Use of Heteroduplex Mobility Assay for Identification and Differentiation of Phytoplasmas in the Aster Yellows Group and the Clover Proliferation Group

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ABSTRACT

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This paper describes the identification and differentiation of phytoplasmas by a highly sensitive diagnostic technique, DNA heteroduplex mobility assay (HMA). Closely related phytoplasma isolates of clover proliferation (CP), potato witches'-broom (PWB), and alfalfa witches'-broom (AWB) were collected from the field from 1990 to 1999. The entire 16S rRNA gene and 16/23S spacer region were amplified by polymerase chain reaction (PCR) from the field samples and standard CP, PWB, and AWB phytoplasmas and were subjected to restriction fragment length polymorphism (RFLP) analysis and HMA. Two subgroups (I and II) of phytoplasmas in the CP group were identified by HMA but not by RFLP analysis. The results were confirmed by 16/23S spacer region sequence data analysis. After HMA analyses of the PCR-amplified

16/23S spacer region, 14 phytoplasma isolates from field samples were classified into two aster yellows subgroups: subgroup I, phytoplasma isolates from China aster (*Callistephus chinensis*) yellows, French marigold (*Tagetes patula*) yellows, cosmos (*Cosmos bipinnatus* cv. Dazzler) yellows, clarkia (*Clarkia unguiculata*) yellows, California poppy (*Eschscholzia californica* cv. Tai Silk) yellows, monarda (*Monarda fistulosa*) yellows, and strawflower (*Helichrysum bracteatum*) yellows; and subgroup II, phytoplasma isolates from zinnia (*Zinnia elegans* cv. Dahlia Flower) yellows, Queen-Annes-Lace (*Daucus carota*) yellows, scabiosa (*Scabiosa atropurpurea* cv. Giant Imperial) yellows, Swan River daisy (*Brachycombe multifida* cv. Misty Pink) yellows, pot marigold (*Calendula officinalis*) yellows, purple coneflower (*Echinacea purpurea*) yellows, and feverfew (*Chrysanthemum parthenium*) yellows. The results indicate that HMA is a simple, rapid, highly sensitive and accurate method not only for identifying and classifying phytoplasmas but also for studying the molecular epidemiology of phytoplasmas.

Phytoplasmas have associated with disease in more than 300 plant species (23) since their discovery in 1967 (12). Inability to culture these organisms in vitro has impeded their detection and characterization. In recent years, phytoplasma diseases have been reported in various vegetables and ornamentals in Canada (2,15,16,21). However, most of these disease-associated phytoplasmas have not been identified and their genetic relationship to other phytoplasmas affecting herbaceous ornamentals, vegetables, and forage crops has not been established.

Clover proliferation (CP), potato witches'-broom (PWB), and alfalfa witches'-broom (AWB) are very important diseases and cause severe economic losses each year in Alberta. Since the association of CP with a phytoplasma was first reported (3), the relationship has been studied by nucleic acid hybridization with DNA probes randomly cloned from CP phytoplasma genomic DNA (8), polymerase chain reaction (PCR) with CP group-specific primers (10,18), and restriction fragment length polymorphism (RFLP) analyses (14,21) of PCR-amplified highly conserved genes. The above studies have revealed that CP, PWB, and AWB phytoplasmas are closely related to each other. This raises the question of whether CP, PWB, and AWB are caused by the same phytoplasma strain because all of the previously described techniques cannot differentiate between them. The answer to this question is important for understanding the epidemiology of these diseases and disease management.

Heteroduplex mobility assay (HMA) is based on the principle that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels proportional to their degree of divergence. Recently, HMA has been reported as a reliable means for identification and classification of phytoplasmas (1,6,29,30,32). The sensitivity of HMA for detecting DNA mutation is higher than any other technique currently available, except for exhaustive DNA sequencing studies (8,9). In this study, HMA analysis of the PCR-amplified 16/23S spacer region was employed to characterize phytoplasmas associated with ornamental plants and to study genetic diversity of CP, PWB, and AWB phytoplasmas under field conditions. The analyses resulted in identification of 14 phytoplasmas in subgroups I and II of the aster yellows (AY) group associated with yellows diseases of ornamental plants in Alberta, Canada. To the best of our knowledge, two subgroups of CP, PWB, and AWB phytoplasmas were identified for the first time in field samples.

MATERIALS AND METHODS

Sources of phytoplasmas. The phytoplasma isolates (from collections in Alberta, Canada and in Germany) used in this study are listed in Table 1. An AY isolate (AY27), a CP isolate, and a PWB isolate, originally collected from diseased field plants in Alberta, were maintained in periwinkle (*Catharanthus roseus* (L.) Don) in the greenhouse and used as standard representative isolates. An AY phytoplasma isolate from apricot (AYA) was provided by E. Seemüller, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany. The other isolates were collected in Alberta, Canada as indicated in Table 1.

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Total nucleic acid was extracted from approximately 1 g of freshly cut midrib tissues of diseased samples as described previously (31). The final pellets were dissolved in 50 µl of Tris-EDTA buffer (10 mM Tris at pH 8.0 and 1 mM EDTA) and diluted to a final concentration of approximately 20 ng/µl of nucleic acid with sterile deionized water.

Primer pairs and PCR amplification. PCR assays were used for detection of various phytoplasmas. The somewhat universal primers P1 (11) and P7 (27) were designed on the basis of the 16S rDNA sequence of Mollicutes and were expected to amplify the entire 16S rRNA gene together with the 16/23S spacer region, a DNA fragment approximately 1,800 bp in length.

Amplifications were performed as described previously (31), with 200 µM each deoxynucleoside triphosphate (dNTP), 1.0 µM each primer, 1.25 units of *Taq* DNA polymerase (Perkin-Elmer, Branchburg, NJ), and 50 ng of template DNA in a 50-µl reaction volume. Thirty-five PCR cycles were conducted in a DNA thermal cycler (2400; Perkin-Elmer Cetus, Norwalk, CT) at the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The final extension step was 10 min at 72°C and the reaction mixture was held at 4°C. Five microliters of the PCR products was analyzed by electrophoresis in a 1.5% agarose gel, and DNA bands were stained in ethidium bromide and visualized with a UV transilluminator.

Nested PCR. To increase the sensitivity of PCR and to detect potential mixed infections, the primer pairs P3/P7 (27) designed to amplify the 16/23S spacer region approximately 320 bp in length, and R16F2n/R2 (13,22) designed to amplify the partial 16S rRNA gene approximately 1,200 bp in length, were used for nested

PCR. PCR products amplified by primers P1/P7 were diluted 0 to 100 times in sterile deionized water and were used as templates for a subsequent series of 35 PCR cycles. PCR parameters for primers R16F2n/R2 were the same as previously described. The following parameters were used for primers P3/P7: denaturation at 94°C for 40 s, annealing at 55°C for 45 s, and extension at 72°C for 50 s.

RFLP analysis of 16S rDNA sequences. The sensitivity of RFLP analysis in differentiating known phytoplasma strains CP, PWB, and AWB was compared with HMA. The partial 16S rDNA sequences (1.2 kb) amplified by nested PCR using primers R16F2n/R2 were analyzed by restriction endonuclease digestion. Five microliters of each PCR product was digested with the following restriction endonucleases according to the instructions of the manufacturer: *AluI*, *HhaI*, *HpaII*, *MseI*, *RsaI*, and *Sau3AI* (Gibco BRL, Gaithersburg, MD). The restriction products were resolved on a vertical 5% polyacrylamide gel followed by staining in ethidium bromide. The DNA bands were then visualized with a UV transilluminator. The molecular weights of the fragments were determined by comparison with the 100-bp DNA ladder (BRL-Life Technologies, Egenstein, Germany).

HMA analysis of 16/23S spacer region. The 16/23S spacer region and 16S rRNA gene amplified by nested PCR from CP, PWB, and AWB phytoplasmas were analyzed by HMA. A 4.5-µl aliquot of the PCR product amplified from AY27 as a reference was combined with 4.5 µl of each PCR product amplified from the various CP, PWB, and AWB phytoplasma isolates. Then, 1 µl of 10× annealing buffer (100 mM Tris-HCl at pH 8.0, 10 mM EDTA at pH 8.0, and 1 M NaCl) was added. One drop of mineral oil was overlaid on the reaction mixture.

TABLE 1. Phytoplasma isolates used in this study

Isolate	Disease name	Host plant	Year isolated	Reference
AWB1	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1998	N/A
AWB2	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1998	N/A
AWB3	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1998	N/A
AWB4	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1998	N/A
AWB5	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1998	N/A
AWB6	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1998	N/A
AWB7	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1998	N/A
AWB8	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1998	N/A
AWB90	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1990	N/A
AWB92	Alfalfa witches'-broom	<i>Medicago sativa</i> L.	1992	N/A
AY27	Aster yellows	<i>Callistephus chinensis</i> L.	1976	(4)
AYA	Apricot aster yellows	<i>Prunus armeniaca</i> L.	1993	(24)
ChAY	China aster yellows	<i>Callistephus chinensis</i> (L.) Nees	1999	N/A
CIY	Clarkia yellows	<i>Clarkia unguiculata</i> Lindl., cv. Passion for Purple	1999	N/A
CoY	Cosmos yellows	<i>Cosmos bipinnatus</i> Cav., cv. Dazzler	1999	N/A
CP	Clover proliferation	<i>Trifolium hybridum</i> L.	1974	(3,5)
CP1	Clover proliferation	<i>Trifolium hybridum</i> L.	1996	N/A
CP2	Clover proliferation	<i>Trifolium hybridum</i> L.	1996	N/A
CP3	Clover proliferation	<i>Trifolium hybridum</i> L.	1996	N/A
CP4	Clover proliferation	<i>Trifolium hybridum</i> L.	1996	N/A
CP5	Clover proliferation	<i>Trifolium hybridum</i> L.	1996	N/A
CP6	Clover proliferation	<i>Trifolium hybridum</i> L.	1996	N/A
CPY	California poppy yellows	<i>Eschscholzia californica</i> Cham., cv. Tai Silk	1999	N/A
FFY	Feverfew yellows	<i>Chrysanthemum parthenium</i> (L.) Bernh	1999	N/A
FMY	French marigold yellows	<i>Tegetes patula</i> L.	1999	N/A
MY	Monarda yellows	<i>Monarda fistulosa</i> L.	1997	(31)
PCY	Purple coneflower yellows	<i>Echinacea purpurea</i> Möench.	1999	N/A
PMY	Pot marigold yellows	<i>Calendula officinalis</i> L.	1999	N/A
PWB	Potato witches'-broom	<i>Solanum tuberosum</i> L.	1974	(7)
PWB1	Potato witches'-broom	<i>Solanum tuberosum</i> L.	1996	N/A
PWB2	Potato witches'-broom	<i>Solanum tuberosum</i> L.	1996	N/A
PWB3	Potato witches'-broom	<i>Solanum tuberosum</i> L.	1996	N/A
PWB4	Potato witches'-broom	<i>Solanum tuberosum</i> L.	1996	N/A
PWB5	Potato witches'-broom	<i>Solanum tuberosum</i> L.	1996	N/A
QALY	Queen-Annes-Lace yellows	<i>Ammi majus</i> L.	1999	N/A
ScY	Scabiosa yellows	<i>Scabiosa atropurpurea</i> L., cv. Giant Imperial	1999	N/A
SFY	Strawflower yellows	<i>Helichrysum bracteatum</i> L.	1999	N/A
SRDY	Swan River daisy yellows	<i>Brachycome multifida</i> (DC.) G.L. Davis, cv. Misty Pink	1999	N/A
ZY	Zinnia yellows	<i>Zinnia elegans</i> Jacq., cv. Dahlia Flower	1999	N/A

Fig. 1. 16/23S spacer region of phytoplasmas amplified by nested polymerase chain reaction from various phytoplasma isolates using primer pair P3/P7 and separated by electrophoresis in a 1.5% agarose gel. M, 100-bp DNA ladder; and H, healthy plant. Aster yellows 27 (AY27), China aster yellows (ChAY), zinnia yellows (ZY), cosmos yellows (CoY), clarkia yellows (CIY), French marigold yellows (FMY), California poppy yellows (CPY), Queen-Annes-Lace yellows (QALY), pot marigold yellows (PMY), Swan River daisy yellows (SRDY), purple coneflower yellows (PCY), scabiosa yellows (SCY), monarda yellows (MY), strawflower yellows (SFY), feverfew yellows (FFY), apricot aster yellows (AYA), clover proliferation (CP), potato witches'-broom (PWB), and alfalfa witches'-broom (AWB1).

After denaturing at 98°C for 4 min, the DNA fragments were hybridized on ice. The hybridization products were separated by electrophoresis in a 5% polyacrylamide gel in 1× Tris-borate-EDTA buffer (0.088 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA) at 200 to 250 V for 3 to 4 h at room temperature in PROTEAN II (Bio-Rad, Hercules, CA). DNA bands were stained in ethidium bromide and visualized under a UV trans-illuminator.

To characterize the phytoplasmas associated with ornamental plants, the 16/23S spacer region amplified from reference phytoplasma strains CP or AY27 was combined with each DNA fragment from the 16/23S spacer region amplified from phytoplasma isolate AYA and from the field samples. All the procedures were the same as described previously.

Nucleotide sequencing. PCR amplified 16/23 spacer region was isolated from a 1% agarose gel, purified by a gel extraction kit (QIAquick; Qiagen Inc., Mississauga, Ontario, Canada) and directly sequenced with a DYEnamic ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech Inc., Cleveland). Primers P3 and P7 were used for cycle sequencing both strands of 16/23S spacer region.

Fig. 2. Restriction fragment length polymorphism analysis of polymerase chain reaction (PCR)-amplified 16S rDNA fragments from various clover proliferation (CP), potato witches'-broom (PWB), and alfalfa witches'-broom (AWB) phytoplasma isolates. PCR products were digested with restriction endonucleases **A**, *AluI*; **B**, *RsaI*; **C**, *HpaII*; **D**, *MseI*; **E**, *Sau3AI*; and **F**, *HhaI*. The restriction products were subjected to electrophoresis in a 5% polyacrylamide gel. M, 100-bp DNA ladder; and AY27, aster yellows 27.

RESULTS

Detection of phytoplasmas in diseased ornamental plants.

Direct PCR and nested PCR were employed to detect phytoplasmas in field samples of ornamental plants collected in Alberta in 1999. For direct PCR with primer pair P1/P7, a DNA fragment approximately 1.8 kb in size was amplified from all samples showing yellows or witches'-broom symptoms. No DNA bands were amplified from the healthy control. Weak bands were observed with field samples of zinnia, cosmos, clarkia, and scabiosa (data not shown). Therefore, following primary amplification, the PCR products were subjected to nested PCR amplification using primers P3/P7 to increase the sensitivity of the PCR and the yield of PCR products for further analyses. DNA fragments, approximately 320 bp in length, were amplified from all diseased samples but not from the healthy control (Fig. 1). Similar DNA fragments were consistently amplified from all other phytoplasma samples used as references in this study by both direct PCR and nested PCR using primers P3/P7.

Finer differentiation of CP phytoplasmas by HMA compared with RFLP analysis. To compare the sensitivity of HMA and RFLP in detecting genetic divergences of phytoplasma isolates collected from the field, the 16S rRNA gene amplified from all CP, AWB, and PWB phytoplasma isolates was analyzed by both HMA and RFLP. In the RFLP analysis of the 16S rRNA gene, the results repeatedly showed that the RFLP profiles of all field samples and standard isolates of CP, PWB, and AWB were identical for all restriction enzymes tested (Fig. 2). However, when the same PCR products (1.2 kb) were analyzed by HMA

using AY27 as a reference, DNA heteroduplex bands at approximately 1,800 bp in addition to the homoduplex bands at approximately 1,200 bp, were observed in a polyacrylamide gel (Fig. 3A). No heteroduplex band was observed for the combination of AY27 × AY27. DNA heteroduplexes formed by 16S rDNA fragment combinations of AY27 and AWB92, CP3, PWB, PWB1, PWB2, or PWB4 migrated faster than those formed by other combinations. The results indicated that the closely related phytoplasmas were successfully differentiated by HMA but not by RFLP analysis.

The 16/23S spacer regions amplified from all phytoplasma isolates of AWB, CP, and PWB were subjected to HMA using AY27 as a reference. Heteroduplex bands at approximately 900 bp were observed for all AWB, CP, and PWB isolates but not for AY27. Similar to the results obtained with HMA profiles by the 1.2-kb 16S rDNA fragment amplified by nested PCR, HMA profiles of 16/23S spacer region revealed that the heteroduplex mobilities of AWB92 and CP3 and PWB, PWB1, PWB2, and PWB4 were identical to each other but differed from the remaining isolates of CP, PWB, and AWB (Fig. 3B). Therefore, two subgroups of phytoplasma isolates of AWB, CP, and PWB were identified on the basis of HMA profiles of the 16/23S spacer region and 16S rRNA gene. Phytoplasma isolates AWB1 to AWB8, AWB90, CP, CP1, CP2, CP4 to CP6, PWB3, and PWB5 were classified as CP subgroup I whereas AWB92, CP3, PWB, PWB1, PWB2, and PWB4 were classified as CP subgroup II. Neither subgroup corresponded with any of the three diseases, because each disease was represented unevenly by phytoplasmas belonging to both HMA groups.

Because the 16/23S spacer regions of AY27 and CP, PWB, and AWB are different in length, two homoduplex bands were observed for the 16/23S spacer genes. The homoduplex band at approximately 320 bp represents the 16/23S spacer region of AY27, whereas the band at approximately 300 bp represents those of CP, PWB, and AWB.

Fig. 3. Heteroduplex mobility assay analysis of **A**, nested polymerase chain reaction (PCR)-amplified 16S rRNA gene and **B**, 16/23S spacer region from clover proliferation (CP), potato witches'-broom (PWB), and alfalfa witches'-broom (AWB) phytoplasma isolates. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. Aster yellow 27 (AY27) was used as a reference. M, 1-kb DNA ladder.

	1	11	21	31	41	51
AWB3	TCTAAGGACTATATATACTAAATCATCATCTTCAGTTTGAAGACTTAAGTAGAAAAAT					
CP1	TCTAAGGACTATATATACTAAATCATCATCTTCAGTTTGAAGACTTAAGTAGAAAAAT					
PWB3	TCTAAGGACTATATATACTAAATCATCATCTTCAGTTTGAAGACTTAAGTAGAAAAAT					
AWB92	TCTAAGGACTATATATACTAAATCATCATCTTCAGTTTGAAGACTTAAGTAGAAAAAT					
CP3	TCTAAGGACTATATATACTAAATCATCATCTTCAGTTTGAAGACTTAAGTAGAAAAAT					
PWB4	TCTAAGGACTATATATACTAAATCATCATCTTCAGTTTGAAGACTTAAGTAGAAAAAT					
	61	71	81	91	101	111
AWB3	AAGTTTTCCTTTTAAGCCCAAGGGCCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA					
CP1	AAGTTTTCCTTTTAAGCCCAAGGGCCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA					
PWB3	AAGTTTTCCTTTTAAGCCCAAGGGCCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA					
AWB92	AAGTTTTCCTTTTAAGCCCAAGGGCCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA					
CP3	AAGTTTTCCTTTTAAGCCCAAGGGCCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA					
PWB4	AAGTTTTCCTTTTAAGCCCAAGGGCCCTATAGCTCAGTTGGTTAGAGCACACGCCTGA					
	121	131	141	151	161	171
AWB3	TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCACCAAAAAAGGTCCTGCTTAAA					
CP1	TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCACCAAAAAAGGTCCTGCTTAAA					
PWB3	TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCACCAAAAAAGGTCCTGCTTAAA					
AWB92	TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCACCAAAAAAGGTCCTGCTTAAA					
CP3	TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCACCAAAAAAGGTCCTGCTTAAA					
PWB4	TAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCACCAAAAAAGGTCCTGCTTAAA					
	181	191	201	211	221	231
AWB3	AAGTTCTTTGAAAAGTAGATAAATCATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAA					
CP1	AAGTTCTTTGAAAAGTAGATAAATCATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAA					
PWB3	AAGTTCTTTGAAAAGTAGATAAATCATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAA					
AWB92	AAGTTCTTTGAAAAGTAGATAAATCATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAA					
CP3	AAGTTCTTTGAAAAGTAGATAAATCATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAA					
PWB4	AAGTTCTTTGAAAAGTAGATAAATCATGCTTTAAAATTTTCATAAAGTTGAAGGAAGTAA					
	241	251	261			
AWB3	GGCATATAGTGGATGCCTTGGCACT					
CP1	GGCATATAGTGGATGCCTTGGCACT					
PWB3	GGCATATAGTGGATGCCTTGGCACT					
AWB92	GGCATATAGTGGATGCCTTGGCACT					
CP3	GGCATATAGTGGATGCCTTGGCACT					
PWB4	GGCATATAGTGGATGCCTTGGCACT					

Fig. 4. Nucleotide sequences of the 16/23S spacer region of alfalfa witches'-broom (AWB), clover proliferation (CP), and potato witches'-broom (PWB) phytoplasmas. The nucleotide sequences of primers P3 and P7 are not included. Phytoplasma isolates of AWB3, CP1, and PWB3 were classified as members of CP subgroup I, whereas AWB92, CP3, and PWB4 were classified as members of CP subgroup II.

Nucleotide sequence of the 16/23S spacer regions of AWB, CP, and PWB phytoplasmas. The approximately 265-bp sequence of the 16/23-spacer region amplified from AWB, CP, and PWB phytoplasmas was determined by directly sequencing both strands (Fig. 4). Analysis of the sequence alignment data revealed that members of CP subgroup I (AWB3, CP1, and PWB3) had identical sequences that differed from those of CP subgroup II (AWB92, CP3, and PWB4) at positions 78, 99, and 237. The sequence data agree with the results obtained by HMA.

Identification and classification of phytoplasmas associated with ornamental plants. Heteroduplexes were formed by combining the 16/23S spacer region amplified from CP and each of the corresponding DNA fragments from the phytoplasmas associated with ornamental plants (Figs. 5 and 6). The phytoplasma isolates from diseased ornamental plants were classified into two AY subgroups by HMA. China aster yellows (ChAY), French marigold yellows (FMY), cosmos yellows (CoY), clarkia yellows (CIY), California poppy yellows (CPY), monarda yellows (MY), and strawflower yellows (SFY) phytoplasmas showed heteroduplex mobilities similar to AY27 when CP was used as a reference and, therefore, were classified as new members of subgroup I together with AY27 (Fig. 5). Phytoplasma isolates Queen-Annes-Lace yellows (QALY), scabiosa yellows (ScY), pot marigold yellows (PMY), Swan River daisy yellows (SRDY), purple coneflower yellows (PCY), zinnia yellows (ZY), and feverfew yellows (FFY) showed HMA profiles identical to each other but different from AY27 and were classified as AY subgroup II (Fig. 5). Having a unique heteroduplex mobility, phytoplasma strain AYA was distinguishable from all members of the two AY subgroups tested in this study. Similar results were obtained when AY27 was used as the reference (Fig. 6). However, the heteroduplexes formed between phytoplasmas AYA and AY27 had very similar mobilities to those formed between AY27 and AY subgroup II (Fig. 6).

DISCUSSION

Phytoplasmas have been classified into 20 major groups on the basis of sequence analysis of the 16S rRNA gene (25). A total of

246 phytoplasmas have been assigned to 20 major groups on the basis of molecular or serological analyses. The AY group is the largest major group, and includes more than 100 phytoplasma isolates. In this study, four phytoplasma isolates from FMY, CoY, CIY, and CPY were identified as members of AY subgroup I and the other four phytoplasma isolates from ZY, QALY, ScY, and SRDY were identified as members of AY subgroup II.

PMY and PCY were found to be associated with phytoplasmas by electron microscopy in Canada in 1997 (15,16). However, the genetic identity of the pathogen was not established at that time. We determined that PMY phytoplasma belongs to AY subgroup II. Previous RFLP analysis of the 16S rDNA fragment amplified from PCY phytoplasma by four restriction enzymes (*AluI*, *HhaI*, *RsaI*, and *Sau3AI*) showed restriction patterns identical to those of AY27 (19,28). In the present study, HMA profiles of the 16/23S spacer region indicated that PCY is different from AY27 and belongs to AY subgroup II. This discrepancy may be due to the inability of RFLP to differentiate PCY from AY27. However, the existence of different types of phytoplasmas in samples from different origins cannot be excluded because different samples were used in these studies.

In this study, we demonstrated that HMA is a highly sensitive diagnostic technique for the differentiation and identification of phytoplasmas. The closely related phytoplasma isolates of CP, PWB, and AWB were not differentiated by RFLP with the restriction endonucleases used in this study. These results are consistent with previous studies in which CP, PWB, and AWB phytoplasmas were classified in the same phylogenetic group based on their identical RFLP profiles (14,17,18,21,22). However, genetic differences between CP, PWB, and AWB phytoplasma isolates were clearly discerned by HMA with the 16/23S spacer region or the 16S rRNA gene. The results provide evidence that HMA is much more sensitive than RFLP analysis in differentiating closely related phytoplasma isolates. The sensitivity of HMA in detecting DNA mutations of phytoplasmas also was demonstrated in previous studies in which a single base pair insertion/deletion or two base pair substitutions in a 500-bp DNA fragment were easily detected (29,30).

Fig. 5. Heteroduplex mobility assay analysis of nested polymerase chain reaction-amplified 16/23S spacer region from various phytoplasma associated with ornamental plants. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. Clover proliferation (CP) was used as a reference. M, 1-kb DNA ladder. Aster yellows 27 (AY27), China aster yellows (ChAY), French marigold yellows (FMY), cosmos yellows (CoY), clarkia yellows (CIY), California poppy yellows (CPY), monarda yellows (MY), strawflower yellows (SFY), Queen-Annes-Lace yellows (QALY), scabiosa yellows (ScY), pot marigold yellows (PMY), Swan River daisy yellows (SRDY), purple coneflower yellows (PCY), zinnia yellows (ZY), feverfew yellows (FFY), and apricot aster yellows (AYA).

Fig. 6. Heteroduplex mobility assay analysis of nested polymerase chain reaction-amplified 16/23S spacer region from various phytoplasma isolates associated with ornamental plants. Heteroduplexes and homoduplexes were separated by electrophoresis in a 5% polyacrylamide gel. Aster yellows 27 (AY27) was used as a reference. M, 1-kb DNA ladder. China aster yellows (ChAY), French marigold yellows (FMY), cosmos yellows (CoY), clarkia yellows (CIY), California poppy yellows (CPY), monarda yellows (MY), strawflower yellows (SFY), Queen-Annes-Lace yellows (QALY), scabiosa yellows (ScY), pot marigold yellows (PMY), Swan River daisy yellows (SRDY), purple coneflower yellows (PCY), zinnia yellows (ZY), feverfew yellows (FFY), and apricot aster yellows (AYA).

In this study, two subgroups of AWB, CP, and PWB phytoplasmas were identified, to our knowledge for the first time, by HMA. Phytoplasmas of both types can cause witches'-broom of alfalfa and potato plants and proliferation of clover plants. The differences between these two subgroups were confirmed by sequence data that showed three single-base pair differences in the 16/23S spacer region between subgroups I and II. These results support a notion that two different populations of phytoplasmas belonging to CP subgroups I and II occur in CP, PWB, and AWB diseases in Alberta.

Our study clearly indicates that HMA is more accurate than RFLP analysis for the classification of phytoplasmas. Although RFLP analyses of highly conserved genes have been useful in constructing a phylogenetic tree of phytoplasmas, RFLP analysis relies on the presence or absence of restriction sites in a DNA fragment and a set of restriction enzymes must be used (13,14,22). DNA sequence analyses for more than 20 phytoplasma isolates in previous studies have indicated that RFLP-based classification of phytoplasmas in certain groups does not fully coincide with the phylogenetic relationships of the organisms based on DNA sequences (20,26). For example, strain AYA and other typical AY strains that were classified in two different major groups by RFLP analyses (24) were found to share sequence homology at a level as high as 99% in the 16S rRNA gene (27). In this study, the 16/23S spacer region of AYA phytoplasma had a unique heteroduplex mobility that was different from that of AY27 (Fig. 5), and thus AYA may be classified as a member of a new AY subgroup, which fully agrees with the results of sequence analysis of the 16S rRNA gene.

Use of different standard phytoplasma strains as references in HMA can increase the sensitivity for detecting minor differences between phytoplasma isolates. In this study, the heteroduplex mobility of AYA was very similar to those of QALY, ScY, PMY, SRDY, PCY, ZY, and FFY when AY 27 was used as a reference (Fig. 6). However, the differences between AYA and others were clearly observed by HMA when CP was used as a reference (Fig. 5). This suggests that two or more references should be used when attempting to differentiate similar phytoplasmas or to detect minor mutations of phytoplasmas.

In conclusion, our results suggest that HMA may offer a useful method for studying the genetic diversity and molecular epidemiology of phytoplasmas by monitoring DNA sequence diversity of a given phytoplasma population and tracking the evolution of the phytoplasmas.

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