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*Full Length Research Paper*

# New hosts of 16SrI phytoplasma group associated with edible *Opuntia ficus-indica* crop and its pests in Mexico

**Leopold Fucikovsky Zak*, María de Jesús Yáñez-Morales*, Iobana Alanis-Martínez and Enrique González-Pérez**

Colegio de Postgraduados, Campus Montecillo, Fitosanidad, Mexico State, Mexico.

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**In Mexico in the region of Nopaltepec in Mexico State, the edible Cactus crop, *Opuntia ficus-indica* is mainly cultivated for prickly pear fruit production. This crop has problems with common pests (insects, mollusks and weeds) which may serve as reservoirs and together with the named Cactus with phytoplasma-like symptoms which through the time inhibit the fruit production, and for this reason, the farmers called these *Opuntia* plants as Planta Macho (male plant). For molecular identification of the probably involved phytoplasma during 2005 and 2006, 38 samples of *Opuntia* plant tissues, fruit and some pests were collected for DNA extraction. By direct and nested PCR, 16S rRNA gene was amplified and sequenced. PCR products were analyzed by RFLP with restriction enzymes and in the sequences restriction sites were mapped. Phylogenetic analysis showed that the same phytoplasma was associated with *Opuntia* crop and its pests (the weeds *Argemone mexicana*, a grass and *Lupinus* sp.; a chinch bugs, *Chelinidea* sp. and the brown garden snail, *Helix aspersa*). Thus the edible Cactus crop and pests represented novel hosts of Cactus male plant Phytoplasma, and was classified as 16SrI Aster yellows group, of the species *Candidatus* phytoplasma asteris. This is the first report of this phytoplasma in Mexico and elsewhere.**

**Key words:** *Argemone*, brown garden snail, chinch bugs, edible Cactus, grasses, *Lupinus*, male plant phytoplasma, Mexico.

## INTRODUCTION

In the region of the great Mexican pyramids in the Teotihuacan Valley, especially near the community of Nopaltepec inside the municipality of Nopaltepec and whose ubication is in the northeast region of Mexico State (Lat. 19º28´20´´ to 19º52´24´´, Long. 98º38´20´´ to 98º46´45´´, at 2300 m asl and weather classified as Bs1k; and which means a semiarid and temperate climate, with rain season during the months of June until October) (SEDUV, 2003), exist approximately 20,000 ha of cultivated and edible Cactus, *Opuntia ficus-indica* (L.) Mill. (Cactaceae family).

This crop is grown mostly for its sweet and refreshing fruits called “tunas” (prickly pear) that are consumed locally and a great quantity is exported to United States of America during the months of August and September each year. The young cladodes free of spines are also consumed locally in different, very tasty preparations. Approximately 15,000 families depend on this plant in this area. There are many larger or smaller areas in Mexico where this Cactus is grown for the same purpose.

It has been estimated (Fucikovsky and Yáñez, 2006) that about 60% of the plants of six years of age and on, do not produce any fruits, or the fruits are very small (Figure 1a) and without taste, born frequently on the flat part of the cladode surface (Figure 1b) instead on the crest. Many cladodes increase in thickness, compared to normal ones (Figure 1c) on the same plant. Plants grow slow and some may be very stunted (Figure 1d). Many plants turn yellowish without production and occupy an unproductive space (Figure 1e). Besides, the affected plants have young or older cladodes in form of a heart (Figure 1f) (Fucikovsky and Yáñez, 2006). The incubation time of the disease and the appearance of the symptoms is unknown, although it was observed in the field that detached diseased cladodes with symptoms give rise to

*Corresponding authors. E-mail: fucikovs@colpos.mx, yanezmj@colpos.mx. Tel: + 55-595-95-20200.


**Figure 1.** Associated Cactus male plant phytoplasma symptoms on edible *O. ficus-indica* crop. Panel shows: (a) atrophied fruits (left), (b) fruits on the flat part of the cladodes surface, (c) cladodes increase in thickness (from center to the left), (d) stunted plant, (e) plants turn yellowish and without fruits, (f) cladodes in form of a heart.

new ones with thick or a heart shape formation in few months.

Besides the above problem, among the *Opuntia* crop there are common pests (such as insects, molluscs and weeds). Some of them are *Argemone mexicana* L. (Papaveraceae), *Ipomoea* sp. (Convolvulaceae), a grass (Gramineae) and *Lupinus* sp. (Leguminosae) (Sánchez-Sánchez, 1980); plus chinch bugs, *Chelinidea* sp. (Hemiptera: Coreidae) and the brown garden snail, *Helix aspersa* Müller (Gastropoda) (Nobel, 2002; Hernández-Gutiérrez, 1993; Pimienta, 1990) which cause problems.

Anteriorly worldwide, ornamental cacti were studied with similar symptoms and phytoplasma was associated. Some of these were ornamentals such as *Opuntia*

*monacantha* in Lebanon (Choueiri et al., 2005); and *Opuntia* sp. and *Zygocactus truncatus* in China (Cai et al., 2007, 2008), and on Cactus in USA (Hodgetts et al., 2008). Also in Mexico phytoplasmas were associated with ornamental cacti such as *Echinopsis subdenudata* and *Opuntia* sp. (Leyva-Lopez et al., 1999; Avina-Padilla et al., 2009). Furthermore on *O. ficus-indica* crop, phytoplasma causing disease was reported in Italy (Granata et al., 2006); and also in Argentina, Chile and South Africa (Granata et al., 2006) and in USA (Bertaccini et al., 2007).

In Mexico phytoplasma-like symptoms on *Opuntia* crop has been described by several authors (Hernández-Gutiérrez, 1993; Nobel, 2002; Pimienta, 1990).

This phytoplasma problem (Fucikovsky and Yáñez, 2006; Hernández-Pérez et al., 2009a, 2009b) has preoccupied the farmers for many years and for this reason, this work was done in order to determine the presence of some microorganism, possibly phytoplasma, in these sterile plants, where the farmers gave it the name Planta Macho (male plant), which indicates that it will not produce practically any fruit when the symptoms are advanced. About the above pest, in addition weeds, sucking insects and snails were also analyzed for phytoplasma detection. We hypothesize that these organism-pests are related to the male plant disease on the edible Cactus crop.

## MATERIALS AND METHODS

### Collection of samples

In the *Opuntia* crop, during 2005 and 2006, 38 samples from healthy and diseased looking plants plus some samples of common pests were collected in the area of Nopaltepec as follows: 15 samples were of cladodes, spines, and pear fruits (seed with the pulp, and peel); 18 samples of weeds (representing four genera), plus three snails and two winged chinch bugs (green and brown). All the samples were preserved at -85°C before laboratory processing.

### DNA extraction

From each sample, DNA was extracted from 0.3 g of previously lyophilized tissues and the protocol of Ahrens and Seemüller (1992) was followed. The DNA quality was verified by electrophoresis on a 1% agarose gel (Ultrapure, Gibco, USA) using TBE buffer, stained, and visualized in a transilluminator (Gel Doc 2000, BIO RAD®, USA). The concentration was quantified in a Perking-Elmer spectrophotometer (Lambda BIO 10, USA).

### PCR amplification

With DNA of each sample, direct PCR followed by nested PCR were conducted. At first PCR universal primers were used, P1 (5`-AAG AGT TTG ATC CTG GCT CAG GAT T-3`) and Tint (5`-TCA GGC GTG TGC TCT AAC CAG C-3`) for amplification of the 16S rRNA gene and the 16S-23S spacer region (Smart et al., 1996). The reaction mixture was 80 ng of DNA, 20 pmols of each primer and a PCR bead (PuRe Taq Ready To-Go, PCR Beads; Amersham Biosciences) in 17 µL of ultrapure sterile water. For nested PCR, the primers were R16F2n (5`-GAA ACG ACT GCT AAG ACT GG-3`) and R16R2 (5`-TGA CGG GCG GTG TGT ACA AAC CCC G-3`) (Lee et al., 1993). The reaction mixture was 1 µL from the dilution 1:30 (1 µL of the direct PCR product in 30 µL of ultrapure sterile water), 20 pmols of each primer and a PCR bead in 20 µL of ultrapure sterile water. The positive control was DNA from *Catharanthus roseus* with Aster yellows phytoplasma and the negative control was ultrapure sterile water.

The PCR amplification was done using a programmable thermocycler (Gene Amp. PCR System mod. 2400, Perkin-Elmer®, USA) with a cycle of initial denaturalization at 94°C for 1 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and an extension at 72°C for 3 min; and a final extension cycle at 72°C for 10 min (White et al., 1990). The DNA quality was verified by electrophoresis on a 1% agarose gel (Ultrapure, Gibco, USA) using TBE buffer, stained, and visualized in a transilluminator (Gel Doc 2000, BIO RAD®, USA). The concentration was quantified in a Perking-Elmer spectrophotometer (Lambda BIO 10, USA). The PCR fragments were visualized as mentioned above. The marker 1 Kb, DNA ladder was used.

### RFLP analyses

Twelve of the nested PCR products with the primers R16F2n/R16R2 were digested with the following restriction enzymes: *Alu*I, *Hha*I, *Hpa*II, *Kpn*I and *MseI*. The restriction products were separated in 8% acrylamid gels and stained with etidium bromide. The DNA patterns of RFLP obtained were compared with those already reported (Lee et al., 1993, 1998). The same above DNA positive control and øX174 RFI DNA *Hae*III digest marker were used.

### Restriction sites map

With four of the restriction endonucleases (*Alu*I, *Hha*I, *Kpn*I and *MseI*) and five representative sequences, two from this study and tree of the 16SrI phytoplasma group aligned, restriction sites were determined by MapDraw of the DNASTAR program (DNASTAR, Inc.).

### DNA sequencing

All the PCR products were cleaned with PCR purification QIAquick kit (Qiagen, USA) before sequencing. The sequence was in two directions with primers R16F2 and R16R2 in a sequenciator ABI PRISM 3700 (Applied Biosystems, USA). Later the sequences were deposited in the NCBI GenBank.

### Sequence similarity

The sequences were analysed by the Lasergene 2001, V. 5 Software (DNASTAR Inc., USA) with the profile mode of one pair by Martinez-NW and multiple aligment by Clustal W Methods. All the sequences were aligned and also the most related sequences were obtained by Blasting in the GenBank data base.

### Phylogenetic analyses

Two evolutionary trees were constructed with 43 sequences (35 download from the NCBI GenBank plus 10 sequences from this study). In one tree (Figure 2A) were included our 10 selected sequences (2 from the crop and 8 from its pests) and which were

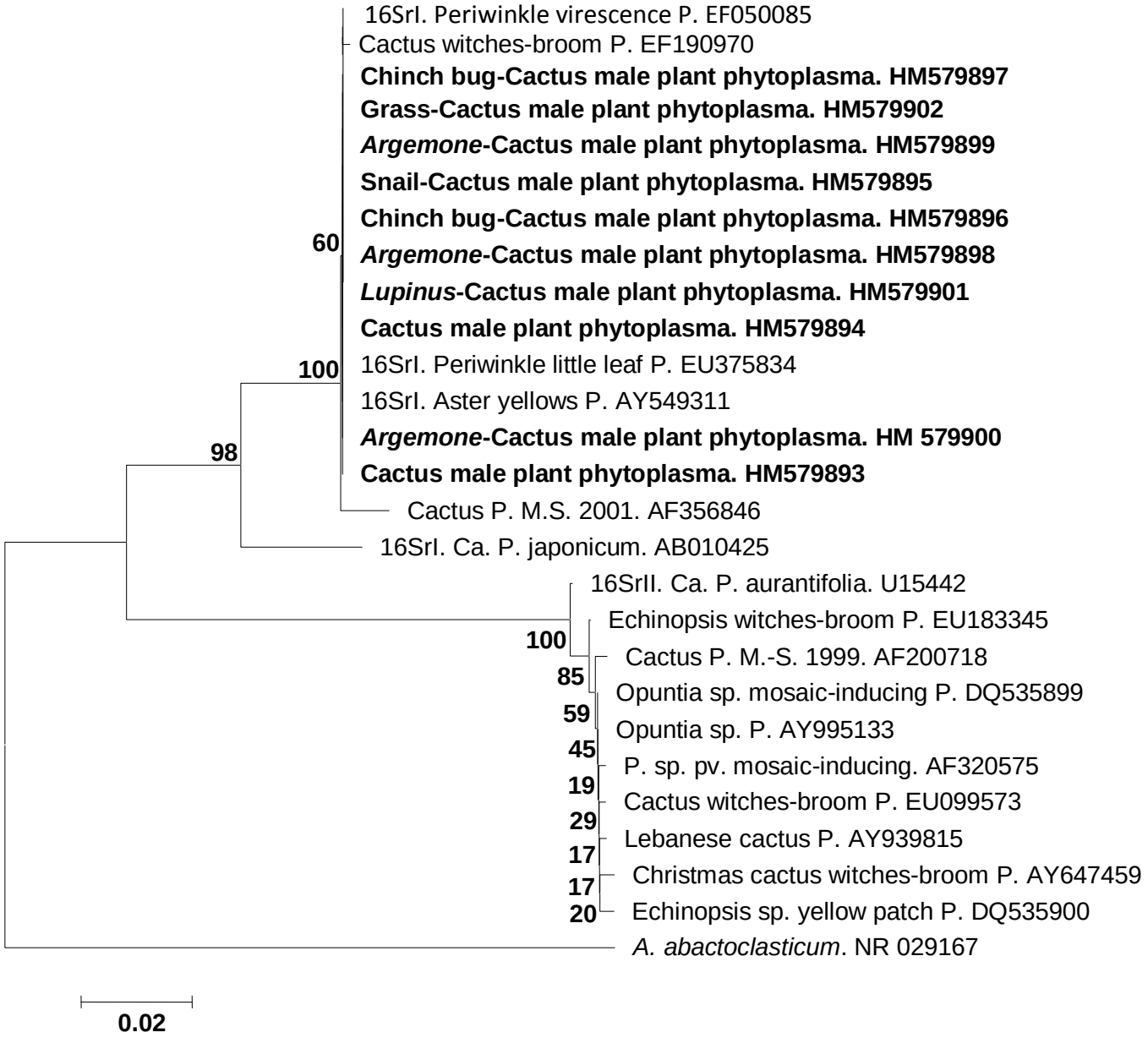


**Figure 2a.** Phylogenetic trees that show the relationships among representative 16S rRNA phytoplasma strains gene sequences and constructed by the neighbour-joining method, *Anaeroplasma* (*A.*) *abactoclasticum* as outgroup and numbers on the branches are bootstraps confidence values. It shows the relationships among 10 strains from this study (all in boldface) of Cactus male plant phytoplasma detected on edible Cactus crop (HM579893, HM579894), and its pests; the weeds *Argemone mexicana* (HM579898, HM579899, HM579900), a grass (HM579902) and *Lupinus* sp. (HM579901); the Hemiptera-Coreidae insect, *Chelinidae* sp. (HM579896, HM579897) and the molluscs, *Helix aspersa* (HM579895); and the reference strains of 16SrI phytoplasma group from GenBank; which included Cactus witches-broom (EF190970) and Cactus phytoplasma (P.) Martínez (M.) Soriano (S.) 2001 (AF356846), plus the species Candidatus (Ca.) Phytoplasma japonicum (AB010425) all of them of the 16SrI group. The reference strains of 16SrII phytoplasma group also from GenBank clustered in a different branch.

deposited in the NCBI GenBank, plus 16 sequences retrieved (9 from cacti and *Opuntia*, three aligned and representing one species of the 16SrI group and one more of a second species of this same group; and one sequence of the 16SrII phytoplasma group and two more from *Echinopsis*, an ornamental Cactaceae, of this same last group). In a second tree (Figure 2B) were included only two of our sequences with high nucleotide fragment length, the ones above mentioned, and 16 additional sequences from each one of 15 representative 16SrI-XV groups (IRPCM, 2004). The trees were constructed with the neighbor-joining algorithm and the confidence was assessed by bootstrap analysis based on 5000 strap replications using MEGA 4.1 software (Kumar et al., 2004). *Anaeroplasma abactoclasticum* (an obligately anaerobic Mollicutes) was the out-group to root each one of the trees (GenBank

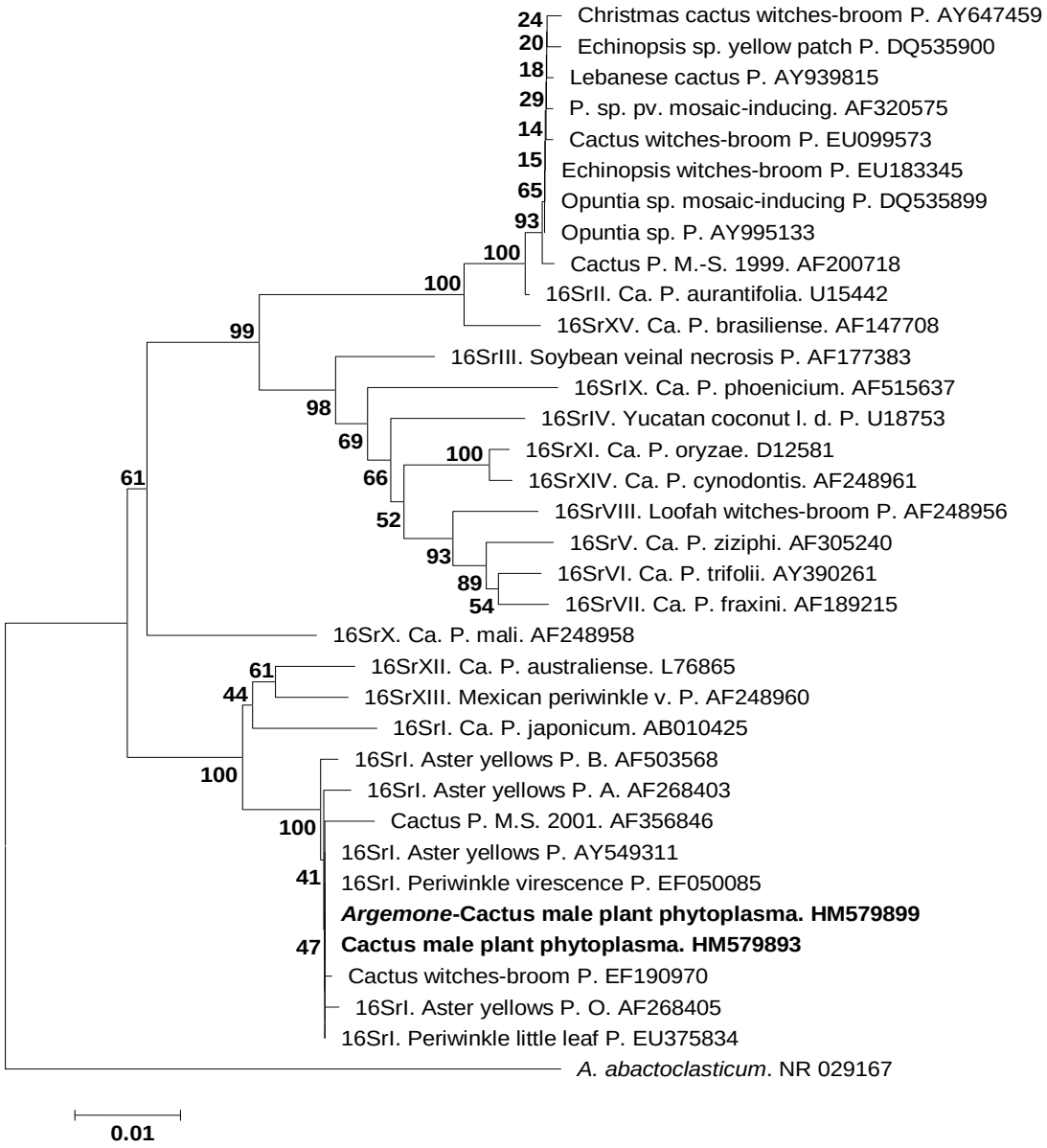


**Figure 2b.** Phylogenetic tree that shows the relationships among representative 16S rRNA phytoplasma strains gene sequences and constructed by the neighbour-joining method, *Anaeroplasma* (*A.*) *abactoclasticum* as outgroup and numbers on the branches are bootstraps confidence values. The 34 sequences reconfirmed the phylogenetic relationship in 16SrI Aster yellows phytoplasma group of two selected strains of this study (HM579893, HM579899) (in boldface) which were grouped in a last branch, while the reference strains of 16SrII group were in the first branch, and the other representative reference strains of 16SrIII-XV phytoplasma groups were also in different branches. GenBank accession numbers are indicated. Bars, 0.01 (B) and 0.02 (A) are substitutions per nucleotide position.

Accesssion NR 029167).

## RESULTS

### DNA amplification

From the 38 samples analyzed, 24 of them were phytoplasma positive. The nested PCR amplification generated DNA fragments of 1.2 kb which were observed by electrophoresis and corresponding to the amplification of the 16S rRNA gene according with the DNA positive control. No band was observed in the negative control. The DNA of these samples were deposited in the Colegio de Postgraduados, Fitosanidad, bacteriology laboratory. On *O. ficus-indica* crop, the phytoplasma was detected

on six symptomatic samples (on a young cladode center with cuticle and a margin with a deformed zone, transition zone between old and young cladode, a cladode, peel and seed with the pulp of an atrophied fruit), plus two samples from apparently healthy plants (apex of a young cladode with true leaves and spines, and base of a cladode joined with an old cladode).

Related to the pests, 16 samples were phytoplasma positive: five of *A. mexicana* (on small apical leaves, young leaves, deformed green-thick petals, and young elongated capsule with seeds), plus three samples of apparently healthy plants (young leaves and young capsule), on *Lupinus* sp. two samples (leaves of chlorotic and stunted plant, and from apparently healthy plant), on grass one positive sample from healthy appearing leaves with apical part of the stem; and on the chinch bugs the three samples were positive and also the two samples of the brown garden snail. No phytoplasma was detected on Convolvulaceae samples.

**PCR amplification and sequence similarity**

The nested PCR products amplified the 16S ribosomal phytoplasma gene of 22 DNA samples. Twenty of them were of 914 to 1233 bp and the other two of 441 bp (nested PCR product from transition zone between old and young cladode) and 454 bp (product from seed with the pulp). The sequences from *Opuntia* and pests shared 99.9 and 100% of similarity index among them. Ten of the high length nucleotide sequences (2 from the crop and 8 from its pests) were deposited in the NCBI GenBank (Accessions numbers HM579893, HM579894, HM579895, HM579896, HM579897, HM579898, HM579899, HM579900, HM579901, HM579902) and used for the construction of trees (Figures 2A and B).

**Phylogenetic analyses**

A selected largest nucleotide sequence of 1233 bp fragment length (Accsession # HM579899) from this study blasted in the GenBank only with 100 phytoplasmas sequences belonging to 16SrI group with 99 and 100% homology. Four aligned representative sequences of this 16SrI group were:

Aster yellows phytoplasma (AY549311), Cactus witches'-broom phytoplasma (EF190970) (of our host family); Periwinkle little leaf phytoplasma (EU375834) and Periwinkle virescence phytoplasma (EF050085) (both of two last sequences related to our DNA positive control).

All of them showed 99.6 to 100% homology with our sequence. The phylogenetic tree (864 nucleotide portion of fragment length) (Figure 2A) had two clusters. Cluster one had two groups and the first group had two subgroups.

In one subgroup (Cluster 1), all the phytoplasma sequences from this study (in boldface) were grouped together; plus the four sequences from the 16SrI phytoplasma group (AY549311, EF050085, EF190970, EU375834) (including the sequence from the ornamental Cactus). In the other subgroup was the sequence of an ornamental Cactus (AF356846) with 98.2% of similarity. In group two was the sequence of a different species (Accession # AB010425) of this same 16SrI phytoplasma group. In Cluster 2, all the sequences of the 16SrII phytoplasma group were grouped (U15442). Here sequences of ornamental cacti and *Opuntia*, plus the only one of *O. ficus-indica* crop in Italy (AY995133) were included.

The other phylogenetic tree constructed with 1072 nucleotide portion of fragment length (Figure 2B) reconfirmed the finding in Figure 2A. One time more the 16SrI phytoplasma group included our sequences (HM579893, HM579899) (boldface) and the ones from ornamental Cactus (AF356846, EF190970); and showed that all of them belong to the same species namely, Aster yellows phytoplasma.

**RFLP analyses**

The restriction patterns with the enzyme *Kpn*I of our *Opuntia* and pests samples and the positive control of 16SrI phytoplasma group were close to the ones previously described (Lee et al., 1993).

**Restriction sites map**

The map (Figure 3) showed equal restriction sites with the three sequences of the 16SrI phytoplasma group (Gundersen et al., 1996) and our sequences (HM579893, HM579899). They had the same restriction site patterns (eight with *Mse*I, four with *Alu*I, and two with *Hha*I and *Kpn*I).

**DISCUSSION**

In Mexico, by direct sequence analysis we identified on *O. ficus-indica* crop and its pests the 16SrI phytoplasma group. Worldwide there are other reports of this phytoplasma group mainly on ornamental Cactaceae in countries such as: China (Accession # EF190970) (Wei et al., 2007; Cai et al., 2008), even in Mexico (Acc. # AF356846) (IRPCM, 2004; Leyva-López et al., 1999); UK (Aster yellows Cactus) (Lee et al., 1998) and in USA (Cactus aster yellows) (Hodgetts et al., 2008). On *Opuntia* crop, in USA a 16SrI-B phytoplasma subgroup was identified on a fruit of *O. ficus-indica* (Bertaccini et al., 2007) and this 16SrI phytoplasma group agrees with our results.

In relation to the 16SrII phytoplasma group, in Italy,

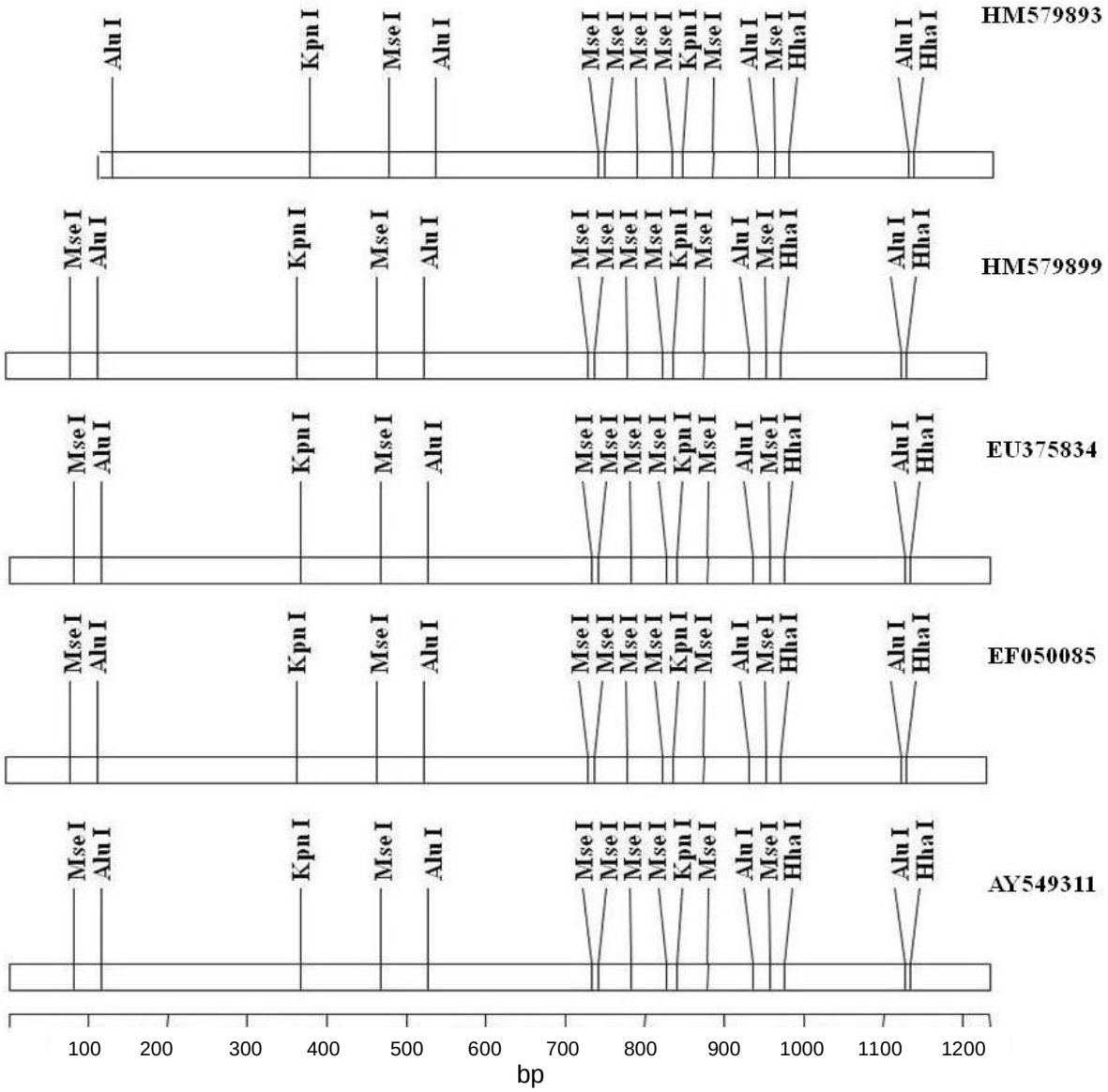


**Figure 3.** Map of the restriction sites in 16SrI phytoplasma group of five representative sequences. HM579893 strain from *O. ficus-indica* crop and HM579899 from the pest-weed *Argemone mexicana*; and the aligned ones (NCBI GenBank) of the 16SrI group, EU375834 (Periwinkle little leaf phytoplasma), EF050085 (Periwinkle virescence phytoplasma) and AY549311 (Aster yellows phytoplasma). Recognition sites were with the endonuclases *Alu*I, *Hha*I, *Kpn*I and *MseI*. Map was generated by DNASTAR program and MapDraw option.

16SrII-C phytoplasma subgroup was identified on *O. ficus-indica* crop (AY995133) (Granata et al., 2006) which differs from our findings in Mexico. However, in this same *Opuntia* crop and a close area to the region of our study, a 16SrII phytoplasma group was recently reported (Hernández-Pérez et al., 2009a, b), although no sequence was available in the NCBI GenBank or indirect proofs such as RFLP were showed. The authors mentioned some Accessions numbers which belong to other authors.

Again this 16SrII group was reported mainly on ornamental cacti in several countries. These are China (Acc. # AY647459; 16SrII-C, Acc. # EU099573) (Cai et al., 2008), Lebanon (Acc. # AY939815) (Choueiri et al., 2005), and in Mexico (Acc. # AF200718, AF320575) (Granata et al., 2006; IRPCM, 2004), (Acc. # DQ535899) (Avina-Padilla et al., 2009), plus one unclassified strain (EU183345) also in Mexico. On the *Opuntia* crop, this phytoplasma group appears to affect the crop fertility gradually, through time (Fucikovsky and Yáñez, 2006), however apparently it is unknown if the fertility on ornamental Cactaceae is also affected.

All these mentioned sequences of both 16SrI and 16SrII groups were in agreement with our phylogenetic

analyses (Figures 2A and B). The fact that in the same host crop we report 16SrI group in Mexico and 16SrII group in Italy should be due to the different geographic regions as it was thought before, that each group has specific geoubication area (Lee et al., 1998). In the cases of two 16Sr phytoplasma groups in a same area (one group in the crop and other in an ornamental Cactaceae), perhaps it means that different groups are adapting in a same area (Wei et al., 2007) or possibly because of the phytoplasma genetic diversity (Cai et al., 2008). In fact, two phytoplasma groups can have the same symptoms, as reported in USA on Cactus pear (Bertaccini et al., 2007) and in China on ornamental cacti (Cai et al., 2008).

From this study, the same 16SrI phytoplasma group was associated with the host crop and its pests. Then there are concerns because, even the tissues of crop and weed plant with healthy appearance gave positive results. This indicates that the phytoplasma is present in both healthy appearing and disease plants. In *Opuntia* crop this finding is important, because the farmers in this region replace diseased or old plants, or plant new crop, by using cladodes from other healthy-looking *Opuntia* plants although it may harbour phytoplasma and then dispersing the microorganism. This possibility was also discussed in Italy on *Opuntia* crop management (Granata et al., 2006). The phytoplasma on weeds also indicates that many plants may act as a reservoir for the phytoplasma that affects Cactus.

Besides, a surprise was encountered when the snails and the chinch bugs resulted also positive for the same phytoplasma. Snails have rasping tongues, feed at night and together with the chinch bugs that are sucking insects (Nobel, 2002), both may well transmit the phytoplasma to the Cactus. In the case of the insect chinch bugs, *Chelinidea* sp. of the Hemiptera order was mentioned that this Hemiptera order is the most successful of insect phytoplasma vectors (Weintraub and Beanland, 2005). Furthermore these chinch bugs were also found frequently on the grass which may also serve as a reservoir of the phytoplasma.

Because of these worldwide cacti phytoplasma diseases among ornamental and crop plantations, the Cactaceae species biodiversity are probably at great risk. We suggest that quarantine officials should fix rules to avoid dispersing this disease and have in mind that some potential vectors mentioned, may also be a problem inside and also among countries because of the movement of the plants.

## Conclusion

In Mexico, Cactus male plant phytoplasma is associated with the edible Cactus crop, *O. ficus-indica* and its pests, and all of these represent a novel phytoplasma hosts. This Cactus male plant phytoplasma was classified as Aster yellows phytoplasma 16SrI group, of the species *Candidatus phytoplasma asteris*. On bases of our knowledge, this is the first report in Mexico and elsewhere, relating this microorganism with edible Cactus and pests, and is an approach to understand this disease and its possible future control.

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