Identification and Phylogenetic Analysis of a New Phytoplasma from Diseased Chayote in Brazil

H. G. Montano, Programa de Biotecnologia Vegetal/Universidade Federal do Rio de Janeiro (UFRJ), Cidade Universitária, Ilha do Fundão, Rio de Janeiro, and UFRRJ/IB, Departamento de Entomologia e Fitopatologia, Brazil, and USDA-ARS, Molecular Plant Pathology Laboratory, Beltsville, MD; **R. E. Davis** and **E. L. Dally**, USDA-ARS, Molecular Plant Pathology Laboratory, Beltsville, MD 20705; **J. P. Pimentel** and **P. S. T. Brioso**, UFRRJ/IB, Departamento de Entomologia e Fitopatologia, C.P. 74585, CEP 23851-970, Seropédica, RJ, Brazil

ABSTRACT

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Chayote (*Sechium edule*) (Cucurbitaceae), also known as vegetable pear, mirliton, or mango squash, is a commercially important vegetable crop in Brazil, where it is affected by chayote witches'-broom disease. Affected plants exhibit witches'-broom growths and other symptoms characteristic of plant diseases caused by phytoplasmas. Since previous electron microscopic studies revealed the association of a phytoplasma with chayote witches'-broom, the present work was aimed at detecting and classifying the phytoplasma that may be the causal agent of the disease. Strains of a phytoplasma belonging to group 16SrIII (X-disease phytoplasma group) were discovered in chayote affected by witches'-broom disease and in diseased plants of *Momordica charantia* that were growing as weeds in fields of chayote in Brazil. On the basis of results from restriction fragment length polymorphism and nucleotide sequence analyses of 16S rDNA, the phytoplasma was classified in a new subgroup, designated subgroup III-J. This classification was supported by a phylogenetic tree constructed by the Neighbor-Joining method.

Additional keywords: bitter gourd, RFLP, 16S rRNA

Chayote (*Sechium edule*) (Cucurbitaceae), also known as vegetable pear, mirliton, or mango squash, is a vegetable crop that originated in Central America and is produced commercially in Brazil, Costa Rica, Italy, Mexico, Puerto Rico, and Northern India (13,24,28). Fruits are nutritious, high in fiber, and a good source of vitamins and minerals. In Brazil, chayote is an economically important species grown mainly in the states of Rio de Janeiro, São Paulo, Pernambuco, Minas Gerais, and Paraná (25). The importance of chayote in the State of Rio de Janeiro is reflected by the fact that it is among the most widely cultivated vegetables in this state. However, the productivity and quality of chayote can be markedly reduced by a malady known as chayote witches'-broom (ChWB) disease (16).

Corresponding author: Robert E. Davis
E-mail: rdavis@asrr.arsusda.gov

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In Brazil, ChWB was first observed in the 1960s in the state of Rio de Janeiro (31). Symptoms exhibited by diseased plants included development of witches'-broom growths, generalized stunting and yellowing, leaf malformation, flower and fruit drop, as well as fruit malformation

(5,19,31,32). Besides Rio de Janeiro State, ChWB has been reported in the states of São Paulo, Minas Gerais, Paraná, and Pernambuco (19). More recently, the disease has been found in the state of Espírito Santo, where it has induced severe losses (5). During the 1970s, electron microscopic studies revealed the association of a phytoplasma (previously termed mycoplasma-like organism, MLO) with ChWB (19,32,33).

The aim of the present work was to detect and classify the phytoplasma that may be the cause of ChWB in Brazil. Our findings revealed that a previously undescribed phytoplasma was associated with the disease and that this phytoplasma was also present in diseased plants of *Momordica charantia* that were growing as weeds in fields of chayote. A preliminary report of part of this work has been published (26).

MATERIALS AND METHODS

Plant samples and reference phytoplasma strains. Symptomatic leaves of chayote were collected from naturally diseased plants exhibiting symptoms of ChWB in two different locations, Campo

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

Phytoplasma	Accession no.	Reference
Chayote witches'-broom [ChWBIII(Ch10)] ^a	AF147706	This paper
Chayote witches'-broom [ChWBIII(Mor5)] ^a	AF147707	This paper
Michigan aster yellows (MIAY)	M30970	23
Peanut witches'-broom (PnWB)	L33765	12
Sweet potato witches'-broom (SPWB)	L33770	12
Western X-disease (WX)	L04682	Unpublished
Canada peach X-disease (CX)	L33733 ^b	12
Clover yellow edge, Canada (CYE = CYE-C)	AF175304	Unpublished
Virginia grapevine yellows (VGYIII)	AF060875	9
Coconut lethal yellows (LY)	U18747	38
Elm yellows (EY1)	AF122910	Unpublished
Clover proliferation (CP)	L33761 ^b	12
Ash yellows (AshY)	AF092209	Unpublished
Pigeon pea witches'-broom (PPWB)	U18763	Unpublished
Apple proliferation (AT)	X68375	36
Rice yellow dwarf (RYD)	D12581	27
Australian grapevine yellows (AUSGY)	L76865	7
(<i>"Candidatus Phytoplasma australiense"</i>)		
Stolbur (STOL)	X76427	36
<i>Acholeplasma laidlawii</i>	M23932	39

^a Sequences determined in this study.

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

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Grande and Mendanha, in the state of Rio de Janeiro. In the same field in Campo Grande where leaves of diseased chayote were collected, we also collected symptomatic leaves of *Momordica charantia* (family Cucurbitaceae), a weed, exhibiting symptoms of yellowing, reduction in size of leaves, and witches'-broom. Reference phytoplasma strains representing various 16S rDNA restriction fragment length polymorphism (RFLP) groups were used in direct comparisons of RFLP patterns 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) phytoplasmas, and the potato witches'-broom (PWB) strain in clover proliferation phytoplasma group (group 16SrVI). Group affiliations are designated according to the classification system of Lee et al. (21). Phytoplasmas AY, SPWB, CX, CYE-C, EY, and PWB were maintained by graft transmission in periwinkle plants (*Catharanthus roseus*) maintained in a greenhouse. GenBank accession numbers of phytoplasmal 16S rDNA sequences used in this study are listed in Table 1.

Nucleic acid extraction. Leaf midribs or whole small leaves (1 g) were excised from diseased plants of chayote and *M. charantia*, and the DNA was extracted following a modification of the procedure of Ahrens and Seemüller (1). Samples were surface-sterilized with 0.5% sodium hypochlorite for 10 min, then rinsed twice in sterile distilled water. Tissues were pulverized in liquid nitrogen, and 4 ml of cold grinding buffer (100 mM K₂HPO₄; 31 mM KH₂PO₄; 10% sucrose; 2% polyvinylpyrrolidone [PVP-40]; 10 mM EDTA, pH 7.6) was added. The slurry was filtered through cheesecloth, and the extract centrifuged at 2,500 × g for 5 min. The supernatant liquid

Fig. 1. Restriction fragment length polymorphism (RFLP) analysis of 16S rDNAs amplified in polymerase chain reactions (PCRs) from naturally diseased plants of chayote (*Sechium edule*) and *Momordica charantia* 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 *Hae*III digest. AY, SPWB, CX, CYE, EY, PWB: digests of DNA from reference phytoplasmas aster yellows (AY), sweet potato witches'-broom (SPWB), Canada X-disease (CX), clover yellow edge (CYE = CYE-C), elm yellows (EY), and potato witches'-broom (PWB) strain of clover proliferation phytoplasma. 10, 11, 12, 13, 14, 2A, 2B, 15: digests of DNAs amplified from diseased plants of chayote. Mor5, digests of DNA amplified from a diseased plant of *M. charantia*. **A, B, C, D:** RFLP patterns following digestion with *Alu*I (**A**), *Mse*I (**B**), *Hpa*II (**C**), and *Rsa*I (**D**), respectively.

was collected and centrifuged at $15,000 \times g$ for 30 min. Nucleic acids were extracted from the pellets by resuspending in 1.5 ml of CTAB buffer (2% hexadecyltrimethylammonium bromide, Sigma Chemical Co., St. Louis, MO); 100 mM Tris-HCl, pH 8; 20 mM EDTA, pH 8; 1.4 M NaCl; 2% PVP-40) and incubating at 60°C , for 30 min. Samples were extracted with chloroform:isoamyl alcohol (24:1) and further centrifuged at $15,000 \times g$ for 5 min. The upper aqueous phase was removed, and nucleic acids were precipitated overnight by the addition to this phase of 0.6 volume of cold isopropanol (-20°C). Nucleic acids were collected by centrifugation at $15,000 \times g$ for 30 min; the nucleic acid pellets were washed with cold 70% ethanol, dried, and resuspended in 200 μl of TE (10 mM Tris-HCl, pH 8; 1 mM EDTA, pH 8). Then 200 μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the solution, which was centrifuged at $15,000 \times g$ for 30 min. The supernatant liquid was recovered, extracted with an equal volume of chloroform, centrifuged at $15,000 \times g$ for 5 min, and the aqueous layer removed and cold absolute ethanol added to precipitate nucleic acids. DNAs were pelleted by centrifugation at $15,000 \times g$ for 30 min, washed with cold 70% ethanol, dried, resuspended in TE, and stored at -20°C . DNA was further purified by the use of GeneCleanIII Kit (Bio101, Vista, CA).

Polymerase chain reaction (PCR) conditions and primers. Two universal phytoplasma primer pairs, P1/P7 (10,35) and R16F2n/R2 (11), were used to prime amplification of phytoplasma 16S rDNA (16S rRNA gene) sequences in nested PCR assays. Products of PCR primed by P1/P7 were diluted 1:50 with HPLC grade water (Sigma) and used as template in PCR primed by R16F2n/R2. Each reaction was performed in 0.5-ml PCR tubes, in a final volume of 25 μl of reaction mixture as previously described (11). DNA fragment size standard was 1 kb ladder (Life Technologies, Gaithersburg, MD). PCRs were carried out for 35 cycles using the following conditions: 1 min (2 min for first cycle) denaturation at 94°C , annealing for 2 min at 55°C , and primer extension for 3 min (10 min in final cycle) at 72°C . Negative controls consisted of reaction mixtures devoid of templates. PCR products were analyzed by electrophoresis through 1% agarose gel, staining with ethidium bromide, and visualization of DNA bands using a UV transilluminator (34).

Nested PCR assays using group-specific primer pairs. DNA products amplified in PCR primed by P1/P7 were diluted 1:50, as aforementioned, and used as template for reamplification in nested PCRs. Nested PCRs were primed by group-specific primer pairs designated R16(I)F1/R16(I)R1 (specific for group 16SrI, aster yellows group phytoplasmas), R16(III)F2/R16(III)R1 (specific for group 16SrIII, X-

Fig. 2. Restriction fragment length polymorphism (RFLP) analysis of 16S rDNAs amplified in nested polymerase chain reactions (PCRs) from three representative phytoplasma strains, two strains (Ch10 and Ch2A) from chayote (*Sechium edule*) and one (Mor5) from *Momordica charantia*. 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 *Hae*III digest. CYE: digest of DNA from reference phytoplasma clover yellow edge (CYE = CYE-C). (A) RFLP patterns following digestion with *Sau* 3AI, *Hinf*I, *Hha*I, and *Bfa*I. (B) RFLP patterns following digestion with *Hae*III, *Eco*RI, *Kpn*I, and *Hpa*I.

disease phytoplasma group), and R16(V)F1/R16(V)R1 (specific for group 16SrV, elm yellows group) (20). Analyses of amplified products were carried out as previously described.

RFLP analyses of amplified phytoplasma DNA. Products from nested PCR primed by R16F2n/R2 were analyzed by single restriction endonuclease digestion with *AluI*, *KpnI*, *ThaI* (Life Technologies), and *BfaI*, *DdeI*, *EcoRI*, *HaeIII*, *HinfI*, *HpaI*, *HpaII*, *MseI*, *RsaI*, *Sau 3AI*, *TaqI*, and *HhaI* (New England Biolabs, Beverly, MA), following instructions by the manufacturers. The products of digestion were analyzed by electrophoresis through a 5% polyacrylamide gel followed by staining with ethidium bromide and visualization of DNA bands with UV transilluminator. DNA fragment size standard used was PhiX174 RF *HaeIII* digest (Life Technologies). The RFLP patterns of phytoplasma DNAs were compared with the RFLP patterns previously published (7,9,14,15,21,22).

Cloning of PCR products and sequencing of DNA. Phytoplasma DNA sequences that had been amplified in PCRs primed by P1/P7 were cloned in *Escherichia coli* using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The cloned 1.8 kbp DNA fragments of rDNA were sequenced by use of automated DNA sequencing. The sequences were assembled into contigs after a minimum of 2× sequencing coverage for each base position. The nucleotide sequences determined in this study were deposited in the GenBank data library; GenBank accession numbers for these sequences are given in Table 1. Other phytoplasma and *Acholeplasma laidlawii* 16S rDNA sequences were obtained from GenBank (see Table 1 for GenBank accession numbers). Alignments of sequences were generated and sequence similarities evaluated by using the DNA-

STAR program Align option (DNASTAR, Inc., Madison, WI). Putative restriction site maps were generated by using the DNA-STAR program MapDraw option.

Phylogenetic analysis. 16S rRNA gene sequences (1.2 kb) from chayote witches'-broom phytoplasma strains Ch10 and Mor5, three other group 16SrIII phytoplasmas, phytoplasma strains representing 10 other groups, and *A. laidlawii* were aligned using Clustal X version 1.63b (37) and DNASTAR. Gaps and incomplete sequences were removed. Cladistic analyses were performed with the computer program Clustal X. Phylogenetic trees were constructed by the Neighbor-Joining method, and the tree was viewed by using TreeViewPPC (30). *A. laidlawii* was selected as the outgroup to root the tree. Bootstrapping was performed 1,000 times for estimation of stability and support for the clades.

RESULTS

Detection and classification of phytoplasmas in diseased chayote. On the basis of phytoplasma-specific DNA amplification in PCR (data not shown), phytoplasmas were detected in all of the eight chayote plants tested exhibiting symptoms of witches'-broom disease. RFLP analysis of PCR-amplified 16S rDNA indicated that the diseased chayote plants contained strains of a phytoplasma belonging to group 16SrIII (X-disease phytoplasma group) (see below).

Presence of a group 16SrIII phytoplasma in all DNA samples was verified by group 16SrIII-specific, nested PCR. PCR assays primed by primer pair R16 (III)F2/R1 yielded an amplified product of approximately 0.8 kb, while no amplified products were observed when PCRs were primed by group-specific primer pair R16(I)F1/R1 or primer pair R16(V)F1/R1 (data not shown).

All of the eight DNA samples tested yielded mutually indistinguishable collective RFLP patterns (Fig. 1). The *AluI*, *MseI*, and *HpaII* RFLP patterns observed for DNA from the eight strains from chayote plants corresponded to RFLP patterns that have been found (21) only in phytoplasma strains belonging to group 16SrIII. Since the collective RFLP patterns indicated that the phytoplasma strains detected in the eight diseased chayote plants were mutually indistinguishable, two of the phytoplasma strains (designated Ch2A and Ch10, derived from two plants, 2A and 10, growing in different fields) were selected as representative for further analysis.

Phytoplasma strains Ch2A and Ch10 were mutually indistinguishable also on the basis of collective RFLP patterns following analysis using eight additional restriction enzymes (Fig. 2 and Table 2). These results lead us tentatively to conclude that these are strains of the same phytoplasma, which we term chayote witches'-broom phytoplasma ChWBIII. The collective RFLP patterns from use of the 12 restriction enzymes distinguished the ChWBIII phytoplasma strains from other phytoplasmas classified in group 16SrIII (Fig. 2 and Table 2). For example, the *HhaI* RFLP patterns distinguished both Ch2A and Ch10 from CYE phytoplasma, the subgroup III-B reference strain (Fig. 2A and Table 2), and the *ThaI* RFLP patterns distinguished both chayote strains from phytoplasma MW1 (Table 2). Comparisons of the collective RFLP patterns with the published RFLP patterns of other phytoplasma 16S rDNAs (7,9,14,15,21,22,) indicated that the ChWBIII phytoplasma is a phytoplasma that has not been described previously. Therefore, we assign ChWBIII phytoplasma to a new subgroup, designated subgroup J (III-J), in group 16SrIII.

Detection and classification of phytoplasmas in diseased *Momordica*. A phyto-

Table 2. Summary of pattern groups produced by restriction fragment length polymorphism (RFLP) analysis of 16S rDNA from group 16SrIII phytoplasmas associated with chayote witches'-broom and other diseases of plants

Phytoplasma strain	16S rRNA group (subgroup) ^a	RFLP pattern obtained with restriction endonuclease indicated ^b										
		<i>MseI</i>	<i>AluI</i>	<i>BfaI</i>	<i>DdeI</i>	<i>HhaI</i>	<i>HpaI</i>	<i>HpaII</i>	<i>Sau 3AI</i>	<i>TaqI</i>	<i>ThaI</i>	<i>RsaI</i>
<i>ChWBIII(Ch10)</i>	III(J)	9	6	1	1	4	1	6	4	1	3	2
<i>ChWBIII(Mor5)</i>	III(J)	9	6	1	1	4	1	6	4	1	3	2
CX	III(A)	8	6	1	1	1	1	5	3	1	3	2
CYE-C	III(B)	9	6	1	1	1	1	6	4	1	3	2
PB	III(C)	10	6	4	4	4	1	6	4	1	4	2
GR1	III(D)	11	6	1	1	4	1	6	4	1	5	2
SP1	III(E)	9	6	1	2	5	1	5	3	1	3	2
MW1	III(F)	9	6	1	1	4	1	6	4	1	4	2
WWB	III(G)	9	6	1	1	1	1	5	3	1	3	3
PoiBI	III(H)	9	6	4	3	1	1	5	3	1	3	2
VGIII	III(I)	9	6	1	1	1	1	5	3	1	3	2

^a System for designating phytoplasma group and subgroup affiliations are after (21). III, group 16SrIII (X-disease phytoplasma group). Subgroups are in parentheses.

^b Each number represents a specific RFLP pattern obtained by use of a given restriction endonuclease. Numbers according to the system of Lee et al. (21); numbers in *italics* designate RFLP patterns determined in the present study. The remaining numbers are from Davis et al. (9) and Lee et al. (21). ChWBIII(Ch10), chayote witches'-broom phytoplasma strain Ch10 from diseased chayote. ChWBIII(Mor5), chayote witches'-broom phytoplasma strain Mor5 from diseased *Momordica charantia*. CX, Canada peach X-disease. CYE-C, clover yellow edge phytoplasma from Canada. PB, pecan bunch. GR1, goldenrod yellows. SP1, spiraea stunt. MW1, milkweed yellows. WWB, walnut witches'-broom. PoiBI, poinsettia branch-inducing phytoplasma. VGIII, Virginia grapevine yellows group 16SrIII phytoplasma.

toplasma was detected in the diseased *M. charantia* plant exhibiting symptoms of witches'-broom disease, on the basis of phytoplasma-specific DNA amplification in PCR (data not shown). RFLP analysis of the 16S rDNA amplified from the phytoplasma did not distinguish it from ChWBIII

phytoplasma and indicated that it also belonged to group16SrIII, new subgroup III-J (Figs. 1 and 2 and Table 2). We consider the phytoplasma to be a strain (termed strain Mor5) of ChWBIII phytoplasma.

Nucleotide sequence, sequence similarities, and putative restriction sites in

Table 3. Sequence similarities among 16S rDNAs from phytoplasmas classified in group 16SrIII (X-disease phytoplasma group)^a

Phytoplasma	Sequence similarity (%) with indicated phytoplasma DNA			
	Mor5	WX	CYE-C	VGyIII
<i>ChWBIII (Ch10)</i>	99.8	98.9	99.0	99.1
<i>ChWBIII (Mor5)</i>		98.7	98.8	99.0
WX			98.6	99.3
CYE-C				98.9

^a Sequence similarities of 1.2-kbp sequences of 16S rDNA were estimated using the Align option of DNASTAR program. The 1.2-kbp sequences extend from the annealing site of polymerase chain reaction (PCR) primer R16F2n through the annealing site of PCR primer R16R2. *ChWBIII*, chayote witches'-broom. WX, western X-disease. CYE-C, clover yellow edge, Canada. VGyIII, Virginia grapevine yellows. Italic type indicates sequence similarities determined in this study.

phytoplasma rRNA operon sequences. Two strains, Ch10 and Mor5, of ChWBIII phytoplasma were selected for nucleotide sequencing of rDNA. Nucleotide sequences determined for the cloned 1.8-kbp DNA fragments from ChWBIII phytoplasma strains Ch10 and Mor5 have been deposited in the GenBank database under accession numbers AF147706 and AF147707, respectively. Sequence similarities among the ChWBIII phytoplasma strains and group 16SrIII reference phytoplasmas ranged from 98.7 to 99.8% (Table 3). Since these values were greater than 95%, the results are consistent with the placement of ChWBIII(Ch10) and ChWBIII (Mor5) in group 16SrIII.

The cloned and sequenced ChWBIII 16S rDNAs were subjected to putative restriction site analysis and compared with each other and with the nucleotide sequences determined previously for fragments from

Fig. 3. Analysis of putative restriction sites in rRNA operon sequences from phytoplasmas belonging to group 16SrIII (X-disease phytoplasma group). Maps were generated using the MapDraw option of the DNASTAR program and manually aligned for comparison of recognition sites for restriction nucleases. ChWBIII(Ch10), chayote witches'-broom phytoplasma strain Ch10 from naturally diseased plant of chayote in Brazil. ChWBIII(Mor5), chayote witches'-broom phytoplasma strain Mor5 from naturally diseased plant of *Momordica charantia* in Brazil. CX, Canada peach X-disease phytoplasma. CYE-C, clover yellow edge phytoplasma from Canada. VGyIII, Virginia grapevine yellows phytoplasma VGyIII. Solid bar represents the 16S rDNA segment amplified in polymerase chain reaction (PCR) primed by primer pair F2n/R2. Arrows indicate restriction sites that differentiate ChWBIII(Ch10) and ChWBIII(Mor5) from the reference phytoplasmas. GenBank accession numbers of the phytoplasma DNAs are in parentheses.

the rRNA operons of Canada X-disease (CX), clover yellow edge (CYE-C), and Virginia grapevine yellows phytoplasma VGYIII (VGYIII) reference phytoplasmas (Fig. 3). Expected fragment sizes based on analysis of putative restriction sites were in excellent agreement with fragment sizes obtained by enzymatic RFLP analysis of amplified DNA. The ChWBIII phytoplasma strains differed from CX, CYE-C, and VGYIII by the presence in the ChWBIII sequences of a *HhaI* site that is absent in the sequences from the other phytoplasmas (see arrows in Fig. 3). ChWBIII further differed from the other phytoplasmas by the presence of a *Sau* 3AI site in the CX and VGYIII sequences that is absent in ChWBIII, by a *HpaII* site present in ChWBIII but absent in CX and VGYIII, and by a *MseI* site present in ChWBIII that is absent in CX. These restriction sites were located within a region (see solid bars in Fig. 3) that corresponds to the 1.2-kbp fragment that is amplified in PCR by primer pair F2n/R2.

Phylogenetic analysis. Phylogenetic analysis of 17 phytoplasmas and *A. laidlawii* yielded a consensus tree (Fig. 4). The bootstrapping values strongly supported most branches, indicating a tree whose

branching order is in general agreement with previous findings (7,12,36,40). Strains representing subgroups A, B, and I from group 16SrIII were included in the analysis, but sequences from strains representing other subgroups in group III were not available. Strains from other phytoplasmas belonging to 10 groups were included for comparison with group 16SrIII strains. Chayote witches'-broom phytoplasma strains give rise to a new branch, not seen in previous phylogenetic trees, indicating that this phytoplasma may represent a new phytoplasma lineage, and supporting the designation of ChWBIII phytoplasma as representative of a new subgroup in group 16SrIII (X-disease phytoplasma group).

DISCUSSION

The findings presented here provide a definitive classification of the phytoplasma associated with chayote witches'-broom disease in Brazil. The results in the present communication are consistent with previous reports indicating that diseased chayote contained a phytoplasma that belonged to group III (17,26). From the results now available, it seems possible that this group III phytoplasma may be capable of induc-

ing symptoms of witches'-broom in chayote. Thus, we have termed the chayote witches'-broom associated phytoplasma as ChWBIII phytoplasma.

The present communication delineates chayote witches'-broom (ChWBIII) phytoplasma as representative of a distinct new subgroup (III-J) in group 16SrIII. Since the phylogenetic tree supported the classification and indicated that chayote witches'-broom phytoplasma may represent a new lineage, this phytoplasma could represent a distinct species, even though the 16S rRNA gene sequence similarities between the chayote witches'-broom phytoplasma strains and other group 16SrIII phytoplasmas were 98.7% or greater. The geographic occurrence of chayote witches'-broom phytoplasma in a region of South America, and its apparent absence from other continents where various other group 16SrIII phytoplasmas occur, are consistent with the concept that this phytoplasma is a separate species.

Interestingly, symptomatic *M. charantia* growing as a weed in a commercial field of chayote was infected by a group 16SrIII phytoplasma, which we tentatively conclude belongs to the same taxon as ChWBIII phytoplasma. These results implicate *Momordica* as a potential source of ChWBIII phytoplasma inoculum for infection of commercial chayote. This reasoning is consistent with a previous suggestion that *M. charantia* may be the main reservoir of the chayote witches'-broom disease agent (31) and with the suggestion that a phytoplasma previously observed in *M. charantia* (19,33) was the same as that responsible for chayote witches'-broom disease (18).

Although the phytoplasma associated with chayote witches'-broom in Brazil was first detected, through electron microscopy, in 1977, no epidemiological investigations of the disease have yet been carried out, and potential insect vectors of the chayote witches'-broom disease pathogens remain unknown. For the design of effective disease management strategies, it will be important to investigate the range of plant species, either wild plants or other cultivated crops, that are capable of serving as alternate hosts for the chayote phytoplasmas. With the new 16S rRNA gene sequence data provided in this report, it will now be possible to search for potential insect vectors and to gain understanding of the spread of the ChWBIII phytoplasma to chayote and other plant species.

In preliminary investigations using PCR primed by phytoplasma universal and/or group-specific primer pairs, and RFLP analyses of PCR-amplified DNA (2,3,8), phytoplasmas affiliated with group III have been reported in cassava (*Manihot esculenta*), eggplant (*Solanum melongena*), corn (*Zea mays*), passionfruit (*Passiflora* sp.), periwinkle (*Catharanthus roseus*), strawflower (*Helichrysum bracteatum*),

Fig. 4. Phylogenetic tree constructed by the Neighbor-Joining method of 16S rRNA gene sequences from 17 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. Roman numerals represent 16S rDNA restriction fragment length polymorphism (RFLP) groups according to ref. 21. III-J: group 16SrIII, subgroup J (this report).

and sunnhemp (*Crotalaria juncea*) from different regions in Brazil. The results indicated that some of the phytoplasmas detected were closely related to subgroup B of group 16SrIII. It is possible that some of the phytoplasma strains noted in those plants belong to the same 16S rRNA subgroup as phytoplasma ChWBIII found in chayote and *Momordica*, but the published data are insufficient to make this determination.

Few phytoplasmas have been previously noted in plants belonging to the family Cucurbitaceae. In addition to the chayote and *Momordica*-infecting phytoplasma in Brazil (this report), the only other phytoplasma thus far identified in Cucurbitaceae was reported in loofah (*Luffa* spp.) in Taiwan. That phytoplasma belongs to group 16SrVIII (loofah witches'-broom phytoplasma group) (22). Witches'-broom diseases of chayote have been reported in Mexico and Taiwan, where phytoplasma cells have been observed by electron microscopy in the phloem of diseased plants (4,29). However, it is not known whether or not these latter diseases in chayote may be caused by the phytoplasma that is associated with chayote witches'-broom in Brazil, by the loofah witches'-broom phytoplasma, or by other phytoplasma taxa. In the future, the use of molecular methods, as used in the present work and in other studies (reviewed in 6), should resolve this issue.

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