Characterization of a Phytoplasma Associated with Frogskin Disease in Cassava

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ABSTRACT

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Cassava frogskin (CFSD) is an economically important root disease of cassava (*Manihot esculenta*) in Colombia and other South American countries including Brazil, Venezuela, Peru, Costa Rica, and Panama. The roots of severely affected plants are thin, making them unsuitable for consumption. In Colombia, phytoplasma infections were confirmed in 35 out of 39 genotypes exhibiting mild or severe CFSD symptoms either by direct or nested-PCR assays employing rRNA operon primer pairs. The CFSD-associated phytoplasmas were identified as group 16SrIII strains by RFLP and sequence analyses of amplified rDNA products, and results were corroborated by PCRs employing group 16SrIII-specific ribosomal RNA gene or ribosomal protein (rp) gene primers. Collectively, RFLP analyses indicated that CFSD strains differed from all phytoplasmas described previously in group 16SrIII and, on this basis the strains were tentatively assigned to new ribosomal and ribosomal protein subgroups 16SrIII-L and rpIII-H, respectively. This is the first molecular identification of a phytoplasma associated with CFSD in cassava in Colombia.

Additional keywords: cassava, frogskin disease, PCR/RFLP analyses, sequencing,

phylogeny.

Cassava frogskin disease (CFSD) is an important disease affecting cassava (*Manihot esculenta* Crantz) roots, whose causal agent has remained unknown for many years despite its economic significance. Recently, it has been reported with increasing frequency in Colombia, Brazil, Venezuela, Peru, Costa Rica, and Panama with an incidence of up to 90% recorded in Colombian commercial fields in the production areas of Valle del Cauca, Cauca, Meta, and of the North Coast (~~34~~35,6). CFSD causes deep lesions in roots, eventually reducing their diameter; therefore in many cassava varieties symptoms are observed only when the plants are harvested. Some varieties may also show leaf symptoms such as mosaic, chlorosis, and curling and/or curvature in leaf margins. However these symptoms are difficult to observe under field conditions, and could be confused with damage from mites, thrips, deficiencies of microelements, viruses (6) or herbicide toxicity, or they can be masked when temperatures are high (>30°C). Characteristic CFSD symptoms in the roots are a woody aspect and a thickened peel that is cork-like, fragile, and opaque. The peel also presents lip-like slits that, when they join, create a net-like or honeycomb pattern. In advanced stages of the disease, the sclerenchyma and parenchyma are brown, instead of white, cream, or pink. In many cases, roots may be very thin and the bases of stems very thick (7). When roots do not tuberize adequately, stems tend to be thicker than normal. Generally, the aerial parts of diseased plants are more vigorous and better developed than those of healthy plants. In contrast, the roots of healthy plants are well developed, with thin, shiny and flexible

peels.

In open fields, the disease may affect plants with different levels of symptom severity in the roots; other plants remain asymptomatic with a few characteristic slits, whether scattered or localized in the root. Depending on the intensity of symptoms in mother plants from which cuttings are produced, the severity and incidence of infected roots per plant increases from one planting cycle to another.

To develop appropriate management strategies for reducing the spread of this disease, a proper identification of possible etiological agent(s) is necessary. Phytoplasmas have been recently detected in CFSD-affected cassava of susceptible commercial varieties, using nested-PCR to amplify a fragment of the 16S rRNA gene, but were not detected in healthy plants from the same varieties harvested from fields free of the disease (2).

Various diseases that induce symptoms in roots in diverse plant species have been reported to be associated with phytoplasmas (8,14,~~27~~28,~~32~~33). The objectives of this study were (i) to show a close association of CFSD symptoms and the presence of phytoplasmas, (ii) to identify and characterize phytoplasmas consistently associated with CFSD-affected roots, leaf midribs, petioles, and stems of different cassava varieties and genotypes from various regions of Colombia, and (iii) to verify transmissibility of phytoplasmas associated with frogskin disease to healthy cassava and periwinkle plants.

MATERIALS AND METHODS

Plant material. Symptomatic cassava plants one year old were collected from 2002 to 2004 from commercial plantations and the CIAT collection in three areas of Colombia

where the of CFSD has been endemic for more than 20 years (North Coast: 9°19´08N, 75°17´46W, 150 m; Valle del Cauca: 3°30´03.51N, 76°21´20.04W, 980 m; and Cauca: 3°04´16.90N, 76°29´57.34W, 1038 m). Leaf, midrib, petiole, stem, and root tissues from samples representing 39 genotypes of cassava grown in the field and/or in the greenhouse were collected. Five plants of each genotype (195 plants total) were sampled and their leaf, stem and root tissues were pooled separately for PCR analyses (not all tissues were sampled from each genotype) giving a total of 76 samples (Table 1). The roots of all 195 plants selected for molecular analysis were examined for symptoms and classified according to a scale of symptom severity modified from (~~28~~29). The ratings were as follows: 0, no symptoms in roots, roots have a thin and flexible peel; 10, very mild symptoms (few fissures or lip-like splits in a limited number of roots; slightly opaque and an inflexible peel); 35, mild symptoms (few fissures or lip-like splits in many roots; an opaque and brittle peel); 65, moderate symptoms (many fissures or lip-like splits in all root parts, some reduction of root size; opaque and brittle peel); 90, severe symptoms (reticulations or honeycombing in several roots, moderate reduction of root diameter/volume; thick, cork-like, and brittle peel); 100, very severe symptoms (reticulation or honeycombing in many roots, severe reduction of root mass, with pronounced woody or fibrous character and a thick, cork-like and brittle peel) (Table 1). Samples from cassava plants obtained by meristem culture *in vitro* were employed as negative controls for PCR assays.

Phytoplasma infected material. The 16S ribosomal sequences from the phytoplasma strains CFSDY15, CFSDY17 and CFSDY29 were amplified and used for the cladistic,

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RFLP, and virtual RFLP analysis. Phytoplasma reference strains representing different 16SrIII subgroups (Fig. 3) maintained in periwinkle [*Catharanthus roseus* (G.) Don.] or isolated from original host plants as nucleic acid and employed for molecular identification were: peach yellows leaf roll (PYLR), Green Valley X disease (GVX), and peach X-disease (CX) (ribosomal subgroup –A); phytoplasma from *Euscelidius variegatus* from Italy (API), ranunculus virescence (RA), plum leptonecrosis (LNI), and clover yellow edge (CYE –C) (ribosomal subgroup –B); golden rod yellows (GRI) (ribosomal subgroup –D); spirea stunt (SPI) (ribosomal subgroup –E); vaccinium witches' broom (VAC), and milkweed yellows (MW1) (ribosomal subgroup –F); walnut witches' broom (WWB) (ribosomal subgroup –G); poinsettia branch-inducing (JRI) (ribosomal subgroup –H); Virginia grapevine yellows (VGY) (ribosomal subgroup –I); chayote witches' broom (ChWB) (ribosomal subgroup –J); strawberry leafy fruit (SFL) (ribosomal group –K); dandelion virescence (DanVir) (ribosomal subgroup –P); black raspberry witches' broom (BRWB7) (ribosomal subgroup –Q); cirsium white leaf phytoplasma (CWL) (ribosomal subgroup –U); and *Solanum marginatum* big bud from Ecuador (SBB).

Nucleic acid extraction. DNA was extracted from 0.4 g of tissue from each plant part according as described previously (19,~~35~~36). Tissue samples were frozen and ground in liquid nitrogen using a sterile mortar and pestle. After the final ethanol precipitation, nucleic acid extracts were resuspended in 30 - 50 µl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) buffer and stored at 4°C until used.

PCR amplification of phytoplasma DNA. Phytoplasma universal primers P1/P7

(11,~~37~~38), were used to amplify DNA from the 16S, the beginning of the 23S rRNA genes plus the spacer region between them (for P1/P7), and primer pairs R16mF2/R16mR1 and R16F2n/R16R2 (20) were used to amplify part of the 16S gene, either in direct or nested-PCR assays. The resulting P1/P7 and R16mF2/R16mR1 amplicons from the primary PCR were diluted 1:29 with sterile HPLC grade water and the second amplification was done with R16F2n/R16R2. Samples amplified directly with R16F2n/R16R2 were diluted 1:50 with sterile water and then subjected to nested-PCR with the group-specific primer pair R16(III)F2/R16(III)R1 (25). The DNA from plants infected with a coffee crispiness phytoplasma strain (CCP) (AY525125) (18) was employed as a positive control, while DNA from healthy cassava or sterile water were included as negative controls in each assay.

The amplifications were carried out as described previously (~~25~~26,~~31~~32) with primer pairs described above and listed in Table 2. Each reaction was 25 µl, with 100 ng of DNA, 1X buffer, 3 mM $MgCl_2$, 1 U *Taq* polymerase (Promega, Madison, WI), 0.8 mM dNTPs (Invitrogen Life Technologies, Carlsbad, CA), and 0.1 µM of each primer (Operon Technologies, Inc., Alameda, CA). The amplified products were analyzed by electrophoresis in a 1.5% agarose gel, visualized by staining with 0.75 µg/ml ethidium bromide and imaged with a Stratagene Eagle Eye® II video system (La Jolla, CA).

Further molecular characterization of CFSD DNA extracts was performed by direct PCR and nested-PCR which specifically amplifies part of the 16SrIII group ribosomal protein operon (about 300 bp) (10), using the primer pair rpL2F3/rp(I)R1A (~~30~~31) for the first amplification followed by the nested primer pair rpIIIF1/rpIIIR1. This PCR was

performed using the conditions described by Schaff *et al*. (~~36~~37) (see footnote to Table 2) and visualized as described above.

Restriction Fragment Length Polymorphism (RFLP) analysis of PCR products. For identification of the phytoplasmas associated with CFSD, the direct and nested-PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis. R16(III)F2/R16(III)R1 derived amplicons were digested with *Rsa*I, *Alu*I, *Mse*I, and *Taq*I restriction endonucleases (New England Biolabs, Beverly, MA, USA), while P1/P7 and R16F2n/R16R2 amplicons were digested with *Hpa*II, *Tru*I, and *Hha*I (Fermentas, Vilnius, Lithuania) following the instructions of the manufacturer. The nested-PCR amplicons obtained with the rpIIIF1/rpIIIR1 primers were analyzed with restriction enzymes *Tru*I, *Alu*I (Fermentas, Vilnius, Lithuania) and *Tsp509*I (New England Biolabs, Beverly, MA, USA). Separation of bands generated from restriction digests was performed in 5% polyacrylamide gels and staining and visualization of DNA was as described above for agarose gels.

Multiple alignments using GenBank sequences of selected phytoplasma strains (clover yellow edge, walnut witches' broom, Virginia grapevine yellows, chayote witches' broom, strawberry leafy fruit, dandelion virescence phytoplasma, and black raspberry witches' broom phytoplasmas) which represented different 16SrIII subgroups were made with CLUSTALX (~~39~~40) and these alignments were used to generate putative restriction site maps of these 16S rRNA gene sequences with the DNASTAR program MapDraw option (DNASTAR Inc.). These maps were manually aligned for comparison to patterns obtained from phytoplasma strain CFSDY15 DNA digested with

endonucleases *Hpa*II, *Mse*I, *Hha*I, and *Sau*3AI (data not presented).

Sequencing of PCR products. R16F2n/R16R2 amplicons from samples CFSDY17 and CFSDY29 (Table 1) were purified using a Qiaquick PCR Purification Kit (Qiagen, GmbH, Hilden, Germany) and cloned in vector pGEM-T Easy and *Escherichia coli* DH5α cells (Invitrogen Life Technologies). Two clones per sample of the 1,200-1,300 bp insert were fully sequenced in both directions (Iowa State University, Office of Biotechnology, Ames, IA, USA). The R16F2n/R16R2 PCR products amplified from samples CFSDY17 and CFSDY29 also were sequenced directly after purification with a DNA-sequencing kit from Applied Biosystems. Sequences were edited and assembled using Sequencher 4.1 software.

Direct sequencing in both directions using primers P1, F1 (9), and P7 was also performed on the P1/P7 amplicon from sample CFSDY15 after processing it with a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The sequences were assembled using Sequencher 4.1 software and compared with selected nucleotide sequences in the GenBank database using BLAST (version Blast N 2.2.18) at the National Center for Biotechnology Information, Bethesda, Maryland, USA.

Cladistic analysis of CFSD phytoplasma sequences. Publicly available 16S rDNA sequences from 43 representative strains of the genus '*Candidatus* Phytoplasma' (23) including strains belonging to different subgroups of the 16SrIII group were retrieved from GenBank and aligned with the 16S ribosomal DNA sequences from CFSDY15, CFSDY17, CFSDY29 using CLUSTALX and BioEdit (21). All sequences before constructing phylogenetic trees, were trimmed to cover only 16SrDNA. Phylogenetic

trees were constructed with Maximum parsimony (MP) analysis using the close neighbor interchange algorithm, with the initial tree created by random addition for 10 replications. Neighbor-joining (NJ) and Minimum evolution (ME) using nucleotide p-distances were performed with MEGA version 4 (~~38~~39). For all methods, bootstrap analysis was also performed and replicated 500 times for estimation of stability and support for the clades; complete deletion option was used for gaps in all analyses. *Acholeplasma laidlawii* (a cultivable *Mollicute*) is phylogenetically related to phytoplasmas and was designated as the out-group to root the trees.

Phytoplasma transmission by grafting and dodder. Transmission experiments were carried out in an insect-proof greenhouse at 20–25°C and 50–90% relative humidity (RH) using naturally infected potted cassava plants as sources of inoculum. Infected donor plants were of the cassava genotype SM909-25, which showed severe symptoms in roots, and leaf chlorosis and curling under greenhouse conditions (23°C and 80% RH), and the genotype CW94-21 from the CW family in CIAT's cassava genetic improvement program, which showed mild disease symptoms under field conditions.

Transmission using dodder plants grown from seeds germinated in an insect proof greenhouse was performed for about two months from a single CFSD symptomatic source plant of each indicated cassava genotype to six two-month-old cassava plantlets obtained from *in vitro* culture and to six five-to-six-week old periwinkle seedlings per trial. The plants were visually inspected every week for three months for the appearance of symptoms.

Graft transmission using leaf midribs, petioles and shoots from each of five

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infected SM 909-25 and CW 94-21 cassava plants *via* cleft, chip-budding and spliced whip-grafts to six cassava and periwinkle plants per variety was also done using donor samples selected for stem diameters similar to those of recipient plants. Graft unions were carefully covered with Parafilm® and a plastic bag for one to three weeks. Plants were incubated in the greenhouse and observed for symptom development for five months as described above.

The presence and identity of phytoplasmas was assessed in all the plants before and after transmission experiments using nested-PCR assays with R16F2n/R16R2 primers followed by nested primers R16(III)F2/R16(III)R1. For positive samples the RFLP analyses and/or the sequencing methods described above were applied for phytoplasma identification.

RESULTS

RFLP analyses of PCR products. Direct PCR with P1/P7 primers and nested-PCR with R16F2n/R16R2 or R16(III)F2/R16(III)R1 primers resulted in the amplification of 1.7, 1.2, and 0.8 kb DNA fragments respectively from the majority of symptomatic cassava plants. The P1/P7 primer pair alone yielded direct amplification only from the SM 1219-9 and SM 909-25 cassava genotypes which were infected with the CSFDY29 and CFSDY~~29~~15 strains respectively (data not shown). Nested PCR protocols detected phytoplasmas in the 25 cassava genotypes that exhibited symptoms of CFSD with a disease rating from 35 to 100 (Table 1). No phytoplasma was detected in genotypes SM1201-5 and GM 228 14 by any PCR assay, while samples from the other 12

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genotypes in this rating group were positive for phytoplasma (Table 1). Genotypes, M Chn2, M Arg 2, ICA Catumare and Manzana (the latter two only in one of the two sampled plants), were asymptomatic and negative for phytoplasmas (Table 1). Negative control samples of cassava from *in vitro* culture were always negative in all direct and nested-PCR assays employed (data not presented).

PCR with the nested primer pair R16(III)F2/R16(III)R1 (Table 1, C) amplified the expected fragment from 97% (30/31) of root samples and 92% (33/36) of leaf samples from plants with symptom ratings between 10 and 100 (Table 1). RFLP analysis of the products obtained with these primers show that all of the cassava samples tested and the control strain CCP yielded mutually indistinguishable RFLP patterns with the four restriction enzymes employed (data not shown). The other test assays (Table 1, A and B) result to be less effective in phytoplasma detection, in particular leaf and root samples yielded, respectively 22% (8/36) and 26% (8/31) of positive results using system A; while 50% (18/36) and 48% (15/31) of positive results were obtained using system B respectively with leaf and root samples.

RFLP patterns of P1/P7 and R16F2n/R16R2 amplicons obtained from the CFSD samples listed in Table 1 showed identical profiles (data not shown); therefore only RFLP data from sample CFSDY15 (obtained from cassava genotype SM 909-25) is presented. Collectively, digestion profiles P1/P7 and R16F2n/R16R2 amplicons generated with restriction enzymes *Hpa*II, *Tru*I, and *Hha*I distinguished CFSDY15 from the indicated phytoplasma reference strains (Figs. 1A,B). The virtual restriction site analysis of the 16S ribosomal gene from strain CSFDY15 agreed with the RFLP analysis results in

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polyacrylamide gels, indicating that this strain was distinguishable from clover yellow edge, dandelion virescence phytoplasma, and black raspberry witches' broom strains by the absence of an *Mse*I site in position 1524. The CFSDY15 strain lacks a *Sau*3AI site, and shows an *Hpa*II site respectively at about position 200 and 800 when compared with walnut witches' broom, and Virginia grapevine yellows sequences; it lacks also a *Hha*I site that is present in chayote witches' broom, strawberry leafy fruit, dandelion virescence phytoplasma, and black raspberry witches'-broom at position 550 and 750. The results of RFLP and virtual RFLP analyses clearly indicate that phytoplasma infecting cassava differs on 16Sr RNA from all other described, and could be assigned to a new 16SrIII-L subgroup.

Nested-PCR using the ribosomal protein group III specific primers (Table 2) produced amplicons of the expected size from both SM 909-25 (CFSDY15) symptomatic cassava samples (leaf and roots), that were employed for identification, and also from selected phytoplasma reference strains belonging to 16SrIII group (data not shown). RFLP analyses with *Tsp509*I and *Alu*I restriction enzymes showed no differences among cassava strain CFSDY15 and most of the reference strains employed; however its *Tru*I profile was unique, indicating that phytoplasmas infecting cassava could be assigned to a new ribosomal protein subgroup rpIII-H (Fig. 2).

Cladistic analyses. Sequencing of the R16F2n/R16R2 amplicons of strains CFSDY17 and CFSDY29, and of the P1/P7 amplicon from CFSDY15 yielded sequences of 1,260, 1,298 bp and of 1,679 bp respectively, which were deposited in GenBank with the accession numbers AY737646, AY737647 and EU346761.

Comment [m9]: How many of the samples presented in Table 1 did you examine with the group III primers? If only "several" yielded a product does that mean that CFSD samples which did not yield a group III product have a different phytoplasma?

Comment [m10]: The 16S should be a highly conserved region. Why are these amplicons not the same size if Y17 and Y29 are the same organism? As explained in material and methods amplicons were sequenced directly what resulted in not equal length of good chromatograms, so sequencing yielded different length sequences while the original amplicons were of the same size.

286 Maximum parsimony analysis of the 16S rRNA gene of CFSD phytoplasmas
287 CFSDY15, CFSDY17 and CFSDY29 strains, and 43 representative strains of the genus
288 '*Candidatus* Phytoplasma' grouped cassava frogskin disease phytoplasma with
289 phytoplasmas belonging to 16SrIII group. Cladistic analysis of 16S rRNA nucleotide
290 sequences by (Fig. 3), neighbor-joining and minimum evolution (data not shown),
291 maximum parsimony methods provided trees with similar topology, so the maximum
292 parsimony results are shown (Fig. 3). Cassava frog skin disease isolates CFSDY17,
293 CFSDY29 and CFSDY15 clustered together as part of a larger cluster composed of
294 members of the 16SrIII group, and their placement in the tree suggests they are closely
295 related to chayote witches' broom and cirsium white leaf phytoplasmas, which belong to
296 16SrIII-J and 16SrIII-U ribosomal subgroups respectively. The bootstrap support for the
297 proposed new ribosomal subgroup 16SrIII-L within the 16SrIII group was 67% in the MP
298 tree, and 67 and 65% in the NJ and ME generated trees (data not shown).
299 **Transmission of cassava frogskin disease phytoplasma.** No specific symptoms were
300 observed in cassava or periwinkle receptor plants inoculated with either transmission
301 method. Based on results from nested-PCR assays, graft transmission occurred in five of
302 six transmissions from infected source SM 909-25 to both periwinkle and cassava, and
303 from source CW 94-21 in four of six and three of six transmissions to cassava and
304 periwinkle, respectively. Dodder transmission occurred only from variety SM 909-25,
305 with one of six and two of six transmissions successful for cassava and dodder
306 respectively (Table 3). The identity of phytoplasmas in the receptor plants was confirmed
307 as CFSD phytoplasmas by sequencing or RFLP assays (data not shown). These

phytoplasmas were indistinguishable from those of the donor plants tested and from those identified in the samples from symptomatic cassava.

DISCUSSION

In this study, the presence of a phytoplasma in tissues of plants exhibiting symptoms of CFSD was confirmed by direct and nested-PCR assays for two phytoplasma genes. RFLP analyses of the 16S rRNA, intergenic spacer sequences and ribosomal protein gene sequences indicated that the phytoplasmas infecting cassava belong to a new ribosomal DNA and ribosomal protein subgroups which are proposed to be named 16SrIII-L and rpIII-H, respectively (~~26~~27).

The cladistic analysis of the 16S rRNA gene confirmed that the 16SrIII ribosomal group is highly supported while the branch support for the newly named 16SrIII-L subgroup within the larger 16SrIII group is considerably weaker. Nevertheless, results supporting creation of ~~other~~ 16SrIII-B, -F, -G, -H, -I, -J, -K, -Q, -P and -U subgroups already reported in the literature (9,24,2~~7~~6,3~~2~~1) had less bootstrap support than the newly proposed 16SrIII-L subgroup. Other studies have shown that the subgrouping system using the 16Sr RNA gene does not have adequate branch support to have significant phylogenetic meaning, but it was shown, however, to have geographic and strain relevance (~~26~~27). Phytoplasmas belonging to the 16SrIII group have been reported in chinaberry (*Melia azedarach*) in Bolivia; in chayote showing witches' broom disease and in tomato showing big bud disease in Brazil; in garlic (*Allium sativum* L.) and in chinaberry (3,17,22) in Argentina, and finally in association with several diseases

including cassava witches' broom in Brazil (5). The detection of closely related phytoplasmas in many crops in South America suggests the presence and wide distribution of specific insect vectors or dissemination *via* propagation material into these different environments.

~~Phytoplasmas were detected in~~ In cassava plants, phytoplasmas were detected with direct PCR amplification~~detection successful~~ only in petioles and midribs of young leaves. Successful detection in roots required a nested-PCR assay. The best sensitivity was obtained with nested PCR, employing the primer pairs R16F2n/R16R2 and followed by group 16SrIII specific primers. This system (Table 1, C) produced 97% and 92% of positive result among the symptomatic root and leaf samples, respectively. The lack of pathogen detection in a few plants phenotypically evaluated as diseased, but with very mild symptoms (rating 10) could be related to the uneven distribution of phytoplasmas *in planta*, as already reported for other species (15).

The lack of specific symptoms in cassava and periwinkle plants infected through dodder or grafting under greenhouse conditions is consistent with our observations that limited symptoms develop on naturally infected greenhouse-grown cassava plants, and may be related to the short period of symptom observation (up to 5 months).

Although, Koch's postulates cannot be fulfilled for nonculturable phytoplasmas, the association of a phytoplasma with the majority of symptomatic cassava plants suggests that it is likely the causal agent of CFSD. Moreover the CFSD root symptoms are similar to those exhibited by lucerne plants severely affected by the Australian lucerne yellows phytoplasma, in which roots of symptomatic plants have a characteristic yellow-brown

discoloration under the taproot periderm (~~33~~34). In sweet potato infected by phytoplasmas and showing sweet potato little leaf disease, symptoms include stunting of the whole plant, and roots are also visibly affected. Depending on the time of infection, cassava tuber yields can also be severely affected and plants infected at early growing stages may not produce harvestable tubers (8). Similarly, observations from other studies indicate carrot phytoplasmas induce, among other symptoms. a reduction in the size and quality of taproots (13,~~27~~28,~~32~~33).

Several diseases reported as being of viral etiology were later associated with phytoplasma infection (12,~~29~~30) and in some cases both viruses and phytoplasmas were found to be together associated with diseases (1,4,16). The reported detection of viruses in CFSD affected plants (6,7) cannot exclude the possibility that additional pathogens can affect symptom expression. More work on the epidemiology of CFSD and on the genetic susceptibility of cassava genotypes to this disease is in progress.

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Table 1. Detection of phytoplasma in cassava samples using nested-PCR.

Disease Rating[a]	No. of Samples	Genotypes	Tissue	PCR Tests A	B	C
100	10	CM 6740-7	Leaf[b]	4/6	4/6	6/6
		CM 849-1	Root	3/4	3/4	4/4
		CM 5460-10	Stem	Nt	Nt	Nt
		M Col 2063 (CFSDT17)				
		CM 9582-24				
		SM 1219-9 (CFSDY29)				
90	15	M Bra 383	Leaf	3/7	5/7	6/7
		Venezolana	Root	4/8	6/8	7/8
		CM 9582-65	Stem	Nt	Nt	Nt
		ICA Nataima				

513			CM 9582-64				
514			CG 6119-5				
515			SM 909-25 (CFSDY15)				
516							
517	65	14	M CR 81	Leaf	1/7	5/7	7/7
518			M Col 634	Root	1/6	4/6	6/6
519			M Per 16	Stem	0/1	1/1	1/1
520			CM 3306-9				
521			Manzana				
522			ICA Catumare				
523			CIAT Parrita				
524							
525	35	8	CM 2177-1	Leaf	0/4	3/4	4/4
526			HMC-1	Root	0/4	2/4	4/4
527			M Per 335	Stem	Nt	Nt	Nt

528			CM 3306-19				
529			CM 4919-1				
530							
531	10	21	M Bra 856-54	Leaf	0/12	1/12	10/12
532			M Bra 882	Root	0/9	0/9	9/9
533			M Cub 74	Stem	Nt	Nt	Nt
534			M Col 1468				
535			M Col 1178				
536			M Bra 325				
537			M Bra 856				
538			SM 1201-5				
539			GM 228-14				
540			M Bra 829				
541			M Bra 839				
542							

543	0	8	M Chn 2	Leaf	0/4	0/4	0/4
544			Manzana	Root	0/4	0/4	0/4
545			ICA Catumare	Stem	Nt	Nt	Nt
546			M Arg 2				

547 [a]0, no symptoms; 10, very mild symptoms; 35, mild symptoms; 65, moderate symptoms; 90, severe symptoms; 100, very severe
548 symptoms.
549 [b]Leaf indicates a mix of leaf blade, midrib and petiole tissue.
550 [c]Number of positive samples/total samples tested. Nt, not tested.
551 [d]A, primers P1/P7 P1/P7+R16F2n/R16R2; B, R16mF2/R16mR1+ R16F2n/R16R2; C, R16F2n/R16R2+R16(III)F2/R16(III)R1.
552
553
554

555 Table 2. Primer combinations employed for PCR analyses of phytoplasmas infecting
556 cassava.

PCR[a] system	Direct PCR	Nested-PCR	Sequences amplified
A	P1/P7	R16F2n/R16R2	16Sr RNA
B	R16mF2/R16mR1	R16F2n/R16R2	16Sr RNA
C	R16F2n/R16R2	R16(III)F2/R16(III)R1	16Sr RNA
D	P1/P7	-	16Sr – 5' of 23S RNA
E	rpL2F3/rp(I)R1A	rpIIIF1/rpIIIR1	ribosomal protein operon (rp gene)

Comment [m11]: Why doesn' this combination of primers have a designation, e.g. 'E'?

557 [a]35 cycles for A, B, C, and D systems, and 40 cycles to rp gene were used with:
558 denaturation 1 min (2 min for the first cycle) at 94°C; annealing 2 min at 50°C (except
559 P1/P7 and R16mF2/R16mR1, 55°C); primer extension 3 min (10 min in final cycle) at
560 72°C.
561

562 Table 3. Results of phytoplasma transmission by grafting and dodder from cassava to
563 cassava and periwinkle.

	Transmission Results	
Donor Sources	SM 909-25	CW 94-21
Dodder transmission to healthy cassava	1/6[a]	0/6
Dodder transmission to healthy periwinkle	2/6	0/6
Grafting to healthy cassava	5/6	4/6
Grafting to healthy periwinkle	5/6	3/6

564 [a] Number of plants in which phytoplasmas were detected / number of receptor plants
565 employed for transmission experiment.
566

Figure legends

Fig. 1. Polyacrylamide gels showing the RFLP profiles of 16S rDNA amplified in direct PCR with primers P1/P7 (A) and in nested-PCR with primers R16F2n/R16R2 (B) from representative phytoplasma strains and from cassava frogskin disease phytoplasma strain CFSDY15. The three gels (left to right) in both panels A and B show banding patterns resulting from *Hpa*II, *Tru*I, and *Hha*I restriction endonuclease digests. *Hpa*II digest of P1/P7 amplicon (panel A) shows that CFSDY15 has a different profile from all other strains while other restriction enzymes on both amplicons did not show specific polymorhisms for the phytoplasma infecting cassava. However comparing collective profiles in panels A and B CFDSY15 only is identical to profile of JRI (ribosomal subgroup III-H). Phytoplasma- strains used for reference digestion patterns: GVX, Green Valley X disease; VAC, vaccinium witches' broom; GRI, golden rod yellows; SPI, spirea stunt; JRI, poinsettia branch-inducing; RA, ranunculus virescence; PYLR, peach yellowleaf roll; CX, peach X-disease; API, phytoplasma from *Euscelidius variegatus* from Italy; SBB, *Solanum marginatum* big bud from Ecuador; and MWI, milkweed yellows. Left and right outer lanes contain PhiX174, marker ΦX174 *Hae*III digested PhiX174 and *Hae*I digested pBR322 marker DNAs, respectively.

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Comment [m12]: This needs to be defined.

Fig. 2. Polyacrylamide gels showing RFLP patterns of phytoplasma DNAs from cassava and selected reference strains amplified in nested-PCR with the rpIIIF1/rpIIIR1 primer pair. The three gels (left to right) show *Tsp509*I, *Alu*I, and *Tru*I restriction endonuclease

digests. CFSDY15 strain shows distinguishable profiles from all other phytoplasma strains employed with both *Tsp509*I and *Tru*I restriction enzymes while *Alu*I do not shows polymorphisms except for strain VAC. Lanes marked CFSDY15 -M, -P and -L contain digested DNA amplified separately from midribs (M), petioles (P) and leaves (L) infected with Cassava frogskin disease phytoplasma strain CFSDY15. Phytoplasma strains (left to right) used for reference digestion patterns are: GVX, Green Valley X disease; VAC, vaccinium witches' broom; GRI, golden rod yellows; SPI, spirea stunt; JRI, poinsettia branch-inducing; API, phytoplasma from *Euscelidius variegatus* from Italy; CX, peach X-disease; SBB, *Solanum marginatum* big bud from Ecuador; MW1, milkweed yellows). Outermost left lane in each gel contains *Hae*III digested PhiX174 marker DNA.

Fig. 3. Phylogenetic tree constructed by maximum parsimony analysis of near full length 16S rDNA sequences from phytoplasma strains CFSDY15, CFSDY17 and CFSDY29 from Colombia and from reference phytoplasmas within the genus '*Candidatus* Phytoplasma'. Acronyms of strains in the 16SrIII group: SFL, strawberry leafy fruit; CYE-C, clover yellow edge; BRWB7, black raspberry witches' broom; MW1, milkweed yellows; DanVir, dandelion virescence; ChWB, chayote witches' broom; CWL, cirsium white leaf phytoplasma; JRI, poinsettia branch-inducing; WWB, walnut witches' broom; VGY, Virginia grapevine yellows; '*Ca*. P. pruni*', '*Candidatus* Phytoplasma pruni', . In triangles one member of each described phytoplasma taxonomic group (23,~~26~~27) was used.

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* - '*Ca.* P. pruni' name proposed at the X International Congress of the International Organization of Mycoplasmology, held in Bordeaux, France, 1994, but not yet formally described, is reported here as incidental citation which do not constitute prior citation, according to rule 28b of the bacteriological code (~~24~~25).

** - Subgroup suggested in this paper.