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Molecular characterization of a phytoplasma of the ash yellows group (16Sr VII-B) occurring in *Artemisia annua* and *Conyza bonariensis* weeds

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SUMMARY

In South America, plants infecting phytoplasmas from different 16Sr groups have been detected and characterized. Among them, 16Sr VII (ash yellows) phytoplasmas have been detected in herbaceous plants. In Brazil, EriWB and RPWB isolates infecting *Erigeron* sp. and *Catharanthus roseus* have been assigned to a new 16Sr VII-B subgroup. In Argentina, 16Sr VII subgroup C was created to enclose the ArAWB phytoplasma which infects alfalfa (*Medicago sativa*) plants. Recently, new isolates from the 16Sr VII group phytoplasmas have been detected in *Artemisia annua* and *Conyza bonariensis* showing typical witches´ broom symptoms. The aim of this work was to perform a molecular characterization of these isolates in order to classify them and associate them to their closest relatives. A restriction analysis on PCR fragments (PCR-RFLP) from the 16S rRNA gene was performed. Sequences of three isolates were obtained to perform a cladistic analysis and to reveal their similarity to other phytoplasmas. Identical PCR-RFLP patterns, high sequence similarity (99.9%) and the cladistic grouping associated the new phytoplasma isolates infecting weeds in Argentina with the 16Sr VII-B subgroup. ArtWB strains represent geographic isolates of the EriWB phytoplasma. This work reveals the wide distribution of 16Sr VII-B phytoplasmas and their capability to infect different host plants.

Key words: Ash yellows phytoplasma, 16Sr VII, geographic distribution.

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RESUMEN

En Sudamérica se han detectado y caracterizado fitoplasmas de diferentes grupos 16Sr. Entre éstos, se detectaron fitoplasmas del grupo 16Sr VII (ash yellows) infec-

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tando diferentes especies de plantas herbáceas. En Brasil, se reportaron los aislamientos EriWB y RPWB infectando *Erigeron* sp. y *Catharanthus roseus*, los que fueron asignados al nuevo subgrupo 16Sr VII-B. En la Argentina, el subgrupo 16Sr VII-C fue creado para incluir al fitoplasma ArAWB que infecta alfalfa (*Medicago sativa*). Nuevos aislamientos de fitoplasmas del grupo 16Sr VII se han detectado en *Artemisia annua* y *Conyza bonariensis* en la Argentina, mostrando típicos síntomas de "escoba de bruja". El objetivo de este trabajo fue caracterizar molecularmente a estos aislamientos para clasificarlos y asociarlos a los fitoplasmas más relacionados. Se analizaron patrones de restricción (PCR-RFLP) del gen 16S de ARNr y se obtuvieron secuencias de tres aislamientos para realizar un análisis cladístico y poder determinar la similitud con otros fitoplasmas. Idénticos patrones de PCR-RFLP, la alta similitud de secuencias (99,9%) y el agrupamiento cladístico resultante asociaron a estos fitoplasmas presentes en malezas en Argentina (ArtWB), al subgrupo 16Sr VII-B. Estos aislamientos representan aislamientos geográficos del fitoplasma EriWB. Este trabajo revela la amplia distribución geográfica de los fitoplasmas del subgrupo 16Sr VII-B y su capacidad de infectar diferentes hospedantes.

Palabras clave: Ash yellows fitoplasma, 16Sr VII, distribución geográfica.

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INTRODUCTION

Phytoplasmas are phytopathogenic wall-less prokaryotes belonging to the Class Mollicutes. They inhabit the phloem tissue of infected plants and cause hundreds of plant diseases in taxonomically unrelated hosts (Mc Coy *et al*., 1989). In nature, phytoplasmas are transmitted by hemipterans, mainly Cicadellidae, which feed on phloem sap (Tsai, 1979). The current classification is primarily based on the analysis of restriction patterns (PCR-RFLP) and nucleotide sequences of highly conserved genomic regions, such as the ribosomal RNA, ribosomal proteins and Tu elongation factor genes. By means of the analysis of restriction patterns of the 16S rRNA gene, fifteen phytoplasma "16Sr" groups have been established, these comprise more than 40 subgroups. This classification coincides, in general, with that resulting from the phylogenetic analysis of the gene sequences mentioned above (Lee *et al*., 1998; Seemüller *et al*., 1998; Lee *et al*., 2000; Lee *et al*., 2004). During the last years, phytoplasmas from different 16Sr groups have been detected and characterized in South America, these groups infect cultivated plant species (Montano *et al*., 2000; Galdeano *et al*., 2004; Conci *et al*., 2005; Jones *et al*., 2005; Galvis *et al*., 2007), ornamental species (Montano *et al*., 2001; Harrison *et al*., 2003; Torres *et al*., 2004) and weeds (Barros *et al*., 2002). Phytoplasma infections can produce severe symptoms in plants and can cause a rapid host death (Harrison *et al*., 1999; Padovan & Gibb, 2001) in some pathologies.

Conyza bonariensis (L.) Cronquist (synonym: *Erigeron bonariensis*) and *Artemisa annua* L. are herbaceous species of the Asteraceae family which grow as weeds in many crops of Argentina. While *C. bonariensis* is a cosmopolitan species native of South America, *A. annua* comes from Asia and has naturalized throughout diverse regions of America and Europe (Zuloaga & Morrone, 1999). In recent years, phytoplasma infection was reported in several *C. bonariensis* and *A. annua* plants showing yellowing and witches'-broom symptoms (Torres *et al*, 2002). Infected plants have been found in peach orchards and vegetable crops in the province of Córdoba, where no phytoplasma infection was detected

in the crops. *C. bonariensis* plants with the same severe symptomatology were also observed in alfalfa (*Medicago sativa* L.) plots in the province of San Juan. In this area, the alfalfa crop is affected by the ArAWB (16Sr VII-C) phytoplasma, which induces witches´ broom symptoms (Conci *et al.*, 2005). Diseased plants develop severe symptoms as crop ages and can reach a leafless stunted aspect, although up to the present there is no record of crop losses due to this pathology.

Considering that infected weeds can be pathogens and vector reservoirs and, given the wide distribution of *A. annua* and *C. bonariensis*, it became necessary to study the etiological agent of the diseases observed in order to establish whether these species represent a potential risk for the crops. The objectives of the present work were to determine the causal agent of the observed symptomatology, and to identify and characterize the pathogen detected in *A. annua* and *C. bonariensis* plants collected from diverse ecological regions of Argentina.

MATERIALS AND METHODS

Phytoplasma DNA source and PCR amplification

Phytoplasma DNA were obtained from *A. annua* and *C. bonariensis* plants with witches'-broom symptoms, collected from vegetable, fruit and alfalfa fields from Monte Cristo, Colonia Caroya, Jesús María (Córdoba) and Guanacache (San Juan). Total DNA was extracted from 21 symptomatic plants (*A. annua,* n=4; *C. bonariensis,* n=17), according to the methodology proposed by Doyle & Doyle (1990). Quality and concentration of the purified nucleic acid were determined by 1% agarose gel electrophoresis in 1X TAE buffer. Phytoplasma presence was determined by PCR with P1/P7 universal primers for phytoplasmas (Deng & Hiruki, 1991; Schneider *et al.*, 1995), which amplify a fragment of 1.8 kb that comprises 16S rDNA gene, 16S-23S intergenic spacer region and 23S rDNA gene, 5' end. PCR reaction was performed in a Biometra TRIO-Thermoblock programmed under the following conditions: 35 cycles of 30 sec denaturizing at 95 °C (3 min for the first cycle), 30 sec at 55 °C and 1 min 40 sec polymerization at 72 °C. DNA from healthy plants was used as negative control for both species. DNA from Ash Y1^T (16Sr VII–A, provided by Dr. E. Seemüller) and ArAWB (16Sr VII–C) (Conci *et al.*, 2005) phytoplasmas was used as reference strain. For the PCR-RFLP analysis, DNA was amplified by nested PCR. Primer pair P1/P7 was used for the first reaction under the above mentioned conditions, PCR product was diluted to 1:100, from which 1 µL was used as target DNA for the following round. For the latter, primers R16F2/R16R2, which are universal for phytoplasmas and amplify a 1.2 kb fragment corresponding to 16S rRNA gene (Lee *et al.*, 1993), were used. Cycling conditions were: 35 cycles of 1 min at 94 °C (3 min for the first cycle), 2 min at 55 °C and 2 min at 72 °C.

Restriction pattern analysis

In order to establish the relationship among these new isolates of phytoplasmas detected in weeds in Argentina (named as artemisia witches´ broom phytoplasma, or ArtWB), the restriction patterns generated by digestion of the 1.2 kb fragments (Lee *et al.*, 1998) of diseased samples were analyzed and compared with those from the reference strains. Digestions for the PCR-RFLP analysis were performed independently with restriction enzymes *Alu*I, *EcoR*I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Mse*I, *Rsa*I, *Sau*3AI and *Taq*I, (New England Biolabs, Beverly, MA, USA), according to the manufacturer's instructions. Digestion products from each reaction were analyzed by 1.5% agarose (Biodynamics SRL, Argentina) + 0.5% Metaphor (BioWittaker Molecular Applications, Rockland, ME, USA) gel, or 8% acrilamide-bisacrilamide (29:1) gel to improve resolution of small or similar size bands. In both cases electrophoresis was performed in 0.5X TBE buffer and DNA was visualized under UV light after staining with ethidium bromide.

Sequence and phylogenetic analysis

With the aim of establishing the relationships among ArtWB and other previously described phytoplasmas, the nucleotide sequence of three isolates (ArtWB I present in *A. annua*; ArtWB II and ArtWB III detected in different *C. bonariensis* samples) were analyzed. For such analysis, the 1.8 kb ribosomal DNA fragment, amplified from each isolate, was cloned into a Bluescript II SK+ plasmid (Stratagene, La Jolla, USA) which was later used to transform *Escherichia coli* DH5a competent cells. From each isolate, one clone was selected to be sequenced by automatic services. The resulting sequences were deposited in data banks under the following accession numbers: ArtWB-I DQ989178; ArtWB-II DQ989179; ArtWB-III DQ989180, and compared with those of other phytoplasmas present in the GenBank (http://www.ncbi.nlm.nih.gov). Sequence similarity among the three phytoplasma isolates and members of groups 16Sr VII (LWB3, AshY1, AshY3, AshY5, RPWB, EriWB and ArAWB), 16Sr VI (BLL) and 16Sr V (FD and EY1), was obtained after the multiple alignment of a 1700 nucleotide region from

the ribosomal RNA operon (Table 1). The alignment was performed using the *Clustal W* option of the program MegAlign (Lasergene Biocomputing software DNASTAR ver. 5, 2001; Madison, WI). The same alignment procedure, in which 19 taxa were included, was followed by the phylogenetic analysis. The shortest tree was searched for by the parsimony principle using the NONA program (Goloboff, 1993). The data set resulting from the sequence alignment was reduced to 244 informative characters, and a heuristic search routine was carried out with the WinClada program (Nixon, 2002). The program parameters applied to reconstruct the phylogeny were as follows: hold 1000, 100 repetitions (mult*100), 5 starting trees per replication (hold/5), random start (random seed=0) and multiple TBR+TBR (mult*max*). A consensus cladogram was generated by the majority rule and a bootstrapping re-sample strategy was followed to establish the node support (1000 repetitions, 10 searches per replication, 5 starting trees per replication, keeping a maximum of 100 trees, with random start and the command "*Don´t do max**"). The cladograms were drawn using WinClada.

RESULTS AND DISCUSSION

In this work, an extended characterization of the phytoplasmas that cause witches'-broom symptoms in *A. annua* and *C. bonariensis* weeds was carried out. PCR amplification of 1.8 kb DNA fragments was obtained from both species showing witches'-broom symptoms when P1/P7 primers were used. Most *A. annua* DNA samples inhibited the PCR amplification, this was achieved with only one of the four collected plants. The purified DNA from all of the *C. bonariensis* plants was amplified without difficulty. No amplification was obtained from asymptomatic plants (results not shown).

The RFLP analysis of the 1.2 kb fragments (R16F2/R16R2) led us to include ArtWB phytoplasma within group 16Sr VII since restriction patterns generated from the digestion with enzymes *Hae*III, *Hpa*II, *Rsa*I, *Hpa*I, *EcoR*I and *Sau3*AI were identical to those of the type isolates AshY1^T (16Sr VII-A) and ArAWB (16Sr VII-C) (data not shown). The patterns of *Hha*I and *Hinf*I enzymes were identical to those of subgroup VII-A, but they were different from the patterns of subgroup VII-C phytoplasmas. On the contrary, the profiles generated by *Alu*I and *Mse*I were identical to those of subgroup VII-C but different from those of subgroup VII-A. Bands generated from the digestion with *Taq*I were different from both VII-A and VII-C subgroups (Figure 1). Restriction patterns of all five enzymes corresponded to those published for subgroup VII-B (Barros *et al.*, 2002; Table 2).

Regarding the nucleotide sequence and the plant

Table 1. Phytoplasma isolates used in this work. GenBank accession numbers and analyzed nucleotide positions are indicated.

Acronym	Name	Accession number	Analyzed region
A. modicum	*Acholeplasma modicum*	M23933	43-1473
A. palmae	*Acholeplasma palmae*	L33734	1-1457
BLWB	black locust witches'-broom	AF244363	36-1746
ChWBIII	chayote witches'-broom	AF147706	36-1744
ChTDIII	china-tree decline	AY081817	36-1745
GDIII	garlic decline	AY081816	25-1730
EY1	elm yellows	AF189214	36-1784
FD	flavescence doree	X765660	3-1747
BLL	brinjal little leaf	X83431	34-1740
ArAWB	Argentinean alfalfa witches´ broom	AY147038	35-1749
RPWB	erigeron witches'-broom phytoplasma isolate RPWB	AF411592	33-1748
EriWB	erigeron witches´ broom	AY034608	36-1751
AshY3	ash yellows 3	AF105315	1-1722
AshY5	ash yellows 5	AF105316	1-1722
AshY1	ash yellows 1	AF092209	1-1722
LWB3	lilac witches´ broom	AF105317	1-1722
ArtWB-I	**artemisia witches´ broom I**	**DQ989178**	**36-1751**
ArtWB-II	**artemisia witches´ broom II**	**DQ989179**	**36-1751**
ArtWB-III	**artemisia witches´ broom III**	**DQ989180**	**35-1750**

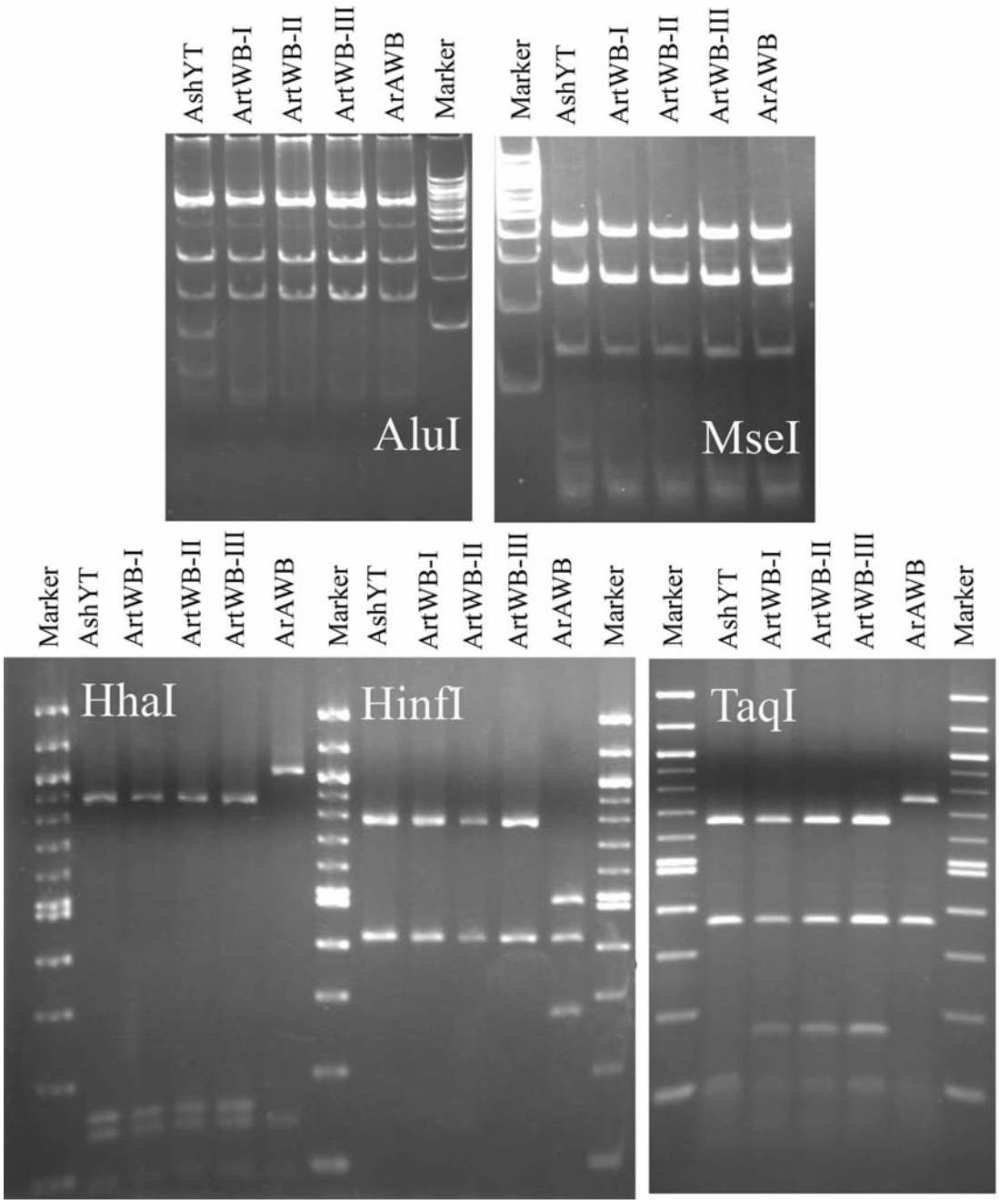

Figure 1. RFLP analysis of 1.2 Kb fragments amplified by nested PCR (P1-P7/ R16F2-R16F2 primers), digested with enzymes *Alu*I, *Mse*I, *Hha*I, *Hinf*I and *Taq*I. M: molecular weight marker (100, 200, 300, 400, 500, 512,600, 700, 800, 900, 1000, 1200 and 1517 bp).

host species, group 16Sr-VII (ash yellows) includes very heterogeneous organisms. Their members' geographical distribution is restricted to the Americas, except for the report of a phytoplasma infecting *Hypericum perforatum* in Italy, which is possibly related to the ash yellows group (Bruni *et al.*, 2005). The subgroup 16Sr VII-A phytoplasmas are responsible for decline symptoms only in woody species of *Fraxinus* and *Syringa* in North America (Griffiths *et al.*, 1999), and have recently been reported to infect grapevines (*Vitis vinifera* L.) in Chile (Gajardo *et al.,* 2005). However, the best characterized phyto-

plasmas belonging to 16Sr VII group in South America correspond to subgroups VII-B and VII-C, and are associated to witches'-broom symptoms of herbaceous species. In Brazil, Barros *et al.* (2002) proposed phytoplasmas EriWB (Erigeron witches´ broom phytoplasma) and RPWB (Rio das Pedras witches´ broom phytoplasma), detected in *Erigeron sp.* and *Catharanthus roseus* (L.) G. Don. plants respectively as members of the new subgroup 16Sr VII-B. In Argentina, witches'-broom symptoms of alfalfa were related to the infection with ArAWB (Argentinian alfalfa witches´- broom) phytoplasma which belongs to group 16Sr VII, subgroup C (Conci *et al.*, 2005). The DNA sequence comparison of isolates ArtWB-I, ArtWB-II and ArtWB-III with those of members of the 16Sr VII group showed the highest similarity with the sequences of subgroup VII-B EriWB and RPWB phytoplasmas (99.9%) followed by subgroup VII-C ArAWB phytoplasma with 98%, and subgroup VII-A members with 97.7% (Table 3). The cladistic analysis through heuristic search resulted in two shortest trees (358 steps), which were resolved in a consensus cladogram (359 steps) with a retention index of 91 (Ri) and a consistency index of 81 (Ci) (Figure 2). The general topology of the resulting cladogram was coincident with current classification schemes (Lee *et al.*, 1998; Seemüller *et al.*, 1998; Griffiths *et al.*, 1999; Barros *et al.*, 2002; Conci *et al.*, 2005). The isolates from North America (subgroup VII-A) were separated with a high bootstrapping support (99%) from those present in South America (subgroups VII-B and VII-C). All the new ash yellows isolates reported in this paper affect herbaceous plants, as were those previously reported in Argentina and Brazil. These findings increase the evidence of differences between subgroup VII-A

Table 2. Restriction patterns that showed differences among 16Sr VII-ash yellows phytoplasma subgroups, generated by the digestion of the 1.2 Kb fragments of the 16S rRNA gene, with enzymes *Taq*I, *Alu*I, *Mse*I, *Hha*I and *Hin*fI.

Phytoplasma	Subgroup	Geographical distribution	Host	Restriction patterns				
				*Taq*I	*Alu*I	*Mse*I	*Hha*I	*Hin*fI
ArtWB-I	**VII-B**	**Argentina**	***Artemisia annua***	**2**	**2**	**2**	**1**	**1**
ArtWB-II	**VII-B**	**Argentina**	***Conyza bonariensis***	**2**	**2**	**2**	**1**	**1**
ArtWB-III	**VII-B**	**Argentina**	***Conyza bonariensis***	**2**	**2**	**2**	**1**	**1**
*EriWB	VII-B	Brazil	*Erigeron* sp.	2	2	2	1	1
*RPWB	VII-B	Brazil	*Catharanthus roseus*	2	2	2	1	1
ArAWB	VII-C	Argentina	*Medicago sativa*	3	2	2	2	2
AshY1	VII-A	USA	*Fraxinus* sp.	1	1	1	1	1

(*) Taken from Barros *et al.* (2002).

Table 3. Sequence similarity of ArtWB I, II and III isolates compared with other members of group 16Sr VII (EriWB, RPWB, ArAWB, AshY3, AshY5, AshY1, LWB3), 16Sr VI (BLL) and 16Sr V (EY1, FD).The analysis was performed over a 1750 nucleotide multiple alignment with ClustalW (DNASTAR Lasergen, 2001).

	ArtWB-I	ArtWB-II	ArtWB-III	EriWB	RPWB	ArAWB	AshY3	AshY5	AshY1	LWB3	BLL	EY1	FD
ArtWB-I	***												
ArtWB-II	99.9	***											
ArtWB-III	99.9	99.9	***										
EriWB	99.9	99.9	99.9	***									
RPWB	99.9	99.9	99.9	99.9	***								
ArAWB	98.0	98.1	98.0	98.0	98.0	***							
AshY3	97.7	97.8	97.7	97.7	97.7	99.6	***						
AshY5	97.7	97.8	97.5	97.7	97.7	97.0	99.8	***					
AshY1	97.7	97.7	97.7	97.7	97.7	96.9	99.7	99.9	***				
LWB3	97.5	97.5	97.5	97.5	97.5	96.7	99.5	99.7	99.8	***			
BLL	95.3	95.4	95.3	95.3	95.3	95.3	95.1	95.2	95.3	95.0	***		
EY1	92.7	92.8	92.7	92.7	92.7	92.8	92.8	92.9	92.7	92.7	92.5	***	
FD	92.7	92.7	92.7	92.7	92.7	92.7	92.8	92.9	92.7	92.7	92.5	98.8	***

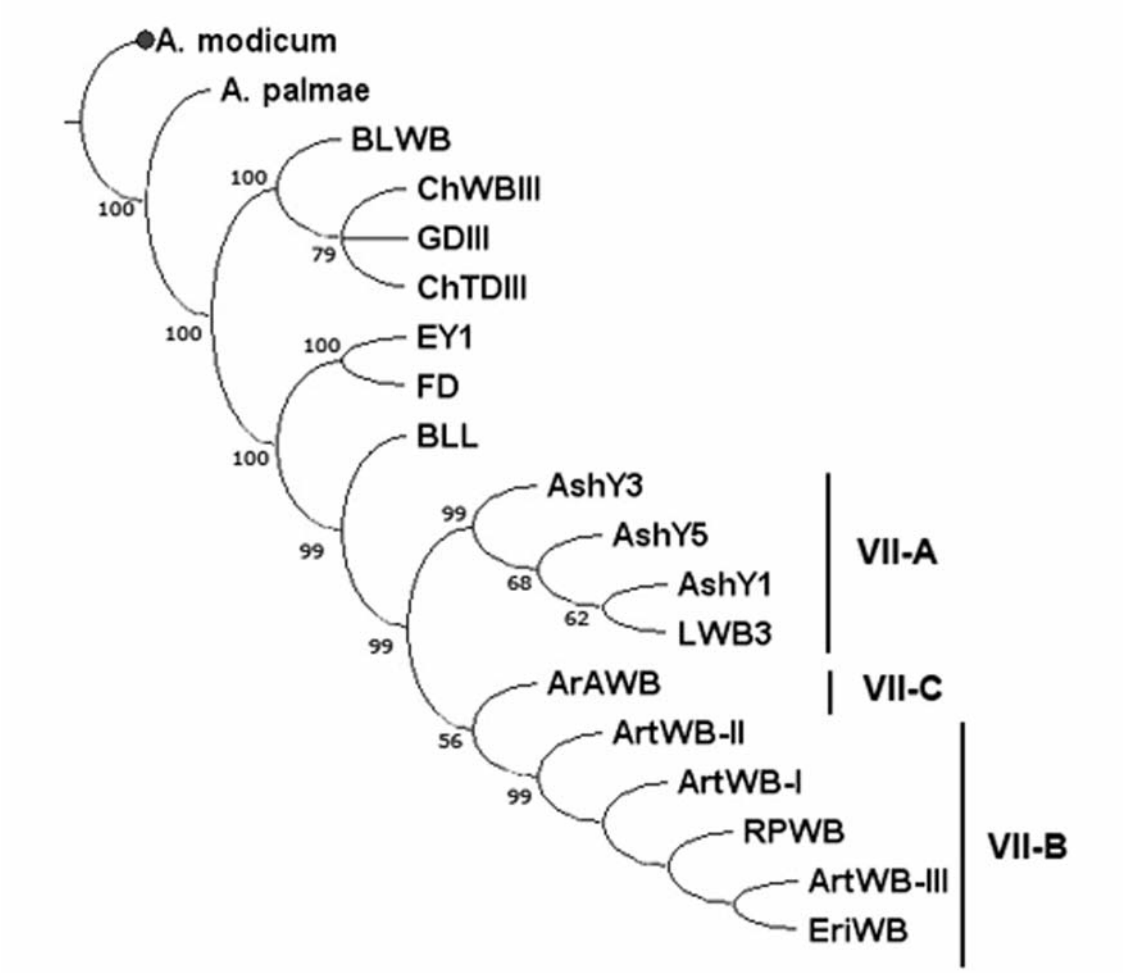

Figure 2. Consensus cladogram generated by the majority rule from two equally parsimonious trees constructed with program NONA. The values shown on each node indicate their support (bootstrapping). Phytoplasma isolates from group 16Sr VII (ash yellows) are pointed out.

phytoplasmas and those of subgroups VII-B and VII-C.

The 16S ribosomal RNA gene has been extensively used for evolution relationships analysis among organisms and has become the basis of the phytoplasma taxonomy (Lee *et al.*, 1998; Seemüller *et al.*, 1998). However, Cummings & Meyer (2005) have pointed out that the phylogenetic relationships obtained from molecular data from a unique gene might result in weak inferences; therefore, the exploration of new genomic regions would be useful to better resolve the grouping of phytoplasmas with a high 16S DNA sequence homology. In this sense, the analysis of the PCR-RFLP patterns of ribosomal proteins and transcription factor genes has led to a more precise classification system (Lee *et al.*, 2004). Such is the case of the identification of at least ten subgroups within the 16Sr I group (aster yellows), which is apparently highly homogeneous with regard to the 16S rRNA gene (Lee *et al.*, 2000; Marcone *et al.*, 2000). It would be interesting to use this analysis strategy to extend the characterization of subgroup VII-B isolates, for which no additional differentiation has been achieved based on the ribosomal RNA operon.

Artemisia annua L. represents a new host species for VII-B subgroup phytoplasmas, in addition to *Conyza bonariensis* (synonym: *Erigeron bonariensis*) and *Catharanthus roseus* which have been reported in Brazil (Barros *et al.*, 2002). Genus *Conyza* seems to be a suitable host for phytoplasmas since *Conyza* species infected not only with ash yellows, but also with X-disease and aster yellow group phytoplasmas have already been reported (Lee *et al.*, 1994; Bian-

chini & Bedendo, 2000; Guzmán, pers. comm., 2005). In Argentina, *A. annua* and *C. bonariensis* are weeds frequently found in many kinds of crops, such as vegetables, fruits and alfalfa, from which the plants reported in this work came. Interestingly, none of the *C. bonariensis* samples associated with alfalfa fields from the endemic area of witches´ broom disease were infected with the ArAWB phytoplasma (16Sr VII-C) which is, until now, the only phytoplasma present within this crop. A similar situation has been reported by Padovan & Gibb (2001) in papaya crops in Australia, where the percentage of weeds infected by the same pathogen as the papaya plants was very low, suggesting that such weeds would not be the source of inoculum. The coexistence of different phytoplasmas in the same plot, infecting crops or weeds, suggests that specific vectors to each pathogen could exist. For this reason, the search for vectors is an important issue to address in order to understand the potential risk of weeds infected with phytoplasmas in close association with crops.

The results presented in this work extend the knowledge of phytoplasmas belonging to VII-B subgroup, demonstrating its wide geographical distribution in South America. Diverse climatic conditions exist from the subtropical center-east of Brazil (San Pablo district), through the semiarid temperate center of Argentina (Córdoba) to the arid west (San Juan). This raises the question whether there is more than one vector insect, or only one species with a wide geographical distribution and polyphagous feeding habit.

CONCLUSIONS

Artemisia annua L. is a new host species for VII-B subgroup phytoplasmas, in addition to *Conyza bonariensis* (*Erigeron bonariensis*) and *Catharanthus roseus*. The results obtained in this work suggest that ArtWB phytoplasmas represent geographic isolates of EriWB phytoplasma (16Sr VII-B). Also, the results demonstrate that phytoplasmas belonging to subgroup VII-B are present in a wider geographical area of South America than it was initially thought. To date, 16Sr VII-B phytoplasmas are only detected in herbaceous hosts.

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