

Can *Amplicephalus funzaensis* Linnavuori 1968 (Hemiptera: Cicadellidae) transmit phytoplasmas to strawberry?

¿Puede *Amplicephalus funzaensis* Linnavuori 1968 (Hemiptera: Cicadellidae) transmitir fitoplasmas a fresa?

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ABSTRACT

Phytoplasmas are plant pathogenic bacteria of the class Mollicutes that lack cell walls, are restricted to the phloem of their plant hosts, are difficult to culture, and are transmitted by insect vectors. Phytoplasmas from 16SrI and 16SrVII groups have been associated with diseases in urban trees in the Bogota plateau and with potato and strawberry crops in Cundinamarca, Colombia. The objective of this work was to evaluate if the vector *Amplicephalus funzaensis* (Hemiptera: Cicadellidae) could transmit phytoplasmas to *Fragaria x ananassa* under semi-controlled conditions. A transmission assay on *F. x ananassa* var. Monterrey was performed with *A. funzaensis* from a population naturally infected with phytoplasmas, whose host was *Cenchrus clandestinus*. Seven months after herbivory by these insects, the plants did not show symptoms associated to phytoplasmas, even though more than one third of the insects used carried phytoplasmas. In total, 120 *A. funzaensis* individuals were tested for the presence of phytoplasmas by molecular methods; of these, 46 (38%) were positive for phytoplasmas, showing the existence of insect populations with a high number of individuals that are a potential source of inoculum for the pathogen transmission. Additionally, for the molecular identification of *A. funzaensis*, a DNA barcode was generated from the *cytochrome c oxidase (COI)* gene.

Key words: insect vectors, DNA barcoding, cytochrome c oxidase, *COI* gene.

RESUMEN

Los fitoplasmas son bacterias patógenas de plantas de la clase Mollicutes que carecen de pared celular, están restringidas al floema de sus hospederos vegetales, son difícilmente cultivables y son transmitidos por insectos vectores. Fitoplasmas de los grupos 16SrI y 16SrVII se han asociado a enfermedades en árboles urbanos de la Sabana de Bogotá y en cultivos de fresa y papa de Cundinamarca, Colombia. El objetivo de este trabajo fue evaluar si el conocido vector *Amplicephalus funzaensis* (Hemiptera: Cicadellidae) podía transmitir fitoplasmas a fresa *Fragaria x ananassa* en condiciones semicontroladas. *A. funzaensis*, naturalmente infectados con fitoplasmas, cuyo hospedero era *Cenchrus clandestinus* se utilizaron para realizar ensayos de transmisión en plantas *F. x ananassa* var. Monterrey. Siete meses posteriores a la herbivoría por estos insectos, las plantas no presentaban síntomas asociados a fitoplasmas, a pesar de que más de un tercio de estos insectos portaban fitoplasmas. En total, en este trabajo se evaluaron 120 individuos de *A. funzaensis* para la presencia de fitoplasmas por métodos moleculares; de estos, 46 (38%) fueron positivos para fitoplasmas, mostrando la existencia de poblaciones de insectos con un número alto de individuos que son fuente potencial de inóculo para la transmisión del patógeno. Además, para la identificación molecular de *A. funzaensis*, se generó un código de barras de ADN partir de una secuencia del gen *citocromo c oxidasa (COI)*.

Palabras clave: insectos vectores, código de barras de ADN, citocromo c oxidasa, gen *COI*.

Introduction

Phytoplasmas are bacteria of the class Mollicutes that lack cell walls and are sensitive to tetracycline. It has been stated that they inhabit the phloem cells of plants or the intestine, haemolymph, salivary glands and other internal organs of some hemipteran insects of the families Cicadellidae, Fulgoromorpha, and Psyllidae (Lee *et al.*, 2000; Weintraub and Beanland, 2006; Hogenhout *et al.*, 2008; Bertaccini

and Duduk, 2009). Phytoplasmas are difficult to cultivate (Contaldo *et al.*, 2016), so their detection, identification and classification are mainly based on the amplification of the 16SrRNA gene (Gundersen *et al.*, 1996) and further analysis by restriction fragment length polymorphism (RFLP) with 17 restriction enzymes that discriminate them into ribosomal groups. To date, 34 16Sr groups are recognized for phytoplasmas (Pérez-López *et al.*, 2016). The genus '*Candidatus Phytoplasma*' was adopted for incompletely

Received for publication: 19 March, 2019. Accepted for publication: 24 February, 2020

Doi: 10.15446/agron.colomb.v38n1.78583

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described, non-cultivable phytoplasmas. A new species within *Candidatus* is created when the sequence of the 16SrRNA gene contains less than 97.5% of nucleotide identity compared to previously described phytoplasma species (IRPCM, 2004).

The presence of phytoplasmas is associated with diseases in crops, ornamentals, fruit and woody plants belonging to different botanical families (Lee *et al.*, 2000; Bertaccini *et al.*, 2014). In Colombia, phytoplasmas from the 16SrIII group were reported in *Coffea arabica*, *Cordia alliodora*, *Physalis peruviana* and *Solanum quitoense* (Galvis *et al.*, 2007; Mejía *et al.*, 2014); 16SrV and 16SrXII in *Solanum tuberosum* (Mejía *et al.*, 2011; Varela and Franco-Lara, 2017); 16SrIII-L in *Manihot esculenta* (Álvarez *et al.*, 2009); 16SrIX in *Catharanthus roseus* (Duduk *et al.*, 2008a), and 16SrI in *Elaeis guineensis*, *Zea mays* and *Musa acuminata* (Duduk *et al.*, 2008b; Álvarez *et al.*, 2014; Aliaga *et al.*, 2018). In Bogota, phytoplasmas of the groups 16SrI and 16SrVII are widely distributed in urban trees such as *Acacia melanoxylon*, *Croton* spp., *Eugenia neomyrtifolia*, *Fraxinus uhdei*, *Liquidambar styraciflua*, *Magnolia grandiflora*, *Pittosporum undulatum*, *Populus nigra*, and *Quercus humboldtii* (Perilla-Henao *et al.*, 2012; Perilla-Henao and Franco-Lara, 2013; Franco-Lara and Perilla-Henao, 2014; Franco-Lara *et al.*, 2017). Phytoplasma diseases in strawberry *Fragaria x ananassa* have been reported in Australia (Padovan *et al.*, 1998; Padovan *et al.*, 2000), the United States (Harrison *et al.*, 1997; Jomantiene *et al.*, 1999), France (Danet *et al.*, 2003), Italy (Bertaccini *et al.*, 1997), and Lithuania (Valiunas *et al.*, 2006). In Colombia, phytoplasma groups 16SrI and 16SrVII have been reported in strawberry varieties Camino Real and Ventana, with symptoms of flower phyllody, virescence of achenes, double sepals and aborted fruits (Perilla-Henao and Franco-Lara, 2014).

Amplicephalus funzaensis Linnavuori 1968 (Cicadellidae: Deltocephalinae) and *Exitianus atratus* Linnavuori 1959 (Cicadellidae: Deltocephalinae) are known vectors of phytoplasma groups 16SrI and 16SrVII in Bogota (Perilla-Henao *et al.*, 2016). The objective of this study was to determine if *A. funzaensis* can transmit phytoplasmas to *Fragaria x ananassa*, and to generate a DNA barcode for this insect species.

Materials and methods

The transmission assays were carried out at the campus of the Universidad Militar Nueva Granada (UMNG), in Cajica, Cundinamarca (4°55'0" N, 74°1'30" W), at an altitude of 2558 m a.s.l., with average annual temperature of 13.9°C

and average annual relative humidity of 87.9% (information obtained in 2016 from the meteorological station at Nueva Granada Campus, when the assays were carried out).

Entomological material

Insects were collected from pastures represented mainly by *Cenchrus clandestinus* using an entomological net and a mouth aspirator. Samplings were performed at the campus UMNG or at the Universidad Nacional de Colombia (UN) campus in Bogota, Colombia (4°38'18.88" N, 74°5'2.76" W). The insects were stored in 90% (v/v) ethanol. Taxonomic identification was based on morphological characters (Linnavuori, 1959; Dietrich, 2005; Silva-Castaño *et al.*, 2019). Before performing the transmission experiments, a natural population of *A. funzaensis* that lived on the grass *C. clandestinus* at the campus UMNG in Cajica was tested for the presence of phytoplasmas. In July 2015, 20 individuals were analyzed by nested PCR to test for phytoplasmas. The *A. funzaensis* used for the transmission assays were captured in April 2016 at the campus UMNG from the population in which the natural infection by phytoplasmas had been recorded. In February and March 2016, another group of twelve *A. funzaensis* belonging to two different populations, UMNG campus and UN campus were used to generate a DNA barcode for this species.

Transmission assays

One hundred adult *A. funzaensis* were collected and maintained for 5 min at 4°C to reduce their mobility and separate females and males, before placing them on strawberry *F. x ananassa* variety Monterrey of approximately 6 months old, grown in an aquaponic culture. The plants were propagated by division of the crown and the new plants were potted individually in 7 kg pots using soil and rice husks (2:1) as substrate. Before the experiment, the plants were kept for three months in insect free cages and were watered every 3 d by drip irrigation and fertilized once a month with Hoagland and Arnon (1950) nutrient solution. To confirm that all the plants were free of phytoplasmas before the transmission assays, nested PCR tests were performed, as described below. Treatment 1 consisted of two replicas with five plants each, and treatment 2 (control) consisted of one replica with five plants that were not exposed to *A. funzaensis* herbivory. Plants of both treatments were kept in independent insect free cages. For treatment 1, each plant was infested with ten male and female adults of *A. funzaensis* captured at the campus UMNG in Cajica, from the population in which phytoplasmas had been previously detected. To guarantee that the insects were in contact with the leaves, two 10 cm X 10 cm veil bags containing five insects each were tied to different leaves of each plant.

When the insects died, 1 week later, they were retrieved and stored at -20°C for molecular testing. Two months post-herbivory, leaves of all the plants of treatments 1 and 2 were sampled and tested for phytoplasmas. The plants were kept for 7 months in observation inside cages and monthly observations were made to register the appearance of symptoms. During these months, watering and fertilization were performed as described before.

DNA extraction

Insect DNA extractions were performed following the methodology described by Hung *et al.* (2004). One *A. funzaensis* individual was ground with liquid nitrogen and 30 μl of lysis buffer (0.1 M Tris-HCl pH 8.0, 0.05 M EDTA, 0.01% (v/v) N-Lauroylsarcosine). After grinding, 970 μl of the same buffer were added. The tissue was treated with 1% (w/v) sodium dodecyl sulphate and 0.08 mg ml^{-1} proteinase K, and incubated at 55°C overnight. The next day, 500 μl of 1:1 equilibrated pH 8.0 phenol and chloroform were added and the sample was centrifuged at 10,000 g for 10 min. The aqueous phase was removed and the nucleic acids precipitated with 1 ml of absolute ethanol for 15 min at -20°C , and then samples were centrifuged at 10,000 g for 25 min. The pellet was washed in 1 ml of 70% (v/v) ethanol. The supernatant was eliminated and the ethanol left to evaporate. Finally, the DNA was resuspended in 50 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and stored at -20°C . The DNA concentration of the samples was quantified in a Qubit (Invitrogen[®]) using the Qubit Assay kit.

The plant DNA extractions were performed using a protocol by Dr. A. Bertaccini (personal communication) and adapted in our laboratory. Extractions started from 0.33 g of leaf midribs. The midribs were ground in liquid nitrogen and extraction buffer I (92 mM $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$, 30 mM KH_2PO_4 , 10% sucrose (w/v), 5% (w/v) PVP-10 and L-ascorbic acid (pH 7.6)). The samples were filtered through sterile cloths, centrifuged at 6500 g for 10 min and resuspended in 1.3 ml of extraction buffer II (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl) with 25 μl of proteinase K (5 mg/ml) and 145 μl of N-Lauroylsarcosine 10% (w/v). Samples were incubated at 55°C for 2 h. The lysate was centrifuged for 10 min at 8,000 g and the pellet discarded. The nucleic acids were precipitated with 0.6 volumes of isopropanol and incubated at 4°C overnight. Next day, the extracts were centrifuged at 8,000 g for 30 min and the pellet resuspended in 990 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0], 24.75 ml of 20% (w/v) sodium lauryl sulphate and 19.8 μl of proteinase K (5 $\mu\text{g ml}^{-1}$)), and incubated at 37°C for 1 h. Later, 175 μl of 5 M NaCl and 140 μl (10% (w/v) cetyl trimethyl ammonium bromide/0.7M NaCl) were added and

the lysate incubated at 65°C for 10 min. Two consecutive extractions were performed, the first with an equal volume of chloroform / isoamyl alcohol (24:1) and the second with an equal volume of chloroform. In both cases the phases were separated by centrifugation at 8,000 g for 10 min and the organic phase discarded. The supernatant was precipitated with 0.6 volumes of isopropanol at 4°C overnight. Finally, the nucleic acids were pelleted by centrifugation at 11,000 g for 20 min and the pellet washed with ethanol 70%. The pellet was resuspended in 50 μl of TE buffer and the DNA was stored at -20°C . Later, the DNA was purified with the PowerClean[®] Pro DNA Clean-Up kit (Mo Bio Laboratories, Inc.) to eliminate other impurities.

Amplification of the *COI* gene of *A. funzaensis* and sequence analysis

The amplification of a region of the *COI* mtDNA gene was performed with the universal insect primers LCO1490/HCO2198 (Folmer *et al.*, 1994). The PCR reactions were conducted at a final volume of 25 μl , with 0.05 U of Taq polymerase (Bioline[®]), 1X reaction buffer, 2.5 mM MgCl_2 , 0.3 mM dNTPs, 0.3 μM of each primer and 50 ng of DNA diluted in deionized water. The thermic profile consisted on a denaturation for 10 min at 95°C , followed by 35 cycles of denaturation for 1 min at 95°C , annealing for 45 s at 40°C , extension for 45 s at 72°C and, a final extension period for 10 min at 72°C . The amplicons were purified with the UltraClean[®] PCR Clean-up Kit (Mo Bio Laboratories, Inc.) and were bi-directionally sequenced in Macrogen, Korea. The sequences were edited with the Geneious R9 software (Biomatters Ltd.) and each individual consensus sequences were built by superposition of the forward and reverse sequences. The consensus sequences were compared using BLASTN with the GenBank database. Additionally, nine *A. funzaensis* sequences obtained in this research, and other Cicadellidae downloaded from the BOLD database (<http://www.barcodinglife.org>) were used to construct a distance tree by the neighbor-joining method, with 1000 bootstrap, based on the Kimura 2 parameter (K2P) model, using the Mega v.6.0 software (Tamura *et al.*, 2013). A sequence of *Psylla galeaformis* (Hemiptera: Psyllidae) was used as out-group. The Barcode Gap analysis of the *COI* gene sequences was performed by comparison of the intraspecific versus the interspecific sequences, obtained from the distance matrix generated in MEGA v.6.0, according to the K2P model.

Detection of phytoplasmas using PCR

The presence of phytoplasmas was tested by PCR in *F. x ananassa* and *A. funzaensis* DNA extracts. To verify that PCR inhibitors were not present in the plant DNA extracts, amplification of rps16 chloroplast intron was performed,

before phytoplasma detection (Oxelman *et al.*, 1997). The PCR reactions were conducted in a final volume of 15 μ l, with 0.05 U of Biolase (Bioline), 1X reaction buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs and 0.2 μ M of primers. Dilutions 1:50 and 1:100 were prepared from the plant DNA extracts in deionized water and 2 μ l were used as template. Deionized water was used as negative control in all the PCR reactions. The thermic profile was: initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 53°C, extension for 2 min at 72°C, and a final extension period for 10 min at 72°C.

The PCR reactions for phytoplasmas were made as explained before. For *F. x ananassa*, 1:20, 1:50 and 1:100 dilutions of the DNA extracts were tested. For the insects, 1:20 dilutions of DNA extracts were analyzed. The PCR with universal primers P1A/P7A (Lee *et al.*, 2004) produced amplicons of ~1800 bp, which were diluted 1:20 and used in nested PCRs with the internal primers R16mF2/R16mR1 (Gundersen and Lee, 1996) to obtain ~1430 bp amplicons. Frequently, when low intensity amplicon bands were observed in nested PCRs, the resulting amplicons were amplified again using internal primers R16F2n/R16R2 (Gundersen and Lee, 1996) using 1:20 dilutions of the amplicon as template. Bands with enough DNA were further analyzed by sequencing or RFLP analyses. In these reactions, the positive control corresponded to fragments of the region 16S-23S of the phytoplasmas Maize bushy stunt - MBS (16SrI-B group) and ash yellows ASHY (16SrVII-A group), previously cloned and maintained in an *Escherichia coli* strain. In the *F. x ananassa* plants and in the *A. funzaensis*, additional nested PCR tests were performed with different primer combinations such as P1A/P7A (Lee *et al.*, 2004) followed by R16F2n/R16R2, and R16mF2/R16mR1 in primary reactions followed by R16F2n/R16R2. The plant DNA extracts were analyzed in 1:50 and 1:100 dilutions and the insect DNA extracts in 1:10 dilutions. The thermic profile was as described before, except for annealing temperatures that depended on the primer pairs used: P1A/P7A, 55°C; R16mF2/R16mR1, 54°C and R16F2n/R16R2, 54°C. The amplicons were separated using standard electrophoresis in 1% agarose gels in TBE buffer 1X (89 mM Tris base, 89 mM boric acid, 2 mM EDTA), stained with ethidium bromide and visualized under UV light.

To determine phytoplasma ribosomal group affiliation, nested PCR amplifications were performed with the R16F2n/R16R2 primers at a final volume of 30 μ l. This volume was divided into four, and each aliquot digested with the restriction endonucleases *Rsa*I, *Mse*I, *Hha*I or *Alu*I

(New England BioLabs). Digestions were conducted at a final volume of 20 μ l, with 0.05 U of enzyme, 1X buffer and 6.5 μ l of the PCR product obtained by nested amplification. The reactions were incubated at 37°C overnight. The restriction products were separated using agarose gel electrophoresis at 3% in TBE buffer. Additionally, the selected amplicons obtained with the primers R16F2n/R16R2 were purified with the UltraClean® PCR Clean-up Kit (Mo Bio Laboratories, Inc.) and sequenced bidirectionally in Macrogen, Korea. The sequence analysis was performed as explained before.

Results

Entomological material

Only specimens of *A. funzaensis*, confirmed by morphological and molecular methods, were included in this study (Fig. 1).

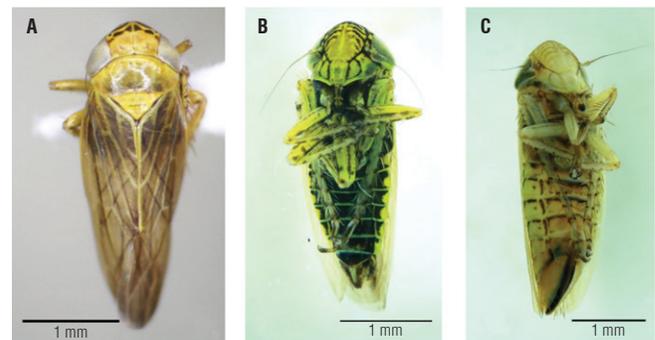


FIGURE 1. *Amplicephalus funzaensis*. (A-B) Adult male, dorsal and ventral views. (C) Female, ventral view.

Transmission assays

At the time of this experiment it was not possible to raise *A. funzaensis* under laboratory conditions, so the transmission experiment was performed with insects from a naturally infected population. In a previous work, a population of *A. funzaensis* infected with 16SrI and 16SrVII phytoplasmas had been found, but before performing the experiment it was tested again for the presence of phytoplasmas. Twelve adults of twenty *A. funzaensis* from that population were found positive for phytoplasmas by nested PCR, but no further RFLP or sequencing analysis were performed because the quantity of the obtained DNA was too small.

For the transmission experiment, a different set of 100 insects were allowed to feed in 10 phytoplasma-free plants, placing 10 insects per plant. DNA extraction is a destructive method, so the detection of the phytoplasmas in these insects was performed after they were allowed to feed on

TABLE 1. Results of transmission assays of phytoplasmas by *Amplicephalus funzaensis* to *Fragaria x ananassa* in semi-controlled conditions.

	<i>A. funzaensis</i> (adults)					Plants <i>Fragaria x ananassa</i>		
	Females		Males		Positive by PCR (b)		Group detected by RFLP/ Total individuals tested (c)	Detection by PCR (d)
	Plants	per plant	per plant (a)	Females	Males			
Plants exposed to herbivory	1	5/10	- 5/10	4	1	16SrI (2/4)	-	
	2	8/10	- 2/10	5	0	16SrI (4/4)	-	
	3	7/10	- 3/10	2	0	16SrI (2/2)	-	
	4	9/10	- 1/10	6	0	16SrI (5/6)	-	
	5	7/10	- 3/10	4	2	16SrI (4/5)	-	
	6	8/10	- 2/10	3	1	16SrI (4/4)	-	
	7	2/10	- 8/10	0	7	16SrI (6/7)	-	
	8	8/10	- 2/10	7	2	16SrVII (1/7)*	-	
	9	9/10	- 1/10	1	0	16SrI (1/1)	-	
	10	7/10	- 3/10	2	1	16SrI (1/3)	-	
Controls Plants not exposed to herbivory	1		0	-	-	-	-	
	2		0	-	-	-	-	
	3		0	-	-	-	-	
	4		0	-	-	-	-	
	5		0	-	-	-	-	
Total	70 Females - 30 Males		34	14				

(a) Number of females and males placed on each tested plant, over total number of insects. (b) Number of females and males positive for phytoplasmas by nested PCR.

(c) Number of positive insects for 16SrI or 16SrVII groups, determined by RFLP with restriction enzymes *RsaI*, *MseI*, *HhaI* and *AluI*, over the total of tested insects.

(d) Detection of phytoplasmas in *F. x ananassa* by nested PCR and double nested PCR 2 months post-inoculation.

(-) Negative for phytoplasmas.

(*) Samples with mixed infections containing 16SrI and 16SrVII.

the test plants. After two months post-herbivory, all the *F. x ananassa* plants were negative for phytoplasmas, regardless of the PCR primer combination used, as were the non-exposed negative control plants. In all the cases, the positive PCR controls generated bands of the expected size and the negatives did not amplify (Figs. 2 A-C). No symptoms associated with phytoplasmas in leaves, flowers, or fruits were observed after 7 months of evaluation (Tab. 1).

The control plants were also negative for the presence of phytoplasmas in tests conducted simultaneously with plants exposed to insects. In all cases, the positive controls generated bands of the expected size and the negatives did not amplify (Figs. 2 A-C).

Of 100 *A. funzaensis* used in the transmission assays, 22 individuals resulted positive for phytoplasmas by nested PCR with primers P1A/P7A followed by R16F2n/R16R2 and 36 individuals were positive with primers R16mF2/R16mR1 and R16F2n/R16R2. Taking into account nested PCR tests with both sets of primers, 48 of 100 individuals that were used in the transmission tests were positive in PCR using primers for phytoplasmas (Fig. 3, Tab.1).

Of the 48 positive insects, 41 were analyzed by RFLP. Of these, 34 were infected with phytoplasmas: 33 were infected with 16SrI group and one simultaneously with 16SrI and 16SrVII groups (Tab. 1). In the others, the RFLP patterns obtained were not consistent with any phytoplasma group profile, suggesting non-specific amplifications (Fig. 4).

DNA barcoded for *A. funzaensis*

Amplicons of the mitochondrial DNA *COI* gene were obtained from 12 individuals, six collected at the UMNG and six at the UN. Consensus sequences of lengths between 667 and 706 bp were obtained from nine individuals (Fig. 5A); the other sequences were illegible. The edited sequences were uploaded in the BOLD database (<http://v3.boldsystems.org/>) (Ratnasingham and Hebert, 2007). The sequences of all the *A. funzaensis* collected in Bogota at the UN showed a conserved haplotype (Sequences ID: COLCI053-18, COLCI054-18, COLCI055-18, COLCI056-18, and COLCI057-18), but the ones collected at the UMNG in Cajica represented three haplotypes. Haplotype 1 was identical to the Bogota sequence (Sequence ID: COLCI049-18); two sequences called haplotype 2 (Sequences ID: COLCI051-18 and COLCI052-18) presented substitutions T

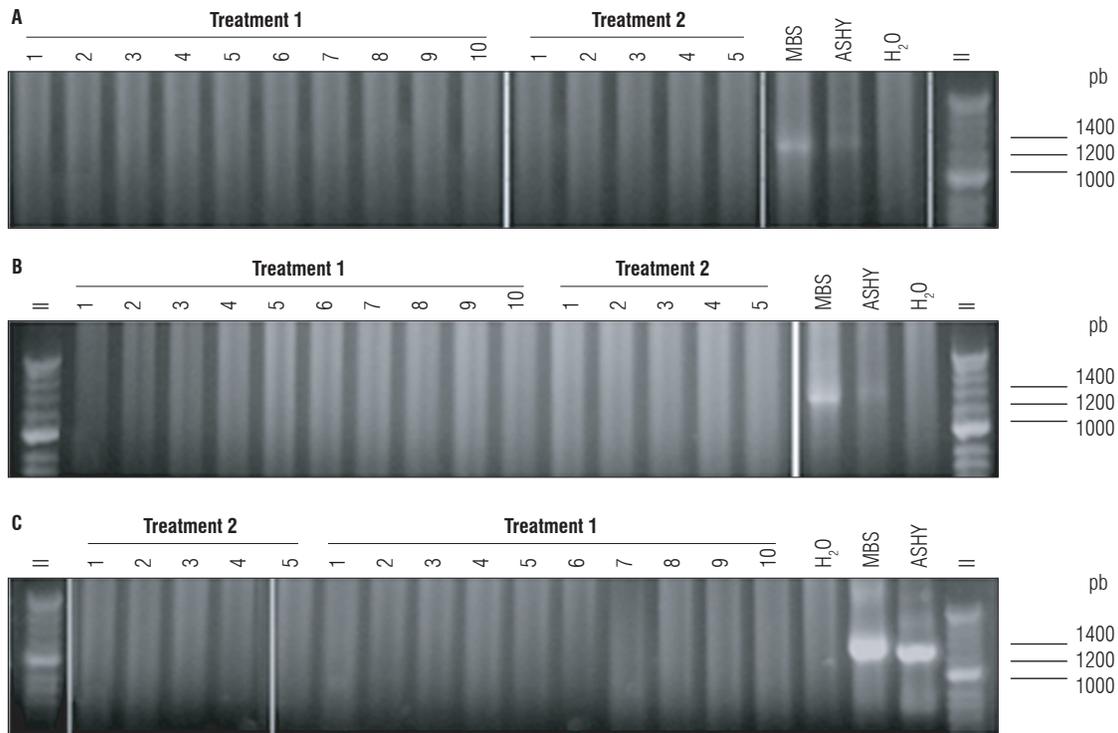


FIGURE 2. Nested and double nested PCR of *Fragaria x ananassa* samples exposed to *Amplicecephalus funzaensis* (Treatment 1) and control plants (Treatment 2). (A) Double nested PCR with primers P1A/P7A, R16mF2/R16mR1 followed by R16F2n/R16R2. (B) Nested PCR with primers P1A/P7A and R16F2n/R16R2. (C) Nested PCR with primers R16mF2/R16mR1 and R16F2n/R16R2. In all the cases, only the result of the last PCR is shown. MBS: Positive control group 16Srl-B. ASHY: Positive control group 16SrVII-A. Negative control: water. II: Molecular weight marker Hyperladder 50 pb (Bioline®).

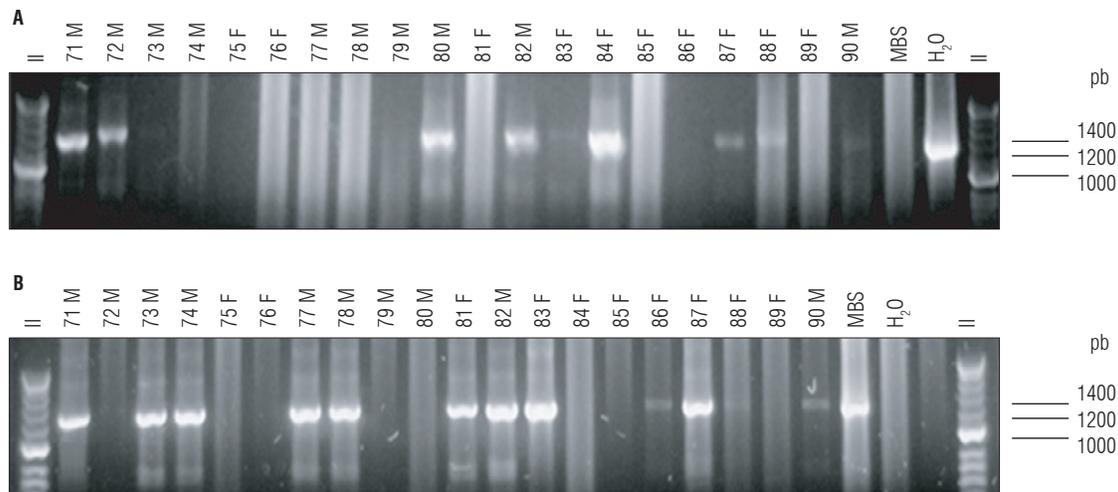


FIGURE 3. Detection of phytoplasmas by nested PCR in the *Amplicecephalus funzaensis* used in transmission assays. (A) Nested PCR with primers P1A/P7A followed by R16F2n/R16R2. (B) Nested PCR with R16mF2/R16mR1 followed by R16F2n/R16R2. Only the result of the nested PCR is shown. The number corresponds to the insect code, F: females and M: males. MBS: Positive control group 16Srl-B. Negative control: water. II: Molecular weight marker Hyperladder 50 pb (Bioline®).

by C at positions 183, A by G in 453 and T by C in 517. In haplotype 3, there was a substitution of G by A in position 342 (Sequence ID: COLCI050-18). The sequence similarity of the *COI* sequences obtained for *A. funzaensis* was over

99%. Sequences for *A. funzaensis* were not found in the BOLD or GenBank databases, therefore, Deltocephalinae sequences showing similarities between 86% and 87% were used for comparison.

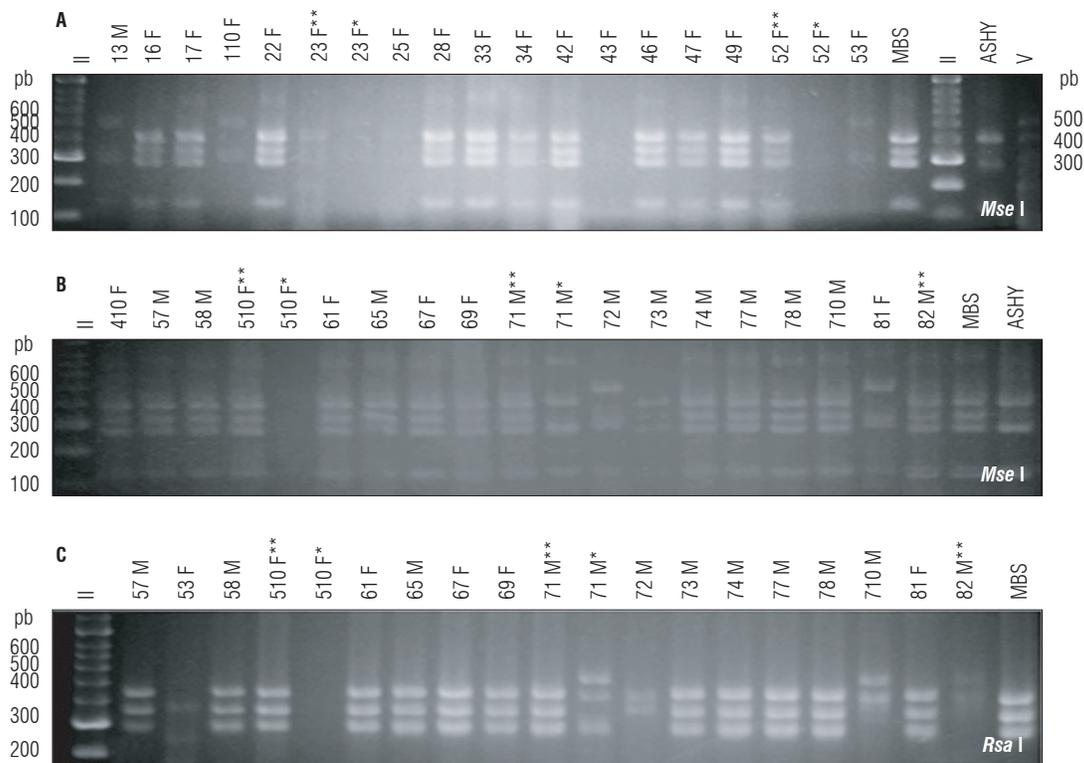


FIGURE 4. Detection of phytoplasmas in *A. funzaensis* used in the transmission assays. (A-B) RFLP with *Mse*I, (C) RFLPs with *Rsa*I, of amplicons obtained by nested PCR with primers P1A/P7A, R16mF2/R16mR1 and R16F2n/R16R2. *Nested PCRs with P1A/P7A followed by R16F2n/R16R2. **Nested PCR with R16mF2/R16mR1 followed by R16F2n/R16R2. The number corresponds to the insect code, F: Females and M: Males. MBS: Positive control group 16SrI-B. ASHY: Positive control group 16SrVII-A. Negative control: Water. II: Molecular weight marker Hyperladder 50 pb (Bioline). V: Molecular weight marker Hyperladder 25 pb (Bioline®).

The nine *A. funzaensis* sequences obtained in this work formed a distinct cluster from other sequences of the *Amplicephalus* genus obtained from BOLD and GenBank. All the sequences clustered by taxa, showing that they had enough differences to separate them as distinct groups (Fig. 5B). The average intraspecific genetic divergence varied between 0.0000 and 0.0245 (mean 0.0048) and the interspecific divergence between 0.1003 and 0.2341 (mean 0.1501). The most divergent species were *P. flavicosta* compared to *A. simplarius* (0.2341), *A. curtisii* compared to *A. littoralis* (0.2233) and *P. flavicosta* compared to *A. littoralis* (0.2233). The less divergent species were *A. littoralis* and *A. simplarius* (0.1003). The *A. funzaensis* sequences were close to *D. balli* (0.1499) and *A. curtisii* (0.1739), and showed the largest divergence with *A. simplarius* (0.2004). The barcode gap analysis showed that for the eight species analyzed, the sequence variability at the intraspecific level was lower than the variability at the interspecific level.

Discussion

A very important aspect of the epidemiology of the diseases associated with phytoplasmas is the identification of their

insect vectors. To do this, the insects nearby the symptomatic plants are collected and their ability to transmit phytoplasmas to healthy host plants is tested (Weintraub and Beanland, 2006). The presence of phytoplasmas in an insect does not guarantee its vector ability; therefore, transmission tests are mandatory (Purcell *et al.*, 1981; Vega *et al.*, 1993). In this work, tests were performed with *A. funzaensis* because it is a known vector of phytoplasma, and it is ubiquitously distributed in the *C. clandestinus* grasslands of the Bogota plateau that frequently surrounds *F. x ananassa* crops (Perilla-Henao *et al.*, 2016; Silva-Castaño *et al.*, 2019).

No evidence was observed of phytoplasma transmission by *A. funzaensis* to *F. x ananassa*. The plants exposed to infected *A. funzaensis* were negative by PCR 2 months post-herbivory and no symptoms were observed for seven months, although the observations performed during the transmission experiment suggested that the insects fed from the test plants and that 36 of the 100 insects used in the assay were infected with phytoplasmas of 16SrI or 16SrVII groups. Several explanations for these results are possible. For instance, that the *F. x ananassa* variety Monterrey

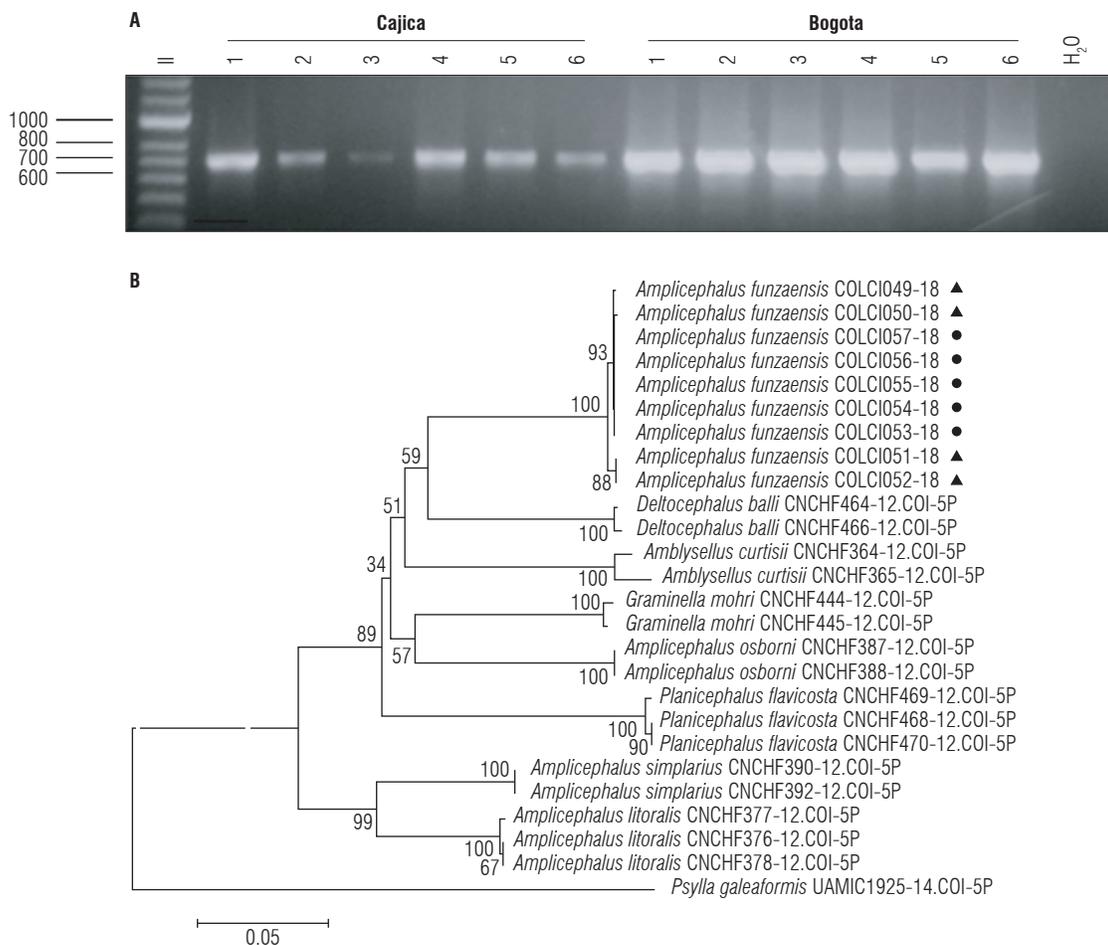


FIGURE 5. (A) Amplification of the *COI* gene of *Amplicephalus funzaensis* by PCR. Negative control: water. II: Molecular weight marker Hyperladder 50 pb (Bioline). (B) Distance tree constructed by neighbor-joining (NJ) based on the Kimura 2 parameter model (K2P) with 1000 bootstrap, using 25 *COI* sequences of the genus *Deltoccephalini*, which includes *Amplicephalus*. The out group was a sequence of *Psylla galeaformis*. Insects collected at UMNG are shown with triangles, and at UN with circles.

plants are not susceptible to phytoplasmas. However, more tests would be necessary to confirm this possibility taking into account that phytoplasma infections in strawberry are widely reported (Padovan *et al.*, 1998; Jomantiene *et al.*, 1999; Padovan *et al.*, 2000; Danet *et al.*, 2003; Valiunas *et al.*, 2006). Furthermore, phytoplasmas of groups 16SrI and 16SrVII have been detected in strawberries of Camino Real and Ventana in Cundinamarca before (Perilla-Henao and Franco-Lara, 2014). Taking into account that the PCR tests were performed 2 months post-herbivory, it is also possible that the plants at that moment were infected but the phytoplasma title was under the PCR detection level. It would have been desirable to test for the presence of phytoplasmas by PCR in the following period, but the plant tissue collected in the subsequent months was lost due to refrigeration problems. Symptoms were not observed in the next seven months in the plants exposed to insects. Furthermore, it has been known that in different plant hosts,

several groups of phytoplasmas can accumulate either in the source tissues, such as in *Euphorbia pulcherrima* and *Cathatantus roseus* with levels of accumulation that can vary one order of magnitude being more concentrated in *C. roseus* than in *E. pulcherrima* (Christensen *et al.*, 2004), or in the case of Australian Papaya Dieback Phytoplasma which accumulated in young leaves but not in fruits (Siddique *et al.*, 1998), making it difficult to select the sampling tissue in an unknown host.

When this research was carried out, a rearing for *A. funzaensis* was unavailable. In consequence, many variables that influence the transmission efficiency were not controlled. The transmission of phytoplasmas by their insect vectors is of the circulative, propagative type, and occurs in three phases (Weintraub and Beanland, 2006; Perilla-Henao and Casteel, 2016). The acquisition access period (APP) is the feeding time necessary for the insect

to acquire phytoplasmas, and the latency period (LP) is the period elapsed between the acquisition and the moment in which the insect is able to transmit phytoplasmas. During LP, the phytoplasmas replicate inside the insect and only when the salivary glands become infected, transmission occurs (Weintraub and Beanland, 2006). The latency period can take as long as 3 weeks (Webb *et al.*, 1999). The inoculation access period (IAP) is the time during which the insects are infective, which in this case is all their lives (Alma *et al.*, 2019). It is known that insects that test positive for phytoplasmas by nested are not necessary vectors (Purcell *et al.*, 1981; Vega *et al.*, 1993); to be able to transmit, phytoplasmas must infect the salivary glands of the insect (Weintraub and Beanland, 2006; Alma *et al.*, 2019). In this case, the DNA extractions performed on the *A. funzaensis* individuals were performed from the whole body and not only from the head, so it is impossible to know if the salivary glands were infected. The efficiency of transmission is also affected by the insect species. *Euscelidius variegatus* (Hemiptera: Cicadellidae) is considered to be an inefficient insect vector of the Crisantemum Yellows (CY) phytoplasma (group 16SrIB), in comparison to *Macrostelus quadripunctulatus* (Hemiptera: Cicadellidae). It has shown that in *E. variegatus* about 75% of the individuals get their salivary glands infected with CY in experimental conditions in comparison to *M. quadripunctulatus* in which 100% of the individuals get infected salivary glands. The transmission efficiency is attributed to the fact that the intestine and salivary gland membranes of *E. variegatus* act as semi-selective barriers to the pass of phytoplasmas, making them less efficient in transmission (Galetto *et al.*, 2009). It was later demonstrated that the CY Amp protein (major antigenic membrane) plays a role in the recognition between the phytoplasma and insect membranes in the early stages of the vector infection (Rashidi *et al.*, 2015). On the other hand, Palermo *et al.* (2001) showed that in infective individuals of *Macrostelus quadripunctulatus* (Hemiptera: Cicadellidae), a very efficient vector of CY, the infected insects have periods in which they were unable to transmit, but a few days later they recovered this ability (Palermo *et al.*, 2001). In this test, the infected *A. funzaensis* were collected from an infected grass patch, but the AAP and LP were not considered. Other variables not considered in this research were the number of insects infected at the beginning of the experiment (Galetto *et al.*, 2009), the host plant from which the phytoplasmas were collected (Palermo *et al.*, 2001), the plant load (Galetto *et al.*, 2014), and the control of temperature which could affect the transmission efficiency (Murrall *et al.*, 1996; Maggi *et al.*, 2014). Recommendations for future experiments include a period of pre-adaptation feeding for the insects before the

transmission tests, and longer observation periods and using nymphs instead of adults, as it is generally considered that they are more efficient vectors. Nymphs and adults can be infected with phytoplasmas but their transmission efficiency varies, even if they feed on the same part of the plant if nymphs are exposed earlier in their lives to phytoplasmas (Weintraub, 2007; Alma *et al.*, 2019).

The potential transmission ability of insect vectors can vary according to their feeding habits on different plant species, the phytoplasma groups that infect the host plants and the susceptibility of the insect to the colonization of the phytoplasmas, in addition to the intimate molecular relationships that occur between insects and phytoplasmas (Palermo *et al.*, 2001; D'Amelio *et al.*, 2007; Galetto *et al.*, 2009; Bosco and D'Amelio, 2010). There is little information about the interactions between phytoplasmas and different AP and LP which compete for tissues and organs (D'Amelio *et al.*, 2007; Bosco and D'Amelio, 2010; Perilla-Henao and Casteel, 2016), as would be the case of the 16SrI and 16SrVII phytoplasmas.

In this experiment, the *A. funzaensis* was collected from a natural population of infected insects growing in grass *C. clandestinus*, a monocot C4 plant, which was placed on strawberry, a dicot C3 plant. It is, therefore, possible that the different diet and nutritional content of the plants, in addition to the stress associated with the experimental conditions found in caged plants inside a greenhouse, may have affected their survival or transmission efficiency.

In total, 120 individuals of *A. funzaensis* were individually tested for the presence of phytoplasmas, including the insects evaluated to test the population used in the transmission experiments, and the insects were allowed to feed on the test plants. Of these individuals, 38% carried phytoplasmas. Of the 100 insects used for the transmission essays, 33 carried phytoplasmas of the 16SrI group and one carried a mix of 16SrI and 16SrVII groups. The existence of *A. funzaensis* populations in which large percentages of insects are infected with phytoplasmas make them a potential risk for dispersion of phytoplasmas in the Bogota plateau.

The groups 16SrI and 16SrVII have been reported as the most common phytoplasma groups in the Bogota plateau infecting trees (Perilla-Henao *et al.*, 2012; Perilla-Henao and Franco-Lara, 2013; Franco-Lara and Perilla-Henao, 2014; Franco-Lara *et al.*, 2017). Furthermore, 17% of the positive samples by nested PCR produced RFLP patterns that were inconsistent with phytoplasmas, confirming that not specific amplification of DNA can occur with the

phytoplasma primers used, as has been reported before (Abeysinghe *et al.*, 2016).

This is the first report of a *COI* DNA barcode sequence for *A. funzaensis*. This sequence allows the separation of this species from other members of the same genus such as *A. litoralis*, *A. osborni* and *A. simplarius*. The two *A. funzaensis* populations studied were separated by a geographical distance of 35 km. The DNA barcode was represented by a major haplotype shared between the two populations and two additional haplotypes that had a few nucleotide substitutions (average genetic distance of 0.0048). This low genetic variability suggests that *A. funzaensis* in the Bogota plateau is a meta-population thriving due to the efficient use of *C. clandestinus* as a host plant, a non-native grass that dominates the landscape. More information is needed to characterize the genetic variability of this species in the zone; in addition, possible differences between its symbionts could enhance its biological efficiency improving its nutritional and reproductive status (Takiya *et al.*, 2006). Among the symbionts, phytoplasmas are known to cause differential effects on the fitness of the insect vectors, in some cases improving their general fitness (Beanland *et al.*, 2000), or on the contrary negatively affecting their life span, fecundity, and progeny size (Bressan *et al.*, 2005).

This work does not provide proof of the ability of *A. funzaensis* to transmit phytoplasmas to *F. × ananassa* and more tests are needed. However, the fact that a high number of insects are infected with three groups of phytoplasmas highlights the need for epidemiological surveillance of crops and plants of the urban landscape of the Bogota plateau.

Acknowledgments

We thank Dr. Michael Robin Wilson of the National Museum of Wales, United Kingdom for helping with the identification of *A. funzaensis*, and Andrés Felipe Silva-Castaño for taking the insect pictures. Also, the gratitude is extended to the Universidad Militar Nueva Granada for funding project CIAS 839.

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