Distinct strains of the re-emergent *Cassava common mosaic virus* (genus: *Potexvirus*) infecting cassava in Argentina

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Cassava common mosaic disease (CCMD) has been reported in all regions where cassava is grown in the Americas and the causal agent, *Cassava common mosaic virus* (CsCMV), has been identified as a mechanically transmitted potexvirus (*Alphaflexiviridae*). In Argentina, cassava is grown mainly in the northeast (NEA) region that shares borders with Brazil and Paraguay. Increasing incidences of CCMD were observed during the years 2014 to 2016 associated with severe leaf mosaic symptoms and yield reductions where the occurrence of CsCMV was confirmed by RT-PCR and sequencing. In this work, the virus has been successfully purified and a double-antibody sandwich (DAS-) ELISA test has been developed from an Argentinean isolate of CsCMV to extend the diagnostics of the disease. A collection of 726 samples was screened and CsCMV was detected with 100% prevalence in the NEA region. Additional co-infecting viruses were detected in some plants (64.4%); in these, CCMD symptoms correlated with CsCMV only, although more severe symptoms could be observed in mixed infected plants. Sequence analysis of the conserved RdRp domain showed a wider diversity of CsCMV isolates. Interestingly, a separate phylogenetic cluster was formed by isolates from the NEA region that only shared 77.1% to 80.3% nucleotide identity with the other clusters. These results indicate the presence of mixed strains occurring in the NEA region and suggest the presence of geographically distinct strains of CsCMV in South America.

Keywords: cassava common mosaic disease, *Manihot esculenta*, re-emergent virus, virus surveillance

Introduction

Cassava (*Manihot esculenta*), a tropical root crop, is the fourth most important source of calories that provides the staple food for an estimated 800 million people worldwide (Legg *et al.*, 2015). Cassava is grown almost exclusively by low-income, smallholder farmers, and it is one of the few staple crops that can be produced efficiently on a small scale. In 2016, estimated world cassava production reached over 270 million tonnes, restoring cassava's status as one of the world's fastest expanding staple crops (Food and Agriculture Organization, 2017).

In Argentina, this crop is exclusively cultivated in the northeast region (henceforth denominated NEA), covering an area of 35 000 to 45 000 ha, where the province of Misiones has the largest cultivated area of about 20 000 to 25 000 ha, followed by Corrientes, Formosa and Chaco with 1880, 1625 and 1000 ha, respectively. In these latter provinces, cassava roots are used for fresh consumption and for animal feed, while in Misiones cassava is also used for industrial starch production (O. Uset, EEA Montecarlo – INTA, Misiones, Argentina, personal communication). The main varieties of cassava for industrial uses are IAC-90 and CA25-1, whereas Rocha, Coloradita and Pomberí varieties are for fresh consumption.

As a vegetatively propagated species, cassava is affected by the accumulation of systemic pathogens in successive crop cycles, with significant negative consequences on yields and on the quality of storage roots (Carvajal-Yepes *et al.*, 2014). There are at least 15 species of viruses that can infect cassava (Legg *et al.*, 2015). In Argentina, at least three viruses have been reported (Zanini *et al.*, 2014; Di Feo *et al.*, 2015). *Cassava common mosaic virus* (CsCMV) is a re-emergent virus significantly affecting cassava production in South America; it has been reported in Venezuela (Chaparro-Martinez & Trujillo-Pinto, 2003), Peru (Costa & Kitajima, 1972; Fernandez *et al.*, 2017), Colombia, Brazil and Paraguay

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(Silva *et al.*, 2011; Calvert *et al.*, 2012; Lozano *et al.*, 2017) and recently in Argentina (Di Feo *et al.*, 2015), close to the Paraná region where it was previously reported. The affected plants develop mosaic and chlorosis in leaves, with increased severity in the subtropical zones of South America due to the prolonged cold periods (Calvert *et al.*, 2012). The effects on reduction in root yield caused by CsCMV can be from 30% to 60% (Costa & Kitajima, 1972; Venturini *et al.*, 2016).

The aim of the present study was to characterize the recent outbreak of the disease in Argentina by determining the incidence and prevalence of the disease. After optimizing the diagnostics of CsCMV by producing an antiserum from an Argentinean isolate, the phylogenetic relationships of virus isolates were analysed with those previously reported in South America (Lozano *et al.*, 2017).

Materials and methods

Survey area, sampling procedure and data collection

The field survey and collection of samples were conducted from 2014 to 2016 in the main cassava-growing provinces of Argentina, including Misiones, Formosa and Chaco. In total, 726 cassava leaf samples of different varieties were collected from 50 lots, in which random samples were picked along a diagonal transect (Table S1). The average area of each lot sampled was 2.6 ha in Misiones, 0.5 ha in Formosa and 0.6 ha in Chaco. All samples collected corresponded to the top youngest leaves of cassava plants. They were stored on ice for transporting and then kept at −80 °C until processing.

Virus isolation, purification and antiserum production

The viral isolate used to produce the antiserum (isolate Cor_6AR; Table S3) was obtained from a sample exhibiting systemic mild mosaic symptoms after mechanical inoculation (using 0.05 M sodium phosphate buffer, pH 7.2) to *Nicotiana benthamiana*. The presence of CsCMV in the inoculated plants was detected by plate-trapped antibody (PTA-) ELISA with serum kindly provided by Eliezer R. Souto (Department of Agronomy, Universidade Estadual de Maringá, Brazil).

Cassava common mosaic virus was purified from 100 g of *N. benthamiana* leaves, 30 days after inoculation (dai), following a previously modified protocol (Izaguirre-Mayoral & Marys, 1995; Silva *et al.*, 2011). Purified virus preparations were examined in a JEOL 1200 transmission electron microscope after negative staining with 2% (w/v) uranyl acetate. Virus concentration was estimated as $(A_{\lambda 260} - A_{\lambda 330})/EC$ using an average extinction coefficient (EC) of 2.84 for potexviruses (Mukhamedzhanova *et al.*, 2011) and the purity was measured by calculating $A_{\lambda 260}/A_{\lambda 280}$ using a NanoDrop 1000 spectrophotometer (Thermo Scientific). A polyclonal antiserum was obtained by giving a New Zealand white rabbit eight multiple intradermal applications on each side of the animal's spine. The injections consisted of 0.15 mg virus emulsified with Freund's complete adjuvant (1 mg mL^{-1}; Vaitukaitis, 1981). Twenty-eight days later, 0.045 mg of purified virus emulsified with incomplete Freund's adjuvant (1 mg mL^{-1}) was injected intramuscularly. Collection of blood samples started 2 weeks after the last injection. The titres were evaluated by nitrocellulose membrane (NCM-) ELISA (Parent *et al.*, 1985), PTA-ELISA (Mowat & Dawson, 1987), double-antibody sandwich (DAS-) ELISA (Clark & Adams, 1977) and by immunosorbent electron microscopy (ISEM) plus decoration (Milne & Leseman, 1978). Healthy and infected leaves were collected from cassava plants maintained in a greenhouse and used as negative and positive controls, respectively.

Determining the incidence, prevalence and severity of CsCMV

For CsCMV detection, DAS-ELISA was performed in polystyrene microtitre plates precoated with IgG 1/1000 (v/v) concentration and alkaline phosphatase-conjugated IgG 1/1000 (v/v) concentration. Approximately 100 mg of fresh tissue was ground in a 1/10 proportion (w/v) with extraction buffer (1× phosphate-buffered saline pH 6.8, 2% polyvinylpyrrolidone, 0.05% Tween 20, 0.02% Na_2SO_3). Cassava plants obtained by *in vitro* culture of meristems (Schaller *et al.*, 2014) were used as negative controls. Samples were considered positive when the absorbance values were higher than the mean value of the negative control readings plus three times their standard deviation. CsCMV incidence was estimated as a percentage of infected plants per lot while the prevalence was calculated as a percentage of infected lots in each province. CsCMV incidence was analysed by generalized linear model under binary distribution (Di Rienzo *et al.*, 2012).

The symptomatology was evaluated for each plant on a four-degree ad hoc scale, where 0 = no symptoms on leaves; 1 = mild mosaic chlorotic blotches on leaves and absence of leaf distortion; 2 = moderate chlorotic blotches on all leaves and leaf distortion; and 3 = severe morphological mosaic spots on all leaves, leaf distortion and reduction of leaf size (Fig. 1). The average degree of severity (ADS) of symptoms per lot was calculated by the equation: $ADS = \Sigma(F_i \times X_i)/n$; where F_i is the frequency of plants with the i-th class of severity scale; X_i is the value of the i-th class of severity scale (0 to 3); and n is the total number of sampled plants. The data were analysed with the software InfoStat (Di Rienzo *et al.*, 2012) by chi-square test for a two-way contingency table, between the leaf symptom expression and the virus presence. The mean of ADS was compared by linear model with varIdent function for province factor. Fisher's least significant difference (LSD) test was used for comparison of means.

RT-PCR, sequencing and phylogenetic analysis

Forty-five samples were chosen randomly from plants collected in Corrientes (experimental field of the Facultad de Ciencias Agrarias, UNNE), Chaco, Formosa and Misiones for sequence analysis. Total RNA was extracted from leaf tissues by the CTAB method (Carvajal-Yepes *et al.*, 2014) and 2–3 μg were used for cDNA synthesis using M-MLV reverse transcriptase (Promega) and random hexamer primers (Invitrogen). A pair of generic oligonucleotides, 1RC/Potex5, was used to amplify a genomic segment of CsCMV as previously described by van der Vlugt & Berendsen (2002). These primers amplify a fragment of 720 nucleotides (nt) of the RNA-dependent RNA polymerase (RdRp) domain. The presence of co-infecting viruses in the samples was checked by RT-PCR using available primers for torradoviruses (Verbeek *et al.*, 2012) and for the *Cassava frogskin-associated virus* (CsFSaV) reovirus (Calvert *et al.*, 2008). Fifteen PCR amplicons were cloned using the pGEM-T Easy vector (Promega) system and sent for sequencing (Macrogen). Virus sequences were identified using BLAST (http://www.ncbi.nlm.nih.gov/) and identities among isolates were calculated by using SDT v. 1.2 (Muhire *et al.*, 2014). Phylogenetic analysis was inferred

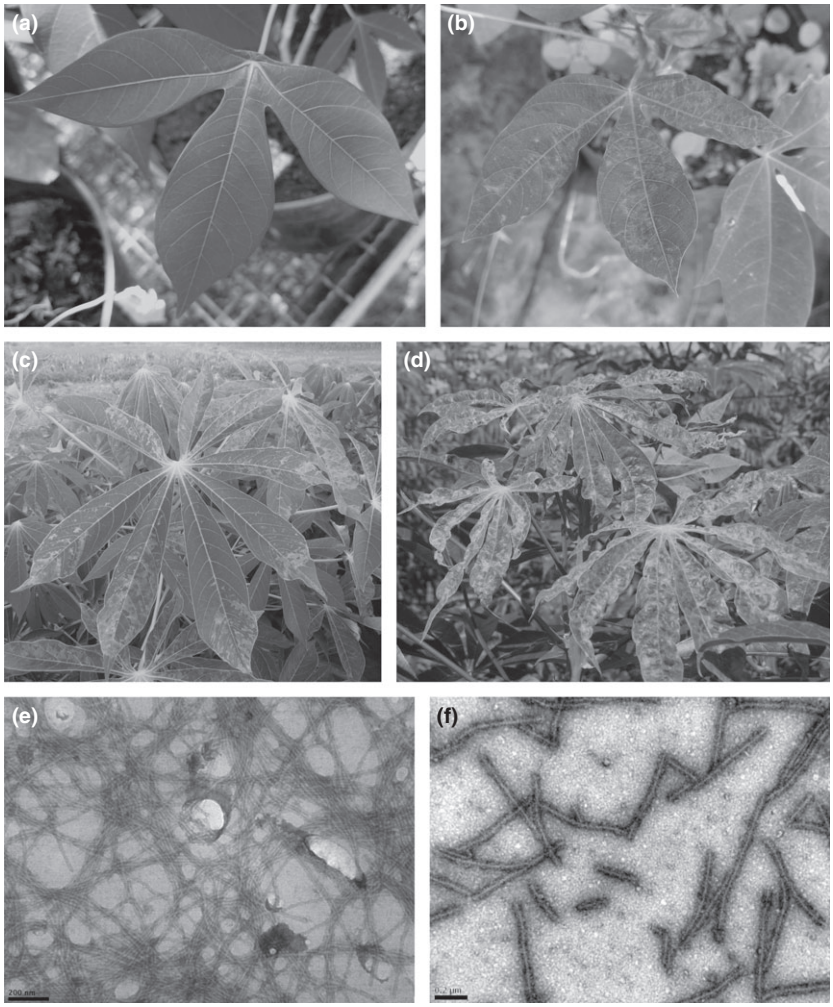

Figure 1 Symptoms and virus purification. Average degree of severity (ADS): (a) 0, no symptoms on leaves; (b) 1, mild mosaic chlorotic blotches on leaves and absence of leaf distortion; (c) 2, moderate chlorotic blotches on all leaves and leaf distortion; (d) 3, severe morphological mosaic spots on all leaves, leaf distortion and reduction of leaf size. (e) *Cassava common mosaic virus* (CsCMV) viral particles observed in a JEOL 1200 electron microscope (×100 000) from the extract used for rabbit immunization and production of the antiserum. (f) Uniformly decorated viral particles from extracts of cassava leaves observed in a JEOL 1200 electron microscope (×80 000) with 1/10 (v/v) antibody coating of CsCMV diluted and 1/1000 (v/v) antiserum-coated grids, negatively contrasted with 2% uranyl acetate.

using the neighbour-joining method (2000 bootstrap replications) and distances calculated using the Poisson correction method taking the number of amino acid (aa) substitutions per site as units. Evolutionary analyses were conducted in MEGA 7 (Kumar *et al.*, 2016). To calculate if there was a correlation between the presence of virus and the expression of mosaic symptoms, the Spearman coefficients were calculated for the most frequent viruses. For each cassava plant, the degree of severity of foliar symptoms was established according to the rating scale described above.

Results

Virus purification and antiserum production

Nicotiana benthamiana plants inoculated with CsCMV showed leaf mosaic symptoms 20 dai and the infection was confirmed by PTA-ELISA. Four fractions containing virus particles were collected after ultracentrifugation of the sucrose gradient. As the $A_{\lambda 260}/A_{\lambda 280}$ ratio for pure viral particles should be 1.09–1.37 (van Regenmortel & Mahy, 2010), the fraction with a ratio of 1.16 and 0.275 mg mL^{-1} concentration was chosen to immunize the rabbit (Fig. 1e).

Serological tests

There was no cross-reaction detected with leaf extracts of uninfected *N. benthamiana* or cassava. ELISA absorbance values at 405 nm for the antiserum dilutions used for PTA-ELISA and DAS-ELISA tests are shown in Table 1. DAS-ELISA was sensitive and reliable in detecting CsCMV in leaf samples collected from infected cassava plants in the greenhouse. For the NCM-ELISA test, a minor nonspecific reaction with the antiserum was established at 1/64 000 (v/v) antiserum dilution. In ISEM tests from CsCMV-affected cassava leaves, filamentous virus particles with a modal length of 480–490 nm, resembling potexvirus virions, were trapped with antibodies against CsCMV at 1/1000 (v/v) dilution. There was a strong decoration of individual CsCMV particles with 1/10 (v/v) antibody dilution (Fig. 1f).

Incidence, prevalence and severity of CsCMV

A total of 726 samples were collected and analysed by DAS-ELISA, and 85.2% of them were positive for CsCMV. The CsCMV incidence by province was

significantly different ($P < 0.0001$), whereas the CsCMV incidence within provinces for year was not meaningful (Table 2). The viral incidence per lot varied between 20% and 100% in Misiones, 57.9% and 100% in Formosa, and was always 100% in Chaco (Table S1; Fig. S1). A prevalence of 100% of CsCMV was detected in the total of lots, provinces and years sampled.

Cassava plants analysed in the provinces of the NEA region showed different degrees of severity of foliar symptoms. Moderate mosaic and leaf distortion in all leaves of the analysed samples were generalized, although the recorded symptoms varied from 0, without leaf symptoms, to 3, with severe mosaic in all leaf lobes, alternating with normal green and patches of intense yellow and marked leaf distortion (Fig. 1). The leaf symptom expression was strongly associated with the presence of CsCMV ($P < 0.0001$), i.e. when the virus was present the percentage of plants that expressed symptoms was higher (98.7%). On the other hand, only 14.2% of plants with symptoms tested negative for CsCMV (Table S2). These latter plants, with symptoms but CsCMV-negative, corresponded to only 8.1% of the total of cassava samples analysed. The percentage of symptomless plants that tested positive for CsCMV corresponded to 1.1% of the total of cassava samples analysed.

The mean ADS was higher than 1.4, but differed significantly among provinces. Chaco had the highest severity of symptoms and the highest incidence of CsCMV (Table 2).

RT-PCR, sequencing and phylogenetic analysis

All samples tested by RT-PCR, except one, were positive for at least one virus. CsCMV was the most frequent (77.8%), followed by CsFSaV (71.7%) and *Cassava torrado-like virus* (CsTLV; 15.6%). Mixed infections occurred in 64.4% of samples, with CsCMV co-infecting with CsFSaV the most frequent (69%), followed by CsCMV co-infecting with CsFSaV + CsTLV (10.3%) and CsTLV co-infecting with CsCMV (6.9%) or CsFSaV (6.9%). Samples with more than one virus exceeded an ADS of 2, whereas in single infections the ADS was 1.5. Correlation between symptom severity and virus presence was found when CsCMV was present in single infection ($R^2 = 0.37$; $P = 0.03$), and the degree of severity was even greater when it was co-infecting with CsFSaV. However, no correlation was found when only CsFSaV was present ($P = 0.76$). Furthermore, correlation between symptom severity and virus presence was found when CsTLV was present ($R^2 = 0.43$; $P = 0.01$). It should be noted that CsTLV was always detected in co-infection. Positive RT-PCR results for the genus *Potexvirus* coincided with the serological detection of CsCMV by DAS-ELISA, except for one sample (isolate Mis_4).

The nt identity of a region corresponding to the conserved RdRp domain (Table S3) varied from 88.7% to 93.1% (99.2% aa) when three Argentinean isolates (Corr_44, For_EC26 and For_EC28) forming a separate group (Fig. 2) were compared. The nt identity between these and available CsCMV sequences varied from 72.5% to 80% (83.6–95.1% aa). The main nt identity observed was with the isolate from the Brazilian state of Paraná (JF913280), from 78.6% to 80% (94.2–95.1% aa), whereas the least nt identity observed was with the Venezuelan isolate (KP663619), from 72.5% to 73.3% (83.6% aa), detected in the euphorbiaceous host chaya (*Cnidoscolus aconitifolius;* Mejías *et al.*, 2015).

Table 1 Detection of *Cassava common mosaic virus* antigen in cassava leaves using PTA- and DAS-ELISA.

PTA-ELISA absorbance values at 405 nm

Antigen		Antiserum dilution (v/v)	
Source	Dilution of leaf sap (w/v)	1/10 000	1/20 000
Cassava	1/10	0.21	0.20
	1/100	0.96	0.61
	1/300	0.69	0.44
Virus-free cassava	1/10	0.05	0.04

DAS-ELISA absorbance values at 405 nm

		IgG dilution (v/v)			
		1/1000		1/1500	
Antigen		IgG conjugate dilution (v/v)			
Source	Dilution of leaf sap (w/v)	1/1000	1/2000	1/1000	1/2000
Cassava	1/10	0.80	0.58	0.64	0.43
	1/100	0.64	0.46	0.48	0.33
Virus-free cassava	1/10	0.05	0.04	0.04	0.05

Table 2 Summary means of the average degree of severity (ADS) and the incidence of CsCMV found in all plants analysed by province.

Province	ADS	SE	CsCMV incidence	SE
Chaco	2.2 a	0.12	1.000 a	0.001
Formosa	1.8 b	0.12	0.955 a	0.012
Misiones	1.4 b	0.15	0.739 b	0.026

Different letters indicate significant differences using Fisher's LSD test at $\alpha = 0.05$.
CsCMV, C*assava common mosaic virus*; SE, standard error.

Discussion

This work highlights the importance of CsCMV early detection in cassava crops in the NEA region of Argentina. Although cassava common mosaic disease (CCMD) has been considered to be of minor importance, current studies indicate it can be associated with up to 30% yield losses in cassava (Venturini *et al.*, 2016). The study here reports an incidence of up to 85.2% of CsCMV in the NEA region, comparable to the 96% incidence reported in the neighbouring Brazilian state of Paraná (Silva *et al.*, 2011). This is a similar situation as reported in the 1970s in Colombia, where up to 90% incidence of

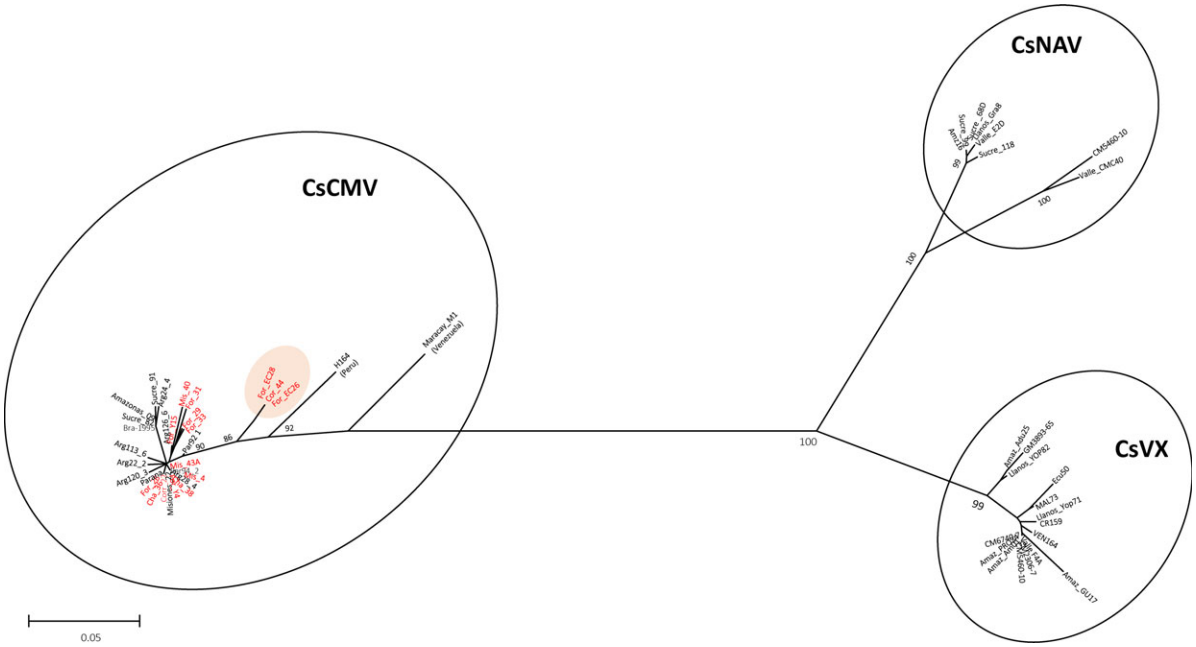

Figure 2 *Cassava common mosaic virus* (CsCMV) RdRp phylogenetics. Phylogenetic relationships among isolates of CsCMV and other potexviruses reported infecting cassava in the Americas. Amino acid sequences analysed correspond to the RdRp domain region at the C-terminal of the replicase protein. Sequence isolates are described in Table S3 and in Lozano *et al.* (2017). All CsCMV sequences were isolated from cassava except for isolate Maracay_M1, which was isolated from chaya in Venezuela (Mejías *et al.*, 2015). The tree was obtained with MEGA 7 using the neighbour-joining method, with 2000 bootstrap replications.

CCMD was recorded (Costa & Kitajima, 1972; Nolt *et al.*, 1991). These results suggest that the management of planting material is unsuitable, as vegetative propagation by cassava stakes supports the inadvertent distribution of systemic pathogens. It is known that CsCMV is easily transmissible through agricultural tools (Calvert *et al.*, 2012), which also contribute to this increase in incidence. The exchange of stakes between provinces and its introduction from bordering countries without phytosanitary control increases the spread of this and other diseases.

Out of all of the viruses detected in the samples, CsCMV associated strongly with the observed CCMD symptoms (98.7%). This result singled out CsCMV as the predominant virus associated with disease in this region, near to the Brazilian Paraná state where CCMD has been known for a long time (Silva *et al.*, 2011). There were 8.1% of cassava plants with symptoms in which no CsCMV was detected. This would indicate the presence of other pathogens affecting these plants. These plants were checked for other cassava viruses reported in South America, and although no correlation with mosaic symptoms was observed, the results of incidence indicate that they contribute to the observed variability in symptom severity and probably also in yield reduction. It is known that mixed viral infections can interact in unexpected ways, including synergisms and antagonisms (Syller, 2012; Adams *et al.*, 2014). Naturally occurring mixed infections have been reported associated with severe cases of cassava mosaic and cassava brown streak diseases in Africa (Legg *et al.*, 2011, 2015; Munganyinka *et al.*, 2018) and cassava frogskin disease in South America (Carvajal-Yepes *et al.*, 2014), where the cumulative effect of each pathogen leads to an increasing effect on yield losses. Furthermore, mixed infections among distinct strains of the same virus species can facilitate the generation of recombination variants showing novel biological features, e.g. more severe variants. One of the best examples is that of novel recombinant strains of cassava mosaic virus in Uganda, which caused a severe pandemic of cassava mosaic disease in Africa in the 1990s (Zhou *et al.*, 1997; Pita *et al.*, 2001). By comparing all available RdRp partial sequences, the present study has already observed a higher diversity of CsCMV isolates than expected. Most isolates grouped with those collected in Colombia, Paraguay, Argentina, Brazil and Peru in previous decades, but a separate cluster was formed by sequences obtained from NEA samples collected from 2014 to 2016 that shared only 72% nt identity (83.6% aa) with sequences in the major group, including a previous strain isolated in the neighbouring Paraná region in 2010 (GenBank JF913280). These results indicate the presence of mixed strains causing CCMD in the NEA region (and probably also in the neighbouring fields of Brazil and Paraguay). The same can be observed for isolates detected recently in Peru (Fernandez *et al.*, 2017) and Venezuela (Mejías *et al.*, 2015), indicating the occurrence of geographically distinct strains of CsCMV (Lozano *et al.*, 2017). However, the differences detected at nt sequence level among distinct isolates of

CsCMV are not sufficient to impede its detection by ELISA. The production of a CsCMV-antiserum from an Argentinean isolate and its use in the early monitoring of the disease in the NEA region has been essential for this work. In Argentina, the indiscriminate entry of infected plant material and its exchange among cassava-producing provinces should be a call for action and increase awareness of the need for better phytosanitary and disease management protocols in the country, such as the search for resistant cassava varieties, the early detection of the virus, prevention of dissemination of infected stakes, and the production of virus-free planting material.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Sampled regions and incidence of *Cassava common mosaic virus* (CsCMV). The lots sampled by province are indicated with points, and sites where some of the CsCMV isolates used to construct the phylogenetic tree of Figure 2 were collected are marked with triangles. Percentages of incidence of CsCMV and cassava common mosaic disease per province are indicated in pie charts for each sampled year. For more information visit www.pestdisplace.org.

Table S1. *Cassava common mosaic virus* incidence and severity of foliar symptoms per lot by province and year sampled.

Table S2. Crosstab for leaf symptom expression and infection with *Cassava common mosaic virus* (CsCMV) in all analysed plants.

Table S3. List of samples tested positive for CsCMV by RT-PCR and DAS-ELISA tests. Some isolates from each province sampled were sequenced (GenBank) and used to construct the phylogenetic tree in Figure 2. Other isolates of CsCMV are described in Lozano *et al.* (2017). ND, not determined.