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Full Length Research Paper

Molecular characterization of Yucatan tomato phytoplasma (Group 16Sr III)

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Tomato (*Lycopersicon esculentum*) is an important vegetable crop in Mexico. Recently, a phytoplasma associated with leaf yellowing and curling, severe stunting and little leaf in tomato plant was identified as Yucatan tomato phytoplasma (16SrIII group). DNAs extracted from tomato leaves with symptoms were examined for the presence of this phytoplasma by nested polymerase chain reaction (PCR). Positive results were obtained in 44% of samples, yielding an rDNA product of 1.25 kb. *In vitro* and *in silico* restriction fragment length polymorphism (RFLP) patterns obtained with endonucleases *Hpa*II, *Mse*I, *Rsa*I and *Taq*I were characteristics of group 16SrIII, according to the classification scheme of phytoplasmas. The pattern with *Alu*I and *Hae*III discriminated between these phytoplasmas and the members of 16SrIII group. Molecular characterization of the causal agent of Yucatan tomato phytoplasma will facilitate the study of this disease's epidemic aspects and its phytosanitary management. In addition, it will contribute to a greater knowledge of the genetic diversity of phytoplasmas present in Mexico.

Key words: Diagnostics, phytoplasma, 16S rDNA, PCR-RFLP.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable crop worldwide. In 2009, Mexico produced 2.04 million metric tons with a value of US$ 1.050 million (SIAP, 2010). In the Yucatan Peninsula, tomato is a particularly important crop for small farmers; however, due to insect pests and diseases, the yield and quality of the fruit are lower than expected, causing heavy economic losses. Recently, phytoplasma diseases of tomato have been reported in the northern and central states of Mexico (Holguin-Peña and Vazquez-Juarez, 2007; Santos-Cervantes et al., 2008). In the Yucatan Peninsula symptoms associated with phytoplasma diseases such as leaf yellowing and curling, severe stunting and little leaf were observed and identified as a phytoplasma belonging to Group 16SrIII (X-disease) (Tapia-Tussell et al., 2010). Phytoplasmas are wall-less bacteria in the class Mollicutes that inhabit plant phloem and are known to cause disease in hundreds of plant species worldwide (Liefting et al., 2004). Although, not all plant species infected with phytoplasmas have disease symptoms, however, infected plants normally show symptoms such as virescence, phyllody, yellowing, witch's broom, leaf roll and generalized decline (Bertaccini et al., 2005).

Until recently, phytoplasmas were identified and classified based on symptomatology, host range, and vector specificity, but these methods are not suitable for revealing genetic relatedness among different phytoplasmas (Lee et al., 2000). DNA-based molecular techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), analysis of PCR products, and sequence analysis have recently been used to detect and differentiate phytoplasma strains (Gundersen et al., 1996; Lee et al., 2000; Leyva-Lopez et al., 2002; Rojas-Martinez et al., 2003; Schneider et al., 1993; Tran-Nguyen et al., 2000). Moreover, as a result of the low titers of phytoplasmas present in infected plant material, it is often appropriate to use nested PCR, and in

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the most widely used test, phytoplasma specific primer pair P1/P7 is used initially, followed by R16F2 and 16R2, which yield a fragment 1.2 kb in length (Anfoka et al., 2003; Hodgetts et al., 2007).

The efficiency of nested PCR has shown that it can re-amplify the direct PCR product in dilutions of 1:60 000 (Khan et al., 2004). Nested PCR can involve the use of group-specific primers for the second round of amplification, for example R16 (I) F1/R1 group-I-specific, R16 (III) F2/R1 group-III-specific and R16(V)F1/R2 group-V-specific primers (Anfoka and Fattash, 2004). However, a system has not yet been devised to identify all the taxonomic groups, and this approach in particular requires more than one PCR step, increasing the chances of contamination between samples; moreover, it does not provide the rapid and simple diagnostic tool required. An alternative to the use of group-specific primers is to digest the 16S PCR products with specific restriction endonucleases, for example *Alu*I, *Hae*III or *Rsa*I. The pattern of cut DNA is viewed using agarose or acrylamide gel electrophoresis and can provide a more informative analysis of the phytoplasma present (Lee et al., 2002).

Meanwhile, there is still very little information available on phytoplasma diseases of tomato in Mexico. Therefore, the aim of this study was to characterize molecularly, a phytoplasma associated with yellows-type disease and little leaf in tomato plant in Yucatan Peninsula identified as Yucatan tomato phytoplasma.

MATERIALS AND METHODS

Tomato plants showing symptoms of severe stunting, rolled leaves and little leaf were collected in the Mexican states of Campeche and Yucatan, in the most important municipalities engaged in the production of this crop. A total of 50 tomato leaf samples were collected during 2008 (Table 1).

Nucleic acid extraction

Total genomic DNA used as templates in PCR reaction was extracted from approximately 1.5 g of cut midrib tissues of tomato plants with disease symptoms, according to a method developed in the GeMBio laboratory and this was used in all the tests performed (Tapia-Tussell et al., 2005).

Phytoplasma detection by PCR

DNA samples used as templates for PCR were first diluted to a volume of 50 ng/µL with sterile deionized water. Amplifications of 16S rRNA were performed in 25-µL reactions volumes, each containing 100 ng of DNA template, 1 µM of each primer, 200 µM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Invitrogen) and 1x PCR reaction buffer (10x: 200 mM Tris-HCl, 500 mM KCL, pH 8.4; Invitrogen) in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer). The phytoplasma universal primer pairs P1 (5′-AAGAGTTTGATCCTGGCTCAGGATT-3′) (Deng and Hiruki, 1991) /P7 (5′-CGTCCTTCATCGGCTCTT-3′) (Smart et al., 1996) were used for first round PCR and the reaction conditions were 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 53°C for 50 s and 72°C for 90 s, and a final extension step of 72°C for 10 min.

Products of P1/P7-primed PCR were diluted 1:20 with sterile deionized water and 3 µL of each dilution then used as the template in the second-round PCR (nested-PCR) using primer pair R16F2 (5′-GAAACGACTGCTAAGACTGG-3′)/R16R2 (5′-TGACGGGC-GGTGTGTACAAACCCCG-3′) (Gundersen and Lee, 1996). For the second round, reaction conditions were 95°C for 5 min followed by 30 cycles of 94°C for 30 s, 56°C for 50 s and 72°C for 90 s, and a final extension step of 72°C for 10 min. PCR amplification products (10 µL) were analyzed by electrophoresis on 1.5% (w/v) agarose gels in 1x Tris-Borate-EDTA □TBE□ buffer. DNA was stained with ethidium bromide and visualized on a UV transilluminator and images were taken with a UVP BioImaging Systems. DNAs extracted from asymptomatic plants and sterile water were used as negative and internal controls respectively, while total genomic DNAs from *Cocos nucifera* and *Catharanthus roseus* were used as positive control.

Restriction fragment length polymorphism analysis

Products of nested-PCR primed by R16F2/R16R2 were digested separately with the restriction enzymes *Alu*I, *Hind*III, *Hpa*II, *Rsa*I and *Taq*I according to the manufacturers' instructions (Invitrogen Life Technologies, USA). PCR-RFLP pattern of digested DNA were analyzed by electrophoresis through 2% agarose gel (NuSieves 3:1) using 0.5 x TBE as running buffer. DNA bands were visualized with a UV transilluminator after gels were stained with ethidium bromide. PCR-RFLP patterns obtained were compared with patterns previously described (Lee et al., 1998).

In silico enzyme digestion

Nine sequences were aligned using the BioEdit sequence alignment program (16SrIII: FJ951625, FJ951628, FJ951626, AY863194, AY863192, AY863193; 16SrII: EU125185, EU125184 and 16SrI: DQ987871, DQ168882). The aligned sequences were exported to the *in silico* restriction analysis and virtual gel plotting program pDRAW32 (AcaClone). Each DNA fragment was digested *in silico* with six distinct restriction enzymes (*Alu*I, *Hae*III, *Hpa*II, *Mse*I, *Taq*I and *Rsa*I) that have been previously used for phytoplasma 16Sr rRNA gene RFLP analysis (Wei et al., 2007). After *in silico* restriction digestion, a virtual 2.0% agarose gel electrophoresis image was generated. The virtual gel image was then captured for subsequent PCR-RFLP pattern comparisons.

Phylogenetic analysis

16Sr-23Sr DNA nucleotide sequences were retrieved from the NCBI website (http:www.ncbi.nim.nih.giov/BLAST/) and used for phylogenetic analyses. Sequences from clones of Yucatan tomato phytoplasma were aligned and compared among themselves and with sequences from other phytoplasmas belonging to 16SrI, 16SrII and 16SrIII groups (Table 2). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and bootstrap replicated 1000 times by using the software MEGA 4.0.2 (Taumara et al., 2007). *Acholeplasma laidlawii* (FJ 590758) was used as out-groups to root the tree.

RESULTS

Phytoplasma detection by PCR

Phytoplasmas associated with yellows-type disease and

Table 1. Sources of tomato leaf samples collected and their response to phytoplasma detection by nested PCR.

Sample	Accession no. (GenBak)	Location	Nested PCR
FTg- 01	-	Tekax, Yucatán	+
FTg- 02	FJ951627	Tekax, Yucatán	+
FTg-03	-	Tekax, Yucatán	+
FTg-04	-	Tekax, Yucatán	+
FTg-05	-	Tekax, Yucatán	+
FTg-06	-	Tizimín, Yucatán	-
FTg-07	-	Tizimín, Yucatán	+
FTg-08	-	Tizimín, Yucatán	-
FTg-09	-	Oxkutzcab, Yucatán	+
FTg-10	-	Oxkutzcab, Yucatán	-
FTg-11	FJ951628	Oxkutzcab, Yucatán	+
FTg-12	-	Oxkutzcab, Yucatán	-
FTg-13	-	Calkiní, Campeche	-
FTg-14	-	Calkiní, Campeche	-
FTg-15	-	Calkiní, Campeche	-
FTg-16	-	Calkiní, Campeche	-
FTg-17	-	Calkiní, Campeche	-
FTg-18	FJ951626	Calkiní, Campeche	+
FTg-19	-	Calkiní, Campeche	-
FTg-20	-	Calkiní, Campeche	+
FTg-21	-	Calkiní, Campeche	-
FTg-22	-	Calkiní, Campeche	-
FTg-23	-	Calkiní, Campeche	+
FTg-24	-	Calkiní, Campeche	+
FTg-25	-	Tekax, Yucatán	+
FTg-26	-	Tekax, Yucatán	+
FTg-27	-	Tekax, Yucatán	+
FTg-28	-	Tekax, Yucatán	+
FTg-29	-	Tekax, Yucatán	+
FTg-30	-	Tekax, Yucatán	+
FTg-31	FJ951629	Hopelchén, Campeche	+
FTg-32	-	Hopelchén, Campeche	+
FTg-33	-	Hopelchén, Campeche	+
FTg-34	-	Hopelchén, Campeche	-
FTg-35	-	Hopelchén, Campeche	+
FTg-36	-	Hopelchén, Campeche	-
FTg-37	-	Tekax, Yucatán	-
FTg-38	-	Tekax, Yucatán	+
FTg-39	-	Tekax, Yucatán	-
FTg-40	-	Tekax, Yucatán	-
FTg-41	-	Tekax, Yucatán	-
FTg-42	-	Conkal. Yucatán	-
FTg-43	-	Conkal. Yucatán	-
FTg-44	-	Conkal. Yucatán	-
FTg-45	-	Conkal. Yucatán	-
FTg-46	-	Conkal. Yucatán	-
FTg-47	-	Conkal. Yucatán	-
FTg-48	-	Calkiní, Campeche	-
FTg-49	-	Calkiní, Campeche	-
FTg-50	-	Calkiní, Campeche	-

Table 2. GenBank accession number of sequences of 16S rDNA used in this work.

Phytoplasma	Group	Accession number	Reference
Montana potato purple top phytoplasma (PPT-MT117-2)	16SrIII	FJ226075	Lee et al., 2009
Montana potato purple top phytoplasma (PPT-MT117-3)	16SrIII	FJ226076	Lee et al., 2009
Tomato big bud phytoplasma (TBB-Br/A)	16SrIII	AY863192	Amaral Mello et al., 2006
Tomato big bud phytoplasma (TBB-Br/B)	16SrIII	AY863193	Amaral Mello et al., 2006
Tomato big bud phytoplasma (TBB-Br/C)	16SrIII	AY863194	Amaral Mello et al., 2006
Tomato little leaf Phytoplasma (ToLL)	16SrI	DQ375238	Santos-Cervantes et al., 2008
Tomatillo little leaf Phytoplasma (Tomatillo LL)	16Sr	DQ987871	Santos-Cervantes et al., 2007
Pepper little leaf Phytoplasma (PeLL)	16SrI	DQ092321	Santos-Cervantes et al., 2008
Pepper little leaf Phytoplasma (PeLL)	16SrI	DQ168882	Unpublished
Tomatillo witches'-broom Phytoplasma (Tomatillo WB)	16SrII	EU125185	Unpublished
Pepper witches'-broom Phytoplasma (PeWB)	16SrII	EU125184	Unpublished
Yucatan tomato Phytoplasma strain FTg-18	16SrIII	FJ951626	Tapia-Tussell et al., 2010
Yucatan tomato Phytoplasma strain FTg-2	16SrIII	FJ951627	Tapia-Tussell et al., 2010
Yucatan tomato Phytoplasma strain FTg-11	16SrIII	FJ951628	Tapia-Tussell et al., 2010
Yucatan tomato Phytoplasma strain FTg-31	16SrIII	FJ951629	Tapia-Tussell et al., 2010

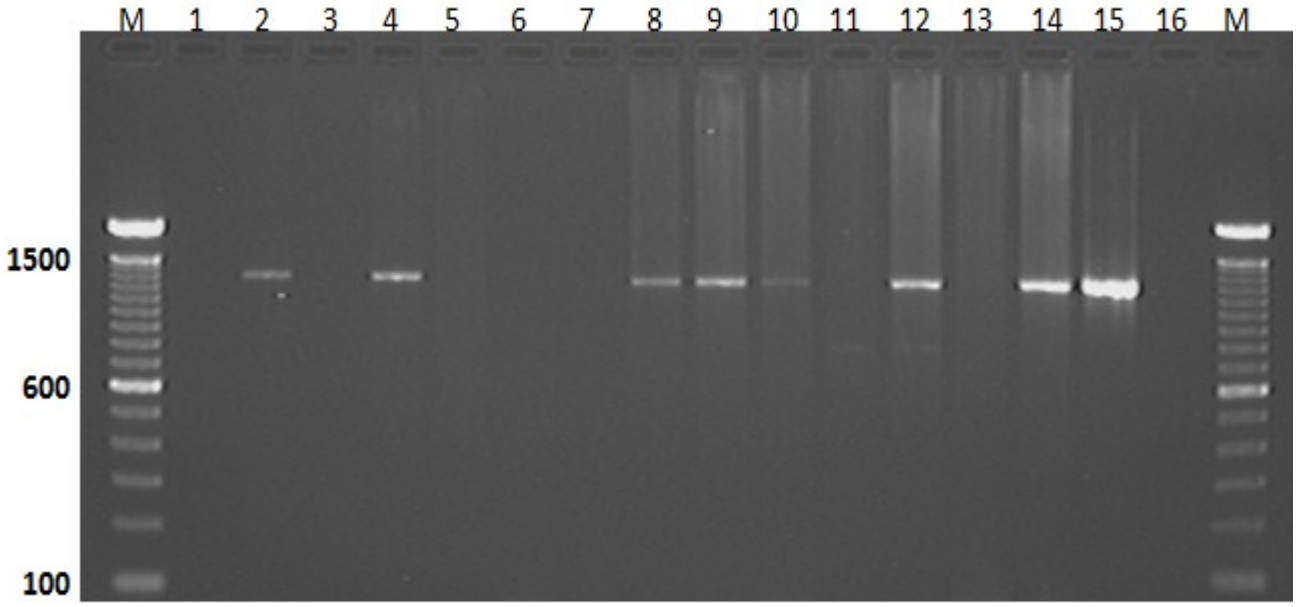

Figure 1. Gel electrophoresis of products from PCR performed on DNA extracted from tomato samples using nested-PCR with primers R16F2/R16R2. Line M: Molecular Marker 100 bp ladder (Invitrogen Life Technologies); lane 1, FTg-06; lane 2, FTg-07; lane 3, FTg-08; lane 4, FTg-09; lane 5, FTg-10; lane 6, FTg-12; lane 7, FTg-13; lane 8, FTg-31; lane 9, FTg-02; lane 10, FTg-33; lane 11, FTg-34; lane 12, FTg-18; lane 13, FTg-36; lanes 14 and 15, positive control (*Cocos nucifera* and *Catharanthus roseus*); lane 16, negative control (healthy plant).

little leaf in tomato plants in Yucatan were detected by nested PCR with two universal primer pairs P1/P7 and R16F2/R16R2 in 22 out of 50 tomato samples (Table 1). Typical bands of 1.2 kb were visualized in agarose gel for DNA extracted from tomato and positive controls (*C. nucifera* and *C. roseus)* (Figure 1). No amplification was observed when DNA from asymptomatic plants was used as template.

In Figure 2A and 2B are shown a diseased plant with characteristic symptoms of yellowing, severe stunting and little leaf, and the tomato growing areas in the Yucatan peninsula respectively. As can be seen in Figure 2B, this phytoplasma was found in all areas except Conkal. Although, disease incidence percentages varied between places, the highest incidences were found in the municipalities with a greater surface area dedicated to this crop, the most affected being the state of Yucatan (48% of samples showed positive), while Campeche registered

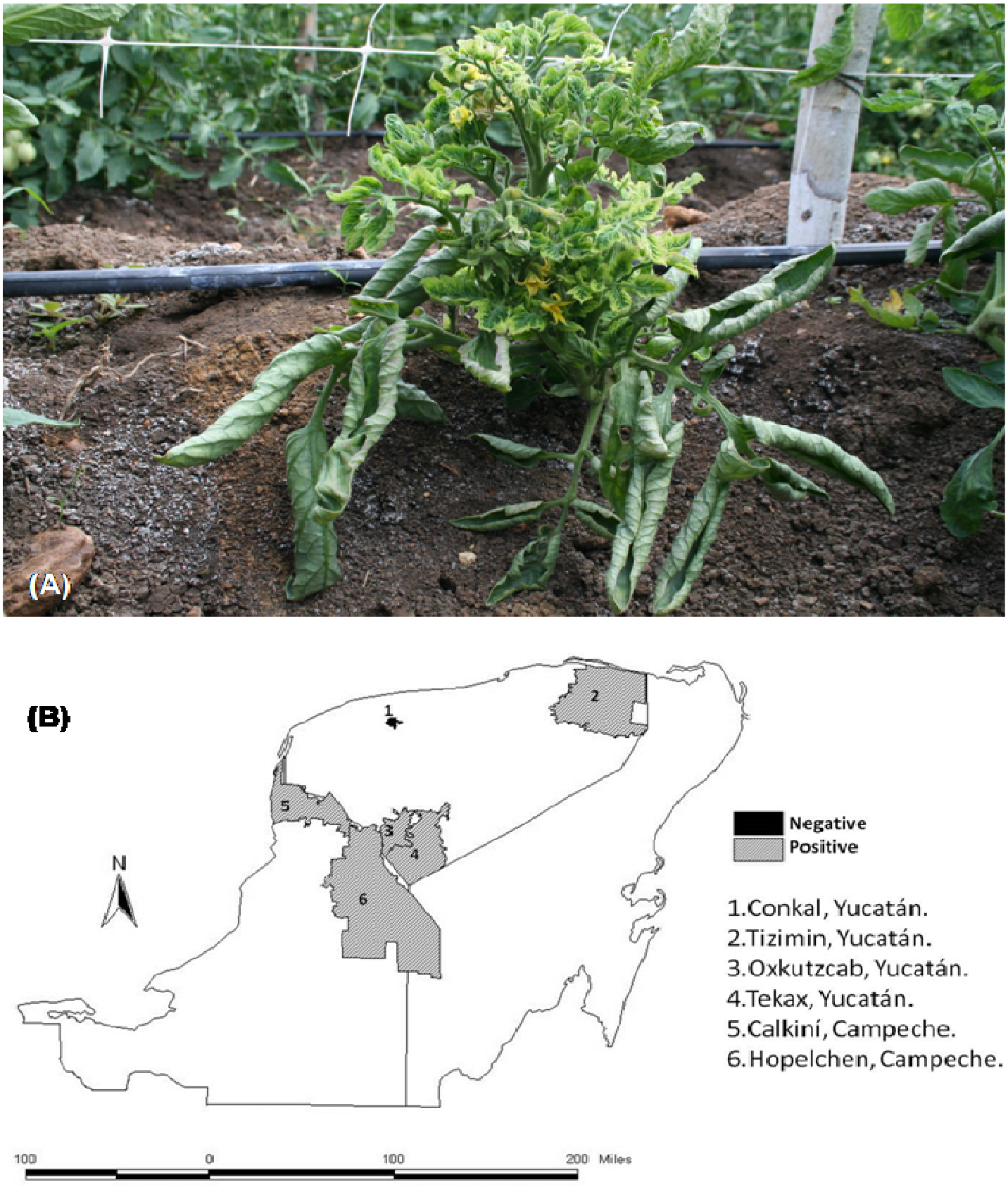

Figure 2. A) Yellowing, severe stunting and little leaf symptoms in tomato. B) Yucatan tomato phytoplasma incidence in tomato growing areas in the Yucatan Peninsula.

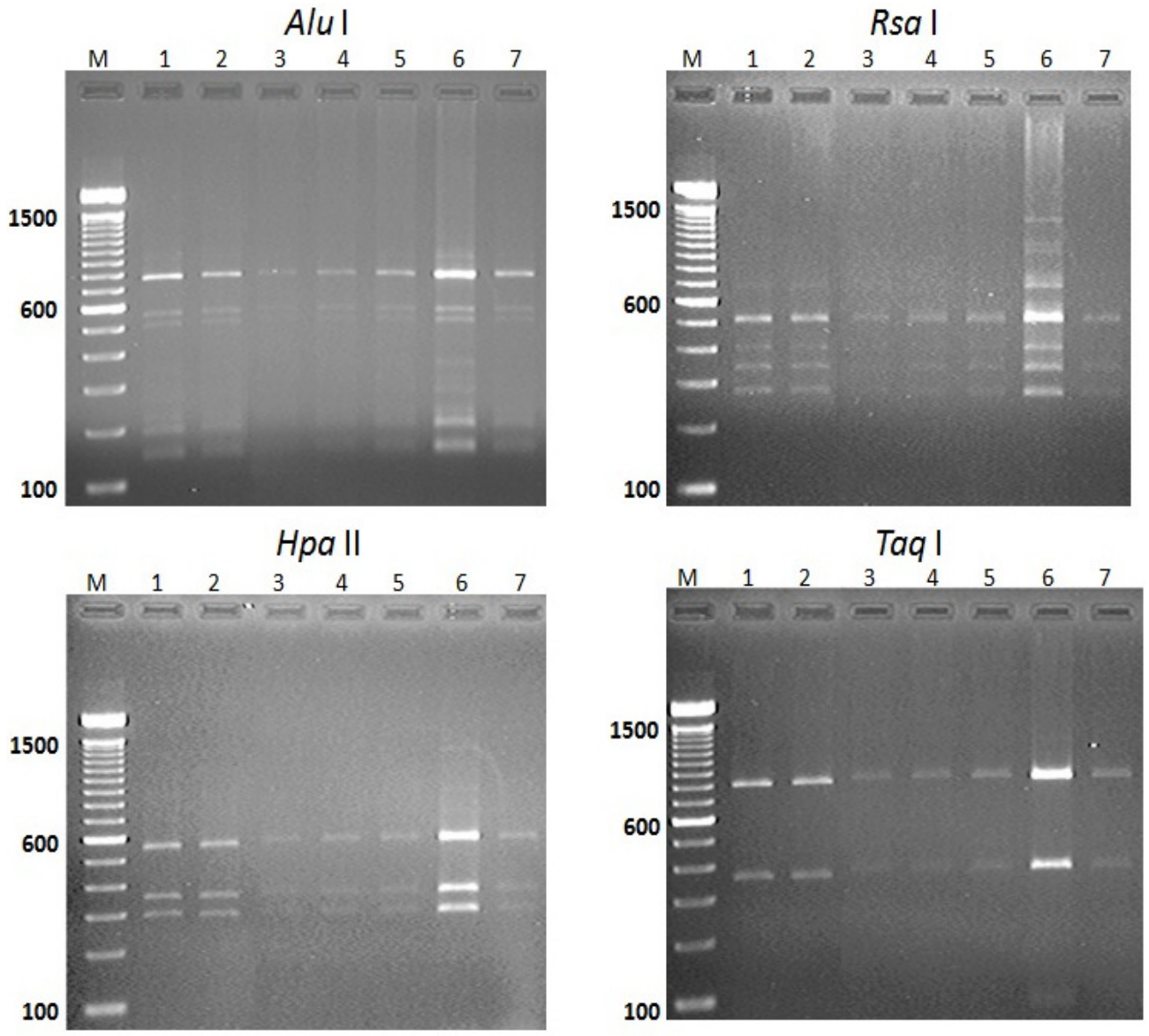

Figure 3. Restriction fragment length polymorphism (PCR-RFLP) patterns of 16S rDNA from Yucatan tomato phytoplasma amplified by nested PCR with primers R16F2/R16R2. DNA products were digested with restriction endonucleases *Alu*I, *Hpa*II, *Rsa*I and *Taq*I. Lane M: Molecular marker 100 bp ladder; lane1, FTg-02; lane 2, FTg-11; lane 3, FTg-18; lane 4, FTg-20; lane 5, FTg-23; lane 6, FTg-31 and lane 7, FTg-38.

slightly less (38%).

Restriction fragment length polymorphism analysis

In all the positive samples, the restriction fragment length polymorphism patterns with endonucleases *Hpa*II, *Rsa*I and *Taq*I (Figure 3) were characteristics of group 16SrIII according to the classification scheme of phytoplasmas (Lee et al., 1998). However, in case of *Alu*I, the patterns resulted were slightly different from the characteristic band patterns of group 16SrIII from those reported by Amaral Mello et al. (2006) in tomato big bud phytoplasma.

In silico enzyme digestion

Restriction fragment length polymorphism patterns with *Mse*I and *Taq*I (Figure 4) were characteristic of group 16SrIII, (Wei et al., 2007). Virtual PCR-RFLP patterns (Figure 4) revealed differences between Yucatan tomato phytoplasma strains and other tomato phytoplasma strains of 16SrIII as well as with 16SrI and 16SrII groups used in this study. FTg31, FTg11 and FTg18 phytoplasma showed *Alu*I, *Hae*III, *Hpa*II and *Rsa*I restriction patterns different from all member of 16SrIII group.

In silico, PCR-RFLP pattern from Yucatan tomato phytoplasma were similar to that previously described for

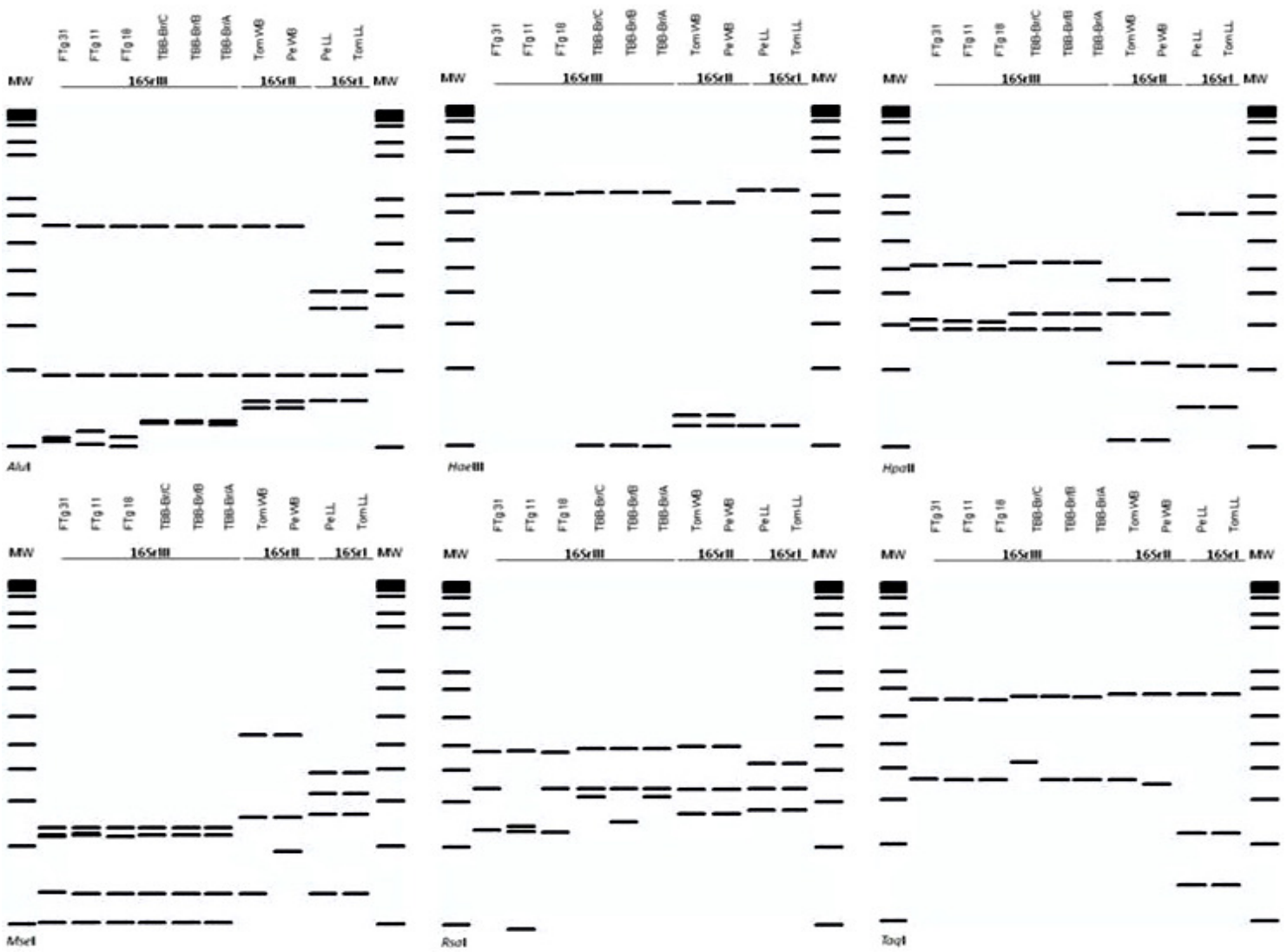

Figure 4. Virtual PCR-RFLP patterns from *in silico* digestions of 16S rRNA gene from nine phytoplasma strains representing the three different groups. MW: 1 kb plus molecular marker.

Tomato big bud phytoplasma (Amaral Mello et al., 2006) and milkweed yellow phytoplasma (Lee et al., 1998), but some different fragments were detected. The *Alu*I pattern differed showing a double band approximately 109 and 115 bp. In case of *Hae*III pattern, the absence of a 100 bp band was characteristic in the strains of Yucatan tomato phytoplasma. The *Hpa*II and *Rsa*I patterns also distinguish between 16SrIII strains, showing differential bands (310 and 233 bp respectively) in the FTg31, FTg11 and FTg18 strains.

Phylogenetic analysis

The 16S rDNA sequence of the four Yucatan tomato phytoplasma accessions (FJ951626 to FJ951628) compared with those of 11 other phytoplasmas of 16SrIII, 16SrII and 16SrI groups (Table 2) and *A. laidlawii* yielding the consensus tree are shown in Figure 5. The bootstrapping values indicated strong support for all branches. Phylogenetic analysis showed close relationship among Yucatan tomato phytoplasma clones that are grouped in the same subclade, which in turn forms a part of a larger clade where the members of the phytoplasma group 16SrIII were grouped.

DISCUSSION

The results of this study indicated that Yucatan tomato phytoplasma was present in 44% of the tomato samples tested. Taking into consideration that the sampled areas are the main producers of this crop in the Yucatan peninsula, the relatively high incidence percentage is

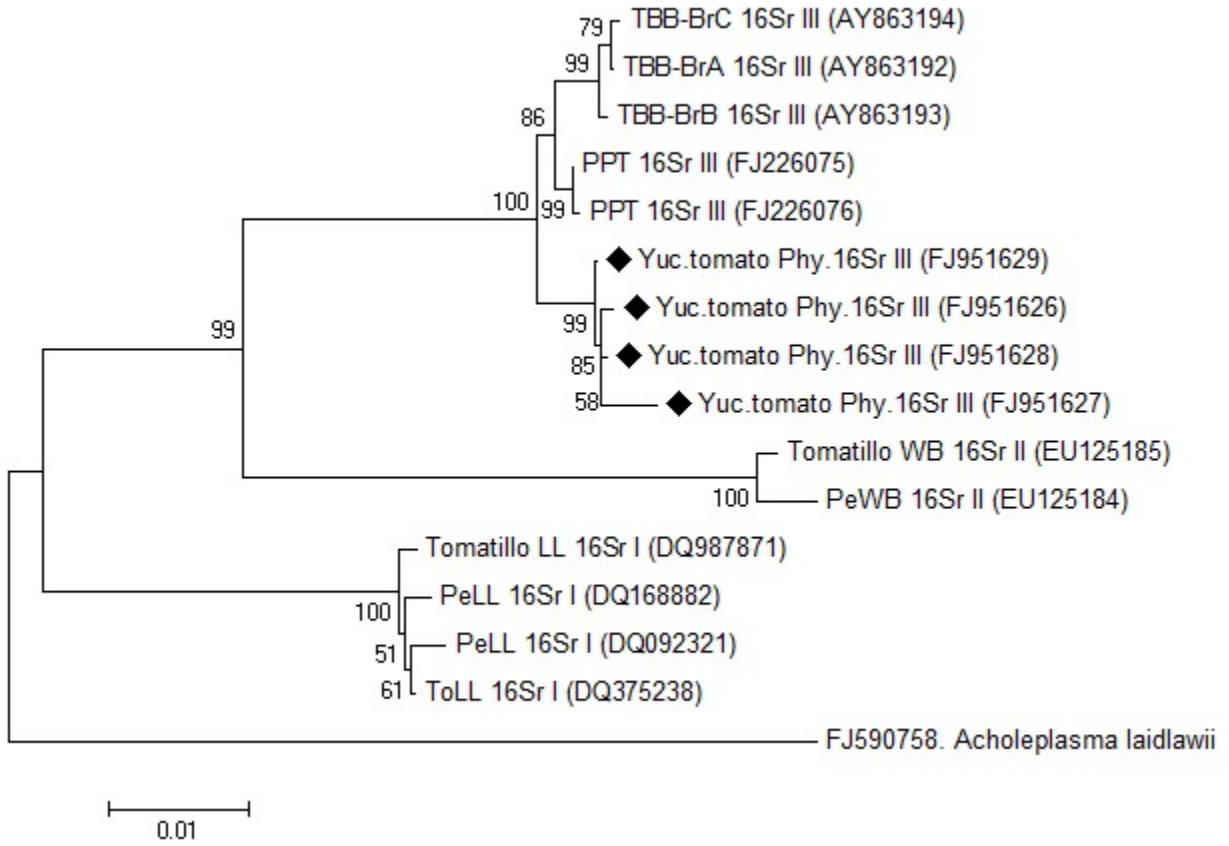

Figure 5. Phylogenetic tree of the 16S rDNA gene sequences of the clones of Yucatan tomato phytoplasma aligned with different groups of tomato phytoplasmas. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

particularly disquieting since its effect on the plants has a direct influence on fruit yield and quality. Further studies focusing on this disease's epidemiology and on its possible vectors, which have yet to be identified, are therefore required. The aim must be to achieve, if not a total control, at least a reduction in its incidence through adequate phytosanitary management, as has been proposed for other crops affected by phytoplasmas (Rojas-Martinez et al., 2003).

The study to characterize the Yucatan tomato phytoplasma causal agent of leaf yellowing and curling, little leaf and severe stunting in tomato was based on *in vitro* and *in silico* RFLP analysis of PCR amplified rDNA using different restriction endonucleasas, as this approach has proved to be useful for Phytoplasma differentiation and classification (Amaral Mello et al., 2006; Santos-Cervantes et al., 2008). Collective RFLP patterns clearly revealed that Yucatan tomato phytoplasma is a member of group 16SrIII (Lee et al., 1998). The patterns from digestion using *Alu*I and *Hae*III were useful in order to differentiate the Yucatan strains from tomato big bud phytoplasmas and milkweed yellows phytoplasma belonging to 16SrIII group (Amaral Mello et al., 2006; Anfoka et al., 2003; Lee et al., 1998; Santos-Cervantes et al., 2008). Virtual RFLP patterns produced by *Mse*I and *Taq*I were the key for group level classification; this is consistent with previous reports (Wei et al., 2007). In addition, this result reinforced the formation of a different subclade with the isolates under study, within the clade of group 16SrIII.

Furthermore, this study to characterize the phytoplasma affecting tomato in the Yucatan peninsula complements previous information on phytoplasmas affecting this crop in other areas of Mexico which have been classified in group 16SrI (Aster yellows) (Holguin-Peña et al., 2007; Santos Cervantes et al., 2008), thus indicating that phytoplasma diseases in tomato are genetically diverse. Different phytoplasmas associated with tomato diseases from 16S rDNA groups have been characterized all over the world using DNA-based techniques and sequence analysis (Anfoka et al., 2003; Santos-Cervantes et al., 2007, 2008), which have proven to be a good tool for detecting and characterizing phytoplasma strains. Hence, the molecular characterization of the causal agent of Yucatan tomato phytoplasma representative of group 16SrIII associated with tomato diseases in this region will facilitate studies concerned

with epidemic aspects of the disease and its control or phytosanitary management, while contributing to a greater knowledge of the genetic diversity of phytoplasmas present in Mexico.

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