

Activity 1. ETIOLOGY: Identification of a reovirus in *Manihot esculenta* affected with cassava frogskin disease.

Abstract

The causal agent of cassava frogskin disease (CFSD) has remained unknown since the disorder was first identified in 1971. Although a viral agent has been suspected, it has been difficult to develop conclusive evidence. Viruslike particles of 45 and 70 nm in diameter were found in partially purified preparation. Nine or more species of ds-RNA were associated with cassava frogskin disease. Through cloning, it was found that these extractions contained ribosomal RNAs which were the dominant cDNA clones produced. The putative protein of one cDNA clone had homology with the P5 protein of rice ragged stunt virus (RRSV). The technique of reverse transcriptase AFLP was used to associate markers with CFSD plants. One AFLP product was consistently associated with affected plants and it was cloned and sequenced. This putative protein of this product has homology with the P1 protein of RRSV. These different experiments lead to the conclusion that a virus in the family reoviridae is infecting cassava. Studies are continuing to understand determine if this virus is a causal agent of cassava frogskin disease.

Introduction

Cassava frogskin disease (CFSD) is a disorder of unknown etiology that affects cassava and was first reported in 1971 from southern Colombia (Hernández *et al.*, 1975). CFSD is endemic in the Amazon regions of Colombia, Peru and Brazil. In the Amazonian region, one common name for CFSD is "Jacare" which translates to caymen. There is evidence that the range of CFSD is increasing and it has spread to other cassava growing regions of Colombia, Brazil, Venezuela and Costa Rica.

In CFSD affected cassava, the root periderm and corky layers enlarge to form raised lip shaped fissures. Roots that are severely affected do not fill with starch. In areas where the disorder is endemic the yield losses can be 100% (Lozano & Nolt, 1989). In a limited number of cassava landraces, CFSD affected plants are stunted and the leaves develop mosaic symptoms. The roots of these varieties are either stunted or have fissure typical of CFSD.

Cassava is propagated vegetatively, and all the plants grown from affected plants will have CFSD. The symptom severity is affected by temperature. In hot dry areas, affected plants may not have symptoms. If stem cuttings from these plants are grown in a cooler area, all the plants will have severe symptoms. In those plants that develop mosaic leaf symptoms, a constant temperature of 30C in a growth room will completely mask the symptoms.

Multiple double stranded RNA species have been associated with CFSD (Cuervo, 1989). This report includes evidence on particles and cDNA clones that have viral homology that were associated with the disease.

Methods

Source of host plants and isolates. The CFSD isolates were collected in Colombia and maintained in greenhouses by vegetative propagation. The isolates of C FSD were collected both in the Andean and Amazonian regions of Colombia. The isolate FSD-14 was maintained in the cassava clone M. Col 113, CMD 40 was collected with symptom of CFSD in Tolima, and FSD-29 was maintained in the landrace Secundina. The isolates of CMD were all maintained in Secundina and were designated CMD-5, CMD-80, CMD-86. The isolate R. Tolima was a regional landrace and 5460-10 was collected from a crossing block at CIAT Palmira, and FSD Amazonia was collected from the Amazonian region of Colombia.

The healthy control plants used throughout these experiments included the variety Secundina that had been subjected to heat therapy and cultured in vitro. After hardening of these Secundina plants, they were maintained in a greenhouse. Secundina is a variety that expresses mosaic leaf symptoms when affected with CFSD. Other varieties that had been tested as free of CFSD were also used as healthy controls.

Sectioning, fixation and staining for the electron microscope. Leaf, stem, petiole and root tissues of cassava were fixed in a 2% glutaraldehyde solution in 0.1M cacodylic acid and 8% paraformaldehyde, pH 7.2, for 12 hours following the method of Karnovsky.(5). The tissues were then treated with a 2% osmium tetroxide solution in water for 1 hour. The dehydration process followed a graded series of alcohol solutions with a final treatment in acetone. The tissues were impregnated using Spurr's resin and sectioned using a Sorval MT6000 ultramicrotome with a diamond knife. The sections were collected on grids coated with formvar and carbon film, stained with lead citrate and uranyl acetate for 5 minutes, and were viewed using a JEM-100SX transmission electron microscope (TEM).

Viral particle purification. Leaf and root tissues from CFSD affected plants were subjected to extraction procedures similar to those used to purify reoviruses (7). In the method that worked best, the plant tissue was extracted in 2 volumes of buffer (0.05 M Na_6HPO_4 , pH 7.8 containing 0.005 M EDTA and 0.01 M Na_2SO_3) and clarified with an equal volume of freon 113. The mixture was filtered and subjected to centrifugation at 7,600 g. and the clarification with freon was repeated. The supernatant was collected and subjected to 90 min. of centrifugation at 58,000 g. The pellet was resuspended in the extraction buffer and finally subjected to 5 hr of centrifugation in a Cs_2SO_4 step gradient (20, 30, 40, and 50%) at 84,000 G. The zones were collected, diluted with extraction buffer, and precipitated using 90 min. of centrifugation at 58,000 G.

AFLPs analysis. The AFLP method (Vos et al. 1995) was implemented by digesting the cDNA using restriction enzymes *EcoRI-MseI* and ligating it with corresponding adaptors. The PCR preamplification PCR was done with selective *MseI*-adaptors. The profile was 30 cycles consisting of 94°C for 30 sec, 55°C for 30 sec, and 1 minute at 72°C. In the cascade PCR reaction, seven combinations of primers (with 2 and 3 selective nucleotide for the *EcoRI* y *MseI* sites respectively) were used. The amplified products were analyzed using a 6% polyacrylamide gel and visualized by silver staining.

Elution and amplification of selected cDNAs. The polymorphic cDNA amplified products were eluted from pieces of the polyacrylamide gels. The selected pieces of the gels were soaked in water for 10 minutes at room temperature then an additional 15 minutes at 65°C. The aqueous portion containing the cDNA was subject to an ethanol precipitation and the pellet was resuspended in sterile water. Five μl of each sample was amplified using 10mM dNTPS, 1X PCR buffer, 2mM MgCl_2 , 0.2 μl de 10U/ μl Taq polymerase final volume of 20 μl . The PCR profiles were the same used in the original amplification and were visualized used agarose gel electrophoresis.

Cloning and sequencing of PCR products. The PCR products were cloned into the TA plasmid (In Vitrogen). Plasmid DNA was purified using WizardTM plasmid purification columns (Promega). Nucleotide sequences were determined using an ABI Prism 377 sequencer (Perkin-Elmer,) by the dideoxynucleotide chain termination procedure (Sanger et al. 1977) using the ABI dye terminator reaction ready kit. The sequence data were analyzed using DNAMAN Version 4.13 (Lynnon Biosoft, Vaudreuil, Quebec).

Results

Viruslike particles and inclusion bodies in plants affected with FSD. Previously we have reported isometric viruslike particles approximately 70 nm in diameter were found in thin sections of leaves, petioles, stems and roots of FSD affected cassava. These viruslike particles have an inner darker-stained core and an outer lighter coat, and were often found in groups or associated with inclusion bodies. Using the electron microscope a study of phloem related tissues in petioles was made. Inclusion bodies with paracrystalline structures were consistently found in the plants, which were affected with cassava frogskin disease (**Figure 1**). These viroplasmalike and the crystalline inclusion bodies were similar to those reported for plant reoviruses. In a survey of our CFSD sources, these viroplasms and crystalline inclusion bodies were consistently found in the phloem associated cells in the petioles of cassava. When viewing the healthy controls, no viroplasmas or crystalline inclusion could be found.

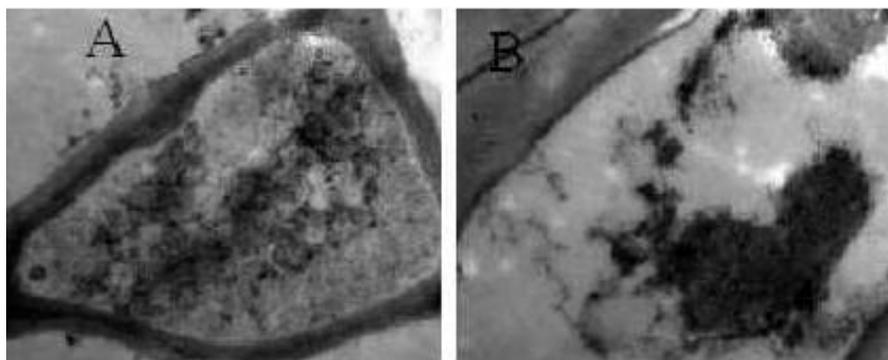


Figure 1. Viruslike inclusion bodies, crystalline structures and viruslike particle found in the phloem associated tissues in petioles of cassava plants affected with cassava frogskin disease. In A there is a viroplasmalike body and in B there is a crystalline inclusion body.

Table 1. Transmission electron microscope study of for signs of cassava frogskin disease.

Source	Disease status	Tissue	Observations
Secundina	Healthy	Petioles	The cell structure appeared normal.
Greenhouse	Healthy	Petioles	The cell structure appeared normal.
CMC 40	Healthy	Petioles	The cell structure appeared normal.
Mexico 95	Healthy	Petioles	The cell structure appeared normal.
Malasia 50	Healthy	Petioles	The cell structure appeared normal.
Paraguay 183	Healthy	Petioles	The cell structure appeared normal.
CMC 40	CFSD	Petioles	Viroplasmas like structures Cystraline virus like inclusions Virus like particles
R-Tolima	CFSD	Petioles	Viroplasmas like structures Cystraline virus like inclusions Filamentosas inclusions
Amazona 16	CFSD	Petioles	Viroplasmas like structures Cystraline virus like inclusions
5460-10	CFSD	Petioles	Viroplasmas like structures Cystraline virus like inclusions
CMD 80	CFSD	Petioles	Viroplasmas like structures
FSD 29	CFSD	Petioles	Viroplasmas like structures Cystraline virus like inclusions

Complimentary DNA cloning analysis and AFLP analyses. The majority of the cDNA clones that were sequenced had a high degree of homology with ribosomal RNAs. An exception was a cDNA clone of 280 nucleotides that contained an open reading frame of 93 bases and had 37% identity and 45% (**Figure 3**) similarity with the P5 protein of rice ragged stunt virus (RRSV).

Because of the difficulties in generating additional cDNA clones that did not have homology with ribosomal RNAs, it was decided to use AFLP analysis to identify potential amplified regions associated with CFSD. In the seven restriction site-primer combinations used to amplify rt-AFLP products from the dsRNA from healthy and affected roots, a total of 37 polymorphic products were identified. Of these 37 products, the 21 were present in the CFSD affected plants and 16 were found in the healthy plants. These products range in size from ca. 190 to 1400 base pairs.

Five different cassava lines have been selected with both healthy and CFSD affected plants. One AFLP product of approximately 230 nucleotides consistently appeared in the CFSD affected plants but was absent in the healthy controls (**Figure 2**).

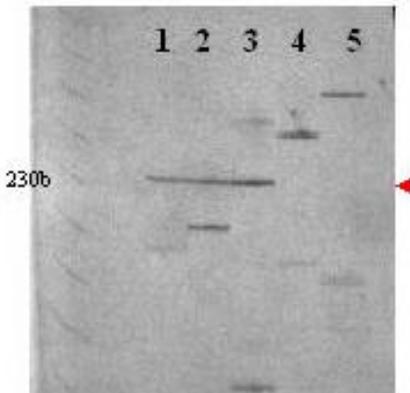


Figure 2. The cDNA-AFLP product found only in cassava frogskin affected plants. Lane 1: Mbra258 CFSD, 2: R. Tolima CFSD, 3: Amazonas 16 CFSD, 4: healthy CMC 40, and 5: healthy Secundina.

This AFLP product was cloned and two independent clones were sequenced. The cDNA clones were 228 nucleotides in length and contained an open reading frame of 72 amino-acids. The putative protein had 50% identity and 70% similarity (**Figure 3**) with a portion of the P1 protein of RRSV.

```

14   LPFRFKNHVINYYKKSSSLKQMIGSDQAVFLPGGLMHYTRGGVVICLEVYVYKNALIIATYGV
    || | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
455  LPLRYKQHVINFRKREMHQKNSNIMTGYLPGGLLHDTLGGIVICLRLDIFEDTVISVYGI

74   WVPYKLDRLIIVMICENGNANIVSRPYL      CFSD S5
    || | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
515   YNGMKLIRLICMICVYSGINIPGRPYF      RRSV S1

915  GSACAESSVMIKMSGKTKPIVKEGSTDLFLADKNFILADPI      CFSD S1
    || | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
17   GSATVEASVILEAAGKRRPSTSEASTNLFHLDNRNFIIADPV      RRSV S1

```

Figure 3. A comparison of the predicted amino acid sequences from two cDNA clones of the virus found in cassava with the proteins encoded by the segment 5 and segment 1 of rice ragged stunt virus.

Specific primers were designed for both the cDNA clones CFSD-S1 and S5. These were used to amplify extractions of total RNA that were made from both healthy and CFSD affected plants. After hybridization with either the CFSD-S1 or S5 label cDNA probe, specific products were detected. The detection was more efficient when total RNA was extracted from the roots of affected plants as compared with the leaves (**Figure 4**). The petioles of the plants were similar to roots as a starting material for the extraction. The consistency of the detection was somewhat

variable, with the same source sometime being positive and other times being negative. In general, the virus could be detected in all isolates of CFSD affected cassava.

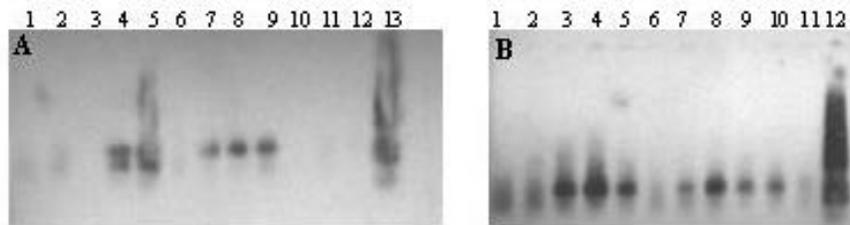


Figure 4. Detection of specific rt-PCR products using the CFSD-S5 cDNA clone as a hybridization probe. In both A and B lanes 1: Healthy Secundina, 2: healthy CMC 40, 3: FSD 5, 4: FSD 80, 5: FSD 86, 6: FES Secundina Cenicana, 7: FSD 5460-10, 8: FSD Mbra 258, 11: FSD Amazon 16. In A: lanes 10: FSD 29, 13 CFSD-S5 plasmid control, and B lane 10: FSD 14, 12: CFSD-S5 plasmid control.

Discussion

Although CFSD does not produce tumors typical of most plant reoviruses, it is characterized by the hyperplasia of the root cortex. The fissures in the cortex of the cassava roots are similar to the longitudinal splitting in maize that is a symptom of infection with maize rough dwarf Fijivirus.

Two cDNA clones isolated for cassava plants affected with frogskin disease have sequence homology with rice ragged stunt in the genera oryzavirus of the family reoviridae. The homology between the RRSV P1 polymerase protein and the P1 protein of the cassava virus were slightly higher than the homology between the P5 structural proteins. Polymerase proteins tend to be more conserve than structural proteins in viruses. Although the amount of sequence data available for the cassava virus was limited, the generation of clones with homology to two separate genomic segments of RRSV was confirmation that a reovirus was infecting cassava.

The use of AFLP to detect genetic differences is well documented and is frequently used as a means to identify differentially expressed genes. Although the genomes of plant viruses are relatively small, the lack of success with standard cDNA made more sensitive methods needed. The successful cloning of a portion of this plant virus genome demonstrates that AFLP techniques may be useful to identify causal agents of other disorders that have remained unknown despite efforts to identify them.

Cassava is a dicotyledon plant and the virus describe is probably a new genera of plant reoviruses. Studies are underway to determine if this reovirus is a causal agent of cassava frogskin disease.

References

- Cuervo, I.M. (1989) Caracterización de los ácidos ribonucleicos de doble cadena (ARN-cd asociados a enfermedades virales en yuca (*Manihot esculenta*, Crantz). Tesis Ing. Agr. Palmira. U. Nacional de Colombia. 96p.
- Francki, R.I.B., Milne, R.G., and Hatta, T. 1985. Plant reoviridae. pages 47-72 in: Atlas of Plant Viruses. Vol. 1. CRC Press, Pr. Inc., Boca Raton FL.
- Fukushi, T., Shikata, E., and Kimura, I. 1962. Some morphological characteristics of rice dwarf virus. *Virology* 18: 192-198.
- Hernández, A., Calderón, H., Zárate, R.D. and Lozano J.C. (1975) El cuero de sapo de la yuca (*Manihot esculenta*, Crantz). *Noticias Fitopatológicas* 4, 117-118.
- Karnovsky, J. M. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. of Cell Biol.* 27: 137A.
- Milne, R. G., and Lovisolo O. 1977. Maize rough dwarf and related viruses. Pages 267-341 in: *Advances in Virus Research*. Vol. 21 M.A. Lauffer, F.B. Bang, K. Maramorosch, and K.M. Smith, eds., Academic Press, Inc. New York, NY.
- Nuss, D. L. and Dall, P. J. 1990. Structural and functional properties of plant reoviruses Pages 249-306 in *Advances in Virus Research* Vol. 38. K. Maramorosch, F.A. Murphy, and A.J. Shatkin, eds., Academic Press, Inc. San Diego, CA.
- Vos, P., Hogers, R., Bleeker, M., Reijnders, M., Van der Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407-4414.

Contributors: L.A. Calvert, M. Cuervo, I. Lozano, N. Villareal, J. Arroyabe.

Activity 2. Molecular-based approach to the differentiation of cassava mealybug species (Homoptera: Pseudococcidae)

Abstract

Two species of mealybugs are important cassava pests in Latin America and Africa: *Phenacoccus herreni* and *Phenacoccus manihoti*. They are morphologically similar but *P. herreni* reproduces sexually while *P. manihoti* reproduces parthenogenetically. The similar morphology has made the determination of these species difficult. Populations of *P. herreni* and *P. manihoti* were tested using RAPDs (randomly amplified polymorphic DNA) analyses. It was easy to identify molecular markers to distinguish these species of mealybug implying that they are not that closely related. To further study the phylogenetic relationships of these mealybugs, a 16S mitochondrial rDNA fragment was cloned and sequenced. Parsimony and distance analyses were performed and the phylogenetic relatedness of these species was determined. The results confirm the placement of *P. herreni* and *P. manihoti* in distinct species and show that the African and Latin American populations of *P. manihoti* are closely related. Moreover, to determine whether or not parthenogenesis in *P. manihoti* is related to *Wolbachia*, populations from five *Phenacoccus* strains comprising three species were tested by PCR using *Wolbachia* specific primers for *ftsZ* gene. Only one strain from *P. herreni* was infected with *Wolbachia* indicating that parthenogenesis in *P. manihoti* is not induced with *Wolbachia*.

Introduction

The cassava mealybugs, *Phenacoccus manihoti* Matile-Ferrero and *Phenacoccus herreni* Cox and Williams (Homoptera: Pseudococcidae), are oligophagous insects living mainly on cassava *Manihot esculenta* Crantz (Euphorbiceae)(Matile-Ferrero, 1977; Cox & Williams, 1981). During the early 1970s, *P. manihoti* was accidentally introduced into Africa from South America spreading rapidly across Afrotropical zone such as Benin, Senegal, Congo, Togo, Gabon and Cameroun in the absence of its natural enemies and caused severe damages (Herren, 1981). In South America, *P. manihoti* is present only in restricted areas of Paraguay, Brazil and Bolivia. In contrast, *P. herreni* is more widely distributed in South America (Bolivia, Brazil, Colombia, French Guiana, Grenada, Guyana, Tobago and Venezuela), but it is not present in Africa (Williams & Granara de Willink, 1992).

These two mealybug species show a high degree of similarity in appearance, particularly in females, and it is difficult to differentiate them at the species level because of the wide variation of morphological characters in both species. The consistent characters that distinguish them is that *P. manihoti* is pink and reproduces by thelytokous parthenogenesis and that *P. herreni* is yellow and bisexual (Cox & Williams, 1981; Williams & Granara de Willink, 1992). Therefore, only observations of live material will resolve the differentiation of these two species. Because no firm evidence by molecular-based approach was done to differentiate them, the question arises as to whether or not *P. manihoti* should be considered to be the same species as *P. herreni*. It is well described that *Wolbachia*, an intracellular rickettsia-like organism, is widespread in arthropods and is known to alter host reproduction causing for example parthenogenesis in Hymenoptera (Stouthamer et al., 1993). Therefore, it is not excluded that the difference in

reproduction between *P. manihoti* and *P. herreni* could be due to the presence of *Wolbachia* symbiont in *P. manihoti*. Attempts to induce parthenogenesis in *P. herreni* by keeping females in the absence of males were not successful (Cox & Williams, 1981; Calatayud, pers. obs.), while females of *P. manihoti* reared in presence of males of *P. herreni* continued to reproduce parthenogenetically (Cox & Williams, 1981).

In this paper, molecular analyses were used to test whether *P. manihoti* and *P. herreni* are distinct species. The ITS region of the mitochondrial 16S rDNA gene has been used in several studies of phylogenetic relationships. Here, we have used RAPD PCR tools in order to identify these mealybugs and to characterize the ITS of the mitochondrial 16S rDNA gene from four populations representing three species of mealybugs in Africa and South America. Furthermore, a polymerase chain reaction (PCR) was used to detect *Wolbachia* in these populations.

Materials and Methods

Insects. All mealybug populations were collected from *Manihot esculenta* which is a host plant for these mealybug species. About 50 adult females of two mealybug species from different locations were supplied for DNA extraction. Populations of *Phenacoccus manihoti* (2 populations), *Phenacoccus herreni* (2 populations) were provided from different geographic zones (Table 1). Adult females of another mealybug species such as *Phenacoccus madeirensis* Green (Homoptera: Pseudococciade), was also collected on cassava from the field at CIAT headquarters and used as positive control for species differentiation. All mealybugs were suspended in 70% ethanol and stored at -20°C until molecular analyses.

DNA extractions. Genomic DNA was extracted from individual mealybugs by the method of Gilbertson et al. (1991). After each insect was homogenized in 10 µl of the extraction buffer, containing 50mM of EDTA (pH 8.0), 500 mM NaCl and 10 mM 2-mercaptoethanol, an additional 190 µl of extraction buffer and 20 µl of 10% aqueous SDS solution were added. The sample was incubated at 65°C for 10 min. Then 20 µl of potassium acetate solution (5 M, pH 5.5) was added and the reaction mix was centrifugation at 15000 rpm for 10 min, 100 µl of pure isopropanol solution was added to the suspension. The solution was centrifuged at 15000 rpm for 10 min, and the pellet was washed by 50 µl of 70% aqueous ethanol solution. After centrifugation at 15000 rpm for 5 min, the pellet was dried by speed vacuum system. The samples were resuspended in sterile water and stored at -20° C.

RAPD PCR analysis. The DNA was amplified using the polymerase chain reaction (PCR). The primer used were Operon H9 (5'-TGTAGCTGGG-3') and H16 (5'-TCTCAGCTGG-3')(Operon, Alameda, CA, USA). Reaction mixtures for PCR amplification consisted of 1.2 units of *Taq* DNA polymerase, 3 mM MgCl₂, 0.15 mM deoxynucleotide triphosphate, 0.8 µM primers, and 10 ng of DNA template in a final volume of 25 µl. The reactions were carried out using programmable thermal controllers (PTC-100, MJ Research, Waltham, MA, USA). The PCR parameters were 94°C for 5 min, 40°C for 2 min and 72°C for 3 min followed by 39 cycles of 94°C for 1 min, 40°C for 1.5 min and 72°C for 2 min. The PCR products were run in agarose gels, stained with ethidium bromide and visualized using UV light.

PCR, cloning and sequence analysis of the 16S mitochondrial rDNA fragment. Mitochondrial 16S rDNA fragment was amplified using the polymerase chain reaction (PCR). The primer 4119 (5'-CGCCTGTTTAACAAAAACAT-3') was the forward primer and primer 4118 (5'-CCGGTCTGAACTCAGATCACGT-3') was the reverse primer (Xiong & Kocher, 1991). The PCR reactions conditions were 30 cycles of 1 min at 95°C, 50 sec at 50°C and 50 sec at 72°C. In the last cycle, the 72°C reaction was for 10 min. The products were purified using the Wizard™ PCR purification columns (Promega, Madison, WI, USA) and were visualized by agarose gel electrophoresis with ethidium bromide. The PCR products were cloned into the plasmid PCR script amp SK(+)-TM (Stratagene, La Jolla, CA, USA). Plasmid DNA was purified using Wizard™ plasmid purification columns (Promega, op. cit.). Nucleotide sequences were determined using an ABI Prism 377 sequencer (Perkin-Elmer) by the dideoxynucleotide chain termination procedure (Sanger et al., 1977) using the ABI dye terminator reaction ready kit. The sequence data were analyzed using DNAMAN Version 4.13 (Lynnon Biosoft, Vaudreuil, Quebec).

Wolbachia detection in *Phenacoccus* populations. Total DNA was extracted as described above from individuals and PCR was carried out with specific *Wolbachia ftsZ* gene primers (Holden et al., 1993). Two kinds of PCR were performed, classical method as described by Holden et al. (1993) and Heddi et al. (1999) and long PCR, which was shown to improve *Wolbachia* DNA amplification (Jeyaprakash & Hoy, 2000).

Results

PCR RAPD Analysis of mealybugs. Populations of *P. herreni*, *P. manihoti* and *P. madeirensis* were tested using the operon primers H9 and H16 in RAPD analyses. For *P. herreni*, populations from Colombia and Brazil were tested, and the amplified products had similar patterns (**Figures 1 a and b**). For *P. manihoti*, populations from Paraguay and the Republic of Congo were tested, and the amplified products had similar patterns. The set of primers amplified nearly identical set of products from both populations. Only one population of *P. madeirensis* was tested as a positive control for species differentiation and the patterns of amplified products for both set of primers were very distinct from the other mealybugs tested.

Wolbachia detection in *Phenacoccus* populations. Five *Phenacoccus* strains, belonging to three species were collected from different locations worldwide and tested by PCR for the presence/absence of *Wolbachia* : 2 *P. herreni* from Colombia and Brazil, 2 *P. manihoti* from Congo and Paraguay and 1 *P. madeirensis* from Colombia. Two repetitions based on two independent DNA extractions were realized on each strain in parallel with a positive control from *Trichogramma*.

All strains were shown to be negative for *Wolbachia* when using the classical PCR method. Long PCR demonstrated that the only *Wolbachia* infected strain is the *P. herreni* from Brazil (data not shown). Neither method of PCR detected *Wolbachia* in *P. manihoti*, *P. madeirensis*, or in *P. herreni* from Colombia. Moreover, it is noteworthy that the *ftsZ* band intensity from *P. herreni*-Brazil sample was very weak as compared to the *Trichogramma* positive control suggesting a low *Wolbachia* density within this strain.

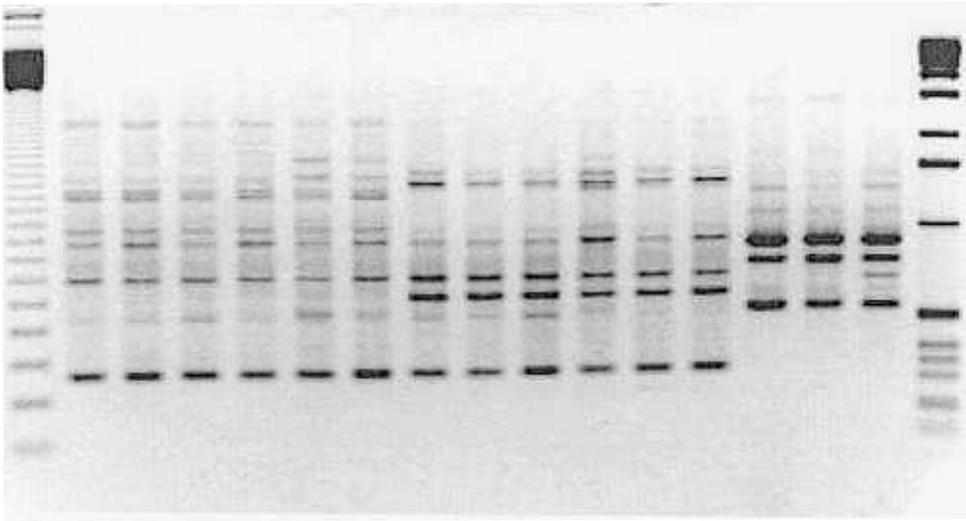


Figure 1a. RAPD PCR products from individual mealybugs with H9 primer. M1: 123 bp Markers, 1 - 3: *P. herreni* CIAT, 4 - 6: *P. herreni* Brasil, 7 - 9: *P. manihoti* Congo (Africa), 10-12: *P. manihoti* Paraguay, 13-15: *P. maderensis* CIAT, M2: 1 Kb Markers.

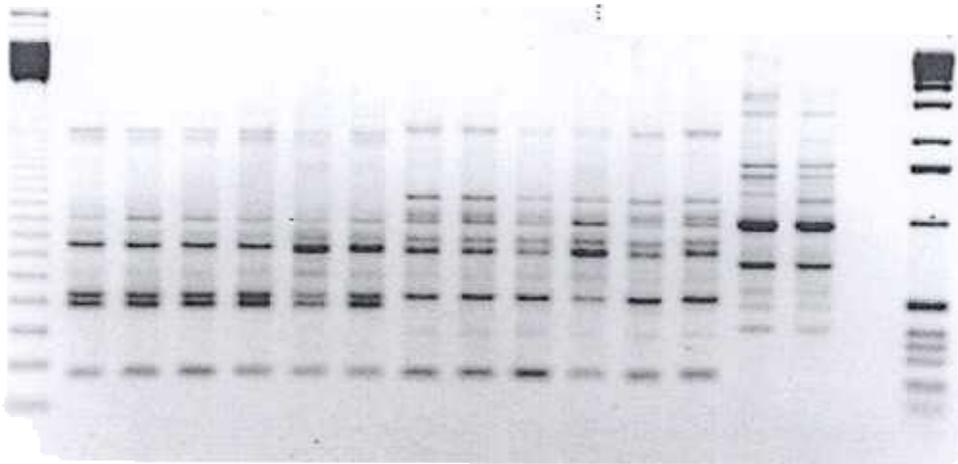


Figure 1b. RAPD PCR products from individual mealybugs with H16 primer. M1: 123 bp Markers, 1 - 3: *P. herreni* CIAT, 4 - 6: *P. herreni* Brasil, 7 - 9: *P. manihoti* Congo (Africa), 10-12: *P. manihoti* Paraguay, 13-15: *P. maderensis* CIAT, M2: 1 Kb Markers.

Discussion

The two primers used by RAPD analysis proved efficient for distinguishing between *P. herreni* and *P. manihoti* species. This implies that they are sufficiently evolutionarily different at the molecular level to produce multiple unique amplified products. Both primers proved also useful in confirming that the populations of *P. herreni* appeared nearly identical using RAPD analyses, and it was concluded that they are the same species. The same conclusion can be done also for the populations of *P. manihoti*. Since it is not easy to distinguish between *P. herreni* and *P. manihoti* using morphological characteristics, the RAPDs are a diagnostic method that can be used for the rapid identification of these species. Also the possibility to distinguish *P. herreni* to *P. manihoti* was additional evidence that these species are different indeed unique species.

Discussion part of 16S mitochondrial rDNA fragment

Wolbachia is a rickettsia-like organism that is widespread in invertebrates and it is estimated to occur in 15-20 % of insect species (Werren, 1997). It has been also found in Crustacea, nematodes and arachnids. It is transmitted vertically throughout the egg cytoplasm but it has also undergone extensive intertaxon transmissions, sometimes between different orders of insects. In arthropods, *Wolbachia* is known to alter host reproduction in mainly four ways : cytoplasmic incompatibility (Breeuwers & Warren, 1990 ; O'Neill & Karr, 1990), parthenogenesis (Stouthamer et al., 1993), feminization of genetic males (Rigaud et al., 1991) and male-killing (Hurst et al., 1999 ; Jiggins et al., 2000). In the *Phenacoccus* genus, *Wolbachia* does not seem to play an important role on the insect reproduction. First because the *Phenacoccus manihoti*, the species that reproduce parthenogenetically, is not infected with *Wolbachia*, as revealed by PCR experiment. Second, the only *Wolbachia* infected strain among the five strains tested is the *P. herreni* from Brazil, which is bisexual. Furthermore, this strain does not seem to harbor a high density of *Wolbachia* as exhibited by the *Trichogramma* positive control. This indicate either a possible incompatibility between *Phenacoccus* and *Wolbachia* or a recent *Wolbachia* contamination of the insect by this bacterium.

Acknowledgements

We thank Bruno Le Rü (IRD) for providing the *P. manihoti* population from Pointe Noire (Kouilou, Congo), Aristoteles Pires de Matos (EMBRAPA) for providing the *P. herreni* population from Cruz das Alma (Bahia, Brazil), and Julio Caballero (MAG) for providing the *P. manihoti* population from San Lorenzo (Central, Paraguay). Thanks are also given to Pilar Hernandez for her technical help. This work was supported in part by the French Ministry of Foreign Affairs.

References

- Beuning, L. L., P. Murphy, E. Wu, T. A. Batchelor and B. A. M. Morris. 1999. Molecular-based approach to the differentiation of mealybug (Hemiptera: Pseudococcidae) species. *J. Econ. Entomol.* 92: 463-472.
- Cox, J. M. and D. J. Williams, 1981. An account of cassava mealybugs (Hemiptera: Pseudococcidae) with a description of a new species. *Bull. Entomol. Res.* 71: 247-258.
- Gilbertson, R. L., M. R. Rojas, D. R. Russell and D. P. Maxwell. 1991. Use of the asymmetric polymerase chain reaction and DNA sequencing to determine genetic variability of bean golden mosaic geminivirus in the Dominican Republic. *J. Gen. Virol.* 72: 2843-2848.
- Heddi, A., A. M. Grenier, C., Khatchadourian, H. Charles and P. Nardon. 1999. Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. *Proc. Nat. Acad. Sci. USA.* 96: 6814-6819.
- Herren, H. 1981. Biological control of the cassava mealybug. *Tropical Roots Crops, Research Strategies for the 1980s - First Triennial Root Crops Symposium, Ibadan (NIG), IITA:* 79-80.
- Holden, P. R., J. F. Y. Brookfield and P. Jones. 1993. Cloning and characterization of an *ftsZ* homologue from bacterial symbiont of *Drosophila melanogaster*. *Mol. Gen. Genet.* 240: 213-220.
- Hurst, G. D. D., M. F. Jiggins, G. Schulenburg, D. Bertrand, S. A. West, I. I. Goriacheva, I. A. Zakhov, J. H. Werren, R. Stouthamer and M. E. N. Majerus. 1999. Male killing *Wolbachia* in two species of insect. *Proc. R. Soc. Lond. B* 266: 735-740.
- Jeyaprakash, A. and M. A. Hoy. 2000. Long PCR improves *Wolbachia* DNA amplification : wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* 9: 393-405.
- Jiggins, F. M., G. D. D. Hurst, C. E. Dolman and M. E. N. Majerus, 2000. High-prevalence male-killing *Wolbachia* in the butterfly *Acraea encedana*. *J. Evol. Biol.* 13: 495-501.
- Matile-Ferrero, D. 1977. A new species infesting cassava in equatorial Africa, *Phenacoccus manihoti* n. sp. (Homoptera, Coccoidea, Pseudococcidae). *Ann. Société Entomol. France.* 13: 145-152.
- O'Neil, S. L. and T. L. Karr. 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature* 348: 178-180.
- Rigaud, T., C. Souty-Grosset, R. Raimond, J. P. Mocquard and P. Juchault. 1991. Feminizing endocytobiosis in the terrestrial crustacean *Armadilidium vulgare* Latv (Isopoda) : recent acquisition. *Endocytobiosis Cell Res.* 7: 259-273.
- Sanger, F., S. Nicklen and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Nat. Acad. Sci. USA* 74: 5463-5467.

Stouthamer, R., J. A. J. Breeuwer, R. F. Luck and J. H. Werren. 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature* 361: 66-68.

Werren, J.H. 1997. Biology of *Wolbachia*. *Ann. Rev. Entomol.* 42: 587-609.

Williams, D. J. and M. C. Granada de Willink, (eds) 1992. Mealybugs of Central and South America. CAB International, Wallingford, UK. 635p.

Xiong, B. and T.D. Kocher. 1991. Comparison of mitochondrial DNA sequences of seven morphospecies of black flies (Diptera : Simuliidae). *Genome* 34: 306-311.

Contributors: L. Calvert, M. Cuervo, A. Heddi, C. Khatchadourian, D.F. Múnera, A.C. Bellotti, P.-A. Calatayud

Training

Descripcion general de la enfermedad de Cuero de Sapo, Municipio de Pescador, Departamento del Cauca, March 2002. Maritza Cuervo. Distribution of 200 pamphlets in this zone.

Distribution of 150 pamphlets of FSD in three courses of cassava production given in Guanipa, Cojedes y Zulia (Venezuela), April 2002.

Conference

Avances en la asociación de un nuevo virus en yuca con la enfermedad del cuero de sapo. XVI Congreso Nacional de Fitopatología y Ciencias afines. Bogotá. 3-5 July 2002.