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Madeleine Karlsson

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Madeleine Karlsson

Supervisor: Docent Anders Kvarnheden (Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences)

Assistant supervisors: Dr. Aldo Rojas (INTA, Nicaragua)
Delfia Isabel Marcenaro Rodriguez (INTA, Nicaragua)

Examiner: Professor Christina Dixelius (Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences)

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Sammanfattning

Produktionen av bönor i Nicaragua påverkas av förekomsten av begomovirus (familj *Geminiviridae*; släkte *Begomovirus*). Virusinfektioner orsakar en minskad avkastning av olika grödor varje år, vilket gör dem viktiga att undersöka. Denna studie var en del av INTAs pågående jobb att undersöka förekomst och diversitet av virus som infekterar bönplantor i Nicaragua. Syftet med denna studie var att identifiera vilka arter av begomovirus som infekterar bönplantor, samt att beräkna graden av så kallade blandade infektioner med olika begomovirus. Växtprover samlades in i två skilda områden i Nicaragua (Jinotega och Nueva Guinea), och observationer som tydde på virusinfektioner gjordes i båda områdena. Rolling circle amplification (RCA) användes för att öka mängden cirkulärt virus-DNA för vidare analys med polymeraskedjereaktion (PCR) och restriktionsanalys (RFLP). PCR med ett universialt primerpar användes för att konfirmera infektion med begomovirus i både Jinotega och Nueva Guinea. Resultaten från restriktionsanalyserna (RFLP) tydde på infektion med mer än ett begomovirus i växtprover från två fält i Jinotega. Sekvensanalyser av RCA-produkten från två växtprover (Jinotega 2a och Nueva Guinea 15c) indikerade även blandad infektion med *Bean golden yellow mosaic virus* (BGYMV) och *Calopogonium golden mosaic virus* (CalGMV) i prov 2a samt infektion med CalGMV i 15c. Eftersom begomovirus infekterar bönor även på atlantkusten bör man överväga att göra upp strategier för att kontrollera begomovirusinfektioner även i detta område. Eftersom spridningen av CalGMV kan vara mer omfattande i Nicaragua än tidigare varit känt, finns det även behov för ytterligare studier om överföringen av virus mellan bönplantor och alternativa värdar, såsom *Calopogonium* sp.

Nyckelord: *bönor, begomovirus, blandade virusinfektioner, rolling circle amplification, restriktionsanalys*

Abstract

The production of common bean in Nicaragua is restrained by the occurrence of begomoviruses (family *Geminiviridae*; genus *Begomovirus*). Infections with viruses are known to cause yield loss every year, making them an important target for investigation. This study was a part of INTA's work to investigate the current prevalence and diversity of viruses in common bean plants in Nicaragua. The objective of this study was to identify species of begomoviruses that infects bean plants, and to estimate the degree of mixed infection of different begomoviruses. Samples were collected from two different areas on both costs of Nicaragua (Jinotega and Nueva Guinea), and observations indicating virus infection were made in both areas. Rolling circle amplification (RCA) was run in order to increase the amount of circular viral DNA and polymerase chain reaction (PCR) with a universal primer pair was used for detection of begomoviruses. Infection with begomovirus was confirmed in both Jinotega and Nueva Guinea. The results from restriction fragment length polymorphism (RFLP) indicated infection with more than one begomovirus in samples from two fields in Jinotega. Sequence analysis of RCA products from two samples (Jinotega 2a and Nueva Guinea 15c) also indicated mixed infection with *Bean golden yellow mosaic virus* (BGYMV) and *Calopogonium golden mosaic virus* (CalGMV) in sample 2a and infection with CalGMV in sample 15c. As begomoviruses are infecting beans of the Atlantic coast, strategies should be considered to control begomovirus infections in this area as well. Since the distribution of CalGMV could be more extensive in Nicaragua than previously known, there is a need for further investigations regarding the transmission of viruses between common beans and alternative hosts, such as the weed *Calopogonium* sp.

Key words: *common bean, begomovirus, mixed viral infections, rolling circle amplification, restriction analysis*

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1. Introduction

1.1 Background

When looking at the important crops of Nicaragua, it becomes clear that the common bean (*Phaseolus vulgaris*) is of great importance for the country. Beans are staple foods, which are eaten every day together with traditional dishes. Together with maize, tomatoes, pepper and cucurbits, beans are the main crop for small-scale holdings (Rojas, 2004). As many as 76% of the population live in rural areas where they make a living from agriculture (FAO). Most agriculture is small scale and thus very vulnerable to crop loss.

In Nicaragua, as well as other Latin American countries with tropical lowlands and mid-altitude valleys, whitefly (*Bemisia tabaci*) transmitted begomoviruses (family *Geminiviridae*; genus *Begomovirus*) are important pathogens of common beans and other vegetable crops (Morales, 2001; Singh & Schwartz, 2010). Begomoviruses are known to be responsible for substantial losses every year, causing a constraint to the bean production. The virus infection can affect plants in many ways, but the most serious damage is on the photosynthesis with reduced yield of starch as a result. Early infection can often result in a total crop loss (Rojas, 2004). Begomoviruses are transmitted to dicotyledonous plants, including beans and other important crops. The genome consists of single stranded-circular DNA, and many begomoviruses have a bipartite genome. This means that it consists of two segments, DNA-A and DNA-B (Fig. 1A), where the size of each component is 2.6-2.7 kb (Ala-Poikela et al., 2005).

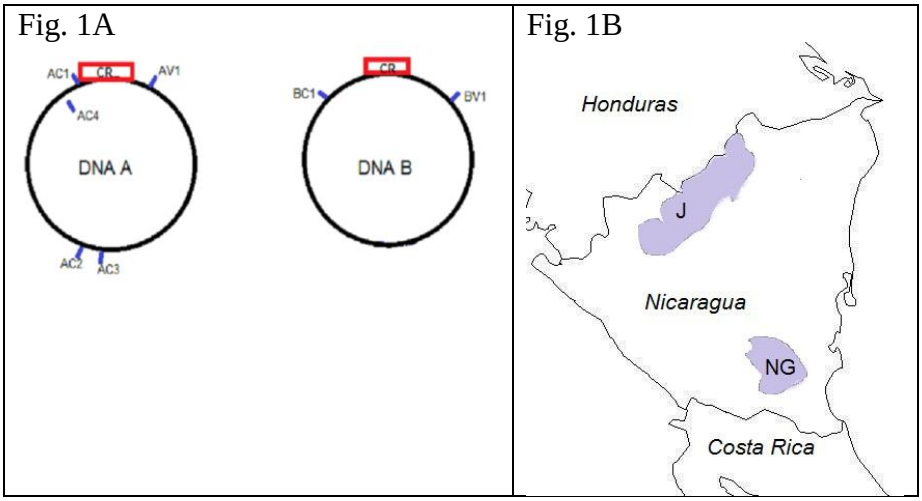


Fig. 1. A. Genome organization of a bipartite begomovirus. Included are the starting positions of the genes AC1, AC4, AC2, AC3, AV1, BC1, BV1 and the common region (CR) of component A and B. **B**. Collection sites in Nicaragua: Jinotega (J) and Nueva Guinea (NG).

An extensive mapping of different begomoviruses in Nicaraguan bean plants have been done by Karkashian et al. (2011). *Bean golden yellow mosaic virus* (BGYMV) was the most common begomovirus found. Symptoms of begomovirus infection in beans include mosaic with yellowing of leaves, reduced growth and malformation, distortion of leaves and pods, and reduction of seed yield and quality (Lapidot & Friedmann, 2000; Bracero & Rivera, 2003). In the recent study by Karkashian et al. (2011), the samples were collected in 2002,

and only covered the western and central part of Nicaragua. The Atlantic coast of Nicaragua has an extensive use of the arable land for bean production and needs to be investigated as well. Also, the work with breeding of new bean varieties, including plants tolerant or resistance against different pathogens, creates the need for new data constantly.

BGYMV was discovered in Nicaragua in the early 70s, but it was not until the 90s it became economically significant. In this period there was an increase of the prevalence of the virus, possibly as a result of moving other plant material into the traditional bean growing areas (Karkashian et al., 2011). The prevalence of *B. tabaci* has also increased in Nicaragua, making the transmission of begomoviruses more frequent as a result of intensive crop monocultures. A new variant of *B. tabaci* has recently been found, called biotype B. This variant is a very effective virus vector and is feeding on several types of crops, which possibly can be a part of the growing problems (Torres-Pacheco et al., 1996; Ala-Poikela et al., 2005**)**.

The occurrence of begomoviruses cannot be reduced by the use of agrichemicals, why a lot of efforts have been put into trying to control the vector *B. tabaci*. The main protection against *B. tabaci* has for many years been the application of insecticides. However, the extensive use has possibly led to an increase in insecticide-resistance in the vector (Torres-Pacheco et al., 1996) and thus contributed to an exponential increase in the *B. tabaci* population and also the incidence of begomoviruses (Morales, 2001). Since insecticides often fail to provide protection against begomoviruses, breeding for resistance has been proven to be a very good and sustainable complementary control method (Morales, 2001). For the practice of breeding to be successful, information on the diversity among the prevalent viruses is essential. There is nevertheless some constrains when working with resistance, for example the occurrence of mixed virus infections in the fields (Ala-Poikela et al., 2005). Mixed virus infections provide the pre-condition for recombination, which is one of the mechanisms behind the formation of new virus strains or species. The new viruses may cause more severe disease compared to the former virus genotypes, adding to the complexity of the situation (Padidam et al., 1999). There is a need for more research in this field since the occurrence of mixed begomovirus infections has not been extensively studied in Central America (Ala-Poikela et al., 2005). Also, different types of beans are requested in different areas for social reasons. Farmers may neglect a resistant cultivar excellent to use in one area if the people living there is unwilling to buy the crop. Consumer preferences of size, shape, colour and taste must be met before commercialization of a new variety can be made, which makes the breeding process more difficult (Singh & Schwartz, 2010). At present, most varieties of beans in Nicaragua are considered resistant against begomovirus infection (pers. mess. Delfia Isabel Marcenaro). However, the rate of evolution among viruses is very high and few cultivars will be able to stay resistant against all virus strains over time (Singh & Schwartz, 2010).

Despite efforts from farmers to manage the viruses, these pathogens continue to cause significant yield loss. The main reason for this is the lack of technical assistance in rural areas (Karkashian et al., 2011). The viruses are continuously evolving and cause great damage to different kinds of crops due to the breakdown of former resistance and the emergence of more aggressive pathogens. This fact gives an indication of the importance of a functioning breeding program since the situation is constantly changing. Today, much research about begomoviruses in crops grown in Nicaragua is performed at El Instituto Nicaragüense de

Tecnología (INTA). The governmentally founded institute is working with both agricultural research and information to the farmers in order to achieve an improved and sustainable food production in the country.

1.2 Objectives

This project was a part of on-going research at INTA that aims to identify and characterize pathogens of different crops in Nicaragua. One objective was to establish the virus status of different bean cultivars in Nicaragua. Extensive work has been carried out by a research group from Costa Rica in order to identify begomoviruses that infect bean plants in different regions of Nicaragua (Karkashian et al., 2011). However, the samples were taken in 2002 and since viruses are constantly evolving, there is always a need for new results. Knowledge about the diversity of viruses infecting crops is essential for the work to produce new resistant varieties as well as other methods for virus control. The counties of Jinotega and Nueva Guinea, located in different parts of Nicaragua, were chosen as collection sites for bean samples.

1.3 Aims of study

1. To establish the prevalence of begomoviruses in Jinotega and Nueva Guinea, Nicaragua.
2. To establish the degree of mixed begomovirus infections in Jinotega and Nueva Guinea, Nicaragua.
3. Identification of begomoviruses with sequencing.

2. Materials and Methods

2.1 Collection of plant samples and field survey

Young leaves were collected from common bean plants of varying ages and varieties during January and February 2012. The samples were collected in 12 different fields in the counties of Jinotega and Nueva Guinea (Table 1; Fig. 1B) from rural small-scale farms, and symptoms were observed. The plants showed a varying degree of symptoms from severe mosaic, stunting and curling of the leaves to very mild mosaic. From three of the fields, one sample without symptoms was collected. The samples were collected in plastic bags, labelled and stored at -20°C until analysis.

2.2 DNA extraction and rolling circle amplification (RCA)

The sap from frozen leaf samples was applied to FTA-cards (Whatman) in order to store and extract total DNA. Ethanol (70%) was added to four small discs from the FTA-card in a PCR-tube, which was centrifuged for 5 minutes. The discs were washed with TE-buffer (EDTA and Tris-HCl pH 8) and FTA purification buffer, and left to dry at room temperature. In order to elute the DNA from the discs, 20 µl of Tris-HCl (pH 8) was added and the purified DNA was stored at -20°C until analysis.

As previously described by Inoue-Nagata et al. (2004), rolling circle amplification (RCA) was run on samples in order to increase the amount of circular viral DNA for further analysis. The method is amplifying all infecting circular DNA in the samples by a single reaction (Haible et al., 2006). RCA on the extracted DNA was carried out using a bacteriophage Φ29 DNA polymerase included in the "Illustra TempliPhi 100 Amplification Kit" (GE Healthcare) according to instructions given by the manufacturer. Five µl of sample buffer was incubated at 95°C for 3 minutes, together with 1 µl DNA-extract. Five µl of reaction buffer and 0.2 µl of enzyme were added subsequently and the mixture was incubated at 30°C for 18 hours. Later, the RCA product was analysed by electrophoresis (100 V) on a 1% agarose gel stained with EtBr. The gel was run in 1X TAE buffer (Tris, acetate and EDTA) for one hour in order to select samples suitable for further analysis.

2.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used in order to detect begomoviruses in the bean samples. The universal primer pair AV494 and AC1048 was used to amplify the highly conserved core region of the coat protein gene *AV1* with the expected size of 576 bp including primers (Wyatt & Brown, 1996). Each reaction contained 1 µl of RCA product, 0.25 µl DreamTaq Polymerase (Fermentas), 0.3 µl dNTP mix, 0.3 µl of each primer (10 mM), 2.5 µl 10x DreamTaq Buffer (Fermentas) and 18.5 µl of sterile water to a total reaction volume of 25 µl. As negative controls, PCR tubes containing only PCR reagents were used. A positive control from a begomovirus infected tomato plant was used when analysing the DNA-extracts at the laboratory of INTA. Viral DNA was initially denatured at 94°C for 3 min and then amplified in a thermal cycler (BioRad) for 34 cycles with the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 1 min and elongation at 72°C for 90 s. These cycles were followed by a final extension at 72°C for 10 minutes. PCR products were analysed by electrophoresis on an agarose gel (1%) stained with EtBr in 1X TAE buffer.

2.4 Restriction fragment length polymorphism (RFLP)

In order to distinguish viral genotypes and detect mixed virus infections, restriction fragment length polymorphism (RFLP) was carried out on the RCA product of PCR positive samples. Two µl of RCA product was digested with six different restriction enzymes: *Eco*RI, *Hpa*II, *Bam*HI, *Hin*dIII, *Sac*I and *Kpn*I (Fermentas). The conditions were essentially according to the manufacturer's instructions. The reaction mix was incubated at 37°C for 1 hour. The reaction was stopped by 20 min of incubation at 65°C (*Eco*RI, *Hpa*II, *Hin*dIII, *Sac*I) or 80°C (*Bam*HI, *Kpn*I). The RFLP products were analysed by electrophoresis (100 V) on a 1% agarose gel in 1× Tris borate-EDTA (TBE) buffer, which was run for 1 hour.

2.5 Cloning and sequencing

DNA fragments of the expected size of begomovirus genome molecules (2.7 kb) obtained by digestion with *Eco*RI for three fields (1, 2 and 15) were cloned in order to provide larger amounts of DNA for the sequencing reaction. Digested DNA was extracted from the gel using the GeneJET gel extraction kit (Fermentas) according to the manufacturer's protocol, and was used for subsequent cloning. Ligation was done with an *Eco*RI-digested and alkaline phosphatase treated pBluescript vector using T4 DNA ligase (Fermentas) according to the

manufacturer's instructions. The mixture was incubated overnight at 14°C. In the transformation step, the cloned plasmids were inserted into DH5α *Escherichia coli* cells. *E. coli* cells (100 µl) were added to Eppendorf tubes and the tubes were incubated on ice for 30 minutes. After heat shocking for 1 min at 42°C and subsequent cooling on ice, 900 µl of LB medium was added and the mixture was incubated with shaking at 37°C for one hour. The bacterial cells were spread on LB agar plates containing ampicillin and X-gal. White/blue screening of the LB agar plates made it possible to select colonies containing the inserted DNA. After allowing the colonies to grow overnight in LB media, the plasmid DNA was extracted following the GeneJET plasmid miniprep kit (Fermentas) protocol. The isolated plasmid DNA was screened with *Eco*RI digestion in order to detect successful cloning. The clones were sent to Macrogen Inc. for sequencing with the primer pair M13F (forward) and M13R (reverse). BLASTn was used to compare the sequences with those available in the NCBI GenBank.

3. Results

3.1 Field survey

Symptomatic bean plants were found in both Jinotega and Nueva Guinea, but in general the fields located in the county of Jinotega had a larger frequency of plants with symptoms. Plants with symptoms were easier to find compared to Nueva Guinea, and the symptoms were in general more prominent. However, plants with severe symptoms were only found in field 1, 2, 4, and 5 (Table 1). In Nueva Guinea, plants with severe symptoms were limited to field 11 and 15. Severe symptoms include strong mosaic pattern on leaves, stunting and curling of leaves (Fig. 2). On plants with very mild symptoms, only a weak mosaic pattern could be seen (Fig. 3). The insect vector *B. tabaci* was seen more frequently in the fields of Jinotega.

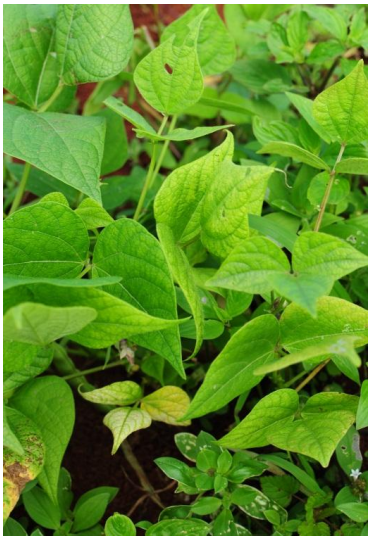
Fig 2	**Fig 3**	
		**Fig. 2.** Bean plant from Nueva Guinea with severe mosaic, stunting and curling of leaves. **Fig 3.** Bean plant from Nueva Guinea with mild mosaic.

Table 1. Samples of common bean collected in Nicaragua during January-February, 2012

Field nr.	County	Field location	Age of plant (days)	Variety	Samples with severe symptoms[a]	No. of samples tested negative with PCR[b]	No. of samples tested positive with PCR[c]
1	Jinotega	Colon Arriba	55	INTA Sequia	4/4	4/4	0/4
2		Colon Abajo	20	INTA Sequia	4/4	1/4	3/4
3		Coyolito	34	INTA Sequia	0/4	1/1	0/1
4		La Esperanza	45	Waspareño	3/3	1/2	1/2
5		La Calmena	45	Waspareño	4/4	1/2	1/2
6		Boniche	90	INTA Rojo	0/4	2/2	0/2
11	Nueva Guinea	Carlos Delgado	45	INTA Cárdenas	1/4	2/2	0/2
13		Los Angeles	35	INTA Rojo	0/4	2/2	0/2
15		La Esperanza	40-45	INTA Rojo	2/4	1/1	1/1
16		La Esperanza	30-35	INTA Cárdenas	0/4	2/2	0/2
17		La Esperanza	30	SEN 46	0/4	3/3	0/3
18		INTA Nueva Guinea	35	INTA Rojo	0/4	2/2	0/2

[a]Strong mosaic, curling and dwarf growth.
[b]Not all samples were tested with PCR.
[c] Not all samples were tested with PCR.

3.2 DNA amplification with RCA and begomovirus detection with PCR

PCR was run on DNA-extracts from all samples in the laboratory of INTA. However, these analyses failed to provide valid results since neither the samples nor the positive control provided a product visible by gel electrophoresis. Further PCR analyses were done after RCA in order to increase the amount of circular DNA in the extractions. Of the samples collected, RCA was run successfully for 28 samples from all 12 fields. The presence of a single high molecular band on the agarose gel, which corresponds to the begomovirus genome, indicates that DNA has been amplified. In some cases, all DNA products remained in the gel wells, which suggests that that RCA reaction could have been successful, but without a high molecular product in the gel. PCR was run with the universal primer pair AV494 and AC1048 for all samples amplified with RCA, in order to amplify the *AV1* gene. PCR product of the expected size ~580 bp, including primers, was obtained for in total 6 samples (Fig. 4; Table 1), indicating infection with begomoviruses.

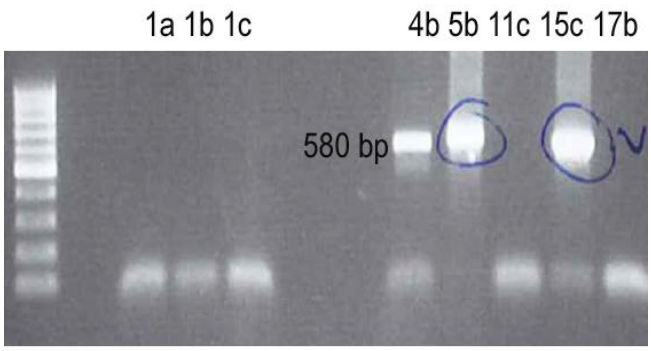


Fig 4. Polymerase chain reaction (PCR) detection of begomovirus infection. PCR products of approximately 580 bp are shown for sample 4b from field 4, 5b from field 5 and 15c from field 15. A 100 kb marker (Generuler DNA Ladder, Fermentas) was used as reference.

3.3 Detection of begomovirus diversity with restriction fragment length polymorphism (RFLP)

RCA-product from samples positive for PCR (Table 1), as well as one sample from field 1 with strong signs of virus infection, was digested with restriction enzymes in order to detect mixed infections with different begomoviruses. RFLP-analysis with *Eco*RI, *Hpa*II, *Bam*HI, *Hin*dIII, *Sac*I and *Kpn*I was performed on one RCA-product each for fields 1, 2, 4, 5, and 15. The RCA-product for a plant from field 17 was digested with *Eco*RI, *Hpa*II, *Bam*HI, and *Kpn*I.

The results for each digestion are described below and summarized in Table 2. Full-length fragments (~2.7 kb), corresponding to one viral DNA-component, were obtained for all samples, with the exception of 17b. Smaller fragments were mainly found for RCA products digested with *Hpa*II, *Bam*HI and *Sac*I, which in some cases could be summed up to the size of a viral component. Also, most digestions left some RCA product undigested, which could correspond to additional virus components. In total, a majority of the digestions did not show any sign of a mixed infection. For most digestions, only one or two viral components were found that corresponded to infection with one type of begomovirus. However, for two of the fields in Jinotega (1a and 2a), mixed infection was indicated with at least one of the restriction enzymes used.

3.3.1 *Eco*RI digestion

Digestion with *Eco*RI gave a full-length fragment for samples 1a, 2a and 15c that correspond to one viral component (Table 2; Fig. 5). For all samples, with the exception of 4b and 17b, undigested RCA product was visible as a high molecular band. For sample 2a, a digestion product with the size of 5.0 kb was found. Fragments of this size are not expected and should probably correspond to something else than begomovirus DNA.

3.3.2 *Hpa*II digestion

A full-length fragment was found for sample 5b after *Hpa*II digestion (Table 2). Two smaller fragments were also found that could correspond to another viral component, when added together. The same situation was the case for samples 2a, 4b and 15c, giving one or more viral components. However, no sample showed signs of more than two viral components, indicating that no mixed infection could be found when using the *Hpa*II enzyme. No digestion pattern was obtained for samples 1a and 17b.

3.3.3 *Bam*HI digestion

Digestion with *Bam*HI revealed a fragment of full-length size for samples 5b and 15c (Table 2), corresponding to one viral component. Also undigested RCA product was present, indicating additional viral components for these samples. For samples 1a, 2a, and 15c, a fragment of 1.1 kb was present. No digestion pattern was obtained for samples 4b and 17b. As no digestion with *Bam*HI gave a restriction pattern indicating more than two viral components, no mixed infection could be found.

3.3.4 *Kpn*I digestion

For most samples, no digestion products of any specific length could be found after *Kpn*I digestion. However, for sample 4b, one full-length fragment as well as one larger fragment with the size of 5.0 kb and undigested RCA product was visible (Table 2). Undigested RCA product was also found for samples 2a, 15c and 17b that could correspond to one or more viral components.

3.3.5 *Sac*I digestion

Digestion with *Sac*I resulted in a fragment corresponding to full-length size for sample 15c (Table 2). Additionally, smaller fragments with the total length of one viral component were found for 1a and 2a and undigested RCA product for 1a, 2a and 4b. No digestion product was obtained for sample 5b.

3.3.6 *Hin*dIII digestion

*Hin*dIII digestion resulted in full-length fragments for all samples tested, except for samples 5b and 15c, for which no RCA-product at all was seen (Table 2). For samples 1a and 2a, a larger fragment of 2.9 kb was present. Two smaller fragments, whose sizes together would correspond to a complete viral component, were found for sample 2a giving one additional component. All positive samples had undigested RCA product present. The results for the *Hin*dIII digestion summarized, suggest that a mixed infection could be present in sample 1a and 2a with more than two viral components.

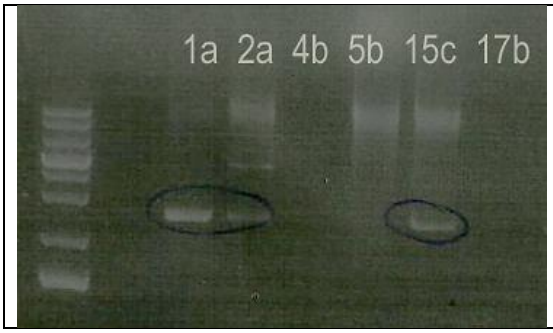


Fig. 5. Digestion of RCA product from samples 1a, 2a, 4b, 5b, 15c and 17b with the restriction enzyme *Eco*RI. Undigested RCA product is visible for sample 1a, 2a, 5b and 15c (upper arrow). Full-length fragments are visible at approximately 2.7 kb for 1a, 2a and 15c (lower arrow).

Table 2. Summary of restriction analysis of RCA products for samples 1a, 2a, 4b, 5b, 15c and 17b. Included in the table are the six different restriction enzymes used and the sizes of the resulting fragments, as well as undigested RCA product. It also shows suggestions of the number of begomovirus DNA-components present in the sample.

Sample	Restriction enzyme	Size of fragments (kb)	No. of begomovirus components	Mixed infection
1a	*Eco*RI	RCA prod[a], 2.7 (2.7)[b]	≥ 2	No
	*Hpa*II	-[c]	-	-
	*Bam*HI	RCA prod, 1.1 (1.1)	≥ 1	No
	*Kpn*I	-	-	-
	*Sac*I	RCA prod, 1.4, 1.3 (2.7)	≥ 2	No
	*Hin*dIII	RCA prod, 2.9, 2.7 (5.6)	≥ 3	Yes
2a	*Eco*RI	RCA prod, 5.0, 2.7 (7.7)	≥ 2	No
	*Hpa*II	2.1, 1.6, 1.4 (6.1)	2	No
	*Bam*HI	RCA prod, 1.1 (1.1)	≥ 1	No
	*Kpn*I	RCA prod	≥ 1	No
	*Sac*I	RCA prod, 1.4, 1.3 (2.7)	≥ 2	No
	*Hin*dIII	RCA prod, 2.9, 2.7, 1.6, 1.1 (8.3)	≥ 3	Yes
4b	*Eco*RI	-	-	-
	*Hpa*II	1.4, 1.6 (3)	1	No
	*Bam*HI	-	-	
	*Kpn*I	RCA prod, 5.0, 2.7 (7.7)	≥ 2	No
	*Sac*I	RCA prod	≥ 1	No
	*Hin*dIII	RCA prod, 2.7 (2.7)	≥ 2	No
5b	*Eco*RI	RCA prod,	≥ 1	No
	*Hpa*II	2.7, 1.6, 0.5 (4.8)	2	No
	*Bam*HI	RCA prod, 2.7 (2.7)	≥ 2	No
	*Kpn*I	-	-	-
	*Sac*I	-	-	-
	*Hin*dIII	-	-	-
15c	*Eco*RI	RCA prod, 2.7 (2.7)	≥ 2	No
	*Hpa*II	2.1, 1.6, 1.1, 0.6 (5.4)	2	No
	*Bam*HI	RCA prod, 2.7, 1.1 (3.8)	≥ 2	No
	*Kpn*I	RCA prod	≥ 1	No
	*Sac*I	2.7 (2.7)	1	No
	*Hin*dIII	-	-	-
17b	*Eco*RI	-	-	-
	*Hpa*II	-	-	-
	*Bam*HI	-	-	-
	*Kpn*I	RCA prod	≥ 1	No

[a] Undigested RCA product in the sample.
[b] Total size of the digested product in kb.
[c] RCA product or its digestion product not visible.

3.4 Begomovirus identification with cloning and sequencing

Of the RCA products showing full-length fragments when investigated with RFLP, the products for three samples were digested with *Eco*RI and cloned (1a, 2a, and 15c). Two clones each for 1a and 2a, and three clones for 15c were sent for sequencing. The clones for 2a and 15c were successfully sequenced and the partial sequences of 800-900 nucleotides were used for initial virus identification. The clones for sample 1a did not contain enough DNA material and gave poor sequencing results. Comparison with publicly available sequences in GenBank using BLASTn provided information needed for preliminary identification (Table 3). The forward sequence reaction for clone 2aA from sample 2a

(Jinotega) showed highest identity (96%) with the common region (CR) on DNA-A of *Calopogonium golden mosaic virus* (CalGMV), while the reverse sequence reaction showed highest identity at 78% to DNA-B of *Tomato mottle Taino virus* (ToMoTV) and *Sida golden mosaic Florida virus* (SiGMFV). Both DNA-A and DNA-B of bipartite begomoviruses have a CR, which is almost identical in sequence. The high identity of the determined sequences to CR of CalGMV and the lower identity to DNA-B of other begomoviruses suggest that the cloned fragment represents the DNA-B molecule of CalGMV, which previously has not been published. Further, the clone 2aE from sample 2a showed 99% nucleotide identity with BGYMV, indicating a mixed infection together with CalGMV and BGYMV. A similar result as for clone 2aA was obtained when analyzing the sequences of 15cA and 15cD from sample 15c (Nueva Guinea), suggesting infection with CalGMV.

Table 3. Results from partial sequencing of cloned RCA products

Sample	Primer	Name	Max. identity
2aA	M13F	Calopogonium golden mosaic virus, replication associated protein (rep) and coat protein (cp) genes (DNA-A)	94%
	M13R	Tomato mottle Taino virus (DNA-B)	78%
2aE	M13F	Bean golden yellow mosaic virus (DNA-A)	99%
	M13R	Bean golden yellow mosaic virus (DNA-A)	99%
15cA	M13F	Calopogonium golden mosaic virus, replication associated protein (rep) and coat protein (cp) genes (DNA-A)	93%
	M13R	Tomato mottle Taino virus (DNA-B)	78%
15cD	M13F	Tomato mottle Taino virus DNA-B	78%
	M13R	Calopogonium golden mosaic virus, replication associated protein (rep) and coat protein (cp) genes (DNA-A)	93%
15cE	M13F	Tomato mottle Taino virus (DNA-B)	78%
	M13R	Vector DNA	99-100%

4. Discussion

Symptoms of virus infection in bean plants could be found both in Jinotega and Nueva Guinea, the collection sites for this study. However, it was obvious that the severity of symptoms was varying among different cultivars, from mild to very severe. Especially in fields 1, 2, 4, and 5 (Jinotega) symptoms were really distinct with strong mosaic pattern on

leaves, stunting and leaf curling. In Nueva Guinea, some plants with strong symptoms could be found in field 11 and 15. In the majority of fields located in Nueva Guinea the incidence of both virus symptoms and whiteflies was lower compared to Jinotega. The results of the field survey could, however, not be confirmed by PCR for all samples, since the detection repeatedly failed without prior amplification of circular DNA with RCA. The results from the field survey in Jinotega, with a high incidence of begomovirus-symptoms, is in line with the former study made by Karkashian et al. (2011), where begomoviruses were found in a majority of tested bean plants. In general, the region of Jinotega, as well as Matagalpa, Chontales, Masaya, and Boaco, has experienced problems with whiteflies and symptoms indicating begomovirus infection (. Delfia Isabel Marcenaro pers communication). The latter regions were not included in this study and a more extensive survey is needed in order to determine the present situation. In Nicaragua, Nueva Guinea is a region where problems with viruses are reported less frequently, but lately technicians from INTA have reported a higher incidence of symptomatic plats. This study is a part of the present work to try to identify pathogens infecting plants in Nueva Guinea. The field survey, as well as lab results, indicates that viruses could be a problem also in this region. Still, in order to really form a picture of the situation among bean cultivars today, the results of this study need to be complemented with more valid PCR results or sequencing for begomovirus detection and identification.

The first round of PCR analyses failed to provide a DNA product visible with gel electrophoresis. This is probably due to insufficient amounts of viral DNA in the extractions, as PCR was successful after prior amplification with RCA. For a reliable diagnosis of begomoviruses, RCA in combination with RFLP has been proven to be easier and better than PCR or antibody detection, especially when the amount of DNA is low (Inoue-Nagata et al., 2004). RCA is amplifying all circular DNA in one reaction, including defective viral DNA molecules (D-DNAs) and presumably satellite DNAs, making the technique highly suitable when analysing geminiviral DNA (Haible et al., 2006; Paprotka et al., 2010a). Compared to PCR with specific or universal primers, detection of DNA components without any previous sequence information is possible when combining RCA with RFLP. However, the time limit of this study made the RCA method unsuitable when screening a large number of samples, why not all samples were analysed. As successful PCR confirmation was not done for all samples, it was not possible to see if plants with very mild or no symptoms were infected with begomoviruses. Still, further analyses with RFLP of RCA products and sequencing of cloned DNA components did provide more information on the diversity of begomoviruses in samples tested.

For a reliable result, several restriction enzymes were used in this study in order to distinguish viral genotypes and detect mixed infection. Of the restriction enzymes used, only *Hpa*II is known as a "four-cutter" meaning that it has a recognition site that is four nucleotides long (Kessler et al., 1985). The remaining restriction enzymes *Eco*RI, *Bam*HI, *Kpn*I, *Sac*I and *Hin*dIII are all "six-cutters". Since the recognition sequence of *Hpa*ll is shorter, it should be able to cut more frequently in a genome. This is useful when identifying the individual virus components because less RCA product is left undigested and the actual number of components can be revealed. Undigested RCA product could possibly contain other viral

components without the restriction site. Another possibility is the presence of D-DNAs from geminiviruses or mitochondrial plasmid DNAs from the plant (Paprotka et al., 2010a). D-DNA represents half a viral genome packed in viral particles that will appear in the gel as a 1.4 kb fragment. Such fragments were found mainly after digestion with *Sac*I. Another possibility is the presence of DNA satellites as smaller fragments or in undigested product. These have previously only been associated with monopartite begomoviruses from Asia and Africa, but have lately been found in connection to bipartite viruses in the New World (Paprotka et al., 2010b). After a number of the digestions, no digestion pattern could be seen. This is not expected as all digestions should give raise to a digestion pattern or undigested RCA-product on the gel. Since the same RCA-product was used for all digestions, this part of the results can only be explained with human error. RFLP indicated that some of the samples collected could be infected with more than one begomovirus. However, without complete sequencing of the product, it is impossible to know what the smaller fragments and the undigested RCA products are, and the suggested numbers of components may be incorrect. In this case, *Hpa*II gives a more clear answer to the actual numbers of viral components and together with *Eco*RI it has previously been shown promising when differentiating between distinct viral DNA components of begomoviruses (Jeske et al., 2010). The degenerate primers AV494 and AC1048, which were used for provisionary begomovirus identification with PCR, have formerly been used for identification of begomoviruses in many different hosts (Wyatt & Brown, 1996; Rojas, 2004). They are used to amplify the core region of the coat protein gene, which including primers, has the size of 576 bp. This fragment is enough for preliminary identification of a begomovirus. However, in order to establish the final classification of a new virus species or strain, the sequence of at least a complete DNA-A component is required. *Eco*RI as a "six-cutter" enzyme was in this study used in order to obtain full-length fragments for cloning and sequencing. The amounts of cloned DNA-fragments were sufficient for providing a successful sequencing reaction, but in order to avoid mutagenic effects, staining of the digested RCA products with EtBr and ultraviolet light could have been omitted (Jeske et al., 2010).

The results of the sequencing indicated that sample 2a from Jinotega was infected with BGYMV and CalGMV. Also, the sample from Nueva Guinea (15c) seemed to be infected with CalGMV. It should be noted that a complete sequence of CalGMV not yet is published, and therefore, the virus is not established as a species by the International Committee on Taxonomy of Viruses. The results of this study are indicating that DNA-B from CalGMV has been isolated, but this needs to be confirmed by further analysis of the complete sequence. If the isolate turns out to be DNA-B of CalGMV, it would be the first complete sequence of this DNA-component. CalGMV has previously been shown to infect bean plants in the northern part of Costa Rica (Díaz et al., 2002). Since the alternative host *Calopogonium* sp. is known to grow in many regions in Central America there is a possibility that CalGMV is present in other regions, as indicated by the results of this study. CalGMV was also found in Nicaragua in the recent study made by Karkashian et al. (2011), but only in the counties of Rivas and Granada. The present study indicates that the distribution of CalGMV could be more extensive. There is a possibility that the samples from Jinotega and Nueva Guinea could be infected with Nicaraguan isolates of CalGMV, but this needs to be confirmed by further

analysis of the complete genome sequences, including DNA-A. It is important to point out that these are only preliminary results of the sequencing. In order to really establish the true identity of the begomoviruses, the full-length sequences of the genomes are needed.

Sequencing was also used to confirm the presence of mixed infection in the samples. Indeed, the sequence analyses for sample 2a together with the restriction analysis indicated that it had a mixed infection. Since mixed virus infection is a pre-condition for recombination it is of great importance to investigate the prevalence of such events in order to study the emergence of new viruses, and more research in this field is needed in Central America. The new biotype of *B. tabaci* is thought to have increased the speed of recombination because it has a wider range of hosts (Padidam et al., 1999). In Nicaragua, different crops susceptible to virus infection are often grown together, which enables viruses to infecting new hosts. Also, many weeds are known to serve as virus reservoirs as in the case of *Calopogonium* sp. and it would be of great interest to investigate weeds growing in the area.

5. Conclusions

This study indicates that infection with begomoviruses is a problem in both the Pacific and the Atlantic regions of Nicaragua. There are also results which suggest that mixed infection is occurring, facilitating possible viral recombination, which needs to be taken seriously. BGYMV, which is known to infect bean plants in Nicaragua, was found in Jinotega, while there were indications of CalGMV infection in both Jinotega and Nueva Guinea. CalGMV has previously been found in Nicaragua, but only partial genome sequences have been determined. More extensive work is needed with the samples of this study in order to possibly obtain the complete sequence of CalGMV. As begomoviruses also are infecting beans of the Atlantic coast, it should be considered for making strategies to control the diseases of this area. Since CalGMV was found in two separate areas of the country, there is a need for further investigation about the transmission between common beans and the weed *Calopogonium* sp.

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Personal contact

Delfia Isabel Marcenaro, INTA, Managua, Nicaragua [23 April 2012]

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Institutionen för växtbiologi och skogsgenetik
Uppsala Biocenter, SLU
Box 7080, Almas Allé 5, 750 07 Uppsala