

Full Length Research Paper

Molecular characterization of an aster yellows phytoplasma associated with proliferation of periwinkle in Malaysia

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Madagascar periwinkle is a common decorative, easy growing and spreading perennial herb. Phyllody, virescence, proliferation, little leaf and yellowing symptoms were observed on periwinkle in Serdang and Banting, Selangor, Malaysia. Polymerase chain reaction (PCR) assays using P1/P7 universal phytoplasma primers and *secA* primers designed for identification and classification of phytoplasma, amplified 1.8 kb fragment that encompasses the entire 16S rRNA gene, the 16S - 23S intergenic spacer region and the beginning of the 23S rRNA gene as well as the 840 bp from part of *secA* gene, respectively. Sequence analysis of the 16S rDNA, 16S - 23S intergenic region and the beginning of the 23S rRNA and *secA* gene PCR products determined that the phytoplasma strain associated with periwinkle proliferation in Malaysia belongs to the '*Candidatus* Phytoplasma asteries' (16Sr I-B) group of phytoplasmas. The virtual restriction fragment length polymorphism (RFLP) analysis with 10 restriction endonuclease enzymes revealed identical patterns to phytoplasmas members of Aster yellows phytoplasma subgroup B. A phylogenetic tree based on 16S rDNA sequences, *secA* gene sequences and virtual RFLP revealed that the periwinkle proliferation phytoplasma is closely related to the subgroup 16SrI-B. Periwinkle proliferation also confirmed which 16SrI-B has wide geographical distribution and host range.

Key words: Periwinkle, aster yellows phytoplasma, polymerase chain reaction, cloning, virtual restriction fragment length polymorphism.

INTRODUCTION

Phytoplasmas are the smallest wall-less prokaryote known plant-pathogenic bacteria associated with diseases in numerous plant species worldwide (Lee et al., 2000).

Yellowing, decline, witches broom, leaf curl, floral virescence and phyllody are the most conspicuous symptoms associated with phytoplasmas, although sometimes infections are asymptomatic. Since phytoplasma cannot be cultured *in vitro*, molecular techniques must be used for their diagnosis and characterization.

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Diagnosis of phytoplasmas is routinely done by polymerase chain reaction (PCR) and can be divided into three phases: total DNA extraction from symptomatic tissue, PCR amplification of phytoplasma-specific DNA and characterization of the amplified DNA by restriction fragment length polymorphism (RFLP) analysis or nested PCR with group-specific primers (Marzachi, 2004).

Abbreviations: PCR, Polymerase chain reaction; RFLP, restriction fragment length polymorphism; UPM, University Putra Malaysia; LB, Luria- Bertani; BLAST, basic local alignment search tool; NCBI, national centre for biotechnological information; PP, periwinkle proliferation; AY, aster yellows.

Based on the computer-simulated RFLP analysis of 16S rRNA gene, all available phytoplasma sequence accessions were classified into 28 16Sr RFLP groups and around 100 subgroups (Wei et al., 2007).

The phylogenetic analysis of *Candidatus* phytoplasma asteries (16SrI) phytoplasmas were carried out based on 16S rDNA, *tuf* gene sequence and *rp* operon (*rpI22* and *rps3* genes) (Lee et al., 2004a). The 16Sr I aster yellows (AR) phytoplasma group with more than 103 phytoplasmas is the largest subclade (Seemüller et al., 1998) and has been separated into 6 subgroups (I-A through I-F) based on 16S rDNA RFLP or putative restriction site analysis (Wei et al., 2007). The number of phytoplasmas placed in various subgroups varies from only one (I-D, I-E, I-F) to several or many; I-B being by far the largest subgroup (Gundersen et al., 1996; Marcone et al., 2000).

Periwinkle (*Catharanthus roseus* (L.) G. Don) is also called vinca or myrtle. *C. roseus* is native to the Indian Ocean island of Madagascar. This herb is now common in many tropical and subtropical regions worldwide. Madagascar periwinkle is a common decorative, easy growing and spreading perennial herb. As many other plants of the dogbane family, the Vincas are poisonous, although mildly. During the 1950s, *C. roseus* was discovered to contain a number of chemicals in the alkaloid class. There are also 2 drugs extracted from it; vinblastine (used in the treatment of Hodgkins' disease) and vincristine (used in the treatment of leukemia) (Duke, 1985).

C. roseus (periwinkle) is a valuable experimental host. It is used as an assay host for phytoplasmas by grafting, dodder or vector transmissions, and they tend to induce specific symptoms (Marwitz, 1990).

Periwinkles are known to be susceptible to the aster yellows (16SrI) group phytoplasma in Argentina (Torres et al., 2004), and have also been found to be naturally infected with spirea stunt (16SrIII-E), peach yellow leaf roll (16SrIII-A), clover proliferation (16SrVI), potato witches' broom (16SrVI-A) (Lee et al., 1998) and Mexican periwinkle virescence (16SrXIII) (Gundersen et al., 1994).

The phytoplasma associated with periwinkle has been previously cited based on dot blot hybridization which belongs to 16Sr I AY group (Khew et al., 1991). However, it has not been sufficiently characterized to enable its assignment to one of the established subgroups (Seemüller et al., 1998). The aim of the present study is to characterize the phytoplasma at the molecular level. Molecular characterization of the 16S rRNA gene plus spacer region and first part of 23S gene, *secA* gene and virtual RFLP were carried out to study the phytoplasma at the molecular level.

MATERIALS AND METHODS

Plant sample collection

Samples of leaf tissue were collected from naturally infected

periwinkle showing phyllody, virescence, proliferation and chlorosis symptoms in Serdang and Banting of Selangor State, Malaysia.

Samples were also collected from symptomless periwinkles. In addition, healthy samples which were grown from seed were collected as a control from glass house at University Putra Malaysia (UPM).

Transmission by grafting

Tests for transmission by grafting were conducted using infected periwinkle plants showing symptoms of phyllody, virescence, proliferation, little leaf and flower abortion. Scions from naturally infected periwinkle were grafted into 7 healthy periwinkles by side grafting. The graft-inoculated area in which the diseased scion was inserted was then bound tightly with plastic tape and was covered with plastic bag for 1 week to minimize dehydration. The grafted plants were then kept in the greenhouse at about 30°C at UPM until symptoms appeared.

Total nucleic acid extractions from plant tissue

Total nucleic acids were extracted using the small scale DNA extraction method of Zhang et al. (1998). Approximately, 0.15 g of fresh tissues were frozen in liquid nitrogen and directly ground to fine powder in a cold mortar. The powder was transferred into a 1.5 ml Eppendorf tube and 800 µl of cetyl trimethyl ammonium bromide (CTAB) buffer was added; preheated at 60°C and incubated at 60°C for 20 min in a water bath shaker. After the incubation, the lysate was cooled to room temperature. Then, extraction was carried out with two-third volume of chloroform-isoamylalcohol at 24:1 ratio and mixture was vortexed vigorously and then centrifuged at 13000 g for 8 min in a Hettich centrifuge model Mikro200. The aqueous phase was collected and transferred to a clean tube and equal volume of -20°C isopropanol was added to precipitate the nucleic acids and then, inverted to mix gently. The mixture was chilled at -20°C for 15 min and then centrifuged at 13000 g for 10 min to precipitate the nucleic acid. Pellets were each rinsed in 70% -20°C ethanol by inverting the tube several times gently and allowed to air-dry. Each pellet was dissolved in 100 µl ultrapure sterile water and stored at -20°C.

PCR analysis

DNA extracts derived from symptomatic and asymptomatic periwinkle were amplified using direct PCR. DNA extracts were analysed initially by direct PCR using the phytoplasma universal primer pairs P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3') (Deng and Hiruki, 1991)/P7 (5'-CGT CCT TCA TCG GCT CTT-3') (Schneider et al., 1995b) derived from the conserved regions of the 16S ribosomal sequence used to amplify phytoplasma ribosomal DNA. Universal primer pair P1/P7 was used to amplify a 1.8 kb fragment that prime the entire 16S rRNA gene, the 16S-23S rDNA intergenic spacer region, tRNA-Ile and the beginning of the 23S rRNA gene. SecAfor1 (5'-GAR ATG AAA ACT GGR GAA GG -3')/SecArev3 (5'-GTT TTR GCA GTT CCT GTC ATN CC-3') (Hodgetts et al., 2008) were used to amplify *secA* gene.

PCR assay was performed in 20 µl volumes. Each PCR reaction mixture contained 1 µl of DNA template, 1 µl of each primer (10 pmols each), 0.4 µl of 10 mM dNTP, 0.6 µl of 25 mM MgCl₂, 2 µl of 10X Taq polymerase buffer and 0.3 µl (0.5 units) Taq DNA polymerase (Fermentas, Inc).

The amplification using P1/P7 primers was performed in a thermocycler (iCycler, Bio Rad) under the following conditions: 1 min (2 min for an initial denaturation step) of denaturation at 94°C, annealing for 2 min at 55°C, extension for 3 min at 72°C for 35

cycles and a final extension at 72°C for 10 min (Lee et al., 1993). PCR conditions for PCR with primer pair SecAfor1/SecArev3 were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 1 min and 30 s and a final extension step of 72°C for 15 min (Hodgetts et al., 2008). Aliquots of 7 µl of each final reaction mixture were electrophoresed through 1% agarose gels in 1 x TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0) as running buffer. DNA products in gels were stained with ethidium bromide (EtBr), visualized by UV transillumination and photographed.

Cloning of PCR products

P1/P7 primed rDNA and SecAfor1/SecArev3 primed *secA* gene products amplified from periwinkle from Serdang, Malaysia were purified from agarose gels using QIAquick gel extraction kit (QIAGEN). The products were ligated into pCR 2.1 vector and 2 µl of the ligation mixture transformed into TOP10 *Escherichia coli* competent cells by heat shock following the manufacturers instructions (Invitrogen, Carlsbad, CA, USA). The products were plated on Luria-Bertani (LB) medium containing kanamycin (5 mg/ml) and 5-bromo-4-chloro-3-indolyl β-D- galactopyranoside (X-Gal) and incubated overnight at 37°C. White colonies were grown in liquid LB containing kanamycin. For sequencing, plasmid DNAs were isolated from transformed bacterial colonies using the QIAprep spin Miniprep kit (QIAGEN, Hilden, Germany). Plasmid DNA was digested with *EcoRI* to confirm the presence of insert DNA and sequences were processed on both strands using automated DNA sequencing machine.

Sequence analysis

The sequence editing was carried out using online analysis tools at <http://molbiol-tools.ca/>. The phylogenetic analysis, nearly full-length 16S rDNA, 16S - 23S intergenic spacer region and *secA* gene sequences isolated from periwinkle phytoplasma and other known phytoplasmas obtained from the Basic local alignment search tool (BLAST) search (Altschul et al., 1990) of the National Center for Biotechnological Information (NCBI) web site at <http://www.ncbi.nlm.nih.gov/> and *Acholeplasma laidlawii* (Tables 1 and 2) as an outgroup species was performed using the program CLUSTAL W (Version 3.1) (Thompson et al., 1994). Phylogenetic trees were constructed by Neighbor-joining method with default values with MEGA version 3.1 software (Kumar et al., 2004) bootstrap values illustrated on the phylogenetic dendrogram and were estimated by the bootstrap sampling method (1000 replicates).

In silico restriction enzyme digestion and virtual gel plotting

Phytoplasma rDNA sequence derived from diseased periwinkle in Malaysia and type strain phytoplasmas from each subgroups include AY witches broom phytoplasma *rrnA* I-A, *Candidatus* phytoplasma asteries I-B, clover phyllody phytoplasma strain CPh I-C, AY phytoplasma strain PaWB I-D, blueberry stunt phytoplasma strain BBS3 I-E and AY phytoplasma strain ACLR-AY I-F (Table 1) as described by Wei et al. (2007) from NCBI were exported to the *in silico* restriction analysis and virtual gel plotting using the pDRAW32 programme developed by AcaClone Software (<http://www.acaclone.com>) as described by Wei et al. (2007) to determine genetic relatedness. Each rDNA sequence was analysed in detail *in silico* by separate digestion with restriction endonucleases *AluI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *MboI*, *MseI*, *RsaI* and *TaqI*. These enzymes were chosen from endonucleases list previously described by Lee et al. (1993b), in phytoplasma classification. After *in silico* restriction digestion, a virtual 4.0% agarose gel electrophoresis image was plotted automatically.

RESULTS

Disease symptoms

A wide range of symptoms were remarked on naturally infected periwinkle. The main symptoms were observed as virescence, phyllody, proliferation, chlorosis, witches broom and noticeably reduction in leaves sizes (Figure 1).

Transmission by grafting

Results of the tests for transmission by grafting indicated that the disease is contagious. The periwinkles grafted with buds from diseased periwinkles, showed phyllody, virescence, proliferation, noticeably reduced leaf size and diminished plant development. The periwinkle plants grafted with buds from healthy periwinkles had no symptoms.

PCR amplification of phytoplasma DNA by universal and *secA* primers

The amplification products of the DNA from infected periwinkles using universal primer set P1/P7 and *secA* primers *secAfor1/secArev3* were the expected size of about 1.8 kb and 830 bp, respectively. No product was amplified from the healthy periwinkles (Figures 2 and 3).

Cloning and sequencing of 1800 and 830 bp fragment

P1/P7 and *secAfor1/rev3* products cloned from periwinkle were compared with the 16S rRNA and *secA* genes sequences in the GenBank database. It revealed a 99% similarity between the sequence of cloned P1/P7 products and 16Srl group phytoplasmas and 99% similarity between the sequence of cloned *secAfor1/Rev3* product and onion yellows phytoplasma, OY-M from I-B.

Phylogenetic analysis

Phylogenetic distance trees were constructed from a data set which included 16S rDNA and *secA* gene sequences of periwinkle proliferation phytoplasma and comparable 16S rDNA sequences of 35 additional strains representing 28 phytoplasma groups and *secA* gene partial sequences of 11 phytoplasma strains from different groups, respectively (Figures 4 and 5).

Periwinkle proliferation (PP) phytoplasma clustered closely with other known aster yellows (16Srl) group strains and verified that PP phytoplasma was evolutionarily closest to subgroup 16Srl-B strains.

Restriction analysis

16SrDNA sequences of PP phytoplasma and type strain

Table 1. Phytoplasma 16S rDNA sequences used as references in this study.

Acronym	Phytoplasma or association disease	16S rRNA group	GenBank accession no.	Reference
AYWB I-A	Aster yellows witchesbroom phytoplasma <i>rrnA</i> I-A	16SrI	NC_007716	Bai et al., 2006
Ca. P. asteris I-B	<i>Candidatus</i> Phytoplasma asteris I-B	16SrI	M30790	Lee et al., 2004a
CP I-C	Clover phyllody phytoplasma strain CPh I-C	16SrI	AF222065	2000 (GenBank submission)
AY I-D	Aster yellows phytoplasma strain PaWB I-D	16SrI	AY265206	2003 (GenBank submission)
Bbs I-E	Blueberry stunt phytoplasma strain BBS3 I-E	16SrI	AY265213	2003 (GenBank submission)
AY I-F	Aster yellows phytoplasma strain ACLR-AY I-F	16SrI	AY265211	2003 (GenBank submission)
PP	Periwinkle proliferation phytoplasma	16SrI	FJ008869	Nejat et al., 2009
Ca. P. aurantifolia	<i>Candidatus</i> Phytoplasma aurantifolia	16SrII	U15442	Zreik et al., 1995
WX	Western X	16SrIII	L04682	1999 (GenBank submission)
CLY	Coconut lethal yellowing phytoplasma	16SrIV	AF498307	Harrison et al., 2002a
Ca. P. ulmi	<i>Candidatus</i> Phytoplasma ulmi	16SrV	AY197655	Lee et al., 2004b
Ca. P. trifolii	<i>Candidatus</i> Phytoplasma trifolii	16SrVI	AY390261	Hiruki & Wang, 2004
Ca. P. fraxini	<i>Candidatus</i> Phytoplasma fraxini	16SVII	AF092209	Griffiths et al., 1999
LWB	Loofah witches- broom phytoplasma	16SrVIII	AF353090	2001 (GenBank submission)
Ca. P. phoenicium	<i>Candidatus</i> Phytoplasma Phoenicium	16SrIX 16SrX	AF515636	Verdin et al., 2003
Ca. P. mali	<i>Candidatus</i> Phytoplasma mali	16SrXI	AJ542541	Seemüller & Schneider, 2004
Ca. P. oryzae	<i>Candidatus</i> Phytoplasma oryzae	16SrXII	AB052873	Jung et al., 2003
Ca. P. australiense	<i>Candidatus</i> Phytoplasma australiense	16SrXII	L76865	Davis et al., 1997
Ca. P. japonicum	<i>Candidatus</i> Phytoplasma japonicum	16SrXIII	AB010425	Sawayanagi et al., 1999
MPV	Mexican periwinkle virescence	16SrXIV	AF248960	2000 (GenBank submission)
Ca. P. cynodontis	<i>Candidatus</i> Phytoplasma cynodontis	16SrXV	AJ550984	Marcone et al., 2004b
Ca. P. brasiliense	<i>Candidatus</i> Phytoplasma brasiliense	16SrXVI	AF147708	Montano et al., 2001
Ca. P. graminis	<i>Candidatus</i> phytoplasma graminis	16SrXVII	AY725228	Arocha et al., 2005
Ca. P. Caricae	<i>Candidatus</i> Phytoplasma caricae	16SrXVIII	AY725234	Arocha et al., 2005
Ca. P. Americanum	<i>Candidatus</i> Phytoplasma americanum	16SrXIX	DQ174122	Lee et al., 2006
Ca. P. castaneae	<i>Candidatus</i> Phytoplasma castaneae	16SrXX	AB054986	Jung et al., 2002
Ca. P. rhamni	<i>Candidatus</i> Phytoplasma rhamni	16SrXXI	X76431	Marcone et al., 2004a
Ca. P. pini	<i>Candidatus</i> Phytoplasma pini	16SrXXII	AJ632155	Schneider et al., 2005
Ph.sp	Phytoplasma sp. strain LDN	16SrXXIII	Y14175	Tymon et al., 1998
BVGYP	Buckland valley grapevine yellows phytoplasma	16SrXXIV	AY083605	Constable et al., 2002
SBSP		16SrI	AF509322	Blanche et al., 2003
WTWBP	Weeping tea witches-broom phytoplasma	16SrXXV	AF521672	2002 (GenBank submission)
ScP1	Sugar cane phytoplasma D3T1	16SrXXVI	AJ539179	2003 (GenBank submission)
ScP2	Sugar cane phytoplasma D3T2	16SrXXVII	AJ539180	2003 (GenBank submission)
DP	Derbid phytoplasma	16SrXXVIII	AY744945	2004 (GenBank submission)
<i>A. laidlawii</i>	<i>Acholeplasma laidlawii</i>	-	M23932	Weisburg et al., 1989

of known aster yellows (16SrI) subgroup phytoplasmas were separately digested with 10 endonuclease and then compared. Digestion with the 10 restriction enzymes

showed the same restriction profile for PP and *Candidatus* phytoplasma asteris from 16SrI-B. However, 3 enzymes profile were different and were revealed among PP and

Table 2. Phytoplasma *secA* sequences used as references in this study

Acronym	Phytoplasma or association disease	<i>secA</i> group	GenBank accession no.	Reference
AYWB	Aster yellows witches'-broom phytoplasma AYWB	I-A	CP000061	Bai et al., 2006
OY-M	Onion yellows phytoplasma OY-M	I-B	AP006628	Oshima et al., 2004
OY	Onion yellows phytoplasma	I-B	AB056578	Kakizawa et al., 2001
PWB	Paulownia witches'-broom phytoplasma strain YL07	I-B	EU665493	(GenBank submission)
AAY	Atypical aster yellows phytoplasma	I-B	EU168720	Hodgetts et al., 2008
CAY	Cactus aster yellows phytoplasma	I-B	EU168721	Hodgetts et al., 2008
PP	Periwinkle proliferation phytoplasma	I-B	FJ176389	Nejat et al., 2009
CP	Clover phyllody phytoplasma	I-C	EU168722	Hodgetts et al., 2008
BcRD	Black currant reversion disease phytoplasma	I-C	EU168723	Hodgetts et al., 2008
ACLL	Apricot chlorotic leafroll phytoplasma	I-F	EU168724	Hodgetts et al., 2008
PPWB	Pigeon pea witches'-broom phytoplasma	IX	EU168746	Hodgetts et al., 2008
MPV	Mexican periwinkle virescence phytoplasma	XIII	EU168753	Hodgetts et al., 2008

**Figure 1.** Naturally infected (left), healthy (center) periwinkle and experimentally infected periwinkle by grafting (right).

phytoplasma strains from subgroups 16Srl-A, C, E and F. The PP phytoplasma differed from aster yellows witches'-broom phytoplasma *rrnA* from 16Srl-A in the *HhaI*, *MseI* and *MboI* profiles. Digests with *HaeIII*, *HhaI* and *MseI* produced different pattern between PP and clover phyllody phytoplasma strain CPh from subgroup 16Srl-C.

HhaI, *HinfI* and *TaqI* digests yielded profiles showing difference between PP and blueberry stunt phytoplasma strain BBS3 from 16Srl-E. In addition, digests with *AluI*, *HhaI* and *RsaI* showed different fragment patterns between PP and aster yellows phytoplasma strain ACLR from 16Srl-F. Furthermore, different fragment profile in 2

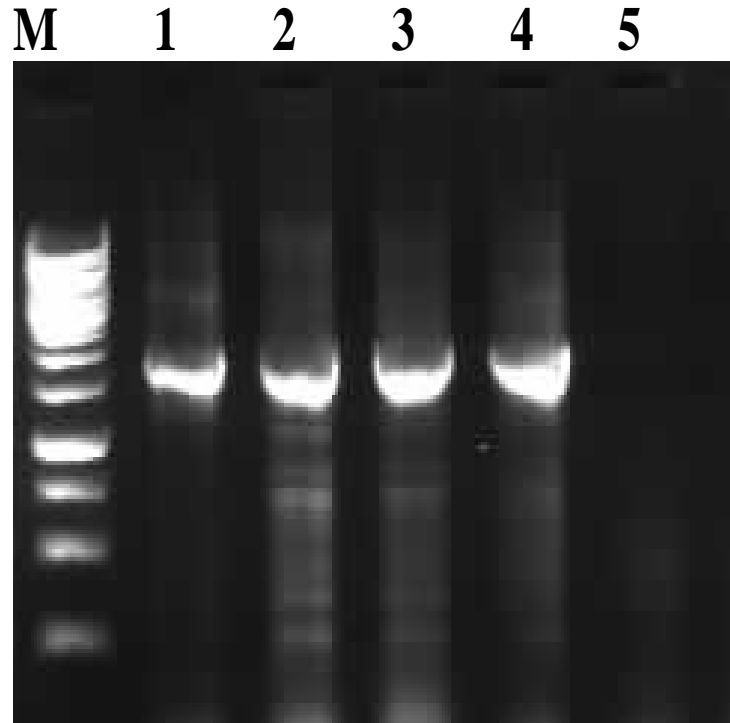


Figure 2. Polymerase chain reaction amplification of naturally infected periwinkle samples with universal primer pairs P1/P7. M: 1 kb ladder; lane 1 - 4: amplification of phytoplasma using P1/P7 primer set from Malaysia; lane 5: healthy periwinkle sample.

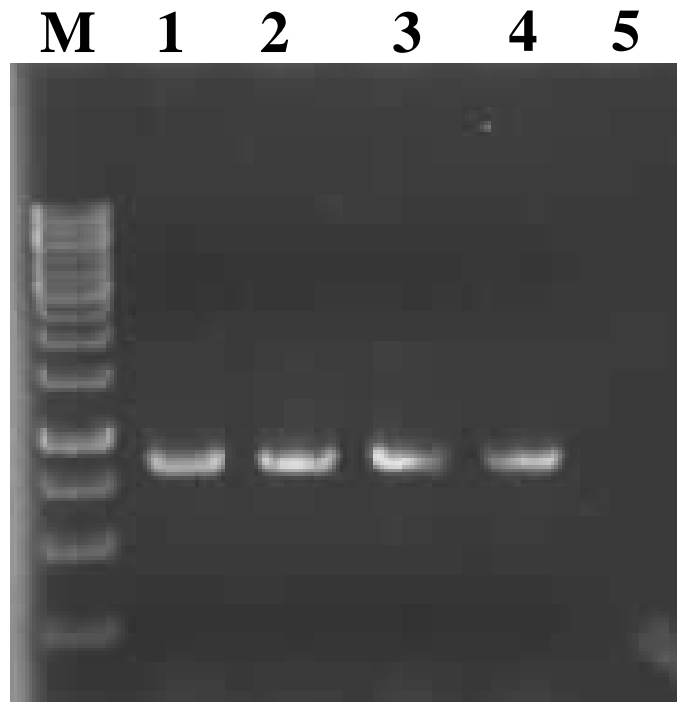


Figure 3. Polymerase chain reaction of naturally infected periwinkle samples with secAfor1/secArev3 primer pairs. M: 1 kb ladder; lane 1 - 4: amplification of phytoplasma secA gene; lane 5: healthy periwinkle sample.

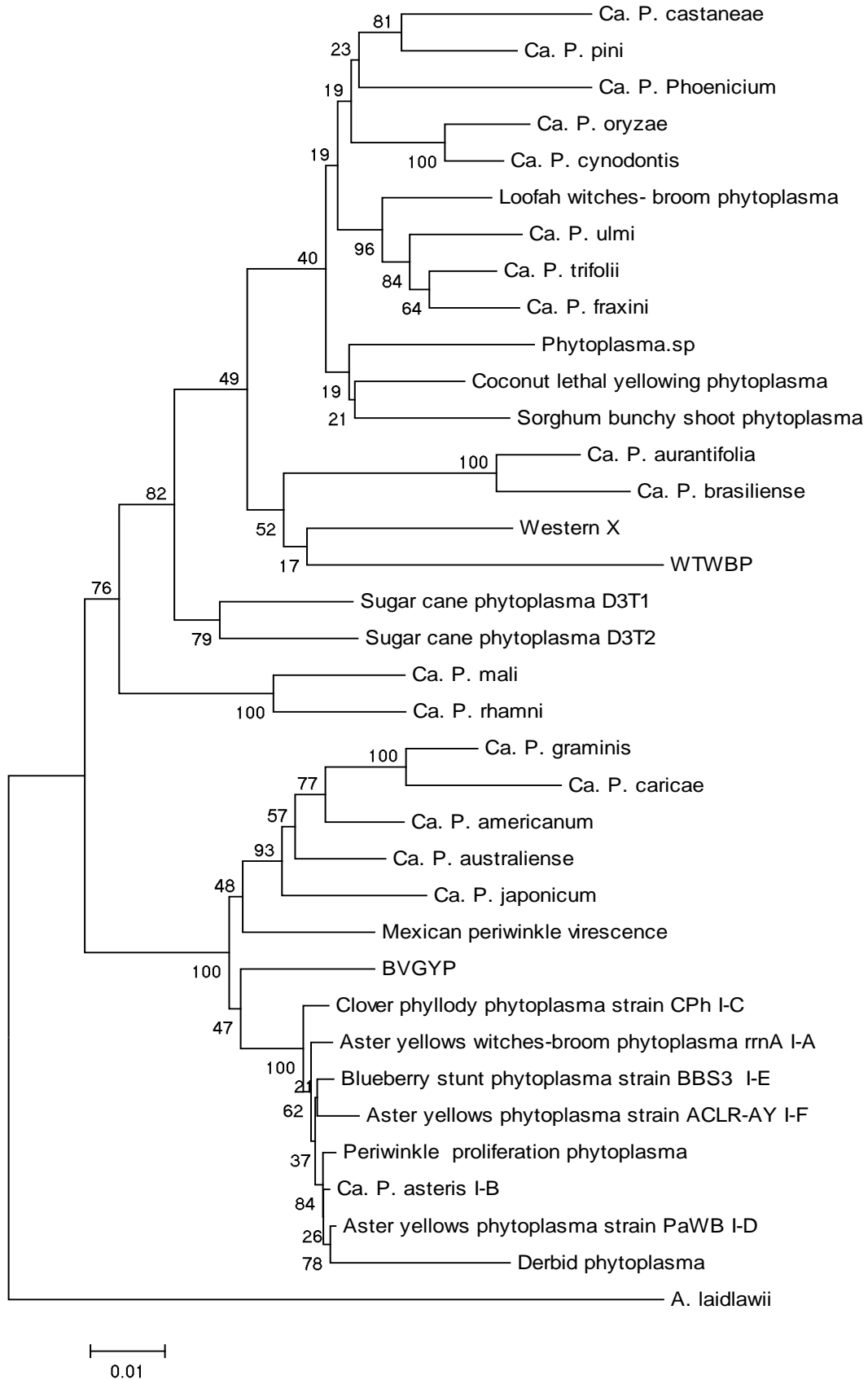


Figure 4. Phylogenetic tree, comparing the 16s rRNA gene sequences of PP phytoplasmas with 35 phytoplasmas and *A. laidlawii* as out group constructed by the Neighbor-Joining method.

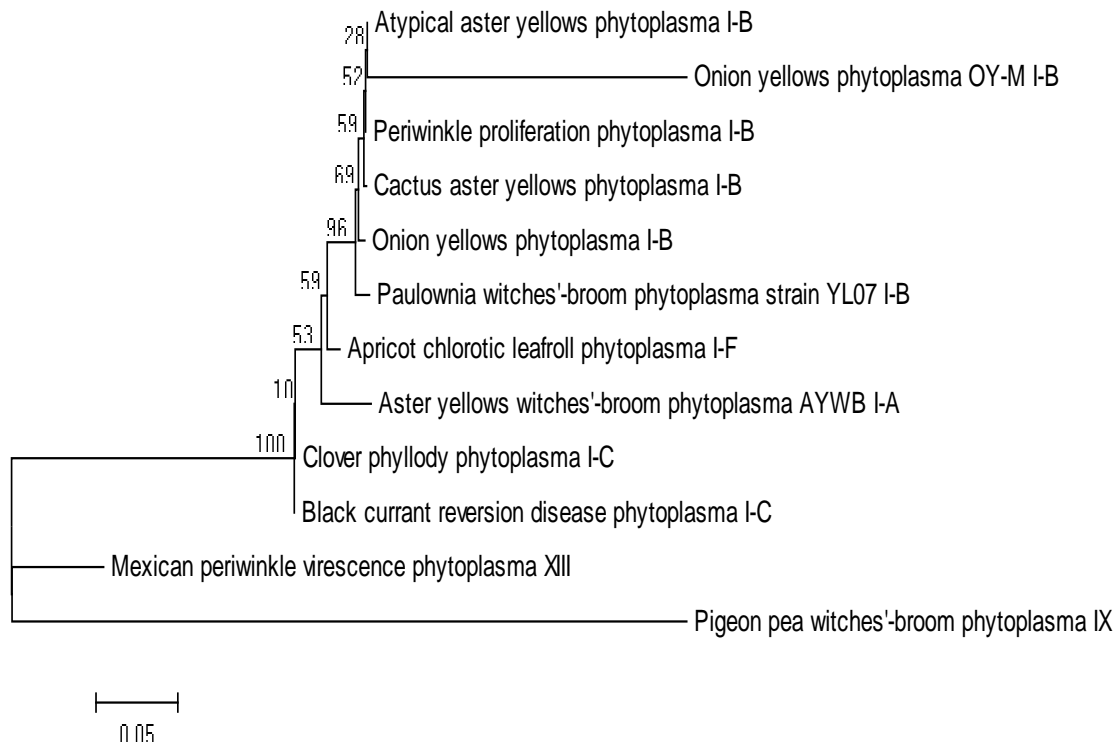


Figure 5. Phylogenetic tree, comparing the *secA* gene sequences of PP phytoplasmas with 11 phytoplasmas constructed by the Neighbor-Joining method.

enzymes (*AluI* and *TaqI*) was shown between PP and aster yellows phytoplasma strain PaWB from subgroup 16Srl-D (Figure 6).

DraI and *EcoRI* showed same fragment patterns among PP and all of the strains from different subgroups.

DISCUSSION

Periwinkle has been demonstrated to be a host for numerous phytoplasmas. Periwinkles are known to be susceptible to the aster yellows (16Srl) group phytoplasma in several countries. Aster yellows phytoplasma has been reported in Malaysia (Khew et al., 1991) and Argentina (Torres et al., 2004). American aster yellows (Lee et al., 1998) and periwinkle virescence (Lee et al., 1991) in North America; the *Catharanthus* virescence (Schneider et al., 1993) in South America; the Italian periwinkle witches'-broom in Europe and the *Catharanthus* virescence from Asia (Marccone et al., 2000) have been reported as members of the 16Srl group.

In the present study phytoplasmas were detected and characterized from naturally infected periwinkle plants grown in different locations in Selangor State, Malaysia. The collected periwinkles exhibited typical symptoms of phytoplasma such as phyllody and virescence.

The pathogenicity of the phytoplasma was confirmed by graft transmission, as they were transmitted from

diseased to healthy periwinkle plants by grafting and the symptoms of experimentally infected periwinkles appeared similar to naturally infected periwinkle.

The association of a phytoplasma with symptoms was also demonstrated through PCR amplification of the 16S rDNA and *secA* genes of periwinkle proliferation phytoplasma characteristic 1.8 kb and 830 bp, respectively. For accurate classification of a phytoplasma strain, it is preferable to sequence 16S rRNA genes. Also, virtual RFLP analysis could serve as a convenient and reliable alternative to conventional RFLP analysis (Wei et al., 2007). In RFLP analysis, a composite pattern is suspected when the sum of the sizes of DNA fragments is greater than the expected size of the F2nR2 region. Furthermore, RFLP analysis of sequence-heterogenous rRNA operons of phytoplasma in mutual isolation could result in erroneous assignment of the same phytoplasma to different 16S rRNA subgroups (Davis et al., 2003). The *secA* gene sequences amplified is offered a further approach to phytoplasma diagnostics and strain identification (Hodgetts et al., 2008).

A phylogenetic tree constructed based on 16S rDNA sequences, *secA* gene sequences and virtual RFLP has revealed the periwinkle proliferation phytoplasma in Malaysia associated with subgroup I-B of aster yellows phytoplasma group.

The aster yellows (AY) group is the largest group of the phytoplasmas (Marccone et al., 2000). The comprehensive

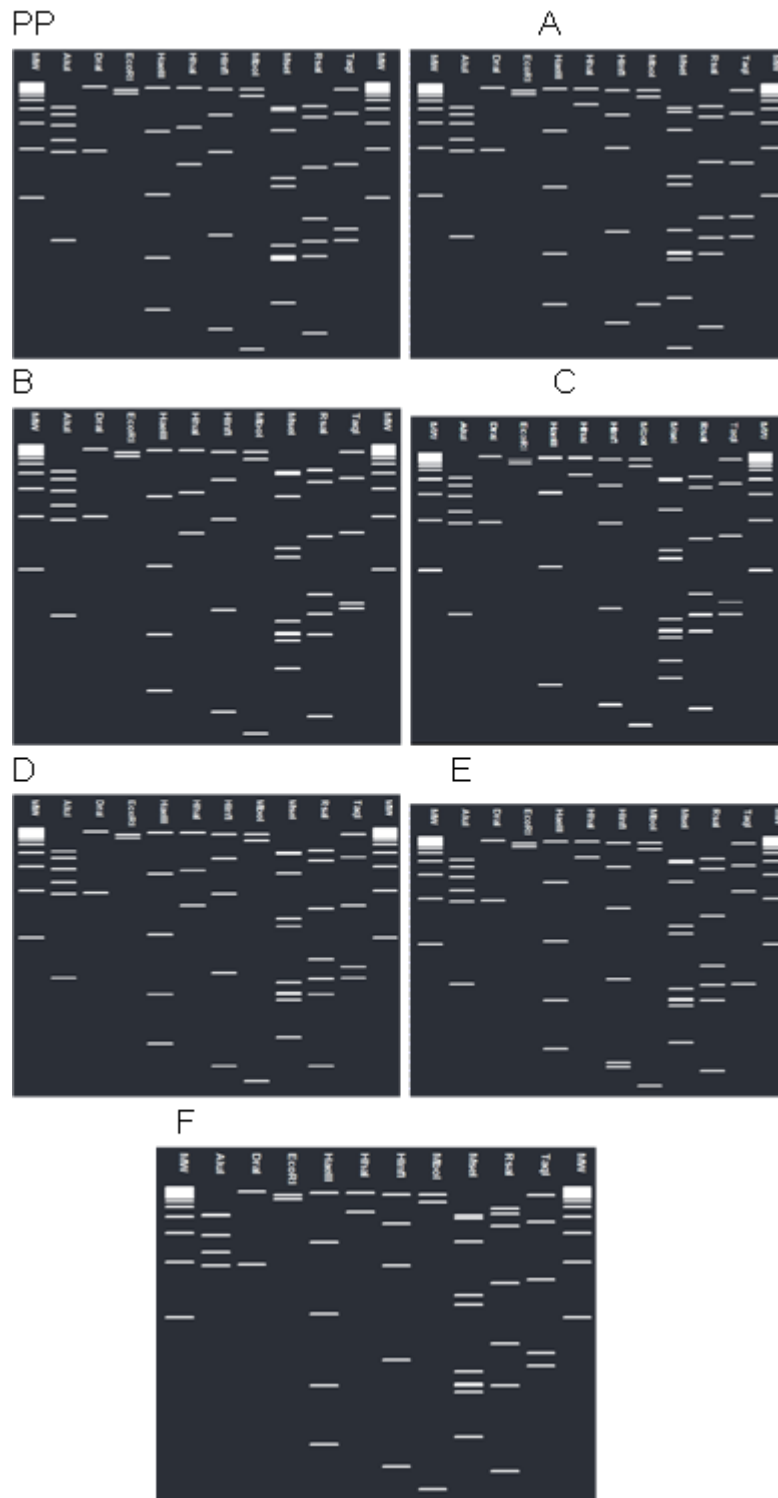


Figure 6. A computer-simulated virtual gel analysis of rDNA PCR products. Comparison of fragment patterns resulting from restriction endonuclease digestion of rDNA sequences from periwinkle proliferation phytoplasma infected periwinkle [PP] and aster yellows witchesbroom phytoplasma *rrmA* (A), *Candidatus* phytoplasma asteris (B), clover phyllody phytoplasma strain CPh (C), aster yellows phytoplasma strain PaWB (D), blueberry stunt phytoplasma strain BBS3 (E) and aster yellows phytoplasma strain ACLR-AY (F) with *AluI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *MboI*, *MseI*, *RsaI* and *TaqI* endonucleases. MW is Promega 100 bp step ladder.

classification of AY-group phytoplasmas appears that subgroup 16SrI-B is by far the most prevalent AY phytoplasma type occurring in America, Europe and Asia (Lee et al., 1992, 1993, 1998; Gundersen et al., 1996; Seemüller et al., 1998; Marcone et al., 2000). The obtained results proved that periwinkle proliferation phytoplasma in Malaysia associated with 16SrI-B group and this consequently demonstrates the wide geographical distribution of the 16SrI-B phytoplasmas.

The major sub groups (16SrI-A-B and -C) have a wide host range and may also occur in the same host. Subgroups 16SrI-A and B induce in periwinkle a wide variety of symptoms such as virescence, phyllody, small and faintly coloured flowers, flower malformations, shortening of internodes, elongation and etiolation of internodes, small and deformed leaves, yellowing and decline (Marcone et al., 2000).

This information will serve as basis for future investigations concerned with epidemiology aspects of the disease, host range study and identifying possible insect vectors through molecular techniques.

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