WALAILAK JOURNAL
http://wjst.wu.ac.th Applied Sciences

Diversity of Phytoplasmas Associated with Several Plants in Western Java-Indonesia

Ariny PRASETYA, Kikin Hamzah MUTAQIN*, Meity Suradji SINAGA and Giyanto

Departement of Plant Protection, Faculty of Agriculture, Bogor Agricultural University, Indonesia

(*Corresponding author's e-mail: kmutaqin@gmail.com)

Received: 14 May 2017, Revised: 4 December 2018, Accepted: 26 January 2019

Abstract

Diseases caused by phytoplasmas have been reported in field crops, ornamentals, and weeds in Indonesia. However, most of phytoplasmas have not been subjected to further identification and thus, they remain unaffiliated with proper classification scheme. More reliable identification of phytoplasmas mostly rely on molecular methods. The aim of this study was to characterize the phytoplasma as the causal agent of naturally infected plants in western Java-Indonesia based on their 16S rRNA nucleotide sequences. Plant exhibiting phytoplasmal symptoms were observed and taken for further molecular examination. Eight plant species from three families in Bogor, Tangerang, and Bekasi, i.e. peanut, soybean, snakebean, *Opuntia* sp., betung bamboo, apus bamboo, Bermuda grass, and digitaria grass (*Digitaria fuscescens*) have been observed and taken as samples for further molecular examination. Nested-PCR with primer pairs P1/P7 followed by R16F2n/R16R2 resulted in amplification of products of approximately 1.2 kb from all symptomatic plant samples tested. BLAST analysis of the nucleotide sequences, phylogenetic analyses, and similarity coefficients derived from RFLP in silico revealed that there were association of a phytoplasma of 16SrII-A subgroup with phytoplasmas identified in peanut, soybean, and snakebean. Such an association showed witches' broom symptoms; 16SrII-C with *Opuntia* sp. causing proliferation and mosaics; and phytoplasmas displaying yellowing and little leaf of two kinds of bamboos and white leaf of Bermuda grass and digitaria grass that were closely related to 16SrXIV-A subgroup. To our knowledge, this is the first report molecular identification of 16SrXIV-A associated with apus bamboo and digitaria grass in Indonesia.

Keywords: Group 16SrII, group 16SrXIV, nested-PCR, sequence

Introduction

Phytoplasmas are bacteria lacking cell walls that inhabit the phloem of plants and are naturally transmitted by phloem-feeding insects. These bacteria have not been successfully cultivated in vitro, and so they are categorized within the genus "*Candidatus* (*Ca*.) Phytoplasma spp.". Phytoplasmas have been associated with several hundred diseases of crops, ornamentals, and weeds all over the world. Plants infected by phytoplasmas exhibit various symptoms, including virescence, phyllody, sterility of flowers, witches' broom, generalized stunting, discolorations of leaves or shoots, leaf curling or cupping, and generalized decline [1].

Previously in Indonesia, phytoplasmas have been found associated with diseases such as legume witches' broom [2], Kalimantan coconut wilt [3], Bermuda grass white leaf, cactus witches' broom, bamboo yellows and sweet potato little leaf [4,5], chilli and tamarillo diseases [6], and *Mimosa pudica* yellows [7]. Phytoplasma infection may result in significant yield loss in some economic crops. However prior to this report, the phytoplasma strain infecting several host plants had not been identified. The

development of identification on phytoplasmas in Indonesia is quite slow compared to other plant pathogens. This may be the result of the inability of the pathogen to grow in vitro, in which the presence is only in the phloem of infected plants.

Initially, phytoplasma diseases have been determined on the basis of the characteristic symptoms and subsequent observation of the pathogen of ultrathin sections of diseased plants [8]. The DAPI staining and scanning electron microscope (SEM) have been used to study phytoplasmas, but they are unable to distinguish the bodies of other micro-organisms or cell components (mitochondria, chloroplast) from phytoplasmas [9]. Molecular-based analyses, in particular the use of polymerase chain reaction (PCR), developed the last two decades have been proven to be more accurate and reliable than other methods previously used for phytoplasma identification [1].

Molecular analysis of 16S rRNA gene sequences has been widely used for differentiation and classification of phytoplasmas. The 16S rRNA provides a useful target for phytoplasma classification due to its conseved and variable regions [10]. Many phytoplasma strains have been classified based on the analysis of the actual and/or virtual electrophoresis gel of 16S rRNA gene restriction fragment length polymorphism (RFLP) patterns [11,12]. The phytoplasma strains within a species should share at least 97.5 % sequence identity within the 16S rRNA gene [11]. Therefore, this study was undertaken to characterize the phytoplasma as the causal agent of naturally infected plants in western Java-Indonesia based on their 16S rRNA nucleotide sequences.

Materials and methods

Plant samples

Towards the end of 2014 and early 2015, plant samples of peanut (*Arachis hypogaea*), soybean (*Glycine max*) and snakebean (*Vigna unguiculata*) showing witches' broom symptoms; betung bamboo (*Dendrocalamus asper*) exhibiting yellowing and little leaves; apus bamboo (*Gigantochloa apus*) with little leaf symptoms were collected in Bogor. The *Opuntia* sp. with proliferation and mosaics was collected in Tangerang. Lastly, the whitening leaves of Bermuda grass (*Cynodon dactylon*) and digitaria grass (*Digitaria fuscescens*) were collected in Bekasi.

Total DNA extraction

Total DNA were extracted from 100 mg of leaves using DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instruction. The concentration of DNA was measured by NanoVue Plus (GE), while a 20 ng μL^{-1} DNA was used for the first PCR reaction.

Amplification of phytoplasma 16S rDNA gene fragments

For the first PCR reaction, primer pairs P1 [13] / P7 [14] which primes a 1800 bp DNA fragment were used. The first round of PCR was set up in 25 µL containing 1 µL of the DNA template, 1 µL of each primer (5 pmol), 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific) and 9.5 µL nuclease free water (Thermo Scientific). First PCR reaction was performed with an initial denaturation at 92 °C for 1 min, followed by 35 cycles (denaturation 95 °C for 1 min, annealing 55 °C for 1 min and extension 72 °C for 1.5 min) and a final extension at 72 °C for 10 min. For the nested PCR reaction, the product of the first round PCR was diluted 1:30 in nuclease free water and was used as a template in place of DNA. The primer pair R16F2n/R16R2 [15] which primes a 1250 bp DNA fragment was used in the nested PCR reaction. The reaction mixture and cycle conditions of nested-PCR were performed the same as the standard PCR. Both PCR amplifications were carried out in Applied Biosystems machine Veriti 96 Well Thermal Cycler. Amplified PCR products (5 µL) were subjected to the electrophoresis in 1 % (w/v) agarose gel containing 4 µL peqgreen (PeqLab) and visualized with a Molecular Imager (BioRadGel Doc^{TM} XR^{+}) equipped with Image Lab^{TM} Software. GeneRuler 1 kb DNA Ladder (Thermo Scientific) was used to estimate the sizes of the PCR products.

Cloning, sequencing and sequence analysis

The R16F2n/R16R2 amplicons from samples obtained in this study were chosen and purified from agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific). They were cloned using PCR Cloning Kit (Thermo Scientific) by ligation into the pTZ57R/T vector and transformed into *Escherichia coli* DH5α competent cells following the manufacturer's instructions (InsTAclone PCR Product Cloning Kit-Thermo Scientific). Plasmids were prepared from transformed bacterial colonies and extracted using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) and sequenced at First Base Asia. The 16S rRNA sequences obtained were compared with reference sequences in GenBank, by Basic Local Aligment Search Tool (BLAST).

Phylogenetic analysis

The obtained sequences obtained were aligned and compared with each other and with 28 reference groups of phytoplasmas using Clustal W. A phylogenetic tree was constructed using the neighbour-joining method implemented in MEGA version 7.0.18 program with 1000 replicates for bootstrap analysis. *Acholeplasma laidlawii* (M23932) was used as an outgroup to root the tree.

In silico RFLP analysis

The trimmed and aligned R16F2n/R16R2 sequences and graminae-infecting phytoplasma groups 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 *Alu*I, *BamH*I, *Bfa*I, *BstU*I, *Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I, previously designed for phytoplasma classification [12]. The virtual RFLP patterns were compared and a similarity coefficient (F) was calculated for each pair of phytoplasma strains according to the formula: F = 2Nxy/(Nx+Ny), in which x and y are the strains of two given phytoplasmas, Nx and Ny. They were the number of bands resulting from enzymatic digestion of strains x and y, respectively, and Nxy is the number of bands shared by the two strains [12].

Results and discussion

Disease symptoms

The major symptom of phytoplasma disease of peanut and soybean was the proliferation of the axillary buds with small leaves resulting in witches' broom appearance. However, the color of the infected leaves generally remained green (**Figures 1A** and **1B**). The symptoms observed in snakebean was the proliferation of the axillary buds with a small leaf curved downward, wavy, and yellowish green (**Figure 1C**). We named the disease as peanut witches' broom, soybean witches' broom, and snakebean witches' broom. The cactus (*Opuntia* sp.) develops disease with symptoms characterized by proliferation and mosaic patterns in the epidermis (**Figure 2**) and is named as cactus witches' broom. This kind of diseases have been recorded in China, Lebanon, Mexico, and Italy [16].

The chlorosis symptoms of leaves were found in betung bamboo with yellow leaves in smaller size and bushy appearance in plant canopy (**Figure 3A**). Apus bamboo (**Figures 3B** and **3C**) showed small leaves with white stripes along the vein, shortened twigs, which made the bamboo clump looked huddled. The disease in betung bamboo is named as bamboo yellowing leaf, while the disease in apus bamboo is named as bamboo little leaf. Phytoplasmas symptoms due to infection, according to Marcone [17], can be non-specific or specific. Non-specific symptoms such as shrink and curl leaves, necrosis of the vascular tissue, vascular tissue enlargement, and dwarfism are common in woody plants. Bamboo plants, known as giant grass, is a plant that grows in a clump, jointed, woody stems, with a hollow in the center. Therefore, the symptoms due to phytoplasma infection on bamboo can vary. The infected Bermuda grass and digitaria grass were observed as small white patches on the ground (**Figure 4**). The infected Bermuda grass showed the display of the whitening of leaves, reduction of the leaf size, and the death of the plants.

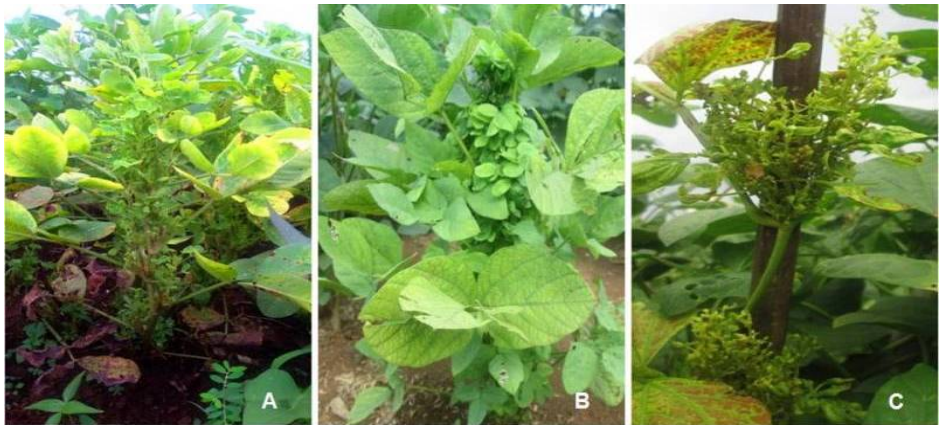

Figure 1 Witches' broom symptoms on peanut (A), soybean (B), and snakebean (C)

Figure 2 Proliferation and mosaic symptoms on *Opuntia* sp.

Figure 3 Small leaves with green yellowish on betung bamboo (A), shortened twigs, small and green leaves on apus bamboo (B), and white stripe along the vein leaves on apus bamboo leaves (C).

Figure 4 Bermuda grass with whitening and little leaf (A) and *D. fuscescens* with whitening and reddenish tip of leaves (B).

PCR amplification

After the first round 35 cycles of amplification from the DNA infected samples, with P1/P7 primer pair, not all samples gave visible amplification products (data not shown). However, amplicons of approximately 1250 bp were visible after the nested PCR assay with primer pair R16F2n/R16R2 from all symptomatic samples (**Figure 5**). Results from the nested-PCR analysis provide evidence that all samples collected were naturally infected with phytoplasma. Negative results revealed in some symptomatic plant samples due to low titre or uneven phytoplasma distribution. According to Gundersen *et al.* [15], nested-PCR assay increased both sensitivity and spesificity of phytoplasma DNA from samples having unusually low titers or inhibitors that may interfere with PCR efficacy. Among the eight different species of plant samples tested, the apus bamboo and digitaria grass had never previously been reported to host a phytoplasma in Indonesia.

Sequence analysis

Comparisons of the 16S rRNA sequences obtained from other phytoplasma 16S rRNA sequences reported in GenBank, using the tool BLAST revealed that phytoplasma detected in peanut, soybean, and snakebean shared 99 % identity with "*Echinacea purpurea* witches' broom phytoplasma" (JN885461), a member of group 16SrII. The phytoplasma detected in *Opuntia* sp. shared 99 % identity with "*Ca.* Phytoplasma aurantifolia" (HG792252), a member of group 16SrII, when compared with BLAST search. The members of the group 16SrII have been previously reported infecting chilli and tamarillo in Indonesia [6]. The group phytoplasma 16SrII is known to be the causal agent of disease in several economic crop plants, including ornamentals.

BLAST comparisons on the phytoplasma detected in betung bamboo shared 95 % identity. Bermuda grass shared 99 % identity with "Bermuda grass white leaf phytoplasma" (AF248961). Likewise, the phytoplasma detected in apus bamboo and digitaria grass shared 99 % identity with "*Ca*. Phytoplasma cynodontis" (EU999999), members of the group 16SrXIV. White leaf of Bermuda grass, caused by a phytoplasma belonging to the 16SrXIV group, has been reported by Rao *et al.* [18], Salehi *et al*. [19] and Omar [20]. "*Ca*. Phytoplasma cynodontis" is also associated with many other grasses and plant species exhibiting white and small leaves, yellows, bushy growth and severe chlorosis symptoms in several countries such as blue grass (*Poa annua*) in Italy [21], golden beard grass (*Chrysopogon aciculatus*) in Korea [22], *Oplismenus burmanii* and *Digitaria sanguinalis* in India [23], *D. scalarum* in Kenya [24], coconut palm (*Cocos nucifera*) [25] and foxtail palm (*Wodyetia bifurcate*) [26] in Malaysia. Though, "*Ca*. Phytoplasma cynodontis" has been previuosly identified from Bermuda grass in Indonesia, but the detail information was not available.

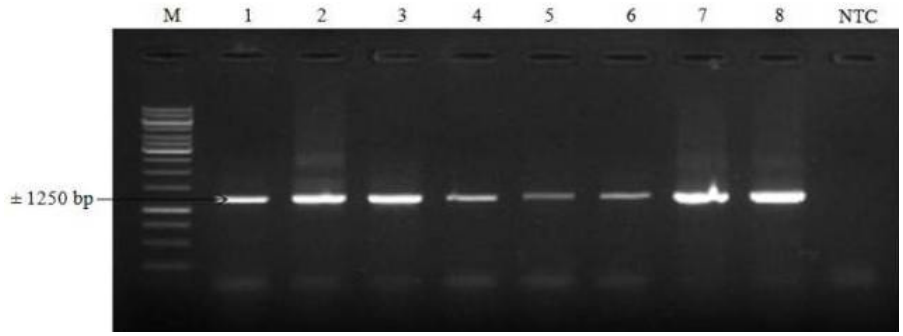

Figure 5 Amplification of phytoplasma rRNA gene products using P1/P7 primers followed by R16F2n/R16R2 primers. Lane (M) = 1 kb ladder (Thermo Scientific); lane (1 - 8) = amplification of phytoplasma from infected peanut, soybean, snakebean, *Opuntia* sp., betung bamboo, apus bamboo, Bermuda grass and digitaria grass; lane (NTC) = no template control/nuclease free water.

Phylogenetic analysis

The phylogenetic analysis of the 16S rRNA sequences obtained with 16S rRNA sequences of the 57 phytoplasmas reference strains from 28 groups of 16S rRNA phytoplasmas and *A. laidlawii* (outgroup) produced the consensus tree as illustrated in **Figure 6**. The phytoplasma identified in peanut, soybean, and snakebean were closely related to phytoplasma reference of subgroup 16SrII-A (*peanut witches' broom phytoplasma*). Witches' broom symptoms of legumes have been reported for a number of years in Indonesia [2], but prior to this report the causal agent of symptoms had not been identified. Results described here showed that a 16SrII-A phytoplasma is causal agent of witches' broom symptoms. The phytoplasma identified in *Opuntia* sp. was most closely related to the phytoplasma reference of subgroup 16SrII-C (*cactus witches' broom phytoplasma*) in the phylogenetic branch of the phytoplasma strains from group 16SrII. This results revealed that the presence of variety of 16SrII subgroup members was identified in crop plants in Indonesia.

The phytoplasma identified in betung bamboo, apus bamboo, Bermuda grass, and digitaria grass were clustered with the subgroup 16SrXIV-A (*Ca*. Phytoplasma cyndontis); however, the phytoplasma identified in betung bamboo revealed far phylogenetically branch related to subgroup 16SrXIV-A, which could explain the differences in symptomatology in betung bamboo. Phytoplasma subgroups can be differentiated based on a range of 95 to 98 % of their 16S rRNA sequence homologies [27]. Sequence similarity comparisons determined that the 16S rRNA sequence of betung yellowing leaf phytoplasma shares only 95 % similarity with group 16SrXIV. However, oligonucleotide sequences of unique region of the 16S rRNA gene of *Ca.* Phytoplasma cynodontis, 5'- AATTAGAAGGCATCTTTTAAT-3' [28], was found. A comparison of 16S rRNA of phytoplasma identified in betung bamboo with representative of subgroup 16SrXIV-A (AJ550984) indicated changes in nucleotide sequences in betung bamboo phytoplasma, including substitution, insertion, and deletion of nucleotides (**Figure 7**). This suggests that the analysis of the complete 16S rDNA sequences included more variable regions like ribosomal proteins to determine the subgroup phytoplasma from betung bamboo. To our knowledge, the occurence of group 16SrXIV causing the disease of bamboo species and digitaria grass is a new record in Indonesia.

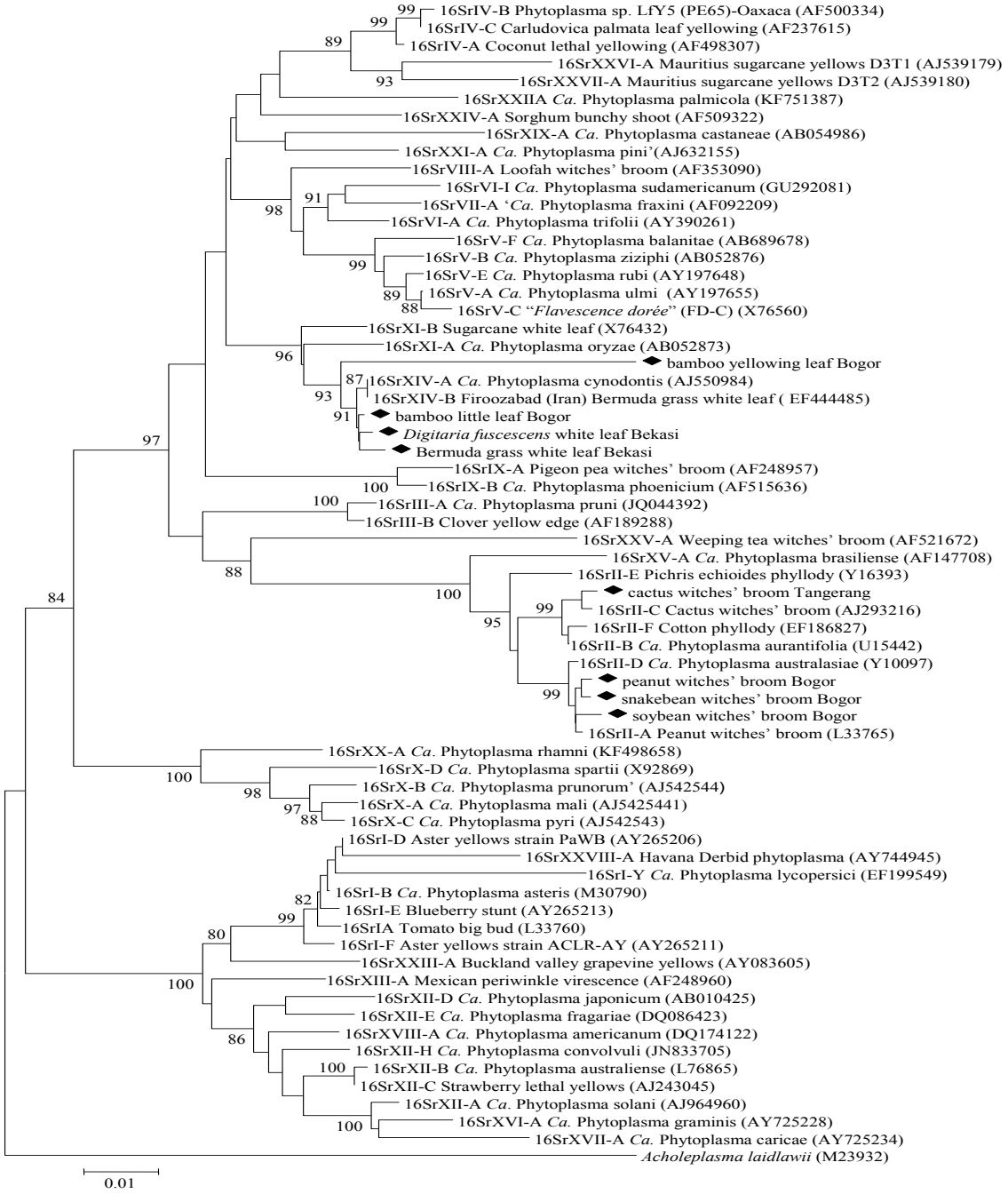

Figure 6 Phylogenetic tree constructed using the neighbour-joining method from 16S rRNA sequence of peanut witches' broom Bogor, soybean witches' broom Bogor, snakebean witches' broom Bogor, cactus witches' broom Tangerang, bamboo yellowing leaf Bogor, Bambu little leaf Bogor, Bermuda grass white leaf Bekasi, and *D. fuscescens* white leaf Bekasi, and selected reference phytoplasma subgroups. *Acholeplasma laidlawii* is the outgroup to root the tree.

```
BBYL       GAAACGACTGCTAAGACTGGATAGGAAATTAGAAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGTATACTTAAAGAGGGGCTTGCGGCACATT 100
16SRrXIV-A GAAACAGTTGCTAAGACTGGATAGGAAATTAGAAGGCATCTTTTAATTTTTAAAAGACCTTTTTCGAAAGGTATACTTAAAGAGGGGCTTGCGGCACATT 100
           *****   ********************************************************************************************

BBYL       AGTTAGTTGGTAGGGTAAAAGCCTACCAAGACCATGATGTGTAGCTGGACTGAGAGGTTGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC 200
16SRrXIV-A AGTTAGTTGGTAGGGTAAAAGCCTACCAAGACTATGATGTGTAGCTGGACTGAGAGGTTGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC 200
           ********************************** ********************************************************************

BBYL       GGGAGGCAGCAGTAGGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAACGCCGCGTGAACGATGAAGTATTTCGGTATGTAAAGTTCTTTTATTGA 300
16SRrXIV-A GGGAGGCAGCAGTAGGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAACGCCGCGTGAACGATGAAGTATTTCGGTATGTAAAGTTCTTTTATTGA 300
           ********************************************************************************************************

BBYL       AGAAGAAAAAATAGTGGAAAAACTATCTTGACGATATTCAATGAATAAGCCCCGGCAAACTATGTGCCAGCAGCCGTGGTAATACATAGGGGGCGAGCGT 400
16SRrXIV-A AGAAGAAAAAATAGTGGAAAAACTATCTTGACGATATTCAATGAATAAGCCCCGGCAAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCGAGCGT 400
           ********************************************************************************** ***********************

BBYL       TATCCGGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTTGGTAAGTCTATAGTTTAATTTCAGTGCTTAACACTGTTCTGCTATAGAAACTATCAGACT 500
16SRrXIV-A TATCCGGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTTGGTAAGTCTATAGTTTAATTTCAGTGCTTAACACTGTTCTGCTATAGAAACTATCAGACT 500
           ********************************************************************************************************

BBYL       AGAGTGAGATAGAGGCAAGTGGAATTCCATGTGTAGCGGTAAAATGCGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTTACTG 600
16SRrXIV-A AGAGTGAGATAGAGGCAAGTGGAATTCCATGTGTAGCGGTAAAATGCGTAAATATATGGAGGAACACCAGAGGCGTAGGCGGCTTGCTGGGTCTTTACTG 600
           ********************************************************************************************************

BBYL       ACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGGAAACTCGGTACTGA 700
16SRrXIV-A ACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAAGTGTCGGGGAAACTCGGTACTGA 700
           ********************************************************************************************************

BBYL       AGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAA 800
16SRrXIV-A AGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAA 800
           ********************************************************************************************************

BBYL       TTCGAAGATACACGAAAAACCTTACCAGGTCTTGACATACTCTGCAAAGCTATAGCAATATAGTGGAGGTTATCAGGGATACAGGTGGTGCATGGTTGTC 900
16SRrXIV-A TTCGAAGATACACGAAAAACCTTACCAGGTCTTGACATACTCTGCAAAGCTATAGCAATATAGTGGAGGTTACCAGGGATACAGGTGGTGCATGGTTGTC 900
           ************************************************************************** ***************************

BBYL       GTCAGCTCGTGTCGTGAGATGTTGGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCAT--TAAGTTGGGCACTCTAAGGTGACTGC 998
16SRrXIV-A GTCAGCTCGTGTCGTGAGATGTTAGG-TTAAGTCCTAAAACGAGCGCAACCCTTGTCACTAGTTGCCAGCATGTTATGATGGGCACTTTAGTGAGACTGC 999
           ********************** ** ********   *****************    ********* ***  ** * ******** **  * ******

BBYL       CGGTGACAAACCGGAAGGAAGGTGGGGGATGACGTCAATTCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGGTACAAAGAGCTG 1098
16SRrXIV-A CAATGAAAAATTGGA-GGAAGGTGAGG-ATCACGTCAAATCATCATGCCCCTTATGATCTGGGCTACAAACGTGATACAATGG-CTGTTACAAAGAGTAG 1096
           *  *** ***  *** ******** ** ** ******* ****************** ********** ***** ******** * * *********  *

BBYL       CAAGACCGCGAGGTGG-AGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGA 1197
16SRrXIV-A CTAAAACGCGAGTTTATAGCCAATCTCATAAAAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAA 1196
           * * * ****** *   *** ************ *  *************** ** ********** ** ******* ******************* *

BBYL       TCAGCATGCCGCGGTGAATACGTTCACGGGGTTTGTACACACCGCCCGTCA 1248
16SRrXIV-A TCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCA 1247
           ******** ***************** ************************
```

Figure 7 Clustal W alignment of 16S rRNA sequences of betung bamboo yellow leaf phytoplasma (BBYL) with 16SrXIV-A reference phytoplasmas (AJ550984) from Genbank

In silico RFLP analysis

The similarity coefficients derived from RFLP analysis were calculated on the basis of in silico restriction site analysis of nucleotide sequences of 16S rRNA genes. The analysis reached the similarity coefficient of 0.98 - 1.00, except in bamboo yellowing leaf, where phytoplasma only had the highest similarity coefficient of 0.78 with 16SrXIV group (data not shown). The phytoplasma strains within a species should share at least 97.5 % sequence identity within the 16S rRNA gene. Conversely, some organisms, despite their 16S rRNA gene sequence being < 97.5 % similar to that of any other '*Ca*. Phytoplasma' species, are not presently described as *Candidatus* species, due to their poor overall characterization [11]. According to Arocha *et al*. [29], silico RFLP analysis has been used as a powerful tool for groups and subgroups phytoplasma identification. The silico RFLP analysis revealed the presence of a single phytoplasma group to each plant species tested. The molecular identification and characterization of a possible agent of phytoplasmal symptoms should be a suitable tool to evaluate the genetic diversity of phytoplasmas not only in Indonesia but also worldwide.

Conclusions

On the basis of this study, it is concluded that the symptoms observed in plant samples collected and reported here are due to the association of phytoplasma. The evidence on the presence of a phytoplasma as the product of the expected size was amplified using phytoplasma 16S rRNA primers in nested-PCR. The subgroup phytoplasma 16SrII-A (peanut witches' broom phytoplasma) is known to be associated with witches' broom disease of peanut, soybean, and snakebean. On the other hand, the proliferation and mosaics of *Opuntia* sp. are caused by the subgroup phytoplasma 16SrII-C. Yellowing and little leaf on bamboos, and also white leaf diseases on bermuda grass and digitaria grass are due to the association of subgroup phytoplasma 16SrXIV-A (*Ca.* Phytoplasma cynodontis).

Acknowledgements

Further thanks to the Ministry of Agriculture BPPSDMP on scholarship study at the Bogor Agricultural University.

References

[1] IM Lee, RE Davis and DE Gundersen-Rindal. Phytoplasma: Phytopathogenic mollicute. *Annu. Rev. Microbiol*. 2000; **54**, 221-55.
[2] M Iwaki, M Roechan, N Saleh, M Sugiura and H Hibino. Identity of mycoplasma-like agents of legume witches' broom in Indonesia. *Contr. Cent. Res. Inst. Agric. Bogor*. 1978; **41**, 1-11.
[3] S Warokka, P Jones and M Dickinson. Detection of phytoplasmas asociated with Kalimantan wilt disease of coconut by the polymerase chain reaction. *Jurnal Littri*. 2006; **12**, 154-60.
[4] KH Mutaqin. 2000, Detection and Comparison of Phytoplasma from Bermuda Grass (*Cynodon* Dactylon (L.) Press.) and Other Hosts using the PCR-RFLP Technique and Its Transmission with Leafhopper. Thesis, Bogor Agricultural University, Bogor, Indonesia.
[5] KH Mutaqin, R Suseno, B Tjahjono and P Hidayat. Molecular detection and transmission studies of phytoplasma originated from Bermuda Grass. *Hayati* 2003; **10**, 66-70.
[6] R Harling, Y Arocha, V Harju, C Tobing, E Boa, P Kelly and R Reeder. First report of 16SrII '*Candidatus* Phytoplasma aurantifolia' infecting chilli and tamarillo in Indonesia. *Plant Pathol*. 2009; **58**, 791.
[7] E Boa, Y Arocha, R Harling, C Tobing, P Kelly and R Reeder. First report of group 16SrI '*Candidatus* Phytoplasma asteris' associated with *Mimosa pudica* yellows in Indonesia. *Plant Pathol*. 2010; **59**, 397.
[8] LN Chiykowski and RC Sinha. Differentiation of MLO disease by means of symptomatology and vector transmission. *Zentralbl. Bakteriol. Hyg. Suppl.* 1989; **20**, 280-7.
[9] J Franova, K Petrzik, F Paprstein, J Kucerova, M Navratil, P Valova, J Nebesarova and H Jakesova. Experiences with phytoplasma detection and identification by different methods. *Bull. Insectol*. 2007; **60**, 247-8.
[10] E Seemuller, C Marcone, U Lauer, A Ragozzino and M Goesch. Current status of molecular classification of the phytoplasmas. *Plant Pathol*. 1998; **80**, 3-26.
[11] IRCPM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *Int. J. Syst. Evol. Microbiol*. 2004; **54**, 1243-55.
[12] W Wei, RE Davis, IM Lee and Y Zhao. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *Int. J. Syst. Evol. Microbiol*. 2007; **57**, 1855-67.
[13] S Deng and C Hiruki. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *J. Microbiol. Meth*. 1991; **14**, 53-61.
[14] B Schneider, E Seemuller, CD Smart and BC Kirkpatrick. *Phylogenetic Classification and Pathogenic Mycoplasma-like Organisms or Phytoplasmas*. *In*: S Razin and JG Tully (Eds.). Molecular and Diagnostic Procedures in Mycoplasmology. Academic Press, London, UK, 1995, p. 369-80.

[15] DE Gundersen and IM Lee. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathol. Mediterr*. 1996; **35**, 144-51.
[16] YH Dewir. Cacti and succulent plant species as phytoplasma hosts: A review. *Phytopathogenic Mollicutes* 2016; **6**, 1-9.
[17] C Marcone. *Movement of Phytoplasmas and the Development of Disease in the Plant*. *In*: PG Weintraub and P Jones (Eds.). Phytoplasmas: Genomes, Plant Hosts and Vectors. CAB International, Wallingford, UK, 2010, p. 114-31.
[18] GP Rao, SK Raj, SK Nehi, S Mall, M Singh and C Marcone. Molecular evidence for the presence of '*Candidatus* Phytoplasma cynodontis' the Bermuda grass white leaf agent, in India. *Bull. Insectol.* 2007; **60**, 145-6.
[19] M Salehi, K Izadpanah, M Siampour and M Taghizadeh. Molecular characterization and transmission of Bermuda grass whıte leaf phytoplasma in Iran. *J. Plant Pathol*. 2009; **91**, 655-61.
[20] AF Omar. Association of '*Candidatus* Phytoplasma cynodontis' with Bermuda grass white leaf disease and its new hosts in Qassim Province, Saudi Arabia. *J. Plant Protect*. 2016; **11**, 101-7.
[21] IM Lee, M Pastore, M Vibio, A Danielli, S Attathom, RE Davis and A Bertaccini. Detection and characterization of a phytoplasma associated with annual blue grass (*Poa annua*) white leaf disease in southern Italy. *Eur. J. Plant Pathol*. 1997; **103**, 251-4.
[22] NKK Win and HY Jung. '*Candidatus* Phytoplasma cynodontis' associates with white leaf disease of golden beard grass (*Chrysopogon acicalatus*). *Trop. Plant Pathol*. 2012; **37**, 76-9.
[23] GP Rao, S Mall and C Marcone. '*Candidatus* Phytoplasma cynodontis' (16SrXIV group) affecting *Oplismenus burmannii* (Retz.) P. Beauv. and *Digitaria sanguinalis* (L.) Scop. in India. *Australas. Plant Dis. Notes* 2010; **5**, 93-5.
[24] OJ Adam, CAO Midega, S Runo and ZR Khan. Molecular determination and characterization of phytoplasma 16S rRNA gene in selected wild grasses from Western Kenya. *J. Plant Pathol. Microb*. 2015; **6**, 1-8.
[25] N Nejat, K Sijam, SNA Abdullah, G Vadamalai and M Dickinson. First report of a 16Sr XIV '*Candidatus* Phytoplasma cynodontis' group phytoplasma associated with coconut yellow decline in Malaysia. *Plant Pathol.* 2009; **58**, 389.
[26] N Naderali, N Nejat, G Vadamalai and YH Tan. First report of two distinct phytoplasma species, '*Candidatus* Phytoplasma cynodontis' and '*Candidatus* Phytoplasma asteris', simultaneously associated with yellow decline of *Wodyetia bifurcata* (foxtail palm) in Malaysia. *Plant Dis*. 2013; **97**, 1504.
[27] IM Lee, DE Gundersen-Rindal, RE Davis and IM Bartoszyk. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol*. 1998; **48**, 1153-69.
[28] C Marcone, B Schneider and E Seemüller. '*Candidatus* Phytoplasma cynodontis', the phytoplasma associated with Bermuda grass white leaf disease. *Int. J. Syst. Evol. Microbiol*. 2004; **54**, 1077-82.
[29] Y Arocha, M Lopez, B Pinol, M Fernandez, B Picornell, R Almeida, I Palenzuela, MR Wilson and P Jones. *'Candidatus* Phytoplasma graminis' and '*Candidatus* Phytoplasma caricae', two novel phytoplasmas associated with diseases of sugarcane, weeds and papaya in Cuba. *Int. J. Syst. Evol. Microbiol*. 2005; **55**, 2451-63.