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MOLECULAR DETECTION AND CHARACTERIZATION OF A PHYTOPLASMA FROM XIANLAJIAO CHILI PEPPER IN SHAANXI PROVINCE, CHINA

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Abstract

Surveys were performed in the main Xianlajiao chili pepper-producing areas of Shaanxi Province to determine whether a phytoplasma was associated with the incidence of Arbuscular Lobular Disease (ALD). During July 2012 and 2013, 92 and 86 chili pepper plants, respectively, showing ALD-like symptoms were collected from 12 counties and the cities of Baoji, Weinan, Xianyang, and Xian. Samples from paulownia trees and plantain with symptoms of paulownia witches' broom and little leaf, respectively, located in pepper fields in Baoji city, Fengxiang county were also collected. Universal DNA primers for amplification of the phytoplasma 16S rRNA gene were used for nested PCR assays and DNA sequencing. Phytoplasma DNA was amplified from two chili pepper plants, the paulownia trees, and the plantain collected from Baoji city. The phylogenetic analysis of 16S rDNA sequences of the phytoplasmas detected in the chili pepper confirmed that these phytoplasmas are members of the 16SrI group "*Candidatus* Phytoplasma asteris". The results of virtual RFLP analysis and sequence homology comparison showed that the two Xianlajiao phytoplasmas have high homology with phytoplasmas 16SrI-B and 16SrI-M. Transmission electron microscopy assays revealed the presence of typical phytoplasma pleomorphic bodies in the phloem of phytoplasma-infected Xianlajiao chili pepper plants. Although our results did not establish a strong association between the presence of the phytoplasma and ALD, the simultaneous detection of a 16SrI phytoplasma in two symptomatic Xianlajiao chili peppers, the paulownia trees, and the plantain suggests a complex epidemiology related to the 16SrI phytoplasma pathogen in these new plant hosts.

Keywords: *Capsicum annuum*; ALD; phytoplasma; 16SrDNA; virtual RFLP

Introduction

The Xianlajiao chili pepper is referred to as 'Keriting' in Switzerland by Syngenta seeds, but is known as Xianlajiao or line pepper in China. Xianlajiao chili pepper is one of the three major types of chili pepper grown in China, with approximately ~430,000 hm^2 planted annually (Zhao, 2003). Based on the research of Bassett (1986), Xianlajiao chili pepper has been shown to be a variety of *Capsicum annuum* L. According to the horticultural classification of pepper varieties developed by Dr. P. G. Smith (Smith & Heiser, 1957), the Xianlajiao may belong to the cayenne group because the fruit type is similar to Cayenne Long Slim, a pepper cultivar from the USA. The main production areas for this pepper in China include Shaanxi, Gansu, Xinjiang, Shanxi, Ningxia, Sichuan, Henan, Guizhou, and Hunan provinces. The general features of the Xianlajiao pepper are: fruits are moderately pungent (about 1900 Scoville Heat Units, SHU) with a length of 12–17 cm and a diameter of 1.0–1.4 cm; the fruits are red, and individual fruits have a fresh weight of 7–12 g; when dried, the fruits have a finely wrinkled surface. Xianlajiao pepper fruits are processed mainly for powder or chili sauce.

For many years, Xianlajiao chili pepper plants with symptoms of Arbuscular Lobular Disease (ALD) were common in China (Zhao, 2003), and this was generally thought to be related to viral infection. In 1974, the Shanghai Biochemistry Institute of Chinese Academy of Sciences first reported mycoplasma-like organisms (MLOs) in mulberry (Zhu, 1985). MLOs are now called phytoplasmas. To date, more than 100 phytoplasma diseases have been reported from China (Luo *et al.*, 2011, Shi *et al.*, 2013) in species such as paulownia, jujube, mulberry, onion, cabbage, lettuce, cowpea, banana, apple, peach, pear, apricot, potato, wheat, and some ornamental plants (Shen *et al.*, 1980, Mou *et al.*, 2011). Diseases such as Paulownia witches' broom, jujube witches' broom, mulberry atrophy disease, wheat blue dwarf, and onion yellow disease are the most common, and are gradually increasing in frequency. Wu *et al*. (1994) identified a new chili pepper MLO disease by microscopic examination, which was the first report of a phytoplasma infecting peppers in China. In the past 20 years, very few reports concerning pepper ALD have been published (Li *et al.*, 2013, Liu *et al.*, 2013), and the relationship between phytoplasma and ALD in pepper warrants investigation.

Phytoplasmas are highly-specialized bacteria that are obligate parasites of plants and insects; they cause diseases of many agriculturally-important plants. Phytoplasmas have reduced genomes and lack cell walls, and, therefore have not been cultured on artificial media. Phytoplasmas were first discovered in 1967 in infected mulberry tissue in Japan (Zhu *et al.*, 1998). Typical symptoms of phytoplasma disease include a bushy "witches' broom" appearance due to reduced apical dominance, leaf yellowing, stunting, green flowers, etc., resulting in small fruits, reduced yields, and even plant death. Phytoplasmas have been identified in >1000 species of plants around the world (Mou *et al.*, 2011). The main methods for identification and characterization of phytoplasma have been enzyme-linked immunosorbent assay (Zhu *et al.*, 1998), optical microscopy (Yuan *et al.*, 1978), fluorescence microscopy (Wang *et al.*, 1999), electron microscopy, 16S rDNA analysis, restriction fragment length polymorphism (RFLP), and DNA sequencing. More recently, the nested PCR technique has been shown to have much higher sensitivity and specificity, and can detect as little as a few

molecules of phytoplasma DNA (Che & Luo, 2006). Lee *et al.* (1993) showed for the first time that nested PCR could effectively discriminate between multiple phytoplasmas from a composite infection within a single host. Thus, this technology has been widely used, especially in pepper phytoplasma detection, and almost all researchers have adopted the use of nested PCR techniques. Electron microscopy is one of the classic ways to detect phytoplasma. Using a healthy plant as a reference, the presence of phytoplasma could be determined based on the site in the plant tissue, and the shape, size, membrane, inclusions, etc. In 1980, Xu identified the existence of an MLO in jujube by electron microscopy (Xu, 1980). Jin *et al.* (1992) identified the existence of an MLO in large Chinese hawthorn. Presently, with the development of molecular detection technology, electron microscopy is usually used as a support method to confirm DNA-based results. In recent years, the relative incidence of phytoplasma disease has increased in China (Li *et al.*, 2013). Observing the symptoms of ALD and using multipoint sampling to detect the presence of phytoplasma infection is important to the further study of this disease.

In this study, leaf samples were collected from pepper plants with ALD-like symptoms in 2012 and 2013, and they were assayed using nested PCR and by transmission electron microscopy (TEM). The aim of the study was to identify the presence of phytoplasmas in Xianlajiao chili peppers with ALD symptoms in the field, and to encourage the continuation of research to elucidate the epidemiological constraints of such diseases in order to support further improvement of disease management and control strategies.

Materials and Methods

Plant material: Plant samples were collected during July 2012 and July 2013. This period coincides with flowering and the maximum expression of ALD symptoms in Xianlajiao pepper. The main symptoms included clustered buds, dwarfing, shortened internodes, smaller leaves, abnormal leaf color, and bulging veins. Two Xianlajiao chili pepper plants that tested positive for phytoplasma are shown in Fig. 1. Sampling locations included the cities of Baoji (Qishan, Fengxiang, Meixian, Fufeng, Qianyang, Longxian), Xianyang (Qianxian, Xingping, Yangling), Weinan (Huaxian, Huayin), and Xian (Zhouzhi). Ninety-two leaf samples were collected in 2012, and a total of 86 leaf samples were collected in 2013. In both years, samples from five symptomless plants were also collected as negative controls. In 2013, there were three paulownia trees (*Paulownia tomentosa* Sieb.) showing symptoms of paulownia witches' broom disease present near the sample collection location (Dan village, Fengxiang). We collected leaf samples from the affected paulownia trees and also a from plantain (*Plantago asiatica*) with unusual leaves in a chili pepper field (samples were not collected at this location in 2012). Leaf samples were taken to the laboratory immediately after collection and stored at −80°C. The positive controls for our experiments were samples of Bermuda grass (*Cynodon dactylon* L. Pers) infected with white leaf disease, which were collected on the south campus of Northwest A&F University in 2012 and are a proven phytoplasma reference control maintained in this laboratory.

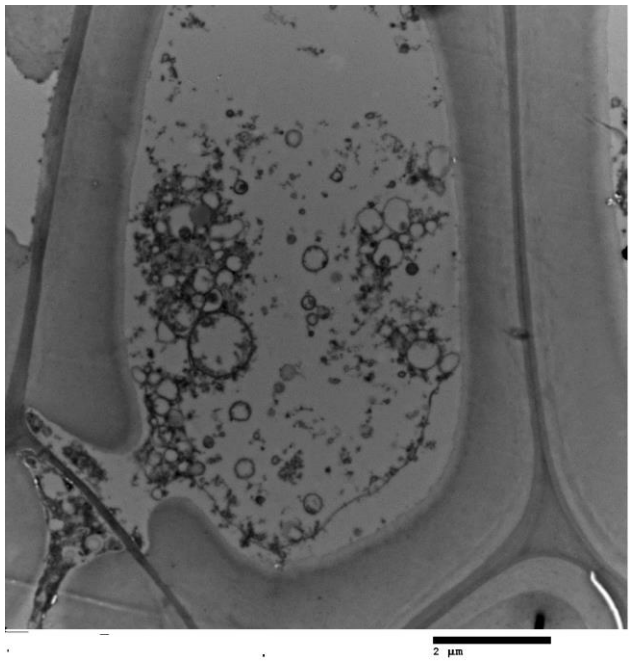

Fig. 1. Typical symptoms of phytoplasma disease in Xianlajiao chili pepper plants.
a: Xianlajiao chili pepper plant #1 that tested positive for phytoplasma, with the symptoms clustered buds, dwarfing, smaller leaves, abnormal leaf, and bulging veins; b: Xianlajiao chili pepper plant #2 that tested positive for phytoplasma, with the symptoms clustered buds, dwarfing, shortened internodes, smaller and abnormal leaf, and bulging veins.

Molecular detection of phytoplasmas in symptomatic leaf samples

DNA extraction: Total DNA was extracted from leaf samples (3 g), using the cetyl trimethylammonium bromide method described by Lee *et al.* (1991).

16S rDNA nested PCR amplification: Total DNA was used as a template in a nested PCR assay with universal primers, P1/P7, that amplify the phytoplasma 16S rRNA gene (Deng & Hiruki, 1991, Smart *et al.*, 1996) for the first PCR round, and primer pair R16F2n/R2 (Gundersen *et al.*, 1996) for the nested reaction. PCR products from the first PCR reaction were diluted 100-fold and a 1 µL sample was used for the nested PCR round. PCR amplifications were performed in final volumes of 25 µL containing 2.5µL 10× buffer (including $MgCl_2$), 2.5 mmol/L of each dNTP, 2 µL template DNA, 10 pmol of each primer, 0.25 µL *Taq* DNA polymerase (1.25 units), and 16.25 µL of sterile deionized water. DNA extracted from phytoplasma-infected Bermuda grass was used as the positive control; DNA from healthy Xianlajiao plants was the negative control; and ultrapure water was the blank control. PCR amplification conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. Nested PCR conditions were similar, but the annealing temperature was increased to 60°C, and extension was at 72°C for 90 s. Nested PCR products (5µL) were checked on 1% agarose gels, stained with ethidium bromide for 10 min, and photographed with a UV light transilluminator (254 nm).

Cloning and sequencing of amplified DNA fragments: 16S rDNA fragments were excised from the agarose gel and purified with a rapid gel extraction kit (Beijing TransGen Biotech Co., Ltd), ligated into the pEASY-T1 Cloning Vector (TransGen Biotech, Beijing, China), and transformed into *Escherichia coli* DH5α. The amplified DNA fragments were sequenced by the Nanjing GenScript Biotechnology Company (Nanjing, China).

16S rDNA nucleotide sequence homology comparison: Alignment of the 16S rDNA sequences amplified from Xianlajiao pepper DNA with other known phytoplasma sequences and construction of the phylogenetic tree were performed using MEGA 5.0 software (Tamura *et al.*, 2011). The neighbor-joining method was used to build the phylogenetic tree, and the tree was bootstrap tested (1,000 replicates) to estimate the level of statistical support for the branches. The aligned and trimmed sequences were exported to the *in silico* restriction analysis and virtual gel plotting program pDRAW32, developed by AcaClone Software (http://www.acaclone.com). Each aligned DNA fragment was digested *in silico* with 17 distinct restriction enzymes that have been routinely used for phytoplasma 16S rRNA gene RFLP analysis. A similarity coefficient was calculated for each pair of phytoplasma strains according to the virtual RFLP patterns (Wei *et al.*, 2007). DNAMAN 6.0 software (Li *et al.*, 2012) was used for sequence homology comparison.

TEM: TEM examination of phytoplasmas in petiole tissues of symptomatic samples was carried out as previously described (Kamińska *et al.*, 2001). The segments were cut into small pieces and fixed. Postfixation in 1% osmium tetroxide was for 2 h at 4°C. The material was then dehydrated, and ultrathin sections on copper grids were stained with uranyl acetate and lead citrate. The grids were examined with an HT7700 (Hitachi Transmission Electron Microscope, Japan) in the Electron Microscope Laboratory of Northwest A&F University.

Results and analysis

16S rDNA nested-PCR assay: The expected DNA fragments of ~1,200 bp were amplified from the positive controls with primer pair R16F2/R16R2 (Bermuda grass infected with white leaf disease is a known phytoplasma reference control). Phytoplasma-specific 16S rDNA fragments were not amplified from healthy Xianlajiao pepper tissue samples collected in 2012 and 2013 using the same primers. The 1,200-bp target DNA fragment was not amplified from 92 Xianlajiao samples showing ALD symptoms collected in 2012. The expected 1,200 bp target DNA fragment was amplified from two symptomatic Xianlajiao samples collected from Dan village in Fengxiang county in 2013 (Fig. 2), but not from the other 84 samples collected in 2013.

Construction and analysis of the phylogenetic tree: The two phytoplasma 16S rDNA sequences obtained in 2013 were aligned with 35 phytoplasma sequences from GenBank, including phytoplasma representatives from the existing 33 RFLP groups (Bertaccini *et al.*, 2014). We used *Acholeplasma laidlawii* and *A. palmae* as the outgroup, and an un-rooted phylogenetic tree was constructed with MEGA5.0 software. As can be seen in Fig. 3, the analysis showed that one of the Xianlajiao phytoplasma sequences (GenBank KF934496) shared homology with the Derbid phytoplasma sequence (GenBank AY744945). These two sequences grouped in a clade that also contained 16S rDNA sequences from the second Xianlajiao phytoplasma (GenBank KF934495), the Aster yellows witches' broom phytoplasma (GenBank NC-007716), and the Buckland valley grapevine yellows phytoplasma (GenBank AY083605).

Virtual RFLP analysis of phytoplasma sequences amplified from Xianlajiao: Based on the results shown in Fig. 3, there is a relatively close phylogenetic relationship between the 16S rDNA sequences from the Derbid phytoplasma (GenBank AY744945), the Aster yellows witches' broom phytoplasma (GenBank NC-007716), the Buckland valley grapevine yellows phytoplasma (GenBank AY083605), and the two Xianlajiao pepper phytoplasmas. The two sequences obtained in this study, all sequences in the 16SrI group, the sequence of the Derbid phytoplasma, and the sequence of the Buckland valley grapevine yellows phytoplasma were analyzed by virtual RFLP (Fig. 4). We also calculated the similarity coefficients between the two Xianlajiao phytoplasmas and other phytoplasmas by comparing the virtual RFLP patterns (Table 1). The similarity coefficients between one Xianlajiao pepper phytoplasma sequence (GenBank KF934495) and the 16SrI-B, 16SrI-C, 16SrI-M and 16SrI-N sequences were the highest; all were 0.938271605. The similarity coefficients between this Xianlajiao phytoplasma, the Derbid phytoplasma, and the Buckland valley grapevine yellows phytoplasma were 0.512195122 and 0.716049383, respectively. For the other Xianlajiao phytoplasma sequence (GenBank KF934496), the similarity coefficients were highest with sequences from groups 16SrI-B, 16SrI-C, 16SrI-M, and 16SrI-N; all were 0.900000000. The similarity coefficient between this Xianlajiao pepper phytoplasmas sequence and the Derbid phytoplasma (GenBank AY744945) was 0.49382716, while the similarity coefficient with and the sequence from the Buckland valley grapevine yellows phytoplasma (GenBank AY083605) was 0.67500000. Overall, our results show that the two Xianlajiao pepper phytoplasma sequences belong to group 16SrI, and have a high level of similarity with 16S rDNA sequences from subgroups B, C, M, and N.

Sequence homology comparison between sequences from the two Xianlajiao phytoplasmas and four 16SrI phytoplasmas: We compared the homology of the two Xianlajiao phytoplasma 16S rDNA sequences with sequences from subgroups I-B, I-C, I-M, and I-N (Fig. 5). Sequence homology was calculated using DNAMAN 6.0. The Xianlajiao phytoplasma 16S rDNA sequence (KF934495) was 99.52% homologous with sequences from subgroups I-B and I-M and 99.44% homologous to sequences from subgroups I-C and I-N. The other Xianlajiao phytoplasma 16S sequence (KF934496) was 99.44% homologous to sequences from subgroups I-B and I-M and 99.36% homologous to sequences from subgroups and I-C and I-N. These results showed that the two Xianlajiao phytoplasmas have higher 16S rDNA sequence homology with phytoplasmas from subgroups I-B and I-M.

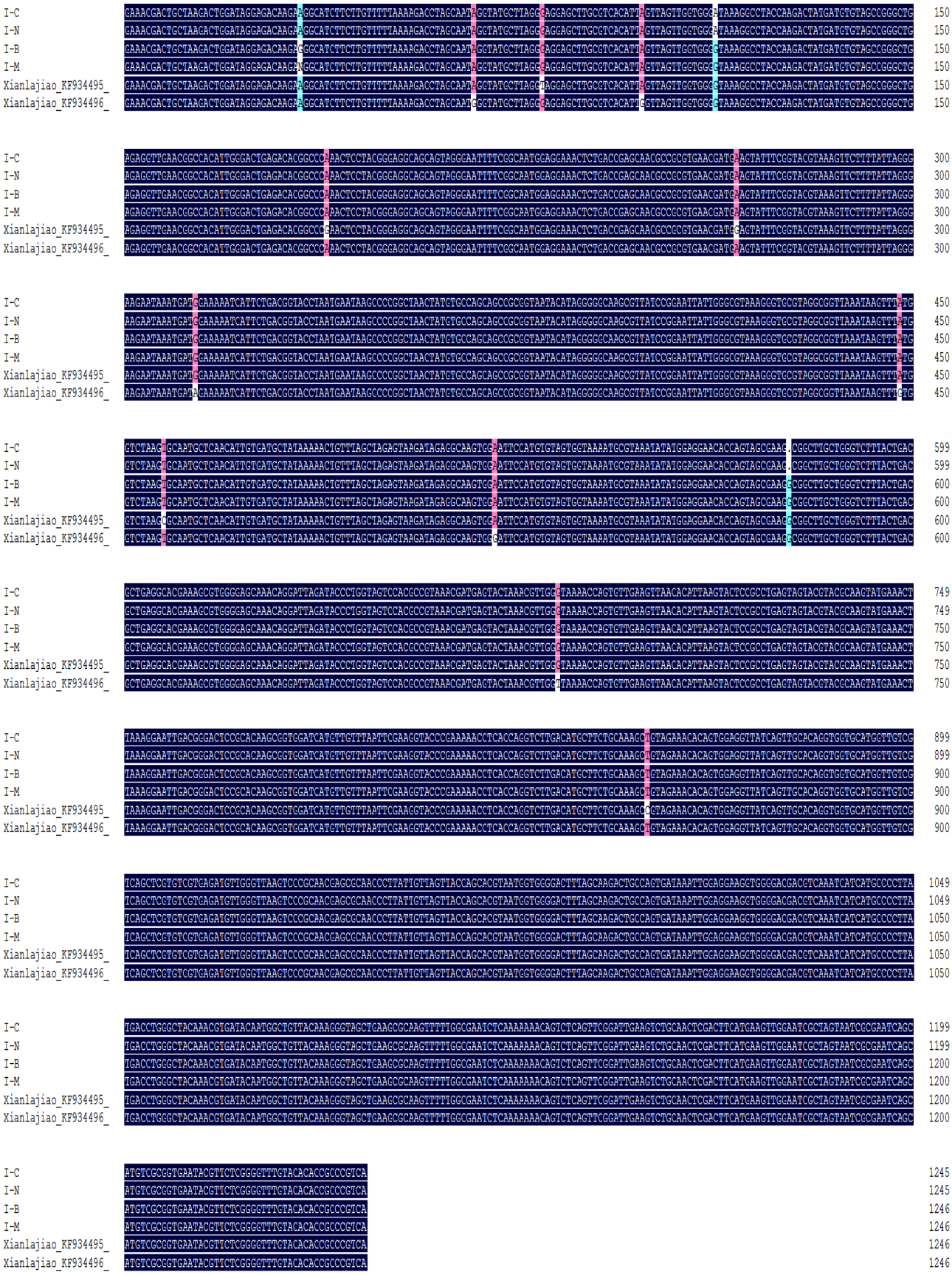

Fig. 2. Results of nested PCR amplification of phytoplasma 16S rDNA.
M: Marker (2000 bp, TransGen Biotech, China); R16F2n/R2 PCR amplifications from phytoplasma-infected pepper showing ALD symptoms (lanes 1 and 2); phytoplasma-infected plantain (lane 3); paulownia tree showing paulownia witches' broom symptoms (lane 4); positive control (lane 5). No PCR amplicons were obtained for the symptomless Xianlajiao peppers (lane 6) and the non-DNA PCR control (lane 7).

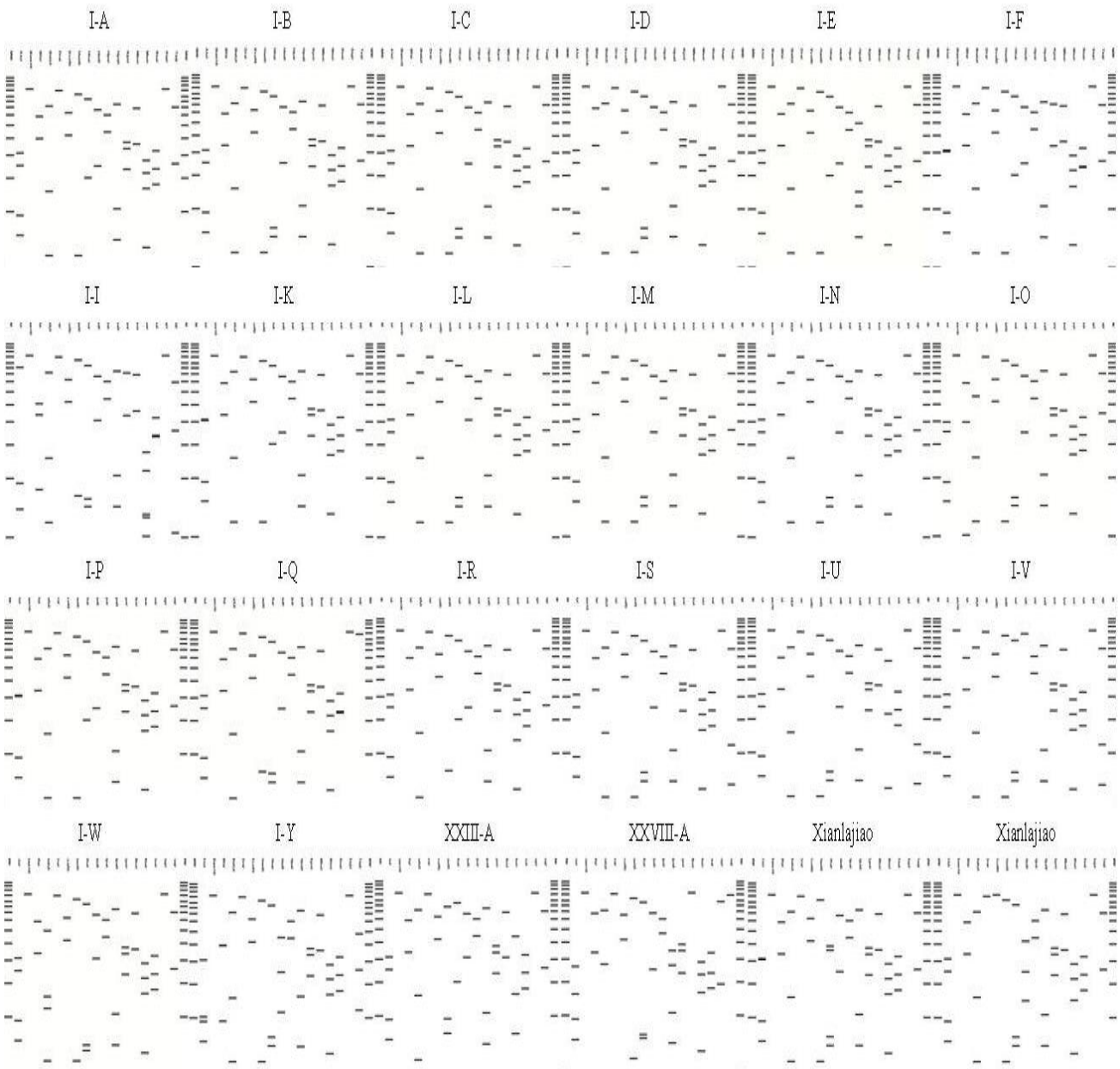

Fig. 3. Phylogenetic tree constructed from phytoplasma 16S rDNA sequences including the two Xianlajiao chili pepper phytoplasmas identified in this study, representatives from the existing 33 RFLP groups, and *Acholeplasma laidlawii* and *A. palmae* as the outgroup. The tree was bootstrap tested (1,000 replicates) to estimate the level of statistical support for the branches.

Discussion

Detection of chili pepper phytoplasma : Phytoplasma disease of chili pepper has been reported from many countries, such as the United States (Randall *et al.*, 2009), Mexico (Lebsky & Poghosyan, 2007, Santos-Cervantes *et al.*, 2008, Lebsky *et al.*, 2011), Costa Rica (Villalobos *et al.*, 2009), Bolivia (Arocha *et al.*, 2010), Italy (Murolo *et al.*, 2010), Turkey (Sertkaya *et al.*, 2007), India (Khan & Raj, 2006), Cuba (Arocha *et al.*, 2007), Lebanon (Choueiri *et al.*, 2007), Australia (Tran-Nguyen *et al.*, 2003), and Spain (Castro & Romero, 2002). The characterized chili pepper phytoplasmas belong to 16Sr groups I (Santos-Cervantes *et al.*, 2008), III (Arocha *et al.*, 2010, Lebsky *et al.*, 2011), VI (Randall *et al.*, 2009), XII (Villalobos *et al.*, 2009), and XII-A (Sertkaya *et al.*, 2007, Murolo *et al.*, 2010). These results show that the chili pepper phytoplasma is common in many parts of the world, although the subgroups vary. In China, Wu *et al.* (1994) identified a new chili pepper MLO disease by microscopic examination, and this was the first report of phytoplasma infecting peppers in China. Subsequently, Wang *et al.* (2010) showed that the 16S rDNA nucleotide sequences from paulownia witches' broom were highly homologous to sequences from the chili pepper phytoplasma; the chili pepper samples were collected close to a paulownia tree with witches' broom disease in Heze, Shandong province. This situation is similar to the present study. Li *et al.* (2013) detected the phytoplasma in chili pepper samples collected from Yangling in Shaanxi province using the universal primer pairs P1/P6 and R16F2n/R16R2 in nested PCR. Similarly, Liu *et al.* (2013) detected phytoplasma in chili pepper samples collected from Yongan in Fujian province.

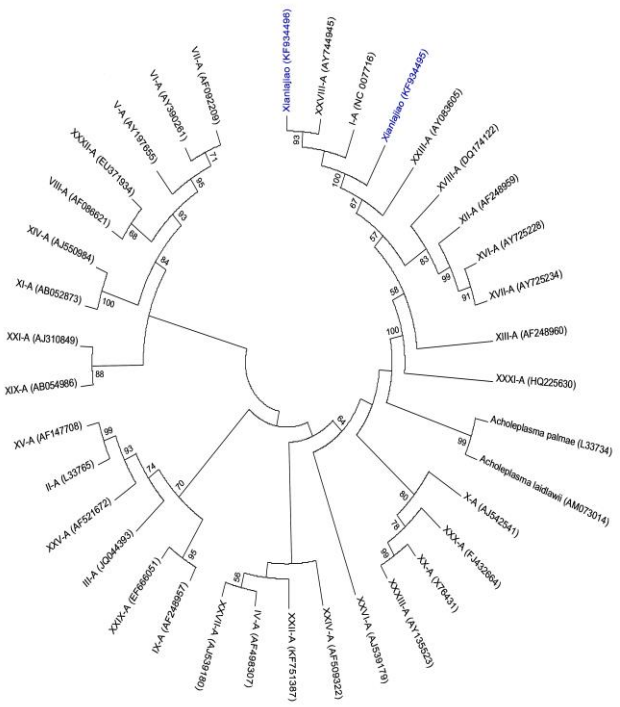

Fig. 4. Virtual restriction fragment length polymorphism (RFLP) patterns of group 16SrI and some other phytoplasma sequences. MW: Marker. *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mse*I, *Rsa*I, *Ssp*I and *Taq*I restriction analysis. The gene IDs used in this figure are, in turn: I-A (NC_007716), I-B (NC_005303), I-C (AY265205), I-D (AY265206), I-E (AY265213), I-F (AY265211), I-I (U96614), I-K (U96616), I-L (AY180957), I-M (AY265209), I-N (AY265205), I-O (AF268405), I-P (AF503568), I-Q (AY034089), I-R (AY102275), I-S (FJ914654), I-U (FJ914650), I-V (FJ914642), I-W (HQ450211), I-Y (EF199549), XXIII-A (AY083605), XXVIII-A (AY744945), Xianlajiao (KF934495) and Xianlajiao (KF934496).

Characterization of Xianlajiao chili pepper phytoplasma: In this study, the results of nested-PCR assay showed the 16S rDNA from Xianlajiao, plantain, paulownia and positive control phytoplasma had similar band size (see Fig. 2). The results of clustering analysis showed that the Derbid phytoplasma (GenBank AY744945, 16SrXXVIII-A), Aster yellows witches' broom phytoplasma (GenBank NC-007716, 16SrI-A) and Buckland valley grapevine yellows phytoplasma (GenBank AY083605, 16SrXXIII-A) belong to a branch with the two Xianlajiao phytoplasmas. Results of virtual RFLP analysis showed that the two Xianlajiao phytoplasmas belong to group 16SrI, and have high similarity with subgroups B, C, M and N. The results of sequence homology comparison between Xianlajiao phytoplasmas and four 16SrI phytoplasmas showed that two Xianlajiao phytoplasmas have their highest homology with phytoplasmas from subgroups I-B and I-M. The above results show that different analysis methods can produce different results. We conclude that the two Xianlajiao phytoplasmas belong to group 16SrI, but we cannot accurately determine whether the subgroup is I-B or I-M.

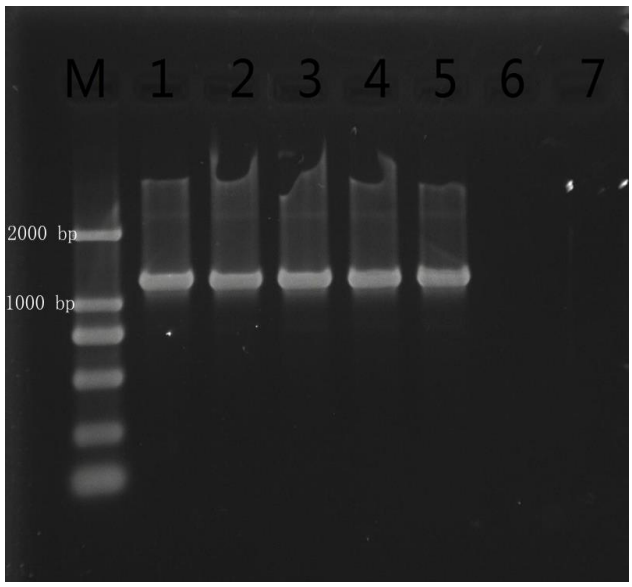

Fig. 5. Sequence alignment and homology comparison between the 16S rDNA fragments from
this study and phytoplasma 16S rDNA nucleotide sequences from GenBank.
I-C, I-N, I-B and I-M are the subgroups of group 16SrI; Xianlajiao KF934495 and Xianlajiao KF934496 are the phytoplasma sequences from the two Xianlajiao chili peppers in this study.

In this study, the chili pepper samples were collected close to a paulownia tree with witches' broom disease; this situation is similar to the study of Wang *et al.* (2010), and Wang's results showed that the 16S rDNA phytoplasma nucleotide sequences from paulownia witches' broom were highly homologous to sequences from the chili pepper phytoplasma. The results of this study showed that there was a high similarity coefficient between chili pepper phytoplasmas and paulownia phytoplasma (I-D, AY265206, (Lee *et al.*, 2004) (see Tab. 1). Therefore, the paulownia trees growing in the same field may be possible source of the Xianlajiao chili pepper phytoplasma.

TEM of Xianlajiao ALD tissue samples: Based on previous descriptions of phytoplasmas being located in phloem tissues, cell shape, and ohter indicators described in references (Shen *et al.*, 1980, Xu, 1980, Jin *et al.*, 1992, Li *et al.*, 2013), combined with experimental electron microscopy images, we identified several phytoplasma-like bodies in Xianlajiao chili pepper plants (arrows in Fig. 6), in which phytoplasma had been detected by nested PCR.

Acknowledgments

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Fig. 6. Transmission electron microscopic identification of phytoplasma pleomorphic bodies in Xianlajiao Arbuscular Lobular Disease samples. The arrows indicate the phytoplasma, the approximate size observed under TEM was about 1.5 × 2 mm.

Table 1. Calculated similarity coefficients between 16S rDNA sequences from the two Xianlajiao pepper phytoplasmas and 22 other phytoplasmas

Phytoplasmas	Similarity coefficient with Xianlajiao phytoplasma KF934495	Similarity coefficient with Xianlajiao phytoplasma KF934496
I-A (NC-007716)	0.850000000	0.810126582
I-B (NC-005303)	0.938271605	0.900000000
I-C (AY265205)	0.938271605	0.900000000
I-D (AY265206)	0.913580247	0.875000000
I-E (AY265213)	0.864197531	0.825000000
I-F (AY265211)	0.860759494	0.769230769
I-I (U96614)	0.626506024	0.609756098
I-K (U96616)	0.900000000	0.810126582
I-L (AY180957)	0.913580247	0.875000000
I-M (AY265209)	0.938271605	0.900000000
I-N (AY265205)	0.938271605	0.900000000
I-O (AF268405)	0.780487805	0.765432099
I-P (AF503568)	0.925000000	0.835443038
I-Q (AY034089)	0.850000000	0.810126582
I-R (AY102275)	0.850000000	0.810126582
I-S (FJ914654)	0.902439024	0.864197531
I-U (FJ914650)	0.864197531	0.850000000
I-V (FJ914642)	0.864197531	0.825000000
I-W (HQ450211)	0.853658537	0.740740741
I-Y (EF199549)	0.767441860	0.729411765
XXIII-A (AY083605)	0.716049383	0.675000000
XXVIII-A (AY744945)	0.512195122	0.493827160

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