An Epidemic of Almond Witches'-broom in Lebanon: Classification and Phylogenetic Relationships of the Associated Phytoplasma

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ABSTRACT

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An epidemic of almond witches'-broom has devastated almond production in Lebanon. Thousands of almond trees have died over the past 10 years due to the rapid spread of the disease. The symptoms, which include early flowering, stunted growth, leaf rosetting, dieback, off-season growth, proliferation of slender shoots, and witches'-brooms arising mainly from the main trunk and roots, resemble those caused by phytoplasmal infections. For the detection of the putative causal agent, nested polymerase chain reaction (PCR) was performed using universal primers (P1/P7, R16mF2/R16mR1, and R16F2n/R16R2) commonly used for the specific diagnosis of plant pathogenic phytoplasmas. Phytoplasmas were readily detected from infected trees with witches'-broom symptoms collected from three major almond growing regions in Lebanon. Restriction fragment length polymorphism (RFLP) analysis of PCR products amplified by the primer pair R16F2n/R16R2 revealed that the phytoplasma associated with infected almonds is similar to, but distinct from, members of the pigeon pea witches'-broom phytoplasma group (16SrIX). A new subgroup, 16SrIX-B, was designated. Sequencing of the amplified products of the phytoplasma 16S rRNA gene indicated that almond witches'-broom (AlmWB) phytoplasma is most closely related to members of the pigeon pea witches'-broom phytoplasma group (with sequence homology ranging from 98.4 to 99.0%). Phylogenetic analysis of 16S rDNA sequences from AlmWB phytoplasma and from representative phytoplasmas from GenBank showed that the AlmWB phytoplasma represents a distinct lineage within the pigeon pea witches'-broom subclade. The same phytoplasma appears also to infect peach and nectarine seedlings.

Stone fruits are affected by several diseases associated with plant pathogenic phytoplasmas. In Europe, phytoplasmas were reported to cause significant loss of production in apricot (apricot chlorotic leaf roll, ACLR) (29), plum (Japanese plum leptonecrosis, JPL) (14), and peach (28,30). Cross-inoculation experiments (11), DNA sequence analysis, and restriction fragment length polymorphism (RFLP) analysis revealed that most known European stone fruit phytoplasmas are genetically similar (3). Therefore, the name European stone fruit yellows (ESFY) has been proposed (27). A recently revised classification scheme for phytoplasmas, based on RFLP analysis of the 16S rDNA, differentiates phytoplasmas into 14 major phylogenetic groups (26). ESFY phytoplasmas belong to group 16SrX (apple proliferation group), subgroup B. ESFY phytoplasmas were also classified as members of the apple proliferation group according to the scheme proposed by Seemüller et al. (33). Other members of this group include apple proliferation (AP) and pear decline (PD) phytoplasmas. In North America, peach yellow leaf roll (PYLR) is associated with a phytoplasma related to PD phytoplasma (22,23,33). ESFY phytoplasmas are only distantly related to X-disease phytoplasmas (X-disease group or 16SrIII group) that are associated with X-diseases of peach (X-disease CX or WX) and of chokecherry (X-disease CCX). Other distantly related phytoplasmas that infect stone fruits include cherry molières disease (MOL) from France (5) and cherry lethal yellows (CLY) from China (36), which belongs to 16SrV (elm yellows group) (26). Almond, another member of the stone fruit family, has been reported to be infected by phytoplasmas in several cases (14,18,25,27,34).

Stone fruits, with almond occupying the largest acreage, represent the major fruit crops grown in Lebanon (13). The area devoted to stone fruit production (23,000 ha) ranks first among fruit trees compared with grape at 15,500 ha, citrus at 15,000 ha, and pome fruits at 12,600 ha (13). In the 1980s and 1990s, the area devoted to almond production in Lebanon increased

Table 1. Detection of phytoplasmas by polymerase chain reaction (PCR) in almond samples collected from different almond growing areas in Lebanon

Sites	Presence of symptoms[a]	Number of samples	Positive by direct PCR[b]	Positive by nested PCR[c]
Furzol (Bekaa)	No	2	0	0
Oussaya[d] (Bekaa)	No	16	0	0
	Yes	5	5	5
Kefraya (Bekaa)	No	2	0	0
Bearzleh (north)	No	1	0	0
Adbel (north)	No	1	0	0
Jbrayel (north)	No	1	0	0
Raskifa[e] (north)	Yes	15	4	9
Bsebaal[e] (north)	No	1	0	0
	Yes	23	6	18
Bserma (north)	Yes	1	0	1
Kfarhazir[f] (north)	No	2	0	2
Bechmezzine[f] (north)	No	3	0	0
Kfaraka[f] (north)	No	4	0	1
Batroun (north)	No	2	0	0
Rachana (north)	No	11	0	0
Tanboureet[e] (south)	No	3	0	0
	Yes	20	4	14
Broumana (ML)[g]	No	3	0	0
Total		116	19	50

[a] Symptoms include early flowering, stunted growth, leaf rosetting, shoot proliferation, and witches'-broom.
[b] Direct PCR with universal primers P1/P7.
[c] Nested PCR with primers P1/P7 followed by primers R16F2n/R16R2.
[d] Symptoms localized in one patch of the orchard.
[e] Symptoms widespread in the area. Samples were collected from well-maintained orchards in Raskifa and from either well or not properly maintained orchards in Bsebaal.
[f] Locations close to infected areas.
[g] Mount Lebanon.

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Table 2. Detection of phytoplasmas by polymerase chain reaction (PCR) in peach and nectarine samples collected from peach growing areas in Lebanon

Sites	Presence of symptoms	Number of samples	Positive by direct PCR[a]	Positive by nested PCR[b]
Peach				
Furzol (Bekaa)	No	1	0	0
Deir El Kamar (Mount Lebanon)	No	2	0	0
Sir (north)	No	2	0	0
Katin (north)	No	2	0	0
Bedjita (north)	No	2	0	0
Hesniyeh(north)	Yes[c]	4	0	0
Ebadieh (north)	No	2	0	0
Kferhata (north)	No	1	0	0
Ain Bakra (north)	No	1	0	0
Bsebaal (north)	Yes[d]	3	0	1
Nectarine				
Bsebaal (north)	Yes[d]	7	4	4
Total		27	4	5

[a] Direct PCR with universal primers P1/P7.
[b] Nested PCR with P1/P7 followed by R16F2n/R16R2.
[c] Leaf roll symptoms.
[d] Symptoms included proliferation of slender shoots, short internodes and small chlorotic leaves, or light green color and seedling decline.

Fig. 1. An almond tree showing extensive shoot proliferation and witches'-broom symptoms.

significantly after some growers in the Bekaa Valley preferred growing almond to grape, cherry, and apricot. An unknown disease associated with almond has led to rapid decline of almond trees in three major almond production regions in Lebanon during the last decade. The first epidemic occurred in the south in the early 1990s. A survey conducted in 1998 to 1999 using enzyme-linked immunosorbent assay (ELISA) showed that most declining trees were free from infection by six of the major stone fruit viruses (20). Very large galls induced by *Agrobacterium tumefaciens* were observed on the roots of a few declining almond trees. These may have accounted for the deaths of many seedlings, but they cannot be the cause of rapid decline of trees over 20 years old. Similar symptoms of almond decline (but without the crown gall) occurred in north Lebanon starting in 1995. The disease spread rapidly, first in the coastal areas to an elevation up to about 500 m, where the majority of almond orchards are located. But a few trees at higher elevations (1,000 m) also showed the characteristic symptoms. A phytoplasma was suspected due to the presence of witches'-broom symptoms arising from tree trunks.

Recent detection techniques for phytoplasmas include ELISA using monoclonal antibodies (7,10), DNA hybridization (9,24), and polymerase chain reaction (PCR) (19,30,31). In this study, we opted for PCR for three main reasons: sensitivity and rapidity of the test and the availability of universal primers for diagnosis of phytoplasmas. The present paper reports the outbreak of an epidemic of almond witches'-broom disease in several almond growing regions in Lebanon and the molecular identification and phylogenetic relationships of a phytoplasma associated with the disease. A preliminary survey indicated that the same phytoplasma caused diseases in some nectarine and peach seedlings with shoot proliferation symptoms in a field adjacent to an infected almond orchard.

MATERIALS AND METHODS

Sources of naturally infected plant materials. Surveys were conducted in the major almond growing areas. Field observations showed that almond plants from seedling stage to over 30 years old were infected. Trees showing symptoms suggestive of phytoplasmal infection were sampled. Leaves and, in some cases, other tissues (barks, petals, and fine roots) were collected for total nucleic acid extraction. The samples were weighed and maintained at 4°C until processing (normally within 2 days) or stored for a longer time at –20°C. In total, 116 samples were collected from different locations (23 from south, 68 from north, and 25 from Bekaa) (Table 1). In addition, leaf samples were collected from asymptomatic or symptomatic peach trees

growing in different areas showing leaf roll symptoms, and from nectarine and peach seedlings with proliferation symptoms grown near an infected almond orchard (Table 2).

Nucleic acid extraction. Leaf midribs were used for nucleic acid extraction from all samples except some from which extraction was also performed using leaf petioles, petals, green bark from proliferating shoots, and fine roots. The small-scale procedure for nucleic acid extraction, described by Zhang et al. (35), was followed with a minor modification of the extraction buffer (CTAB buffer: 2% CTAB, 1.4 M NaCl, 20 mM EDTA, 1% polyvinylpyrrolidone (PVP), 0.2% mercaptoethanol, 100 mM Tris-HCl, pH 8.0). About 100 mg of midrib or other tissue types were immersed in liquid nitrogen and ground using a pestle attached to an electrical drill. The nucleic acid pellet was washed with 80% ethanol, air-dried, suspended in 50 µl of sterile water, and maintained at –20°C until use.

Primers and PCR amplification. Three pairs of universal primers previously designed for the detection of phytoplasmas were used in nested PCR. The first PCR run was done either with primer pair P1/P7 (32), which primes a fragment, approximately 1,800 bp, that extends from the 5′ end of the 16S rDNA to the 5′ region of the 23S rDNA, or with primer pair R16mF2/R16mR1, which primes a 16S rDNA fragment of approximately 1,435 bp. Nested PCR was performed with primer pair R16F2n/R16R2 (15). Amplifications were performed with an Icycler thermocycler (Bio-Rad Laboratories, Hercules, CA) in 20-µl reactions containing 200 mM each of the four dNTPs, 0.5 µM of each primer, 2 mM $MgCl_2$, 1× polymerase buffer, 1 unit *Taq* (Abgene, Surrey, UK) polymerase and 1 to 2 µl of sample nucleic acid (about 10 to 20 ng). Parameters used for 35 cycle PCRs were: denaturation at 94°C for 30 s (2 min for the first cycle), annealing at 50°C for 30 s, and extension at 72°C for 2 min (7 min for the last cycle). One µl of the diluted (1:40) PCR products from the first amplification was used as templates for the nested PCR. The amplified products were analyzed by electrophoresis in 1.2% agarose gel containing 5 µg of ethidium bromide per ml.

RFLP analysis of nested PCR amplified 16S rDNA. PCR products amplified using primer pair P1/P7 or R16mF2/R16mR1 followed by primer pair R16F2n/R16R2 were analyzed by single restriction endonuclease digestion with the following enzymes according to the instructions of the manufacturer: *Alu*I, *Bfa*I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Kpn*I, *Rsa*I, *Taq*I, and *Tha*I (Life Technologies, Gaithersburg, MD), and *Mse*I and *Sau*3A (New England Biolabs, Beverly, MA). The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel and stained in ethidium bromide. DNA bands were visualized with a UV transilluminator. Putative restriction site maps of pigeon pea witches'-broom (PPWB, AF248957), *Knautia arvensis* phyllody (KAP, Y18052), and almond witches'-broom (AlmWB) phytoplasmas were generated by using the DNASTAR program MapDraw option (DNASTAR, Madison, WI).

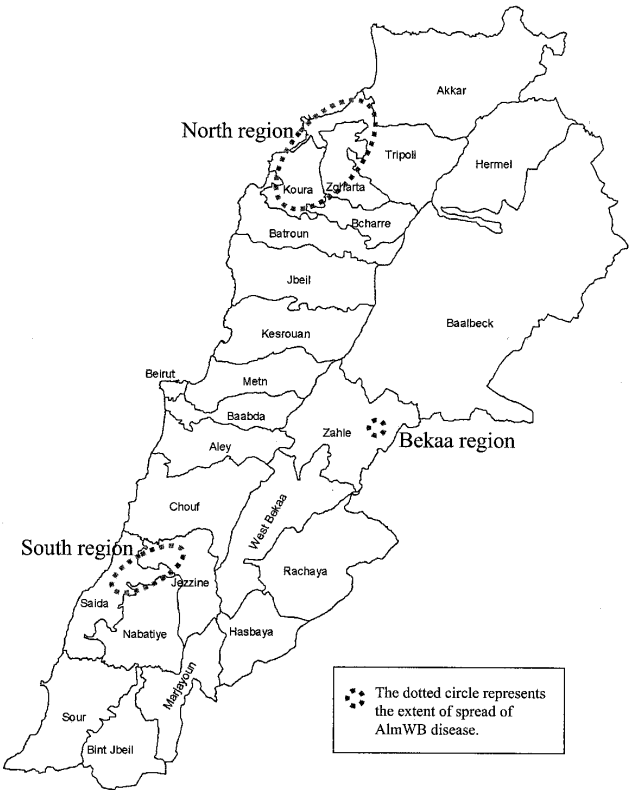

Fig. 2. Map of Lebanon with names of casas indicated, showing the extent of spread of almond witches'-broom disease in three geographical regions (dotted line).

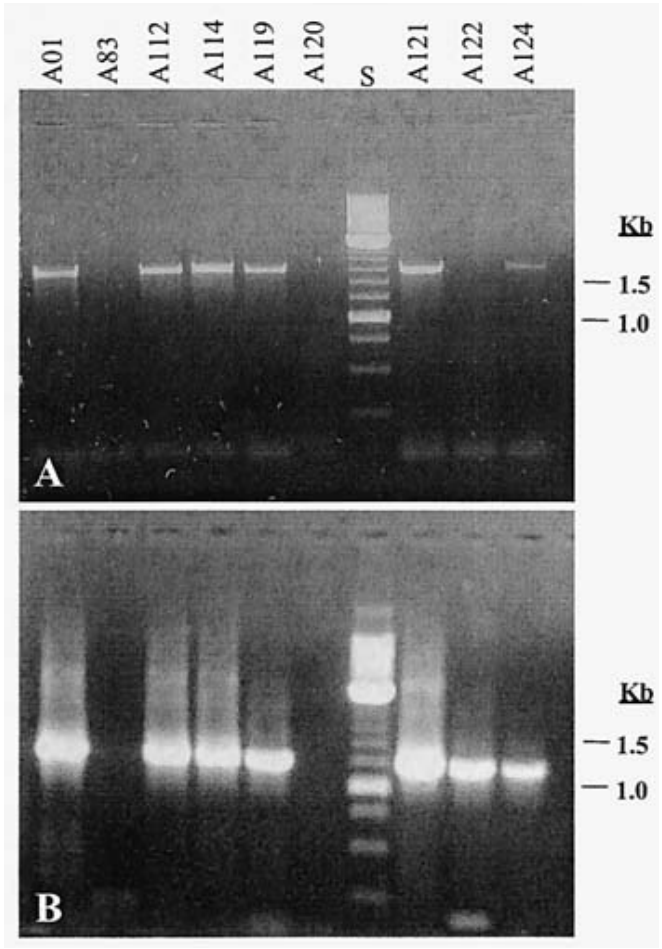

Fig. 3. Direct and nested polymerase chain reaction (PCR) amplifications of phytoplasma 16S rDNA from almond samples suspected of being infected with phytoplasma, collected from different locations. Direct PCR was performed **A,** using primer pair P1/P7; this was followed by a nested PCR amplification, **B,** using the primers R16F2n/R16R2. PCR products were separated by electrophoresis through a 1.2% agarose gel. Lanes 1 and 2 resulted from samples (A01 and A83) collected from Bekaa; 3 and 4 (samples A112, A114) from the south; 5, 6, 8, 9, and 10 (samples A119, A120, A121, A122, A124, respectively) from the north. Lane S, 250 bp DNA ladder.

Phylogenetic analysis. The amplified fragments (1.8 kb) obtained by PCR using primer pair P1/P7 were purified using Qiaquick PCR Purification Kit (Qiagen, Valencia, CA) and cloned in *Escherichia coli* using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. P1/P7 PCR amplified products from four almond samples (A121-1, A121-B from north, A114 from south, and A01 from Bekaa region), one peach (P244), and one nectarine (N231) were cloned. Sequencing was performed with an automated DNA sequencer (ABI Prism Model 377) at the Center for Agricultural Biotechnology, University of Maryland, College Park. The nucleotide sequence was aligned with highly homologous sequences in GenBank and EMBL databases using the Gapped BLAST program (4) and performing gapped and ungapped analyses. Partial sequences (1.5 kb) of 16S rDNA from AlmWB phytoplasma and representative phytoplasma strains available in GenBank were aligned separately by using CLUSTAL, version 5, and DNASTAR's Laser Gene software (DNASTAR). Cladistic analyses were performed with PAUP (phylogenetic analysis using parsimony) version 4.0 written by D. L. Swofford (University of Illinois) on a Power Mac G4. Uninformative characters were excluded from analyses. A phylogenetic tree was constructed by a heuristic search via random stepwise addition implementing the tree bisection and reconnection branch-swapping algorithm to find the optimal tree(s). *Acholeplasma laidlawii* was selected as the out-group to root the tree. The analysis was replicated 1,000 times. Bootstrapping was performed to estimate the stability and support for the inferred clades. The cloned nucleotide sequences were deposited as accessions AF390136 (AlmWB1 = A01), AF390137 (AlmWB2 = A121-A), AF455038 (AlmWB3 = A121-B), AF455039 (AlmWB4 = A114), AF455040 (AlmWB-P1 = P244), and AF455041 (AlmWB-N1 = N231).

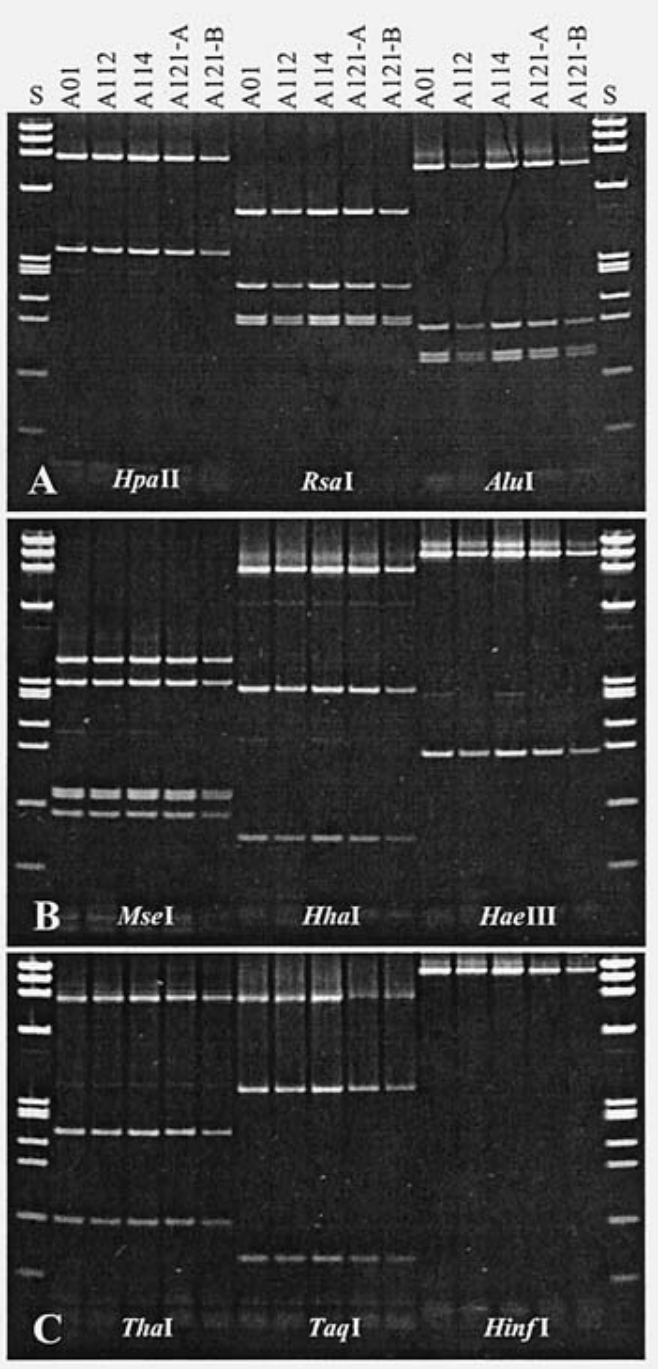

Fig. 4. Restriction fragment length polymorphism (RFLP) analyses of phytoplasma 16S rDNA sequences (R16F2n/R16R2 nested polymerase chain reaction [PCR] products) amplified from five different almond samples showing witches'-broom symptoms. The samples were collected from different regions: Bekaa (A01), South (A112, A114), and North (A121-A, A121-B). DNA products were digested with **A,** *Hpa*II, *Rsa*I, and *Alu*I; **B,** *Mse*I, *Hha*I, and *Hae*III; and **C,** *Tha*I, *Taq*I, and *Hinf*I, and separated by electrophoresis through a 5% polyacrylamide gel. Lane S, ΦX174 RFI DNA *Hae*III digest, fragment sizes (bp) from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, 72. The first PCR was performed with P1/P7 primer pair.

Survey and symptoms. During the survey, no almond trees showing yellowing and leaf roll symptoms were observed. However, in three regions (Bekaa, north, and south), declining almond trees were observed. The most characteristic symptom found was the proliferation of shoots at several points on the main trunk with an appearance of witches'-broom (Fig. 1). Hence, almond witches'-broom (AlmWB) was adopted as the disease name. As the disease advanced, witches'-brooms emerged on many scaffold branches. Discussion with farmers indicated that the first symptom observed was an early flowering (20 to 30 days earlier than normal), and sometimes witches'-brooms appeared on the main trunk. In the second year, the lower branches showed extensive proliferation with smaller leaves, light green in color, and shoots became stunted with short internodes (rosetting). The yield of fruits on infected almond branches was greatly reduced. The fruits produced were small, dark in color, with shriveled seeds. The trees declined rapidly and died within 3 to 4 years after the initial appearance of symptoms. All cultivated varieties seemed equally susceptible, although some variation in symptoms occurred. Many trees showed stunted growth with short internodes and small leaves; others showed proliferation of several lateral individual slender branches, mostly with an upright growth but without witches'-broom. Even though wild almond (*Prunus orientalis*) also gets severely infected, a delay in the appearance of symptoms was often observed. During the year 2000, off-season growth started in late October and flowering of infected trees started in mid-November.

The earliest report of this disease was from South Lebanon. Farmers dated it back to the early 1990s and claimed that over six thousand almond trees died in several villages located in that region. In North Lebanon (Caza of Zghorta), the introduction of the disease was first recorded as recently as 1995. Since that time, it has spread very rapidly to cover the entire region from the coastal areas to an elevation of 500 m where the majority of almond orchards are located, but symptoms also were observed at higher elevations (1,000 m). The disease then spread to neighboring cazas of Koura and Tripoli (Fig. 2). Most almond trees in this region have died or are dying (over 65,000 trees). So far, no symptoms have been observed in the plain of Akkar, north of Tripoli, one of the major almond production areas. In the third region (in the Bekaa Valley, the major stone fruit growing area), the disease seems to be restricted. Only a group of a few almond trees in one orchard was infected; but the disease did not seem to be spreading to adjacent cherry trees in the same region. However, in the north region in a field and a nursery located near an infected almond orchard, the same phytoplasma appeared to be responsible for diseases in peach and in nectarine seedlings (1 to 5 years old), with symptoms suggestive of phytoplasma infection (Table 2).

PCR amplification. Fifty-seven percent of leaf samples collected from symptomatic almond trees from orchards located in the Bekaa (Oussaya), in the south (Tamboureet), and in the north (Raskifa, Bsebaal, Bserma) gave positive results in PCR tests. The additional tests performed in a few symptomatic trees using bark, petals, and fine roots gave positive results consistent with those obtained with leaf tissues. DNA fragments of expected sizes were amplified in extracts prepared from infected trees but not from asymptomatic trees collected from most noninfected areas (data not shown). The approximate sizes of amplified products were 1,800 bp with primers P1/P7, 1,435 bp with primers R16mF2/R16mR1, and 1,200 bp with primers R16F2n/R16R2. In some cases, nested PCR (with primers P1/P7 or R16mF2/R16R1 followed by a second run of PCR using primers R16F2n/R16R2) was needed to produce detectable PCR products (Table 1, Fig. 3). Four samples from nectarine seedlings and one sample from peach seedlings showing proliferation tested positive (Table 2). These symptomatic samples were collected from the same area (Bsebaal) in which almond trees were heavily infected (Tables 1 and 2). No positive tests were obtained from asymptomatic peach trees or from trees showing leaf roll symptoms.

Classification of almond witches'-broom phytoplasma. The almond witches'-broom (AlmWB) phytoplasma 16S rDNA sequences (1.2 kb) amplified by nested PCR, using the second primer pair R16F2n/R16R2, were subjected to RFLP analysis. Collective RFLP patterns of five AlmWB 16S rDNA sequences, representatives of Bekaa (A01), south (A112, A114), and north (A121-A, A121-B), based on analyses with 12 restriction enzymes were identical and were most similar to those previously published for 16S rDNAs from members of the pigeon pea witches'-broom (PPWB) phytoplasma group (16,21,26). The RFLP patterns of AlmWB phytoplasma strains resolved by single digestion with *Mse*I, *Hpa*II, and *Tha*I (Fig. 4), and with *Bfa*I, *Kpn*I, and *Sau*3A (data not shown), were identical to those of PPWB phytoplasma. But the patterns resolved by digestions with the other six enzymes (*Rsa*I, *Alu*I, *Hha*I, *Hae*III, *Taq*I, and *Hin*fI) were distinct from those of PPWB phytoplasma (Fig. 4). RFLP analysis (with eight restriction enzymes) of phytoplasmas amplified from symptomatic peach (P244) and nectarine (N231) samples and a reference PPWB phytoplasma strain revealed that phytoplasma strains P244 and N231 were indistinguishable from AlmWB phytoplasma (A112) and distinct from PPWB phytoplasma (Fig. 5). The close relatedness among almond, peach, and nectarine strains was confirmed by analysis of cloned 16S rDNA sequences. The sequence homology among AlmWB phytoplasma strains from almond collected in three different geographical regions, peach, and nectarine strains ranged from 99.6 to 99.9%. The sequence variations were random.

Maps of putative restriction sites of the latter six enzymes were in agreement with the patterns obtained by enzymatic analysis on AlmWB and PPWB phytoplasma strains (Fig. 6). The sequence variation between the two strains involved at least eight different restriction sites in the portion of 16S rDNA amplified by R16F2n/R16R2 primer pair. Between AlmWB and *Knautia arvenis* phyllody (KAP) phytoplasmas, the sequence variation is smaller, involving only four different restriction sites (Fig. 6).

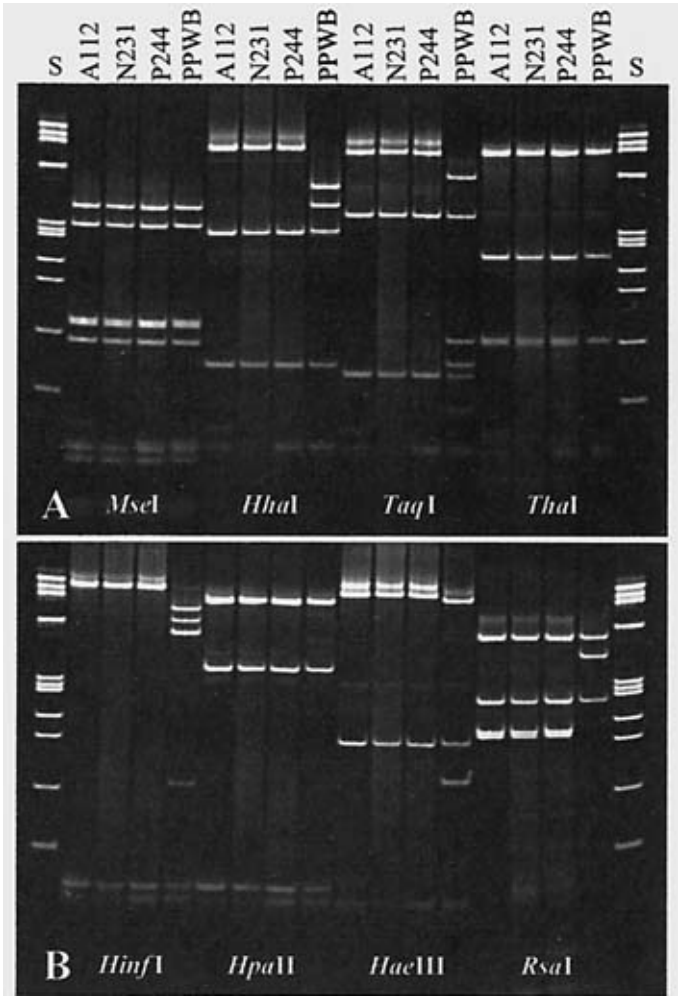

Fig. 5. Restriction fragment length polymorphism (RFLP) analyses of phytoplasma 16S rDNA sequences (R16F2n/R16R2-nested polymerase chain reaction [PCR] products) amplified from almond (A112), peach (P244), nectarine (N231), and a reference phytoplasma strain pigeon pea witches'-broom (PPWB). DNA products were digested with **A,** *Mse*I, *Hha*I, *Taq*I, and *Tha*I; and **B,** *Hin*fI, *Hpa*II, *Hae*III, and *Rsa*I, and separated by electrophoresis through a 5% polyacrylamide gel. Lane S, ΦX174 RFI DNA *Hae*III digest, fragment sizes (bp) from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, 72. The first PCR was performed with P1/P7 primer pair.

Based on the RFLP patterns, the new AlmWB phytoplasma was designated a new subgroup 16SrIX-B in the pigeon pea witches'-broom group (16SrIX). KAP phytoplasma could represent another new subgroup, provided that the putative restriction sites can be confirmed by RFLP analysis.

Phylogenetic analysis. BLAST searches were done on regions of the 16S rRNA gene (about 1.5 kbp). Results showed that the AlmWB phytoplasma was most closely related to members of pigeon pea witches'-broom phytoplasma group: pigeon pea witches'-broom phytoplasma (accession AF248957) (with 98.4% identity), *Picris echioides* yellows (accession Y16389) (with 98.7% identity), *Knautia arvensis* phyllody (accession Y18052) (with 99.0% identity), and Vigna little leaf phytoplasma (accession Y15866) (with 96.5% identity). However, it was distantly related to the other reported phytoplasmas infecting stone fruits such as peach X-disease, peach yellow leaf roll, and European stone fruit yellows (accession X68374) (less than 87% identity). Phylogenetic analysis of near-full-length 16S rRNA gene sequences from 62 representative phytoplasma strains and three *Acholeplasma* species yielded 624 equally parsimonious trees. All the trees resolved similar major phylogenetic groups or subclades identified in previous studies (26,33). A representative tree that most resembled the consensus tree was shown in Figure 7, indicating that AlmWB phytoplasma represents a distinct lineage within the pigeon pea witches'-broom subclade.

DISCUSSION

In this study, we have done an extensive survey on the spread of almond witches'-broom in three major almond production areas in Lebanon (Fig. 2). Recently, Choueiri et al. (8) reported the presence of almond witches'-broom only on a few trees in the Bekaa region. Our results indicated that almond witches'-broom has spread into all three regions (Bekaa, north, and south regions). PCR tests revealed the presence of phytoplasmas in all almond trees with the witches'-broom symptom (100% correlation) and in most trees where stunting, rosetting, and dieback were the main symptoms. Overall, 57% symptomatic samples tested positive for phytoplasma. We have found the detection sensitivity could be further improved by using the commercial kit Phytopure (Amersham Pharmacia, England) for DNA extraction.

RFLP and phylogenetic analyses of PCR-amplified 16S rDNA sequences showed that almond witches'-broom (AlmWB) in all three major almond-growing regions is caused by a new phytoplasma, consistent with the report by Choueiri et al. (8) that a phytoplasma related to PPWB phytoplasma was associated with almond witches'-broom disease. Based on RFLP analysis of 16S rDNA, the AlmWB phytoplasma was designated a new subgroup 16SrIX-B within PPWB phytoplasma group (16SrIX). AlmWB phytoplasma is only distantly related to the other reported phytoplasmas that infect stone fruit trees: ESFY and PYLR (belonging to members of 16SrX group), X-disease (16SrIII group), and CLY (16SrV group). The finding underscores the genetic diversity of phytoplasma strains that infect stone fruits. The diversity seems to correlate with the geographical distributions of the causal phytoplasmas.

The ESFY and other stone fruit phytoplasmas have been shown to infect apricot, peach, plum, almond, and cherry (11,12,17,22,25,26). These phytoplasmas have caused extensive damage in the production of peach, apricot, and plum in Europe and elsewhere (5,14,28,29). But the disease incidence in almond caused by these phytoplasmas is relatively limited (14,18,25,34). Our study has revealed that a lethal almond disease widespread in Lebanon is attributed to a new phytoplasma that has never been described to be associated with stone fruits grown elsewhere. As ESFY phytoplasma in Europe is involved in several stone fruit yellows diseases, in Lebanon AlmWB phytoplasma appears to also attack peach and nectarine seedlings in areas where almond witches'-broom is prevailing.

The rapid spread of the disease that affected young and old trees in both well-managed and completely neglected orchards (not plowed or pruned) suggested the involvement of an efficient aerial vector. However, during our survey, we were not able to find leafhoppers or psyllids on almond trees except for *Asymmetrasca* (*Empoasca*) sp. in one almond orchard in the south and on other stone fruit seedlings in nurseries in several regions. However, the majority of leafhoppers in this genus do not feed on phloem tissue and were not reported as vectors of phytoplasma. It is quite possible that the vector of almond witches'-broom feeds mainly on weeds and passes only transiently on the lower branches of almond trees. Therefore, it would be difficult to find the vector on almond trees. In Europe, the vector of ESFY was unknown until 1998, when Carraro et al. (6) reported that the plum tree psyllid *Cacopsylla pruni* was identified as the vector of this phytoplasma. But the psyllid may not likely be a vector candidate for AlmWB phytoplasma that is only distantly related to ESFY and related phytoplasmas (members of apple proliferation group or 16SrX). In Lebanon, over 150 species of leafhoppers were identified, and among them at least 10 species were reported as phytoplasma vectors in the literature (1,2). At the present time, we are attempting to trap different leafhopper species found in weeds or crops growing in the orchards and their vicinity.

Almond is an important nut crop grown worldwide; among major almond production countries are the United States (California), southern European countries, and central Asia. AlmWB phytoplasma poses a great threat to almond production due to rapid decline of affected almond trees, to its ability to rapidly spread by a yet un-

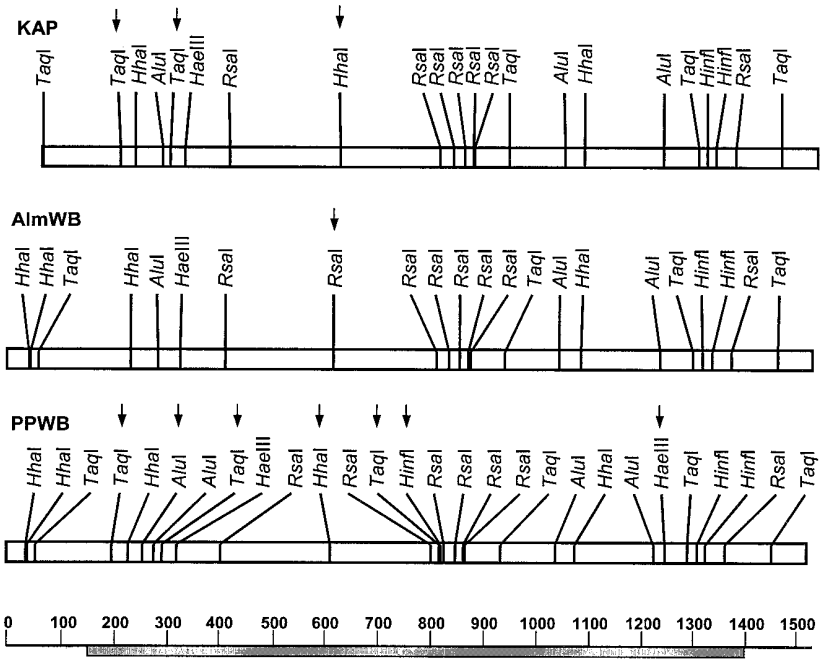

Fig. 6. Maps of putative restriction sites in the nucleotide sequence of 16S rRNA from *Knautia arvensis* phyllody (KAP), almond witches'-broom (AlmWB) and pigeon pea witches'-broom (PPWB) phytoplasmas. The solid bar on the scale represents the region amplified by primers R16F2n/R16R2. Arrows indicate the sites that are different between AlmWB phytoplasma and each of the two reference phytoplasma strains KAP (Y18052) and PPWB (AF248957).

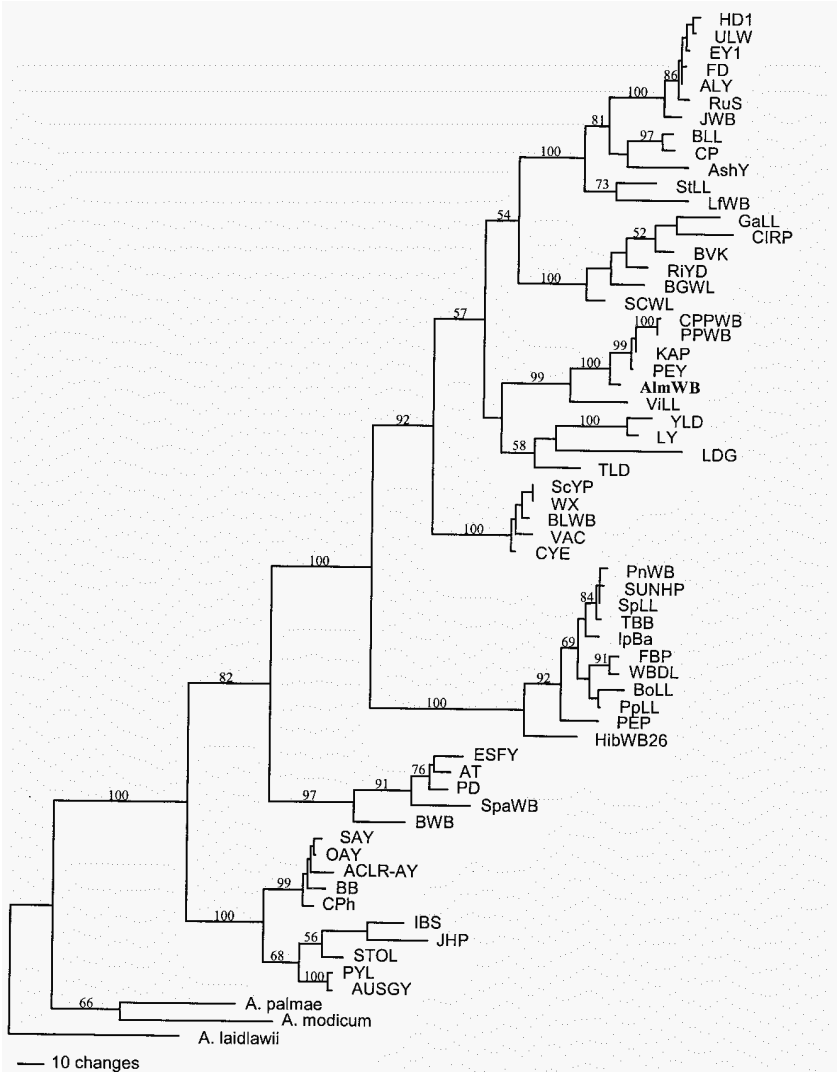

Fig. 7. Phylogenetic tree constructed by parsimony analysis of near-full-length 16S rDNA sequences from 62 phytoplasmas and three *Acholeplasma* spp., employing *A. laidlawii* as the outgroup. Branch lengths are proportional to number of inferred character state transformations. Bootstrap values (measures of support for inferred subclade) are shown on branches. Phytoplasma strain abbreviations and sequence accession numbers: HD1, hemp dogbane phytoplasma (AF122912); ULW, elm witches'-broom/France (X68376); EY1, elm yellows/America (AF189214); FD, Flavescence dorée (X76560); ALY, alder yellows (Y16387); RuS, rubus stunt (Y16395); JWB, jujube witches'-broom (AF305240); BLL, brinjal little leaf (X83431); CP, clover proliferation (L33761); AshY, ash yellows ("*Candidatus* Phytoplasma fraxini") (AF189215); StLL, stylosanthes little leaf (Y17055); LfWB, loofah witches'-broom (AF248956); GaLL, phytoplasma strain GaLL/Australia (Y15865); CIRP, cirsium phyllody (X83438); BVK, from leafhopper *Psammotettix cephalotes* (X76429); RiYD, rice yellow dwarf (D12581); BGWL, Bermuda grass white leaf (Y16388); SCWL, sugarcane white leaf (X76432); CPPWB, Chinese pigeon pea witches'-broom (AF028813); PPWB, pigeon pea witches'-broom (AF248957); KAP, *Knautia arvensis* phyllody (Y18052); PEY, *Picris echioides* yellows (Y16389); AlmWB, almond witches'-broom (AF390136); ViLL, vigna little leaf (AJ289195); YLD, Yucatan coconut lethal decline (U18753); LY, coconut lethal yellowing (U18747); LDG, coconut lethal yellowing/Ghanaian Cape; TLD, Tanzanian lethal decline of coconut (X80117); ScYP, sugarcane yellows phytoplasma (AF056095); WX, western X disease (U54992); BLWB, black locust witches'-broom (AF244363); VAC, vaccinium witches'-broom (X76430); CYE, clover yellow edge (AF175304); PnWB, peanut witches'-broom (L33765); SUNHP, sunn hemp witches'-broom (X76433); SpLL, sweet potato little leaf (AJ289193); TBB, Australian tomato big bud ("*Candidatus* Phytoplasma australasia") (Y08173); IpBa, *Ipomea batatis* (Y90591); FBP, faba bean phyllody (X83432); WBDL, witches'-broom of lime ("*Candidatus* Phytoplasma aurantifolia") (U15442); BoLL, phytoplasma strain BoLL/Australia (Y15863); PpLL, pigeon pea little leaf (AJ289191); PEP, *Picris echioides* phyllody (Y16393); HibWB26, hibiscus witches'-broom ("*Candidatus* Phytoplasma brasiliense") (AF147708); ESFY, European stone fruit yellows (X68374); AT, apple proliferation (X68375); PD, pear decline/Germany (X76425); SpaWB, spartium witches'-broom (X92869); BWB, buckthorn witches'-broom (AF244363); SAY, western severe aster yellows (M86340); OAY, *Oenothera* aster yellows (M30790); ACLR-AY, aster yellows from apricot (X68338); BB, tomato big bud/Arkansas (L33760); CPh, clover phyllody (L33762); IBS, Italian bindweed stolbur (Y16391); JHP, Japanese hydrangea phyllody ("*Candidatus* Phytoplasma japonicum") (AB010425); STOL, stolbur of pepper (X76427); PYL, phormium yellow leaf (U43569); AUSGY, Australian grapevine yellows ("*Candidatus* Phytoplasma australiense"); *Acholeplasma palmae* (L33734); *A. modicum* (M23933); *A. laidlawii* (M23932).

known vector, to its high pathogenicity to young and old trees, and to its adaptation to several microclimates, from the coastal region up to an elevation of about 1,000 m. Furthermore, we are now continuing to investigate the extent of cross-infection by AlmWB phytoplasma to other stone fruits and the damage that it may cause. Pest risk analysis shows that the potential economic impact of almond witches'-broom would be very high in the major almond producing countries if AlmWB phytoplasma and its vector are introduced. It is recommended that national, regional, and international action be taken to eradicate or prevent the further spread of this epidemic disease. However, for a successful eradication, it is important to identify the alternative hosts, the vector, and the mode of transmission. Our results call for a more strict certification and quarantine measures on the movement of stone fruit germ plasm. The diagnostic techniques developed for phytoplasma detection should be standardized and used routinely in certification of stone fruits.

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