Phylogenetic analysis of *Tomato mosaic virus* from *Hemerocallis* sp. and *Impatiens hawkeri*

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ABSTRACT

Duarte, L.M.L., Alexandre, M.A.V., Rivas, E.B., Cattai, M.B., Soares, R.M., Harakava, R., Fernandes, F.M.C. Phylogenetic analysis of *Tomato mosaic virus* from *Hemerocallis* sp. and *Impatiens hawkeri*. ***Summa Phytopathologica***, v.33, n.4, p.409-413, 2007.

The culture and commercialization of ornamental plants have considerably increased in the last years. To supply the commercial demand, several *Hemerocallis* and *Impatiens* varieties have been bred for appreciated qualities such as flowers with a diversity of shapes and colors. With the aim of characterizing the tobamovirus isolated from *Hemerocallis* sp. (tobamo-H) and *Impatiens hawkeri* (tobamo-I) from the USA and São Paulo, respectively, as well as to establish phylogenetic relationships between them and other *Tobamovirus* species, the viruses were submitted to RNA extraction, RT-PCR amplification, coat-protein gene sequencing and phylogenetic analyses. Comparison of tobamovirus homologous sequences yielded values superior to 98.5% of identity with *Tomato mosaic virus* (ToMV) isolates at the nucleotide level. In relation to tobamo-H, 100% of identity with ToMV from tomatoes from Australia and Peru was found. Based on maximum likelihood (ML) analysis it was suggested that tobamo-H and tobamo-I share a common ancestor with ToMV, *Tobacco mosaic virus*, *Odontoglossum ringspot virus* and *Pepper mild mottle virus*. The tree topology reconstructed under ML methodology shows a monophyletic group, supported by 100% of bootstrap, consisting of various ToMV isolates from different hosts, including some ornamentals, from different geographical locations. The results indicate that *Hemerocallis* sp. and *I. hawkeri* are infected by ToMV. This is the first report of the occurrence of this virus in ornamental species in Brazil.

Additional Keywords: maximum parsimony, maximum likelihood, *Tobamovirus* subgroups, ornamental plants

RESUMO

Duarte, L.M.L., Alexandre, M.A.V., Rivas, E.B., Cattai, M.B., Soares, R.M., Harakava, R., Fernandes, F.M.C. Análise filogenética de *Tomato mosaic virus* isolado de *Hemerocallis* sp. e *Impatiens hawkeri*. ***Summa Phytopathologica***, v.33, n.4, p.409-413, 2007.

O cultivo e comercialização de plantas ornamentais têm aumentado consideravelmente nos últimos anos. Para suprir a demanda comercial, diversas variedades de *Hemerocallis* sp. e *Impatiens hawkeri* têm sido desenvolvidas pelas qualidades apreciáveis como flores com diversidade de formas e cores. Com o objetivo de caracterizar o tobamovirus isolado de *Hemerocallis* sp. (tobamo-H) e *Impatiens hawkeri* (tobamo-I) provenientes dos EUA e São Paulo, respectivamente, assim como estabelecer relações filogenéticas entre os isolados e outras espécies de *Tobamovirus*, foram realizados extrações do RNA, RT-PCR, seqüenciamento do gene da capa protéica e análises filogenéticas. Quando foram comparadas seqüências homólogas de tobamovirus, valores superiores a 98,5% de identidade, ao nível de nucleotídeos, foram obtidos com isolados do *Tomato mosaic virus* (ToMV). Em relação ao tobamo-H, foi encontrado 100% de identidade com o ToMV isolado de tomateiro da Austrália e do Peru. Com base em análise de máxima verossimilhança (MV), sugere-se que o tobamo-H e tobamo-I compartilham um ancestral comum com ToMV, *Tobacco mosaic virus*, *Odontoglossum ringspot virus* e *Pepper mild mottle virus*. A topologia da árvore reconstruída a partir de MV mostra um grupo monofilético, sustentado por 100% de "bootstrap", formado por vários isolados de ToMV de diferentes hospedeiras, incluindo ornamentais, a partir de diferentes localizações geográficas. Os resultados indicam que *Hemerocallis* sp.e *I. hawkeri* estão infectados pelo ToMV, sendo esse o primeiro relato desse vírus em ornamentais, no Brasil.

Palavras-chave adicionais: máxima parcimônia, máxima verossimilhança, subgrupos de *Tobamovirus*, plantas ornamentais

Ornamental horticulture has experienced a marked growth in the past 40 years and has developed into one of the major economic branches of modern agriculture (19).

Although the states of Santa Catarina, Rio Grande do Sul, Minas Gerais, Rio de Janeiro, Pernambuco and Ceará produce ornamentals for sale, São Paulo State is the main producer with US$ 9.0 million in exports of live plants and floricultural products in 2001 (9). Nevertheless, there was an increase in the importation of ornamental plants to Brazil in 2004 compared to 2003 (15), posing the risk of virus introduction. Among the introduced plants, *Hemerocalis* sp. (Hemerocallidaceae) from the USA showing virus-like symptoms was maintained in quarantine pending the results of phytossanitary analyses, which detected the presence of a virus.

Other ornamentals which deserve mention are *Impatiens*

hawkeri and *I. walleriana* (Balsaminaceae) which are among the most marketable species, due to the introduction of a wide range of new flower colors and forms (19).

Since *Impatiens* and *Hemerocallis* species are vegetatively propagated and constantly introduced, viruses tend to appear and spread quickly in the crop.

Several viruses are described as infecting *Impatiens* species, including *Impatiens necrotic spot virus*, *Tomato spotted wilt virus*, *Tobacco streak virus*, *Tobacco ringspot virus*, *Helenium virus S* and *Cucumber mosaic virus* (CMV) (19). In Brazil, flexuous particles (6), CMV (7), *Tomato mosaic virus* (ToMV) (8) and an unidentified potyvirus (5) have been reported, although there have been no previous reports of *Hemerocallis* virus in Brazil.

The present study identified a tobamovirus isolated from *Hemerocallis* sp. from the USA and compared its sequence with another tobamovirus previously reported (8) infecting *I. hawkeri* from São Paulo State, Brazil, with emphasis on phylogenetic analysis.

MATERIALS AND METHODS

Virus source, RNA extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The tobamovirus was isolated from *Hemerocallis* sp. (tobamo-H) showing necrotic spots, and from *I. hawkeri* (tobamo-I) exhibiting mosaic and leaf deformation. Viral RNA was extracted from infected leaves by using the Trizol LS reagent according to the manufacturer's instructions (Gibco BRL).

Twenty pmols of the primers PS1 (3) and PAS2 (8) were employed under RT-PCR conditions according to Alexandre *et al*. (3).

Sequencing

Amplified products of tobamo-H and tobamo-I were purified using the CONCERT Rapid Gel Extraction Kit according to the manufacturer's instructions (Gibco-BRL) and sequenced using the ABI Prism Big Dye Terminator System (PE Applied Biosystems) and the primers PS1 and PAS2.

Sequence analyses

A fragment of 480bp corresponding to the complete coat protein (CP) gene of tobamo-H and tobamo-I was analyzed. The sequences were submitted to the Basic Local Alignment Search Tool (Blast 2.0) (http://www.ncbi.nlm.nih.gov/BLAST/) and aligned with *Tobamovirus* species, ToMV isolates and *Tobacco rattle virus* (TRV) using the program CLUSTAL X or by eye with the Se-Al program, Version 1.0 alpha 1 (24). The degree of identity between them was determined by using the PAUP* v. 4.0b10 program (26). Moreover, the tobamo-H and tobamo-I nucleotide sequences of the CP gene was aligned with *Tobacco mosaic virus* isolated from *Petunia* sp. (TMV-p) and 9 ToMV isolates.

Phylogenetic analyses

Sequences from 23 *Tobamovirus* species or isolates including those from tobamo-H and tobamo-I as well as from 9 isolates from ToMV were subjected to phylogenetic analyses. Swofford´s (26) PAUP* v. 4.0b10 with heuristic search and equal weighting in the maximum parsimony (MP) analysis was employed. Bootstrap values were obtained using the branch-and-bound method and computed after 1000 resamplings followed by an MP reconstruction. The likelihood ratio test (LRT) (12) for the nucleotide substitution models comparison was performed using Modeltest v. 3.06 (23). The tree reconstruction was also performed under maximum likelihood (ML) criterion using PAUP*.

RESULTS AND DISCUSSION

The *Tobamovirus* genus consists of 23 definitive species (18, 21). However, in recent years at least 3 new species have been proposed: Zucchini green mottle mosaic virus (ZGMMV) (27), Cucumber fruit mottle mosaic virus (CFMMV) (4) and Nigerian tobacco latent virus (NTLV) (16). *Wasabi mottle virus* – WMoV (Crucifer tobamovirus), *Hibiscus latent Singapore virus* – HLSV (Hibiscus virus S) (28) and *Hibiscus latent Fort Pierce virus* – HLFPV (Florida hibiscus virus) (1, 14) were recently accepted as definitive species by the International Committee on Taxonomy of Viruses (21).

In Brazil, the occurrence of only TMV, ToMV and *Odontoglossum ringspot virus* (ORSV) has been reported in ornamental plants (3, 8, 20, 25).

The primers PS1 (3) and PAS2, designed based on the partial sequence of tobamovirus isolated from *I. hawkeri* (8), were able to amplify a fragment with 480 nucleotides corresponding to the complete coat protein (CP) gene of tobamoviruses isolated from *Hemerocallis* sp. and *I. hawkeri* here named tobamo-H and tobamo-I, respectively. The sequences from tobamo-H and tobamo-I were submitted to GenBank, and the accession numbers are DQ230836 and AY063743, respectively.

The BLAST search revealed significant alignment of the tobamo-H and tobamo-I with the CP of ToMV, TMV and *Pepper mild mottle virus* (PMMoV).

Analyses involving ToMV sequences from several hosts were made because a high identity with tobamo-H and tobamo-I sequences was observed (Table 1). Thus, values superior to 98.5% were verified for ToMV isolates, including those from the ornamental plants *Camellia* sp. (AJ 417701), *Hibiscus rosa-sinensis* (11) and *Eustoma russellianum* (13). It is worth mentioning that an identity of about 86% with the Brazilian ToMV sequence was observed (Table 1), supporting the suggestion that the virus might be considered an intermediary strain of ToMV (22).

In relation to the tobamo-H sequence, 100% of identity with ToMV isolated from tomatoes from Australia and Peru was found. It is important to note that the *Hemerocallis* was imported from the USA and hence the plant was already introduced infected.

In the MP analysis of the nucleotide sequences of tobamo-H, tobamo-I and other tobamoviruses, using *Tobacco rattle virus* (TRV) as the out group, only one tree was found with 2266 steps. When the analysis was carried out with ToMV isolates, using the sequence from the TMV-p as the out group two most-parsimonious trees with 567 steps were found.

Based on LRT with tobamovirus sequences, the model HKY presented the best likelihood value of - lnL = 8488.0928. The distribution of rates at variable sites yielded a gamma with the shape parameter alpha = 0.8800. The statistical tests yielded the following nucleotide frequencies among the sequences: A (0.2594), C (0.1889), G (0.2323) and T (0.3194). The tree reconstructed under the LRT criterion is shown in Figure 1A.

Tobamo-H and tobamo-I revealed a monophyletic group with ToMV-Br supported by a bootstrap value of 94% (data not shown). In the tree reconstructed after ML methodology the studied viruses share a common ancestor with ToMV-Br, TMV-p, ORSV-Br and PMMoV (Fig. 1A).

ML analysis also revealed two groups sharing a common ancestor:

Table 1 – Identity between coat protein gene of *Tobamovirus* isolated from *Hemerocallis* sp. (tobamo-H) and from *Impatiens hawkeri* (tobamo-I), compared with other *Tobamovirus* species or *Tomato mosaic virus* (ToMV) isolates at the nucleotide level

	Identity (%)	
Virus species or isolates	**Tobamo-H**	**Tobamo-I**
CGMMV (NC001801)*	46.6	46.6
CFMoMV (NC002633)	44.2	44.0
FrMV (AF165884)	52.1	52.3
HLFPV (AF395898)	51.5	51.3
HLSV (AY250831)	48.3	48.3
KGMMV(NC003610)	44.4	44.2
NTLV (AY137775)	66.2	65.2
ObPV (DI3438)	62.4	62.2
ORSV-br (AF515606)	68.6	68.4
PaMMV (X72586)	64.7	64.5
PMMoV (M81413)	66.8	66.7
RMV (AF185272)	51.1	51.1
SHMV (J02413)	47.7	47.7
TMV-p (AY29262)	76.4	76.1
Tobamo-H (DQ230836)	__	99.8
Tobamo-I (AY063743)	99.8	__
ToMV-Aus (NC 002692)	100.0	99.8
ToMV-Br (AF411922)	85.9	85.8
ToMV-Cam (AJ417701)	98.7	98.5
ToMV-Chi (AJ011934)	99.8	99.6
ToMV-Ger (AJ429086)	98.7	98.5
ToMV-Hib (AY313136)	99.4	99.1
ToMV-Lis (AY383730)	99.4	99.1
ToMV-Per (NC004573)	100.0	99.8
ToMV-pot (AF260730)	99.4	99.1
TMGMV (AF132907)	65.6	65.4
TVCV (U03387)	50.9	50.9
ZGMMV (NC003878)	45.3	45.1
WMoV (NC003355)	51.9	51.9
YoMV (U30944)	51.1	51.1

*GenBank accession number. CGMMV = *Cucumber green mottle mosaic virus*; CFMoMV = Cucumber fruit mottle mosaic virus; FrMV = *Frangipani mosaic virus*; HLFPV = *Hibiscus latent Fort Pierce virus*; HLSV = *Hibiscus latent Singapore virus*; KGMMV = *Kyuri green mottle mosaic virus*; NTLV = Nigerian tobacco latent virus; ObPV = *Obuda pepper virus*; ORSV-Br = *Odontoglossum ringspot virus*-Brazilian isolate; PaMMV = *Paprika mild mottle virus*; PMMoV = *Pepper mild mottle virus*; RMV = *Ribgrass mosaic virus*; SHMV = *Sunn-hemp mosaic virus*; TMV-p = *Tobacco mosaic virus*-petunia isolate; ToMV-Aus = *Tomato mosaic virus*- Australia; ToMV-Br = Brazilian isolate; ToMV-Cam = *Camellia* isolate; ToMV-Chi = from China; ToMV-Ger = from Germany; ToMV-Hib = *Hibiscus* isolate; ToMV-Lis = Lisianthus isolate; ToMV-Per = from Peru; ToMV-pot = potato isolate; TMGMV = *Tobacco mild green mosaic virus*; TVCV = *Turnip vein-clearing virus*; ZGMMV = Zucchini green mottle mosaic virus; WMoV = *Wasabi mottle virus*; YoMV = *Youcai mosaic virus*

subgroup I (Solanaceae hosts) proposed by Lartey (17) and the monophyletic group formed by Brassicaceae, Curcubitaceae and Malvaceae viruses (Fig. 1A).

Three main tobamovirus groups have been proposed based on genomic location of their origin of virion assembly, structure of the junction between movement protein and CP open reading frames, phylogenetic position, and botanical family of the hosts (17, 2). The hosts of subgroup I are mainly solanaceous, those of subgroup II are cucurbits and legumes, and those of subgroup III are cruciferous.

The tree topology obtained from the data suggested that the lineage from Brassicaceae hosts (Subgroup III) had a divergence more basal than those from cucurbitaceous. On the other hand, the tree topology presented by Adkins (1) shows that cucurbit subgroup lineage seems to be the most basal divergence, probably between or within the *Cucumber green mottle mosaic virus* (CGMMV) and *Sunn-hemp mosaic virus* (SHMV) (10).

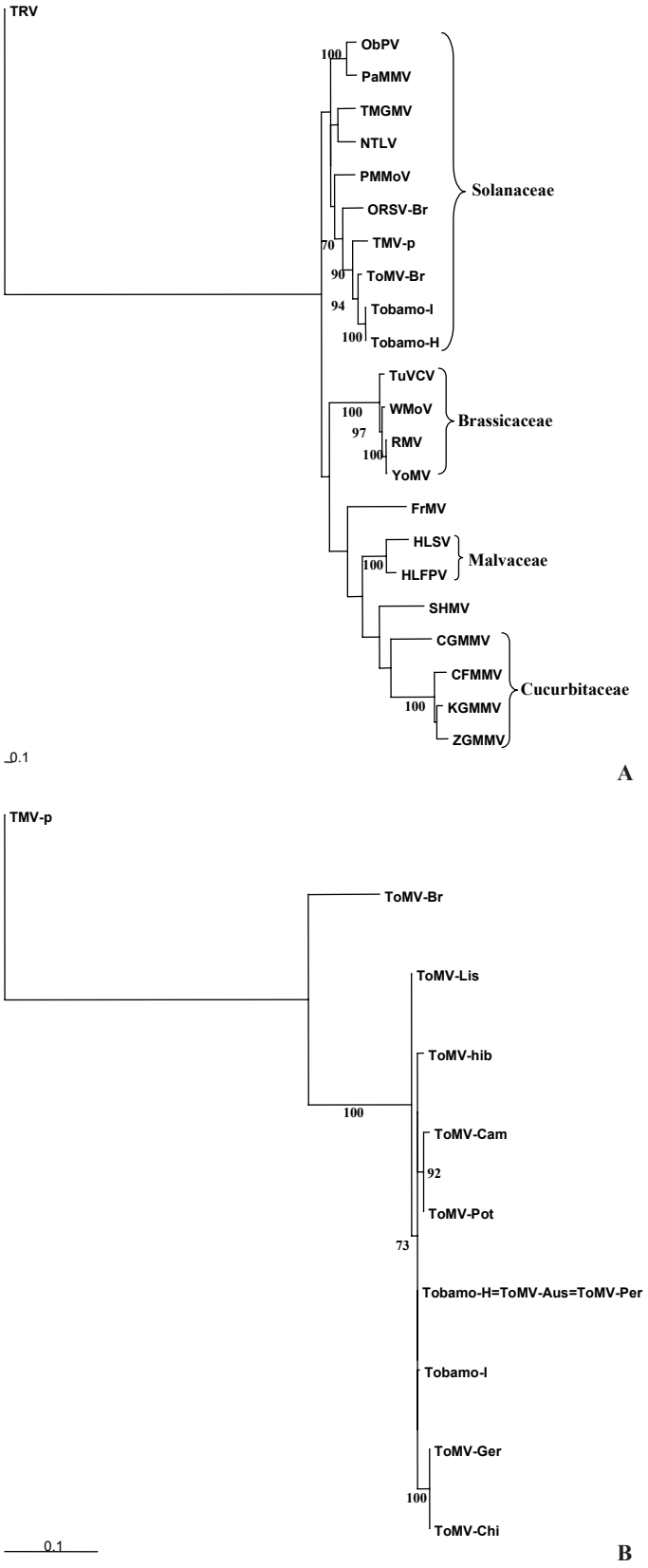

Figure. 1 – Maximum likelihood phylogram of coat protein sequences of: **A** – *Tobamovirus* species. *Tobacco rattle virus* (TRV) used as out group. **B** – *Tomato mosaic virus* (ToMV) isolates. *Tobacco mosaic virus*-petunia (TMV-p) isolate used as out group. Bootstrap values are indicated near the branches. GenBank accession number, as well as the name of viruses are indicated in Table 1, TRV (AJ272199)

Based on our phylogenetic analyses, HLSV and HLFPV clustered with 100% bootstrap and made a monophyletic group with cucurbit viruses, *Frangipani mosaic virus* (FrMV) and SHMV. This analysis corroborated with results obtained by Adkins (1) who proposed that HLSV and HLFPV could be new viruses belonging to the malvaceous subgroup.

In relation to ToMV sequences, the nucleotide substitution model obtained was also HKY, with likelihood value of - lnL = 1344.4966, gamma with the shape parameter alpha = 0.4270 and nucleotide frequencies among the sequences A (0.2958), C (0.2034), G (0.2086) and T (0.2922). The tree reconstructed under LRT criterion is shown in Figure 1B.

With the aim of comparing tobamo-H and tobamo-I with isolates from different hosts including ornamental and from different regions of the world, phylogenetic analyses based on MP (data not shown) and ML methodology were made. The tree topology proposed allowed us to point out that ToMV-Br lineage divergence was most basal in relation to the other taxa. Based on ML analysis using TMV-p as the out group, the monophyletic group formed by ToMV isolated from lisianthus and other isolates, including those from *Hemerocallis* (tobamo-H) and *Impatiens* (tobamo-I) are strongly supported by a bootstrap value of 100% (Fig. 1B). Tobamo-H share a common ancestor with the monophyletic group formed by tobamo-I, and the ToMV isolated from tomatoes from Australia, China, Germany and Peru (Fig. 1B).

Based on the results, the present report describes the occurrence of an infection by ToMV (subgroup I) in two non-solanaceous plants, *Hemerocallis* sp. (Hemerocallidaceae) and *I. hawkeri* (Balsaminaceae). It is important mentioning that this is the first report of ToMV in ornamental species in Brazil.

The ability of ToMV to infect *Camellia*, *E. russellianum* (lisianthus), *Hemerocallis*, *Hibiscus* and *Impatiens* can be explained by the virus jumping from a solanaceous host to another family, indicating a relaxed constraint involving the host plant in the subgroup I proposed by Lartey (17). Gibbs (10) suggested that the ability of *Ribgrass mosaic virus* to prosper in both brassicas and plantains, like the gene sequences encoding their movement-and/or coat protein junction, may be a "derived" rather than an "ancestral" state.

The jumping of virus lineage has also been used to explain the evolution of the tobamovirus subgroup III (17). In the present case, the jumping is observed inside the subgroup I, which is significant for the management of economically important plants especially those vegetatively propagated. Since ToMV is a very infective virus, rigid phytosanitary measures must be used to prevent its transmission. Thus, the incidence of virus infections in stocks will decrease dramatically and will improve the quality standards for impatiens species.

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