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GENETIC DIVERSITY AND EVOLUTION

How To Be a Successful Monopartite Begomovirus in a Bipartite-Dominated World: Emergence and Spread of Tomato Mottle Leaf Curl Virus in Brazil

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ABSTRACT Begomoviruses are members of the family *Geminiviridae*, a large and diverse group of plant viruses characterized by a small circular single-stranded DNA genome encapsidated in twinned quasi-icosahedral virions. Cultivated tomato (*Solanum lycopersicum* L.) is particularly susceptible and is infected by >100 bipartite and monopartite begomoviruses worldwide. In Brazil, 25 tomato-infecting begomoviruses have been described, most of which are bipartite. Tomato mottle leaf curl virus (ToMoLCV) is one of the most important of these and was first described in the late 1990s but has not been fully characterized. Here, we show that ToMoLCV is a monopartite begomovirus with a genomic DNA similar in size and genome organization to those of DNA-A components of New World (NW) begomoviruses. Tomato plants agroinoculated with the cloned ToMoLCV genomic DNA developed typical tomato mottle leaf curl disease symptoms, thereby fulfilling Koch's postulates and confirming the monopartite nature of the ToMoLCV genome. We further show that ToMoLCV is transmitted by whiteflies, but not mechanically. Phylogenetic analyses placed ToMoLCV in a distinct and strongly supported clade with other begomoviruses from northeastern Brazil, designated the ToMoLCV lineage. Genetic analyses of the complete sequences of 87 ToMoLCV isolates revealed substantial genetic diversity, including five strain groups and seven subpopulations, consistent with a long evolutionary history. Phylogeographic models generated with partial or complete sequences predicted that the ToMoLCV emerged in northeastern Brazil >700 years ago, diversifying locally and then spreading widely in the country. Thus, ToMoLCV emerged well before the introduction of MEAM1 whiteflies, suggesting that the evolution of NW monopartite begomoviruses was facilitated by local whitefly populations and the highly susceptible tomato host.

IMPORTANCE Worldwide, diseases of tomato caused by whitefly-transmitted geminiviruses (begomoviruses) cause substantial economic losses and a reliance on insecticides for management. Here, we describe the molecular and biological properties of tomato mottle leaf curl virus (ToMoLCV) from Brazil and establish that it is a NW monopartite begomovirus indigenous to northeastern Brazil. This answered a long-standing question regarding the genome of this virus, and it is part of an emerging group of these viruses in Latin America. This appears to be driven by widespread planting of the highly susceptible tomato and by local and exotic whiteflies. Our extensive phylogenetic studies placed ToMoLCV in a distinct strongly supported clade with other begomoviruses from northeastern Brazil and revealed new insights into the origin of Brazilian begomoviruses. The novel phylogeographic analysis indicated that ToMoLCV has had a long evolutionary history, emerging in northeastern Brazil >700 years ago. Finally, the tools used here (agroinoculation system and ToMoLCV-

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specific PCR test) and information on the biology of the virus (host range and whitefly transmission) will be useful in developing and implementing integrated pest management (IPM) programs targeting ToMoLCV.

KEYWORDS begomovirus, geminivirus, monopartite begomovirus, phylogeography, tomato (*Solanum lycopersicum*), tomato-infecting begomovirus

The family *Geminiviridae* comprises a large and diverse group of plant-infecting viruses that have a small circular single-stranded-DNA (ssDNA) genome encapsidated in a twinned quasi-icosahedral virion (ca. 18 by 30 nm) (1). Many of these viruses cause economically important diseases of dicotyledonous and monocotyledonous crops (2). The family is composed of 14 genera defined based upon nucleotide sequence identity and phylogeny, genome organization, host range, and insect vector (1). The majority of species (>445) belong to the genus *Begomovirus*, and all infect dicotyledonous plants and are transmitted by whiteflies of the cryptic species complex *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (3, 4). These viruses are found mostly in tropical and subtropical regions of the world and cause a wide range of symptoms in field and vegetable crops (typically stunting and distorted growth and leaves with some combination of curling, crumpling, yellow mosaic/mottle, interveinal chlorosis, and vein swelling and purpling, as well as fruits of smaller size and poor quality) (2, 5–7). Whitefly transmission occurs in a circulative nonpropagative mode (4, 5), although transovarial transmission and organ (salivary glands)-specific replication of the monopartite begomovirus tomato yellow leaf curl virus (TYLCV) suggest that a more intricate virus-vector interaction may exist (8–10).

Begomoviruses have a phylogeographic distribution in which most viruses classified as Old World (OW) species possess a monopartite genome (~2.8 kb), whereas New World (NW) species mostly possess a bipartite genome of ~5.2 kb (3). Monopartite begomoviruses are phloem limited and not mechanically transmissible, with those from the OW possessing an additional gene (V2) and usually associated with small circular ssDNA satellites, e.g., the ~1.4 kb betasatellites that are required for pathogenicity and are encapsidated by the helper begomovirus capsid protein (CP) (3, 11–14). In contrast, bipartite begomoviruses have two ~2.6-kb DNA components (DNA-A and DNA-B) that are both required for development of typical disease symptoms. The DNA-A and DNA-B components are individually encapsidated (3, 15–17). Satellite DNAs are not necessary for symptom development and are not typically associated with these viruses. These components differ in sequence, except for a shared ~200-nucleotide (nt) common region (CR), which is >90% identical for cognate components of a species. The CR contains the origin of replication and promoter-like elements (18, 19). Some bipartite begomoviruses are not phloem limited and are mechanically transmissible (16, 20, 21).

The phylogeographic distribution of crop-infecting begomoviruses has become less clear, partly because movement of plant materials has resulted in long-distance spread. The most notorious of these is introduction and establishment of the OW monopartite TYLCV in the NW (4, 22, 23), and the introduction of the NW bipartite squash leaf curl virus (SLCuV) into the Middle East has caused increased economic losses in cucurbit crops (4). Indigenous NW monopartite begomoviruses were discovered associated with cultivated tomato (*Solanum lycopersicum* L.), e.g., tomato leaf deformation virus (ToLDeV) in Peru and Ecuador (24–26), tomato leaf curl purple vein virus (ToLCPVV) in Brazil (27), and tomato twisted leaf virus (ToTLV) in Venezuela (28). Finally, satellite DNAs (alphasatellites and deltasatellites) have been associated with a small number of NW bipartite begomoviruses (29–33).

In Brazil, begomovirus disease symptoms have been described in weeds and crops (common bean [*Phaseolus vulgaris* L.] and tomato) since the 1930s (34, 35) and were associated with indigenous *B. tabaci* populations (36). Bean golden mosaic caused by bean golden mosaic virus (BGMV) became economically important in Brazil, whereas

tomato crops were largely unaffected (35). However, following the introduction and establishment of the invasive Middle East-Asia Minor 1 (MEAM1 = biotype B) species of *Bemisia tabaci* in the early 1990s, there was an explosion of begomovirus diseases of tomato in Brazil (35, 37). MEAM1 whiteflies, which are highly polyphagous and reproduce at high rates, have mediated emergence and spread of begomovirus diseases of tomato worldwide, typically through evolution of local progenitor viruses (4, 5, 35, 38). The new begomovirus diseases in Brazil were associated with 25 novel, locally evolved tomato-infecting begomoviruses, most of which have a bipartite genome consistent with their NW origin. The most important are tomato severe rugose virus (ToSRV), which occurs in the central production regions, e.g., the states Goiás (GO), Minas Gerais (MG), and São Paulo (SP), and tomato mottle leaf curl virus (ToMoLCV), which occurs in the northeastern (NE) region, e.g., the states Piauí (PI), Bahia (BA), Pernambuco (PE), and Ceará (CE) (35, 39, 40). These begomovirus diseases dramatically impacted tomato production in Brazil, including a shift in production from the NE to the central region of the country (35).

ToMoLCV was first identified in the late 1990s in association with outbreaks of begomovirus disease symptoms (stunting and leaf curling, mottle, and vein swelling) in tomato in NE Brazil (BA, PE, Paraíba [PB] and Rio Grande do Norte [RN]) (41). However, ToMoLCV has yet to be fully characterized, including the nature of the genome, genetic diversity of the virus, and role in begomovirus disease of tomato in Brazil (41). The International Committee on Taxonomy of Viruses (ICTV) recognized ToMoLCV as a member of a new bipartite begomovirus species in 2013 based on sequence comparisons of partial DNA-A component sequences of PCR-amplified fragments (41, 42). The subsequent detection of DNA-A and DNA-B sequences in tomato mottle leaf curl disease (ToMoLCD) samples collected from the Federal District in 2004 was used to suggest that ToMoLCV has a bipartite genome (43). However, infectivity studies with infectious clones were not conducted, and analyses of ToMoLCD samples from other locations in Brazil failed to reveal evidence of a DNA-B component (39, 40, 44).

Here, we show that ToMoLCV is an indigenous NW monopartite begomovirus. The key line of evidence was the development of ToMoLCD symptoms in tomato plants agroinoculated with the cloned ToMoLCV genomic DNA. This fulfilled Koch's postulates for ToMoLCD and is fully consistent with the failure to detect a DNA-B component in ToMoLCD samples from 10 states of Brazil. Evidence is presented that ToMoLCV has a long evolutionary history in NE Brazil, and subsequently spread widely in the country. Thus, ToMoLCV found an evolutionary path to become a successful monopartite begomovirus in a bipartite-dominated world.

RESULTS

In order to determine the nature of the ToMoLCV genome and to conduct a comprehensive genetic analysis of ToMoLCV in Brazil, we first generated a collection of ToMoLCV isolates from tomato plants with typical ToMoLCD symptoms and from archival DNA extracts from such samples. Isolates of ToMoLCV were initially identified in infected samples based on (i) amplification of the expected-size ~1.1-kb fragment in PCR tests with the degenerate DNA-A primer pair PAL1v1978/PAR1c496 and (ii) the sequence of this fragment having >91% identity with any available ToMoLCV sequences. Based on these criteria, 35 isolates were recovered from the A samples, which were collected by us in surveys conducted during 2013 to 2016 (Fig. 1A to D). Thirteen isolates were recovered from DNA extracts of the begomovirus collection at Embrapa Vegetables (B samples), and 8 from the DNA extracts of the C samples. These 56 ToMoLCV isolates were from 10 states of Brazil, mostly in the NE, and were collected from 2002 to 2016 (see Table S1 in the supplemental material).

Nature of the ToMoLCV genome. In PCR tests with four degenerate DNA-B component primer pairs, the expected-size fragments were not amplified from DNA extracts of the 56 ToMoLCV isolates, whereas these fragments were amplified from a DNA extract of tomato leaves infected with the bipartite begomovirus ToSRV (data not

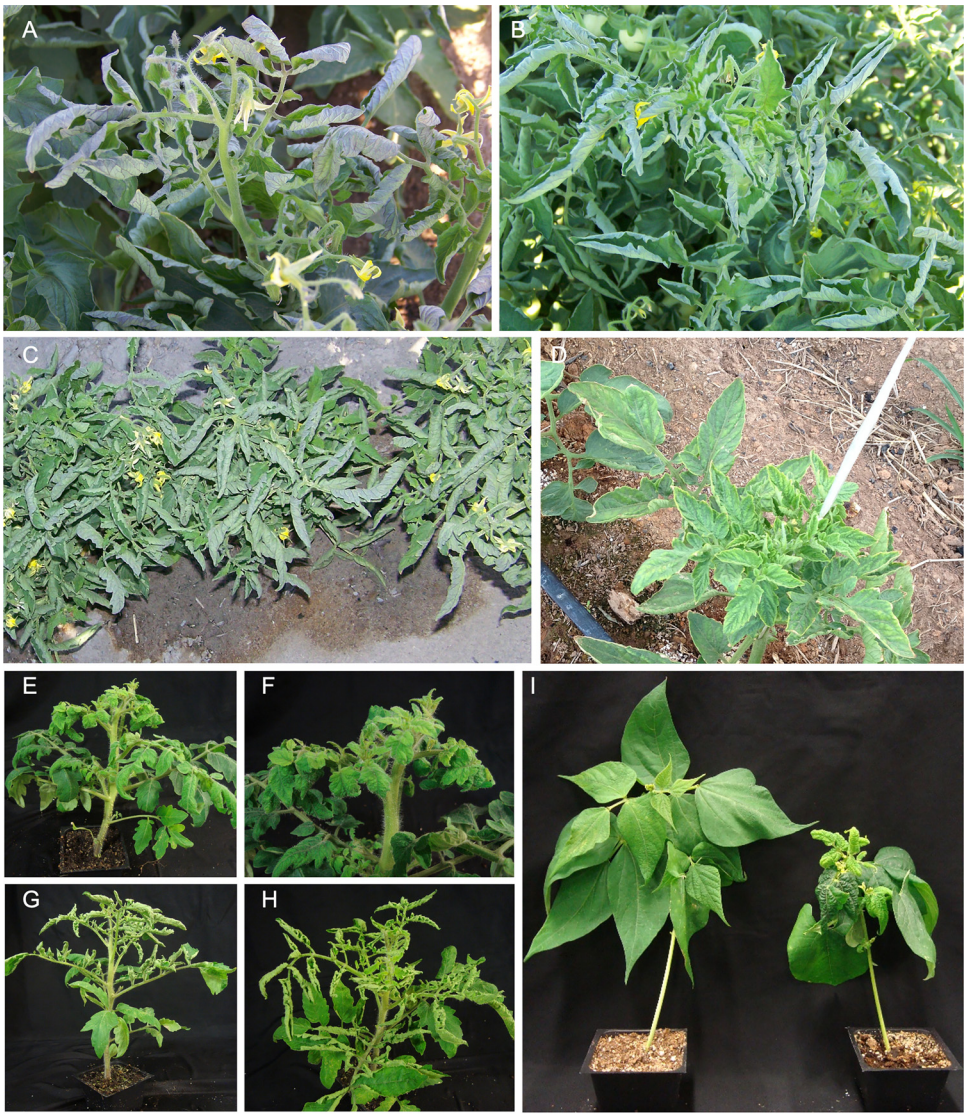

FIG 1 Symptoms of ToMoLCD caused by tomato mottle leaf curl virus (ToMoLCV). Typical (diagnostic) symptoms of ToMoLCD include stunting and erect upright growth and leaves with striking upcurling and rolling and vein swelling and purpling. (A to C) ToMoLCD symptoms observed in commercial processing tomato fields near Jaíba, Minas Gerais State, Brazil, in 2015 (A and B) and near Guadalupe, Piauí State, in 2014 (C). (D) Atypical symptoms, i.e., more similar to those caused by bipartite begomoviruses in Brazil, caused by ToMoLCV infection in a fresh market tomato plant in a commercial field in Sinop, Mato Grosso State, in 2016. (E and F) Symptoms induced in tomato plants (cultivar Glamour) agroinoculated with the infectious cloned multimeric genomic DNA of ToMoLCV isolate BR-BA-11; (G and H) symptoms induced in plants of processing tomato cultivar HMX3887; (I) symptoms induced in the common bean cultivar Topcrop (infected plant on the right and mock-inoculated plant on the left).

shown). These results suggested that these 56 ToMoLCV isolates do not possess a DNA-B component or that the DNA-B component is so divergent that these degenerate DNA-B primers do not anneal properly.

We next used rolling-circle amplification (RCA) and restriction fragment length polymorphism (RFLP) analysis with the four-base-cutting enzyme MspI, which is not based on sequence-specific amplification, to estimate the ToMoLCV genome size. Eight patterns were generated for the 56 isolates, but the fragment sizes added up to ~2.5 kb (Fig. S1). The frequency of the ToMoLCV RCA/RFLP patterns varied, and they were named 1 to 8 in order of most to least common (i.e., pattern 1 was most common). Comparisons revealed that pattern 1 was associated with ToMoLCV isolates from BA (BA strain group), the next two most common patterns (2 and 3) with isolates from PI

(PI strain group), and pattern 4 with isolates from MG (MG strain group). For the other patterns (patterns 5 to 8), the number of isolates was too small to make any associations. An equivalent RCA/RFLP analysis with MspI performed with the DNA extract of ToSRV-infected tomato leaves revealed six fragments that added up to ~5.2 kb, as expected for the bipartite genome of this virus. Thus, these results are fully consistent with the failure to detect a DNA-B component in the PCR tests and suggest that the genome of these ToMoLCV isolates may be composed of a single genomic DNA.

Digestion of RCA products with six-base-cutting enzymes revealed that the genomic DNA of 36 isolates was linearized by ApaI, whereas that of the other 20 isolates was linearized by NdeI (Table S1). These enzymes were then used to clone the ToMoLCV genomic DNA of all 56 isolates. The complete genome sequences were determined and the accession numbers for each isolate are presented in Table S1.

Analyses of the genomic DNA sequences of ToMoLCV isolates. Pairwise comparisons were next performed with sequences of the 56 ToMoLCV isolates collected in the present study and 31 ToMoLCV complete sequences retrieved from the GenBank. In Table S4, results are presented for comparisons made between BR-BA-11 (used here as an exemplary isolate) and the other 86 ToMoLCV genomic DNA sequences. The results of the complete sequence comparisons revealed identities ranging from 92.1 to 99.3% and indicated that these are isolates or strains of ToMoLCV (i.e., above the 91% species threshold). Using the 94% strain demarcation threshold, there are 42 isolates that can be divided into 20 with sequences nearly identical to those of the exemplary isolate (>97% identity across genes) and 22 divergent isolates (DIs) with lower identities (94.4 to 96.3%) due to 10 to 15% divergence in CR, C1, and C4 sequences (Table S4). For the 45 isolates that were classified as strains (<94% identity with the BR-BA-11 sequence), the level of divergence in these genes was greater (Table S4). For all 87 ToMoLCV isolates, the V1 (CP), C2 and C3 genes were more conserved (>92% identities). The C4 identities revealed an interesting feature in that the nucleotide sequences were highly conserved (~94 to 100%), whereas the amino acid sequences were substantially more divergent (82.5 to 100%), especially for strains (82.5 to 89.7%). However, the size of the C4 proteins of all 87 isolates is 98 amino acids (aa); all possess the N-terminal myristoylation domain identified in the C4 of TYLCV and associated with membrane targeting (45), and the amino acid composition is consistent with the C4s of ToMoLCV being intrinsically disordered proteins (IDP) (46).

The heat map of the Sequence Demarcation Tool (SDT) analysis of the 87 ToMoLCV genomic DNA sequences revealed identities ranging from 90.7 to 99.0% (Fig. S2). The sequence of each isolate had at least one value equal to or greater than the ICTV-recognized species threshold of 91% identity (6) to another ToMoLCV sequence. Thus, the SDT analysis also shows that the 87 sequences represent isolates of the species *Tomato mottle leaf curl virus*. Furthermore, the heat map also divided the isolates into 5 or possibly more strain groupings (isolates with sequences <94% sequence identity with those of other isolates).

Taken together, these results revealed a relatively high level of genetic diversity among ToMoLCV isolates and evidence of recombination (Table S4). To represent the diversity of the species in sequence comparisons, we selected 6 sequences as "core" ToMoLCV isolates for further studies. The isolates were chosen based on sequence divergence, geographical origin, and SDT strain groupings. The core isolates are BR-BA-11, BR-PI-14, BR-PB44-14, BR-MG1.1_5-15, BR-CE24_2-02, and BR-DFPADFM-04. The genomic DNA sequences of the core isolates were used in the phylogenetic analyses (species evolutionary relationships and the tanglegram).

Investigation of the genetic and biological properties of ToMoLCV isolates from two states of NE Brazil. The core isolates BR-BA-11 and BR-PI-14 had different RCA/RFLP patterns and origins and were selected for more extensive characterization. ToMoLCV BR-BA-11 has a rare RCA/RFLP pattern (type 8) and was collected in BA in 2011, whereas BR-PI-14 had the most common pattern (type 1) and was collected in PI in 2014. The sequence of the genomic DNA of both isolates is 2,630 nt, the size of a NW begomovirus genomic DNA/DNA-A component (3). The genome organization of

the ToMoLCV genomic DNAs of these isolates is similar to that of the genomic DNA/DNA-A component of NW begomoviruses, i.e., one virus-sense gene (V1) that encodes the CP and four complementary-sense genes (C1, C2, C3, and C4) that encode replication-associated protein (Rep), transcription activation protein (TrAP), replication enhancer protein (REn) and C4 protein, respectively. Finally, the CP amino acid sequences have the N-terminal motif PWRsMaGT and the REn proteins have the C-terminal motif AVRFATDK, both of which are characteristic of NW begomoviruses (47, 48). Taken together, these results indicate that ToMoLCV is a NW monopartite begomovirus.

The intergenic region (IR) between the start codons of the C1 and V1 open reading frames (ORFs) is 346 nt for both isolates and contains the characteristic geminivirus stem-loop structure with the conserved nonanucleotide sequence and nicking site (TAATATT↓AC), high-affinity Rep binding sites (iterons), the C1 TATA box, and the G box (49) (Fig. S3), GGTGTattGGGGT (iterons are underlined), as well as an upstream inverted repeat of the first iteron (ACACC). Both isolates also have the same iteron-related domain (IRD) amino acid sequence in the N terminus of Rep, including the FRIH motif, which is predicted to interact with the GGKGT core iteron (18), including the GGTGT and GGGGT iterons of ToMoLCV (Fig. S3). Results of comparisons and alignments of the IR and Rep IRD nucleotide and amino acid sequences, respectively, showed that there were 4 iterations of the core iterons and 8 iterations of the IRD motif, which were correlated with the iteron groups (data not shown).

Infectivity and host range experiments. To test the hypothesis that the ToMoLCV genomic DNA is infectious and can induce ToMoLCD and that ToMoLCV is a monopartite begomovirus, we next performed infectivity tests in which the multimeric cloned genomic DNA of ToMoLCV BR-BA-11 and ToMoLCV BR-PI-14 were each delivered into plants via agroinoculation. In initial experiments, *Nicotiana benthamiana* Domin. plants inoculated with the genomic DNA of either isolate were stunted, and leaves developed relatively mild downward curling and crumpling. Agroinoculated tomato plants (cultivar Glamour) were stunted, and leaves showed strong downward curling, mottling, and crumpling (Fig. 1E and F). The rates of infection (>95%) and symptoms were similar for both isolates. ToMoLCV infection in symptomatic leaves of *N. benthamiana* and tomato plants was confirmed based on the amplification of the expected-size ~1.3-kb DNA fragment in PCR tests with the ToMoLCV-specific primer pair. No symptoms were observed in control plants agroinoculated with the empty vector (EV), and no DNA fragment was amplified from newly emerged leaves of these plants. These results established the infectivity and pathogenicity of the genomic DNAs and supported the hypothesis that ToMoLCV is a NW monopartite begomovirus.

However, the symptoms induced by ToMoLCV in the tomato cultivar Glamour were not typical of ToMoLCD in the field, i.e., strong upward curling and rolling and striking vein swelling and purpling of leaves, and were more similar to those induced by bipartite begomoviruses such as ToSRV. This conundrum was resolved in the host range experiments. Here, plants of the processing tomato cultivar HMX3887 developed the typical ToMoLCD symptoms, i.e., stunting, erect upright growth and leaves with strong upward curling and rolling and striking vein swelling and purpling by 15 days postinoculation (dpi) (Fig. 1G and H). Notably, the symptoms induced in cultivar HMX3887 were similar to ToMoLCD symptoms observed in the field in Brazil in 2014 and 2015 (Fig. 1A to C, G, and H).

In other crop and indicator plants, similar results were obtained with both isolates (Table 1). Four categories of symptoms/infection were observed: (i) severe symptoms (e.g., stunting and leaf curling, crumpling, mosaic/mottle, and vein swelling and purpling), (ii) mild symptoms (slight downward leaf curling and crumpling, and vein swelling), (iii) symptomless infection, and (iv) absence of symptoms and infection. As previously noted, severe but different symptoms were observed in tomato cultivar Glamour (Fig. 1E and F) and cultivar HMX3887 (Fig. 1G and H). In addition, common bean (cultivar Topcrop) developed severe stunting and leaf curling, crumpling, and mottling (Fig. 1I). No recovery from these severe symptoms was observed (up to 40 dpi). Mild symptoms were observed in *N. benthamiana* and *Datura stramonium* L. (downward leaf curling and crumpling). ToMoLCV

TABLE 1 Host range and symptoms induced in plants agroinoculated with the cloned genomic DNA of ToMoLCV isolates BR-BA-11 and BR-PI-14

	BR-BA-11		BR-PI-14	
Plant species	**Infectivity[a]**	**Symptoms[b]**	**Infectivity[a]**	**Symptoms[b]**
Solanum lycopersicum cv. Glamour	43/44	Severe	45/46	Severe
Solanum lycopersicum cv. HMX3887	15/15	Severe	15/15	Severe
Nicotiana benthamiana	20/21	Mild	18/20	Mild
Nicotiana glutinosa	29/40	None	27/41	None
Nicotiana tabacum cv. Havana	12/29	None	17/31	None
Nicotiana tabacum cv. Turkish	14/32	None	15/32	None
Nicotiana tabacum cv. Samsun	23/40	None	25/39	None
Datura stramonium	13/27	Mild	33/35	Mild
Capsicum annuum cv. Cayenne	0/17	Not infected	0/20	Not infected
Solanum melongena cv. Black Beauty	0/26	Not infected	0/18	Not infected
Phaseolus vulgaris cv. Topcrop	27/30	Severe	28/29	Severe
Cucurbita pepo cv. Small Sugar	21/27	None	17/27	None
Chenopodium quinoa	0/29	Not infected	0/29	Not infected
Chenopodium amaranticolor	0/24	Not infected	0/24	Not infected

[a]Number of infected plants/total number inoculated. Infected plants were determined based on appearance of symptoms at 21 dpi and detection of viral DNA in newly emerged leaves by PCR tests with a ToMoLCV-specific primer pair (ToMoLCV373c/1694v). Results represent data from three independent inoculations.

[b]Symptoms were visually rated at 21 dpi. Plants were classified as having severe symptoms of stunting and leaf curling, crumpling, mottling, and vein swelling and purpling; mild symptoms of slight to moderate stunting and downward curling, crumpling, and mild vein swelling of leaves; or no obvious symptoms.

infection was confirmed in plants with severe (category i) and mild (category ii) symptoms by PCR tests with the ToMoLCV-specific primer pair.

Symptomless plants were infected (category iii) or not infected (category iv) based on results of PCR tests. Category iii hosts included *Nicotiana glutinosa* L., *Nicotiana tabacum* L. (cultivars Havana, Turkish, and Samsun), and pumpkin (*Cucurbita pepo* L. cv. Small Sugar). Rates of infection ranged from 41% for tobacco cultivar Havana to 77% for pumpkin and were lower than those in hosts that developed severe symptoms (>90%) (Table 1). The species that were not infected included *Chenopodium quinoa* Willd., *C. amaranticolor* Coste & Reyn., eggplant (*Solanum melongena* L.) cultivar Black Beauty, and pepper (*Capsicum annuum* L.) cultivar Cayenne.

In Brazil, the carioca-type common bean cultivar Pérola is preferred and is widely grown (50). This variety is also recalcitrant to agroinoculation. Therefore, the multimeric clone of ToMoLCV BR-BA-11 was used to bombard hypocotyls of seedlings of cultivar Pérola and Topcrop (18 seedlings/cultivar). None of these seedlings developed symptoms, whereas all 18 cultivar Topcrop seedlings bombarded with the infectious cloned DNA-A and DNA-B components of BGMV developed golden mosaic symptoms (data not shown). This demonstrated that the bombardment procedure was successful. However, the failure of ToMoLCV to infect bombarded cultivar Topcrop seedlings indicates that ToMoLCV does not infect common bean via this inoculation method.

Taken together, the infectivity of the ToMoLCV genomic DNA and induction of ToMoLCD symptoms fulfilled Koch's postulates for this disease and demonstrated that ToMoLCV is a NW monopartite begomovirus. Furthermore, ToMoLCV mostly infected species in the family Solanaceae, especially tomato, but also infected plants in the families Fabaceae (common bean) and Cucurbitaceae (pumpkin), though less efficiently.

Mechanical transmission. Some bipartite begomoviruses are readily mechanically transmissible, such as tomato mottle virus (ToMoV), but monopartite begomoviruses are typically not mechanically transmissible (3). In the experiments to assess this property for ToMoLCV, tomato seedlings (cultivar Glamour) mechanically inoculated with sap prepared with symptomatic ToMoLCV-infected tomato leaf tissue did not develop symptoms, nor was ToMoLCV DNA detected in newly emerged leaves at 15 dpi (18 plants inoculated). In contrast, all tomato seedlings mechanically inoculated with sap prepared with symptomatic ToMoV-infected tomato leaf tissue developed golden yellow mosaic symptoms in newly emerged leaves by 15 dpi, consistent with the sap transmissibility of this virus. Thus, these

TABLE 2 Whitefly (*Bemisia tabaci* MEAM1) transmission of ToMoLCV BR-BA-11 and BR-PI-14[a]

	BR-BA-11		BR-PI-14	
Plant species	**Infectivity[b]**	**Symptoms[c]**	**Infectivity[b]**	**Symptoms[c]**
Solanum lycopersicum cv. H9553	8/13	Severe	3/9	Severe
Phaseolus vulgaris cv. Pérola	3/10	None	0/10	Not infected
Phaseolus vulgaris cv. Topcrop	1/4	Mild	2/4	Mild

[a]*Bemisia tabaci* MEAM1 whiteflies were given an IAP of 48 h, following an AAP of 48 h on tomato (cultivar H9553) leaves infected with ToMoLCV BR-BA-11 or BR-PI-14 by agroinoculation.
[b]Number of infected plants/total number inoculated. Infection was determined in newly emerged leaves of all inoculated plants by PCR tests with a ToMoLCV-specific primer pair (373c/1694v) 15 days after removal of whiteflies. Results are from three independent experiments for tomato (cultivar H9553) and two independent experiments for common bean (cultivars Pérola and Topcrop).
[c]Symptoms were visually evaluated 15 days after removal of whiteflies. Plants with severe symptoms showed stunting and leaf curling, crumpling, green mottle, and vein swelling and purpling; whereas plants with mild symptoms showed mild downward leaf curling, crumpling, and vein swelling.

results indicate that ToMoLCV is not mechanically transmissible to tomato, consistent with a monopartite begomovirus.

Progeny virus of the ToMoLCV infectious clones is transmitted by *Bemisia tabaci* MEAM1. In nature, begomoviruses are transmitted by species of the *B. tabaci* complex (4, 5, 38). In Brazil, the MEAM1 species is currently predominant (35). Therefore, we next investigated the capacity of MEAM1 whiteflies to transmit progeny virus derived from the infectious cloned genomic DNAs of ToMoLCV BR-BA-11 and BR-PI-14. In these experiments, tomato plants (cultivar H9553) exposed to whiteflies provided a 48-h acquisition access period (AAP) on leaves of tomato plants infected with each ToMoLCV isolate developed typical ToMoLCD symptoms in newly emerged leaves by 15 dpi. Rates of transmission were 62% for ToMoLCV BR-BA-11 and 33% for BR-PI-14 (Table 2). Common bean seedlings (cultivar Topcrop) exposed to whiteflies viruliferous for ToMoLCV developed symptoms of mild mosaic, with rates of transmission of 25% for ToMoLCV BR-BA-11 and 50% for BR-PI-14. None of the cultivar Pérola seedlings exposed to whiteflies viruliferous for ToMoLCV developed symptoms. Control plants exposed to nonviruliferous whiteflies did not develop symptoms.

In PCR tests with the ToMoLCV-specific primer pair, the expected-size ~1.3-kb fragment was amplified from leaf samples of all symptomatic tomato and common bean plants. Symptomless infection was detected in 30% of cultivar Pérola plants exposed to whiteflies viruliferous for ToMoLCV BR-BA-11 (Table 2). No fragment was amplified from leaf samples of equivalent plants exposed to nonviruliferous whiteflies (Table 2). This established that a NW monopartite begomovirus is transmitted from plant to plant by MEAM1 whiteflies and induces ToMoLCD in whitefly-inoculated plants.

ToMoLCV is a member of a new lineage of NW begomoviruses and is composed of multiple strains. We next investigated the relationship of ToMoLCV with other begomovirus species. Sequence comparisons were initially performed with the complete genomic DNA sequences of the six ToMoLCV core isolates and sequences representing a wide range of begomoviruses (Table S6). The highest identities (77 to 79%) were with crop- and weed-infecting bipartite begomoviruses from NE Brazil (Table S6). Notably, identities with other NW monopartite begomoviruses ranged from 70 to 76%. These results are consistent with ToMoLCV being a distinct species that originated in NE Brazil.

The tree showing species evolutionary relationships is presented in Fig. 2 and comprises 58 complete sequences, including (i) the six ToMoLCV "core" isolates; (ii) the 10 begomoviruses with the highest identities with ToMoLCV BR-BA-11; (iii) DNA-A components of 21 tomato-infecting begomoviruses from Brazil; (iv) NW monopartite tomato-infecting begomoviruses; (v) representative members of the NW begomovirus lineages Abutilon mosaic virus (AbMV), Boerhavia golden mosaic virus (BoGMV), and squash leaf curl virus (SLCuV); and (vi) selected OW monopartite tomato-infecting species (Table S5). These sequences were placed into six strongly supported (posterior probability [pp], 1.0) clades, five of which correspond to the previously recognized AbMV, Brazil, BoGMV, and SLCuV NW lineages and the OW lineage (Fig. 2). The other strongly supported clade

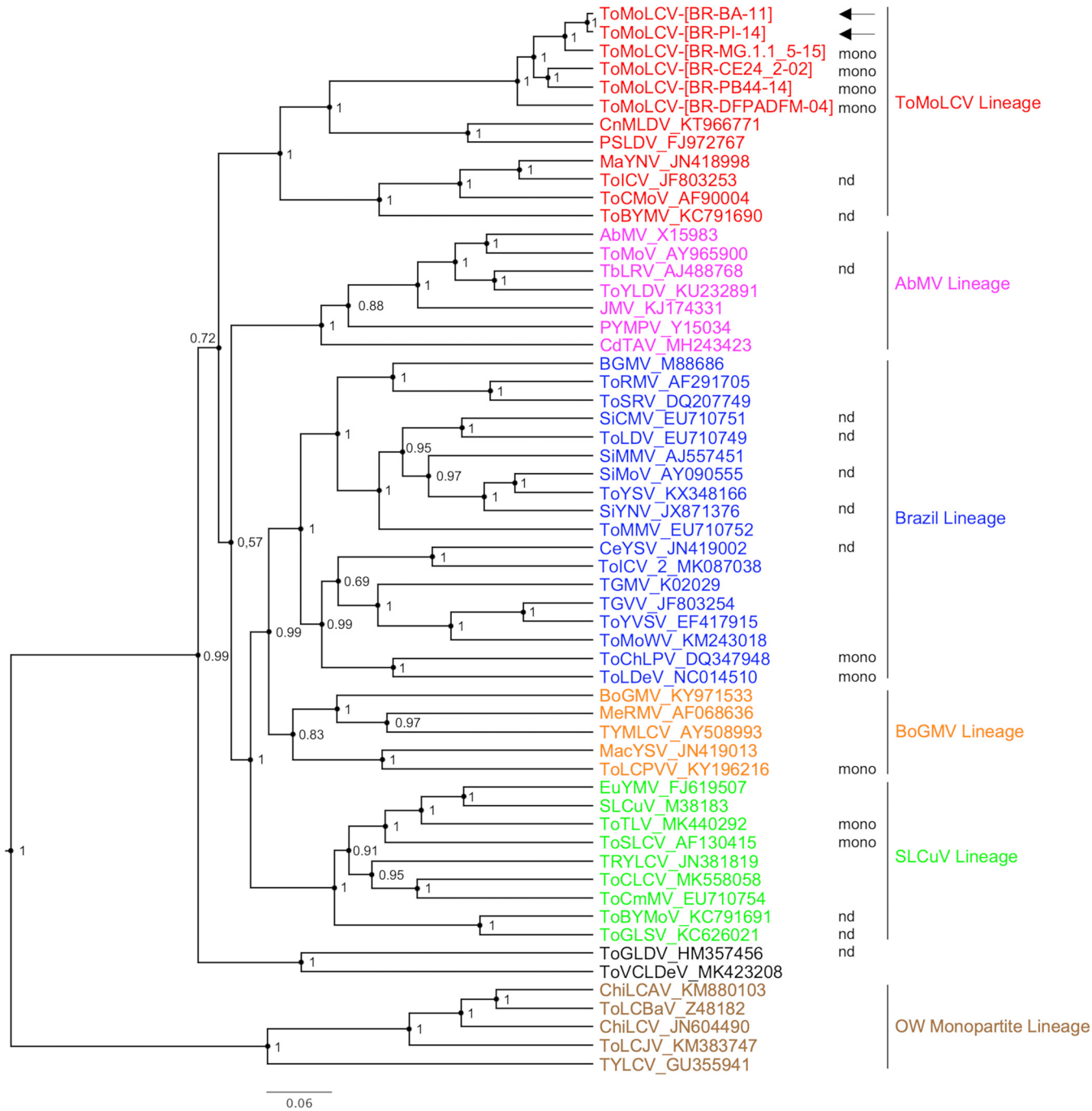

FIG 2 Bayesian phylogenetic consensus tree generated from an alignment of the complete nucleotide sequences of six ToMoLCV core isolates representing genetic diversity in the species; the 10 begomoviruses with highest identities with ToMoLCV based on a BLASTn search performed with the sequence of the ToMoLCV BR-BA-11 genomic DNA; 25 tomato-infecting begomoviruses from Brazil (some included in the second set); New World (NW) monopartite tomato-infecting begomoviruses; representative species of NW begomovirus lineages Brazil (blue), AbMV (magenta), BoGMV (orange), and SLCuV (green); and selected Old World (OW) monopartite tomato-infecting species (brown), for a total of 58 sequences. The ToMoLCV BR-BA-11 and BR-PI-14 isolates extensively characterized in the present study are indicated with arrows. Except for ToMoLCV, the sequence of one exemplary isolate of each species was used. Genome structure is indicated by "mono" (monopartite), "nd" (not determined), and no designation (bipartite). The phylogenetic analysis was performed with BEAST v.2.5. Branch strengths were evaluated by Bayesian posterior probabilities; nodes with values of >0.5 are shown. The length of horizontal branches corresponds to the rate of nucleotide substitution. GenBank accession numbers are given. See Table S5 for full virus names.

included the ToMoLCV core isolates and crop- and weed-infecting begomoviruses from NE Brazil (Fig. 2). Furthermore, within this lineage, the ToMoLCV isolates were placed in a strongly supported (pp, 1.0) subclade. The other members of this clade have bipartite or yet-to-be-characterized genomes and were placed in two other subclades (Fig. 2). These

results suggest that these viruses from NE Brazil constitute a distinct lineage of NW begomoviruses, for which the name "ToMoLCV lineage" is proposed.

In this phylogenetic tree, NW monopartite begomoviruses were placed in most lineages. For example, ToLDeV from Peru and Ecuador and tomato chino La Paz virus (ToChLPV) from Mexico were placed in the Brazil lineage and are related to bipartite tomato-infecting begomoviruses from Brazil, whereas the tomato severe leaf curl virus (ToSLCV) from Guatemala and ToTLV from Venezuela were placed in the SLCuV lineage. The OW monopartite tomato-infecting begomoviruses were placed in a strongly supported clade that was distantly related to those of the NW viruses.

Finally, of particular interest was the placement of ToLCPVV, another NW monopartite begomovirus species from NE Brazil (PI), in the BoGMV lineage. This lineage had a single member (BoGMV) known to occur only in the Dominican Republic (51). Macroptilium yellow spot virus (MacYSV) from Brazil and tomato yellow margin leaf curl virus (TYMLCV) from Venezuela also were placed in the strongly supported (pp, 0.83) BoGMV lineage. Moreover, the BoGMV and Brazil lineages were placed in a large strongly supported (pp, 0.99) clade, consistent with the evolutionary relationship between viruses in these lineages. Together, these results indicate that ToMoLCV is part of a newly identified lineage of NW begomoviruses and provide more evidence that NW monopartite begomoviruses are highly diverse and polyphyletic.

We next investigated the ToMoLCV strain relationships of the 87 ToMoLCV isolates. As shown in Fig. 3, the 87 isolates were placed into five strongly supported (pp, $\geq$0.85) strain groups, which are named with the abbreviation of the state from which the majority of isolates were collected. These included the previously described DF strain group (43) and four new groups: MG, BA, PI, and PB (Fig. 3). The previously described PE strain group, which had a single isolate collected in 2004, was changed to the MG strain group to reflect the fact that the greatest number of isolates were collected from this state (43). It is worth noting that seven isolates of the PB strain group were placed in the BA strain group in other analyses, indicating a more complex genetic relationship for these isolates (Fig. 3; Fig. S2).

The strain groups have a strong geographical association, especially isolates in the DF and PB groups, which may represent early founder events and subsequent diversification. This association is less clear for the other strain groups, in which there is considerate mixing; e.g., ToMoLCV BR-PI-14 was placed in the BA strain group, and a cluster of 4 isolates from MG were placed in the PI strain group (Fig. 3). The PI strain group was divided into two strongly supported clades, and members of this strain group were detected in the BA and PB strain groups. This suggested that PI may be a center of ToMoLCV diversity. The BA strain group is the most diverse and contains isolates from 8 states of Brazil, indicating extensive spread from this state. Finally, the PI and DF strain groups were placed in a strongly supported (pp, 0.98) clade, whereas the MG, BA, and PB groups were placed in another strongly supported (pp, 0.98) clade, suggesting an earlier separation event that was followed by the diversification into the five recognized strain groups (Fig. 3).

Tanglegram reveals evidence of convergent evolution and recombination in the C4/AC4 gene. Tanglegrams are used to compare two phylogenetic trees constructed with the same operational taxonomic units (OTUs). Bridges (lines) are drawn between the same OTUs, thereby providing insight into viral evolution and/or recombination (52). Thus, we merged the species evolutionary relationships tree (Fig. 2) and the tree generated with the C4/AC4 amino acid sequences. Note that tomato bright yellow mottle virus and Sida mottle virus lack this gene and were not included in the tanglegram analysis.

The tanglegram analysis showed that OTUs belonging to the ToMoLCV lineage were placed in well-supported clades in similar positions in the trees. Putative recombination events (REs) are revealed by placement of C4/AC4 sequences in lineages different from those in the species relationship tree. In this analysis, RE events were revealed in all lineages to various levels. This was seen with the topology of the ToMoLCV lineage, in which the ToMoLCV isolates were placed in a strongly supported clade in the species relationship

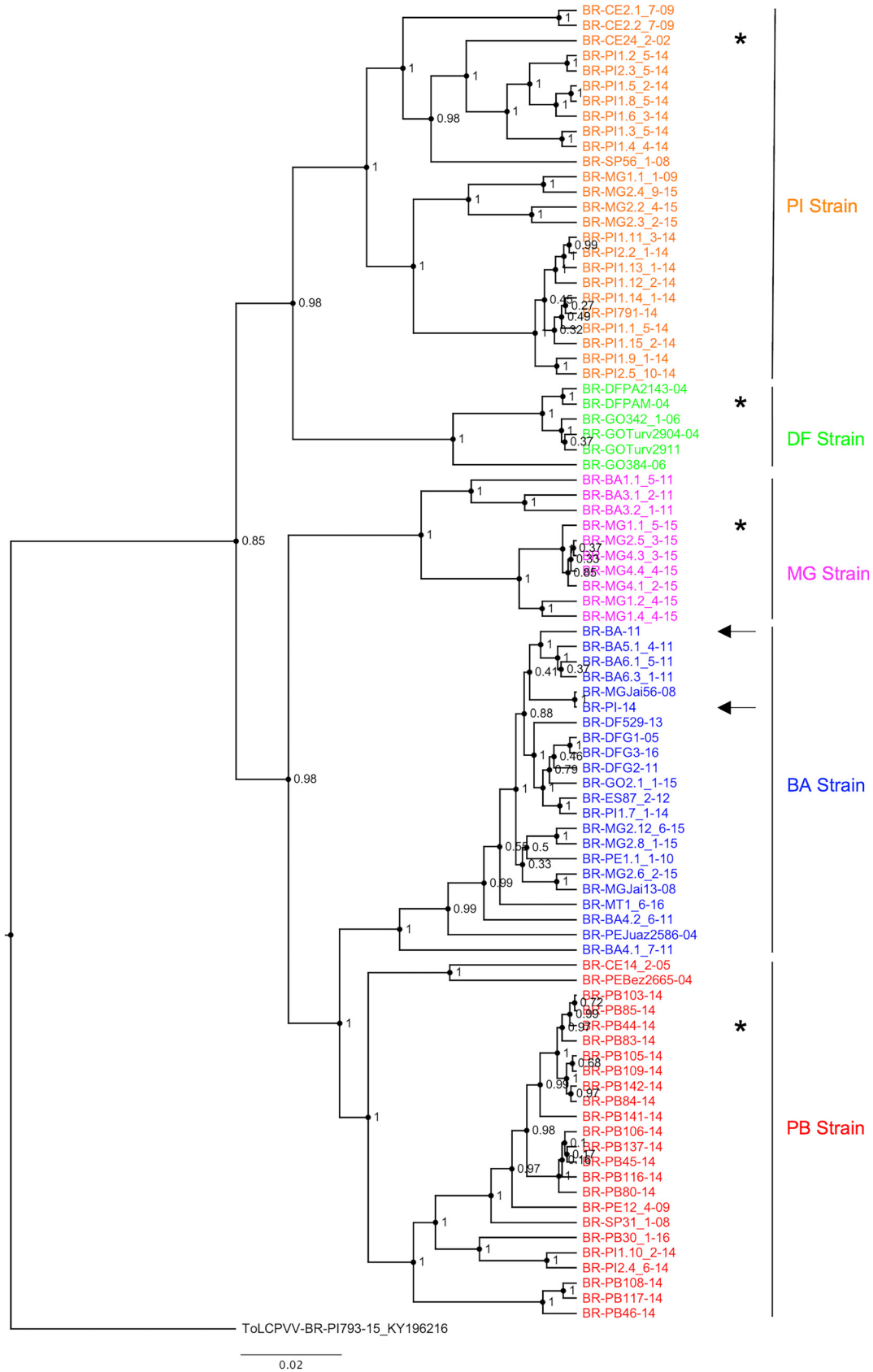

FIG 3 Phylogenetic consensus tree showing the relationship among the complete sequences of the genomic DNA of 87 isolates of ToMoLCV collected from 10 states of Brazil between 2002 and 2016. The ToMoLCV isolates BR-BA-11 and BR-PI-14

(Continued on next page)

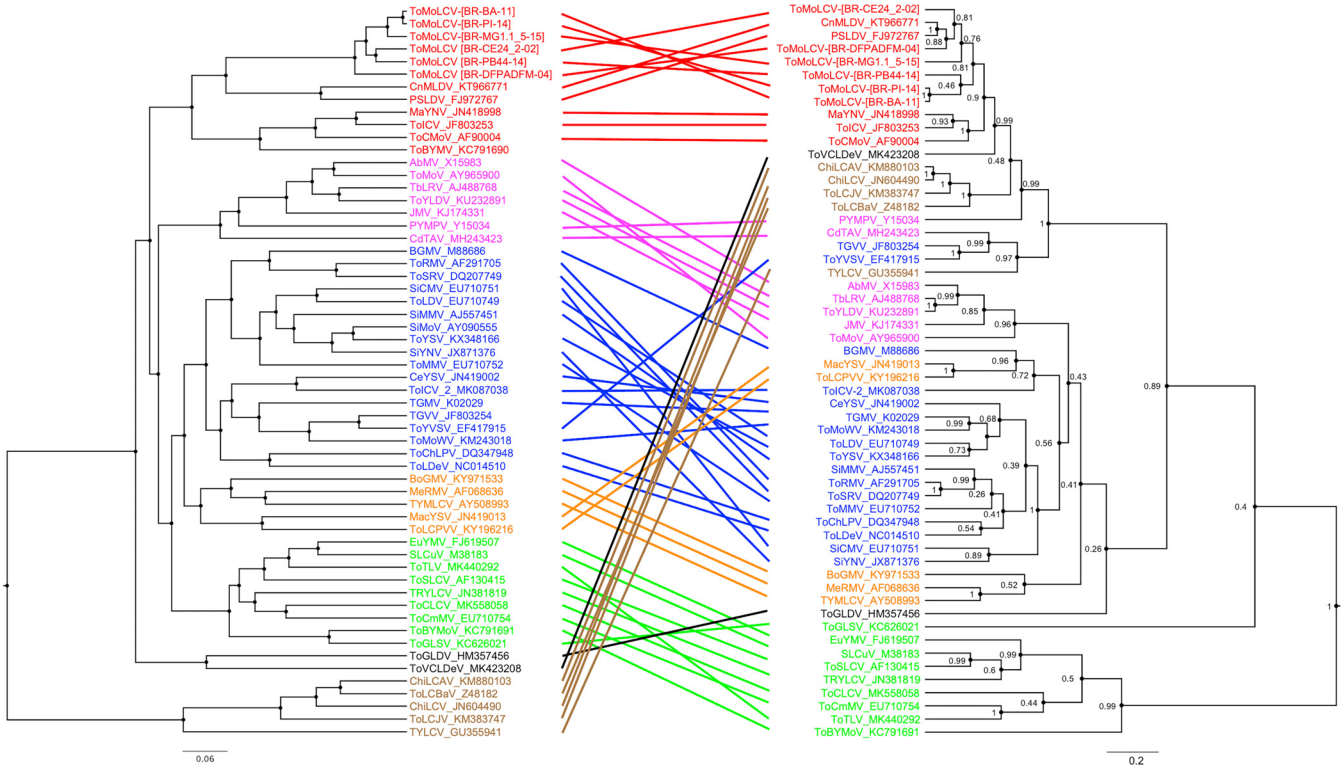

FIG 4 Tanglegram displaying combined phylogenetic consensus trees generated with the complete nucleotide sequences of the genomic DNA/DNA-A components (left) and the amino acids of the C4/AC4 proteins (right) of six ToMoLCV core isolates representing the genetic diversity in the species; the 10 begomoviruses with the highest identities with the ToMoLCV BR-BA-11 genomic DNA based on a BLASTn search; 25 tomato-infecting begomoviruses from Brazil (some included in the second group); New World (NW) monopartite tomato-infecting begomoviruses; representative species of the NW begomovirus lineages Brazil (blue), AbMV (magenta), BoGMV (orange), and SLCuV (green); and selected Old World (OW) monopartite tomato-infecting species (brown), for a total of 58 sequences. Except for ToMoLCV, the sequence of an exemplary isolate of each species was used. The phylogenetic analysis was performed with BEAST v.2.5. Branch strengths were evaluated by Bayesian posterior probabilities and are shown only for the C4/AC4 tree (see Fig. 2 for values for the tree generated with the genomic DNA/DNA-A components). The length of horizontal branches corresponds to the rate of nucleotide or amino acid substitution. GenBank accession numbers are given. See Table S5 for full virus names.

tree, whereas in the C4 tree, the ToMoLCV isolates were placed in a larger, poorly supported clade that included other begomovirus of the lineage (Fig. 4). This is suggestive of recombination or convergent evolution within the clade. The ToMoLCV C4 sequences were placed into a larger well-supported (pp, 1.0) clade that included begomoviruses from four lineages: (i) ToMoLCV, (ii) AbMV (potato yellow mosaic Panama virus [PYMPV] and chino del tomate Amazonas virus [CdTAV]), (iii) Brazil (tomato golden vein virus [TGVV] and tomato yellow vein streak virus [ToYVSV]), and (iv) viruses of the OW lineage (Fig. 4). The placement of NW and OW begomoviruses in this large C4/AC4 clade further contributed to the incongruence of these trees. Moreover, the placement of C4/AC4 sequences in different lineages than the species relationship tree based on the complete nucleotide sequence is fully consistent with the location of the C4/AC4 gene in the recombinational hot spot (20, 53). For example, an RE in the genomic DNA of the NW monopartite ToLCPVV that resulted in the acquisition of the AC4 gene of MacYSV was identified (27). As previously noted, ToLCPVV and MacYSV were placed in the BoGMV lineage, whereas they were placed together with BGMV in the Brazil lineage in the C4/AC4

FIG 3 Legend (Continued)

were extensively characterized and are indicated with arrows, whereas the other four core isolates used for phylogenetic analyses (Fig. 2 and 4) are indicated with asterisks. Five strain groups were identified: Piauí (PI) (orange), Federal District (DF) (green), Minas Gerais (MG) (magenta), Bahia (BA) (blue), and Paraíba (PB) (red). The genomic DNA of the New World monopartite begomovirus ToLCPVV from Brazil was used as an outgroup. The phylogenetic analysis was performed with BEAST v.2.5. Branch strengths were evaluated by Bayesian posterior probabilities and are shown at the nodes. The length of horizontal branches corresponds to the rate of nucleotide substitution. See Tables S1 and S3A in the supplemental material for ToMoLCV accession numbers.

tree, reflecting this recombination event that likely occurred before the diversification of these viruses (Fig. 4).

Recombination analyses. A major factor contributing to begomovirus diversity is recombination. Evidence for recombination in the ToMoLCV isolates was initially revealed from the pairwise sequence comparisons. Moreover, the potential recombinant sequences (IR, C1, and C4) pointed to the recombinational hot spot in the begomovirus genomic DNA/DNA-A component. To further investigate this, an alignment was generated with partial CR/IR sequences of the ToMoLCV core isolates and tomato chlorotic mottle virus (ToCMoV) isolates. As shown in Fig. S3, three REs were identified: RI, RII, and RIII. Consistent with these results, the Recombination Detection Program (RDP4) analyses performed with three data sets also revealed numerous REs (data not shown).

For the present study, we focused on RE RIII, which was detected in isolate BR-DFPADFM-04 (Fig. S3), because it may explain how a DNA-B component was detected in association with an isolate of ToMoLCV (43). RE RIII was also detected with the RDP analyses carried out with data sets 1 and 2. For example, with data set 2, which included the 58 begomoviruses used for the evolutionary relationships tree, RE RIII was detected with GeneConv, Bootscan, MaxChi, Chimaera, SIScan and 3Seq programs with RDP, and with *P* values of 3.61×10^{-22}, 4.26×10^{-25}, 2.34×10^{-12}, 1.37×10^{-15}, 4.71×10^{-15}, and 1.77×10^{-24}, respectively. Similar results were obtained with data set 1 (data not shown). The RE RIII event was detected in all six ToMoLCV isolates of the DF strain group, which were collected in DF and GO in 2004 and 2006 (Fig. 3). The event involved a 414-nt fragment spanning positions 2199 to 2613 and is in the recombinational hot spot. It includes sequences of the 5′ end of the Rep gene, the complete C4/AC4 gene, and the 5′ end of the CR. The recombinant sequence encodes the N-terminal region of the Rep protein with the IRD and contains the portion of the CR/IR (122 nt) with the corresponding Rep high-affinity binding site (Fig. S3) (53). The major parent was ToMoLCV, whereas the minor parent was ToCMoV, a bipartite tomato-infecting begomovirus also from NE Brazil. Furthermore, the alignments in Fig. S3 show that the iterons and IRD of the DNA-A and DNA-B components of ToMoLCV BR-DFPADFM-04 (43) are identical to those of ToCMoV (Fig. S3). Thus, the predicted recombinant Rep encoded by BR-DFPADFM-04 would direct the replication of the genomic DNA as well as the ToCMoV DNA-B component, thereby generating a bipartite ToMoLCV. Further evidence came from sequence comparisons showing that the complete nucleotide sequence of the ToMoLCV BR-DFPADFM-04 DNA-B component is 93.5% identical to that of the ToCMoV DNA-B component. It is important to note that after the preliminary evidences that ToMoLCV was a monopartite virus, the complete DNA-B sequence of ToMoLCV BR-DFPADFM-04 DNA-B was removed from GenBank.

Cluster analysis of the ToMoLCV population in Brazil. A cluster-based approach (using the program Structure) was used to infer the ancestry of ToMoLCV isolates through the identification of subpopulations (SPs). This analysis revealed seven SPs (SPs 1 to 7) and evidence of admixing (complex ancestry). Each SP was composed of isolates that share the largest percentage of ancestry (Fig. 5). In general, comparisons revealed that the SPs contained isolates with similar geographic origins and from the same strain group. However, there were exceptions, which may be explained by mixed ancestry that is not detected with SDT.

SP1 has 10 isolates, all from the MG strain group. Notably, three isolates were collected in BA, suggesting long-distance spread. The isolates collected in MG in 2015 had similar ancestry, whereas those from BA were substantially different and had extensive admixing. SP2 is composed of 16 isolates, all from the PB strain group. Of these isolates, 14 were collected in 2014 and have similar ancestry, whereas two isolates collected in 2008 and 2009 from SP and PE, respectively, had mixed ancestry, as did two isolates collected in 2014 (Fig. 5). Moreover, this mixed ancestry (shown in yellow in Fig. 5) was also found in the other SPs, but no isolates had this ancestry entirely (Fig. 5). This ancestry may reflect a progenitor virus. SP3 is composed of 6 isolates all from the DF strain group. These were collected in DF and GO in 2004 and 2006, and one had mixed ancestry with SP4. Three isolates are in SP4, two collected from CE in

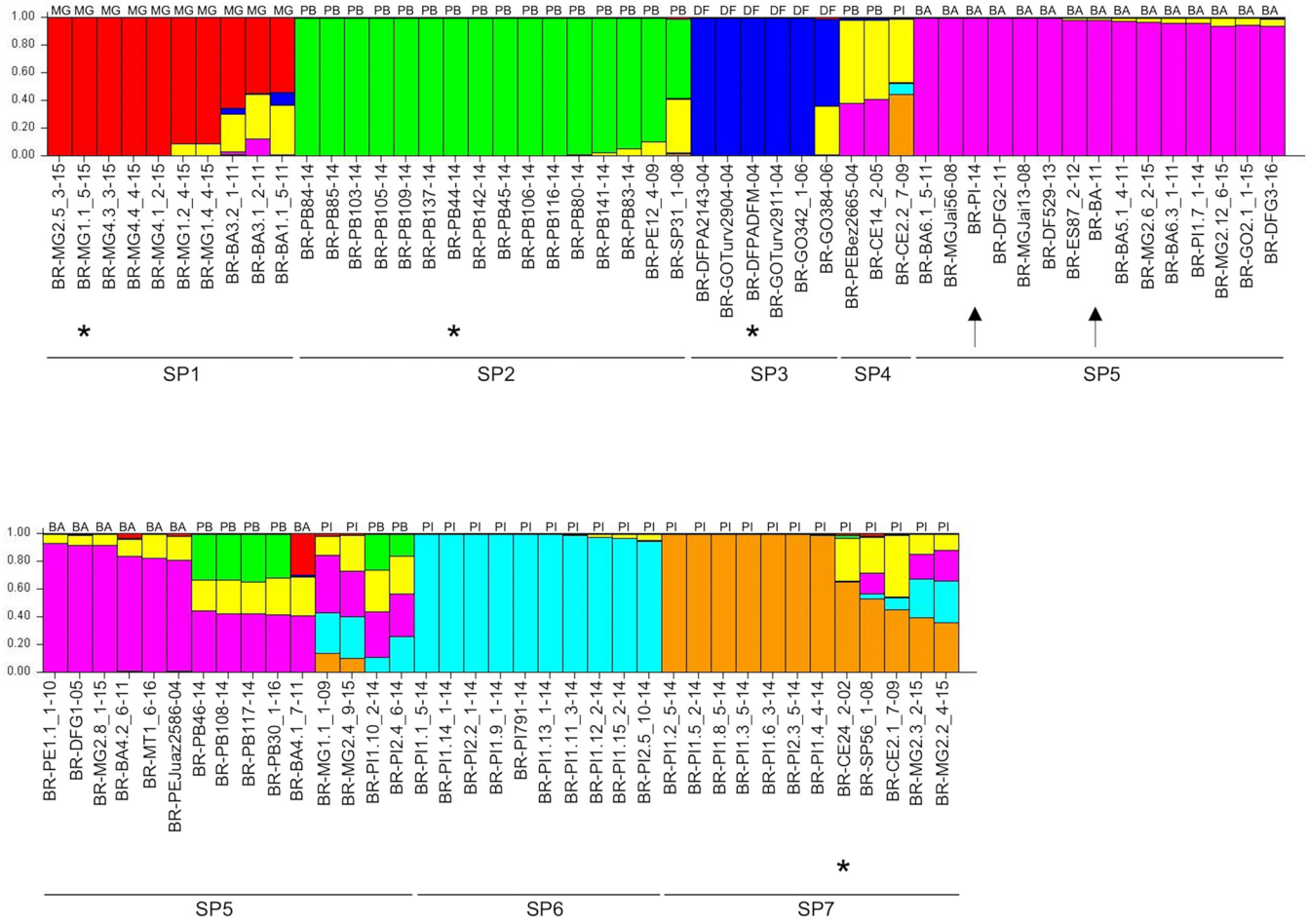

FIG 5 Cluster analysis showing subpopulations of ToMoLCV in Brazil identified with Structure. Each isolate is indicated with a vertical bar of *K* colors, where *K* is the number of subpopulations identified; in this case, *K* is 7. Isolates are sorted according to *Q* (the estimated membership coefficients for each individual). The ToMoLCV BR-BA-11 and BR-PI-14 isolates extensively characterized in the present study are indicated with arrows, whereas the other four core isolates used in phylogenetic analyses (Fig. 2 and 4) are indicated with asterisks. The strain group of each isolate (Fig. 3) is indicated at the top of the bars as follows: PI, Piauí; DF, Federal District; BA, Bahia; MG, Minas Gerais; and PB, Paraíba. See Tables S1 and S3A for ToMoLCV accession numbers.

2005 and 2009 and one from PE in 2004. These isolates have complex ancestry involving four SPs, and each was from a different strain group (PI, MG, and PB).

SP5 is the largest and most diverse SP, with 30 isolates from three strain groups (BA, PB, and PI). Most (22/30) isolates are from the BA strain group, including the exemplary isolates ToMoLCV BR-BA-11 and BR-PI-14, which were extensively characterized in the present study. The other eight isolates in this SP were from PB (six isolates collected from 2014 to 2016) and PI (two isolates collected in 2009 and 2015). All of these as well as one isolate from BA (BR-BA-4.1_7-11) showed substantial admixing; e.g., two isolates came from the PI strain group and have complex ancestries from SP4 (12.7 and 24.8%), SP6 (18.4 and 14.1%), and SP7 (25.2 and 26.4%). SP6 has 10 isolates from the PI strain group with nearly the same ancestry, and these corresponded to isolates of one of the subclades of the PI strain group (Fig. 3). However, SP6 ancestry was detected in isolates of SP4, SP5, and SP7, revealing substantial interaction among SPs. SP7 is composed of 12 isolates all from the PI strain group, and these correspond to the other subclade of the PI strain group as well as other isolates with substantial admixing.

ToMoLCV phylogeography. The 87 full-length sequences of the ToMoLCV genomic DNA were trimmed to include 12 partial sequences dating back to 1997, when this virus was first identified. BEAST v.2.5 software was used to plot the year and location of each of the isolates with the spherical phylogeography tool (Fig. 6). The emergence of ToMoLCV was predicted to have occurred in BA and to have emerged ~700 years ago

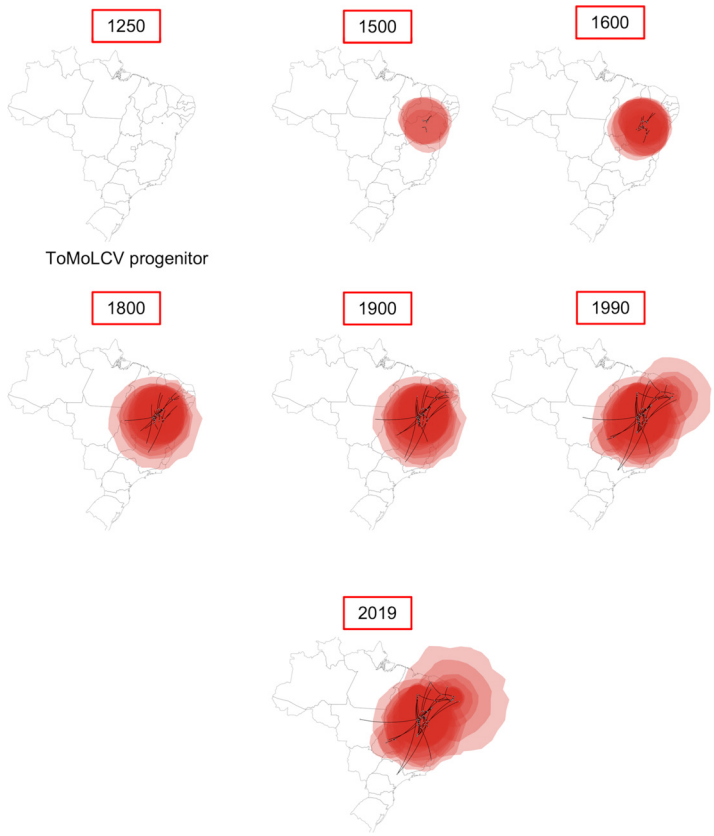

FIG 6 Spatiotemporal dynamics of the emergence and spread of ToMoLCV in Brazil as predicted by BEAST v.2.5 with the spherical phylogeography parameters based on the dates and places of sample collection. For this analysis, 99 partial sequences of ToMoLCV isolates collected from 1997 to 2016 were used. The phylogenetic analysis was performed with BEAST v.2.5. Branch strengths were evaluated by Bayesian posterior probabilities with 95% HPD. The red polygons indicate the geographic range of the virus, whereas the most significant migration pathways are represented by black lines. See Tables S1 and S3A and B for ToMoLCV accession numbers.

with 95% highest posterior density (HPD). Note that a similar prediction was made when the analysis was performed with the 87 complete sequences, though the locations were better defined (Fig. S4). Subsequent spread and diversification occurred locally in NE Brazil for hundreds of years. In the early 1800s, ToMoLCV was predicted to have spread to other states of NE Brazil, i.e., CE, PB, PE, PI, and RN. By the mid- to late 1900s, ToMoLCV had spread to other parts of Brazil, including the major tomato-producing states of GO and MG and as far west as MT (Fig. 6).

DISCUSSION

Although ToMoLCV has been associated with begomovirus disease outbreaks in tomato in Brazil since the mid-1990s, the nature of the viral genome and its role in ToMoLCD have remained unclear (35, 41). Here, we demonstrate that ToMoLCV is an indigenous NW monopartite begomovirus that induces a range of symptoms in tomato, including those associated with ToMoLCD. Several lines of evidence support this conclusion. First, a DNA-B component was not detected in 56 ToMoLCD samples from 10 states of Brazil in which infection with the ToMoLCV DNA-A (genomic DNA) was confirmed. Second, RCA/RFLP results revealed a genome size of ~2.6 kb, which is consistent with the failure to detect a DNA-B component, and a genomic DNA of a NW monopartite begomovirus (54). Final conclusive evidence was the capacity of the cloned ToMoLCV genomic DNA to induce typical ToMoLCD symptoms in agroinoculated plants of the processing tomato cultivar HMX3887. Because these symptoms were indistinguishable from those observed in

ToMoLCV-infected processing tomato plants in the field in Brazil (Fig. 1A to C, G, and H), these results fulfilled Koch's postulates for ToMoLCD. Importantly, we established that progeny virus, derived from the cloned infectious ToMoLCV genomic DNA, was transmitted from plant to plant by MEAM1 whiteflies. This has not been reported for a NW monopartite begomovirus and shows that the V2 gene product of OW monopartite begomoviruses is not required for whitefly transmission. Together, these results are fully consistent with ToMoLCV causing the ToMoLCD outbreaks observed in Guadalupe (PI) in 2014 and Jaíba (MG) in 2015. Furthermore, a recent study on local diversity and prevalence of tomato begomoviruses over 13 years (2003 to 2016) in central Brazil suggested that ToMoLCV is becoming more common than ToSRV (40).

Results of infectivity (agroinoculation and particle bombardment) and whitefly transmission experiments revealed that ToMoLCV has a relatively narrow host range, mostly within the family Solanaceae, and is well adapted to tomato. A number of important aspects of the biology of the virus were also established. First, ToMoLCV induced different disease phenotypes in the two tomato cultivars tested, i.e., bipartite begomovirus symptoms in the fresh market cultivar Glamour (Fig. 1E and F), and typical ToMoLCD in the processing cultivar HMX3887. This phenomenon was also observed in the field in Mato Grosso State in 2016, where symptoms of bipartite begomovirus infection in a fresh market variety were determined to be caused by ToMoLCV (Fig. 1D). Thus, there are cultivar-specific differences in the ToMoLCV-host interaction that impact symptom development and complicate virus diagnosis based on symptoms. Furthermore, these results suggest that these different disease phenotypes may be associated with fresh market versus processing tomato cultivars. In terms of diagnostics, tomato plants with any begomovirus disease symptoms in Brazil should be tested for ToMoLCV, and the specific PCR test will be useful for this purpose. Second, ToMoLCV induced severe symptoms in the highly susceptible common bean cultivar Topcrop (Andean gene pool) but was poorly infectious and failed to induce symptoms in the carioca-type cultivar Pérola. This cultivar is widely grown in Brazil and possesses Middle American gene pool heritage, which is associated with begomovirus resistance (55). Thus, our results suggest that ToMoLCV is unlikely to pose a threat to common bean production in Brazil, and this is supported by the lack of reports of ToMoLCV associated with begomovirus disease of common bean. However, because a small number of cultivar Pérola plants inoculated with viruliferous whiteflies developed a symptomless ToMoLCV infection, it is possible such plants could serve as reservoirs for ToMoLCV. This should be investigated with whitefly transmission experiments.

Genome analyses and sequence comparisons strongly suggest that ToMoLCV is a NW monopartite begomovirus that is highly divergent from previously characterized begomoviruses, including other NW monopartite begomoviruses, e.g., ToLDeV, ToLCPVV, ToTLV, ToSLCV, and ToChLPV (4, 26–28). Furthermore, because these NW monopartite begomoviruses cause similar symptoms in tomato (stunting and distorted upright growth with upcurled leaves with vein swelling and purpling), this represents an example of local evolution of tomato-infecting begomoviruses in South America. This was mediated by the whitefly supervector and this highly susceptible host (3, 4). The independent evolution of these viruses was further revealed in the species evolutionary relationship tree (Fig. 2), in which monopartite viruses were placed in each of the NW begomovirus lineages (4, 26–28). The results showing that ToMoLCV was most closely related to bipartite begomoviruses from NE Brazil and that these viruses were placed in the highly supported ToMoLCV lineage strongly suggested that ToMoLCV emerged in NE Brazil (Fig. 2). Evidence of a long period of isolation and diversification of this lineage comes from the presence of genetically divergent (~20%) monopartite and bipartite members that infect a range of plant species. Finally, a relationship between the ToMoLCV and AbMV lineages was also revealed, and demonstrates that Brazilian begomoviruses have emerged from more than one lineage.

Further evidence of begomovirus diversification in NE Brazil comes from results showing the independent evolution of the monopartite begomovirus ToMoLCV and

ToLCPVV, which is a recombinant virus that emerged from progenitors from the ToMoLCV and BoGMV lineages (27, 51). Moreover, expansion of the BoGMV lineage, which previously contained only BoGMV from the Dominican Republic (51), revealed evolutionary relationships with Brazilian begomoviruses, including MacYSV, MeRMV, and ToLCPVV (Fig. 2). Furthermore, an evolutionary relationship between these lineages was also revealed. These results further show the polyphyletic nature of Brazilian begomoviruses and the possibility that the BoGMV lineage arose in South America.

The tanglegram analysis revealed evidence of extensive recombination/convergent evolution in the C4/AC4 amino acid sequences within and among lineages, including the OW begomoviruses (Fig. 4). This is fully consistent with the frequent exchange of C4/AC4 genes via recombination in the hot-spot region and C4/AC4 being an IDP, which are flexible and promiscuous (i.e., they interact with multiple host targets) and evolve rapidly (26, 46). The disordered nature of C4/AC4s allows a high rate of nonsynonymous substitutions and strong positive selection (26, 46). However, in the case of C4, which overlaps the more conserved C1/AC1 gene (under purifying selection), mutations in the DNA sequence are constrained (46). Thus, the differences in the divergence of the ToMoLCV C4 nucleotide (low) and amino acid (high) sequences likely reflect these constraints, and it will be of interest to investigate this mechanism of C4 protein diversification.

The C4/AC4 proteins have different properties, including localization to the plasma membrane; playing a role in nuclear/cytoplasmic shuttling and cell-to-cell movement; binding to receptor-like kinases; suppression of transcriptional and posttranscriptional gene silencing; manipulation of host defenses, including salicylic acid-dependent responses; as effectors for resistance genes; and as pathogenicity factors/symptom determinants (26, 45, 56, 57). Although the function(s) of the ToMoLCV C4 remains to be determined, it appears essential for pathogenicity (J. O. Souza, unpublished data) and the size (98 aa) and N-terminal myristoylation domain associated with membrane targeting are conserved among isolates (45, 46). The high rate of evolution and genome flexibility of the C4/AC4 may explain the phylogenetic relationship between the C4 proteins of NW and OW monopartite tomato-infecting begomoviruses, something previously observed for ToLDeV from Peru (26). The presence of an OW-related C4 in all members of the ToMoLCV lineage could be due to convergent evolution over a long period of time and geographic separation (26). This indicated that this event occurred early in the evolutionary history of these viruses, perhaps even involving recombination between NW and OW progenitors before continental drift (3, 11, 12, 54).

The sequence comparisons, ToMoLCV strain phylogenetic tree and Structure analysis revealed a substantial degree of genetic diversity in the ToMoLCV population in Brazil (Fig. 3 and 5; Fig. S3). The five strain groups (Fig. 3) and seven SPs (Fig. 5) showed a similar strong geographic association with states of NE Brazil, consistent with emergence and diversification of ToMoLCV in this region. There was also evidence of subsequent founder events and associated local spread and diversification (Fig. 3). Evidence of recombination playing a role in ToMoLCV evolution came from results of sequence, phylogenetic, Structure, and RDP analyses. Moreover, this information served as the basis for the selection of the core isolates and the partial IR/CR alignment that revealed three REs in the hot spot region (Fig. S3).

The RE RIII (Fig. S3) was further characterized because it provided an explanation for how a DNA-B component was associated with an isolate of ToMoLCV (43). This RE was in the hot-spot region of isolates of the DF strain group/SP3 collected in 2004 and 2006 and involved the acquisition of the Rep IRD and high-affinity binding site from the DNA-A component of ToCMoV, a bipartite tomato-infecting member of the ToMoLCV lineage (Fig. 2). The resulting hybrid ToMoLCV/ToCMoV genomic DNA with ToCMoV replication specificity would mediate the replication of the ToCMoV DNA-B component. Thus, the resulting bipartite ToMoLCV was generated via recombination and then pseudorecombination, which most likely occurred in tomato plants coinfected with ToCMoV and ToMoLCV, which are sympatric in NE Brazil. Finally, the result showing that the sequence of the

ToMoLCV DNA-B component (43) is 93.5% identical to those of ToCMoV isolates was further evidence that these are components of the same virus (Fig. S3).

The failure to detect a DNA-B component in ToMoLCV isolates collected from DF in 2005 and 2011 may suggest that the acquisition of the ToCMoV DNA-B component did not provide a selective advantage. This may reflect the adaptation of the monopartite ToMoLCV to the phloem of tomato plants and a reduced need for cell-to-cell movement (58). Alternatively, the function(s) of the AC4 of the bipartite ToCMoV may be incompatible with the products of the monopartite genome of ToMoLCV. It should be noted that acquisition of a DNA-B component did provide a selective advantage for the monopartite OW Sri Lanka cassava mosaic virus (59). Further studies involving infectious clones (mixed infections, pseudorecombination, and passage through plants) and focusing on the C4 gene/protein should be conducted to gain more insight into factors involved in the evolution of monopartite viruses from bipartite ones.

The phylogeographic analysis indicated that ToMoLCV emerged in NE Brazil ~700 years ago, well before the introduction of cultivated tomato and MEAM1 whiteflies. This is fully consistent with the long evolutionary history of ToMoLCV revealed in the present study. In this evolutionary scenario, the progenitor of ToMoLCV was an indigenous bipartite begomovirus that infected noncultivated plants and was spread by indigenous whiteflies. As the bipartite ToMoLCV progenitor was spreading and diversifying locally, cultivated tomato was introduced into Brazil (late 1800s to early 1900s). This allowed the introduction of an infectious movement-competent DNA-A component (26–28, 58) into tomatoes, leading to the evolution of NW monopartite begomoviruses well adapted to the phloem of this highly susceptible host. This whitefly-transmissible monopartite tomato-infecting virus was further spread by indigenous whiteflies and, eventually, by human activities, i.e., movement of infected tomato plants. The intensification of tomato cultivation in NE Brazil in 1970 to 1995 further facilitated the build-up and evolution of ToMoLCV, as well as its spread to the south-central states of Brazil (Fig. 6). Together, our results are consistent with the hypothesis that NW monopartite begomoviruses evolved from DNA-A components of a NW bipartite progenitors, following introduction into the highly susceptible tomato host, initially by indigenous whiteflies and then by the invasive polyphagous MEAM1 supervector (60–62).

In conclusion, we show that ToMoLCV is a NW monopartite begomovirus, thereby explaining the failure to detect a DNA-B component in tomato plants with ToMoLCD. Definitive evidence came from the induction of ToMoLCD symptoms by the cloned genomic DNA of ToMoLCV, thereby fulfilling Koch's postulates. We further demonstrate that ToMoLCV is transmissible by MEAM1 whiteflies, but not mechanically. Evidence is provided that ToMoLCV emerged and diversified in NE Brazil as part of the ToMoLCV lineage and has now spread to all tomato-growing states of Brazil. The ToMoLCV population in Brazil is composed of multiple strains and subpopulations, consistent with a long evolutionary history. Indeed, the phylogeographic analysis predicted that ToMoLCV emerged >700 years ago in NE Brazil, and this was most likely from the DNA-A component of a NW bipartite progenitor that was introduced into highly susceptible tomatoes by indigenous whiteflies. The extensive distribution of ToMoLCV in Brazil was likely mediated by the MEAM1 supervector (40). Our results indicate a need to emphasize management of ToMoLCV in Brazil, including identification of resistant germplasm, e.g., using the agroinoculation system, and implementation of tomato-free periods within the context of an IPM program (2).

MATERIALS AND METHODS

Virus isolates and DNA extraction. Tomato leaf samples from plants with ToMoLCD symptoms, i.e., stunting and distorted and upright growth and leaves with curling, mosaic/mottle, vein swelling, and purpling (Fig. 1A to C), were collected during surveys of commercial tomato fields conducted from 2013 to 2016 (A samples, Table S1). Total genomic DNA was extracted from these samples as described by Dellaporta et al. (63).

In addition, total genomic DNA extracts previously prepared from leaves exhibiting ToMoLCD-like symptoms were obtained from the begomovirus collection at Embrapa Vegetables, Brasília, Brazil (B samples), and from samples collected by Rita de Cássia Pereira Carvalho (University of Brasília) and Leonardo S. Boiteux

(Embrapa Vegetables) (C samples) (Table S1). For the B and C samples, DNA extracts were prepared with a cetyltrimethylammonium bromide (CTAB) method (64).

Begomovirus detection, cloning, and sequencing. The begomovirus genomic DNA/DNA-A component was detected by PCR tests with the degenerate primer pair PAL1v1978/PAR1c496 (65). DNA fragments (~1.1 kb) were purified with a gel purification kit (GE Healthcare Bio-Sciences, Marlborough, MA) and directly sequenced with the PAL1v1978 primer. To detect the DNA-B component in samples confirmed to be infected with the ToMoLCV DNA-A component based on PCR and sequencing, two approaches were used. In the first, a series of PCR tests were performed with four degenerate DNA-B primer pairs (PCRc1/PBL1v2040, PCRc1/PBL1v2039, PCRc2/PBL1v2040, and PCRc2/PBL1v2039) (65). The second approach was restriction fragment length polymorphism (RFLP) analysis of DNA products generated by RCA with ϕ29 DNA polymerase (TempliPhi; GE Healthcare Bio-Sciences, Marlborough, MA) with the four-base-cutting MspI. The RCA/RFLP analyses reveal the number and size of begomovirus DNAs infecting sample (66, 67). Products of these digestions were analyzed by gel electrophoresis in 0.8% agarose gels.

To obtain full-length clones, RCA products were digested with six-base cutting restriction enzymes, including AccI, ApaI, BamHI, BssHII, ClaI, EcoRI, EcoRV, HincII, HindIII, KpnI, NdeI, PstI, SacI, SalI, SmaI, SpeI, XbaI, and XhoI, to identify those that linearized the genomic DNA; i.e., an ~2.6-kb fragment was observed. These fragments were cloned into pBlueScript II KS(+) (68) or pSL1180 (69) digested with the appropriate enzyme. Recombinant plasmids were identified by restriction enzyme digestion analysis. Selected clones were sequenced by primer walking (Table S2) at Macrogen, Inc. (Seoul, South Korea), or Quintara Biosciences (San Pablo, CA, USA), and sequences were assembled with the Staden package (70).

Production of multimeric clones. Two ToMoLCV isolates were selected for further characterization, including infectivity studies: one from Bahia (BR-BA6.2-11 in plasmid pBS11_BA6.2), referred to here as ToMoLCV BR-BA-11, and the other from Piauí (BR-PI2.1-14 in plasmid pBS14_PI2.1), referred to here as ToMoLCV BR-PI-14. For ToMoLCV BR-BA-11, a dimeric clone was generated in pCAMBIA0380 (71, 72) by partially digesting RCA products with ApaI and cloning the ~5.2-kb dimer to generate the recombinant plasmid pCam-ToMoLCV2.0-BR-BA-11, which was transformed into *Agrobacterium tumefaciens* (strain C58C1) with the freeze-thaw method (73). Transformants with the cloned dimer were identified by restriction enzyme digestion.

For ToMoLCV BR-PI-14, the full-length monomer was cloned from RCA products digested with ApaI to generate pBS-14_PI2.1. The ~2.2-kb fragment containing the IR was released from pBS-14_PI2.1 by digestion with ApaI and BamHI and cloned into pBluescript II KS(+) digested with the same enzymes to generate pBS-ToMoLCV0.8-BR-PI-14. The full-length monomer was released from pBS-14_PI2.1 with ApaI and cloned into ApaI-digested pBS-ToMoLCV0.8-BR-PI-14 to generate pBS-ToMoLCV1.8-BR-PI-14. This recombinant plasmid was then digested with PvuII, and the ~4.8-kb fragment with the ToMoLCV multimer was cloned into pBin19 (74) digested with SmaI to generate pBin-ToMoLCV1.8-BR-PI-14. Transformation of *A. tumefaciens* and identification of recombinant plasmids were as described above.

Infectivity and host range experiments. The infectivity of the multimeric cloned ToMoLCV genomic DNAs in pCam-ToMoLCV2.0-BR-BA-11 and pBin-ToMoLCV1.8-BR-PI-14 was initially assessed by agroinoculation of 3- to 4-wk-old tomato (cultivar Glamour) and *N. benthamiana* plants with *A. tumefaciens* cell suspensions (optical density at 600 nm = 1.0) delivered into stems just beneath the shoot apex by needle puncture inoculation (60).

A partial host range experiment was then conducted by agroinoculation of 3- to 4-wk-old plants of tomato (cultivar HMX3887), common bean cultivar Topcrop, *N. benthamiana*, tobacco (*N. tabacum* cv. Havana, Turkish, and Samsun), *N. glutinosa*, eggplant cultivar Black Beauty, pepper cultivar Cayenne, pumpkin cultivar Small Sugar, *D. stramonium* L., *C. quinoa*, and *C. amaranticolor*. Three independent experiments were performed with a minimum of five plants of each species. Controls were equivalent plants agroinoculated with the EV pCAMBIA0380.

The infectivity of the multimeric clones in the carioca-type common bean cultivar Pérola was determined by particle bombardment inoculation because this variety has a Middle American gene pool heritage and is recalcitrant to agroinoculation (55, 75, 76). Hypocotyls of germinating seedlings were bombarded with gold particles coated with DNA of the multimeric clone in pCam-ToMoLCV2.0-BR-BA-11 using a PDS-1000/He system (DuPont) as described previously (77). The positive control consisted of the infectious cloned DNA-A and DNA-B components of BGMV from Brazil (78), whereas the negative control consisted of gold particles alone. Three independent bombardments were performed, each with six seedlings per treatment. Bombarded seedlings were planted in soil and grown in a controlled environment chamber (model PGR15; Conviron, Winnipeg, MB, Canada) maintained at 25°C with a 16-h photoperiod, irradiance levels of ~700 to 1,000 μmol m^{-2} s^{-1}, and ~50% RH. Inoculated plants were observed for disease symptoms, and results were recorded at 21 dpi.

Mechanical transmission. Mechanical (sap) transmission was performed as previously described (79). Sap was prepared by grinding symptomatic leaf tissue from tomato (cultivar Glamour) plants agroinoculated with ToMoLCV BR-BA-11 in ice-cold 0.1 M phosphate buffer (pH 7.2) at a 1:10 (wt/vol) ratio. Sap was rub-inoculated onto leaves of 3- to 4-wk-old tomato plants (cultivars Glamour and H9553). The positive control consisted of equivalent plants rub-inoculated with sap prepared with symptomatic leaf tissue from tomato plants (cultivar Glamour) agroinoculated with the sap-transmissible bipartite begomovirus ToMoV (60). The negative control consisted of plants inoculated with 0.1 M phosphate buffer (pH 7.2). Three independent inoculations were performed with a minimum of five plants per treatment.

Testing plants for ToMoLCV infection. For the infectivity experiments, samples of newly emerged leaves of all plants were collected for PCR tests for ToMoLCV infection. Total DNA was extracted (63), and PCR tests performed with the ToMoLCV-specific primer pair ToMoLCV373c (5′-CTT CAC AGC CCT

TTG GTA CAT CGG-3′) and ToMoLCV1694v (5′-GTG ATG TCA TCA ATG ACG TTG TAC TCC G-3′), which direct the amplification of an ~1.3-kb DNA fragment of the ToMoLCV genomic DNA.

Whitefly transmission. A colony of nonviruliferous MEAM1 whiteflies maintained on cabbage plants (*Brassica oleracea* L. var. capitata cultivar União) at Embrapa Vegetables was used for transmission experiments. Tomato plants (cultivar H9553) at the two- to three-leaf stage (3 to 4 wks old) were agroinoculated with ToMoLCV BR-BA-11 or BR-PI-14. By 15 dpi, plants that had developed symptoms of ToMoLCD and tested positive for ToMoLCV infection were used as source plants for whitefly transmission experiments. Here, 15 nonviruliferous adult whiteflies were placed into a 50-mL plastic tube with a detached ToMoLCV-infected leaf with the petiole inserted into 0.8% water-agar. Whiteflies were given a 48-h AAP, and the (presumed) viruliferous whiteflies were removed by aspiration and transferred into a cage containing a single uninfected tomato plant (cultivar H9553) at the two-three leaf stage or an ~10-day-old common bean plant of cultivar Topcrop or Pérola for a 48-h inoculation access period (IAP). After the IAP, the whiteflies were removed by aspiration. The same procedure was performed with nonviruliferous whiteflies fed on leaves from uninfected tomato plants. Fifteen days later, plants were observed for symptoms and newly emerged leaves were sampled. Total DNA was extracted from these samples with the CTAB method (64), and PCR tests with the ToMoLCV-specific primer pair ToMoLCV373c/1694v were performed.

Sequence and phylogenetic analyses. Pairwise sequence comparisons were performed with MUSCLE within the Sequence Demarcation Tool (SDT) v.1.2. (80). Amino acid and nucleotide sequence alignments and comparisons were performed with MUSCLE v.3.5 within the Geneious Prime 2020.1.1 software (Biomatters Ltd., New Zealand). Sequence alignments were exported as Nexus files. The best-fit model of nucleotide or amino acid substitution was determined with the program ModelTest-NG (81). To identify ToMoLCV strain groups, the function creates data sets (based on percent identities) in SDT with a maximum sequence identity of 100% and a minimum sequence identity of 94% (6).

Three phylogenetic analyses were performed, and trees were constructed using Bayesian inference performed with BEAST v.2.5 (82). The data sets were as follows: (i), complete nucleotide sequences of the DNA-A components and genomic DNAs of NW and OW begomoviruses (referred to as the species evolutionary relationships tree); (ii), complete nucleotide sequences of the genomic DNAs of the 87 ToMoLCV isolates generated in the present study (56 isolates) (Table S1) and 31 sequences recovered from GenBank (Table S3A); and (iii), the combination of the species evolutionary relationship tree and a tree generated with the C4/AC4 amino acid sequences of these viruses (tanglegram). For these analyses, a constant population size was assumed, with a log-normal relaxed molecular clock (83). The Markov chain Monte Carlo (MCMC) simulation was run for 100,000,000 generations and sampled at 10,000 trees. The maximum-clade-credibility trees were made by using Tree-Annotator v.2.5.1 and discarding the first 25% of the MCMC chains as burn-in with a posterior probability (pp) limit of 0.5 and with an HPD of 95%. Trees were edited in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). CIPRES Science Gateway was used to host the running processes of ModelTest-NG and BEAST (84). A tanglegram that combined the phylogenetic analyses of the complete nucleotide and C4/AC4 amino acid sequences was generated with Dendrocope3 (85) and edited with Inkscape software.

Detection of recombination with RDP. Detection of recombination events was carried out with the RDP version 4 (86). Data sets were generated with the following: 1, full-length sequences of DNA-A components and genomic DNAs of NW and OW tomato-infecting begomoviruses, including those from Brazil and the ToMoLCV isolates characterized in the present study (258 sequences); 2, the 58 complete nucleotide sequences of DNA-A components and genomic DNAs of NW and OW begomoviruses used in the species evolutionary relationships tree; and 3, the complete nucleotide sequences of the genomic DNAs of the 87 ToMoLCV isolates.

Determination of the genetic structure of the ToMoLCV population in Brazil. Full-length sequences of the genomic DNAs of the 87 ToMoLCV isolates were analyzed with the program Structure v.2.3.4 (87, 88) to gain insight into the genetic structure of the population. The subpopulation selection ($K = 1$ to 10) was performed using 1,000,000 Markov chain steps after a burn-in period of 100,000 steps. The likelihood estimates for each K value were compared based on the maximum log probability of data ln$P$(D) to determine the best-supported number of subpopulations.

Phylogeography analysis of ToMoLCV DNA sequences. To estimate the location and time of emergence of ToMoLCV, the software BEAST v.2.5 was used to infer the spherical phylogeography of the virus based on the dates and locations of sample collection. Because only partial sequences, corresponding to the ~1.1-kb fragment amplified with the PAR1c496/PAL1v1978 primer pair, were available for isolates collected in 1997 (Table S3B) (41), the 87 full-length ToMoLCV sequences were trimmed to align with these partial sequences. A database with these 99 partial sequences and the associated year, city, and state of collection was generated. Nucleotide sequence alignments and the determination of the best-fit model of nucleotide substitution were as described above. Geographical coordinates were obtained from Google Earth and transformed from degrees, minutes, and seconds into decimal degrees of latitude and longitude.

The pattern of spread of ToMoLCV was reconstructed using GEO_Sphere for Spherical Phylogeography in BEAST v.2.5 (82). The phylogeographic analysis used confidence intervals of >95%. Processed trees were visualized using the SPREAD program and Google Earth to produce a graphical animation of the spread of ToMoLCV in the key markup language (kml) file format (89). These kml files contain information that reveals the routes and times of virus spread and can be visualized with Google Earth. The software SpreaD3 was used to create a web-based visualization of the kml format viewed in Google Earth to generate snapshots of the video (90).

Data availability. Tomato mottle leaf curl virus isolates complete nucleotide sequences are available at NCBI GenBank with the following accession numbers: BR-BA-11, ON419900; BR-PI-14, ON419944; BR-PI1.1_5-14, ON419926; BR-PI1.10_2-14, ON419934; BR-PI1.11_3-14, ON419935; BR-PI1.12_2-14, ON419936; BR-PI1.13_1-14, ON419937; BR-PI1.14_1-14, ON419938; BR-PI1.15_2-14, ON419939; BR-PI1.2_5-14, ON419927; BR-PI1.3_5-14, ON419928; BR-PI1.4_4-14; ON419929; BR-PI1.5_2-14, ON419930; BR-PI1.6_3-14, ON419947; BR-PI1.7_1-14, ON419931; BR-PI1.8_5-14, ON419932; BR-PI1.9_1-14, ON419933; BR-PI2.2_1-14, ON419940; BR-PI2.3_5-14, ON419941; BR-PI2.4_6-14, ON419942; BR-PI2.5_10-14, ON419943; BR-GO2.1_1-15, ON419906; BR-MG1.1_5-15, ON419909; BR-MG1.2_4-15, ON419910; BR-MG1.4_4-15, ON419911; BR-MG2.12_6-15, ON419918; BR-MG2.2_4-15, ON419912; BR-MG2.3_2-15, ON419913; BR-MG2.4_9-15, ON419914; BR-MG2.5_3-15, ON419915; BR-MG2.6_2-15, ON419916; BR-MG2.8_1-15, ON419917; BR-MG4.1_2-15, ON419919; BR-MG4.3_3-15, ON419920; BR-MG4.4_4-15, ON419921; BR-MT1_6-16, ON419922; BR-CE2.1_7-09, ON419901; BR-CE2.2_7-09, ON419902; BR-MG1.1_1-09, ON419908; BR-PE1.1_1-10, ON419924; BR-BA1.1_5-11, ON419892; BR-BA3.1_2-11, ON419893; BR-BA3.2_1-11, ON419894; BR-BA4.1_7-11, ON419895; BR-BA4.2_6-11, ON419896; BR-BA5.1_4-11, ON419897; BR-BA6.1_5-11, ON419898; BR-BA6.3_1-11, ON419899; BR-CE24_2-02, ON419904; BR-CE14_2-05, ON419903; BR-GO342_1-06, ON419907; BR-SP31_1-08, ON419945; BR-SP56_1-08, ON419946; BR-PE12_4-09, ON419925; BR-ES87_2-12, ON419905; BR-PB30_1-16, ON419923.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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