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A phytoplasma representative of a new subgroup, 16SrXIII-E, associated with Papaya apical curl necrosis

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Abstract Papaya apical curl necrosis (PACN) has frequently been observed in several Brazilian states. Affected plants exhibit foliar chlorosis, curvature of the apex, shortening of the internodes leading to bunching of the crown leaves, necrosis of the young apical parts, leaf drop, and dieback. Naturally infected plants were sampled and subjected to PCR assays, which confirmed that a phytoplasma was associated with the disease. Sequencing of the 16S rRNA gene, conventional and computer-simulated RFLP analyses, and phylogenetic analysis allowed the determination of the PACN phytoplasma as a representative of a new subgroup, designated 16SrXIII-E. The phytoplasmas of various 16Sr groups, including 16SrI, 16SrII, 16SrX, 16SrXII, and 16SrXVII, are known to be involved in anomalies in papaya plants in several countries. However, the present study reports, for the first time, the occurrence of a 16SrXIII phytoplasma in association with a papaya disease.

Keywords Dieback · Mollicutes · Phytoplasma classification · Phloem bacteria

Brazil ranks second among the world's largest producers of papaya (*Carica papaya* L.), predominantly in the states of Bahia and Espírito Santo. In these areas, the lack of knowledge about the etiology of an important disease known as Papaya Apical Curl Necrosis (PACN) has prevented the adoption of specific control measures (Ventura et al. 2004).

PACN symptoms usually appear 5 months after papaya seedlings are transplanted in the field. The young leaves of naturally infected plants exhibit chlorosis, which then progresses to necrosis, followed by apical curvature of the top of the stem. The top leaves wither and drop off, the petioles and internodes of the top region become shorter, leading to bunching of the crown leaves. Internally, vessel necrosis may be observed in the apical region. Total defoliation, extensive apical necrosis, and plant death are observed in the advanced stages of the disease. Two diseased plants are illustrated in Fig. 1. Incidence levels of up to 75 % may occur, depending on the cultural practices and plant cultivar (Ventura et al. 2004). Epidemiological studies have shown an aggregated spatial arrangement of symptomatic plants, indicating the possible involvement of vectors of a biotic systemic agent (Ventura et al. 2004).

Diseases that result in symptoms resembling PACN have been described in association with phytoplasmas in several countries. However, distinct phytoplasmas are associated with these diseases despite the similarity in symptoms. A representative of the 16SrXII group was identified in Australia (Gibb et al. 1996), Israel (Gera et al. 2005), and Taiwan (Bau et al. 2011); whereas a phytoplasma belonging to group 16SrII was found in Ethiopia (Arocha et al. 2007). In Cuba, phytoplasmas affiliated with the groups 16SrXVII (Arocha et al. 2005), 16SrI, and 16SII (Acosta et al. 2013) were

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Fig. 1 Plants showing some symptoms of Papaya Apical Curl Necrosis: young leaves with chlorosis, necrotic parts and bunching of the crown leaves

detected. Phytoplasmas of the 16SrI and 16SrII groups were also identified in India (Rao et al. 2011; Verma et al. 2012).

Phytoplasmas are wall-less prokaryotes and obligate parasites that inhabit phloem cells (Wei et al. 2007). According to Wei et al. (2007), phytoplasmas are unculturable in a cell-free medium; for this reason, the distinction and classification of this type of bacteria using the biochemical and phenotypic criteria usually employed for the classification of culturable microorganisms are impossible. Thus, a scheme of classification into groups and subgroups has been widely adopted to classify phytoplasmas and is based on the molecular characterization of genetic diversity (Lee et al. 1998; Wei et al. 2007). In Brazil, representatives of various groups (16SrI, 16SrIII, 16SrVII, 16Sr IX, and 16SrXV) have been identified; these are predominantly phytoplasmas affiliated with the 16SrI and 16SrIII groups.

The present work investigated the association of phytoplasma with PACN in papaya and classified this phytoplasma into a system of groups and subgroups based on the 16S rRNA gene.

The apical parts of the stems of five symptomatic papaya plants in 2006 and five in 2012 were sampled from fields in Espírito Santo State, Brazil. Similar samples were also collected from asymptomatic plants grown in the field and in an insect-proof greenhouse for use as negative controls. Maize bushy stunt (MBS) phytoplasma (16SrI group) served as a positive control. Each phytoplasma from each sample was considered a strain.

The total nucleic acids were extracted employing a commercial kit (Dneasy Plant Mini—Qiagen Inc.). For the detection of phytoplasmas, nested PCR reactions were performed using the P1/Tint primers (Deng and Hiruki 1991), followed by R16F2n/R16R2 (Gundersen and Lee 1996), as described previously by Gundersen and Lee (1996). The products were analyzed after 1 % agarose gel electrophoresis and Sybr Safe staining (Invitrogen). The bands were visualized using a UV transilluminator. The DNA fragment size standard was a 1-kb ladder (Invitrogen).

The PCR results revealed that 1.2-kb DNA fragments were only amplified from the symptomatic papaya samples. Identical fragments were also generated for the positive control. Conversely, no fragment was produced using the extracts of asymptomatic and healthy papaya plants.

A conventional RFLP analysis was performed using nested PCR products digested with the enzymes *Alu*I, *Hinf*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mse*I, and *Rsa*I (Invitrogen). The RFLP patterns were compared with previously reported patterns (Lee et al. 1998). Maize bushy stunt phytoplasma (MBS) was used as a reference.

The 16S rRNA gene of seven strains was sequenced. Amplified DNA fragments of 1.2 kb were purified and cloned into competent *Escherichia coli* cells of strain DH5alpha using the pGEM Easy Vector System I (Promega). The sequences were assembled using the PHPH (http://bioinformatica.cenargen.embrapa.br/phph/) and Bioedit programs (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A majority consensus sequence was selected

for each strain based on three sequenced clones. The PACN-Br1 and PACN-Br2 sequences and PACN-Br3 and PACN-BR4 sequences were chosen as representatives of the strains from the samples collected in 2006 and 2012, respectively. A computer-simulated RFLP analysis was performed using these sequences and those belonging to distinct subgroups of the 16SrXIII group available in GenBank. Each sequence was digested with 17 enzymes, as described by Wei and collaborators (2007); *in silico* restriction analysis and virtual RFLP plotting was performed using pDRAW32 software (http://www.acaclone.com). The virtual restriction patterns were compared, and a similarity coefficient (F) was calculated for each pair of phytoplasma strains, as published in a previous work (Wei et al. 2007). Because the virtual restriction patterns and (F) values were identical for the PACN-Br1, PACN-Br2, PACN-BR3, and PACN-4 strains, the sequence of PACN-Br2 was selected to represent the phytoplasma associated with papaya plants.

A phylogenetic tree was constructed using DNA sequences belonging to phytoplasmas classified into distinct groups, the sequences of strains representative of the subgroups that constitute the 16SrXIII group and the sequences of strains PACN-BR1 and PACN-Br2, using MEGA4 (http://www.megasoftware.net/mega4/mega.html) software with the neighbour-joining method.

The collective RFLP patterns generated by the seven restriction enzymes in the digestions of the rDNA amplicons from the strains found in the papaya plants were indistinguishable from each other. The RFLP patterns using *Alu*I, *Hinf*I, *Hpa*II, *Kpn*I, *Mbo*I, and *Rsa*I were identical to those described for the Mexican periwinkle virescence (MPV) phytoplasma, which is the reference for phytoplasmas of the 16SrXIII group (Lee et al. 1998). However, the patterns of the amplified PACN rDNA digested with the enzyme *Mse*I were similar, but not identical, to those of MPV phytoplasma rDNA, indicating that the PACN and MPV phytoplasmas are distinct (Fig. 2). The patterns generated by the MBS phytoplasma used as a control in this study (Fig. 2) were coincident with the reference profiles previously published for this phytoplasma (Lee et al. 1998). This evidence supports our results obtained using an RFLP analysis for the phytoplasma from papaya, as it was not possible to include rDNA from 16SrXIII as a reference in our assays.

The restriction patterns generated by digestion with 17 endonucleases confirmed that the PACN phytoplasma is a member of the 16SrXIII group. The sequence of the PACN phytoplasma shared 99 % similarity with the phytoplasmas associated with MPV (16SrXIII-A/GenBank AF248960) and China-tree yellows (CbY1) (16SrXIII-C/GenBank AF495882). The sequence similarity between the PACN phytoplasma and Strawberry green petal phytoplasma (16SrXIII-B/GenBank U96614) and Potato purple top-PPT/SINPV phytoplasma (16SrXIII-D/GenBank FJ914647) was 98 %. The sequences from strains PACN-Br1, PACN-Br2, PACN-Br3, and PACN-Br4 were deposited in GenBank under accession numbers JQ792171, EU719111, JX893518, and JX893519, respectively. Although the sequence of PACN-Br1 was divergent from the other three sequences for some nucleotides, the restriction patterns generated were indistinguishable.

Based on the virtual RFLP patterns, the similarity coefficients (F) calculated for the PACN phytoplasma in relation to the other members of 16SrXIII ranged from 0.82 to 0.92 (Table 1). These values strongly indicated that the PACN phytoplasma is divergent from the phytoplasmas that are representative of the subgroups comprising the 16SrXIII group and may be considered as a member of the new subgroup 16SrXIII-E. The tree generated by the phylogenetic analysis is shown in Fig. 3. The branching confirmed that the PACN phytoplasma is clustered in the 16SrXIII group but emerges from a branch that is different from those of the other phytoplasmas of this group.

The initial diagnosis based on symptoms was confirmed by PCR assays, demonstrating the association of a phytoplasma with PACN. The previous epidemiological evidence (Ventura et al. 2004) indicating that symptoms could be associated with a biotic agent and that the spatial arrangement of affected plants was type aggregated are in agreement with our results, which showed the consistent presence of phytoplasmas in the diseased plants.

The values of the similarity coefficients (F) for the delineation of new phytoplasma 16Sr subgroup lineages (Wei et al. 2007) allowed the classification of the PACN phytoplasma as a representative of a new subgroup of 16SrXIII. According to Wei et al. (2007), a new subgroup is recognized when a phytoplasma strain presents an F value equal to or lower than 0.97 in comparison to those of all of the existing representative strains of a given group. The F values of the PACN phytoplasma ranged from 0.82 to 0.92 in relation to the phytoplasmas belonging to the different subgroups of 16SrXIII (Table 1), leading us to conclude that this phytoplasma represents a distinct new subgroup. The phylogenetic analysis, revealing that the PACN phytoplasma is a representative of

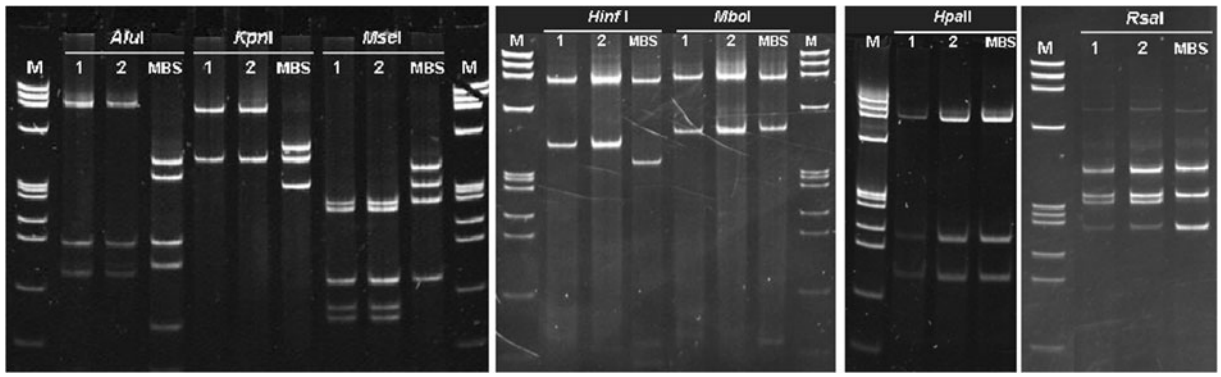

Fig. 2 Conventional RFLP patterns of 16S rDNA from strains PACN-1 and PACN-2, representatives of the phytoplasma found in symptomatic papaya plants. Products from nested PCR with primers P1/Tint and R16F2n/R16R2 were digested with restriction enzymes *Alu*I, *Kpn*I, *Mse*I, *Hinf*I, *Mbo*I, *Hpa*II, and *Rsa*I. Lane M: fragment size standard (*phi*X 174RF *Hae*III); lane 1: strain PACN-Br1; lane 2: strain PACN-Br2; lane MBS: Maize Bushy Stunt phytoplasma, used as a reference

a divergent subgroup that represents a new branch for 16SrXIII, also gave support to our findings.

Although the present study revealed a phytoplasma of the 16SXIII group, new subgroup 16SrXIII-E, in association with papaya in Brazil, the symptoms of the disease are very similar to those described previously for the dieback disease associated with a 16SrXII phytoplasma in Australia (Gibb et al. 1996). It is known that different phytoplasmas may induce similar symptoms in the same host (Wei et al. 2007), and this is also true for papaya diseases. Therefore, dieback-like diseases have been associated with diverse phytoplasmas. In Cuba, representatives of group 16SrI were characterized in plants exhibiting symptoms of dieback (Acosta et al. 2013), whereas members of groups 16SrI and 16SrII (Acosta et al. 2013) and a phytoplasma of group 16SrXVII (Arocha et al. 2005) were identified in plants exhibiting bunchy top disease symptoms. In Ethiopia, a phytoplasma of the 16SrII group was found in papaya with symptoms of dieback (Arocha et al. 2007), and phytoplasmas belonging to groups 16Sr I and 16SrII were present in plants that exhibited some typical symptoms of dieback in India (Rao et al. 2011; Verma et al. 2012). A representative of the 16SrXII group was first identified as causing dieback in Australia (Gibb et al. 1996); later a phytoplasma belonging to this same group was reported in plants with some symptoms related to dieback in Israel (Gera et al. 2005) and Taiwan (Bau et al. 2011).

A small number of plant hosts have been recorded for 16SrXIII phytoplasmas worldwide. However, these hosts belong to a reduced number of distinct botanical families,

Table 1 Similarity coefficients (F) derived from virtual 16S rRNA gene fragment RFLP patterns of the phytoplasma associated with PACN identified in this study and reference phytoplasmas belonging to group 16SrXIII

Phytoplasma	16S rDNA group-subgroup affiliation	MPV	Strawberry Green Petal	ChTY- CbY1	PPT-SINPV	PACN
MPV	16SrXIII-A	1.00				
Strawberry Green Petal	16SrXIII-B	0.93	1.00			
ChTY-CbY1	16SrXIII-C	0.84	0.85	1.00		
PPT-SINPV	16SrXIII-D	0.93	0.96	0.83	1.00	
PACN	16SrXIII-E	0.92	0.91	0.82	0.90	1.00

MPV Mexican periwinkle virescence

ChTY China-tree yellows

PPT Potato purple top

PACN Papaya apical curl necrosis

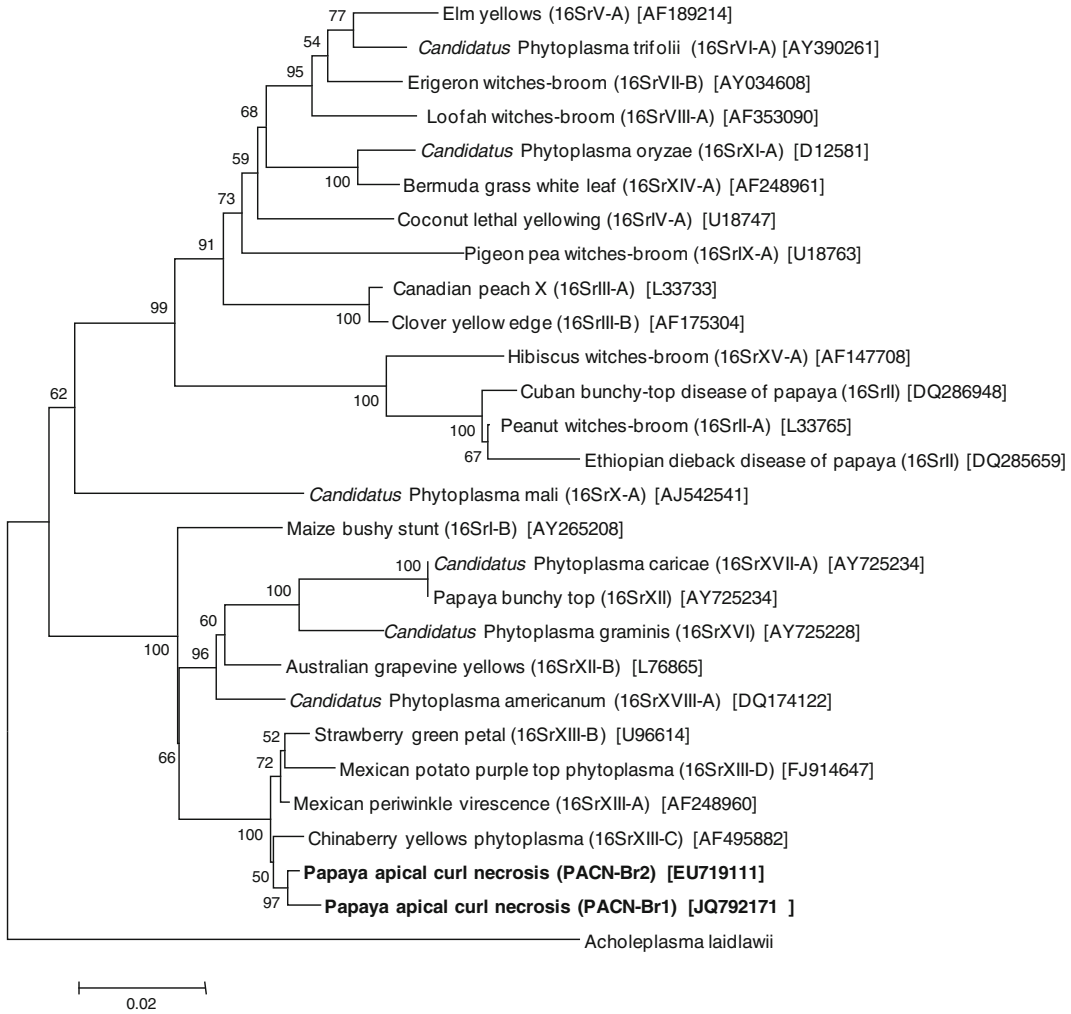

Fig. 3 Phylogenetic tree generated by 16S rDNA sequences of the phytoplasma associated with Papaya Apical Curl Necrosis (PACN), a member of group 16SrXIII, and phytoplasmas affiliated with distinct groups by the neighbour-joining method. *Acholeplasma laidlawii* was used as an outgroup. Subgroups are indicated in parentheses. The numbers on the branches are confidence values of bootstrap support. *Acholeplasma laidlawii* ATCC 23206 was included as outgroup, and bootstrapping was performed 1000 times

such as Apocynaceae (periwinkle, *Catharanthus roseus*) (Lee et al. 1998), Meliaceae (China-tree, *Melia azedarach*) (Harrison et al. 2003), Rosaceae (strawberry, *Fragaria x ananassa*) (Jomantiene et al. 1998), and Solanaceae (potato, *Solanum tuberosum*) (Santos-Cervantes, et al. 2010). The results obtained in the present study showed that the family Caricaceae can also be included as a new host for the 16SrXIII group. Although phytoplasmas affiliated with diverse groups (16SrI, 16SrIII, 16SrVII, 16Sr IX, and 16SrXV) have been identified in association with numerous diseases in Brazil, this is the first paper that reports the presence of a representative of group 16SrXIII. In addition to other divergent subgroups previously reported in other countries, the occurrence of the new subgroup 16SrXIII-E in Brazil suggests a wide geographic distribution of the members of the 16SrXIII group in North and South America. Furthermore, the discovery of the 16SrXIII-E subgroup, firstly described in this study, allows the expansion of the knowledge of the genetic diversity within the 16SrXIII group.

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