'*Candidatus* Phytoplasma brasiliense', a new phytoplasma taxon associated with hibiscus witches' broom disease

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***Hibiscus rosa-sinensis* is a valuable ornamental species widely planted in Brazil. Many plants are affected by witches' broom disease, which is characterized by excessive axillary branching, abnormally small leaves, and deformed flowers, symptoms that are characteristic of diseases attributed to phytoplasmas. A phytoplasma was detected in diseased *Hibiscus* by amplification of rRNA operon sequences by PCRs, and was characterized by RFLP and nucleotide sequence analyses of 16S rDNA. The collective RFLP patterns of amplified 16S rDNA differed from the patterns described previously for other phytoplasmas. On the basis of the RFLP patterns, the hibiscus witches' broom phytoplasma was classified in a new 16S rRNA RFLP group, designated group 16SrXV. A phylogenetic analysis of 16S rDNA sequences from this and other phytoplasmas identified the hibiscus witches' broom phytoplasma as a member of a distinct subclade (designated subclade xiv) of the class *Mollicutes*. A phylogenetic tree constructed on the basis of 16S rRNA gene sequences was consistent with the hypothesis that there was divergent evolution of hibiscus witches' broom phytoplasma and its closest relatives (members of 16S rRNA RFLP group 16SrII) from a common ancestor. On the basis of unique properties of the DNA from hibiscus witches' broom phytoplasma, it is proposed that it represents a new taxon, namely '*Candidatus* Phytoplasma brasiliense'.**

Keywords: phytoplasma, classification, 16S rDNA

INTRODUCTION

Botanical species belonging to the genus *Hibiscus* are widely distributed in the Brazilian landscape and are employed both as small trees and as shrubs (Penna, 1983; Blossfeld, 1984). However, a disease attributed to a phytoplasma has become a major problem in *Hibiscus* culture. In Brazil, witches' broom disease of *Hibiscus* was first reported in São Paulo State in plants of *Hibiscus rosa-sinensis* L. The disease was characterized by symptoms of witches' broom, i.e. leaf yellowing and malformation, as well as by short internodes (Vicente *et al.*, 1974). Later, the disease was

observed, in the State of Rio de Janeiro, in plants of the same species; they displayed similar symptoms and premature dropping of flowers (Davis, 1995; Kitajima, 1994; Kitajima *et al.*, 1984). The disease has also been observed in naturally infected plants of *H. rosa-sinensis* in Brasília, Distrito Federal (P. S. T. Brioso, unpublished). In Australia, an unidentified phytoplasma has been reported to be associated with a witches' broom disease of *Hibiscus heterophyllus*, an Australian native species that is also grown commercially (Hiruki, 1987).

In the present study, we investigated a phytoplasma associated with hibiscus witches' broom disease in Brazil (Montano & Brioso, 1997). The results have defined the relationship between this phytoplasma and phytoplasmas representing '*Candidatus* Phytoplasma species' and putative species worldwide. In this communication, we report results from the amplification of rRNA operon sequences from hibiscus witches' broom

Abbreviations: AY, aster yellows; CX, Canadian peach X; CYE (= CYE-C), clover yellow edge; EY, elm yellows; PPWB, pigeon pea witches' broom; PWB, potato witches' broom; SPWB, sweet potato witches' broom.

The GenBank accession number for the phytoplasma strain HibWB26 sequence determined in this study is AF147708.

phytoplasma templates by the use of phytoplasma-specific PCRs and the cloning and analysis of amplified DNA. The results include RFLP analyses of 16S rDNA and interpretation of the findings in the context of a revised classification scheme (Lee *et al.*, 1998) that contains 14 16S rRNA gene groups, each of which represents at least one putative *Phytoplasma* species (Gundersen *et al.*, 1994). We also report, for the first time, the nucleotide sequence of a segment of the hibiscus witches' broom phytoplasma rRNA operon, and describe sequences that are unique in this phytoplasma. Our data led us to propose that the hibiscus witches' broom phytoplasma is taxonomically unique among known phytoplasmas.

METHODS

Plant samples and reference phytoplasma strains. Symptomatic leaves of *H. rosa-sinensis* L. (*Malvaceae*) were collected in the state of Rio de Janeiro from two naturally infected

plants showing witches' broom symptoms. Reference phytoplasma strains representing various 16S rDNA RFLP groups were used in direct comparisons of RFLP patterns obtained in this study. These references and their 16S rRNA group affiliations were as follows: aster yellows (AY; group 16SrI), sweet potato witches' broom (SPWB; group 16SrII), Canadian peach X (CX; group 16SrIII), clover yellow edge (CYE = CYE-C) from Canada (group 16SrIII), elm yellows (EY; group 16SrV), potato witches' broom (PWB; group 16SrVI) and pigeon pea witches' broom (PPWB; group 16SrIX) phytoplasmas. Group affiliations are designated according to the classification system of Lee *et al.* (1998). Phytoplasmas AY, SPWB, CX, CYE-C, EY, PWB and PPWB were maintained by graft transmission in plants of periwinkle [*Catharanthus roseus* (L.) G. Don] maintained in a greenhouse.

Nucleic acid extraction and PCR. Leaf midribs or whole small leaves (1 g) were excised from diseased plants of *H. rosa-sinensis*. DNA was extracted according to Montano *et al.* (2000) for *Hibiscus* and according to Davis *et al.* (1997) for periwinkle. Nested PCRs primed by phytoplasma universal

Table 1. GenBank accession numbers of phytoplasma 16S rRNA gene sequences used in this study

Phytoplasma	Accession no.	Reference
Hibiscus witches' broom (HibWB26)	AF147708	This paper
Michigan aster yellows (MIAY)	M30970	Lim & Sears (1989)
Peanut witches' broom (PnWB)	L33765	Gundersen <i>et al.</i> (1994)
Sweet potato witches' broom (SPWB)	L33770	Gundersen <i>et al.</i> (1994)
Sunn hemp witches' broom (SUNHP)	X76433	Schneider <i>et al.</i> (1995a)
Witches' broom disease of lime (WBDL) (<i>Candidatus</i> <i>Phytoplasma aurantifolia</i>)	U15442	Zreik <i>et al.</i> (1995)
Faba bean phyllody (FBP)	X83432	Schneider <i>et al.</i> (1995a)
Sweet potato little leaf (SPLL)	X90591	Padovan <i>et al.</i> (1996)
Italian alfalfa witches' broom (IAWB)	Y16390	Seemüller <i>et al.</i> (1998)
Picris echioides phyllody (PEP)	Y16393	Seemüller <i>et al.</i> (1998)
Papaya mosaic (PpM) (<i>Candidatus</i> <i>Phytoplasma australasia</i>)	Y10096	White <i>et al.</i> (1998)
Papaya yellow crinkle (PpYC) (<i>Candidatus</i> <i>Phytoplasma australasia</i>)	Y10097	White <i>et al.</i> (1998)
Western X-disease (WX)	L04682	Seemüller <i>et al.</i> (1994)
Canada peach X-disease (CX)*	L33733	Gundersen <i>et al.</i> (1994)
Clover yellow edge, Canada (CYE = CYE-C)	AF175304	Staniulis <i>et al.</i> (2000)
Chayote witches' broom [ChWBIII(Ch10)]	AF147706	Montano <i>et al.</i> (2000)
Chayote witches' broom [ChWBIII(Mor5)]	AF147707	Montano <i>et al.</i> (2000)
Coconut lethal yellows (LY)	U18747	Tymon <i>et al.</i> (1998)
Tanzanian coconut lethal disease (LD)	X80117	Tymon <i>et al.</i> (1998)
Elm yellows (EY1)	AF189214	Unpublished
Clover proliferation (CP)*	L33761	Gundersen <i>et al.</i> (1994)
Ash yellows (AshY1) (<i>Candidatus</i> <i>Phytoplasma fraxini</i>)	AF189215	Unpublished
Loofah witches' broom (LfWB)	L33764	Gundersen <i>et al.</i> (1994)
Pigeon pea witches' broom (PPWB)	U18763	Unpublished
Apple proliferation (AT)	X68375	Seemüller <i>et al.</i> (1994)
Rice yellow dwarf (RYD)	D12581	Namba <i>et al.</i> (1993)
Australian grapevine yellows (AUSGY) (<i>Candidatus</i> <i>Phytoplasma australiense</i>)	L76865	Davis <i>et al.</i> (1997)
Stolbur (STOL)	X76427	Seemüller <i>et al.</i> (1994)
<i>Acholeplasma laidlawii</i>	M23932	Weisburg <i>et al.</i> (1989)

* For analyses in the present work, sequences L33733 and L33761 were modified by insertion of corrected bases in place of n characters in the original GenBank sequences, and sequence L33733 was modified by correction of base assignments in positions 633 and 676–679 (R. E. Davis & E. L. Dally, unpublished).

Fig. 1. Left: naturally infected *Hibiscus rosa-sinensis* exhibiting symptoms of witches' broom. Right: *H. rosa-sinensis* showing no symptoms of disease.

primer pairs P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995b) and F2n/R2 (Gundersen & Lee, 1996) were carried out according to Jomantiene *et al.* (1998a).

RFLP analyses of amplified phytoplasma DNA. Products from nested PCRs primed by R16F2n/R2 were analysed by single restriction endonuclease digestion with *Alu*I, *Hha*I and *Kpn*I (Life Technologies) and *Bfa*I, *Dra*I, *Hae*III, *Hinf*I, *Hpa*I, *Hpa*II, *Mse*I, *Rsa*I, *Sau*3A1 and *Taq*I (New England Biolabs) according to the instructions of the manufacturers. The products of digestion were analysed by electrophoresis through a 5% polyacrylamide gel, followed by staining with ethidium bromide and visualization of DNA bands with a UV transilluminator. The DNA fragment size standard used was PhiX174 RF *Hae*III digest (Life Technologies). The RFLP patterns of phytoplasma DNAs were compared with previously published RFLP patterns (Davis *et al.*, 1997, 1998; Jomantiene *et al.*, 1998a, b; Lee *et al.*, 1993, 1998; Montano *et al.*, 2000).

Cloning of PCR products and sequencing of DNA. A phytoplasma DNA sequence that had been amplified in a PCR primed by P1/P7 was cloned in *Escherichia coli* by using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. The cloned 1.8 kb DNA fragment was sequenced by automated DNA sequencing. The sequence was assembled after a minimum of 2 × sequencing coverage for each base position. The GenBank accession number for the nucleotide sequence determined in this study is AF147708 (Table 1).

Nucleotide sequence accession numbers. The GenBank accession numbers of the DNA sequences from hibiscus witches' broom phytoplasma, 27 phytoplasma strains representing 12 16S rRNA phytoplasma groups, and *Acholeplasma laidlawii* used in this study are listed in Table 1.

Sequence similarity, similarity coefficient calculations and putative restriction site analysis. Alignments of sequences were generated and sequence similarities evaluated by using the DNASTAR program ALIGN option (DNASTAR). Putative restriction site maps were generated by using the DNASTAR program MAPDRAW option. Similarity coefficients (*F*) be-

tween the 16S rDNA of hibiscus witches' broom phytoplasma and the 16S rDNAs of other phytoplasmas were calculated according to Nei & Li (1979) on the basis of putative restriction site maps. *F* was calculated as $F = 2N_{xy}/(N_x + N_y)$, where *x* and *y* are the strains of two given phytoplasmas, *N_x* and *N_y* are the numbers of fragments resulting from digestion by 10 enzymes in strains *x* and *y*, respectively, and *N_{xy}* is the number of fragments shared by the two strains.

Phylogenetic analysis. The 16S rRNA gene sequences (1.2 kb in size) from hibiscus witches' broom phytoplasma (strain HibWB26), 20 phytoplasma strains representing 11 16S rRNA phytoplasma groups, and *A. laidlawii* were aligned using CLUSTAL X version 1.63b (Thompson *et al.*, 1997) and DNASTAR. Gaps and incomplete sequences were removed. Analyses were performed with the computer program CLUSTAL X. Phylogenetic trees were constructed by the neighbour-joining method and the tree was viewed by using TREEVIEWPPC (Page, 1996). *A. laidlawii* was selected as the outgroup to root the tree. Bootstrapping was performed 1000 times for estimation of stability and support for the clades.

RESULTS

Detection and classification of phytoplasma in naturally diseased *Hibiscus*

The diseased *Hibiscus* plants exhibited symptoms including witches' broom, leaf yellowing, malformation and reduced size of leaves, as well as short internodes and proliferation of axillary shoots (Fig. 1). Amplification of phytoplasma 16S rDNA in nested PCRs primed by phytoplasma universal primer pairs indicated that both of the diseased *Hibiscus* plants, designated plants 24 and 26, respectively, were infected by phytoplasmas (data not shown). These results are consistent with preliminary reports of phytoplasma detection in diseased *Hibiscus* plants in Brazil (Barros *et al.*, 1997, 1998; Montano & Brioso, 1997). The

Fig. 2. RFLP analysis of 16S rDNAs amplified in PCRs from naturally diseased plants of *H. rosa-sinensis* in Brazil. The first PCR was primed by primer pair P1/P7, and this was followed by reamplification of target DNA in a nested PCR primed by primer pair F2n/R2. Lane S: fragment size standard, PhiX174 RFI DNA *HaeIII* digest. Lanes AY, SPWB, CX, CYE, EY, PWB, PPWB: digests of DNAs from the reference phytoplasmas [aster yellows (AY), sweet potato witches' broom (SPWB), Canada X-disease (CX), clover yellow edge (CYE = CYE-C), elm yellows (EY), potato witches' broom (PWB) strain of clover proliferation phytoplasma, and pigeon pea witches' broom (PPWB)]. Hib24 and Hib26: digests of DNAs amplified from diseased plants of *H. rosa-sinensis*. (a) RFLP patterns after digestion with *AluI* and *MseI*. (b) RFLP patterns after digestion with *HpaII* and *RsaI*.

phytoplasma strains detected in plants 24 and 26 were designated strains HibWB24 and HibWB26, respectively.

Initially, strains HibWB24 and HibWB26 were characterized by RFLP analysis using four restriction enzymes (Fig. 2). The HibWB phytoplasma strains could not be distinguished from one another on the basis of *AluI*, *MseI*, *HpaII* and *RsaI* RFLP patterns of

the 16S rDNAs. The *AluI* RFLP pattern of 16S rDNA from the strains was very similar to the patterns of DNA from the CX and CYE (both of which are members of group 16SrIII) and SPWB (a member of group 16SrII) phytoplasmas but differed from the RFLP patterns of DNA from AY (group 16SrI), EY (group 16SrV), PWB (group 16SrVI) and PPWB (group 16SrIX) phytoplasmas (Fig. 2a). However, *MseI* and *HpaII* RFLP patterns of 16S rDNA from HibWB24 and HibWB26 differed from those of all reference phytoplasmas used in this study (Fig. 2, Table 2). The *MseI* and *HpaII* RFLP patterns of 16S rDNA from the HibWB phytoplasma strains were unique and have not been reported previously (Lee *et al.*, 1993, 1998; Davis *et al.*, 1997, 1998; Jomantiene *et al.*, 1998a, b; Montano *et al.*, 2000) for any phytoplasma. The *RsaI* RFLP pattern from HibWB24 and HibWB26 was indistinguishable from the pattern of 16S rDNA from PPWB (Fig. 2b).

Both strains were further characterized by RFLP analysis using an additional nine restriction enzymes, including *BfaI*, *DraI*, *HaeIII*, *HhaI*, *Sau3AI*, *TaqI*, *HinfI*, *HpaI* and *KpnI*. HibWB24 and HibWB26 were not distinguished from one another on the basis of RFLP patterns (Table 2). Fig. 3 shows the *BfaI*, *DraI*, *HaeIII*, *HhaI*, *Sau3AI* and *TaqI* RFLP patterns of DNA from strain HibWB26. RFLP patterns from the use of *HinfI*, *HpaI* and *KpnI* are not shown. A summary of all pattern groups produced by RFLP analysis of 16S rDNA from hibiscus witches' broom phytoplasma strains HibWB24 and HibWB26 is presented in Table 2. The two strains were mutually indistinguishable on the basis of collective RFLP patterns of 16S rDNA. Although the hibiscus witches' broom phytoplasma strains appeared to be similar in

Fig. 3. RFLP analysis of 16S rDNAs amplified in nested PCRs from two reference phytoplasma strains and strain HibWB26 from *H. rosa-sinensis* after digestion with *BfaI*, *DraI*, *HaeIII*, *HhaI*, *Sau3AI* and *TaqI*. Abbreviations are the same as for Fig. 1.

Table 2. Summary of pattern groups produced by enzymic RFLP analysis of 16S rDNA from strains of hibiscus witches’ broom and reference phytoplasmas

Strain abbreviations are as shown in Table 1. Designations of phytoplasma group and subgroup affiliations are according to Lee *et al.* (1998). I, II, III, V, VI, IX are 16S rRNA groups. Subgroups are in parentheses. Hibiscus witches’ broom phytoplasma strains are the only known members of group 16SrXV and are placed in subgroup A. Groups 16SrII and 16SrIX were included because RFLP patterns of 16S rDNA from hibiscus witches’ broom phytoplasma appeared to be most similar to these phytoplasmas. Group 16SrIII strains were included because of the recent description of group 16SrIII phytoplasma strains [ChWBIII(Mor5) and ChWBIII(Ch10)] in Brazil (Montano *et al.*, 2000). Groups 16SrI, 16SrV and 16SrVI were included for comparisons. Each RFLP pattern number represents a specific pattern obtained by using a given restriction endonuclease. The numbers are according to the system of Lee *et al.* (1998). Numbers in bold indicate RFLP patterns determined in this study. Italicized numbers indicate RFLP patterns not previously described. The remaining numbers are from Lee *et al.* (1998) and Montano *et al.* (2000).

Phytoplasma strain	16S rRNA group (subgroup)	RFLP pattern obtained with the restriction endonuclease indicated											
		<i>Mse</i> I	<i>Alu</i> I	<i>Bfa</i> I	<i>Hha</i> I	<i>Hpa</i> I	<i>Hpa</i> II	<i>Sau</i> 3A I	<i>Taq</i> I	<i>Rsa</i> I	<i>Dra</i> I	<i>Hae</i> III	<i>Hin</i> II
HibWB24	XV(A)	28	4	2	3	1	10	2	1	4	1	6	2
HibWB26	XV(A)	28	4	2	3	1	10	2	1	4	1	6	2
AY	I(B)	1	1	2	2	1	1	1	1	1	1	1	1
PnWB	II(A)	6	4	3	3	1	3	2	2	2	1	3	2
SPWB	II(A)	6	4	3	3	1	3	2	2	2	1	3	2
WBDL	II(B)	7	4	3	3	1	4	2	1	2	1	3	2
FBP	II(C)	7	4	3	3	1	3	2	1	2	1	3	2
SPLL	II(D)	6	5	—	—	—	3	—	—	2	—	—	—
CX	III(A)	8	6	1	1	1	5	3	1	2	1	4	2
CYE-C	III(B)	9	6	1	1	1	6	4	1	2	1	4	2
ChWBIII(Ch10)	III(J)	9	6	1	4	1	6	4	1	2	—	4	2
ChWBIII(Mor5)	III(J)	9	6	1	4	1	6	4	1	2	—	4	2
EY	V(A)	14	9	1	2	1	7	4	1	5	1	5	2
PWB	VI(A)	15	10	1	7	1	7	4	1	6	1	5	2
PPWB	IX(A)	17	12	1	9	1	7	4	4	4	2	6	4

terms of some RFLP patterns to members of group 16SrII (peanut witches’ broom phytoplasma group) and to PPWB, both strains were distinguished from all group 16SrII phytoplasmas and PPWB on the basis of collective RFLP patterns. The collective RFLP patterns of amplified 16S rDNA from the hibiscus witches’ broom phytoplasma strains also differed from the patterns described previously for other phytoplasmas. On the basis of the RFLP patterns, the hibiscus witches’ broom phytoplasma was classified in a new group, designated group 16SrXV. As the collective RFLP patterns indicated that the phytoplasma strains from diseased *Hibiscus* were mutually indistinguishable, strain HibWB26 was selected as being representative, and was used for further analysis.

Nucleotide sequence similarities

Sequence similarities between the 1.2 kb sequences of 16S rDNA (equivalent to fragments amplified in PCRs primed by R16F2n/R16R2) from the HibWB phytoplasma and phytoplasmas affiliated with group 16SrII, group 16SrIII (subgroups III-A and III-J) and group 16SrIX (PPWB phytoplasma) were determined. Strains in group 16SrII and the PPWB phytoplasma

were included because RFLP patterns of 16S rDNA from hibiscus witches’ broom phytoplasma appeared to be most similar to these phytoplasmas. Group 16SrIII strains were included because of the recent description of group 16SrIII phytoplasma strains [ChWBIII(Mor5) and ChWBIII(Ch10)] in Brazil (Montano *et al.*, 2000).

Sequence similarities between 16S rDNA from hibiscus witches’ broom phytoplasma and phytoplasmas within group 16SrII ranged from 94.4% (between HibWB26 and SPLL phytoplasma) to 96.4% (between HibWB26 and PpYC and PpM phytoplasmas). The sequence similarities between the 16S rDNAs of hibiscus witches’ broom phytoplasma and WX, ChWBIII(Mor5) and ChWBIII(Ch10) phytoplasmas (members of group 16SrIII) were 88.8, 89.0 and 89.2%, respectively. The sequence similarity between the 16S rDNAs of HibWB phytoplasma and PPWB was 87%. The sequence similarities between the 16S rDNAs of hibiscus witches’ broom phytoplasma and two phytoplasmas, *pichris echinoides* phyllody (PEP) and Italian alfalfa witches’ broom (IAWB) phytoplasmas, which are phylogenetically closely related to the group 16SrII phytoplasmas (Seemüller *et al.*, 1998) but which have not been classified in the 16S rRNA grouping system of Lee *et al.* (1998), were 96.4 and 96.2%, respectively.

Table 3. Similarity coefficients derived from RFLPs based on putative restriction site analysis of nucleotide sequences of 16S rDNAs of hibiscus witches’ broom phytoplasma and other selected phytoplasmas

Abbreviations are as in Table 1.

Phytoplasma	16S rRNA group affiliation	HibWB26	PnWB	SPWB	WBDL	FBP	SUNHP	SPLL	PpYC	CX	CYE	PPWB
HibWB26	XV	1.00										
PnWB	II	0.81	1.00									
SPWB	II	0.78	0.97	1.00								
WBDL	II	0.77	0.88	0.87	1.00							
FBP	II	0.80	0.93	0.92	0.97	1.00						
SUNHP	II	0.77	1.00	0.97	0.88	0.93	1.00					
SPLL	II	0.82	0.91	0.88	0.91	0.94	0.91	1.00				
PpYC	II	0.85	0.94	0.91	0.90	0.97	0.92	0.97	1.00			
CX	III	0.55	0.53	0.54	0.48	0.55	0.53	0.59	0.59	1.00		
CYE	III	0.58	0.50	0.51	0.50	0.54	0.50	0.63	0.60	0.88	1.00	
PPWB	IX	0.59	0.49	0.50	0.44	0.49	0.50	0.49	0.48	0.53	0.55	1.00

sequenced HibWB phytoplasma DNA were in excellent agreement with the fragment sizes obtained by enzymic RFLP analysis of amplified DNA. HibWB phytoplasma was distinguished by the presence, in sequence AF147708, of a *Bfa*I site that is absent in the 16S rDNA of all group 16SrII phytoplasmas mapped, and by the presence of an *Hpa*II site that is absent in sweet potato little leaf phytoplasma 16S rDNA. HibWB phytoplasma was further distinguished by the absence, in HibWB rDNA, of several putative restriction sites that are present in rRNA operon sequences of the group 16SrII phytoplasmas.

Similarity coefficients

Similarity coefficients derived from RFLPs were calculated on the basis of putative restriction site analyses of nucleotide sequences of 16S rDNA (Table 3). Hibiscus witches’ broom phytoplasma 16S rDNA was compared with that of phytoplasmas from 16S rRNA groups that were included in the analysis of sequence similarities described above. Examination of Table 3 as a whole reveals that coefficients of similarity among members of the same 16S rRNA RFLP group, for example group 16SrII, are no less than 0.87. The coefficient of similarity between the 16S rDNAs of strains CX and CYE in group 16SrIII is 0.88. The coefficients of similarity for 16S rDNAs between HibWB26 and 10 phytoplasma strains belonging to groups 16SrII, 16SrIII and 16SrIX ranged from 0.55 (between the HibWB26 and CX phytoplasmas) to 0.85 (between the HibWB26 and PpYC phytoplasmas). The coefficients of similarity for 16S rDNAs between HibWB and strains classified in group 16SrII ranged from 0.77 to 0.85. These results clearly distinguished HibWB phytoplasma from phytoplasmas of group 16SrIII (represented by CX and CYE phytoplasmas) and group 16SrIX, as well as from phytoplasmas classified in group 16SrII. The findings support rec-

ognition of the HibWB phytoplasma as representative of a new 16S rRNA gene group, 16SrXV, as designated above.

Phylogenetic analysis

Phylogenetic analysis of 21 phytoplasmas and *A. laidlawii* yielded the tree illustrated in Fig. 5. Strains from phytoplasmas belonging to 11 16S rRNA RFLP groups were included for comparison with the hibiscus witches’ broom (HibWB26) phytoplasma. The bootstrapping values strongly supported most branches (Fig. 5), indicating a robust tree whose branching order is in good agreement with previous findings (Gundersen *et al.*, 1994; Seemüller *et al.*, 1994; Davis *et al.*, 1997; White *et al.*, 1998). However, the hibiscus witches’ broom (HibWB26) phytoplasma gives rise to a new branch, designated subclade xiv (Fig. 5). This subclade designation builds upon previously published phylogenetic analyses in which 13 phytoplasma subclades were specified (Lee *et al.*, 1998). Although it is most closely related to peanut witches’ broom and related phytoplasmas, it is evident that hibiscus witches’ broom phytoplasma represents a new lineage.

Sequences unique to phytoplasmas in the 16S rRNA gene of hibiscus witches’ broom phytoplasma, strain HibWB26

The 16S rRNA gene sequence from the HibWB phytoplasma was aligned with sequences from 24 other phytoplasmas that represented the 12 16S rRNA RFLP groups for which sequences were available. This analysis revealed that the HibWB phytoplasma contains sequences unique to phytoplasmas and sequences that distinguish the HibWB phytoplasma from other phytoplasmas. Seven sequences previously reported to be unique to phytoplasmas (Gundersen *et al.*, 1994;

Fig. 5. Phylogenetic tree constructed using the neighbour-joining method with 16S rRNA gene sequences from 21 phytoplasmas and *Acholeplasma laidlawii*, employing *A. laidlawii* as the outgroup. The branch lengths are proportional to the numbers of inferred character state transformations. The numbers on the branches are bootstrap (confidence) values. Phylogenetic subclade xiv (identified in this study) is indicated. WB, witches' broom. Other abbreviations are the same as in Table 1.

Davis *et al.*, 1997), i.e. ACUGGA at positions corresponding to 153–158 in sequence AF147708, UUU-UAAAAG at positions 185–193, GUGU at positions 274–277, UGGAGG at positions 365–370, AUCAG at positions 1011–1015, UAGC at positions 1233–1236 and AGUU at positions 1310–1313, were found in the 16S rRNA of the HibWB phytoplasma. The GGCAAG sequence (corresponding to positions 651–656 in the AF147708 sequence) – another sequence unique to phytoplasmas (Gundersen *et al.*, 1994) – has G at position 654 in the 16S rRNA of the HibWB phytoplasma and at the corresponding position in the 16S rRNA of phytoplasmas classified in group 16SrII.

Five sequences were present only in the 16S rDNA of the HibWB phytoplasma and distinguished this organism from other phytoplasmas. The sequence 5'-CAG-3' at positions 575–577 of the HibWB DNA (AF147708) differed at two to three positions from corresponding sequences from phytoplasmas in other subclades, and the sequence 5'-ACTTTG-3' at

positions 630–635 differed from the sequences in other phytoplasmas at three to five positions. The sequence 5'-GAAAAAGAAAG-3' at positions 162–172 differed from other sequences at one to five base positions, the sequence 5'-TCTTTCTTT-3' at positions 176–184 differed from other sequences at two to five base positions, and the sequence 5'-GTCAAAAC-3' at positions 822–829 differed from the sequences in other phytoplasmas at one to four base positions.

DISCUSSION

This communication provides evidence that a previously undescribed phytoplasmal organism is associated with hibiscus witches' broom disease in Brazil. Amplification of phytoplasma-characteristic DNA fragments from diseased plants indicated the association of a phytoplasma with the disease, and the presence in the 16S rDNA of sequences unique to phytoplasmas established that the organism was a phytoplasma. The data also indicated that this phytoplasma is unique among all previously described phytoplasmas. The hibiscus witches' broom phytoplasma was distinct on the basis of RFLP analysis of amplified rRNA operon sequences, analysis of nucleotide sequence data for sequence similarities and putative restriction sites in the DNA, and phylogenetic analysis of 16S rDNA sequences by using the neighbour-joining method. On the basis of comparisons of the RFLP patterns of 16S rDNA from hibiscus witches' broom phytoplasma with the patterns of reference strains used in this study and with results reported elsewhere (Lee *et al.*, 1993, 1998; Davis *et al.*, 1997; Jomantiene *et al.*, 1998a, b; Montano *et al.*, 2000), we classified hibiscus witches' broom phytoplasma as a member of a new 16S rRNA gene group, designated group 16SrXV. Our phylogenetic analysis of 16S rRNA gene sequences indicated that the hibiscus witches' broom phytoplasma forms a distinct subclade, which we have designated subclade xiv and which differs from other phytoplasma subclades.

This and other work (Montano *et al.*, 2000; R. E. Davis & E. L. Dally, unpublished) has indicated the presence, in South America, of several phytoplasma lineages not found in other regions. These findings are consistent with the concept that a unique ecology and geographic separation provided conditions favourable for phytoplasmal divergence from phytoplasma lineages in other regions of the world. Thus, the ecology and geographical location of Brazil may have provided conditions that favoured evolution of the distinct hibiscus witches' broom phytoplasma.

Each phytoplasmal 16S rRNA RFLP group or subgroup corresponds to a distinct subclade within the monophyletic clade that represents all phytoplasma organisms which descended from an *Acholeplasma*-like ancestor within the class *Mollicutes* (Gundersen *et al.*, 1994; Lee *et al.*, 1998; White *et al.*, 1998). The

taxonomic rank of species has been proposed for each of the several distinct subclades (Gundersen *et al.*, 1994). Our data clearly establish placement of the hibiscus witches’ broom phytoplasma within a distinct subclade, namely subclade xiv. In addition, our phylogenetic analysis confirmed the conclusion, based on an RFLP analysis of rDNA, that the hibiscus witches’ broom phytoplasma represents a 16S rRNA group that is distinct from those of other phytoplasmas. The phylogeny inferred from the phylogenetic tree indicates that the hibiscus witches’ broom phytoplasma represents a lineage distinct from those of other phytoplasmas.

It has not been possible to obtain any phytoplasmas in axenic culture, but a means of describing and naming putative taxa such as the phytoplasmas has been described (Murray & Schleifer, 1994). Thus, to facilitate reference to unique phytoplasmal lineages and to have names by which distinct phytoplasmas can be known, the convention of ‘*Candidatus* Phytoplasma species’ has been adopted. The names ‘*Candidatus* Phytoplasma aurantifolia’, ‘*Candidatus* Phytoplasma australiense’, ‘*Candidatus* Phytoplasma australasia’, ‘*Candidatus* Phytoplasma japonicum’ and ‘*Candidatus* Phytoplasma fraxini’ have been proposed for phytoplasmas associated with different diseases of plants (Zreik *et al.*, 1995; Davis *et al.*, 1997; White *et al.*, 1998; Griffiths *et al.*, 1999; Sawayanagi *et al.*, 1999). We propose that the hibiscus witches’ broom phytoplasma be designated a new, distinct ‘*Candidatus*’ species, ‘*Candidatus* Phytoplasma brasiliense’, with the following description: ‘*Candidatus* Phytoplasma brasiliense’ [(Mollicutes) NC; NA; O; NAS (GenBank no. AF147708); oligonucleotide sequences of unique regions of the 16S rRNA gene are 5'-GAAAAAGAAAG-3', 5'-TCTTTCTTT-3', 5'-CAG-3', 5'-ACTTTG-3' and 5'-GTCAAAAC-3'; P (*Hibiscus*, phloem); M].

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