A Phytoplasma Belonging to a 16SrIII-A Subgroup and dsRNA Virus Associated with Cassava Frogskin Disease in Brazil

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Abstract

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Cassava frogskin disease (CFSD) is a particular threat in cassava because symptoms remain hidden until harvest and losses can be total. The information related to the etiological agent of this disease is contradictory, because some authors believe it is caused by phytoplasmas while others believe that it is caused by a virus. In order to refine detection protocols and to characterize organisms associated with CFSD in Brazil, 32 symptomatic and 20 asymptomatic cassava plants were collected in Minas Gerais state. Total DNA was extracted and used for nested polymerase chain reaction (PCR) to detect phytoplasmas. Because endophytic *Bacillus* spp. led to false positives, primers were designed to facilitate the detection of phytoplasma in the presence of

bacteria. In addition, double-stranded (ds)RNA was extracted from tubers and used in reverse-transcription PCR for the detection of the RNA-dependent RNA polymerase gene from Cassava frogskin virus segment 4. The detected phytoplasma was identified as belonging to the group 16SrIII-A by restriction fragment length polymorphism (RFLP), sequencing, and RFLP in silico. This is the first report of a phytoplasma belonging to the 16SrIII-A group associated with cassava plants, the first molecular characterization of a phytoplasma associated with CFSD in Brazil, and a first report of phytoplasma and a dsRNA virus (possible reovirus) co-infecting cassava plants with CFSD symptoms.

Cassava (*Manihot esculenta* Crantz; Euphorbiaceae) is one of the main food staples in developing countries and is of great socio-economic importance, in particular to resource-poor farmers and in areas subject to drought. Brazil is the third-largest producer of cassava in the world and the crop is grown all over Brazil (12). Diseases, including those caused by viruses and phytoplasmas, are major limiting factors for production in Brazil (35). In Brazil, the first identification of phytoplasma as a disease-causing agent in cassava occurred in 1944 in the city of Lins, São Paulo. That set of symptoms was named cassava witches'-broom disease (34). However, the etiology of cassava witches'-broom disease was only determined many years later (13). The disease has subsequently spread throughout Brazil, leading to significant crop losses. Because cassava stems are the source of planting material for the next cycle, control of disease spread has been difficult (13). Cassava frogskin disease (CFSD) was first discovered in Colombia in 1971 (15). It may cause major losses in cassava yield, mainly affecting tubers: deep lesions, reduced diameter, and increased woodiness. Symptoms on the aerial parts of the plant are uncommon (1,2). The etiology of CFSD needs to be elucidated. Alvarez et al. (1) detected a phytoplasma associated with CFSD; it was identified by nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) as belonging to the phytoplasma ribosomal group 16SrIII and ribosomal protein group rpIII-H (2). However, Calvert et al. (5) detected a double-stranded (ds)RNA virus in the phloem of cassava plants with CFSD through visualization of isometric viral particles by electron microscopy and comparison of fragments corresponding to the RNA-dependent RNA polymerase (RdRp) gene with other reoviruses (5). The results presented by Calvert et al. (5) suggest that a virus probably belonging to the *Reoviridae* family is associated with CFSD. However, each of

these two studies was directed at the detection of a single pathogen, with no investigation of the possibility of simultaneous occurrence of both pathogens.

Phytoplasmas are small pathogenic prokaryotes without cell wall, surrounded by a single membrane and having pleomorphic cells (9). They possess a single circular or linear chromosome and may additionally contain up to four different plasmids. The chromosome has reduced size compared with the ancestral gram-positive bacteria (3,6,21,28,38). They are restricted to the phloem of their host plants and multiply inside the insect vectors (15). Recently, its cultivation in axenic culture has become possible (7), although details about the medium are not available. The most common symptoms seen in diseases caused by phytoplasmas are (i) phyllodes, (ii) witches'-broom, (iii) virescence, (iv) dwarfism, (v) sterility of floral organs, (vi) wrinkled leaves, and (vii) yellowing (4). The distribution and accumulation of phytoplasmas in host plants can be quite variable (33). A low concentration of microorganisms in certain tissues, as well as the presence of inhibitors in the host tissue, may render their detection difficult (18). An additional problem related to the detection of phytoplasmas is the presence of *Bacillus* spp. on the plant surface or endophytically, because certain genera of bacteria have a high nucleotide sequence homology with the 16S ribosomal (r)DNA phytoplasma (25). The presence of these bacteria can interfere in the amplification reactions, leading to false positives and making it difficult to detect the pathogen (16). Moreover, the presence of different operons in the 16 rRNA gene may hamper its characterization (24), which is based on the comparison of the restriction patterns of 16S rDNA fragments using different restriction enzymes (22).

Viruses belonging to the *Reoviridae* family have icosahedral particles ranging between 60 and 80 nm in diameter. Their genome is composed of 9 to 12 segments of linear dsRNA, depending on the genus. Conserved proteins such as RdRp and capsid proteins are used to distinguish members of different genera. Members of the family *Reoviridae* that infect plants can replicate in both plants and leafhoppers (19).

Despite the presence of cassava plants showing characteristic symptoms of the CFSD in Brazil, little is known about the presence of a phytoplasma or a virus associated with these symptoms

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in cassava. In the present article, cassava plants with symptoms of CFSD collected in Minas Gerais, Brazil, were analyzed for the presence or absence of virus and phytoplasma in order to make the diagnosis of this disease more accurate. Primers were designed to allow the detection of phytoplasma in cassava in the presence of endophytic bacteria. Additionally, the molecular characterization of the phytoplasma was performed through amplification of 16S rDNA, followed by RFLP analysis, nucleotide sequencing, and phylogenetic analysis. The presence of a dsRNA virus was confirmed through reverse transcription PCR, cloning, and sequencing. The analyses showed that plants with symptoms of CFSD had both a phytoplasma, characterized as belonging to 16SrIII-A group, and a virus from the *Reoviridae* family. This is the first report of a phytoplasma belonging to 16SrIII-A group in cassava plants, the first reported case of a virus associated with CFSD in Brazil, and also the first reported case of the joint presence of a phytoplasma and a virus associated with CFSD.

Materials and Methods

Sources of symptomatic and asymptomatic cassava. Fifty-two cassava plants were collected from a single field in Mociminho (Jaíba, Minas Gerais, Brazil), where CFSD is prevalent. Of these plants, 32 had obvious symptoms of the disease while 20 were asymptomatic. Samples were collected from tubers, stems, and leaves of each plant for analysis. A healthy cassava plant was collected from Viçosa, Minas Gerais, where CFSD has never been reported, for use as a negative control in the detection of phytoplasmas and viruses.

DNA extraction. Total DNA was extracted from all cassava samples (tubers, stems, and leaves) following the protocol of Doyle and Doyle (10), with some modifications. Approximately 100 mg of plant tissue, mainly from midribs and phloem regions, was macerated in liquid nitrogen and then transferred to 1.5-ml microtubes. To each microtube, 800 µl of 2× cetyltrimethylammonium bromide extraction buffer and 0.2% β-mercaptoethanol, preheated to 60°C, were added. The microtubes were vortexed and incubated at 65°C for 60 min, inverted every 10 min in order to homogenize the suspension. After incubation, 600 µl of chloroform/isoamyl alcohol (24:1) was added to each microtube. The solution was homogenized by inverting the tubes and they were centrifuged for 10 min at 14,000 rpm. The upper (aqueous) phase was transferred to a new microtube (approximately 540 µl). To these new microtubes, 540 µl of cold isopropanol was added. The microtubes were left overnight at -20°C for greater precipitation of nucleic acids. After incubation, the microtubes were centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and the pellet formed was washed twice with 1 ml of 80% ethanol. The pellet was resuspended in 500 µl of 1 M NaCl. The microtubes were incubated for 60 min at 4°C and centrifuged at 14,000 rpm for 10 min. The pellet formed was washed twice with 1 ml of 80% ethanol. After evaporation of all the ethanol, the pellet was resuspended in 60 µl of TE plus RNase. The microtubes were stored at -20°C for later use.

Phytoplasma detection. We performed a nested PCR using universal primers for phytoplasma detection, P1 and P7, followed by the amplification with primers FTP (F) and FTP (R), developed in this study to make possible the detection in the presence of endophytic bacteria (Table 1) (8,23,32). These primers amplified an 816-bp fragment from the 16S rDNA of this phytoplasma, without

unspecific amplifications (see Results). The program used for the amplification with primers P1/P7 had 30 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C; for the primers FTP(F)/(R), the program had 35 cycles of 1 min at 94°C, 1 min at 46°C, and 1 min at 72°C. Both programs had a single initial step of 1 min at 94°C and a final step of 7 min at 72°C. The amplified product in the first reaction (P1/P7) was used as template for the second PCR at a dilution of 1:15 in Milli-Q water, using the primers FTP(F) and FTP(R).

Two cassava plants with symptoms of CFSD were kept in the greenhouse (samples 31 and 32) and used to amplify a 1,200-bp 16S rDNA fragment used for the taxonomical classification. A nested PCR was performed using the primers P1/P7, followed by R16F2n/R2 (Table 1), in order to obtain these sequences. The first reaction product (1 µl) was used as a template for the second reaction. The program used for the primers R16F2n/R2 had 35 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C, with only one initial step of 1 min at 94°C and a final step of 7 min at 72°C.

PCRs for phytoplasma detection were performed using the DNA amplification kit GoTaq Master Mix (Promega Corp.) according to the manufacturer's instructions. PCRs for sequencing were performed using the Platinum Taq DNA Polymerase High Fidelity (Invitrogen) according to the manufacturer's instructions. The final product amplified in the PCRs was subjected to agarose gel electrophoresis and, subsequently, the gels were stained with ethidium bromide and photographed under ultraviolet light.

dsRNA extraction and cDNA synthesis. dsRNA was extracted from the phloem region of all cassava tubers, using the viral dsRNA extraction kit (iNTRON Biotechnology), according to the manufacturer's instructions. For the cDNA synthesis, we used a primer R1067 complementary to the 3' end of the coding strand (Table 1), 250 ng of dsRNA, and 200 U of SuperScript III RT (Invitrogen), according to the manufacturer's instructions, with a modification in the denaturation temperature (98°C).

Viral detection. For the viral detection, we performed PCR using the primers F110 and R1067 (Table 1), which amplifies a fragment of approximately 950 bp, corresponding to the partial RdRp gene sequence of a possible reovirus. The program used for these primers had 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C. After 35 cycles, a single step of 72°C for 10 min was added for a final extension. The PCRs were performed using the Platinum Taq DNA Polymerase High Fidelity (Invitrogen), according to the manufacturer's instructions. The final product amplified in the PCRs was subjected to agarose gel electrophoresis, and the gels were stained with ethidium bromide and photographed under ultraviolet light.

Cloning and sequencing of fragments. For cloning, the fragments generated by PCR were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), according to manufacturer's recommendations. The purified fragments were ligated into the pGEM T Easy Vector System I (Promega Corp.) and transformed to *Escherichia coli* DH5α competent cells (31). After cloning, the plasmidial DNA was extracted from the *E. coli*-transformed cells using the Illustra Kit Plasmidprep Mini Spin Kit (GE Healthcare), according to manufacturer's recommendations. The cloned fragments were sent for sequencing at MacroGen Inc.

RFLP in silico analysis. A restriction analysis in silico of the 1,200-bp sequences obtained by amplification of a 16S rDNA using the primers R16F2n/R2 was performed using the online tool iPhyClassifier (39). The in silico restriction was made with the 17 commonly used restriction enzymes for RFLP analysis of phytoplasmas. The enzymes were *AluI*, *BamHI*, *BfaI*, *Bsh1236* (*BstUI*), *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *MboI*, *MseI*, *RsaI*, *SspI*, and *TaqI*. A comparison of restriction patterns of the sequences obtained with the patterns described in literature was made in order to identify the group to which this phytoplasma belongs. We calculated the coefficients of similarity (F) for each pair of isolates used for the analysis, through the iPhyClassifier (39), following the formula $F = 2N_{xy}/(N_x + N_y)$, where $2N_{xy}$ is the number of bands in common in the profile between the two isolates

Table 1. Primers used in polymerase chain reactions

Primers	Nucleotide sequence	Reference
P1	5' AAG AGT TTG ATC CTG GCT CAG GAT T 3'	8
P7	5' CGT CCT TCA TCG GCT CTT 3'	32
R16F2n	5' GAA ACG ACT GCT AAG ACT GG 3'	23
R16R2	5' TGA CCG GCG GTG TGT ACA AAC CCC G 3'	23
FTP(F)	5' AAG GTA TAC TTA AGG AGG 3'	This study
FTP(R)	5' TTT CCT GAT AAC CTC CA 3'	This study
F110	5' TGG CCG GGA GAA CAA TAA TA 3'	5
R1067	5' GCG AAG TAA GTT CCG TCG TT 3'	5

and N_x and N_y are the numbers of bands existing in the profile of each isolate.

Phylogenetic analysis of 16S rDNA and RdRp partial sequences. The two 1,200-bp 16S rDNA nucleotide sequences generated were aligned and compared with other sequences of 65 phy-

toplasmas representative of different groups and subgroups within the group 16SrIII, including sequences of the phytoplasmas responsible for cassava witches'-broom disease (CaWB Br1 and CaWB Br2) and CFSD (CFSD1, -2, and -3) (Table 2). The nucleotide sequence of *Acholeplasma palmae* was used as an outgroup

Table 2. List of phytoplasmas used for phylogenetic analysis, with the classification and GenBank accession number (<http://www.ncbi.nlm.nih.gov>)

Phytoplasma	Group/subgroup	Access number
CaWB Br01	16SrIII-B	GU193976
CaWB Br02	16SrIII-B	GU193977
CFSD3	16SrIII-L	EU346761
CFSD2	16SrIII-L	AY737646
CFSD1	16SrIII-L	AY737647
CFSD Br1	16SrIII-A	...
CFSD Br2	16SrIII-A	KF667079KF667080NC_007716
' <i>Candidatus</i> Phytoplasma asteris'	16SrI	16
' <i>Ca.</i> Phytoplasma aurantifolia'	16SrII	U15442
' <i>Ca.</i> Phytoplasma australasiae'	16SrII	L33765
16SrIII_A	16SrIII-A	L33733
16SrIII_B	16SrIII-B	AF189288
16SrIII_C	16SrIII-C	FJ376626
16SrIII_D	16SrIII-D	FJ376627
16SrIII_F	16SrIII-F	AF510724
16SrIII_G	16SrIII-G	AF190226
16SrIII_J	16SrIII-J	AF147706
16SrIII_K	16SrIII-K	AF274876
16SrIII_L	16SrIII-L	EU169138
16SrIII_M	16SrIII-M	FJ226074
16SrIII_N	16SrIII-N	FJ376629
16SrIII_O	16SrIII-O	AF370120
16SrIII_P	16SrIII-P	AF370119
16SrIII_Q	16SrIII-Q	AF302841
16SrIII_R	16SrIII-R	AF373105
16SrIII_S	16SrIII-S	L04682
16SrIII_T	16SrIII-T	FJ231728
16SrIII_U	16SrIII-U	HM589213
' <i>Ca.</i> Phytoplasma palmae'	16SrIV	AF498307
' <i>Ca.</i> Phytoplasma cocostanzaniae'	16SrIV	X80117
' <i>Ca.</i> Phytoplasma ulmi'	16SrV	AY197655
' <i>Ca.</i> Phytoplasma ziziphi'	16SrV	AY197650
' <i>Ca.</i> Phytoplasma vitis'	16SrV	AB052876
' <i>Ca.</i> Phytoplasma rubi'	16SrV	AY197642
' <i>Ca.</i> Phytoplasma trifolii'	16SrVI	AY390261
' <i>Ca.</i> Phytoplasma sudamericanum'	16SrVI	GU292081
' <i>Ca.</i> Phytoplasma fraxini'	16SrVII	AF092209
Loofah witches'-broom	16SrVIII	AF353090
' <i>Ca.</i> Phytoplasma phoenicium'	16SrIX	AF515636
' <i>Ca.</i> Phytoplasma prunorum'	16SrX	AJ542544
' <i>Ca.</i> Phytoplasma pyri'	16SrX	AJ542543
' <i>Ca.</i> Phytoplasma mali'	16SrX	AJ542541
' <i>Ca.</i> Phytoplasma spartii'	16SrX	X92869
' <i>Ca.</i> Phytoplasma oryzae'	16SrXI	AB052873
' <i>Ca.</i> Phytoplasma fragariae'	16SrXII	DQ086423
' <i>Ca.</i> Phytoplasma japonicum'	16SrXII	AB010425
' <i>Ca.</i> Phytoplasma australiense'	16SrXII	L76865
' <i>Ca.</i> Phytoplasma solani'	16SrXII	AF248959
Mexican periwinkle virescence'	16SrXIII	AF248960
' <i>Ca.</i> Phytoplasma cynodontis'	16SrXIV	AJ550984
' <i>Ca.</i> Phytoplasma brasiliense'	16SrXV	AF147708
' <i>Ca.</i> Phytoplasma graminis'	16SrXVI	AY725228
' <i>Ca.</i> Phytoplasma caricae'	16SrXVII	AY725234
' <i>Ca.</i> Phytoplasma americanum'	16SrXVIII	DQ174122
' <i>Ca.</i> Phytoplasma castaneae'	16SrXIX	AB054986
' <i>Ca.</i> Phytoplasma rhamnii'	16SrXX	X76431
' <i>Ca.</i> Phytoplasma pini'	16SrXXI	AJ632155
Nigerian coconut lethal decline	16SrXXII	Y14175
Buckland valley grapevine yellows	16SrXXIII	AY083605
Sorghum bunchy shoot	16SrXXIV	AF509322
Weeping tea tree witches'-broom	16SrXXV	AF521672
Mauritius sugarcane phytoplasma D3T1	16SrXXVI	AJ539179
Mauritius sugarcane D3T2	16SrXXVII	AJ539180
Havana Derbid	16SrXXVIII	AY744945
' <i>Ca.</i> Phytoplasma omanense'	16SrXXIX	EF666051
' <i>Ca.</i> Phytoplasma tamaricis'	16SrXXX	FJ432664
' <i>Ca.</i> Phytoplasma costaricanum'	16SrXXXI	HQ225630
' <i>Ca.</i> Phytoplasma malaysianum'	16SrXXXII	EU371934
<i>Acholeplasma palmae</i>	...	L33734

for analysis. Sequence alignment was performed using the program Muscle (11) implemented in the program Mega 5.5 (37). The phylogenetic analysis was performed using Bayesian inference (MB) through the program Mr. Bayes, based on the Monte Carlo Markov Chain (30). The model used was previously selected by Akaike Information Criterion through the program MrModeltest V.2 (Nylander, Upsala University). Ten million generations were used. Sampling of trees was carried out every 1,000 generations, generating 10,000 trees. In total, 2,500 trees (25%) were excluded for the calculation of posterior probability and it was verified if there was convergence of cold chains. Trees were also constructed using neighbor-joining (NJ) and maximum likelihood (ML), using the program Mega 5.5 (*data not shown*). The trees were rooted using the sequence of *Acholeplasma palmae* as outgroup, because they belong to the same family of the genus 'Candidatus Phytoplasma' (Acholeplasmataceae).

The nucleotide sequence of RdRp obtained was aligned and a pairwise comparison was made with other sequence of the RdRp gene of Cassava frogskin virus, available in GenBank (DQ139870.1).

Results

Detection of phytoplasma in the samples collected. In total, 52 cassava plants, with and without symptoms of CFSD (Fig. 1), were collected and examined for the presence of phytoplasma in tubers, stems, and leaves, and it was possible to detect the phytoplasma in different tissues examined. Nested PCRs of all collected samples, using the primers P1/P7 and R16F2n/R2, generated fragments of 1,200 bp, as expected for phytoplasmas. However, the sequencing results showed that the fragments belonged to different *Bacillus* spp. (endophytic in cassava), particularly *Bacillus megaterium*. Due to the high sequence homology among phytoplasmas and *Bacillus* spp. found on plant surfaces or endophytically, the

detection of phytoplasma in the collected samples with universal primers commonly used for diagnosis (R16F2n/R2) was not possible. In order to overcome this problem, new primers were designed by comparing the sequences of the phytoplasma associated with CFSD present in the database and sequences of *B. megaterium* obtained from the amplification of cassava samples (Fig. 2). The new primers amplified a fragment of 816 bp (Fig. 3). They were named FTP(F) and FTP(R) and were used for the detection of phytoplasma in all cassava samples collected. Two of these fragments were cloned and then sequenced in order to confirm that the amplified fragment belonged to the phytoplasma.

Nested PCRs using primers P1/P7 in the first reaction and primers FTP(F)/(R) in the second reaction gave consistent results. In all, 100 and 50% of symptomatic and asymptomatic plants, respectively, were positive for the presence of phytoplasmas. In the group of symptomatic plants, 21.9% of tuber samples, 68.8% of stem samples, and 87.5% of leaf samples were positive. In the group of asymptomatic plants, none of the tuber samples, 45% of stem samples, and 30% of the leaf samples was positive (Tables 3 and 4).

Viral detection in samples. All 52 samples were used for a dsRNA virus detection. A virus, probably from the *Reoviridae* family, was also detected in some of symptomatic and asymptomatic tubers, as previously demonstrated by Calvert et al. (5). In all, 50 and 20% of symptomatic and asymptomatic plants, respectively, were positive for the presence of virus in tubers (Tables 3 and 4). A fragment with expected size (isolate CFSV_Br1, accession number KF667081) and three nonexpected size fragments (i.e., nonspecific amplification) were cloned, sequenced, and analyzed. The nucleotide sequences of a fragment with expected size had 98.7% identity with the RdRp of Cassava frogskin virus segment 4 (DQ139870.1). Nonspecific amplification products were cloned and sequenced, and their sequences were found to correspond to mitochondrial sequences.

Fig. 1. Typical symptoms observed in cassava plants with cassava frogskin disease. **A** and **C**, Cassava tubers with deformities, deep injuries, looking woody and with reduced diameters. **B**, Tuber of healthy cassava plant, showing smooth surface and without deformations.

two representatives of the phytoplasma associated with cassava witches'-broom (CaWB1 and -2), the two clones obtained in this work, and a nucleotide sequence of *A. palmarum* as the outgroup. The trees generated by different methods (NJ, ML, and MB) showed similar topology; therefore, only the MB tree is presented (Fig. 6).

Branches well supported by posterior probability values (≥ 0.95) grouped all representatives of the 16SrIII group and the two clones obtained in this work, supporting the RFLP classification. It can be observed that the two clones obtained in this work were grouped with representative of the 16SrIII-L and close to 16SrIII-A, 16SrIII-G, and 16SrIII-S subgroups but are far from the representatives of CFSD from Colombia and from representatives of cassava witches'-broom disease.

Discussion

Many symptoms caused by phytoplasmas reflect changes in the plant's hormonal balance (36). With CFSD, symptoms in foliage are unusual and plants do not show typical symptoms of phytoplasma infections such as virescence, phyllody, wrinkling, dwarfism, sterility of flowers, or witches'-broom (1,2). Woody-type tubers, with thick bark and reduced diameter, may be related to increased levels of lignin and suberin in tuber tissue, as previously reported in apple trees infected by phytoplasma (26).

It is quite common for contaminant microorganisms to interfere in the PCR in the process of detection of phytoplasmas (16). Due to this problem, additional care must be taken to avoid the occurrence of contamination, such as surface sterilization of plant tissues prior to DNA extraction and the use of filter tips, gloves, and contaminant-free water. The use of different combinations of primers described in the literature can be a solution if all the procedures

listed above are insufficient. Another alternative consists of designing new primers based on existing nucleotide sequences, as carried out in this article.

The presence of phytoplasmas in the tissues of cassava plants has been proven here through nested PCR using different combinations of primers. The detection by nested PCR with the primers P1/P7 followed by FTP(F)/(R) was quite efficient, with a positive result in 100% of the symptomatic plants and 50% of asymptomatic plants. It is noteworthy that even asymptomatic plants had a high incidence of phytoplasmas. Despite the presence of phytoplasmas, the absence of symptoms may be related to plant age at the moment of infection, as observed by Rapussi et al. (29) in cauliflower plants infected by a phytoplasma. If the phytoplasma is already present in the cassava stakes at planting, it will have enough time to accumulate in the tissues of the host, thus affecting the whole process of tuber formation. If infection occurs later in the growing cycle, the accumulation of the phytoplasma might not be enough to support the occurrence of visible symptoms, although still enough to allow detection.

Leaves and stems were the best tissues for detection. Detection of phytoplasma in the tubers was not very efficient. The presence

Table 4. Percentage of symptomatic, asymptomatic, and total plants infected with phytoplasma, virus, both, or neither

Infected with	Symptomatic	Asymptomatic	Total plants
Phytoplasma	50	40	46.2
Both	50	10	3.8
Virus	0	10	34.6
None	0	40	15.4

Table 3. Detection of phytoplasma and possible reovirus in different tissues of 32 symptomatic and 20 asymptomatic cassava plants collected in the city of Jaíba, Minas Gerais, Brazil^a

Sample	Symptomatic plants				Asymptomatic plants			
	Phytoplasma			Virus	Phytoplasma			Virus
	Leaf	Stem	Tuber	Tuber	Leaf	Stem	Tuber	Tuber
1	+	+	-	-	-	-	-	+
2	+	+	-	+	+	-	-	-
3	+	+	+	+	-	-	-	-
4	+	+	-	+	-	-	-	-
5	+	-	+	-	-	-	-	-
6	+	-	-	+	+	+	-	-
7	+	+	-	-	-	-	-	-
8	+	+	-	+	-	-	-	-
9	-	+	-	+	-	-	-	-
10	+	+	-	+	-	-	-	-
11	+	-	-	+	+	+	-	-
12	+	-	-	-	-	+	-	+
13	+	+	-	-	-	-	-	+
14	+	-	-	+	-	+	-	-
15	+	-	-	-	-	+	-	-
16	+	-	-	+	-	+	-	+
17	+	+	-	+	+	+	-	-
18	-	+	-	+	-	-	-	-
19	-	+	-	-	+	+	-	-
20	+	+	-	-	+	+	-	-
21	+	+	-	-	...	...	...	...
22	+	+	+	-	...	...	...	...
23	+	+	+	-	...	...	...	...
24	+	-	-	+	...	...	...	...
25	+	+	+	+	...	...	...	...
26	+	-	-	-	...	...	...	...
27	+	+	-	-	...	...	...	...
28	+	-	-	-	...	...	...	...
29	-	+	-	-	...	...	...	...
30	+	+	-	+	...	...	...	...
31	+	+	+	+	...	...	...	...
32	+	+	+	+	...	...	...	...
Σ Positives	28	22	7	16	6	9	0	4
Positives (%)	87.5	68.8	21.9	50	30	45	0	20

^a Presence (+) or absence (-) of specific polymerase chain reaction product.

of PCR inhibitors may be one of the factors that explain the lower detection rate in the tuber tissues (14). To reduce the interference of these inhibitors in the PCR, a dilution of the DNA extract is recommended prior to the first reaction and between the two nested PCRs (17). Even with the implementation of these dilutions, the

Fig. 4. Restriction fragment length polymorphism pattern in silico generated by the nucleotide sequences from the phytoplasmas (CFSD_Br1 and CFSD_Br2) obtained from cassava plants showing cassava frogskin disease (CFSD) symptoms. M = phiX174 digested with HaeIII.

detection rate in the tubers was still very low. Due to the fact that phytoplasmas are distributed and accumulated heterogeneously in plants, detection should be tried using samples extracted from different parts of the plant to ensure the reliability of the results.

The molecular characterization was performed by RFLP and RFLP in silico analyses in sequences generated by R16F2n/R2 primers. The first characterization of a phytoplasma associated with CFSD, in Colombia, suggested the inclusion of this phytoplasma in a new subgroup called 16SrIII-L (2). However, the restriction pattern generated by the sequence determined by R16F2n/R2 primers by RFLP in silico analysis shows no differences when compared with the restriction pattern of the 16SrIII-B subgroup phytoplasma, which contains the phytoplasmas associated with cassava witches'-broom disease (CaWB Br1 and CaWB Br2) (13). The sequences generated in this study were compared with the sequence of phytoplasma associated with CFSD described previously (2). Although these have the same *MseI* cleavage pattern, they differed in the cleavage pattern of the enzymes *MboI* and *HpaII*. This excludes the possibility of belonging to the same subgroup. The RFLP and RFLP in silico analyses of fragments generated by R16F2n/R2 primers of the phytoplasma characterized in this work show that it belongs to a variant of 16SrIII-A subgroup. This is the first report of a phytoplasma belonging to the 16SrIII-A subgroup in cassava plants and also the first reported case and molecular characterization of the phytoplasma associated with CFSD in Brazil.

The characterization based on only a part of the sequence of the 16S rDNA and the separation of phytoplasma subgroups is still very subjective, because it shows no relationship to biological characteristics of the pathogen, its host, or the type of symptoms caused. Differences of only a few nucleotides in these sequences

Fig. 5. Two-dimensional plot representing the similarity coefficient among 25 16SrIII phytoplasmas, including two sequences obtained in this work (CFSD_Br1 and CFSD_Br2), three sequences of phytoplasma associated with cassava frogskin disease (CFSD) in Colombia, and two sequences of a phytoplasma associated with Cassava witches'-broom disease in Brazil (CaWB_Br1 and CaWB_Br2).

do not seem to be able to solve problems in the separation of these phytoplasmas. One example is the phytoplasmas associated with cassava. The phytoplasmas responsible for cassava witches'-broom disease and the phytoplasma related to the CFSD in Colombia have the same restriction fragment pattern of 16S rDNA and have cassava as a host in common but cause very different symptoms (2,13). Although the phytoplasma characterized in this work and that which is associated CFSD in Colombia has the same host and

the same type of symptoms, they have distinct cleavage patterns of 16S rDNA and, thus, are characterized differently. The inability to cultivate these organisms makes their characterization difficult. Increased knowledge of the relationships with insect vectors, interaction with its hosts, and larger number of genes or genomes being sequenced of several phytoplasma groups will bring greater clarity and greater support for the classification of this group of plant pathogens.

Fig. 6. Phylogenetic relationships between sequences of fragments from different groups of phytoplasmas and 16SrIII subgroups, amplified by primers R16F2n/R2, through Bayesian inference (implemented in MrBayes v.2, with model GTR+I+G and 10 million generations). *Acholeplasma palmarum* was used as outgroup. Support for the nodes is presented as filled circles (posterior probabilities from 0.95 to 1.0) or open circles (posterior probabilities from 0.85 to 0.94). CFSD1, CFSD2, and CFSD3 are sequences of phytoplasma associated with cassava frogskin disease (CFSD) in Colombia, classified as 16SrIII-L by the authors (2) and CaWB_Br1 and CaWB_Br2 are sequences of phytoplasma associated with Cassava witches'-broom disease in Brazil, classified as 16SrIII-B by the authors (13). Asterisks indicate 16SrIII-A obtained in this work.

A virus, probably belonging to the *Reoviridae* family, was also detected in some of symptomatic and asymptomatic tubers, as previously demonstrated by Calvert et al. (5). Symptomatic plants presented only phytoplasma or both phytoplasma and virus. Asymptomatic plants presented only phytoplasmas, only virus, or none or both pathogens (Tables 2 and 3). Interestingly, in most of the roots in which the virus was detected, it was not possible to detect phytoplasma. These results may suggest a possible spatial separation between the two organisms due, for example, to the preference of colonization by certain tissues or organs (20,27). The results also suggest that the symptoms of CFSD are caused only by phytoplasmas, because of their presence in all symptomatic plants even without the presence of virus. However, a synergistic interaction may also be occurring, leading to an increase in disease severity in plants in which the virus is also present. Transmission tests should be performed to confirm the etiology of the disease.

In summary, the data obtained with this research prove the existence of a phytoplasma and a putative reovirus associated with cassava plants presenting the CFSD in the town of Jafba, Minas Gerais, Brazil. This phytoplasma was characterized as belonging to the 16SrIII group and subgroup A, based on sequence of 16S rDNA. This is the first report of a phytoplasma belonging to the 16SrIII-A subgroup associated with cassava plants and the first molecular characterization of a phytoplasma causing CFSD in Brazil. The virus found showed high similarity to a *Reoviridae* family. This is the first report of a virus associated with CFSD in Brazil, and also the first report of the presence of both phytoplasma and virus in cassava with CFSD symptoms.

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