced from both directions using forward and reverse sequencing-primers. DNA fragments were subjected to automated sequencing (ABI 3130xl Genetic Analyzer, Applied Bio. Refgen Biyoteknoloji Ltd. şti., Ankara, Turkey). Computer-assisted analyses of nucleotide sequences were assembled using the MEGA7 program (Kumar et al. 2018). P89 adhesin gene sequences of the local *Spiroplasma citri* isolates were separately aligned using Clustal X 1.81 (Thompson et al. 1997). Finally, the phylogenetic tree was constructed by the neighbor-joining method using NJplot and Bootstrap analysis with 1000 replicates on MEGA7.

3. Results and Discussion

The PCR results showed that none of the samples produced amplicons by using the primer pairs targeting the spiral in gene because of low titer of pathogen in plants like in the result of Yokomi et al. (2008); whereas the same samples reacted positively to the PCR assay using the P89f/r and P58-6f/4r primer pairs targeting the putative P89 adhesin gene and the P58 putative adhesin-like gene. In fact, the seven *Spiroplasma citri* assayed isolates (TR-26, midrib from Navelina; TR-26f, columella from Navelina fruit; TR-27, midrib from Navelina fruit; TR-28, midrib from Navelina trees; TR-28f, columella from Navelina fruit; TR-29, midrib from the tested Navelina trees in the field and TR-31, midrib collected from Riored in greenhouse) evidenced clearly the relative expected bands of 707bp (Fig. 2) and 450 bp sizes (Fig. 3) in the agarose gel. As reported by Yokomi et al. (2008), this can be related probably to the field conditions of hot summer months which have an impact on the *Spiroplasma citri* concentrations. These contradicting results are highlighting the importance of using the P89 and P58 primer pairs which were designed in order to overcome the limitations of the sensibility of spiralin primers for *Spiroplasma citri* detection and were claimed to be the most efficient tools for the *Spiroplasma citri* detection (Yokomi et al. 2008).

Furthermore, the obtained nucleotide sequence of the putative P89 adhesin gene from the Turkish *Spiroplasma citri* infected isolates TR-26, TR-27, TR-28, Tr-29, TR-31 were registered on NCBI (National Center for Biotechnology Information) database GenBank Accession N°MK685865, MK685866, MK685867, MK685868, MK685869, analyzed using the BLAST program and compared with other *Spiroplasma citri* nucleotide sequences. Nucleotide sequence analysis of the p89 gene from the Turkish isolates were identical 100% to each other and revealed a high percentage of similarity among sequences with the Mexican isolate 356.46 (KT377390.1) that represents a *Spiroplasma citri* isolate extracted from insect vector (Swisher et al. 2016).

From the comparison with the sequences retrieved from GenBank, a neighbor-joining phylogenetic tree was constructed using MEGA7 based on the partial nucleotide sequences of the P89 gene (Fig. 4). It is important to highlight that only a few nucleotide sequences targeting the P89 and P58 genes are registered in the NCBI database. The partial genomic sequences of the five Turkish isolates showed consistent clustering.

They all reveal a close relationship between each other and the phylogenetic analysis grouped these Turkish isolates TR26, TR 27, TR28, TR29, TR31 in the same main branch with the Mexican isolates 356.46 (KT377390.1), 16.13 (KT377379.1), 356.47(KT377391.1), Algerian isolate G (LN908965.1), Italian isolate Apulia-TN (HE649967.1) and Spanish isolates F12 (KP067956.1), 273-14 (KP258171.1). Whereas the Egyptian Giza-man isolate, Moroccan (GII3) isolate, and both American isolates were grouped together in another clade. At least two various clades can be separated out of this tree. The all Turkish isolates are the same strain and they have a common ancestor. Geographical clustering among these isolates can be observed.

Spiroplasma citri is one of the most important diseases in citrus orchards especially for navel orange group and grapefruits orchards in the East Mediterranean region of Turkey (Çağlayan 1987; Güllü 1989; Çınar et al. 1993; Kersting et al. 1993). Nowadays, it continues spreading in the region, and it is transmitted by leafhopper, *Circulifer haematoceps*, but graft transmission rate is very low (Korkmaz et al. 1993; Kersting et al. 1997). The several studies were conducted epidemiology of the pathogen,especially about its vectors and host plants (Kersting and Başpınar 1995; 1997; Kersting et al. 1997; Sertkaya and Çınar 2002) were generally based on ELISA tests. *Sorghum halepense* (L.) Pers. was accepted important host plant for *Spiroplasma citri* after tested by ELISA test (Uygur et al. 1991), Although it was not possible to culture the pathogen (Sertkaya and Çınar 2002). The similar results also recorded for

Amaranthusgraecizans L., *Capsella bursa-pastoris* (L.) Medik., *Chenopodium album* L., *Cynodon dactylon* (L.) Pers., *Cyperus rotundus* L. (Uygur et al. 1991), *Salsola kali* L. and *Crepisec hinops* (L.) All., *Digitaria sanguinalis* (L.) Scop. (Kersting et al. 1992). Current information and detection techniques which clearly showed here may help more to understand the relationship among *Spiroplasma citri*, host plant, and *Circulifer haematoceps*. Moreover, considering the presence and wide distribution in Turkish orchards of *Spiroplasma citri* isolates, genetically close to borne insect vector Mexican, Algerian, Spanish and Italian *Spiroplasma citri* isolates, make more important to understand its epidemiology. The information collected in the frame of this study could convince the Turkish institutions, to adopt urgent actions, by more research about its continuous spread reasons and main host plant for both *Spiroplasma citri* and *C. haematoceps*. This information may help to create more effective control tactics to prevent disease spreading.

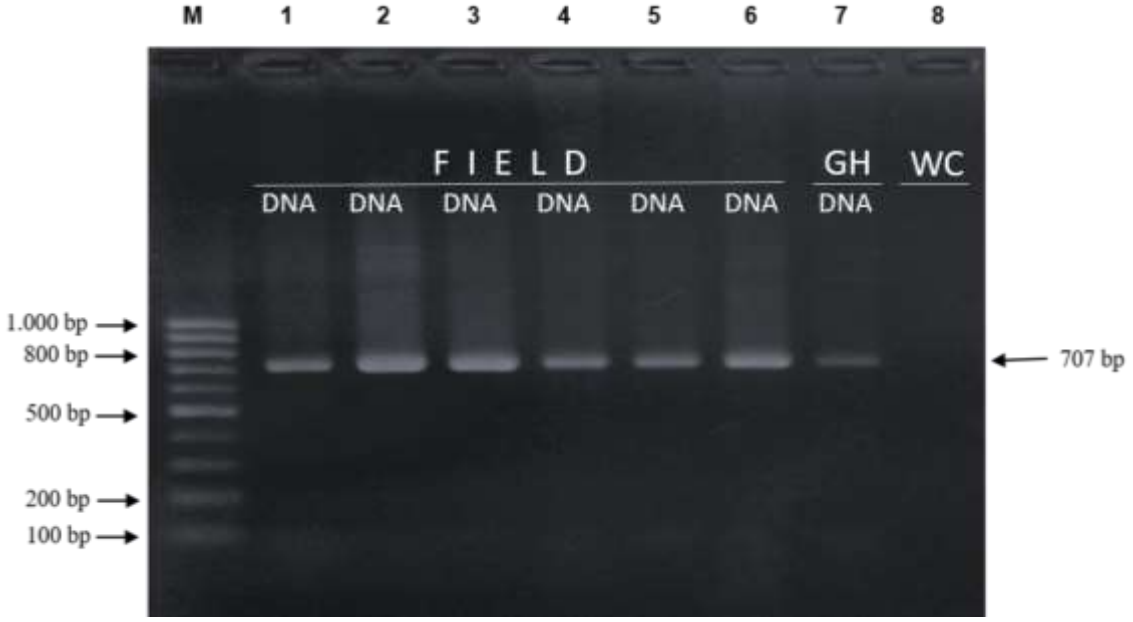

Figure 2. Polymerase chain reaction amplicons (707 bp) produced using P89-r and P89-f primers for PutativeP89 adhesine gene with *Spiroplasma citri* DNA extracted directly from plant tissue on trees in orchard and greenhouse source plants. M; 100 bp DNA marker, 1 (TR-26), 2(TR-26f), 3 (TR-27), 4 (TR-28), 5 (TR-28f), 6 (Tr-29), 7 (TR-31 as positive control), WC; water control.

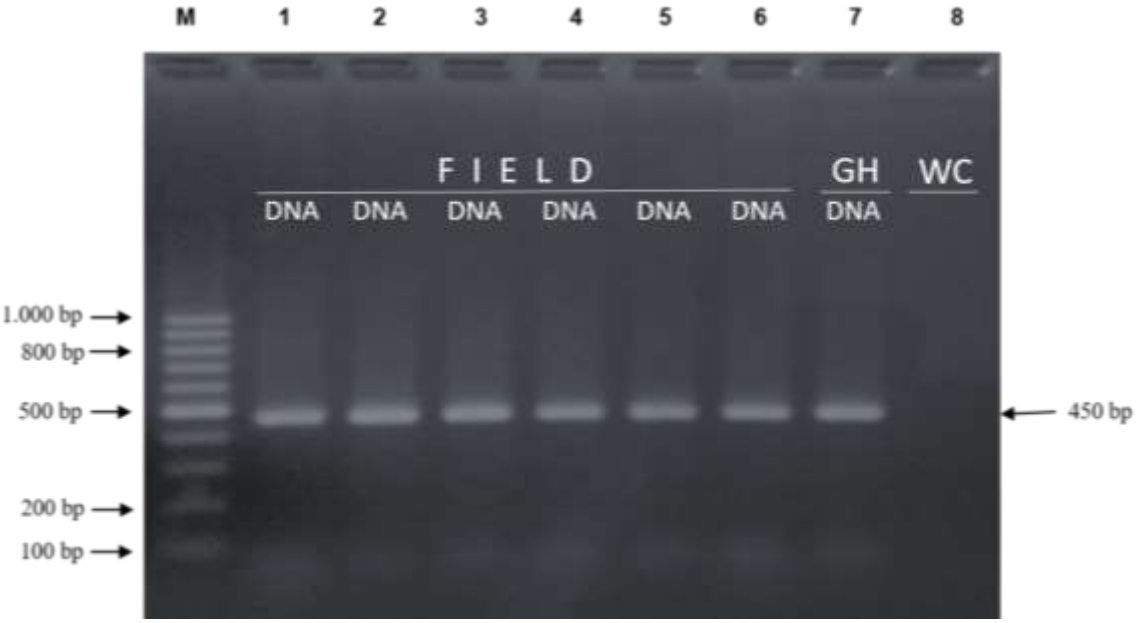

Figure 3. Polymerase chain reaction amplicons (450) produced using P58-6f and P58-4r primers for Putative P58 adhesine-like gene with *Spiroplasma citri* DNA extracted directly from plant tissue on trees in orchard and greenhouse source plants. M; 100 bp DNA marker, 1 (TR-26), 2 (TR-26f), 3 (TR-27), 4 (TR-28), 5 (TR-28f), 6 (Tr-29), 7 (TR-31), WC; water control.

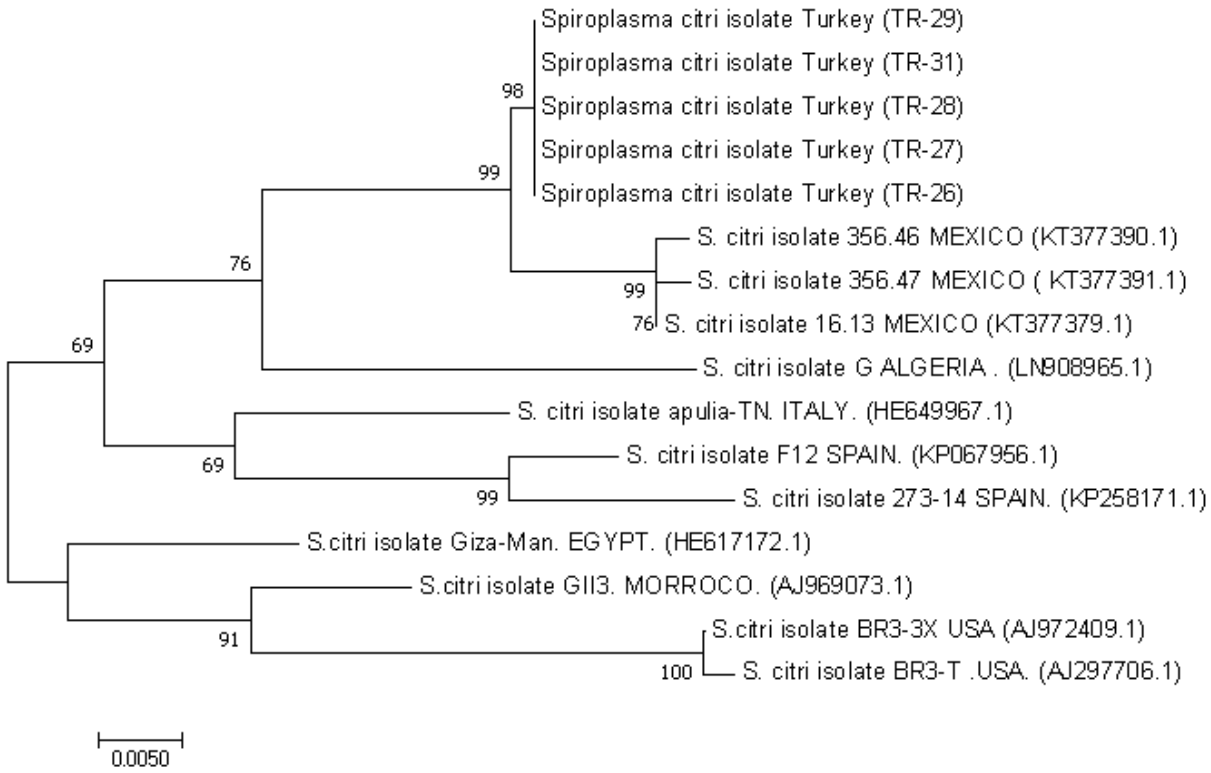

Figure 4. Phylogenetic tree based on P89 sequence analysis of 16 nucleotide sequences of *Spiroplasma citri* strains. The neighbor-joining method using MEGA 7 was utilized, Bootstrap values (percentage) for 1000 replicates are indicated at the main branches are shown on trees.

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