LENIN CELIANO PAZ CARRASCO

SPECIES DIVERSITY, GENETIC STRUCTURE AND VARIABILITY OF BEGOMOVIRUS POPULATIONS INFECTING TOMATOES IN ECUADOR

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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Olinto Liparini Pereira	Luiz Cláudio Vieira da Cunha
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(Orientador)

"Não há transição que não implique um ponto de partida, um processo e um ponto de chegada. Todo amanhã se cria num ontem, através de um hoje. De modo que o nosso futuro baseia-se no passado e se corporifica no presente. Temos de saber o que fomos e o que somos, para sabermos o que seremos."

(Paulo Freire)

I dedicate this dissertation to my wife Mayra del Rocio and my children Maylen and Lenin by the goals we set out to reach everyone with our ideals and the struggle of everyday life in the moments when I was in my academic studies and came to understand. No doubt the love that we profess was the architect for our success.

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To the Administrative staff of the Department of Plant Pathology.

To my country, Ecuador.

SUMÁRIO

RESUMO

PAZ CARRASCO, Lenin Celiano, D.Sc., Universidade Federal de Viçosa, junho de 2013. **Diversidade de espécies, estrutura e variabilidade genética de populações de begomovírus infectando tomateiro no Equador.** Orientador: Francisco Murilo Zerbini Júnior. Coorientadora: Claudine Márcia Carvalho.

O gênero *Begomovirus* (família *Geminiviridae*) inclui vírus com genoma de DNA de fita simples, que infectam espécies de plantas dicotiledôneas e são transmitidas pela mosca-branca *Bemisia tabaci*. Doenças causadas por begomovírus são economicamente importantes por causarem grandes perdas em culturas relevantes em regiões tropicais e subtropicais. No Equador, apesar de relatos de infestação por *B. tabaci* desde a década de 1990, apenas recentemente ocorreu o primeiro relato de um begomovírus, *Tomato leaf deformation virus* (ToLDeV, também presente no Peru) em tomateiro. O ToLDeV é o primeiro begomovírus monossegmentado originário das Américas, e sua presença no Equador ressalta a necessidade de um levantamento da ocorrência de begomovírus em tomateiro nesse país. O estudo de populações do ToLDeV pode fornecer informações relevantes sobre a evolução desse begomovírus monossegmentado e sobre o processo de adaptação desse vírus a um novo hospedeiro. Amostras de tomateiro e de plantas daninhas foram coletadas em 2010 e 2011 em seis províncias do Equador, e genomas completos de begomovírus foram clonados e sequenciados utilizando-se uma estratégia baseada em amplificação por círculo rolante. Nenhuma amostra positiva para begomovírus foi detectada nas províncias de Chimborazo e Galápagos. A absoluta maioria das amostras positivas coletadas nas províncias de Guayas, Loja, Manabíe Santa Elena estavam infectadas com o ToLDeV. Uma amostra de Manabí apresentava infecção tripla com ToLDeV, *Rhynchosia golden mosaic Yucatan virus* (RhGMYuV) e um isolado recombinante entre os dois. Um novo begomovírus foi detectado em uma outra amostra de

tomateiro de Manabí. Amostras de *Rhynchosia* sp. das províncias de Guayas e Manabí estavam infectadas pelo RhGMYuV. Sequências de 67 isolados de ToLDeV obtidos de tomateiro foram utilizadas para identificação de haplótipos e para inferir relacionamento com isolados do Peru. Duas subpopulações claramente estruturadas foram identificadas (Equador e Peru) com evidência de migração entre elas. A menor diversidade haplotípica foi encontrada para o gene C4, em ambas as subpopulações. Os índices de variabilidade genética da subpopulação do Peru foram muito maiores do que os da subpopulação do Equador. O gene C4 apresentou a maior frequência de mutação em ambas as subpopulações. Encontrou-se evidência de seleção negativa ou purificadora atuando sobre todos os genes virais. Testes de neutralidade foram significativos e negativos para os genes CP, Rep, TrAP e C4 da subpopulação do Equador, indicando uma expansão recente. Todos os isolados do Equador apresentam um evento de recombinação incluindo os genes Rep, TrAP, REn e a região intergênica. Em conjunto, os resultados indicam um efeito fundador atuando na subpopulação do Equador, que está sofrendo um rápido processo de expansão e diferenciação.

ABSTRACT

PAZ CARRASCO, Lenin Celiano, D.Sc., Universidade Federal de Viçosa, June, 2013. **Species diversity, genetic structure and variability of begomovirus populations infecting tomatoes in Ecuador.** Adviser: Francisco Murilo Zerbini Júnior. Co-adviser: Claudine Márcia Carvalho.

The genus *Begomovirus* (family *Geminiviridae*) contains viral species with ssDNA genomes, affecting dicotyledonous plants and transmitted by the whitefly *Bemisia tabaci*. Viral diseases caused by begomoviruses are of economic importance due to their adverse effects on the production of tropical and subtropical crops. In Ecuador, despite reports of significant infestations of *Bemisia tabaci* in the late 1990's, only very recently has a begomovirus, *Tomato leaf deformation virus* (ToLDeV, also present in Peru), been reported in tomato. ToLDeV is the first monopartite begomovirus originated in the Americas, and its presence in Ecuador highlights the need for a wider survey of tomato-infecting begomoviruses in this country. The study of ToLDeV populations may provide insights into the evolution of this monopartite virus and its ongoing adaptation to a new host. Tomato and weed samples were collected in 2010 and 2011 in six provinces of Ecuador, and begomovirus genomes were cloned and sequenced using a rolling-circle amplification-based approach. No begomovirus-positive samples were detected in the provinces of Chimborazo and Galapagos. Most begomovirus-positive tomato samples from the provinces of Guayas, Loja, Manabi and Santa Elena were infected with ToLDeV. One sample from Manabí had a triple infection with ToLDeV, *Rhynchosia golden mosaic Yucatan virus* (RhGMYuV) and a recombinant isolate between the two. A new begomovirus species was detected in another tomato sample from Manabí. Samples of *Rhynchosia* sp. from the provinces of Guayas and Manabi were infected by RhGMYuV. Sequences of 67 ToLDeV isolates from tomato were used to identify haplotypes and infer

genetic relationships with Peruvian isolates. Two ToLDeV subpopulations (Ecuador and Peru) were well structured and presented minor allelic migration. The lowest haplotype diversity was displayed by the C4 gene for both subpopulations. Genetic variability indices were generally much higher for the Peruvian subpopulation in comparison with the Ecuadorian subpopulation. The C4 gene had the highest mutation frequency in both subpopulations. There was evidence of negative or purifying selection acting on each of the viral genes, thus preserving protein function. Tests of neutrality were significant and negative for the CP, Rep, TrAP and C4 genes of the subpopulation from Ecuador, indicating that it is undergoing expansion. All isolates from Ecuador and one isolate from Peru showed a recombination event encompassing the Rep, TrAP and REn genes and the intergenic region. Together, the results indicate a founder effect acting upon the Ecuadorian ToLDeV subpopulation, which is undergoing rapid expansion and differentiation.

GENERAL INTRODUCTION

Geminiviruses are circular single stranded DNA (ssDNA) plant viruses encapsidated in twinned icosahedral capsids (Zhang *et al.*, 2001). These pathogens infect a wide range of cultivated and non-cultivated hosts and are responsible for important worldwide epidemics in plants (Rojas *et al.*, 2005b). In Africa, cassava mosaic and maize streak diseases are the major biotic constraints to cassava and corn production, respectively (Bosque-Perez, 2000; Legg & Thresh, 2000). A complex of begomoviruses (whitefly-transmitted geminiviruses) causing the tomato yellow leaf curl disease is responsible for heavy losses in tomato cultivation in the Western Mediterranean basin (Picó *et al.*, 1996; Rojas *et al.*, 2005b). In the Americas, diseases caused by begomoviruses have significantly impacted tomato and bean production since the 1980's (Brown & Bird, 1992; Gilbertson *et al.*, 1993a; Blair *et al.*, 1995; Polston & Anderson, 1997; Morales & Jones, 2004).

The family *Geminiviridae* is divided into seven genera: *Becurtovirus*, *Begomovirus*, *Curtovirus*,*Eragrovirus*, *Mastrevirus*, *Topocuvirus* and *Turncurtovirus* based on host range, type of insect vector, genome organization and phylogenetic relationships (Brown *et al.*, 2012; Hernández-Zepeda *et al.*, 2013). The genus *Begomovirus* is the most economically important and harbors the largest number of species in the family (Fauquet *et al.*, 2005). Begomoviruses from the "Old World" (Europe, Asia and Africa) have one or two genomic components (known as mono- and bipartite begomoviruses, respectively), and are often associated with circular ssDNA molecules designated alphasatellites (previously DNA-1) and betasatellites (previously DNA β) (Briddon *et al.*, 2003; Briddon & Stanley, 2006). Alphasatellite genomes are similar to the genomic component named DNA-R of the ssDNA nanoviruses, and contain an open reading frame (ORF) encoding a replication-associated protein (Rep), followed by an adenine rich sequence and a stem-loop

structure which includes the origin of replication (Briddon & Stanley, 2006). Alphasatellites can replicate autonomously, but require the helper begomovirus for systemic infection and insect transmission (Saunders & Stanley, 1999; Saunders *et al.*, 2000; Saunders *et al.*, 2002). Alphasatellites have been recently identified in Brazil and Venezuela in association with the bipartite begomoviruses *Cleome leaf crumple virus* (ClLCrV), *Euphorbia mosaic virus* (EuMV) and *Melon chlorotic mosaic virus* (MeCMV) (Paprotka *et al.*, 2010b; Romay *et al.*, 2010). Betasatellites are entirely dependent on their helper viruses for replication, movement and insect transmission. Betasatellite genomes contain an ORF, named βC1, which encodes a protein involved in the induction of symptoms and suppression of post-transcriptional gene silencing (Cui *et al.*, 2004; Cui *et al.*, 2005; Briddon & Stanley, 2006).

Begomoviruses from the "New World" (the Americas) have two genomic components known as DNA-A and DNA-B, each 2,500-2,600 nucleotides (nt) in size (Fauquet *et al.*, 2005). These components do not share significant sequence identity, except for a ~200 nt sequence known as the common region (CR) which includes the origin of replication (Hanley-Bowdoin *et al.*, 1999). The DNA-A component of bipartite begomoviruses encodes five proteins: the replication-associated protein (Rep), which is the initiator of the rolling-circle replication mechanism and has nucleic acid binding, endonuclease and ATPase activities (Fontes *et al.*, 1992; Orozco *et al.*, 1997); the trans-activating protein (Trap), a transcriptional factor of the CP and NS genes and also a suppressor of post-transcriptional gene silencing (Sunter & Bisaro, 1992; Voinnet *et al.*, 1999; Wang *et al.*, 2005); the replication-enhancer protein (Ren), a viral factor required for optimal replication (Sunter *et al.*, 1990; Pedersen & Hanley-Bowdoin, 1994); the coat protein (CP), responsible for encapsidation of the ssDNA genome and also essential for insect transmission (Briddon *et al.*, 1990; Hofer *et al.*, 1997); and the C4 protein. Two

proteins are encoded by the DNA-B: the nuclear shuttle protein (NSP) and the movement protein (MP), which are involved in intra- and intercellular viral movement, respectively (Noueiry *et al.*, 1994).

After the viral particles are inoculated into a host cell by the insect vector, the viral ssDNA spontaneously dissociates from the capsid (Lazarowitz, 1992). Then, the ssDNA genome is transported to the nucleus and converted by host enzymes into a double-stranded DNA (dsDNA) intermediate, termed replicative form (RF). The viral genome is replicated using the RF as a template in a rolling-circle mechanism, similar to the replication of the ssDNA bacteriophages φX174 and M13 (Stenger *et al.*, 1991; Stanley, 1995).

The origin of replication (*ori*) is located in the common region in both genomic components. The sequence of the *ori* is conserved among components of the same virus, but variable among species, except for a ~30 nt sequence conserved among all species (Davies *et al.*, 1987; Lazarowitz, 1992). This sequence comprises an inverted GC-rich repeat which forms a hairpin structure carrying a nonanucleotide sequence [5'-TAAT(A,G)TT//AC-3'] found in all geminiviruses (Heyraud-Nitschke *et al.*, 1995; Orozco & Hanley-Bowdoin, 1998). Cleavage of the nonanucleotide is essential to the initiation of replication and is performed by the Rep protein (Fontes *et al.*, 1994; Laufs *et al.*, 1995; Orozco *et al.*, 1998). The sequence-specific binding of the Rep protein to the viral genome involves the recognition of two short direct repeat sequences and at least one inverted repeat known as iterons (Arguello-Astorga *et al.*, 1994). Following Rep binding, the dsDNA:Rep complex is stabilized by the Ren protein and host factors. Then, the Rep protein cleaves the nonanucleotide located in the hairpin, initiating rolling-circle replication (Gutierrez, 1999). The Rep catalytic domain (located at the N-terminal portion of the protein) includes three conserved motifs in proteins involved in rolling-

circlereplication (motif I: FLTY, motif II: HxH and motif III: YxxxV) (Ilyina & Koonin, 1992) and the iteron-related domain (Arguello-Astorga & Ruiz-Medrano, 2001).

Movement of plant viruses within the host can be divided into two processes: cell-to-cell movement via plasmodesmata, and long distance movement in which the virus moves systemically through the plant's vascular system. Begomoviruses replicate in the cell nucleus and thus require an additional transport step from the nucleus to the cytoplasm, which is performed by NSP (Palmer & Rybicki, 1998). The MP associates with the cell membrane and alters the size exclusion limit of the plasmodesmata, mediating the transport of the viral genome (Noueiry *et al.*, 1994). The NSP and MP proteins act cooperatively to mediate intra- and intercellular viral DNA movement (Sanderfoot & Lazarowitz, 1995), allowing the virus to infect the host systemically.

Two models have been proposed to explain the intracellular movement of begomoviruses (Levy & Tzfira, 2010). In the first model, known as "couple-skating"(Kleinow *et al.*, 2008), NSP transports the viral ssDNA or dsDNA from the nucleus to the cell periphery, and MP acts in the plasmodesmata to facilitate cell-to-cell movement of the NSP-DNA complex (Sanderfoot & Lazarowitz, 1995; Frischmuth *et al.*, 2004; Frischmuth *et al.*, 2007; Kleinow *et al.*, 2008). In the second model, known as "relay-race", NSP transports the dsDNA from the nucleus to the cytoplasm. Then, the dsDNA associates with MP, and the dsDNA-MP complex moves cell-to-cell through plasmodesmata (Noueiry *et al.*, 1994; Rojas *et al.*, 1998). MP and NSP recognize the viral DNA in a size- and form-specific manner (Rojas *et al.*, 1998; Gilbertson *et al.*, 2003). The CP is required for long distance movement of monopartite, but not bipartite, begomoviruses (Rojas *et al.*, 2005a).

Begomovirus populations exhibit high molecular variability (Ariyo *et al.*, 2005; Ge *et al.*, 2007; Silva *et al.*, 2011;2012). The main evolutionary processes shaping the

molecular variability of plant virus populations are mutation and recombination (García-Arenal *et al.*, 2003; Seal *et al.*, 2006; Lima *et al.*, 2013). Viruses with divided genomes, including the bipartite begomoviruses, may also evolve by pseudorecombination (or reassortment), in which whole genomic components are exchanged amongst distinct viruses without intermolecular recombination (García-Arenal *et al.*, 2001). Experimental evidence of the ability of begomoviruses to form viable pseudorecombinants has been obtained in laboratory settings (Gilbertson *et al.*, 1993b; Sung & Coutts, 1995; Andrade *et al.*, 2006), although there are limited reports of their natural occurrence under field conditions (Paplomatas *et al.*, 1994; Pita *et al.*, 2001).

Mutation is the primary source of variability in plant virus populations (Roossinck, 1997; García-Arenal *et al.*, 2001;2003) and previous studies have shown that ssDNA viruses may evolve as quickly as RNA viruses which use an error-prone RNA-dependent RNA polymerase for replicating their genomes (Drake, 1991; Shackelton *et al.*, 2005; Shackelton & Holmes, 2006; Duffy *et al.*, 2008). Geminiviruses exhibit high levels of within-host molecular variability (Isnard *et al.*, 1998; Ge *et al.*, 2007; Van Der Walt *et al.*, 2008) and there is evidence that the rapid evolution of geminiviruses might be, at least in part, driven by mutational processes acting specifically on ssDNA (Duffy *et al.*, 2008; Harkins *et al.*, 2009).

An experiment to assess the within-host molecular variability of the begomovirus *Tomato yellow leaf curl China virus* (TYLCCNV) under controlled conditions revealed an average mutation frequency of 3.5×10^{-4} and 5.3×10^{-4} after a 60-day infection period in *Nicotiana benthamiana* and tomato (*Solanum lycopersicum* L.), respectively (Ge *et al.*, 2007). Additionally, substitution rates were estimated for temporally sampled *Tomato yellow leaf curl virus* (Duffy & Holmes, 2008) genome sequences. The substitution rates (2.88×10^{-4} subs/site/year for the full-length genome and 4.63×10^{-4} subs/site/year for the

CP) were similar to those of RNA viruses (Holland *et al.*, 1982; Domingo & Holland, 1997; Roossinck, 2003). These high substitution rates were validated for the bipartite begomovirus *East African cassava mosaic virus* (Duffy & Holmes, 2009), suggesting that they are representative of multiple begomoviruses. The d_N/d_S ratios calculated for all coding sequences analyzed in these studies were lower than 1.0, indicating purifying selection and, consequently, that the substitutions rates were not overestimated due to adaptive selection, reflecting a rapid mutational dynamics for these viruses (Duffy & Holmes, 2008; 2009).

Recombination is the process by which a DNA or RNA segment becomes incorporated into a different strand during their replication (Padidam *et al.*, 1999). Recombination is a common evolutionary process acting on geminivirus genomes (Padidam *et al.*, 1999), and seems to contribute heavily to the standing molecular variability of begomoviruses, increasing their evolutionary potential and local adaptation (Harrison & Robinson, 1999; Padidam *et al.*, 1999; Berrie *et al.*, 2001; Monci *et al.*, 2002; Lima *et al.*, 2013). The high recombination frequency in this group of viruses can be partly explained by the existence of a putative strategy of recombination-dependent replication (RDR) (Jeske *et al.*, 2001; Preiss & Jeske, 2003) in addition to the well-documented rolling-circle replication (RCR) (Saunders *et al.*, 2001). Furthermore, the frequent occurrence of mixed infections (Torres-Pacheco *et al.*, 1996; Harrison *et al.*, 1997; Sanz *et al.*, 2000; Pita *et al.*, 2001; Ribeiro *et al.*, 2003; García-Andrés *et al.*, 2006; Davino *et al.*, 2009) in which more than one virus can simultaneously replicate in the same nucleus (Morilla *et al.*, 2004), also favours the occurrence of recombination.

Recombination events have been directly implicated in the emergence of new begomovirus diseases and epidemics in cultivated hosts (Zhou *et al.*, 1997; Pita *et al.*, 2001; Monci *et al.*, 2002). In fact, the devastating epidemics of cassava mosaic disease

caused by *East African cassava mosaic virus* (EACMV) in Uganda and neighboring countries (Zhou *et al.*, 1997; Pita *et al.*, 2001), the TYLCV epidemics in the Western Mediterranean basin (Monci *et al.*, 2002; García-Andrés *et al.*, 2006; 2007), and the epidemics of *Cotton leaf curl virus* (CLCuV) in Pakistan (Zhou *et al.*, 1997; Idris & Brown, 2002) were all caused by begomovirus complexes including at least one recombinant virus.

A comparative analysis using genomic sequences of ssDNA viruses from several families revealed a conserved, non-random pattern of distribution of recombination breakpoints (Lefeuvre *et al.*, 2009). Although the mechanistic aspects of recombination in ssDNA viruses remain unknown (Padidam *et al.*, 1999), this non-random distribution of recombination breakpoints is conserved amongst mono- and bipartite viruses, with hot spots in the 5'-portion of the Rep gene and the 5'-end of the common region (Lefeuvre *et al.*, 2007a; 2007b). Evidence suggests that the recombination breakpoints tend to occur outside or on the periphery of coding sequences. In agreement, a reduced number of breakpoints have been found within genes encoding structural proteins, such as the CP gene (Lefeuvre *et al.*, 2007a). These results suggest that natural selection acting against viruses expressing recombinant proteins is an important determinant of the non-random distribution of recombination breakpoints in most ssDNA viruses (Lefeuvre *et al.*, 2009). Furthermore, it has been shown that recombination events that preserve co-evolved intra genome interactions (protein-protein and/or protein-DNA) are also favored by selection (Martin *et al.*, 2011).

In addition to the already existing set of information about full-length genome sequences of begomoviruses (available in public databases), the advent of the rolling-circle amplification technique using phagephi29 DNA polymerase (Inoue-Nagata *et al.*, 2004) has provided new possibilities, such as the characterization of novel begomoviruses

infecting a number of cultivated and non-cultivated hosts (Castillo-Urquiza *et al.*, 2008; Varsani *et al.*, 2009; Paprotka *et al.*, 2010a; Albuquerque *et al.*, 2012) and viral population analysis on a genomic scale (Haible *et al.*, 2006; Silva *et al.*, 2011; 2012; Rocha *et al.*, 2013).

Begomoviruses are widespread in the Americas, and several new species have been described in different countries since the 1980's (Brown & Bird, 1992; Polston & Anderson, 1997; Morales & Anderson, 2001; Ribeiro *et al.*, 2003). The main affected crops are beans, peppers and tomatoes. In tomatoes, an inordinately large number of begomoviruses have been described, with distinct species predominating in specific geographical regions (Nahkla *et al.*, 1994; Roye *et al.*, 1999; Rojas *et al.*, 2005a; Marquez-Martin *et al.*, 2011; 2012; Zambrano *et al.*, 2011; Rocha *et al.*, 2013).

In the 1990's in Ecuador, alarming whitefly infestations occurred in various cultivated and non-cultivated species (Valarezo *et al.*, 2004). It was determined that *Aleurotrachelus socialis*, *Bemisia tabaci*, *B.tuberculata*, *Tetraleurodes* sp., *Trialeurodes vaporariorum* and *Trialeurodes* sp. were the species that came to predominate in different agricultural locations. *B. tabaci* preferentially colonized peanuts, cotton, tomatoes, beans (both *Phaseolus lunatus* and *P. vulgaris*), *Sesamum indicum* and *Cynara viridula*. *A. socialis* was found in peppers, *T. vaporariorum* in tomatoes and beans, and *Tetraleurodes* sp. in beans and cassava (Valarezo *et al.*, 2004). Additionally, direct damage was reported due to the B biotype of *B. tabaci* on crops located on the Pacific coast of Ecuador. For example, up to 10,000 ha of soybean were destroyed in Guayas and Los Rios provinces. At the time, no reports of begomoviruses had been made in Ecuador. However, the new species *Tomato leaf deformation virus* (ToLDeV) was described in neighbouring Peru in 2011 (Marquez-Martin *et al.*, 2011)and was detected in Ecuador in tomato fields in the same year (Melgarejo *et al.*, 2013).

ToLDeV was shown to constitute the first case of a truly monopartite begomovirus native to the Americas, with a genomic organization homologous to the DNA-A component of New World bipartite begomoviruses. The virus is not transmitted by mechanical procedures, with a tissue tropism limited to the phloem. The CP and C4 proteins act as long distance movement proteins, with an extra activity for C4 as a suppressor of RNA silencing. In addition, tomato genotypes with the *Ty-1* gene are tolerant to infection by the virus (Melgarejo *et al.*, 2013; Sanchez-Campos *et al.*, 2013).

The present research was meant to assess the diversity of begomoviruses infecting tomatoes in Ecuador, as well as, the genetic variability of viral populations. The specific objectives were: (1) to carry out a country-wide survey of the diversity of begomovirus species infecting tomatoes and associated weeds in Ecuador; (2) to determine the population genetic structure and variability of *Tomato leaf deformation virus* (ToLDeV), the predominant begomovirus affecting tomato crops in Ecuador.

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CAPÍTULO 1

Begomovirus species diversity in tomato crops and weeds in Ecuador and the detection of a recombinant isolate of *Rhynchosia golden mosaic Yucatan virus* infecting tomato

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Begomovirus species diversity in tomato crops and weeds in Ecuador and the detection of a recombinant isolate of *Rhynchosia golden mosaic Yucatan virus* infecting tomato

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Abstract

Viral diseases caused by begomoviruses are of economic importance due to their adverse effects on the production of tropical and subtropical crops. In Ecuador, despite reports of significant infestations of *Bemisia tabaci* in the late 1990's, only very recently has a begomovirus, *Tomato leaf deformation virus* (ToLDeV, also present in Peru), been reported in tomato. ToLDeV is the first monopartite begomovirus originated in the Americas, and its presence in Ecuador highlights the need for a wider survey of tomato-infecting begomoviruses in this country. Tomato and weed samples were collected in 2010 and 2011 in six provinces of Ecuador, and begomovirus genomes were cloned and sequenced using a rolling-circle amplification-based approach. Most tomato samples from the provinces of Guayas, Loja, Manabi and Santa Elena were infected with *Tomato leaf deformation virus* (ToLDeV). One sample from Manabí had a triple infection with ToLDeV, *Rynchosia golden mosaic Yucatan virus* (RhGMYuV) and a recombinant isolate

between the two. A new begomovirus species was detected in another tomato sample from Manabi. Samples of *Rhynchosia* sp. from the provinces of Guayas and Manabi were infected by RhGMYuV. These results indicate the prevalence of ToLDeV in tomatoes in Ecuador, but also the presence of other viruses, albeit at a much lower frequency.

The *Geminiviridae* family is characterized by a unique particle morphology of twinned, incomplete icosahedra and a genome of circular, single stranded DNA (ssDNA). This family has seven genera: *Becurtovirus*, *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Mastrevirus*, *Topocuvirus* and *Turncurtovirus*, based on host range, type of insect vector, genome organization and phylogenetic relationships [2, 6]. Begomoviruses infect dicots, are transmitted in a persistent circulative manner by the whitefly *B. tabaci* (Hemiptera: Aleyrodidae) and have either a monopartite genome which is usually associated with satellite DNA molecules [1] or a bipartite genome (DNA-A and DNA-B) [2].

The emergence of new begomovirus species in tomato has been reported in the last twenty-five years in several Latin American countries [3, 8, 9, 13, 15-17]. In Ecuador, despite reports of significant infestations of *B. tabaci* in the late 1990's [22], only very recently has a begomovirus, *Tomato leaf deformation virus* (ToLDeV), been reported in tomato [11]. Strikingly, ToLDeV, which had previously been reported in neighbouring Peru [8], was shown to be the first monopartite begomovirus originated in the Americas [11, 20]. Its presence in Ecuador highlights the need for a wider survey of tomato-infecting begomoviruses in this country.

In the years 2010 and 2011, leaf tissue from individual tomato plants displaying symptoms of viral infection such as yellow mosaic and leaf distortion was collected in the provinces of Chimborazo (27 samples), Galapagos (65), Guayas (87), Loja (41), Manabi (96) and Santa Elena (80). Weeds present in and around the tomato fields were also

collected in Guayas (9 samples) and Manabi (27). Each sample was placed between two sheets of paper and pressed to complete dehydration. Dehydrated tissue was transferred to kraft paper envelopes (23 x 16 cm) for final storage at room temperature and kept within a thermal container.

The total DNA of each of the dried samples was extracted using a CTAB procedure [4] and used as a template for rolling-circle amplification (RCA) with the use of the phi29 phage DNA polymerase, a procedure that amplifies circular ss or dsDNA molecules such as begomovirus genomes and their replicative forms, respectively [7]. Amplified products were digested with the four-base cutter restriction enzyme *Msp*I and those yielding fragments that added up to 2.6 or 5.2 kbp were selected for further digestion with various restriction enzymes, to identify those that cleave the viral components at a single site. The enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I and *Spe*I generated the expected monomers, which were ligated into pBLUESCRIPT II KS+ (Stratagene) and transformed into *Escherichia coli* DHα5 according to standard protocols [19]. Clones from the same sample presenting different band patterns after digestion with *Hae*III were selected and completely sequenced by primer walking (Macrogen, Inc., Seoul, South Korea).

Sequences were initially analyzed using the Blast*n* algorithm for preliminary species assignment according to the recommendations of the *Geminiviridae* Study Group of the International Committee on Taxonomy of Viruses [2, 5]. Percent identities with the most closely related species were calculated with the program SDT (Species Demarcation Tool) version 1.0 [12]. Genomic sequences were aligned with the Muscle module implemented in MEGA 5.0 [21]. Phylogenetic trees were constructed using Bayesian inference performed with MrBayes v.3.0b4 [18]with the nucleotide substitution model selected by MrModeltest v. 2.2 [14] in the Akaike Information Criterion (AIC). The analyses were carried out running 30,000,000 generations and sampling at every 1,000

steps to produce a posterior tree distribution. The first 6,000,000 generations were discarded as burn-in. Trees were visualized using the FigTree program v1.3.1. Recombination detection was performed with RDP3 [10] using default parameters. To omit unreliable signals, only recombination events supported by at least four out of the seven methods implemented in RDP3 were considered.

A total of 71 clones were obtained from 46 begomovirus-positive tomato samples: 25 clones from samples collected in Guayas; 25 from Manabi; 19 from Santa Elena; and two from Loja (begomovirus infection was not detected in samples from Chimborazo and Galapagos). Pairwise nt identities among 11 isolates (one isolate from each sampling location) were >94.39% with ToLDeV isolates from Peru (Suppl. Figure S1A). Therefore, ToLDeV was the predominant begomovirus infecting tomatoes in those four provinces of Ecuador in 2010 and 2011. Phylogenetic reconstruction based on the DNA-A indicated that ToLDeV isolates from Ecuador formed a monophyletic cluster which included two Peruvian isolates (Figure 1). The remaining isolates from Peru formed two additional clusters (Figure 1).

Sample #338, collected at Rocafuerte, province of Manabi, had a mixed infection. One clone from this sample (EC:Roc338.1:11) showed >98% with ToLDeV (Suppl. Figure S1A). A second clone (EC:Roc338.3:11) displayed 91.8% nt identity with *Rhynchosia golden mosaic Yucatan virus* (RhGMYuV, accession number EU021216), and <88% nt identity with other species (Suppl. Figure S1A). A clone obtained from sample #333 collected at the same location (EC:Roc333.1:11) displayed 99% nt identity with EC:Roc338.3:11 and 91.9% nt identity with RhGMYuV (Suppl. Figure S1A). Therefore, these two clones correspond to a distinct strain of RhGMYuV. Phylogenetic reconstruction based on the DNA-A indicated that isolates EC:Roc333.1:11 and EC:Roc338.3:11 form a monophyletic cluster with RhGMYuV with 79% bootstrap support (Figure 1).

Interestingly, these two isolates cluster with *Tobacco yellow crinkle virus* (TbYCV) and RhGMYuV in Bayesian trees based on the CP and Rep nt sequences respectively (Figure 2).

A third DNA-A component was cloned from sample #338, EC:Roc338.2:11, with 93.1% nt identity with EC:Roc338.3:11 cloned from this same sample. Recombination analysis indicated a strongly supported recombination event in this component, with the major parent identified as EC:Roc338.3:11 and the minor parent identified as EC:StE240.1:11, a ToLDeV isolate from the province of Santa Elena. The recombination breakpoints were established at nts 267-1218, corresponding to most of the CP and a small portion of the REn and TrAP genes (Suppl. Figure S2). The recombination event has strong phylogenetic support: the Rep-based tree places EC:Roc338.2:11 in a monophyletic branch (99% bootstrap support) with the RhGMYuV isolates, while the CP-based tree places the isolate in a monophyletic branch (also with 99% bootstrap support) with the ToLDeV isolates (Figure 2). Thus, we obtained a recombinant isolate of RhGMYuV and one of its parental isolates from sample #338.

Begomovirus clones were also obtained from six samples of *Rhynchosia* sp. collected at the provinces of Guayas (EC:Yag8.1:11) and Manabi [EC:StA19.1:11, EC:StA23.1:11, EC:StA24.1:11 (Suppl. Fig. S3), EC:StA25.1:11, EC:Roc30.1:11]. DNA-A and DNA-B components were cloned in all cases, except EC:StA23.1:11, from which the DNA-B was not cloned. All six DNA-A components showed >91% nt identity with RhGMYuV (Suppl. Figure S1A). The five DNA-B components showed 85-86% nt identity with RhGMYuV (Suppl. Figure S1B). Phylogenetic reconstruction based on the full-lenght DNA-A placed all weed isolates in a monophyletic cluster together with RhGMYuV (Figure 1). The same topology was observed for CP- and Rep-based trees (Figure 2). These results indicate that these weed samples were all infected with isolates of RhGMYuV.

One interesting aspect of the two begomoviruses found in tomatoes in Ecuador (ToLDeV and RhGMYuV) is that they have nearly identical iterons, suggesting that viable pseudorecombinants could be formed between the single ToLDeV component (which is analogous to a DNA-A) and the RhGMYuV DNA-B. For the ToLDeV isolates the iterons comprise two imperfectly conserved direct repeats (GGTGT/GGAGT) and one inverted repeat (ACACC). The corresponding sequences for RhGMYuV are GGTGT/GGTGT and ACACC (Figure 3). Curiously, the RhGMYuV isolates have an additional direct repeat (GGTGT) downstream from the stem-loop sequence at the origin of replication and an additional (imperfect) inverted repeat (ACACC) between the TATA box and the stem-loop (Figure 3).

A begomovirus distinct from ToLDeV and RhGMYuV was isolated from tomato sample #222 (isolate EC:Roc222:11), collected in the province of Manabi. The cloned DNA-A showed a maximum nt identity of 81.2% with *Tomato golden leaf distortion virus* (ToGLDV, accession number HM357456) (Suppl. Figure S1A). This level of identity suggests that EC:Roc222:11 corresponds to a new begomovirus species. Strikingly, this isolate clustered with begomoviruses from Brazil in the DNA-A-based phylogenetic tree (Figure 1). Its iterons are completely different from those of ToLDeV and RhGMYuV, consisting of three direct and two inverted repeats (GGAG and CTCC, respectively) upstream of the origin of replication, as well as an additional downstream direct repeat (Figure 3). A cognate DNA-B component was not cloned. Further characterization of this isolate, including the production of an infectious clone, should elucidate whether it corresponds to a *bona fide* begomovirus.

We conclude that tomato crops in Ecuador, specifically in the provinces of Guayas, Loja, Manabi and Santa Elena, are affected primarily by the monopartite begomovirus ToLDeV. Two samples from Manabi province were infected also by RhGMYuV. A

natural recombinant RhGMYuV isolate and its major parent were detected in a tomato sample from Manabi. A possible new begomovirus species was detected in a tomato sample also from Manabi. Our results also indicate that the widespread weed *Rhynchosia* sp. may act as a reservoir for RhGMYuV.

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Figure legends

Figure 1. Phylogenetic reconstruction, using Bayesian inference, based on full-length DNA-A nucleotide sequences of the begomoviruses from Ecuador described in this study (clones obtained from tomato and weed samples indicated in red and green, respectively) and additional begomoviruses from the Americas. Nodes with bootstrap support equal to or higher than 90% are indicated by filled circles, and those with values between 75 and 89% by empty circles.

Figure 2. Phylogenetic reconstruction, using Bayesian inference, based on (**A**) CP, and (**B**) Rep nucleotide sequences of the begomoviruses from Ecuador described in this study (clones obtained from tomato and weed samples indicated in red and green, respectively) and additional begomoviruses from the Americas. Nodes with bootstrap support equal to or higher than 90% are indicated by filled circles, and those with values between 75 and 89% by empty circles.

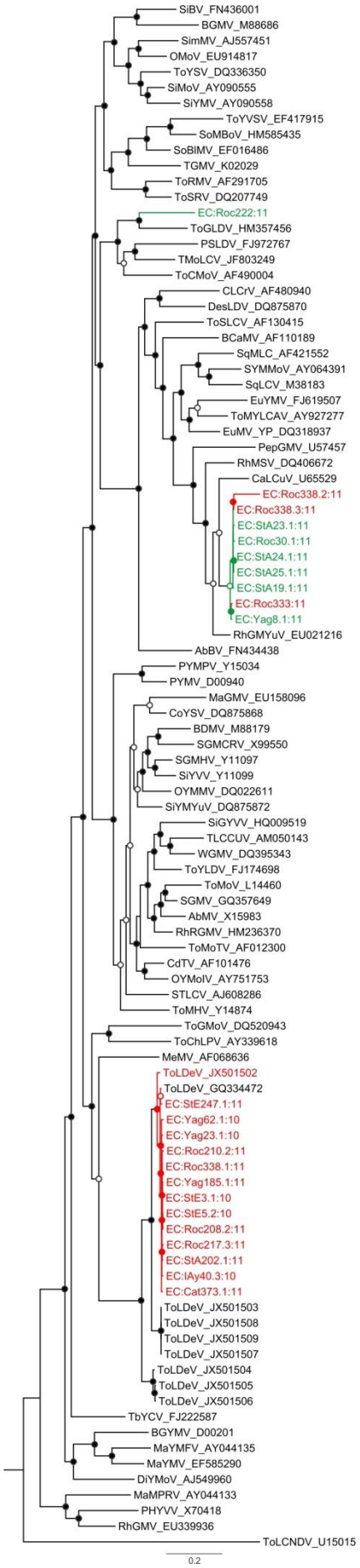
Figure 3. Alignment of the intergenic region (IR) sequences of isolates of ToLDeV, RhGMYuV and EC:Roc222:11 included in the study showing the iterons (gray shadowed boxes). Horizontal arrows represent their position and orientation upstream and dowstream of the Rep gene TATA box (bold underlined). The vertical black arrow indicates the nicking site for the initiation of viral replication. Nucleotides in blue indicate positions in which the sequence of the DNA-B IR differs from that of the DNA-A IR.

Supplementary Figure S1. Pairwise percent nucleotide sequence identities between the DNA-A (A) and DNA-B (B) of the begomoviruses from Ecuador described in this study and the most closely related begomoviruses.

Supplementary Figure S2. Recombination analysis performed using the GENECONV module of the RDP3 package. Putative recombinant blocks are detected based on pairwise identities in a 50-nucleotide sliding window along each alignment. Each putative recombinant block is represented by a rectangle, with the height proportional to the *p*-value. The shadowed box in the plot indicates the recombinant region between ToLDeV and RhGMYuV. The numbers indicate nucleotide positions in the alignment and the exact positions of recombination breakpoints.

Supplementary Figure S3. *Rhynchosia* sp. collected at Santa Ana, province of Manabi (1°8'48.4"S, 80°22'55.9"W), from which the clone EC:StA24.1:11 was obtained.

Figure 1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SoMBoV_HM585435
SoBIMV_EF016486
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ToRMV_AF291705
ToSRV_DQ207749
EC:Roc222:11
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PSLDV_FJ972767
TMoLCV_JF803249
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DesLDV_DQ875870
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BCaMV_AF110189
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OYMMV_DQ022611
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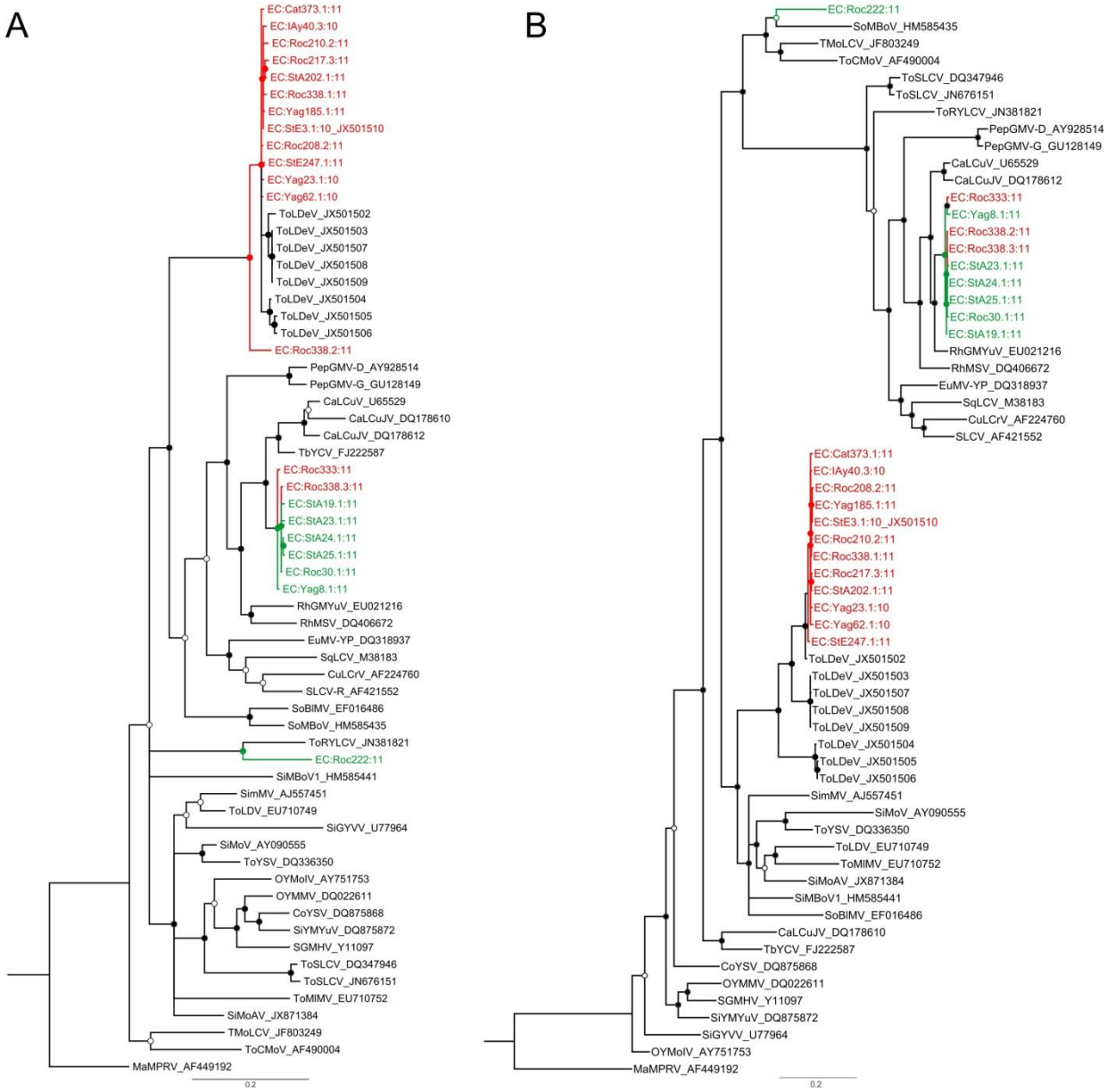
Figure 2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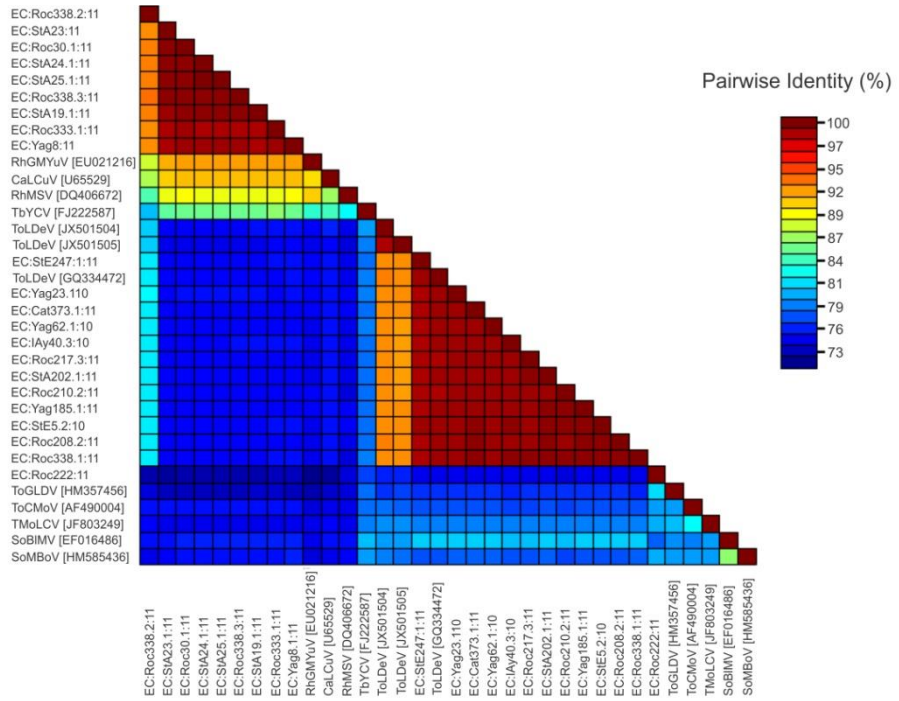
RhGMYuV_EU021216
RhMSV_DQ406672
EuMV-YP_DQ318937
SqLCV_M38183
CuLCrV_AF224760
SLCV_AF421552
EC:Cat373.1:11
EC:IAy40.3:10
EC:Roc208.2:11
EC:Yag185.1:11
EC:StE3.1:10_JX501510
EC:Roc210.2:11
EC:Roc338.1:11
EC:Roc217.3:11
EC:StA202.1:11
EC:Yag23.1:10
EC:Yag62.1:10
EC:StE247.1:11
ToLDeV_JX501502
ToLDeV_JX501503
ToLDeV_JX501507
ToLDeV_JX501508
ToLDeV_JX501509
ToLDeV_JX501504
ToLDeV_JX501505
ToLDeV_JX501506
SimMV_AJ557451
SiMoV_AY090555
ToYSV_DQ336350
ToLDV_EU710749
ToMIMV_EU710752
SiMoAV_JX871384
SiMBoV1_HM585441
SoBIMV_EF016486
CaLCuJV_DQ178610
TbYCV_FJ222587
CoYSV_DQ875868
OYMMV_DQ022611
SGMHV_Y11097
SiYMYuV_DQ875872
SiGYVV_U77964
OYMoIV_AY751753
MaMPRV_AF449192
0.2

Figure 3

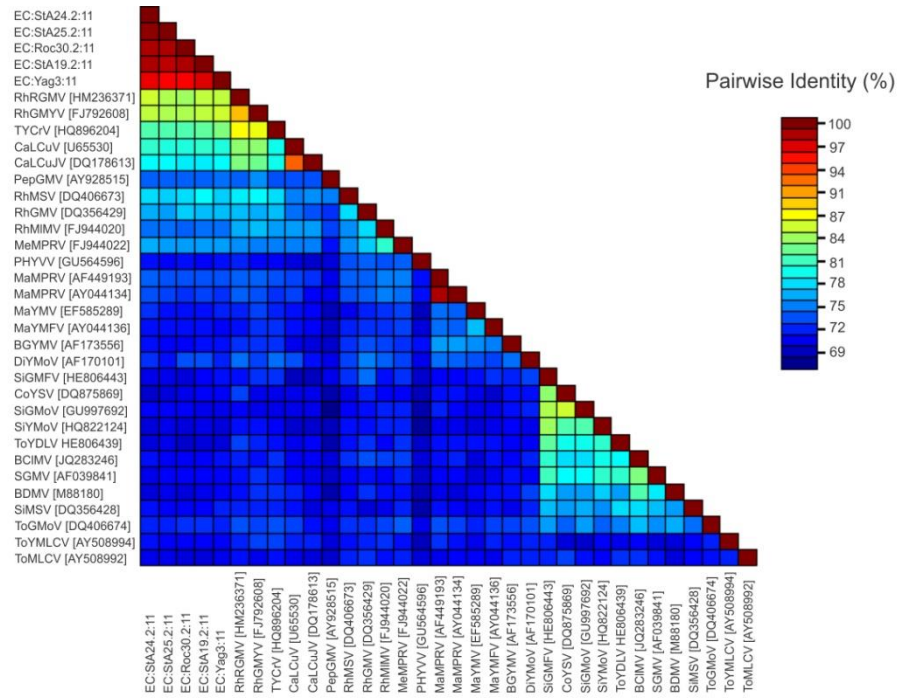

ToLDeV
RhGMYuV
EC:StE5.2:10
EC:Yag23.1:10
EC:IAy40.3:10
EC:Yag62.1:10
EC:Yag185.1:11
EC:StA202.1:11
EC:Roc208.2:11
EC:Roc210.2:11
EC:Roc217.3:11
EC:StE247.1:11
EC:Roc338.1:11
EC:Cat373.1:11
EC:Yag3:11
EC:Yag8.1:11
EC:StA19.1:11
EC:StA19.2:11
EC:StA23:11
EC:StA24.1:11
EC:StA24.2:11
EC:StA25.1:11
EC:StA25.2:11
EC:Roc30.1:11
EC:Roc30.2:11
EC:Roc333:11
EC:Roc338.2:11
EC:Roc338.3:11
EC:Roc222:11

Supplementary Figure S1

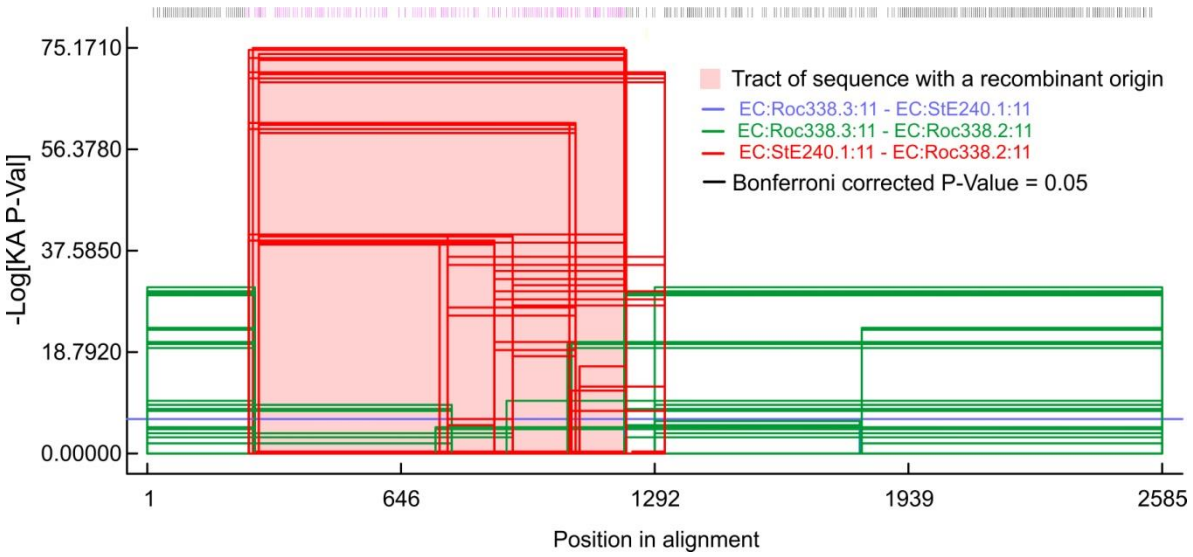
A



B



Supplementary Figure S2



Supplementary Figure S3

CAPÍTULO 2

Genetic variability and population structure of *Tomato leaf deformation virus*, a monopartite begomovirus infecting tomatoes in Ecuador

Paz-Carrasco, L.C., Castillo-Urquiza, G.P., Lima, A.T.M., Ramos-Sobrinho, R., Hora-Júnior, B.T., Silva, J.C.F., Melgarejo, T.A., Rojas, M.R., Gilbertson, R.L., Mizubuti, E.S.G. & Zerbini, F.M. Genetic variability and population structure of *Tomato leaf deformation virus*, a monopartite begomovirus infecting tomatoes in Ecuador. Virology, *in preparation*.

Genetic variability and population structure of *Tomato leaf deformation virus*, a monopartite begomovirus infecting tomatoes in Ecuador

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ABSTRACT

The genus *Begomovirus* of the *Geminiviridae* family contains viral species affecting tropical and subtropical dicotyledonous plants and transmitted by the whitefly ***Bemisia tabaci***. Begomoviruses can have mono- or bipartite ssDNA genomes, and until the recent description of the monopartite *Tomato leaf deformation virus* (ToLDeV), all begomoviruses from the Americas were thought to be bipartite. ToLDeV is the main species that infects tomatoes in Ecuador. The study of ToLDeV populations may provide insights into the evolution of this monopartite virus and its ongoing adaptation to a new host. In this study we investigated the population structure and genetic variability of ToLDeV from Ecuador. Sequences of 67ToLDeV isolates were used to identify haplotypes and infer genetic relationships with Peruvian isolates. Phylogenetic analysis of the DNA-A component confirmed that ToLDeV is related to begomoviruses from the New World. Two ToLDeV subpopulations (from Ecuador and Peru) were well structured and presented minor allelic migration. The lowest haplotype diversity was displayed by the C4 gene for both subpopulations. Genetic variability indices were generally much higher for the Peruvian subpopulation in comparison with the Ecuadorian subpopulation. The C4 gene had the highest mutation frequency in both subpopulations. There was evidence of negative or purifying selection acting on each of the viral genes, thus preserving protein function. Tests of neutrality were significant and negative for the CP, Rep, TrAP and C4 genes of the subpopulation from Ecuador, indicating that it is undergoing expansion. All isolates from Ecuador and one isolate from Peru showed a recombination event encompassing the Rep, TrAP and REn genes and the intergenic region (IR). Together, the results indicate a founder effect acting upon the Ecuadorian subpopulation, which is undergoing rapid expansion and differentiation.

INTRODUCTION

The *Geminiviridae* family is comprised of viruses with circular, single-stranded DNA (ssDNA) genomes encapsidated within geminate particles ~18×30 nm in size(Brown et al., 2012). The family is divided into seven genera (*Becurtovirus, Begomovirus, Curtovirus, Eragrovirus, Mastrevirus, Topocuvirus* and *Turncurtovirus*) based on genomic structure, phylogenetic relationships, host range and vector specificity (Brown et al., 2012; Hernández-Zepeda et al., 2013).

Viruses in the genus *Begomovirus* are transmitted by the whitefly *Bemisia tabaci*, infect dicotyledonous plants and have genomes composed of one or two ssDNA molecules (mono- or bipartite begomoviruses), each with ~2,600 nucleotides (nt) (Brown et al., 2012). The genomes of monopartite begomoviruses have two genes in the virion sense encoding the coat protein (CP) and the AV2 protein involved in virus movement, and four genes in the complementary sense encoding proteins involved in viral replication (Rep and REn), trans-activation (TrAP) and suppression of host defense responses (TrAP and AC4) (reviewed by(Rojas et al., 2005). Bipartite begomoviruses have a DNA-A component which is homologous to the single component of the monopartite viruses, plus a DNA-B component encoding two proteins involved in viral movement (MP and NSP) (Rojas et al., 2005). An intergenic region (IR) contains a stem-loop structure with a conserved nonanucleotide sequence [TAAT(A,G)TT//AC] which constitutes the viral origin of replication (Rojas et al., 2005).

Begomoviruses in the Old World are mostly monopartite, often associated with satellite DNA molecules (Briddon and Stanley, 2006). Until very recently it was thought that all New World begomoviruses were bipartite, with a distinguishing feature from their OW counterparts being the absence of the AV2 gene in the DNA-A (Brown et al., 2012; Rojas et al., 2005). However, a begomovirus present in Peru and Ecuador, *Tomato leaf*

deformation virus (ToLDeV), has been shown recently to be a truly NW monopartite virus, lacking the AV2 gene (Melgarejo et al., 2013; Sanchez-Campos et al., 2013).

Begomoviruses infect tropical and subtropical crops, involving considerable economic losses by specifically affecting herbaceous dicotyledonous plants (Briddon and Markham, 2000; Patil and Fauquet, 2009; Polston and Anderson, 1997; Varma and Malathi, 2003). In Latin America the most severely affected crops have been tomatoes, peppers and beans (Morales and Anderson, 2001). In most countries (*eg*, Brazil, Cuba, Mexico, Venezuela) a large number of viral species have been described (Banuelos-Hernandez et al., 2012; Fiallo-Olive et al., 2013a; Fiallo-Olive et al., 2013b; Fiallo-Olive et al., 2012; Martinez-Zubiaur et al., 1998; Renteria-Canett et al., 2011; Rocha et al., 2013), while in other countries such as Uruguay a single species (in this case, *Tomato rugose yellow leaf curl virus*, ToRYLCV) seems to predominate, at least in cultivated hosts (Marquez-Martin et al., 2012). Regardless, one common feature of these viruses is their high degree of genetic variability (Albuquerque et al., 2012; Marquez-Martin et al., 2012; Rocha et al., 2013).

Several studies have established the high intra- and interspecific variability of begomoviruses. Mutation frequencies and nucleotide substitution rates estimated for *Tomato yellow leaf curl virus* (TYLCV), *Tomato yellow leaf curl China virus* (TYLCCNV) and *East African cassava mosaic virus* (EACMV) are similar to those determined for RNA viruses (~10^{-4} substitutions per site per year) (Duffy and Holmes, 2008; Duffy and Holmes, 2009; Ge et al., 2007). Recombination is considered the most significant source of their genetic variability (Lefeuvre et al., 2007; Lefeuvre et al., 2009; Lima et al., 2013; Martin et al., 2011; Monci et al., 2002; Monjane et al., 2012; Padidam et al., 1999; Pita et al., 2001).

The recent description of ToLDeV in Peru and Ecuador (Marquez-Martin et al., 2011; Melgarejo et al., 2013) could be correlated with reports of high whitefly infestations since the 1990's (Valarezo et al., 2004), assuming a recent transfer of the virus from wild, non-cultivated hosts to tomato by the vector. The study of this viral population thus offers a unique opportunity to capture the process of adaptation to a new host.

The aim of this study was to assess the genetic structure and variability of ToLDeV populations from Ecuador in naturally infected *Solanum lycopersicum* plants, elucidating potential factors that shape the variation and evolution of this viral species.

MATERIAL AND METHODS

Sample collection and storage

During the years of 2010 and 2011, 396 tomato samples displaying virus-like symptoms such as chlorosis, mosaic, yellow leaf curl, stunting and yellow spots were collected in the provinces of Chimborazo (27 samples),Galapagos (65), Guayas, (87), Loja (41), Manabi (96), and Santa Elena (80). Each one of the samples was georeferenced and, after collection, was kept inside pieces of paper that were folded and pressed using a wood press. Samples were dried in this manner and stored at room temperature as herbarium-like specimens until analyzed.

Cloning and sequencing of full length genomes

Total plant DNA was extracted from the dried leaf samples using a CTAB protocol (Doyle and Doyle, 1987). Viral genomes were amplified by rolling-circle amplification (RCA) using the DNA polymerase of phage phi29 (Inoue-Nagata et al., 2004). RCA products were digested with *Msp*I to generate different patterns of DNA fragments.

Selection of samples with the presence of a begomovirus was conducted by identifying band patterns that added up to ~2,600 nt. Samples selected based on these criteria were digested with *Kpn*I, *Sac*I and *Hin*dIII to generate monomers of the viral genome which were cloned into pBluescript II KS(+) (Stratagene) previously digested with same enzyme. Viral inserts were completely sequenced by primer walking at Macrogen, Inc. (Seul, South Korea). All genome sequences were organized to begin at the nicking site in the invariant nonanucleotide at the origin of replication (5'-TAATATT//AC-3').

Sequence analysis

Pairwise sequence comparisons to determine the taxonomic position of each isolate were performed using the program SDT v.1 (Muhire et al., 2013), including all ToLDeV sequences available in GenBank as well as sequences of the most closely related viruses according to a BLAST search. Nucleotide sequences from Ecuadorian isolates were aligned with the Muscle algorithm implemented in MEGA 5.10 (Tamura et al., 2011). The program DnaSPv. 5.10.01 (Librado and Rozas, 2009)was used to identify haplotypes. Only one sequence of each haplotype from a given sample was used in subsequent analyses.

Phylogenetic analysis

Multiple sequence alignments of the complete genomes were prepared using Muscle. Phylogenies were reconstructed using Bayesian inference. The program MrModeltest v. 2.2 (Nylander, 2004) was used to provide a model of nucleotide substitution with the best fit for each data set. The analyses were carried out in MrBayes v. 3.0b4 (Ronquist and Huelsenbeck, 2003) by running two chains with 30,000,000 generations each, and trees were sampled every 1,000 generation resulting in 60,000 trees. After discarding the first 20% of samples as burn-in, the remaining 48,000 trees were used

for calculating posterior probabilities in the consensus tree. Trees were visualized and edited with the FigTree program (tree.bio.ed.ac.uk/software/figtree) and CorelDraw X3 (Corel Corporation), respectively.

Recombination analysis

Selected haplotypes of ToLDeV from Ecuador plus nine isolates from Peru retrieved from GenBank were aligned using Muscle. The detection of recombination events was carried out with Recombination Detection Program (RDP) v.3.42 (Martin et al., 2010). The analyses were performed with default settings and Bonferroni-correction, with a cut-off of $p<0.05$. Only the recombination events detected by more than four of the seven methods implemented in RDP3 (RDP, GENECONV, Chimaera, MaxChi, BootScan, SiScan and 3Seq) were considered to be reliable.

Population subdivision

All ToLDeV haplotypes (63sequences from Ecuador plus nine sequences from Peru) were used for testing population subdivision with the model-based Bayesian clustering algorithm implemented in STRUCTURE 2.3.3. To select the number of clusters that best represented population structure, twenty independent runs of 1 to 20 subpopulations (K=1 to 20) were performed using 1,000,000 Markov chain Monte Carlo steps after a burn-in period of 100,000 steps. All 20 independent replicates were grouped to determine the highest modal value of the distribution ΔK with the Evanno method implemented in the program STRUCTURE HARVESTER(Earl and Vonholdt, 2012), to determine the most likely K.

The DnaSPv5.10.10 program was used to determine Hudson's K_S, K_T,K_{ST} andS_{nn}, and Wright'sF_{ST} indices. K_S is a weighted average of K_1 and K_2 (the average number of

differences between sequences from subpopulations 1 and 2, respectively), K_T represents the average number of differences between two sequences regardless of their subpopulation, and $K_{ST}=1-(K_S/K_T)$. Under the null hypothesis (no genetic differentiation), $<K_{ST}>$ (the observed value of K_{ST}) is expected to be near 0, and the null hypothesis is rejected if $<K_{ST}>$ is too high or supported by a small *p*-value (<0.05). A small value of $<K_S>$also leads to rejection of the null hypothesis. S_{nn} is a measure of how often the "nearest neighbors" (in sequence space) of sequences are from the same location in geographic space. S_{nn} is expected to be near 1 when the populations at the two localities are highly differentiated (high subpopulation differentiation) and near 0.5(panmixis) when the populations at the two localities are part of the same panmictic population. Wright's F_{ST} index of genetic differentiation is a measure that determines the complete level of genetic divergence among subpopulations. Its value is interpreted qualitatively: 0-0.05 indicates little differentiation; 0.05-0.15, moderate differentiation; 0.15-0.25, great differentiation; and above 0.25, very great genetic differentiation among subpopulations.

Descriptors of genetic variability

DnaSPv5.10.01was used to estimate the main descriptors of genetic variability for each population, based either on complete genomes or individual ORF's.

Neutrality tests

The DnaSPv5.10.01 program was also used to calculate Tajima's D, Fu and Li's *F** and *D**, and Fu's F_S, to determine whether the structured populations deviate from neutrality (which assumes a constant population size, no recombination and no migration).The ratio of nonsynonymous substitutions per nonsynonymous site (d_N) and synonymous substitutions per synonymous site (d_S) was calculated according to the single-

likelihood ancestor counting (SLAC) and partitioning approach for robust inference for selection (PARRIS) methods available in the Datamonkey server(www.datamonkey.org), to estimate the occurrence of positive and negative selection at amino acid sites in the CP, Rep, TrAP, REn and C4 open reading frames.

RESULTS

Incidence of ToLDeV in tomato fields in Ecuador

Overall, 12% of the samples (46/396) were positive for the presence of ToLDeV, and these generally corresponded to samples with leaf roll symptoms. However, there was considerable variation in incidence among the different provinces, from zero in Chimborazo and Galapagos (27 and 65 samples, respectively) to 22.5% (18/80) in Santa Elena. The percentage of positive samples was 2.4% in Loja (1/41), 13.5% in Manabi (13/96), and 16% in Guayas (14/87). It must be pointed out that these numbers do not reflect the true incidence of the virus, which is likely to be lower since only symptomatic samples were collected.

Selection ofhaplotypes

Sixty-seven isolates were obtained from the 46 positive samples (Suppl. Table S1). All isolates were clearly classified as ToLDeV (>99.15% identity with the ToLDeV sequences in GenBank). Sixty-three isolates were selected as haplotypes, and were used for the subsequent analyses.

Phylogenetic analysis

Phylogenetic reconstruction using Bayesian inference and including only the single (DNA-A-like) component of ToLDeV isolates from Ecuador and Peru was consistent with geographical subdivision, as the isolates were clearly clustered according to their country of origin (Figure 1). Moreover, the isolates from Peru formed two strongly supported subgroups: one including the three isolates collected in 2010, and another including four isolates collected in 1998 (Figure 1).

Overall, we observed a small genetic distance among the Ecuadorian isolates. The Ecuadorian isolates were clustered in two groups. One group includes three isolates from Santa Elena(EC:StE:247.1:11, EC:StE:254.1:11, EC:StE:254.2:11) and, interestingly, the isolate PE:PT1:To:03 from Carabayllo, Peru. The second group included the remaining isolates. Except for a group of 13 isolates from Yaguachi (province of Guayas) which formed a strongly supported cluster, the isolates in this second group did not cluster based on location of year of collection. Most monophyletic clusters within this group included only a few isolates, and in many cases these isolates came either from the same sample or from different regions. One interesting case were two isolates obtained from the same sample (EC:Roc:208.1:11 and EC:Roc:208.2:11 from Manabi), which did not cluster together: EC:Roc:208.1:11 formed a cluster with two isolates from sample 210 from the same location, while EC:Roc:208.2:11 clustered with two isolates from Santa Elena (EC:StE:243.1:11 and EC:StE:244.1:11). More significantly, all isolates of Ecuador and the PE:PT1:To:03 isolate formed a strongly supported monophyletic cluster with the PA98-1-1 isolate (from Ica, Peru) (Figure 1).

Recombination analysis

A single recombination event was detected for all63isolatesfromEcuadorandfor thePE:PT1:To:03 isolate from Peru.The event was detected by six of the seven methods implemented in RDP3 (the only method that did not detect the event was RDP), and was stronglysupportedby a *p* value of 3.48 x 10^{-19}(SiScan).The major parental sequence was not identified in the data set, and the minor parental sequence is related to the PA98-1-1 isolate from Peru. The recombinant region is located at nucleotide positions 1,220 to 2,529, corresponding to the Rep, C4, TrAP and REn genes and the intergenic region (IR).

Subdivision of the ToLDeV population

The value of the ΔK probability was high (4.8×10^{10}), structuring *K*=2 populations, Ecuador and Peru (Figure 2). Evidence of migration from Peru to Ecuador was observed for isolates EC:StE239.1:11 and EC:StE247.1:11 from Santa Elena and EC:StA201.1:11 from Manabi. Isolates PA98-1-1 and PE:PTI:To:03 (from Peru) were found to be part of the Ecuadorian subpopulation. The values obtained for K_{ST}, S_{nn} and F_{ST}(0.26583, 0.98611 and 0.42929, respectively) indicated that the Ecuadorian and Peruvian subpopulations are genetically differentiated. Together, these results strongly support population subdivision between Ecuador and Peru.

Genetic variability of ToLDeV populations

Genetic variability indices were determined for the entire population (Ecuador and Peru, 72 isolates) and for each subpopulation (Ecuador, 63 isolates; Peru, 9 isolates). Analysis of the complete genome indicated the existence of 70 haplotypes, with a haplotype diversity of 0.999. For each gene of the entire population, haplotype diversity ranged from 0.790 to 0.987, indicating a very high degree of variability (Table 1). The

subpopulation from Ecuador presented 61 haplotypes for the complete genome (haplotype diversity of 0.999), and the Peruvian subpopulation presented 9 haplotypes, with a haplotype diversity of 1.00 (Table 1).

Nucleotide diversity (π) of the complete genome for the entire population was 0.01889 (Table 1). The Peruvian subpopulation had a highest π compared to the Ecuadorian subpopulation (0.05174 vs. 0.00853, respectively, a 6-fold difference; Table 1), indicating remarkable differences in terms of genetic variability between them. Nucleotide diversity values for all genes were also higher for the population from Peru compared to the one from Ecuador, and even more so for the complementary-sense genes (Rep, TrAP, REn and C4; Table 1).Mutation frequencies were determined for each gene, and for both subpopulations the C4 gene had the highest mutation frequency (1.17×10^{-3} and 2.20×10^{-2} for the Ecuadorian and Peruvian subpopulations, respectively) (Table 1). As observed for nucleotide diversity, all genes of the Peruvian subpopulation had a higher mutation frequency compared to the Ecuadorian subpopulation. Together, these results indicate a significantly higher degree of genetic variability for the Peruvian subpopulation compared to the Ecuadorian subpopulation.

Occurrence of selection on the populations

The values of the d_N/d_S ratio were <1.0 for all genes, indicating the occurrence of negative selection (Table 2). However, the value observed for the TrAP gene was very close to 1.0 (0.913917), and no negatively selected sites were determined for this gene by SLAC (Table 2). It thus appear that the TrAP gene is under neutral selection. In the CP, Rep, REn, and C4 genes, 14, 4, 2 and 1 sites (codons), respectively, were determined to be under negative selection (Table 2). Results obtained by PARRIS confirmed the absence of sites under positive selection for each gene (*data not shown*).

Neutrality tests

Forthe ToLDeV subpopulation from Ecuador, significant negative values were obtained for the CP, Rep and C4 genes for Tajima's *D*, Fu and Li's *D**and *F** tests (Table 3), indicating a recent expansion of the subpopulation. Tajima's *D* and Fu and Li's *F** were also significant and negative for the TrAP gene, and Tajima's D for the REn gene. For Tajima's D, this established that the TrAP and REn genes are experiencing a recent bottleneck. For Fu and Li's *F**, it suggests the existence of polymorphism in the TrAP gene subjected to a background of selection, i.e., that the frequent removal of deleterious variants can also result in the occasional removal of neutral linked variation, particularly in low-recombining regions (Barreiro and Quintana-Murci, 2010).

In the subpopulation from Peru the only significant (positive) values were obtained for the C4 gene for Fu and Li's *D**and *F** tests, indicating an excess of intermediate-frequency alleles which could be product of population bottlenecks, structure and/or balancing selection.

DISCUSSION

A single tomato geminivirus, *Tomato leaf deformation virus* (ToLDeV), has been reported in Ecuador (and the same virus has been reported in neighboring Peru), even though heavy infestations by *B. tabaci* biotypes A and B have been reported in the country since the 1990's (a possible geminivirus infection in tomatoes was mentioned in Ica, Peru, but without getting to a definitive confirmation (Morales and Anderson, 2001). This is in contrast to the situation in most Latin American countries, such as Brazil, Cuba, Mexico and Venezuela, where several begomovirus species have been reported in solanaceous crops (Martinez-Zubiaur et al., 1998; Nava et al., 2006; Ribeiro et al., 2003; Zambrano et al., 2011). Even more strikingly, ToLDeV is the only New World monopartite

begomovirus found to date (Melgarejo et al., 2013; Sanchez-Campos et al., 2013). Our previous results (Paz-Carrasco et al., *unpublished*) have confirmed the prevalence of ToLDeV in Ecuador, and also revealed the presence of *Rhynchosia golden mosaic Yucatan virus* (RhGMYuV) in weeds and, to a lesser extent, in tomato. The recent emergence of ToLDeV, its rapid dissemination in tomato crops, and its atypical molecular properties make it an interesting case study for begomovirus evolution and adaptation to a new host.

The most evident result from our analysis was the genetic differentiation between the two ToLDeV subpopulations from Ecuador and Peru. Begomoviruses are known to be phylogenetically related based on geographical location rather than host, which suggests that local evolution, probably due to geographical isolation, leads to speciation. The fact that a recently emerging virus such as ToLDeV already exists as genetically distinct subpopulations suggests that this process takes place in a short time frame. Tomato-infecting begomoviruses in Brazil are also segregated based on geographical location, and a number of species, such as *Tomato common mosaic virus* (ToCmMV), are also genetically structured (Rocha et al., 2013). Interestingly, each ToLDeV subpopulation is structured in additional subpopulations, a situation demonstrated in other investigations (Melgarejo et al., 2013; Sanchez-Campos et al., 2013). In our case, we defined the geographical structure of the two subpopulations by phylogenetic analysis and by the algorithm implemented in Structure. It seems that ToLDeV has its own evolving history in each subpopulation.

The occurrence of migration from the subpopulation of Peru to the subpopulation of Ecuador could be explained by the existing winds in the coast of the Equatorial Pacific in certain times of the year which allow long distance transfers of viruliferous whiteflies. Such long distance transport was observed in the case of *Bean golden yellow mosaic virus* (BGYMV), originally present in Puerto Rico, and which was detected in southern Florida

within two months after the passage of hurricane Andrew (Polston and Anderson, 1997). This form of migration is favoured as there is no evidence of a tomato seedling trade between the two countries, although the movement of vegetable products through the border could be a vehicle for entry of viruliferous whiteflies into Ecuador. Independently of how the migration took place, the evidence suggests that the Peruvian subpopulation acted as the founder of the Ecuadorian ToLDeV subpopulation.

Comparative analysis of the two subpopulations indicated a bottleneck effect accompanied by a rapid increase of each subpopulation. However, based on haplotype and nucleotide diversity, the Peruvian subpopulation is more stable with an older evolving history compared to the subpopulation of Ecuador. This provides additional support for the hypothesis that the subpopulation of Ecuador was founded by the subpopulation of Peru followed by a bottleneck effect.

The nucleotide diversity of the entire ToLDeV population (Ecuador and Peru) based on the CP and Rep genes was relatively low in relation to the monopartite *Cotton leaf curl virus* (CLCuV) (Sanz et al., 1999), but marginally higher when the CP, Rep and C4 genes were compared with those from the monopartite *Tomato yellow leaf curl virus*-Mild[RE] (Delatte et al., 2007).Notably, the subpopulation of ToLDeV of Ecuador presented lower nucleotide diversity then the bipartite begomoviruses *Blainvillea yellow spot virus* (BlSV), *Cleome leaf crumple virus* (ClLCrV), *Macroptilium yellow spot virus* (MaYSV), *Tomato severe rugose virus* (ToSRV) and ToCmMV (Lima et al., 2013; Rocha et al., 2013; Silva et al., 2011)and still low compared with the ToLDeV subpopulation from Peru. Compared to the previous studies of ToLDeV isolates from Peru (Melgarejo et al., 2013; Sanchez-Campos et al., 2013), the Rep and C4 genes of the Peruvian subpopulation had equivalent levels of nucleotide diversity, but the Ecuadorian subpopulation had considerably lower values. This low nucleotide diversity in the

subpopulation of Ecuador is further evidence that this subpopulation has its own limited evolutionary history confined exclusively to its environment without presenting geographic genetic divergence in their different genes of the various isolates.

Another relevant aspect is that the Rep gene was the most variable of both subpopulations, displaying the highest number of segregated sites and the higher mutation frequency. Extensive sequence comparisons have indicated the Rep gene to be one of the most variable amongst begomovirus species (Padidam et al., 1995), but few studies have investigated its intraspecies variation. Although essential for viral replication, the Rep protein must be under lower selective constraints compared to the coat protein, which has structural and vector transmission roles. The mutation frequency of the C4 gene was higher in the Peruvian than in the Ecuadorian subpopulation. Only one out of the 86 codons in this gene was found to be under negative selection, possibly removing nonsynonymous substitutions that could be affecting the functionality of the protein as to promote the movement of the virus and suppression of host defenses. Interestingly, the C4 gene was shown to be under positive selection in a previous investigation (Melgarejo et al., 2013).

The coat protein (CP), which besides being the only structural protein is also involved in interaction with the whitefly and in long distance movement of the virus in the plant, displayed the lowest value of d_N/d_S, as observed for other begomoviruses (Duffy and Holmes, 2008) and other ssDNA viruses (Grigoras et al., 2010). The ToLDeV CP gene showed low variability, similarly to the monopartite CLCuV (Sanz et al., 1999) and the bipartite MaYSV (Lima et al., 2013), most likely due to negative selection being exerted on the5% of its codons. In another study, 4.2% of the codons were found to be under positive selection, with 93.1% of the codons being under negative selection (Melgarejo et al., 2013).

Interestingly, all isolates from the Ecuadorian subpopulation, as well as one isolate from the Peruvian subpopulation (PE:PT1:To:03), were found to be recombinants in a previous study (data not shown) with *Rhynchosia golden mosaic Yucatan virus* (RhGMYuV) as a parent. These results indicated that the recombinant fragment encompasses the Rep, TrAP and REn genes and the IR. Previous reports have also indicated a recombinant nature for ToLDeV, but failed to identify the parental viruses (Marquez-Martin et al., 2011; Melgarejo et al., 2013). Recombination promotes to emergence of new begomovirus diseases (Padidam et al., 1999). One clear example of an aggressive recombinant begomovirus was detected in Uganda between *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) with an extremely severe epidemic that devastated cassava fields and caused food shortages and famine in a number of districts where the crop was the major staple (Pita et al., 2001). Whether ToLDeV gained evolutionary advantages following the recombination event with RhGMYuV remains to be determined.

In conclusion, we have characterized a population of the predominant tomato-infecting begomovirus in Ecuador, ToLDeV. Our results reinforce the hypothesis that it emerged in the Americas as a monopartite virus, that the Ecuadorian population shares this evolutionary event with Peruvian isolates, and that these two (sub) populations are genetically distinct, with the Peruvian subpopulation possibly having founded the Ecuadorian subpopulation. The low nucleotide diversity and the highest number of haplotypes can establish that the subpopulation of Ecuador is undergoing a bottleneck effect accompanied by a rapid population growth with accumulation of mutations. The total population is in negative selection removing dexterous codons that could be interfering with the functionality of the viral proteins. Future work to verify the presence of ToLDeV in other tomato-producing provinces, mainly in the Andean region of the country,

will help to establish the evolutionary mechanisms that maybe acting with greater force upon the virus. Another aspect that is temporarily help evaluate materials resistant tomato for monopartite TYLCV, which palliation could counteract the effect of ToLDeV and excessive use of insecticides to control whiteflies. Knowledge of host plants help ToLDeV program management of these plant species with the whitefly population dynamics in all tomato producing areas in Ecuador.

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Table 1. Genetic variability indices estimated for the complete genome and for the CP, Rep, TrAP, REn and C4 genes of the *Tomato leaf deformation virus* (ToLDeV).

Population	Gene	L[2]	H	Hd	s	k	π	σ_π	η	θ_W	θ_W/site	No. of mutations	Mutation frequency
All	Genome	2601	70	0.999	523	48.642	0.01889	0.00341	0.05003			589	3.14×10^{-3}
(n=72)[1]	CP	756	49	0.987	94	6.899	0.00913	0.00090		19.394	0.026	103	1.89×10^{-3}
	Rep	1050	60	0.994	252	25.454	0.02438	0.00554		51.992	0.050	290	3.84×10^{-3}
	TrAP	390	39	0.948	61	5.374	0.01378	0.00160		12.585	0.032	65	2.31×10^{-3}
	REn	399	33	0.888	53	4.422	0.01108	0.00144		10.935	0.027	57	1.98×10^{-3}
	C4	258	22	0.790	60	6.333	0.02455	0.00659		12.379	0.048	66	3.55×10^{-3}
Ecuador	Genome	2601	61	0.999	301	22.03	0.00853	0.00036	0.02723			321	1.96×10^{-3}
(n=63)	CP	756	43	0.987	71	4.933	0.00653	0.00048		15.067	0.020	77	1.62×10^{-3}
	Rep	1050	52	0.993	123	8.425	0.00802	0.00044		26.101	0.025	131	1.98×10^{-3}
	TrAP	390	31	0.932	42	3.635	0.00932	0.00101		8.913	0.023	44	1.79×10^{-3}
	REn	399	25	0.854	35	2.792	0.00700	0.00095		7.427	0.019	36	1.43×10^{-3}
	C4	258	19	0.745	18	1.328	0.00515	0.00065		3.820	0.015	19	1.17×10^{-3}
Peru	Genome	2601	9	1.000	288	133.583	0.05174	0.00738	0.04629			309	1.32×10^{-2}
(n=9)	CP	756	6	0.833	31	12.194	0.01613	0.00246		11.406	0.015	32	4.70×10^{-3}
	Rep	1044	8	0.972	168	82.389	0.07892	0.01145		61.813	0.059	180	1.90×10^{-2}
	TrAP	390	8	0.972	22	8.417	0.02158	0.00274		8.095	0.021	24	6.84×10^{-3}
	REn	399	8	0.972	21	6.861	0.01720	0.00290		7.727	0.019	23	6.40×10^{-3}
	C4	258	4	0.750	48	24.222	0.09388	0.01342		17.661	0.068	51	2.20×10^{-2}

[1]n: number of isolates.

[2] L: length of the sequence in nucleotides; H: number of haplotypes; Hd: haplotype diversity; s: total number of segregating sites; k: average number of nucleotide differences between sequences; π: average number of nucleotide differences per site; σ_π: standard deviation of the average number of nucleotide differences per site; η: total number of mutations; θ_W: Watterson's estimate of the population mutation rate, θ.

Table 2. Selection pressure on different genomic regions of the *Tomato leaf deformation virus* (ToLDeV) population (Ecuador and Peru, n=72), determined with the SLAC method implemented in the Datamonkey server.

Gene	d_N/d_S	Negatively selected sites	*p*-value
CP	0.11247	18	0.0470949
		22	0.0470949
		46	0.0770462
		79	0.0371088
		85	0.0183345
		94	0.0470949
		98	0.0470949
		118	0.0470949
		120	0.0470949
		129	0.0102202
		151	0.0470949
		182	0.037037
		202	0.0470949
		206	0.0603092
Rep	0.338544	6	0.0471239
		9	0.0338763
		20	0.037037
		45	0.0702014
TrAP	0.913917	*	*
REn	0.343334	22	0.049483
		35	0.0488859
C4	0.634406	49	0.0427925

* no negatively selected sites found.

Table 3. Results of the neutrality test for the CP, Rep, Trap, Ren and C4 genes of *Tomato leaf deformation virus* (ToLDeV) population.

Population	**Gene**	**Tajima's *D***	**Fu and Li's *D****	**Fu and Li's *F****	**Fu's F_S**
All	CP	-2.30225**	-3.32443**	-3.49583**	-39.916
(n=72)	Rep	-2.00152*	-1.01222 (NS)	-1.70225 (NS)	-23.982
	TrAP	-2.00502*	-2.13369 (NS)	-2.49596*	-26.305
	REn	-2.07305*	-2.15323 (NS)	-2.54153*	-20.070
	C4	-1.79099 (NS)	0.28418 (NS)	-0.63721 (NS)	-2.799
Ecuador	CP	-2.39044**	-4.21337**	-4.20100**	-41.113
(n=63)	Rep	-2.42811**	-4.29945**	-4.27103**	-47.433
	TrAP	-2.02897*	-2.01229 (NS)	-2.41258*	-22.607
	REn	-2.07705*	-1.55865 (NS)	-2.09143 (NS)	-16.613
	C4	-2.05208*	-3.25156*	-3.35930**	-16.708
Peru	CP	0.17956 (NS)	-0.09150 (NS)	-0.02901 (NS)	2.050
(n=9)	Rep	1.26701 (NS)	1.33115 (NS)	1.47905 (NS)	2.781
	TrAP	-0.23271 (NS)	-0.32946 (NS)	-0.34315 (NS)	-1.416
	REn	-0.93773 (NS)	-0.90977 (NS)	-1.02745 (NS)	-1.893
	C4	1.48346 (NS)	1.47846*	1.66166*	8.743

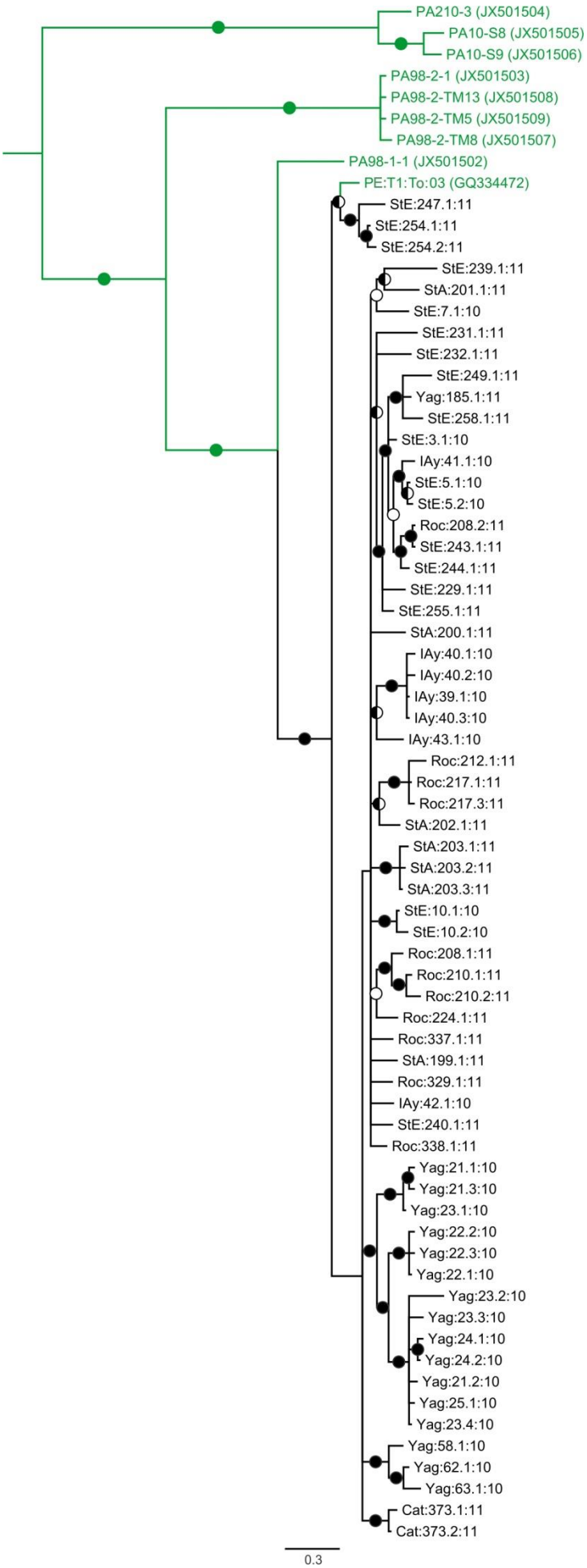
Tajima's *D*, Fu and Li's *D** and *F** measure the departure from neutrality for all mutations in a DNA region. These tests asume constant population size, no recombination, and no migration. Fu's *Fs* tests whether the departure from neutrality is the result of population growth or background selection. NS, not significant; *p<0.05; **0.01<p<0.02

Figure legends

Figure 1. Phylogenetic consensus tree, obtained by Bayesian inference, based on the complete nucleotide sequences of *Tomato leaf deformation virus* (ToLDeV) isolates from Ecuador (in black) and Peru (in green).Nodes with posterior probabilities equal or higher than 95% are indicated by filled circles, those between 80 and 94% by half-filled circles, and those between 50 and 79% by empty circles.

Figure 2. Structure bar plot indicating the probability (y-axis) of each isolate from *Tomato leaf deformation virus* (ToLDeV) belonging to different *K*=2 subpopulations, where each subpopulation is indicated by red (Ecuador) and green (Peru). Each vertical bar represents one isolate belonging to one of the two subpopulations.

Figure 1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PA10-S8 (JX501505)
PA10-S9 (JX501506)
PA98-2-1 (JX501503)
PA98-2-TM13 (JX501508)
PA98-2-TM5 (JX501509)
PA98-2-TM8 (JX501507)
PA98-1-1 (JX501502)
PE:T1:To:03 (GQ334472)
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StE:254.1:11
StE:254.2:11
StE:239.1:11
StA:201.1:11
StE:7.1:10
StE:231.1:11
StE:232.1:11
StE:249.1:11
Yag:185.1:11
StE:258.1:11
StE:3.1:10
IAy:41.1:10
StE:5.1:10
StE:5.2:10
Roc:208.2:11
StE:243.1:11
StE:244.1:11
StE:229.1:11
StE:255.1:11
StA:200.1:11
IAy:40.1:10
IAy:40.2:10
IAy:39.1:10
IAy:40.3:10
IAy:43.1:10
Roc:212.1:11
Roc:217.1:11
Roc:217.3:11
StA:202.1:11
StA:203.1:11
StA:203.2:11
StA:203.3:11
StE:10.1:10
StE:10.2:10
Roc:208.1:11
Roc:210.1:11
Roc:210.2:11
Roc:224.1:11
Roc:337.1:11
StA:199.1:11
Roc:329.1:11
IAy:42.1:10
StE:240.1:11
Roc:338.1:11
Yag:21.1:10
Yag:21.3:10
Yag:23.1:10
Yag:22.2:10
Yag:22.3:10
Yag:22.1:10
Yag:23.2:10
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Yag:24.2:10
Yag:21.2:10
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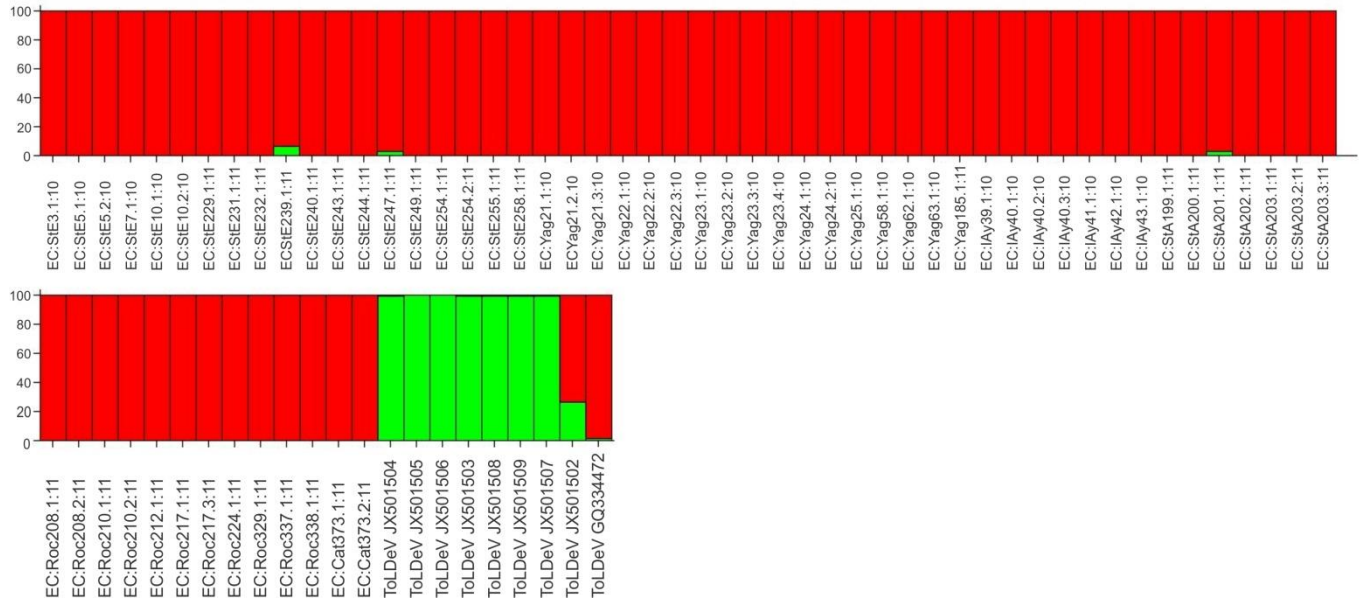
Cat:373.2:11
0.3

Figure 2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ECYag21.2.10
EC:Yag21.3:10
EC:Yag22.1:10
EC:Yag22.2:10
EC:Yag22.3:10
EC:Yag23.1:10
EC:Yag23.2:10
EC:Yag23.3:10
EC:Yag23.4:10
EC:Yag24.1:10
EC:Yag24.2:10
EC:Yag25.1:10
EC:Yag58.1:10
EC:Yag62.1:10
EC:Yag63.1:10
EC:Yag185.1:11
EC:IAy39.1:10
EC:IAy40.1:10
EC:IAy40.2:10
EC:IAy40.3:10
EC:IAy41.1:10
EC:IAy42.1:10
EC:IAy43.1:10
EC:StA199.1:11
EC:StA200.1:11
EC:StA201.1:11
EC:StA202.1:11
EC:StA203.1:11
EC:StA203.2:11
EC:StA203.3:11
100
80
60
40
20
0
EC:Roc208.1:11
EC:Roc208.2:11
EC:Roc210.1:11
EC:Roc210.2:11
EC:Roc212.1:11
EC:Roc217.1:11
EC:Roc217.3:11
EC:Roc224.1:11
EC:Roc329.1:11
EC:Roc337.1:11
EC:Roc338.1:11
EC:Cat373.1:11
EC:Cat373.2:11
ToLDeV JX501504
ToLDeV JX501505
ToLDeV JX501506
ToLDeV JX501503
ToLDeV JX501508
ToLDeV JX501509
ToLDeV JX501507
ToLDeV JX501502
ToLDeV GQ334472

Supplementary Table S1. Isolates of *Tomato leaf deformation virus* (ToLDeV) obtained from tomato samples collected in four provinces of Ecuador during 2010 and 2011. Underlined isolates were removed from the data set as their sequences were identical to those of other isolates.

Year of collection	Province	County	Location of collection	GPS coordinates	Isolate name
2010	Guayas	Yaguachi	El Cóndor	2° 8' 6.0678" S 79° 40' 6.9888" W	EC:Yag21.1:10
		Yaguachi	El Cóndor	2° 8' 6.0678" S 79° 40' 6.9888" W	EC:Yag21.2:10
		Yaguachi	El Cóndor	2° 8' 6.0678" S 79° 40' 6.9888" W	EC:Yag21.3:10
		Yaguachi	El Cóndor	2° 8' 5.9094" S 79° 40' 9.57" W	EC:Yag22.1:10
		Yaguachi	El Cóndor	2° 8' 5.9094" S 79° 40' 9.57" W	EC:Yag22.2:10
		Yaguachi	El Cóndor	2° 8' 5.9094" S 79° 40' 9.57" W	EC:Yag22.3:10
		Yaguachi	El Cóndor	2° 8' 5.7804" S 79° 40' 9.7896" W	EC:Yag23.1:10
		Yaguachi	El Cóndor	2° 8' 5.7804" S 79° 40' 9.7896" W	EC:Yag23.2:10
		Yaguachi	El Cóndor	2° 8' 5.7804" S 79° 40' 9.7896" W	EC:Yag23.3:10
		Yaguachi	El Cóndor	2° 8' 5.7804" S 79° 40' 9.7896" W	EC:Yag23.4:10
		Yaguachi	El Cóndor	2° 8' 5.8302" S 79° 40' 9.4182" W	EC:Yag24.1:10
		Yaguachi	El Cóndor	2° 8' 5.8302" S 79° 40' 9.4182" W	EC:Yag24.2:10
		Yaguachi	El Cóndor	2° 8' 5.8302" S 79° 40' 9.3894" W	EC:Yag25.1:10
		Yaguachi	El Cóndor	2° 8' 5.8302" S 79° 40' 9.3894" W	EC:Yag25.2:10
		Yaguachi	La Palma	2° 13' 3.7992" S 79° 40' 9.2604" W	EC:Yag58.1:10
		Yaguachi	La Palma	2° 13' 3.6588" S 79° 40' 9.231" W	EC:Yag62.1:10
		Yaguachi	La Palma	2° 13' 3.6402" S 79° 40' 9.2094" W	EC:Yag63.1:10
		Isidro Ayora	Las Mercedes	1° 59' 1.6404" S 80° 10' 1.509" W	EC:IAy39.1:10
		Isidro Ayora	Las Mercedes	1° 59' 1.5612" S 80° 10' 1.491" W	EC:IAy40.1:10
		Isidro Ayora	Las Mercedes	1° 59' 1.5612" S 80° 10' 1.491" W	EC:IAy40.2:10
		Isidro Ayora	Las Mercedes	1° 59' 1.5612" S 80° 10' 1.491" W	EC:IAy40.3:10
		Isidro Ayora	Las Mercedes	1° 59' 1.431" S 80° 10' 1.4196" W	EC:IAy41.1:10
		Isidro Ayora	Las Mercedes	1° 59' 1.3194" S 80° 10' 1.2792" W	EC:IAy42.1:10
		Isidro Ayora	Las Mercedes	1° 59' 1.2984" S 80° 10' 1.2894" W	EC:IAy43.1:10
	Santa Elena	Santa Elena	Chanduy-Río Verde	2° 16' 18.1482" S 80° 43' 23.3832" W	EC:StE3.1:10
		Santa Elena	Chanduy-Río Verde	2° 16' 21.5142" S 80° 43' 23.0736" W	EC:StE5.1:10
		Santa Elena	Chanduy-Río Verde	2° 16' 21.5142" S 80° 43' 23.0736" W	EC:StE5.2:10
		Santa Elena	Chanduy-Río Verde	2° 16' 13.3206" S80° 43' 22.461" W	EC:StE7.1:10
		Santa Elena	Chanduy-Río Verde	2° 11' 26.934" S80° 43' 45.7536" W	EC:StE10.1:10
		Santa Elena	Chanduy-Río Verde	2° 11' 26.934" S80° 43' 45.7536" W	EC:StE10.2:10
2011	Guayas	Yaguachi	San Jacinto	2° 10' 24.9" S79° 42' 35.5" W	EC:Yag185.1:11
	Loja	Catamayo	La Era	3° 59' 40.24" S 79° 18' 13.68" W	EC:Cat373.1:11
		Catamayo	La Era	3° 59' 40.24" S 79° 18' 13.68" W	EC:Cat373.2:11

Supplementaty Table S1 (cont.)

Year of collection	Province	County	Location of collection	GPS coordinates	Isolate name
2011	Manabí	Santa Ana	San Ignacio-Colón	1° 06' 43.5" S80° 24' 71.3" W	EC:StA199.1:11
		Santa Ana	San Ignacio-Colón	1° 06' 43.6" S80° 24' 71.5" W	EC:StA200.1:11
		Santa Ana	San Ignacio-Colón	1° 06' 42.8" S80° 24' 72.6" W	EC:StA201.1:11
		Santa Ana	San Ignacio-Colón	1° 06' 25.2" S80° 24' 44.3" W	EC:StA202.1:11
		Santa Ana	San Ignacio-Colón	1° 06' 25.2" S80° 24' 44.3" W	EC:StA202.2:11
		Santa Ana	San Ignacio-Colón	1° 06' 43.2" S80° 24' 73.2" W	EC:StA203.1:11
		Santa Ana	San Ignacio-Colón	1° 06' 43.2" S80° 24' 73.2" W	EC:StA203.2:11
		Santa Ana	San Ignacio-Colón	1° 06' 43.2" S80° 24' 73.2" W	EC:StA203.3:11
		Rocafuerte	El Cordón-Danzarín	0° 54' 42.5" S80° 23' 44.5" W	EC:Roc208.1:11
		Rocafuerte	El Cordón-Danzarín	0° 54' 42.5" S80° 23' 44.5" W	EC:Roc208.2:11
		Rocafuerte	Recta de Rocafuerte	0° 54' 64.1" S80° 25' 63.1" W	EC:Roc210.1:11
		Rocafuerte	Recta de Rocafuerte	0° 54' 64.1" S80° 25' 63.1" W	EC:Roc210.2:11
		Rocafuerte	Recta de Rocafuerte	0° 54' 62.7" S80° 25' 63.7" W	EC:Roc212.1:11
		Rocafuerte	Recta de Rocafuerte-Charapotó	0° 55' 06.7" S80° 26' 34.9" W	EC:Roc217.1:11
		Rocafuerte	Recta de Rocafuerte-Charapotó	0° 55' 06.7" S80° 26' 34.9" W	EC:Roc217.2:11
		Rocafuerte	Recta de Rocafuerte-Charapotó	0° 55' 06.7" S80° 26' 34.9" W	EC:Roc217.3:11
		Rocafuerte	Recta de Rocafuerte-Charapotó	0° 55' 06.7" S80° 26' 34.9" W	EC:Roc217.4:11
		Rocafuerte	Recta de Rocafuerte-Charapotó	0° 55' 11.2" S80° 26' 34.9" W	EC:Roc224.1:11
		Rocafuerte	Danzarín	0° 54' 26.0" S8O° 23' 24.2" W	EC:Roc329.1:11
		Rocafuerte	Danzarín	0° 54' 26.8" S80° 23' 24.8" W	EC:Roc337.1:11
		Rocafuerte	Danzarín	0° 54' 27.6" S80° 23' 25.7" W	EC:Roc338.1:11
	Santa Elena	Santa Elena	Chanduy-Río Verde	2° 17' 44.9" S80° 43' 45.2" W	EC:StE229.1:11
		Santa Elena	Chanduy-Río Verde	2° 17' 46.3" S80° 43' 45.3" W	EC:StE231.1:11
		Santa Elena	Chanduy-Río Verde	2° 17' 45.5" S80° 43' 48.5 W	EC:StE232.1:11
		Santa Elena	Chanduy-Río Verde	2° 17' 44.8" S80° 43' 45.0" W	EC:StE239.1:11
		Santa Elena	Chanduy-Río Verde	2° 17' 45.0" S80° 43' 44.6" W	EC:StE240.1:11
		Santa Elena	El Azúcar	2° 17' 14.0" S80° 35' 29.0" W	EC:StE243.1:11
		Santa Elena	El Azúcar	2° 17' 13.4" S80° 35' 30.8" W	EC:StE244.1:11
		Santa Elena	El Azúcar	2° 17' 13.7" S80° 35' 29.0" W	EC:StE247.1:11
		Santa Elena	Chanduy-Río Verde	2° 17' 47.0" S80° 43' 43.5" W	EC:StE249.1:11
		Santa Elena	Chanduy-Río Verde	2° 17' 45.6" S80° 43' 47.4" W	EC:StE254.1:11
		Santa Elena	Chanduy-Río Verde	2° 17' 45.6" S80° 43' 47.4" W	EC:StE254.2:11
		Santa Elena	Chanduy-Río Verde	2° 17' 46.6" S80° 43' 47.4" W	EC:StE255.1:11
		Santa Elena	Chanduy-Río Verde	2° 17' 48.3" S80° 43' 49.9" W	EC:StE258.1:11