Arch Virol (2011) 156:2063–2069
DOI 10.1007/s00705-011-1070-4

BRIEF REPORT

Molecular and biological characterization of a new Brazilian begomovirus, euphorbia yellow mosaic virus (EuYMV), infecting *Euphorbia heterophylla* plants

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Received: 25 April 2011 / Accepted: 1 July 2011 / Published online: 22 July 2011

Abstract To date, no begomovirus has been fully characterized from *Euphorbia heterophylla*, a widely distributed weed, in Brazil. Here, we show the occurrence of a new begomovirus on *E. heterophylla* plants showing bright yellow mosaic. The bipartite viral genome was cloned from 10 samples, and all clones are almost identical to each other (95.6-98.8% nucleotide sequence identity). The DNA-A sequences shared a maximum nucleotide sequence identity of 87.3% with euphorbia mosaic Peru virus (EuMPV) and thus were classified as belonging to a novel begomovirus species, tentatively named Euphorbia yellow mosaic virus (EuYMV). The EuYMV DNA-B sequences share a maximum nucleotide sequence identity of 56.2% with a euphorbia mosaic virus (EuMV) isolate from Mexico. Phylogenetic analysis demonstrated that this new virus belongs to a different lineage than EuMV isolates from Central America.

Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) have a circular, single-stranded DNA encapsidated in twinned icosahedral particles and are transmitted by the whitefly *Bemisia tabaci* [27]. Begomoviruses native to the Americas have a bipartite genome that consists of two DNA genomic components (DNA-A and DNA-B) of ca. 2.6 kb. They cause serious diseases in a number of economically important crops, mostly in tropical and subtropical regions [21]. The most severely affected crops in

The sequences reported in this paper have been deposited in the GenBank database under the accession numbers FJ619507 (NC0125530), FJ619508 (NC012554), FJ756669, FJ756670, FJ756671, FJ756672, FJ756673, FJ756674, FJ756675, FJ756676, FJ756677 and FJ756678.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-011-1070-4) contains supplementary material, which is available to authorized users.

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Brazil are beans and tomatoes [12, 31]. Following the introduction and rapid dissemination of the B biotype of *B. tabaci* in Brazil in the mid-1990s, novel begomoviruses have emerged as important pathogens of tomato [14, 24] and, to a lesser extent, pepper plants [2, 24]. It is believed that the transfer of indigenous begomoviruses present in wild and weed hosts became possible after the spread of the more polyphagous B biotype, which quickly displaced the previously existing A biotype [19].

Reports of begomovirus infection in weeds and other wild plants in Brazil can be found in the literature as early as the 1950s [7, 8]. *Euphorbia heterophylla* (synonym of *E. prunifolia*) is a native species present in the tropical and subtropical Americas, as well as other tropical regions around the world [30]. It is considered to be a weed of great economic importance, causing yield losses in soybean and peanut crops [5, 29]. *E. heterophylla* is ubiquitous in the Southern, Southeastern and Mid-Western regions of Brazil [9]. Whitefly-transmitted begomoviruses have been reported to cause a mosaic disease on *E. heterophylla* plants in Brazil [7] and Venezuela [10], and the bipartite begomovirus euphorbia mosaic virus (EuMV) has been associated with a similar disease in other countries, such as Puerto Rico [3, 4], Peru [26], Jamaica [6] and the Yucatan Peninsula [16] and Jalisco state [15] in Mexico. In 1950, Costa and Bennet [7] carried out a thorough study on a yellow mosaic disease in Brazil in which it was found that a begomovirus was most likely the causal agent, but the genome sequence of this virus isolate is still not known. The complete molecular characterization of a begomovirus infecting *Euphorbia* spp. was not carried out until 2007, when the complete genome sequence of a EuMV isolate from the Yucatan Peninsula of Mexico was reported [16]. Here, we report the complete nucleotide sequences and host range of a new bipartite begomovirus infecting *E. heterophylla* in Brazil and its phylogenetic relationship to other EuMV isolates.

Ten samples of *E. heterophylla* plants exhibiting typical symptoms of begomovirus infection were collected in different locations in Goias state and the Federal District in central Brazil from 2002 to 2009 (Table 1). Total DNA was isolated from these samples as described [11]. The presence of a begomovirus was initially confirmed by PCR using the universal primers PAL1v1978 and PAR1c496 [25]. Begomovirus genomic DNA was cloned into pBluescript KS+ (Stratagene) using rolling-circle amplification essentially as described by Inoue-Nagata et al. [18]. Inserts were sequenced by primer walking, and cognate components were identified based on their high sequence identity in the common region.

DNA-A nucleotide sequences were initially subjected to a BLAST search [1] for preliminary species assignment based on the 89% threshold level established by the *Geminiviridae* Study Group of the ICTV [27]. Additional pairwise comparisons were made with Clustal V, included in MegAlign (DNASTAR Inc., Madison, WI, USA) using default parameters [17]. The deduced amino acid sequences for the seven viral ORFs (Rep, Trap, Ren, AC4 and CP in DNA-A; MP and NSP in DNA-B) were also compared. Phylogenetic analysis was carried out with MEGA version 4 [28] using the neighbour-joining method. Recombination analysis was performed using the nine methods included in the RDP 3.0 package [20] with default settings.

To determine the experimental host range of the begomovirus isolate, DNA-A and DNA-B clones were used in biolistic inoculations carried out according to

Table 1 Location, year of collection and full-length begomovirus clones obtained from *Euphorbia heterophylla* samples collected in Central Brazil from 2002 to 2009

Collection site	Year of collection	Sample code	Clones	
			DNA-A	DNA-B
Goiás				
São Miguel do Passa Quatro	2002	AB403	EuYMV-GO [BR:GO:AB403:02]	–
São Miguel do Passa Quatro	2002	AB404	EuYMV-GO [BR:GO:AB404:02]	–
Acreúna	2005	AB3540	–	EuYMV-GO [BR:GO:AB3540:05]
Itaberaí	2007	5082	EuYMV-GO [BR:GO:AB5082A:07]	EuYMV-GO [BR:GO:AB5082B:07]
Santo Antônio de Goiás	2008	LE	EuYMV-GO [BR:GO:LEA:08]	EuYMV-GO [BR:GO:LEB:08]
Luziânia	2009	8675	EuYMV-GO [BR:GO:8675:09]	–
Morrinhos	2009	9027	EuYMV-GO [BR:GO:9027:09]	–
Distrito Federal				
Taquara	2002	ABA3	EuYMV-GO [BR:DF:ABA3:02]	–
Planaltina	2007	AB5818	EuYMV-GO [BR:DF:AB5818:07]	–
Taquara	2009	8880	EuYMV-GO [BR:DF:8880:09]	–

Paplomatas et al. [22]. Each viral genome insert was separated from the plasmid by restriction enzyme digestion and recovered from agarose gels. The DNA was re-ligated after incubation with T4 DNA ligase and subjected to amplification by RCA to produce large amounts of double-strand genomic concatamers for biolistic inoculation. Plants of twelve different species were inoculated, and susceptible hosts were identified based on symptom phenotype and PCR amplification of viral DNA-A fragments using universal oligonucleotides [25]. Two weeks after inoculation, DNA was extracted from each plant and analyzed by PCR to confirm the infection. Negative controls consisted of seedlings that were mock-inoculated with buffer for each test species.

The 10 collected samples were PCR-positive, and 12 clones corresponding to full-length genomic components (nine DNA-A and three DNA-B) were obtained from all samples (Table 1). The nine DNA-A clones shared 95.6-98.8% nucleotide sequence identity (Supplementary Table S1), and the three DNA-B clones were 95.8-96.5% identical. One clone for each component was arbitrarily selected for further genomic analysis. The full genome sequence of isolate LE was deposited in GenBank under the accession numbers FJ619507 (DNA-A) and FJ619508 (DNA-B).

The DNA-A from the begomovirus isolate LE is 2609 nt long and shows the highest identity (87.3%) to the recently described euphorbia mosaic Peru virus (EuMPV, accession AM886131), isolated from an *Euphorbia* sp. sample collected in Peru in 2007 [26] (Table S1). Its positive or virion-sense strand contains one ORF corresponding to the *CP* (AV1) gene (750 nt), and the negative or complementary-sense strand contains four ORFs corresponding to the *Rep* (AC1, 1080 nt), *Trap* (AC2, 390 nt), *Ren* (AC3, 399 nt) and *AC4* (363 nt) genes. When the nucleotide sequences of individual DNA-A ORFs were compared, the highest identity was always found with the EuMPV isolate: 87.6% for AV1, 88.7% for AC1, 94.6% for AC2, 95.2% for AC3 and 89% for AC4 (data not shown).

The LE DNA-B is 2578 nt long and shows the highest identity (56.2%) with a euphorbia mosaic virus (EuMV) isolate from the Yucatan Peninsula, Mexico (it is noteworthy that there is no DNA-B sequence available for the EuMPV isolate from Peru). The virion-sense strand contains one ORF corresponding to the *NSP* (BV1) gene (771 nt), and the complementary-sense strand contains the ORF corresponding to the *MP* (BC1) gene (885 nt). When individual ORFs from the DNA-B were compared, the highest identity was always found with EuMV-YP: 69.4% for BV1 and 74.2% for BC1 (data not shown).

Both common regions (CRs) contain the typical features of the begomovirus origin of replication, including the high-affinity Rep-binding sites known as iterons (three

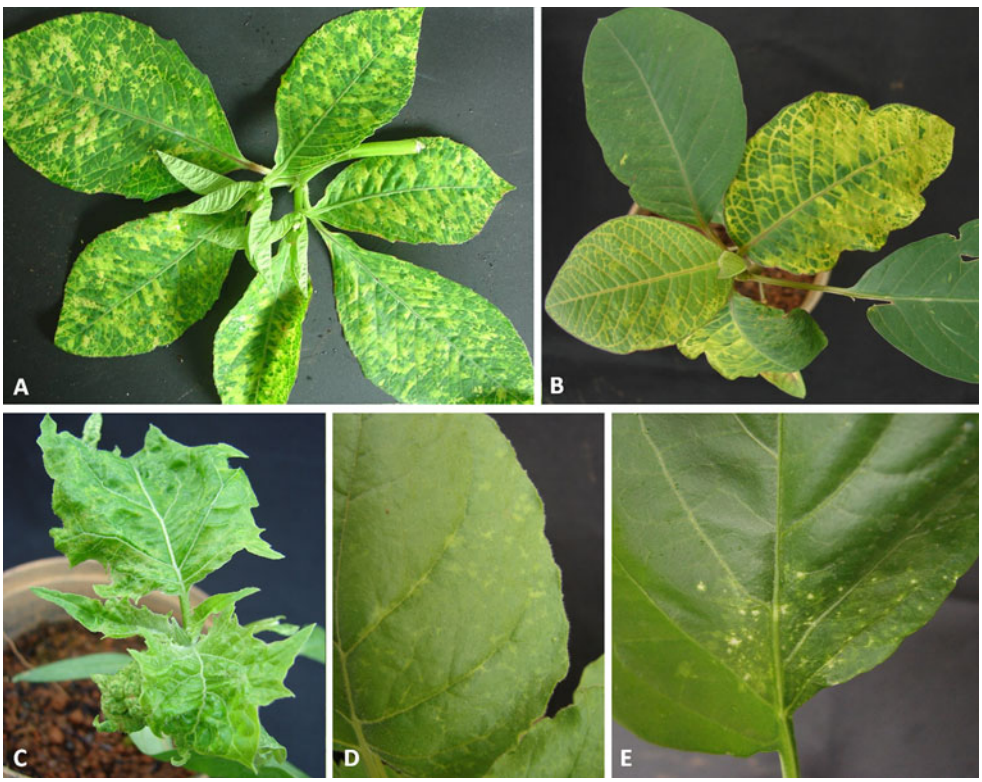
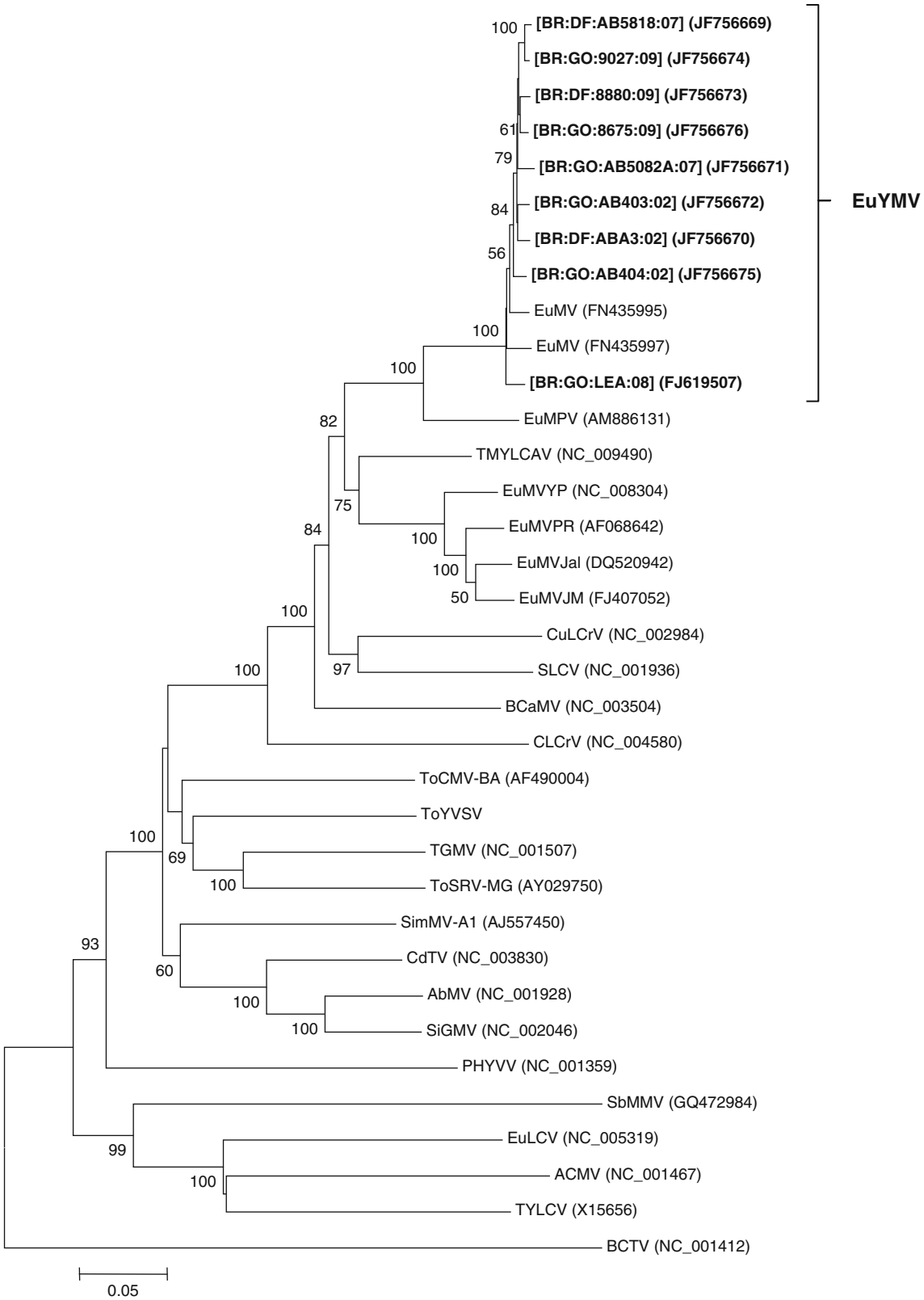


Fig. 1 (**A**) Symptoms induced by EuYMV in naturally infected *Euphorbia heterophylla* plants. (**B**-**E**) Symptoms induced by EuYMV in experimentally infected plants. (**B**) *Euphorbia heterophylla*; (**C**) *Datura stramonium*; (**D**) *Nicotiana benthamiana*; (**E**) *Capsicum annuum* 'Ikeda'



direct repeats, TGGTGTCC, and two inverted repeats, GGACACCA) and the stem-loop structure, which includes the conserved nonanucleotide (TAATATTAC) sequence (Supplementary Figure S1). The DNA-B intergenic region contains a 45-nt segment derived from the cognate *Rep* gene (100% identity). Such elements are present in EuMV-Jal,

◀ **Fig. 2** Phylogenetic tree showing the relationship of EuYMV DNA-A to other begomoviruses from the New and Old Worlds. The tree was constructed by the neighbour-joining method with the MEGA4 program (2,000 bootstrap replications). Accession numbers are shown in the tree. Acronyms and viruses used in the analysis are as follows: AbMV, abutilon mosaic virus (NC_0019280); ACMV, African cassava mosaic virus (NC_001467); BCaMV, bean calico mosaic virus (NC_003504); BCTV, beet curly top virus (NC_001412); CdTV, chino del tomate virus (NC_003830); CLCrV, cotton leaf crumple virus (NC_004580); CuLCrV, cucurbit leaf crumple virus (NC_002984); EuMPV, euphorbia mosaic Peru virus (AM886131); EuMV-Jal, euphorbia mosaic virus-Jalisco (DQ520942); EuMV-JM, euphorbia mosaic virus-Jamaica (FJ4070520); EuMV-MGS1, euphorbia mosaic virus-Mato Grosso 1 (FN435995); EuMV-MGS2, euphorbia mosaic virus-Mato Grosso 2 (FN435997); EuMV-PR, euphorbia mosaic virus-Puerto Rico (AF068642); EuMV-YP, euphorbia mosaic virus- Yucatan Peninsula (NC_0083040); EuLCV, euphorbia leaf curl virus (NC_005319); EuYMV, euphorbia yellow mosaic virus (NC_0125530); PHYVV, pepper huasteco yellow vein virus (NC_001359); SbMMV, soybean mild mottle virus (GQ472984); SiGMV, sida golden mosaic virus (NC_002046); SimMV-A1, sida micrantha mosaic virus-A1 (AJ5574500); SLCV, squash leaf curl virus (NC_001936); TGMV, tomato golden mosaic virus (NC_001507); ToCMV-BA, tomato chlorotic mottle virus-Bahia (AF490004); ToSRV-MG, tomato severe rugose virus-Minas Gerais (AY029750); ToYVSV, tomato yellow vein streak virus (EF417915); TMYLCAV, tomato mild yellow leaf curl Aragua virus (NC_009490); and TYLCV, tomato yellow leaf curl virus (X15656). Beet curly top virus (BCTV) was used as an out-group

EuMV-YP, EuMV-JM and TMYLCAV [15], with differences in length, position from the cognate *Rep* gene and nucleotide identity.

According to the current ICTV criteria for the demarcation of begomovirus species [13], the 87.3% identity between the full-length DNA-A sequences of LE and EuMPV (the closest virus) indicates that LE and all other begomovirus isolates described here are members of a novel begomovirus species, for which we propose the name Euphorbia yellow mosaic virus (EuYMV). This name is derived from the intense yellow mosaic seen on leaves of infected plants (Figure 1a).

A phylogenetic tree based on the full-length DNA-A of EuYMV and all bipartite begomoviruses was constructed (data not shown), but only a few representative sequences of each major clade were kept in the alignment to enable a thorough analysis of the tree (Fig. 2). EuYMV was placed in the squash leaf curl virus (SLCV) clade, which comprises viruses spread from the southern USA to Brazil. A close relationship between EuYMV and EuMV isolates was observed, as well as with tomato mild yellow leaf curl Aragua virus (TMYLCAV). These viruses could be considered to comprise a sub-lineage within the SLCV clade, widely distributed in the Americas. Incidentally, the begomovirus isolates MGS1 and MGS2 from Brazil (accession no. FN435995 and FN435997, respectively), currently classified as EuMV [23], are clearly more closely related to EuYMV (95.4-97.6% identity) than to EuMV isolates (77.2-79.2% identity) (Table S1). These two isolates should be reclassified as EuYMV. Surprisingly (for the recombination-prone begomoviruses), analysis with the RDP program found no evidence of recombination events among EuYMV isolates.

To determine the host range and infectivity of the cloned viral DNAs, several plants were inoculated via particle bombardment (Table 2). The clones were able to infect the original host, *E. heterophylla* (Fig. 1b), as well as three solanaceous species (*Datura stramonium*, *Nicotiana benthamiana* and *Capsicum annuum*). On *E. heterophylla* plants (Fig. 1b), the virus caused interveinal chlorosis, yellow mosaic and stunting, similar to field-infected plants. *D. stramonium* plants were highly susceptible, with strong symptoms of yellow mosaic, blistering, stunting and leaf distortion (Fig. 1c). *N. benthamiana* plants showed mild but clear symptoms of mosaic (Fig. 1d) and blistering. *C. annuum* 'Ikeda' showed small chlorotic spots in the basal portion of uninoculated leaves (Fig. 1e), but the plants later became asymptomatic, although PCR positive. The isolate LE was not detected by PCR, nor did it cause any visible

Table 2 Infectivity and symptoms induced by EuYMV clones upon inoculation on experimental plants

Host plant	Infectivity[a]	Symptoms[b]
Capsicum annuum L. 'Ikeda'	1/4 (25%)	–
Capsicum chinense Jacq. 'PI 159236'	0/4	–
Chenopodium quinoa Willd.	0/7	–
Datura metel L.	0/8	–
Datura stramonium L.	7/7 (100%)	LD, YM, B
Euphorbia heterophylla L.	7/8 (87.5%)	LD, YM, VC, S
Nicotiana benthamiana Domin.	1/8 (12.5%)	MM, B
Nicotiana rustica L.	0/8	–
Nicotiana tabacum L. 'TNN'	0/8	–
Phaseolus vulgaris	0/6	–
Physalis pubescens L.	0/8	–
Solanum lycopersicum L.	0/8	–

[a] Infectivity: number of infected plants (PCR-positive)/ number of inoculated plants

[b] Symptoms: LD: leaf distortion, YM: yellow mosaic, B: blistering, MM: mild mosaic, VC: vein clearing, S: stunting

symptoms in the other eight species used in this study (Table 2).

The goal of this study was the characterization of *Euphorbia* viruses from Brazil, and for this purpose, several samples were obtained in one of the major agricultural areas of the country. Begomovirus infection was invariably correlated with symptomatic plants. Sequence analysis of full-length DNA-A components indicated that only one begomovirus, tentatively named euphorbia yellow mosaic virus (EuYMV), was present in the samples. No other begomovirus could be isolated from the samples. The low sequence diversity among these isolates indicates that it has been co-evolving with *Euphorbia* plants for a long time. Begomoviruses have been reported on this host in Brazil as far back as the 1950s [7]. Unfortunately, no samples from this period remain in storage, and thus it is not possible to verify the identity of the begomoviruses described at that time. Intriguingly, our EuYMV isolates are more closely related to Peruvian and North American viruses than to Brazilian begomoviruses. This observation prompted us to hypothesize that EuYMV was introduced into Brazil, maybe brought with a vegetatively propagated ornamental relative of *E. heterophylla* such as *E. pulcherrima* or *E. milii*. Although it is able to infect a range of solanaceous plants, it has not yet been reported in plants other than *Euphorbia*. Therefore, both the importance of *E. heterophylla* plants as virus reservoir for economically important crops and the economic importance of EuYMV itself remain to be determined.

Acknowledgments We are grateful to Elsa Oliveira Paranaguá e Lago Nogueira, Oneilson Medeiros and Élcio Vieira da Silva (*in memoriam*) for their technical assistance. This work was supported by grants from Embrapa and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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