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RESEARCH ARTICLE

Distribution and diversity of begomoviruses in tomato and sweet pepper plants in Costa Rica

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Abstract

Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) have emerged as important plant pathogens in tropical and subtropical regions worldwide. Although these viruses were reported during the 1970s in Costa Rica, they are still poorly known. Therefore, the objective of this study was to analyse the diversity and distribution of begomoviruses in commercial tomato and sweet pepper fields from different agricultural production systems of the major growing regions of Costa Rica. A total of 651 plants were randomly sampled from greenhouses and open field crops during 2011 and 2012 in three different geographical locations. The bipartite begomoviruses *Tomato yellow mottle virus*, *Tomato leaf curl Sinaloa virus* and *Pepper golden mosaic virus*, and the monopartite begomovirus *Tomato yellow leaf curl virus* were detected in the collected samples. The complete genome of isolates from each species was cloned and sequenced. The frequency of detection of these four begomoviruses in the analysed samples ranged from 0 to 9%, the presence, and the prevalent virus varied largely according to the geographical location, the host (tomato and pepper), and the production system (greenhouses or open fields). An association between geographical region and begomovirus species was observed suggesting that in Costa Rica the heterogeneity on climate, topography and agricultural system might influence the distribution of begomovirus species in the country. A broader survey needs to be conducted to confirm it, although these preliminary results may contribute to the management of begomoviruses in Costa Rica.

Introduction

Begomoviruses (genus *Begomovirus*; family *Geminiviridae*) are transmitted by the whitefly (Hemiptera = Aleyrodidae) *Bemisia tabaci* and constitute the largest genus of plant viruses. The genus comprises more than 300 species distributed worldwide (http://ictvonline.org/). Members of the family *Geminiviridae* are characterised by a circular, single-strand DNA genome, consisting of one (monopartite) or two (bipartite) components of 2.6–3 kb encapsidated in twinned icosahedral particles of about 22 mm × 38 nm (Brown *et al.*, 2012, 2015). Begomoviruses reported from the Old World group in a phylogenetic clade separated from most of the bipartite and monopartite begomoviruses present in the New World (Márquez-Martín *et al.*, 2011; Melgarejo *et al.*, 2013; Brown *et al.*, 2015).

From the early 1990s, numerous begomoviruses have emerged as pathogens causing significant economic losses throughout the world (Navas-Castillo *et al.*, 2011). Several factors are associated with the increased incidence, diversity and distribution of begomoviruses, such as the introduction of susceptible plants into regions where these viruses are endemic, high biological and genetic variability of the viruses, commercial trade of infected

plant materials, or changes in the population of the insect vector, crop cultivation systems and/or climate factors (Rojas *et al.,* 2005; Seal *et al.,* 2006; Nawaz-ul-Rehman & Fauquet, 2009).

Globally, tomato (*Solanum lycopersicum*) and sweet pepper (*Capsicum annuum*) are frequently infected by begomoviruses (Moffat, 1999; Navas-Castillo *et al.,* 2011), and studies over the past 25 years from Latin America, southern United States and the Caribbean have reported the emergence of numerous begomoviruses in these two crops (Brown & Bird, 1992; Morales & Anderson, 2001; Zambrano *et al.,* 2007; Márquez-Martín *et al.,* 2011; Melgarejo *et al.,* 2013; Herrera-Vásquez *et al.,* 2016; Inoue-Nagata *et al.,* 2016). Nakhla *et al.* (2005) conducted a survey to identify the begomoviruses present in tomatoes in Central America. They described the occurrence of at least eight bipartite species, among them *Tomato severe leaf curl virus* (ToSLCV), *Tomato mosaic Havana virus* (ToMHaV) and what they called Tomato mild mottle virus (ToMiMoV) in Honduras; ToSLCV, *Tomato golden mottle virus* (ToGMoV), the putative ToMiMoV, ToMHaV, *Pepper golden mosaic virus* (PepGMV), ToLCSiV and *Pepper huasteco yellow vein virus* (PHYVV) in Guatemala; and *Tomato yellow mottle virus* (ToYMoV) and *Tomato leaf curl Sinaloa virus* (ToLCSiV) in Costa Rica. Since the 1970s, high worldwide incidence of begomoviruses has resulted in dramatic yield losses and economic damage (Navas-Castillo *et al.,* 2011; Rybicki, 2015). For example, in South America, since the 1970s *Bean golden mosaic virus* has been causing serious losses in beans (Gálvez & Castaño, 1976; Faria & Maxwell, 1999; Morales & Anderson, 2001; Sobrinho *et al.,* 2014). Furthermore, from the mid-1990s, an increased incidence of begomoviruses was observed in tomato crops of the major growing regions of Latin America, causing up to 100% yield loss (Ribeiro *et al.,* 1998; Paz-Carrasco *et al.,* 2014; Chinnaraja *et al.,* 2016; Geraud-Pouey *et al.,* 2016; Macedo *et al.,* 2017).

In Costa Rica, the presence of different begomovirus species has been reported in horticultural production systems since the 1970s (Gámez, 1970; Lotrakul *et al.,* 2000; Nakhla *et al.,* 2005; Castro *et al.,* 2013; Barboza *et al.,* 2014, although there is no recent information about the genetic diversity in crops like tomato and sweet pepper. Therefore, in this study we analysed: (a) the begomovirus species that are currently infecting tomato and sweet pepper crops in Costa Rica and (b) their distribution according to the production system, geographical location and host plant. To evaluate these aspects, field and greenhouse surveys were conducted and tomato and sweet pepper samples were randomly collected and analysed for begomovirus infection over 2 years in commercial crops of the major growing areas of Costa Rica.

Material and methods

Sample collection

Field surveys were conducted every 6 months during the dry (January–April) and rainy (July–September) seasons of 2011 and 2012 in three major tomato and sweet pepper production areas of Costa Rica = Cartago, Grecia and Zarcero. At each location, four independent collection sites were selected at the first sampling date from greenhouses and open field production areas. The selected sampling sites were monitored during the whole survey and visited when tomato or pepper crops were present. For sampling, at least eight individual tomato and/or sweet pepper plants were randomly selected from each sampling site. These samples (one individual sample per plant) consisted of the top young leaves of each plant (cultivars without resistance/tolerance genes), which were then dried and stored individually at 4°C until analysed.

DNA extraction and hybridization

Total nucleic acids were extracted from each sample by using a modification of the Dellaporta method described by Rojas *et al.* (1993). Begomovirus presence was preliminarily analysed by dot blot hybridization where aliquots of the total nucleic acids extract (TNA) were heated and denatured and then applied to a nylon membrane (Pall Corporation, Glen Cove, New York, USA). Then TNAs were fixed with ultraviolet light calibrated to 50 MJ and the hybridization signal was detected with autoradiograms after the detection process. For universal begomovirus detection, a generic probe of 400 nt representing the coat protein (CP) gene, was produced by amplification by PCR using the primer pair PBGGTv647/PBGGTc1048 (Potter *et al.,* 2003) and the *Bean golden yellow mosaic virus* (BGYMV) DNA-A clone BGYMV-Gt (kindly supplied by Dr. Pilar Ramirez, University of Costa Rica) as the template. The PCR products were labelled with alkaline phosphatase (Amersham Pharmacia, Piscataway, New Jersey, USA) and hybridizations were conducted at low stringency (55°C) conditions according to the Gene Image Alkphos Direct Labelling and Detection System (Amersham Pharmacia, Piscataway, New Jersey, USA) specifications.

Rolling circle amplification and cloning strategy

Putative begomovirus DNA molecules present in samples positively detected by hybridization using the universal probe were amplified by rolling circle amplification (RCA) using Illustra TempliPhi DNA Sequencing Template Amplification Kit (GE Healthcare, Little Chalfont, UK). To determine the diversity of the begomoviruses present

Table 1 Primers used for probe synthesis for dot blot hybridization analyses

Virus[a]	Forward Primer	Position in the Genome[b]	Reverse Primer	Position in the Genome[b]	Product Size (nt)	Template (GenBank Accession Number)[c]
PepGMV	5′-AATGATTTTGGTGGC AGTGG-3′	2482–2463	5′-TCCCGCTTAACCATTTTGAA-3′	202–221	358	KY064011
ToLCSiV	5′-TTAACCGATGGCAT TTTGGT-3′	2486–2467	5′-GCATCACGCTTAGGCATTTT-3′	194–213	339	KY064013
TYLCV	5′-GGCATGTTGAAATG AATTGG-3′	2630–2611	5′-TGGGGACCAAGTATATAAAGACAA-3′	106–129	280	KF533856
ToYMoV	5′-ACACCAATTGGGTTC CTCTC-3	2482–2463	5′-CGCTTGGGCATTTTGAATTA-3′	160–179	271	KY064010

[a]*Pepper golden mosaic virus* (PepGMV), *Tomato leaf curl Sinaloa virus* (ToLCSiV), *Tomato yellow mottle virus* (ToYMoV) and *Tomato yellow leaf curl virus* (TYLCV).
[b]Nucleotide positions correspond to the DNA-A component for PepGMV, ToLCSiV, ToYMoV or genomic DNA (TYLCV) sequences obtained in this study, for which GenBank accession numbers are indicated.
[c]Clone and GenBank accession number of the corresponding DNA-A sequence used to design the specific primers.

in the samples, RCA products were digested with *Hpa*II endonuclease (New England BioLabs, Ontario, Canada) and digestion patterns were visualised on 1% agarose gels (Haible *et al.,* 2006).

In order to obtain full-length clones of the begomoviruses present, the RCA product from at least one sample exhibiting each distinct *Hpa*II digestion pattern was selected and digested with a set of restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, *Sac*II, *Spe*I and *Xba*I) in order to identify enzymes with unique restriction sites that could be used to clone the full-length genome components (~2.6 kb), following the method described by Inoue-Nagata *et al.* (2004). The restricted fragments corresponding to putative full-length monomers were cloned into the vector pBluescript SK(+) (Stratagene, La Jolla, California, USA). After transformation into *Escherichia coli* DH5α, recombinant plasmids with inserts of the expected sizes were selected and the insert completely sequenced by primer walking performed at Macrogen, Inc. (Seoul, South Korea).

Sequence analysis

Sequences were assembled using Staden program (Bonfield *et al.,* 1995), and the open reading frames (ORFs) analysed using the ORF Finder program (https://www.ncbi.nlm.nih.gov/orffinder/). Sequence similarity searches were performed using BlastN to compare the obtained sequences with sequences available in public sequence databases (Altschul *et al.,* 1990). Full-length sequence pair-wise comparison was performed, and the colour-coded matrix was produced using the species demarcation tool SDT v1.2 program (http://web.cbio.uct.ac.za/SDT; Muhire *et al.,* 2014). All sequences from bipartite and monopartite begomoviruses were compared with the closest sequences from GenBank (http://www.ncbi.nlm.nih.gov/).

Phylogenetic relationships were inferred for the obtained sequences, including the sequences of American begomoviruses detected in tomatoes and sweet peppers available in GenBank (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using Muscle algorithm implemented in MEGA 6.0 (Tamura *et al.,* 2013) and a phylogenetic tree constructed. A maximum likelihood tree was constructed with 3000 bootstrap replications, and evolutionary distances computed using the Kimura 2-parameter method and expressed as the number of base substitutions per site using MEGA 6.0.

Specific hybridization

For the detection of specific begomovirus species, probes directed to the intergenic region (IR) were obtained by PCR for the bipartite begomoviruses ToYMoV, PepGMV, ToLCSiV and the monopartite begomovirus TYLCV. The probes were produced based on genomic (for monopartite begomoviruses) or DNA-A (for bipartite begomoviruses) clones obtained in this study (ToYMoV accession no. KY064010, ToLCSiV accession no. KY064013, TYLCV accession no. KF533856 and PepGMV accession no. KY064011) using specific primer pairs (Table 1). PCR products (*c.* 300 bp) were labelled as indicated previously and hybridizations were conducted at high stringency (75°C) conditions, following Gene Image Alkphos Direct Labelling and Detection System specifications (Amersham Pharmacia). Unlabelled PCR amplified DNA fragments corresponding to each probe were used as positive controls, and two negative controls (water and DNA extracted from a healthy plant) were included in each hybridization.

Statistical modelling

For the positive samples obtained from the dot-blot screening of samples collected in the survey, three preliminary analyses were conducted based on contingency tables = (a) a comparison of the begomovirus proportion by host plant species (tomato and sweet pepper), taking into account the collection region (Cartago, Grecia and Zarcero); (b) proportion of begomoviruses in tomato and sweet pepper according to geographical location and type of production; and (c) comparison of begomovirus species proportion and geographical region (Cartago, Grecia and Zarcero). Analyses were conducted in R statistical software, version 3.1.0 (http://www.r-project.org/). Data obtained from the specific hybridization analyses of samples collected in the survey were classified using a presence or absence matrix (proportion data), and a database was compiled including information about the virus species (vs), geographical location (l), production system (g = greenhouse or field), crop host (h = tomato or sweet pepper), and sampling date (d). The latter referred to sampling month, where month = 0 defined the first sampling time. Multinomial regression models were constructed to examine differences in the probability of detection as a function of the independent factors. In total, 20 models were compared in order to quantify how individual factors predicted begomovirus prevalence in tomato and sweet pepper. Model selection was based on model deviance and Akaike's information criterion (AIC), as well as biological interpretation.

Results

Detection of begomoviruses

A total of 651 leaf samples (Table S1, Supporting Information) from tomato and sweet pepper plants were collected during 2011 and 2012. Both commercial tomato and sweet pepper crops were surveyed in all visited regions. Plants were sampled at random and the putative viral infection symptoms observed in the collected plant were recorded. The symptoms included severe to mild mosaic, leaf chlorosis, upward or downward curling of leaves, veinal chlorosis and/or plant stunting (Fig. 1 and Table S1).

Using a begomovirus universal probe, 58 (9%) positive samples were detected by hybridization. The proportion of begomovirus-positive samples varied from 0% to 26% depending on the region and host species considered. In sweet pepper samples, a begomovirus was detected in 6.6% (24 positive samples from 360) over the entire sampling period; 5.8% (11/189) in 2011 and 7.6% (13/171) in 2012. In tomato, the overall proportion of virus detection was 11.7% (34/291), with an evident increase in 2012 [23% (28/121)] compared to 2011 [3.5% (6/170)] (Table 2 and Table S1).

Identification of the begomovirus species

In order to determine the identity of the putative begomoviruses, the viral DNA was amplified using RCA from each positive sample. Based on restriction fragment length polymorphism (RCA-RFLP) analysis conducted using *Hpa*II, eight different patterns were detected (data not shown). Full-length genome components from at least one sample exhibiting each RCA-RFLP pattern were cloned and full-length sequences were obtained (see below). Four begomoviruses (PepGMV, ToLCSiV, TYLCV and ToYMoV) were identified in these samples. Hybridization tests were then performed to evaluate the prevalence of these viruses in the collected samples. A specific probe was designed for each begomovirus based on the obtained clones and all 651 samples were analysed to determine the proportion of samples infected by each virus (Table 2). Although the viruses PepGMV and ToYMoV share 80% genome wide nucleotide identity, the probes obtained were highly specific, and no cross-reaction was observed.

The begomoviruses were highly host-specific, with different virus species detected in tomato or pepper and a wider diversity observed in tomato (ToLCSiV, TYLCV and ToYMoV) than in sweet pepper (PepGMV) (Table 2). Mixed infection of begomoviruses was observed in only one case, in contrast to results obtained from surveys conducted in other countries (Davino *et al.,* 2009; Urbino *et al.,* 2013). Thus, only one single mixed infection was detected, with co-infection of ToLCSiV and ToYMoV in a tomato sample from the Grecia region (Table S1). The double infection was detected by species-specific hybridization, however, by RCA-RFLP the pattern of DNA profile was similar to a single infection of ToYMoV (data not shown).

There was a differential distribution of begomoviruses depending on the geographical region (Fig. S1). In Cartago, only PepGMV was detected in the 18.2% of sweet pepper plants that tested positive for begomovirus infection. In Zarcero, 25.8% of the tomato sampled plants were infected by a begomovirus, and all of them with ToYMoV. In contrast, in Grecia, although begomovirus infection was detected in only 8.9% of the tomato samples, three begomoviruses were identified in the area = ToLCSiV in 54.5% of the positive samples, TYLCV in 36.4% and ToYMoV in the remaining 9.1%. The first two viruses were only detected in this region. It should be noted that TYLCV was first detected in Costa Rica during this survey (Barboza *et al.,* 2014).

Based on this 2-year survey on begomoviruses, an attempt to correlate the virus distribution and the type

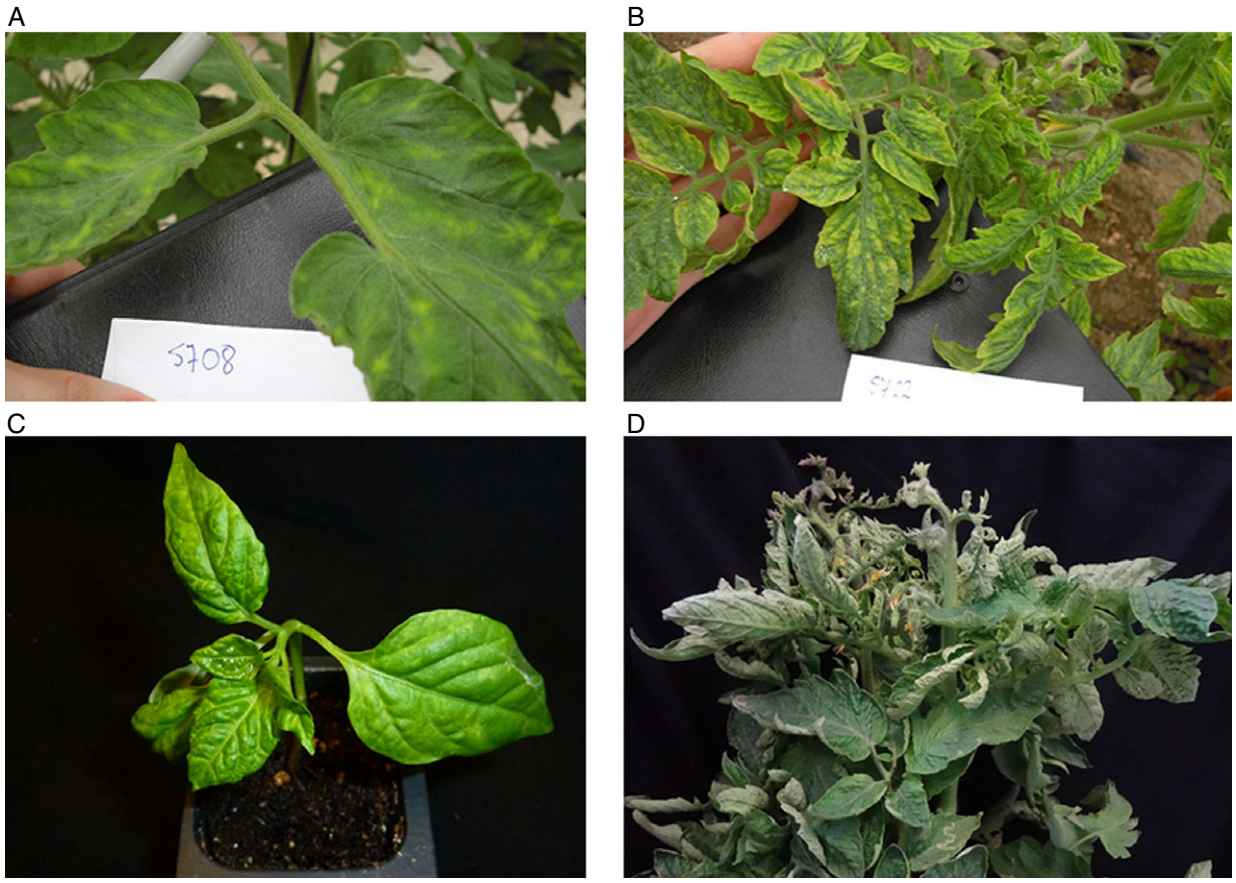

Figure 1 Symptoms observed in tomato and sweet pepper plants sampled in field and greenhouses. Tomato plant infected by *Tomato yellow mottle virus* (ToYMoV) with interveinal chlorosis, rugosity and mild leaf curling (A), tomato plant infected by *Tomato leaf curl Sinaloa virus* (ToLCSiV) with strong interveinal chlorosis, stunting and leaf curling (B). Sweet pepper plant (C) exhibiting veinal clearing and rugosity after infection with *Pepper golden mosaic virus* (PepGMV). Tomato plant with leaf curling, distortion and stunting associated with infection by *Tomato yellow leaf curl virus* (TYLCV) (D).

of production was performed. Contingency table analysis indicated a significant difference ($P < 0.0001$) based on production type (greenhouse versus open field) for ToYMoV (77.4% in greenhouses and 3.7% in open fields; Fisher's P-value < 0.0001). Significant differences were found for the other begomoviruses = PepGMV (12.9% in greenhouses, 74% in open fields, Fisher's P-value = 0.002); ToLCSiV (0% detection in greenhouses and 22.2% in open fields, Fisher's P-value = 0.025). The exception was TYLCV (9.6% in greenhouses and 3.7% in

Table 2 Proportion of samples infected with begomoviruses in tomato and sweet pepper plants in Costa Rica. Infection with isolates of the begomovirus species detected in this study [*Pepper golden mosaic virus* (PepGMV), *Tomato leaf curl Sinaloa virus* (ToLCSiV), *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow mottle virus* (ToYMoV)] in commercial tomato and sweet pepper samples, considering the total number of samples analysed in the survey conducted during 2011 and 2012 and the region from which they were collected (Cartago, Grecia and Zarcero)

Region/Crop	Tomato					Sweet Pepper				
Probe	PepGMV	ToLCSiV	TYLCV	ToYMoV	Total	PepGMV	ToLCSiV	TYLCV	ToYMoV	Total
Cartago	0 (0/75)[a]	0 (0/75)	0 (0/75)	0 (0/75)	0 (0/75)	18.2 (24/132)	0 (0/132)	0 (0/132)	0 (0/132)	18.2 (24/132)
Grecia	0 (0/123)	4.8 (6/123)	3.2 (4/123)	0.8 (1/123)	8.9 (11/123)	0 (0/134)	0 (0/134)	0 (0/134)	0 (0/134)	0 (0/134)
Zarcero	0 (0/93)	0 (0/93)	0 (0/93)	25.8 (24/93)	25.8 (24/93)	0 (0/94)	0 (0/94)	0 (0/94)	0 (0/94)	0 (0/94)
Total	0 (0/291)	2 (6/291)	1.3 (4/291)	8.5 (25/ 291)	(35/291)	6.6 (24/360)	0 (0/360)	0 (0/360)	0 (0/360)	6.6 (24/360)

[a]Proportion of samples infected with begomoviruses (number of positive samples/number of analysed samples in hybridization using begomovirus-specific probes).

Table 3 Proportion of begomoviruses according to the region of Costa Rica. *Pepper golden mosaic virus* (PepGMV), *Tomato leaf curl Sinaloa virus* (ToLCSiV), *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow mottle virus* (ToYMoV) proportion among the begomovirus-infected tomato and pepper samples in Cartago, Grecia and Zarcero during the survey conducted in Costa Rica in 2011 and 2012 based on specific hybridization

Begomovirus	Cartago	Grecia	Zarcero	*P*-Value
PepGMV	100 (24/24)	0 (0/10)	0 (0/24)	4.464e−15
ToLCSiV	0 (0/24)	60 (6/10)	0 (0/24)	0.004
TYLCV	0 (0/24)	40 (4/10)	0 (0/24)	0.0072
ToYMoV	0 (0/24)	10 (1/10)	100 (24/24)	0.089

open fields, Fisher value = 0.6), in which no significant differences were obtained. Although the low number of positive samples for each field limited the robustness of the analysis, the results suggest that the cultivation type might influence the diversity of the begomoviruses.

Distribution of begomovirus according to region was also analysed, and based on the contingency table analysis significant differences ($P < 0.0001$) were observed between the regions (Table 3). The proportion of different begomoviruses in virus-positive samples ranged from 0 to 100%. For example, PepGMV was detected in 100% of the samples in Cartago, whereas it was not detected in Grecia and Zarcero. Presence of ToLCSiV and TYLCV was only detected in Grecia, with proportions of 60% and 40%, respectively. ToYMoV infections were detected more frequently in the northern part of the Central Valley, in a proportion of 10% in Grecia and 100% in Zarcero, whereas it was not detected in the Cartago region.

Genetic diversity of begomoviruses

Based on RCA-RFLP analysis, different RFLP patterns were identified among the samples that tested positive for begomoviruses. At least one sample from each pattern was used to clone the genome of the begomovirus present and 1–2 clones were obtained from each viral DNA to determine their full-length sequence. Four begomoviruses were identified = PepGMV, which exhibited five close but distinct RCA-RFLP patterns, and ToLCSiV, TYLCV and ToYMoV, each with one single pattern. One sample was used to characterise ToLCSiV, five for PepGMV, and two each for TYLCV and ToYMoV with a total 20 DNA components characterised, 13 of DNA-A and 7 of DNA-B (Table S1).

For PepGMV, four full-length DNA-A components [sample #4961 (accession no. KY064011, KY064012), #5867 (accession no. KY064017, KY064018)], and four DNA-B [#4643 (accession no. KY064023), #4644 (accession no. KY064022), #5867 (accession no. KY064019), #5860 (accession no. KY064024)] clones were sequenced. For ToLCSiV, two full-length DNA-A (#4371, accession no. KY064013, KY064014) and two DNA-B (#4371, accession no. KY064020, KY064025) components were sequenced; for TYLCV, four full-length genome components were cloned and sequenced [#5240 (accession no. KF533855, KF533857, KF533856) and #5241 (accession no. KY064016)]. Finally, for ToYMoV, three full-length DNA-A [#5245 (accession no. KY064009) and #5249 (accession no. KY064010, KY064015)] and one DNA-B (#5249, accession no. KY064021) components were obtained (Table S1).

The four characterised DNA-A sequences of PepGMV shared 99% nucleotide sequence identity each other, and 98% identity with PepGMV-[CR] (accession no. AF149227) (Fig. S2). *In silico* analysis of the *Hpa*II restriction pattern of these sequences suggested that point mutations in the restriction endonuclease specific region in a fraction of the viral population and differences in the concentration of DNA-A and DNA-B molecules would be the cause of the observed five restriction profiles.

The three characterised DNA-A sequences of ToYMoV share among them 99 to 100% identity and 98% nucleotide identity with the ToYMoV reference sequence (accession no. KC176780). Sequences representing clones of ToLCSiV (accession no. KY064013 and KY064014) DNA-As share 99% identity with each other and 99% and 98% with the ToLCSiV reference sequence (accession no. AJ608286), respectively.

The four characterised TYLCV genomes share 99% nucleotide sequence identity each other, and the same identity with the reference sequence *Tomato yellow leaf curl virus* SH3 (accession no. FN256258). The Costa Rican isolate of TYLCV exhibited the closest relationship (99% nucleotide identity) with isolates of the Israeli strain (TYLCV-IL) from Japan and China [isolates Japan:Haruno:2005 (accession no. AB192966) and CN:Sh3:08 (accession no. FN256258), respectively]. Remarkably, a lower identity was found with TYLCV isolates of surrounding countries. Thus, the TYLCVs from Guatemala (accession no. GU355941), Mexico (accession no. EF210554) or Cuba (accession no. KM926626) showed a 97–98% nucleotide identity to the isolates from Costa Rica, which could suggest a separate TYLCV introduction in Costa Rica.

The four PepGMV DNA-B sequences obtained share approximately 98% nucleotide identity among each other, whereas when compared to isolate PepGMV-Ser (accession no. AY928517) they share 93 to 94% nucleotide identity. Sequence of DNA-B ToYMoV [CR] 5245 (accession no. KY064021) shares 97% nucleotide identity with ToYMoV DNA-B, accession no. KC176781. The two sequences of ToLCSiV DNA-B exhibit 99% identity between the Costa Rican isolates, while they share

97% identity with the reference Nicaraguan ToLCSiV sequence (accession no. AJ508783). This result suggests that the PepGMV, ToYMoV, ToLCSiV and TYLCV species found in Costa Rica have homogeneous populations of closely related isolates. It became apparent that the Costa Rican begomoviruses characterised here are highly similar, but slightly divergent to those reported in neighbouring countries.

The phylogenetic analysis of the DNA-A sequences resulted into a clear clustering of the sequences reported here with those found in the Americas (Fig. 2). The DNA-A sequences of PepGMV from Costa Rica grouped with the sequence reported by Lotrakul *et al.* (2000) in surveys carried out in tabasco pepper during 1997 in the southwest region of Costa Rica (accession no. AF149227) and with that from tabasco pepper collected in Sinaloa, Mexico, in 1989 (accession no. AY928516) (Brown *et al.*, 2005). They were also closely related to the Mexican PepGMV-D (accession no. AY928514) and PepGMV isolate Guanajuato (accession no. GU128148), but clustered in a distinct group from the remaining Mexican isolates (Fig. 2A). The DNA-B sequences grouped similarly to the DNA-A sequences (Fig. 2B).

ToYMoV DNA-A and DNA-B sequences grouped closely together with those from the ToYMoV isolated from the Central Valley of Costa Rica (accession no. KC176780). Similarly, the DNA-A and DNA-B sequences for ToLCSiV grouped closely with those from Nicaragua. Finally, the TYLCV sequences from Costa Rica grouped with those of isolates of the IL strain, closely related to those isolates reported in Asia and Mexico, and less related to that of other Central American isolates.

Models of begomovirus infection

In order to try to understand the begomovirus occurrence and to help predicting the risk of infection of a tomato and sweet pepper plant in Costa Rica, 20 models were examined to quantify which factors best-explained differences in their deviance values (Table 4). The additive model of virus species as a function of location (l), growth condition (g), host (h) and collection date (d) was found to be the model that best explained the probability of virus infection, based on lowest deviance value, AIC, and biological relevance. Based on this model the probability of begomovirus infection was calculated for each region and season (from March 2011 to September 2012) (Fig. 3). The highest probability of begomovirus was observed for tomato in greenhouse production and sweet pepper in open field production, considering the three geographical regions. In the case of ToLCSiV, there was a minimal increase in risk at the beginning of the dry season in Grecia for tomato grown under open field condition. In contrast, in Zarcero the probability of ToYMoV infection in tomato greenhouse production increased over time. For PepGMV, the risk was the highest in Cartago at the beginning of 2011, compared to 2012, while in Grecia the risk of TYLCV infection in greenhouse tomato production during 2012 was low.

Discussion

The current study is the first large scale survey on begomoviruses in tomatoes and peppers grown in Costa Rica since 1994–1996, when Nakhla *et al.* (2005) carried out a survey and molecular characterisation of a number of tomato infecting begomoviruses in Central America and Costa Rica. Our study provides new information for a better understanding of diversity of begomoviruses present in the country, as well as the distribution and occurrence of different viruses. As indicated by Inoue-Nagata *et al.* (2016), a poor knowledge of the aetiology of virus diseases may result in inefficient control measures against whitefly-transmitted viruses and the implementation of inappropriate cultural practices. Thus, for example, maintenance of bridge crops where there are overlapping periods of production can result into a continuous maintenance of begomovirus inoculum (Ali-Shtayeh *et al.*, 2014; Macedo *et al.*, 2017). We showed in our study that so far there is a geographical and a host species structure regarding the begomovirus population present in Costa Rica, which should be taken into account when designing management programmes.

In order to analyse large amounts of samples, the use of molecular hybridization was useful to identify different begomoviruses. First, the use of a generic probe based on the conserved CP ORF sequence and a low stringency condition enabled the detection of all begomoviruses, including those of undescribed viruses. Then, specific probes were produced for the preliminarily detected begomoviruses based on the IR, and used at a high stringency hybridization condition. Following this procedure, dot blot hybridization was a useful technique for the analysis of hundreds of samples and was consistent with the results obtained with the RCA-RFLP analysis and genome sequencing. Therefore, the use of dot blot hybridization and specific probes can be considered for future surveys without the need of sequencing all the samples. This technique was employed to detect different begomovirus species in Central America and other regions (Nakhla *et al.*, 2005; McLaughlin *et al.*, 2008; Fortes *et al.*, 2016), and with adequate calibration controls can be used in breeding programmes to select resistant and/or tolerant genotypes (e.g. García-Cano *et al.*, 2008).

The combination of dot blot hybridization, RCA-RFLP, cloning and sequencing techniques applied in tomato

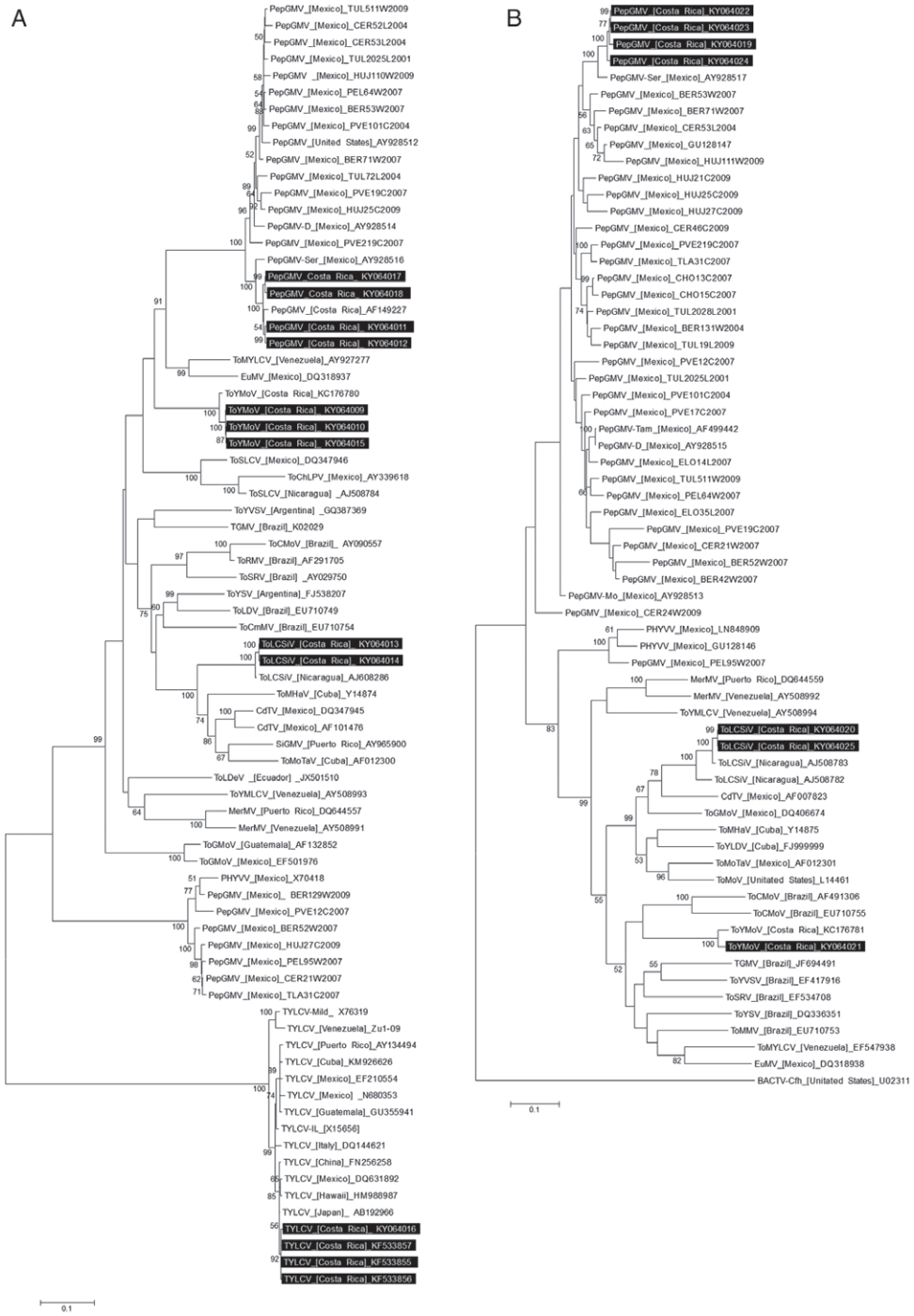

Figure 2 (A) Phylogenetic relationships of the complete DNA-A of isolates of Costa Rican begomoviruses (*Tomato yellow mottle virus* (ToYMoV), *Pepper golden mosaic virus* (PepGMV), *Tomato leaf curl Sinaloa virus* (ToLCSiV) and *Tomato yellow leaf curl virus* (TYLCV) found in tomato and sweet pepper crops with representative sequences of begomoviruses reported in the Americas, and TYLCV sequences from Asia and America. (B) Phylogenetic relationships of the full-length DNA-B of isolates of Costa Rican begomoviruses (ToYMoV, PepGMV and ToLCSiV) found in tomato and sweet pepper crops with representative sequences of isolates of begomoviruses reported in the Americas. Sequences were aligned (using Muscle) and the Maximum likelihood tree was constructed with 3000 bootstrap replications, and evolutionary distances computed using the Kimura 2-parameter method (K2P) and expressed as the number of base substitutions per site using MEGA 6.0. Only bootstrap values higher than 50% are shown. The bar indicates the substitutions per site and in the black box the sequences obtained from the current work in Costa Rica.

Table 4 Comparison of fitted models for predicting the risk of begomovirus occurrence in tomato and sweet pepper in Costa Rica

	Model	Deviance	AIC
1	Multinomial	530.9	538.9
2	vs ~ d	470.7	495.7
3	vs ~ g	470.7	486.7
4	vs ~ d + g	418.8	442.8
5	vs ~ d + g + d * g	417.8	449.8
6	vs ~ h	453.9	469.9
7	vs ~ l	402.0	426.0
8	vs ~ d + l	344.6	376.5
9	vs ~ d + l + s * d	344.3	392.3
10	vs ~ l + g	365.7	397.7
11	vs ~ l + g + l *	365.7	405.7
12	vs ~ l + g + l	310.7	350.7
13	vs ~ l + g	344.5	376.5
14	vs ~ l + d	344.3	392.3
15	vs ~ l + h	401.5	425.5
16	vs ~ g + h	373.0	397.0
17	vs ~ g + l	365.7	397.7
18	vs ~ g + l + g * l	365.7	405.7
19	vs ~ h + g * h	367.2	399.2
20	vs ~ l + g + h + d	207.4	255.4

[a]Virus species (vs), collection date (d), growth condition (g), season (s), host (h), and location (l).

and pepper samples revealed a complex begomovirus species diversity in Costa Rica similar to that observed in countries of the region (McLaughlin *et al.,* 2008; Geraud-Pouey *et al.,* 2016; Herrera-Vásquez *et al.,* 2016). Presence of isolates of the bipartite begomovirus ToLCSiV and ToYMoV and the monopartite begomovirus TYLCV was detected infecting tomato in Costa Rica. In sweet pepper, only PepGMV was found. Although no tomato infected with PepGMV was detected in this survey, infections of this virus have been reported in tomato under natural conditions (Ala-Poikela *et al.,* 2005), or after artificial inoculation via particle bombardment (Brown *et al.,* 2005). Therefore, additional work is needed to understand the ability of PepGMV isolates present in Costa Rica to infect tomato plants.

Our results indicate that in general tomato was more severely affected by begomovirus infections than sweet pepper. The number of positive samples in sweet pepper increased by only 1.8% throughout the sampling period, whereas in tomato the proportion increased by 19.5%. The growing season and type of production appeared to be related with virus distribution, in the case of ToYMoV and PepGMV. For example, for ToYMoV, relatively high proportion was detected in tomato greenhouse production in Zarcero during 2012, which is an important information considering that the last visual report of this virus in Costa Rica was from 2008 (Hilje & Stansly, 2008). Therefore, we recommend the development of a continuous monitoring and testing programme in order to understand the evolution of ToYMoV occurrence, as this information is important for developing effective management strategies to reduce its impact in the Central Valley.

For PepGMV, a high proportion of infected plants was detected in sweet pepper production in the Cartago region, especially under open field conditions during both years of the study. In this latter case, our results were similar to those obtained in Nicaragua by Ala-Poikela *et al.* (2005), where they observed that PepGMV was widespread in pepper, chilli pepper and cashew plants. In a previous work from Costa Rica, Lotrakul *et al.,* (2000) also found Tabasco and Habanero plants infected with this virus (PepGMV was found to be synonymous to the former Texas pepper virus and Serrano golden mosaic virus). PepGMV has also been reported in other countries of the New World, such as south western of United States, Mexico, Guatemala, Honduras and Belize, infecting sweet pepper (Torres-Pacheco *et al.,* 1996; Lotrakul *et al.,* 2000; Nakhla *et al.,* 2005; McLaughlin *et al.,* 2008). Taken together, these data suggest that PepGMV has a regional importance, particularly in Cartago region of Costa Rica. The impact of PepGMV in sweet pepper production should be evaluated and a breeding programme targeting the development of cultivars that are resistant to this virus is needed in Costa Rica.

On tomatoes, the low proportion observed for both ToLCSiV and TYLCV should not be ignored, especially because ToLCSiV has been reported infecting tomato in Costa Rica in samples collected in 1994 and 1996, and in other neighbouring countries (Brown *et al.,* 1993; Nakhla *et al.,* 2005; Herrera-Vásquez *et al.,* 2015).

The low frequency of mixed infections observed in this study might be explained by the geographical and host structure found in the begomovirus population, and also by the general low proportion of begomoviruses in the field. Geographical barriers such as differences in elevation among sampled regions can tentatively explain the present distribution of the begomoviruses in Costa Rica. Our results are in agreement with those of Khan *et al.,* (2013), who found that mountains can be physical barriers to the movement of insects and affect the occurrence of vector-transmitted viruses. However, the global warming and the increasing spread of whiteflies in the country (Hilje & Morales, 2008; Guevara-Coto *et al.,* 2011) may result in a consistent expansion in begomovirus incidence and diversity, and thus in the occurrence of mixed infections.

The genome analysis of the begomoviruses found in Costa Rica indicated that they have a close genetic relationship (93–100% nucleotide sequence identity) with isolates of begomoviruses already reported from Costa

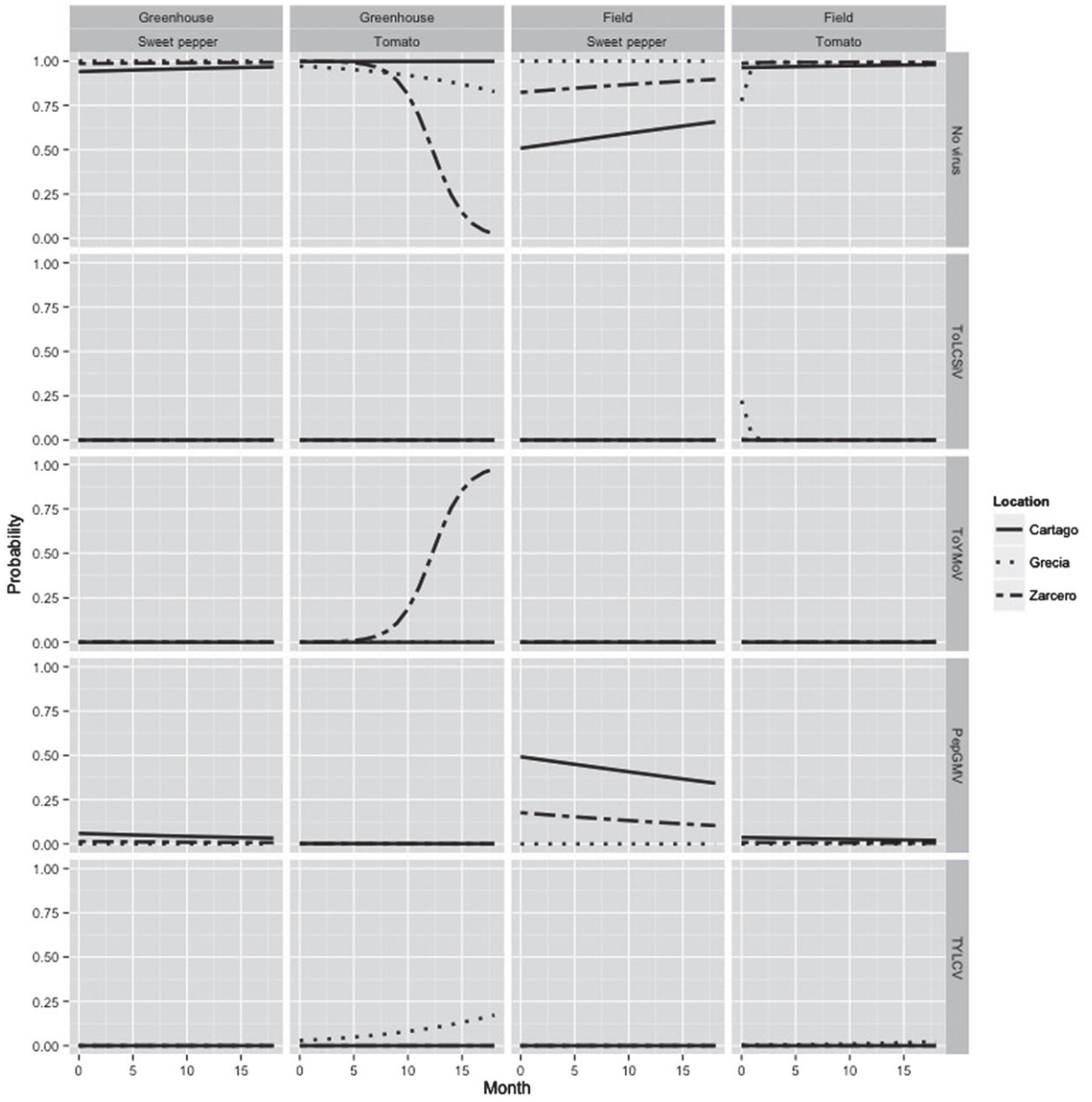

Figure 3 Probability of begomovirus infection in three different geographic regions (Cartago, Grecia and Zarcero). Values near one in the "y" axis show a high probability and values near zero indicate a low probability. Probability values were calculated for: no virus infection, *Tomato leaf curl Sinaloa virus* (ToLCSiV), *Pepper golden mosaic virus* (PepGMV), *Tomato yellow mottle virus* (ToYMoV), and *Tomato yellow leaf curl virus* (TYLCV) over a period of 18 months, beginning in March 2011 until September 2012.

Rica or nearby countries (Nicaragua, Mexico, Guatemala, USA). Based on sequence comparison, and according to ICTV guidelines for begomovirus classification (Brown *et al.,* 2015), the isolates detected in the current study grouped with already described begomovirus species (ToLCSiV, TYLCV, ToYMoV and PepGMV). It is interesting to note that pairwise comparison with begomovirus sequences available in GenBank supported that the ToYMoV isolates from Costa Rica are closely related to the ToYMoV reference isolate (accession no. KC176780 and KC176781) and all belonged to a separate begomovirus species as already mentioned by Morales *et al.* (2001). DNA-A sequences of ToYMoV isolates share a maximum nucleotide identity of 84% with that of *Tomato mild yellow leaf curl Aragua virus* [Venezuela:10:2003] (accession no. AY927277) and following guidelines for

begomovirus classification (Brown *et al.,* 2015) ToYMoV is currently accepted as a separated begomovirus species. This virus has recently been reported infecting tomatoes in Panama according to partial sequences deposited in GenBank (accession no. KP318654 and KP318653) (Herrera-Vásquez *et al.,* 2016) indicating that it may be widespread in Central America.

Tomato yellow leaf curl disease associated with TYLCV infections is one of the most devastating diseases of tomato in tropical and subtropical regions of the world (Mabvakure *et al.,* 2016) and as shown here, it was detected causing infections in Costa Rica. Tomato cultivars that are resistant to TYLCV are available commercially (Vidavski *et al.,* 2008; Pereira-Carvalho *et al.,* 2015), so that, they can be evaluated against TYLCV and other begomoviruses found infecting tomato under Costa Rican growth conditions.

In the present survey, three begomoviruses were found infecting tomatoes. Based on this, it is recommended that begomovirus-resistant/tolerant commercial cultivars are tested for resistance against these viruses. This is especially important for the Central Valley, which is the major vegetable production region of the country. Production of infectious clones will assist tomato breeding programmes for resistance to begomoviruses, and is the next objective of our group. In pepper, as already reported in Costa Rica (Lotrakul *et al.,* 2000; Castro *et al.,* 2013), only isolates of the begomovirus PepGMV were detected. Studies on the host range of the Costa Rican isolate of PepGMV should be conducted.

It was shown that the risk for begomovirus infection as a function of geographical region, crop and growth condition was higher in greenhouse production systems compared with open field for tomatoes, whereas in sweet pepper the risk was higher under field conditions. Therefore, it is recommended that control efforts are focused on these two possibilities.

It is important to comment that to understand the begomovirus occurrence and to help predicting the risk of infection of a tomato and sweet pepper plant in Costa Rica, 20 models were examined with the data available from the present study. However, for more accurate results, this study needs to be improved by increasing the number of infected samples considered from the different regions, host species and growth conditions, and by adding other variables such as = type of cultivars grown, presence of neighbouring crops and wild plants acting as sources, weather factors (wind, rain and altitude), the transplant preparation and original, or presence of the vector, among others.

Overall, the results of our research indicate that begomovirus infections may become important in Costa Rica and that a number of begomoviruses coexist under natural epidemics. It is important to understand the diseases that these begomoviruses cause according to the virus species, growth conditions, season, crop host, location and host ecology, as this will help to define long-term biological research priorities to enable an effective management of these diseases. Also, it is important to perform periodic surveys and analyses of infections as this will allow the detection of shifts in virus distribution, or the emergence or introduction of new begomoviruses.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (A) Distribution and number of samples infected with isolates of begomovirus species detected in this study according to the crop (tomato and sweet pepper). (B) In yellow *Pepper golden mosaic virus* (PepGMV) distribution, in blue *Tomato yellow leaf curl virus* (TYLCV), the black star is the distribution of *Tomato leaf curl Sinaloa virus* (ToLCSiV), and in purple *Tomato yellow mottle virus* (ToYMoV), the black points indicate sites from which samples were collected during 2011 and 2012.

Fig. S2. Two-dimensional pairwise identity colour matrix with pairwise nucleotide sequence identities calculated using SDT v1.2. (A) Comparison of DNA-A (for bipartite begomovirus) or genome (for *Tomato yellow leaf curl virus*) full-length sequences obtained in this study with the closest related sequences. (B) Comparison of DNA-B full-length sequences obtained in this study with that of related bipartite begomoviruses *Tomato leaf curl Sinaloa virus* (ToLCSiV), *Pepper golden mosaic virus* (PepGMV), *Tomato yellow mottle virus* (ToYMoV) and *Tomato yellow leaf curl virus* (TYLCV).

Table S1. Tomato and sweet pepper samples collected and analysed in this study. Samples for which DNA-A (A) and/or DNA-B (B) were characterised are indicated and GenBank accession numbers of the sequences deposited provided. Symptoms for positive samples also are provided