LARISSA GOULART ZANARDO

CARACTERIZAÇÃO BIOLÓGICA, MOLECULAR E ANÁLISE DA VARIABILIDADE GENÉTICA DE *Cowpea mild mottle virus* (CPMMV) EM SOJA NO BRASIL

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Magister Scientiae*.

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SUMÁRIO

RESUMO

ZANARDO, Larissa Goulart, M.Sc., Universidade Federal de Viçosa, Fevereiro de 2013. **Caracterização biológica, molecular e análise da variabilidade genética de *Cowpea mild mottle virus* (CPMMV) em soja no Brasil.** Orientador: Francisco Murilo Zerbini Júnior. Coorientadoras: Claudine Márcia Carvalho e Gloria Patricia Castillo Urquiza.

A partir do ano 2000 plantas de soja nos campos dos estados da Bahia, Goiás, Maranhão, Mato Grosso, Minas Gerais, Paraná e Tocantins foram descritas apresentando sintomas da doença da necrose da haste da soja. Os sintomas eram variados, alguns mais suaves outros mais severos. A doença foi associada ao *Cowpea mild mottle virus* (CPMMV, família *Betaflexiviridae*, gênero *Carlavirus*). Nesse estudo foi proposta a realização da caracterização biológica, molecular e análise da variabilidade genética de isolados de CPMMV, causando sintomas da necrose da haste em soja nos campos de diferentes estados produtores do Brasil. O estudo foi realizado com amostras coletadas nos estados da Bahia, Goiás, Maranhão, Mato Grosso, Minas Gerais e Pará. Os isolados causaram uma variedade de sintomas em soja cv. CD206, isolados brandos e severos foram observados. O genoma completo de 6 isolados foi sequenciado e adicionalmente a sequencia parcial de outros 12 isolados foi também determinada (ORF2-3'terminal). Nenhum isolado brasileiro de CPMMV, independente do hospedeiro, havia sido totalmente sequenciado até esse trabalho. As caracterizações biológica e molecular mostraram que os seis isolados brasileiros, cujos genomas foram completamente determinados, pertencem a uma nova estirpe de CPMMV, distinta daquela à qual pertence o único isolado de CPMMV previamente sequenciado, oriundo de Gana na Àfrica. A ORF1 (RdRp) desses seis isolados brasileiros apresentou valores de identidade de sequencia (60-61% para nt e 58-69% para aa), inferiores ao estabelecido pelo Comitê Internacional de Taxonomia de Vírus (ICTV), quando foram comparados com o isolado Gana de CPMMV. A ORF5 (CP), no entanto, apresentou valores de identidade (79% para nt e 95-96% para aa) superiores ao estabelecido pelo ICTV quando foram comparados com o isolado Gana. Ambas as proteínas são utilizadas para classificar isolados do gênero *Carlavirus* em uma mesma espécie. As comparações par-a-par e análises filogenéticas mostraram que os isolados brasileiros são altamente relacionados entre si e distintos de isolados de outras espécies do gênero *Carlavirus*. As árvores filogenéticas construídas com as sequencias parciais dos genomas não mostraram agrupamentos com base em região geográfica ou ano de coleta, porém agrupamentos com base nos sintomas foram observados para as árvores construídas com a sequência parcial (ORF2-3'terminal), ORF2 (TGB1), ORF5 (CP) e ORF6 (NABP). Além do relacionamento entre os isolados de CPMMV, foi demonstrado através do sequenciamento parcial, que existem variações entre os isolados brasileiros. Evidencias de duas possíveis estirpes de CPMMV no Brasil foram encontradas, com variações moleculares e biológicas entre os isolados de ambas as estirpes. Eventos de recombinação foram identificados ao longo do genoma dos isolados, e eles ocorreram principalmente na ORF1, região da polimerase, e com menor frequência em outras regiões genoma. Com esse trabalho foi verificado que o critério taxonômico, que define as espécies do gênero *Carlavirus*, pode ser falho em casos em que apenas a sequencia parcial é determinada, se apenas a ORF1 tivesse sido determinada durante o estudo poderíamos propor que os nossos isolados pertencem à uma nova espécie do gênero *Carlavirus.* Além disso, ficou clara a necessidade de se determinar a ocorrência de transmissão por semente dos isolados de CPMMV brasileiros, pois a transmissão por sementes e/ou a alta capacidade de dispersão e voo da mosca branca *Bemisia tabaci* podem ter contribuído para a dispersão do vírus nos diferentes estados produtores de soja. Esses fatos justificam o não agrupamento dos isolados com base em região geográfica ou ano de coleta.

ABSTRACT

ZANARDO, Larissa Goulart, M.Sc., Universidade Federal de Viçosa, February 2013. **Biological characterization, molecular and analysis of genetic variability of *Cowpea mild mottle virus* (CPMMV) in soybean in Brazil.** Advisor: Francisco Murilo Zerbini Júnior. Co-advisers: Claudine Márcia Carvalho and Gloria Patricia Castillo Urquiza.

Since 2000, field soybean plants in the states of Bahia, Goiás, Maranhão, Mato Grosso, Minas Gerais, Paraná and Tocantins have been described with symptoms of soybean stem necrosis disease. The symptoms are varied, some milder other severe. The disease has been associated with *Cowpea mild mottle virus* (CPMMV, family *Betaflexiviridae*, genus *Carlavirus*). This study is aimed at the biological characterization and molecular and genetic analyses of genetic variability of isolates of CPMMV that cause symptoms of stem necrosis in soybean fields of different producing Brazil states. The study was conducted with samples collected in the states of Bahia, Goiás, Maranhão, Mato Grosso, Minas Gerais and Pará. The isolates caused a variety of symptoms in soybean cv. CD206, and mild and severe isolates were observed. The complete genomes of six isolates were sequenced and additionally the partial sequence of another 12 isolates was also determined (ORF2-3'end). No Brazilian CPMMV isolate, from any host, had been entirely sequenced until now. Biological and molecular characterization showed that the six Brazilian isolates, whose genomes have been completely determined, belong to a new CPMMV strain distinct from that which belongs to the only isolated CPMMV previously sequenced from Ghana in Africa. The ORF1 (RdRp) of these six Brazilian isolates showed values of sequence identity (60-61% to 58-69% for nt and aa), less than that set by the International Committee on Taxonomy of Viruses (ICTV), when they were compared with a Ghanaian CPMMV isolate. ORF5 (CP), however, showed identity values (79% to 95-96% for nt and aa) greater than those established by the ICTV when they were compared with the Ghanaian isolate. Both proteins are used to classify isolates of the genus *Carlavirus* in the same species. Pairwise comparisons and phylogenetic analysis showed that Brazilian isolates are highly related to each other and distinct from isolates of other species of the genus *Carlavirus*. The phylogenetic trees constructed with partial sequences of the genomes did not show groupings based on geographic region or collection year, but groupings based on symptoms were observed for trees constructed with partial sequences (ORF2-3'end), ORF2 (TGB1), ORF5 (CP) and ORF6 (NABP). Besides the relationship between isolates CPMMV demonstrated by partial sequencing, there are variations between the Brazilian isolates. Evidence of two possible CPMMV strains were found in Brazil with biological and molecular variations between isolates of both strains. Recombination events were identified in genome of the isolates, and they occurred mainly in the ORF1 region of the polymerase, less frequently in other regions of the genome. With this study it was found that the taxonomic criteria, which define the genus *Carlavirus* may fail in cases where only a partial sequence is determined: if only the ORF1 had been determined during the study we could propose that our isolates belong to a new species of the genus *Carlavirus*. In addition, there is clearly the need to determine the occurrence of seed transmission in Brazilian CPMMV isolates, since the transmission by seed and/or whitefly *Bemisia tabaci*, with high dispersibility may have contributed to the spread of the virus in different soybean producing states. These facts justify the non-grouping of isolates based on geographic region or collection year.

INTRODUÇÃO GERAL

A família *Betaflexiviridae* é constituída por seis gêneros: *Capillovirus*, *Carlavirus*, *Citrivirus*, *Foveavirus*, *Trichovirus* e *Vitivirus* (King *et al.*, 2011). Vírions da família *Betaflexiviridae* são filamentosos com 12-13nm de diâmetro e 600-1000 nm de comprimento, variando de acordo com o gênero. São monopartidos, com genoma de RNA fita simples sentido positivo (ssRNA). Possuem na extremidade 5' terminal um 'cap' (m7GpppG) e na extremidade 3' terminal a cauda Poli(A). A tradução das fases abertas de leitura ('Open Reading Frames' - ORFs), é feita através de mRNAs subgenômicos, e até seis proteínas podem ser geradas desde a extremidade 5' até a 3' do genoma viral (Adams *et al.*, 2004; Martelli *et al.*, 2007; King *et al.*, 2011). Há uma grande diversidade no número e na natureza dos genes virais que são expressos próximos a região 3' através de mRNAs subgenômicos. Estes genes codificam proteínas do movimento viral como as da superfamília p30-like (gêneros *Capillovirus*, *Citrivirus*, *Trichovirus* e *Vitivirus*), proteínas do bloco triplo de genes (TGB) (gêneros *Carlavirus* e *Foveavirus*), proteínas de ligação a ácidos nucléicos (NABP), a proteína do capsídeo (CP) e outras proteínas encontradas em alguns membros da família (Figura 1) (Martelli *et al.*, 2007; King *et al.*, 2011). Apesar da variedade de proteínas encontradas para os gêneros dessa família a RNA polimerase dependente de RNA (RdRp) e a proteína do capsídeo (CP) formam uma unidade taxonômica coerente (Adams *et al.*, 2004).

A organização genômica típica varia entre os gêneros (Figura 1), e até entre espécies de um mesmo gênero. A primeira e maior ORF é sempre o gene da replicase, cujo tamanho varia de 190 a 250 kDa (King *et al.*, 2011). Nesta sequência está contido os domínios metiltransferase, RNA helicase e RNA polimerase dependente de RNA comuns a todos os vírus pertencentes á família (Figura 1)

(Koonin & Dolja, 1993; King *et al.*, 2011). Em seguida estão as ORFs responsáveis por codificar proteínas relacionadas ao movimento viral. Dois gêneros da família *Betaflexiviridae*, *Carlavirus* e *Foveavirus*, possuem um conjunto de três ORFs (ORF2, 3 e 4), que se sobrepõem parcialmente, conhecido como bloco triplo de genes (‘triple gene block’ - TGB), envolvido no movimento célula-á-célula e a longa distância (Morozov & Solovyev, 2003; Martelli *et al.*, 2007; King *et al.*, 2011). Adicionalmente, foi demonstrado que a TGBp1 (ORF2) do *Potato virus* M (PVM, gênero *Carlavirus*) suprime o silenciamento sistêmico (Senshu *et al.*, 2011). Os outros quatro gêneros possuem uma única ORF que codifica uma proteína da superfamília 30K. A última ou penúltima ORF (ORF5) é a responsável por codificar a proteína do capsídeo (CP), já que alguns gêneros, *Carlavirus*, *Trichovirus* e *Vitivirus,* o genoma viral apresenta uma sexta ORF, cujo produto é uma proteína rica em cisteína (‘cysteine-rich protein’ - CRP) com atividade de ligação à ácido nucleico (‘nucleic acid-binding activity’ - NABP) (Martelli *et al.*, 2007; King *et al.*, 2011). Foi demonstrado para o *Grapevine virus A* (GVA, gênero *Vitivirus*) (Zhou *et al.*, 2006) e para o PVM que a proteína NABP ou CRP é capaz de suprimir o silenciamento de RNA sistêmico e local (Senshu *et al.*, 2011).

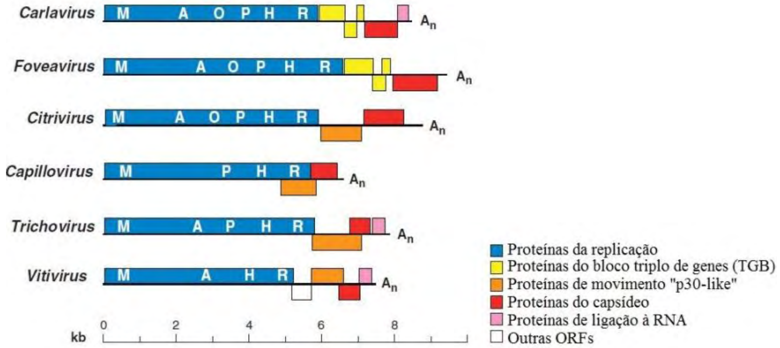

Figura 1: Representação esquemática dos genomas de espécies pertencentes aos gêneros que compõe a família *Betaflexiviridae*. As barras coloridas representam as proteínas codificadas por cada uma das possíveis fases de leitura aberta (‘Open Reading Frame’ - ORFs). Os genomas virais podem atingir até 9000 nucleotídeos e seis ORF’s. Domínios da replicase: M-metiltransferase, A-alkB, O-otu-like peptidase, P-papain-like protease, H-RNA helicase e R-RNA polimerase dependente de RNA. Reproduzido de Martelli et al. (2007) com modificações.

Os flexivírus de modo geral infectam uma variedade de hospedeiros selvagens e cultivados incluindo plantas herbáceas, dicotiledôneas lenhosas e com menos frequência monocotiledôneas (Martelli *et al.*, 2007). Os capillovírus, citrivírus, foveavírus, trichovírus e os vitivírus tendem a infectar plantas lenhosas, já os carlavírus infectam preferencialmente plantas herbáceas, podendo ser assintomáticos ou sintomáticos, com o mosaico um dos sintomas principais (Martelli *et al.*, 2007). Os membros da família *Betaflexiviridae* tem sido descritos em vários hospedeiros, mas a gama de hospedeiros é restrita para membros individuais de cada gênero (King *et al.*, 2011).

Todas as espécies da família podem ser transmitidas via inoculação mecânica. Os capillovírus, citrivírus e foveavírus não possuem vetor conhecido, porém são capazes de ser transmitidos via enxertia, material propagativo e apenas os capillovírus via sementes. Os carlavírus podem ser transmitidos por afídeos, na grande maioria, ou por mosca branca, no caso do *Cowpea mild mottle virus* (CPMMV) (Naidu *et al.*, 1998; Almeida *et al.*, 2005; King *et al.*, 2011), do *Melon yellowing-associated virus* (MYaV) (Nagata *et al.*, 2005) e do *Cucumber vein-clearing virus* (Menzel *et al.*, 2011). Os trichovírus são também transmitidos por material propagativo e enxertia e são os únicos transmitidos por ácaros (King *et al.*, 2011). Já os vitivírus podem ser transmitidos por material propagativo, cochonilhas e afídeos (King *et al.*, 2011).

Os carlavírus replicam-se nas células parênquimais do hospedeiro e por isso são mais facilmente transmitidos que os vírus restritos ao floema (Martelli *et al.*, 2007). Os sintomas induzidos por carlavírus variam de muito leve à grave, dependendo da variedade das plantas, das condições ambientais, das espécies ou estirpes de vírus, como verificado em especial para viroses da batata (Nie *et al.*, 2008). Muitas vezes pode ser observado a ocorrência de infecções assintomáticas (Martelli *et al.*, 2007).

No Brasil entre as espécies do gênero *Carlavirus* encontradas estão o *Potato virus S* propagado em batata (*Solanum tuberosum*) (Gaspar *et al.*, 2008; Duarte *et al.*, 2012), *Cole latent virus* que infecta couve (Gaspar *et al.*, 2008), o *Cowpea mild mottle virus* (CPMMV) em plantas de feijão (Costa *et al.*, 1983) e soja (Almeida *et al.*, 2003; Almeida *et al.*, 2005; Almeida, 2008; Gaspar *et al.*, 2008), *Garlic common latent virus* em alho (Fajardo *et al.*, 2001), *Lily symptomless virus* em lírio (Rivas, 2010) e o *Melon yellowing-associated virus* (MYaV) em plantas de melão (Nagata *et al.*, 2005). Até o ano de 2005 o CPMMV era considerado o único carlavírus transmitido pela mosca branca *Bemisia tabaci* (Munyappa & Reddy, 1983; Naidu *et al.*, 1998; Adams *et al.*, 2004). Nesse ano, porém o MYaV foi descrito como também sendo transmitido por esse vetor (Nagata *et al.*, 2005). A incidência e os danos causados por *B. tabaci* aumentaram exponencialmente a partir da década de 70, em associação ao grande aumento da área plantada com soja especialmente porque a soja é um excelente hospedeiro de *B. tabaci*. A não adoção de medidas de controle permite que as populações de insetos atinjam níveis altíssimos.

Na safra de 2000/01 plantas de soja na região de Morrinhos e Goiatuba, estado de Goiás, apresentavam sintomas de nanismo, queima do broto e necrose da haste. As necroses eram severas e levavam as plantas à morte. Os sintomas foram associados à necrose da haste, cujo agente etiológico é o CPMMV. Novamente, em 2001/02 sintomas similares apareceram em plantas de soja em Barreiras, estado da Bahia. Em 2002, um novo surto ocorreu devastando os campos do estado de Goiás, nas regiões de Acreúna, Quirinópolis e Porteirão. Cerca de 1000 ha apresentaram perdas totais, ocasionando um prejuízo de 600 mil dólares. Na safra 2002/2003 o

CPMMV foi encontrado em Sorriso (MT), Balsas (MA), Palotina (PR) e Goiânia, Luziânia e Vianópolis (GO) (Almeida *et al.*, 2003; Almeida *et al.*, 2005).

Os hospedeiros naturais do CPMMV incluem espécies da família *Fabaceae*. Sua ocorrência tem sido relatada na Ásia (Irã, Israel, Taiwan e Tailândia) (Antignus & Cohen, 1987; Tavasoli *et al.*, 2009), África (Costa do Marfim, Gana, Nigéria, Tanzânia) (Brunt & Kenten, 1973; Thouvenel *et al.*, 1982; Mink & Keswani, 1987; Menzel *et al.*, 2010), Brasil (Costa *et al.*, 1983; Almeida *et al.*, 2005). Na Argentina foi relatada a ocorrência em feijão e soja na Província de Salta (Rodríguez-Pardina *et al.*, 2004; Laguna *et al.*, 2006). Recentemente, foi encontrado infectando feijão de corda (*Vigna unguiculata* subsp. *Sesquipedalis*) na Venezuela (Brito *et al.*, 2012). No Brasil o vírus já foi encontrado em diversos estados produtores de soja: Bahia, Goiás, Maranhão, Mato Grosso, Minas Gerais, Paraná e Tocantins (Almeida, 2008). Entre os estados produtores apenas o Rio Grande do Sul ainda não apresentou relatos da doença.

Os sintomas causados por carlavírus variam de acordo com o hospedeiro que está sendo infectado e a época do ano. O CPMMV em feijão caupi (*Vigna unguiculata*) causa manchas cloróticas nas folhas primárias e distorção das folhas, em amendoim provoca lesões necróticas, anéis cloróticos, com posterior clorose sistêmica (Brunt & Kenten, 1973). Em soja e feijão comum (*Phaseolus vulgaris*) pode causar uma diversidade de sintomas como: clorose e mosaico nas folhas, necrose apical, distorção e nanismo (Brunt & Kenten, 1973; Iwaki *et al.*, 1982; Almeida *et al.*, 2003; Almeida, 2008; Tavasoli *et al.*, 2009). Em campo, os sintomas iniciais da virose da soja não chamam a atenção dos produtores, apenas próximo à floração e surgimento das vagens é que eles tornam-se mais evidentes (Almeida, 2008). É após esse período que são observadas a queima do broto e a necrose das hastes, que terminam na morte das plantas e consequente perda de produção (Almeida, 2008).

Alguns isolados causam sintomas severos (necroses) outros já mais brandos (mosaico e bolhas no limbo), podendo às vezes ser assintomáticos. Os sintomas distintos apresentados pelos isolados de CPMMV sugerem a existência de estirpes distintas ou mesmo mais de uma espécie de carlavírus.

A variabilidade genética para o CPMMV ainda não foi estudada, nem sua história evolutiva. Na verdade poucos isolados foram sequenciados parcialmente, e apenas um isolado teve seu genoma completamente determinado (Menzel *et al.*, 2010). A maior parte dos trabalhos envolvendo o CPMMV relata a identificação viral, sua caracterização biológica, o sequenciamento parcial do genoma e a identificação do vetor (Brunt & Kenten, 1973; Iwaki *et al.*, 1982; Thouvenel *et al.*, 1982; Costa *et al.*, 1983; Munyappa & Reddy, 1983; Jeyanandarajah & Brunt, 1993; Almeida *et al.*, 2005; Laguna *et al.*, 2006; Marubayashi *et al.*, 2010). Apesar de ter sido identificado há cerca de 40 anos atrás (Brunt & Kenten, 1973), muito pouco se sabe sobre esse vírus no Brasil e no mundo, porém o vírus tem sido encontrado com frequência nos campos brasileiros de soja. Levando em consideração que o Brasil é o segundo maior produtor de soja do mundo, que o vírus é disseminado pela mosca branca *B. tabaci* e que já foi encontrado em diversos campos de estado produtores de soja, maiores perdas econômicas poderão ocorrer se o vírus continuar a ser disseminado pelos campos de soja.

Evidências de variação genética em vírus de plantas foram reportadas em 1926, quando se observou possivelmente que diferentes variantes causariam diferentes sintomas, citado por (Garcia-Arenal *et al.*, 2001). As variações genéticas surgem de erros que ocorrem durante a replicação dos genomas virais (Garcia-Arenal *et al.*, 2001). As mutações representam a fonte primária de variação genética em que a seleção natural e a deriva genética atuam (Drake & Holland, 1999). Nesse contexto, a mutação corresponderiam ao processo de incorporação de nucleotídeos na fita recém formada, que durante a replicação do ácido nucléico estão ausentes na fita molde (Garcia-Arenal *et al.*, 2001). As taxas de mutação são importantes para o entendimento da estrutura genética de populações ao longo do tempo e consequentemente o curso da evolução. Os vírus de RNA possuem taxas de mutação superiores as determinadas para os vírus de DNA (Drake & Holland, 1999). Para vírus de DNA a taxa de mutação estimada é de 10^{-8} a 10^{-6} substituições por nucleotídeo por infecção celular (s/n/c) e para vírus de RNA a taxa de mutação estimada é de 10^{-6} a 10^{-4} s/n/c (Sanjuan *et al.*, 1999).

Novos variantes virais podem surgir também a partir da recombinação. No contexto da infecção viral, a recombinação seria o processo pelo qual segmentos do genoma são trocados entre segmentos de nucleotídeos de diferentes variantes genéticos durante o processo de replicação, resultando em trocas genéticas (Garcia-Arenal *et al.*, 2001). Compreender o papel da recombinação na evolução dos vírus de planta é altamente importante. Há evidências crescentes sobre a contribuição da recombinação da variabilidade genética em vírus de RNA, isso inclui uma variedade de vírus de senso positivo. As taxas de recombinação variam amplamente e as causas dessas variações ainda não são claras (Chare & Holmes, 2006).

Conhecer as características moleculares e biológicas de um dado vírus e a sua variabilidade genética é altamente importante, pois essas informações permitem conhecer características do patógeno que podem contribuir para a adoção de medidas preventivas contra a virose no campo. Assim, o estudo realizado nesse trabalho visa caracterizar biológica e molecularmente isolados de CPMMV obtidos de soja, e avaliar a variabilidade genética do CPMMV.

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CHAPTER 1

MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF *Cowpea mild mottle virus* ISOLATES INFECTING SOYBEAN IN BRAZIL AND EVIDENCE OF RECOMBINATION

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Molecular and biological characterization of *Cowpea mild mottle virus* isolates infecting soybean in Brazil and evidence of recombination

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Short title: Characterization of CpMMV isolates infecting soybean

Key words: CpMMV; *Carlavirus*; phylogeny; recombination.

Abstract

We report the biological and molecular characterization of six isolates of a new *Cowpea mild mottle virus* strain (CPMMV; *Carlavirus, Betaflexiviridae*). Soybean plants with mosaic and stem necrosis were collected in Bahia, Goiás, Mato Grosso and Minas Gerais states, Brazil. Complete genomes of the CPMMV isolates are 8,180-8,198 nucleotides (nt) long, excluding the 3'-polyadenylate tail, and have 67-68% nt sequence identity with a Ghanaian isolate of CPMMV, the only CPMMV isolate for which the genome previously has been sequenced. The replicase has only 60-61% nt sequence identity with the Ghanaian CPMMV isolate, and the coat protein is highly conserved (79% nt sequence identity and 95-96% amino acid sequence identity). The high CP identity and the phylogenetic analyses supported the classification of the Brazilian isolates as CPMMV. Biological and molecular differences with the Ghanaian CPMMV isolate were found and indicated that the six

isolates represent a distinct CPMMV strain denominated as CPMMV-BR. Furthermore, we show that recombination occurred mainly in the polymerase gene, and may occur less frequently in other regions of the CPMMV genome.

Introduction

Cowpea mild mottle virus (CPMMV, family *Betaflexiviridae*, genus *Carlavirus*) is a serious problem in Brazilian soybean (*Glycine max*), causing dwarfing, chlorosis, vein clearing/mosaic, leaf and stem necrosis (Iwaki *et al.*, 1982; Almeida *et al.*, 2003; Almeida, 2008; Tavasoli *et al.*, 2009). The virus was originally reported infecting cowpea (*Vigna unguiculata*) in Ghana (Brunt & Kenten, 1973; Tavasoli *et al.*, 2009) and was subsequently found causing mosaic and leaf crinkling in soybean in Thailand (Iwaki *et al.*, 1982) and the Ivory Coast (Thouvenel *et al.*, 1982). At around the same time, it was reported in Brazil in common bean (*Phaseolus vulgaris*) cv. Jalo (Costa *et al.*, 1983). Much later, in the 2000-2001 growing season, soybean plants showing symptoms of stem necrosis caused by CPMMV were observed in the state of Goiás. Over the next two years, new outbreaks occurred in the states of Mato Grosso, Bahia, Maranhão and Paraná (Almeida *et al.*, 2003), several thousands of kilometers apart.

CPMMV has a single-stranded, positive-sense RNA genome of 8,127 nucleotides polyadenylated at the 3'end, with a typical organization of genus *Carlavirus* in six open reading frames (ORFs) and a short UTR at the 5' and 3' termini (Martelli *et al.*, 2007; Menzel *et al.*, 2010; Adams *et al.*, 2012). ORF1 encodes the putative RNA-dependent RNA polymerase (RdRp). ORFs 2, 3 and 4 encode the triple gene block (TGB1, TGB2, TGB3, respectively), essential for virus movement (Martelli *et al.*, 2007; Adams *et al.*, 2012). ORF5 encodes the coat protein

(CP) while ORF6 encodes a nucleic acid-binding protein (NABP) with a zinc finger motif (Koonin *et al.*, 1991; Lukhovitskaya *et al.*, 2009). The filamentous virus particles can be found in the cytoplasm of palisade, mesophyll, parenchyma and epidermal cells in soybean and *Nicotiana clevelandii* (Brunt *et al.*, 1983). These particles form aggregates in the form of sheets or bundles and often brush-like inclusions (Brunt *et al.*, 1983; Almeida *et al.*, 2003; Almeida *et al.*, 2005). CPMMV is transmitted in a non-persistent manner by the whitefly *Bemisia tabaci* (Munyappa & Reddy, 1983; Jeyanandarajah & Brunt, 1993; Naidu *et al.*, 1998; Almeida *et al.*, 2005; Marubayashi *et al.*, 2010; Brito *et al.*, 2012). Seed transmission appears to be dependent on the viral isolate: for a Ghanaian isolate of CPMMV, seed transmission occurred in soybean, cowpea and with lower frequency in common bean (Brunt & Kenten, 1973); in Venezuela it was demonstrated that CPMMV can be transmitted by yardlong bean seeds (Brito *et al.*, 2012); but Almeida et al. (2005) reported that a Brazilian CPMMV isolate was not transmitted by soybean seeds.

To date, only one CPMMV isolate, from cowpea has been completely sequenced (Menzel *et al.*, 2010). This is probably the same isolate as that previously characterized biologically (Brunt & Kenten, 1973). Only partial sequences of CPMMV from soybean have been reported (Almeida *et al.*, 2005; Tavasoli *et al.*, 2009). Several partial sequences designated as CPMMV are available in GenBank, including the coat protein and/or NABP regions and movement proteins. Full molecular and biological characterization of Brazilian CPMMV isolates is important for a better understanding of this emerging pathogen in soybean crops. The aims of this study were to sequence the full genome of a range of CPMMV isolates collected from soybean, to determine their molecular and biological characteristics, and to compare the genetic variability between these isolates and the previously reported Ghanaian CPMMV isolate from cowpea.

Material and methods

Viral isolates and host range studies

The six CPMMV isolates used in this study were obtained from soybean plants that showed dwarfing and stem necrosis. Three isolates, CPMMV:BR:GO:01:1, CPMMV:BR:BA:02 and CPMMV:BR:MT:02:, were obtained from samples previously collected respectively in Goiatuba (Goiás state – 2000-2001 season, coordinates 18°0’40” S, 49°22’10” W), Barreiras (Bahia state – 2001-2002 season, coordinates 12°8’54” S, 44°59’33” W), and Sorriso, (Mato Grosso state – 2001-2002 season, coordinates 12°33’31” S, 55°42’51” W) (Almeida *et al.*, 2003). The other three isolates, CPMMV:BR:MG:09:2, CPMMV:BR:MG:09:3 and CPMMV:BR:GO:10:5, were obtained from samples collected in Capinópolis (coordinates 18°40’48” S, 49°33’58” W) and Tupaciguara (coordinates 18°36’12” S, 48°41’25” W), Minas Gerais state – 2008-2009 season; and in Cristalina (Goiás state – 2009-2010 season, coordinates 16°46’4” S, 47°36’47” W), respectively. All collected samples were stored in a freezer at -80° C, in order to preserve the original samples. For the study all six isolates were inoculated and maintained in a greenhouse by mechanical inoculation onto soybean cv. CD206 using 0.1 M phosphate buffer, pH 7.2, with 0.1% sodium sulfite.

To rule out the occurrence of mixed infections, the presence of begomoviruses and of *Soybean mosaic virus* (SMV, genus *Potyvirus*) was checked. The presence of begomoviruses was evaluated by total DNA extraction (Dellaporta *et al.*, 1983) followed by PCR amplification using the universal oligonucleotides

PBL1v2040/PCRc1 (Rojas *et al.*, 1993). Infection by SMV was tested by indirect ELISA using a specific polyclonal antiserum produced by one of the authors (AMRA).

For host range tests, the isolates were mechanically inoculated on to plants of 22 species/cultivars belonging to the families Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae and Solanaceae (Table 1). Inoculated plants were kept in a greenhouse with average daily temperatures of 26 ± 2^{o}C. Symptoms were recorded until 28 days post-inoculation (dpi). Systemic top leaves were used for indirect ELISA tests (Clark *et al.*, 1986) using a polyclonal antiserum (Carvalho *et al.*, 2013) to confirm CPMMV infection in these plants. Additionally, plants showing only local symptoms on inoculated leaves were used for indirect ELISA.

RT-PCR and cloning

Total RNA was extracted from 100 mg of leaf tissue of soybean cv. CD206 systemically infected with each of the six CPMMV isolates (21 dpi), using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription (RT) was performed using Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's protocol, using 500 ng of total RNA and the CPMMV primers (40 μM) (Suppl. Table S1). The primers were designed based on the alignment of carlavirus sequences available in GenBank and on the sequence of the Ghanaian isolate of CPMMV (Suppl. Table S2). PCR amplifications were carried out using Platinum *Taq* polymerase (Invitrogen) and forward and reverse primers (Suppl. Table S1). All amplifications consisted of 35 cycles of the following profile: 94ºC for 1 min, annealing at the temperatures listed in Suppl. Table S1, elongation at 72ºC for 1–2 min, depending on the expected size of the fragment to be amplified, and a final extension step at 72ºC for 10 min. Amplified PCR products were gel-purified using the Ilustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), ligated into the pGEM-T Easy Vector (Promega) and transformed into *Escherichia coli* DH5α cells. Plasmid DNA was purified from recombinant clones using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced in both directions with universal primers (M13F/M13R) at Macrogen (Seoul, South Korea). The sequence of the 5'-end of the viral genome was determined using the Rapid Amplification of complementary DNA (cDNA) Ends (RACE) kit, version 2.0 (Invitrogen) according to the manufacturer's protocol, using primer Race R (Suppl. Table S1).

Sequence analysis and phylogeny

The sequences were assembled using DNA BASER Sequence Assembler v.3.5 (Heracle Biosoft) and the ORFs were located using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The complete genomes were deposited in GenBank [accession no. KC884244 (CPMMV:BR:MG:09:2), KC884245 (CPMMV:BR:MG:09:3), KC884246 (CPMMV:BR:MT:02:1), KC884247 (CPMMV:BR:BA:02), KC884248 (CPMMV:BR:GO:01:1), KC884249 (CPMMV:BR:GO:10:5)]. The nucleotide (nt) sequences of the RdRp (ORF1) and CP (ORF5) were initially submitted to a BLAST search for preliminary species assignment based on the 72% threshold level established for the *Carlavirus* genus (Adams *et al.*, 2012). Additional pairwise nt sequence comparisons were performed by p-distance using MEGA v. 5 (Tamura *et al.*, 2011), and amino acid sequence comparisons were performed using DNAMAN v. 7.0 (Lynnon Biosoft) using the quick alignment method with default parameters. The transmembrane domains of the proteins were estimated using SMART (http://smart.embl.de/) (Letunic *et al.*, 2012). The prediction of putative nuclear localization signals were estimated using cNLS Mapper (http://nls-mapper.iab.keio.ac.jp) (Kosugi *et al.*, 2009).

Nucleotide sequences used in phylogenetic and recombination analyses were aligned using the MUSCLE (Edgar, 2004) module in MEGA v. 5. Phylogenetic analyses were performed using carlavirus sequences from GenBank and the six isolates obtained in this study (Suppl. Table S2). For the phylogenetic tree of the complete genomes, the 5’UTR, 3’UTR and intergenic regions were removed from the alignment and overlapping coding regions were maintained. Phylogenetic trees were constructed by Bayesian inference and Markov Chain Monte Carlo (MCMC) simulation implemented in MrBayes v. 3.0 (Ronquist & Huelsenbeck, 2003), with the evolution models selected by MrModeltest v. 2.2 (Nylander, 2004) using the Akaike Information Criterion (AIC). The MCMC simulation was run for 10 million generations and was sampled every 500 steps, resulting in 20,000 saved trees. Burn-in was set at 2 million generations, leaving 16,000 trees from which the 50% majority rule consensus trees and posterior probabilities were calculated. The trees were viewed using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/)

Recombination analysis

Detection of potential recombinant sequences, identification of likely parental sequences and localization of possible recombination break points were performed using the Recombination Detection Program RDP v.3.44 (Martin *et al.*, 2010). A multiple comparison-corrected P-value cutoff of 0.05 was used throughout. The analysis included only full CPMMV nucleotide sequences. Only recombination events which were detected by three or more of the seven methods implemented in RDP 3.44 were considered.

Results

Biological properties of CPMMV isolates

After excluding the occurrence of begomoviruses and SMV in the plants in which the CPMMV isolates were being maintained (data not shown), we proceeded with the biological characterization. The Brazilian CPMMV isolates were able to infect a limited number of host plants (Table 1). None of the isolates infected the representatives of the families Amaranthaceae and Cucurbitaceae (Table 1). Of the seven cultivars tested, belonging to three species of family Fabaceae, three were systemically infected by all isolates (Table 1). One of these was soybean cv. CD206, in which a range of symptoms were observed: three isolates caused mosaic and/or crinkled leaves associated with lower disease severity (Figure 1C-E), while the other three isolates caused severe symptoms such as leaf and stem necrosis and bud blight (Figure 1F-K). Soybean cv. Pintado was also infected by all isolates but in this cultivar the infection was consistently symptomless. Meanwhile, common bean cv. Jalo was consistently infected, with mosaic symptoms, by all isolates (Table 1 and Figure 1L). Common bean cv. Manteigão was infected only by isolates CPMMV:BR:GO:01:1 and CPMMV:BR:MT:02:1, and cowpea cv. B7 Gurguéia was infected only by isolates CPMMV:BR:BA:02 and CPMMV:BR:GO:01:1, suggesting that they could be used as differential hosts (Table 1 and Figure 1M).

For the representatives of the Chenopodiaceae and Solanaceae families, only local symptoms in the inoculated leaves were observed (Table 1 and Figure 1A, B, N, O and P). Additionally, a biological characteristics exclusive to the Brazilian

CPMMV isolates was the induction of chlorotic local lesions in *N. benthamiana* (Figure 1N) and *N. debneyi* (Figure 1P), respectively. In the cases of CPMMV:BR:BA:02 and CPMMV:BR:GO:01:1, a further characteristic was the induction of necrotic local lesions and dwarfing in *N. glutinosa* (Table 1 and Figure 1O).

Genomic sequence of CPMMV

The complete genomic sequences of the Brazilian CPMMV isolates were assembled from overlapping clones of RT-PCR and RACE products. They range in size from 8,180 to 8,198 nucleotides (nt), excluding the 3’-terminal poly(A) (Table 2). The nt sequence identity values of the complete genomes among the Brazilian isolates are very high, ranging from 93 to 99%, but are far lower when compared to the Ghanaian isolate (HQ184471), at 67-68%.

The genomes are organized into six putative ORFs, as for other species in the genus *Carlavirus*. The 5’-untranslated region (UTR) is 72-94 nt in length, while the 3’ UTR is 36-89 nt (Table 2). The nucleotide identity values for the 5'-UTR and 3'-UTR regions range from 94-100% and 98-100%, respectively, among the Brazilian isolates, but again are lower when comparing Brazilian isolates with the Ghanaian isolate, at 79-80% and 89-91%, respectively.

ORF1 of all isolates encodes a putative replicase protein, with four conserved motifs: methyltransferase, C23 peptidase (cysteine endopeptidase of single-stranded RNA viruses), RNA helicase and RNA-dependent RNA polymerase. The RdRp is the most conserved ORF among Brazilian isolates (Figure 2). However, identity values between the Brazilian isolates and the Ghanaian isolate are lower than 72% (nt) and 80% (aa) (Figura 2).

ORF2 is separated from ORF1 by an intergenic region of 28 nt, except for isolate CPMMV:BR:GO:01:1. The ORF2 of this isolate shows two possible initiation codons. The first is situated within ORF1 and the second is separated from ORF1 by an intergenic region of 28 nt, with 234 aa residues (Table 2). ORF2 of the other five Brazilian isolates encodes a polypeptide of 231 aa. ORF3 and ORF4 of Brazilian CPMMV isolates showed the same size (Table 2). These three ORFs comprise the triple gene block (TGB). The TGB1 protein (ORF2) contains a P-loop NTPase domain and a RNA helicase domain in all Brazilian CPMMV isolates. For TGB2 protein (ORF3), two transmembrane domains (residues 12-34 and 69-91 or 75-92, in CPMMV:BR:GO:01:1) were predicted in all Brazilian isolates For TGB3 protein (ORF4) a transmembrane domain (residues 4-26) was predicted in all Brazilian CPMMV isolates. High levels of nt and aa identity were observed among the Brazilian CPMMV isolates, and lower levels between Brazilian and Ghanaian isolates, for TGB (Figure 2).

The CP (ORF5) contains a flexivirus domain that is required for genome encapsidation and the motif GLGVPTE at aa positions 120-126 in all Brazilian CPMMV isolates. This motif is conserved in all carlavirus species. ORF5 is separated from ORF4 by a second intergenic region of 14-15 nt for all isolates (Table 2). It is the most conserved region between the Ghanaian CPMMV isolate and all Brazilian isolates (identity values higher than 72% nt and 80% aa) (Figura 2). These results indicate that all six isolates reported in this study belong to the species *Cowpea mild mottle virus*.

ORF6 encodes a nucleic acid binding protein (NABP) with a motif for a putative C-4-type zinc finger and an adjacent putative nuclear localization signal (NLS) defined by YARKRR(A/S)KII with a basic motif (RKRR) for all Brazilian isolates. ORF6 showed the same size for all Brazilian CPMMV isolates except CPMMV:BR:GO:01:1 (Table 2). High identity values were found among Brazilian isolates, and lower values in the comparisons of Brazilian isolates with the Ghanaian isolate (Figura 2).

Sequence comparisons between CPMMV isolates and other carlaviruses showed low levels of identity for all ORFs (Suppl. Figure S1). The *Cucumber vein-clearing virus* (CuVCV) was the carlavirus that showed the highest sequence identity mainly to the ORF2 and ORF5 of CPMMV isolates (Suppl. Figure S1E).

Phylogenetic analyses of complete genomes showed that the Brazilian isolates cluster with the Ghanaian CPMMV isolate with a posterior probability of 1.0 (Figure 3), confirming their close relationship. Analyzing this cluster, it is clear that the Brazilian isolates are highly related to each other and that the isolate CPMMV:BR:GO:01:1 is most distinct amongst them. Additionally, phylogenies were reconstructed for each ORF, and in all cases the Brazilian CPMMV isolates were placed in the same cluster with the Ghanaian isolate (Suppl. Figure S2). The topology of the phylogenetic trees changed slightly depending on the ORF (Suppl. Figure S2), but the close relationship among CPMMV isolates was sustained. The Brazilian isolates formed a monophyletic group for all ORFs. The longer branch length separating CPMMV:BR:GO:01:1 from the other Brazilian isolates for all ORFs, except ORF1, suggests that these regions are more divergent for this isolate (Suppl. Figure S2). Overall, phylogenetic analyses indicated a close relationship between Brazilian and Ghanaian CPMMV isolates, supporting the idea that these isolates belong to the same viral species. The species of carlavirus closest to CPMMV was CuVCV, evidenced by the tree of ORF2, ORF3 and mainly ORF5 (Suppl. Figure S2B, C and E).

Recombination analyses

The analysis of the six Brazilian and the single Ghanaian CPMMV genomes revealed five putative recombination events (Figure 4). All detected recombination events had Brazilian isolates as possible recipients, but the Ghanaian isolate was identified as the putative donor (or a close relative) in one event. Recombination was detected mainly in ORF1 (RdRp). Only one event involved ORFs 2, 3 and 4 (TGB) and ORF5 (CP).

Two recombination events (events 3 and 4) involving a large portion of ORF1 of isolates CPMMV:BR:BA:02 and CPMMV:BR:GO:01:1 were supported by all methods in the RDP package (Figure 4). Recombination event 3 showed CPMMV:BR:MT:02:1 as the putative major parent and an unknown isolate as the minor parent, which led to recombinant isolate CPMMV:BR:BA:02. Recombination event 4 showed an unknown isolate as major parent and CPMMV:BR:MG:09:3 as the putative minor parent, which led to recombinant isolate CPMMV:BR:GO:01:1. The Ghanaian isolate (accession # HQ184471) showed up as a possible minor parent for recombination event 1, which occurred in all isolates except CPMMV:BR:GO:01:1 (Figure 4). It is important to note that the possible parents suggested by the RDP analyses, probably, can not be the actual donors. The parents suggested by recombination analysis may be most closely related to the true parental isolates.

Phylogenetic trees based on the recombinant or non-recombinant portions were constructed using Bayesian inference to determine whether the recombination events were supported. This analysis showed that topological incongruence occurred with all recombinant portions of the recombination events identified by RDP3, while for the non-recombinant portion the same groupings observed for the complete genome were maintained (data not shown). These results support our data and reinforce the occurrence of recombination events.

Discussion

Stem necrosis of soybean caused by CPMMV is a recent problem in Brazilian soybean. Although its causal agent was reported in common bean in the 1980's, the occurrence of CPMMV-associated stem necrosis was observed in soybean only 20 years later. Few studies have been carried out with CPMMV, and only one isolate from cowpea has been completely sequenced. Considering the economical importance of soybean in Brazil and the fact that CPMMV is transmitted by the widespread *B. tabaci*, stem necrosis could become an extremely important disease. This study was performed to gather information on soybean isolates of CPMMV.

Interestingly, different symptoms were observed in association with different CPMMV isolates. The virus can also cause a symptomless infection in some hosts. This ability to cause symptomless infections is a characteristic of carlaviruses (Martelli *et al.*, 2007). For cv. CD206, variable symptoms suggest the existence of necrotic and mild virus isolates. The different symptoms caused are an important aspect of these CPMMV isolates. It is too early to ascribe a genetic basis for the necrotic and mild phenotypes, but we suspect that the differences may be associated with the TGB proteins, for two reasons. First, in the case of *Pepino mosaic virus* (PepMV) (genus *Potexvirus*, family *Alphaflexiviridae*), a virus with a genomic organization similar to carlaviruses, mild isolates became necrotic following a point mutation at amino acid 67 of the TGB3 protein (Hasiów-Jaroszewska *et al.*, 2011). Secondly, the TGB was the region of the genome where we found the lowest identity values among our CPMMV isolates.

Overall, the symptoms induced by Brazilian CPMMV isolates were distinct from those induced by the Ghanaian isolate of CPMMV in plants of the family Fabaceae. The Ghanaian isolate, obtained from cowpea, was able to infect several soybean cultivars causing vein mosaic and leaf chlorosis occasionally followed by apical necrosis (Brunt & Kenten, 1973). The Brazilian isolates caused mild mosaic in cowpea cv. B7 Gurguéia, while infection by the Ghanaian isolate in cowpea was symptomless or characterized by mild chlorotic mottle or conspicuous chlorosis (Brunt & Kenten, 1973). In bean, the Brazilian isolates caused mosaic in cv. Jalo and a symptomless infection in cv. Manteigão, while the Ghanaian isolate caused a characteristic chlorotic spotting or faint chlorotic lesions in different cultivars (Brunt & Kenten, 1973).

The induction of local symptoms in representatives of the families Chenopodiaceae and Solanaceae and the inability to infect *G. globosa* (fam. Amaranthaceae) are generally in line with results from the Ghanaian CPMMV isolate (Brunt & Kenten, 1973). However, some relevant differences were also observed: the Ghanaian isolate is able to infect *Nicotiana clevelandii* and causes a local infection in *G. globosa* (Brunt & Kenten, 1973), and an Israeli CPMMV isolate was able to infect tomato, *Datura stramonium* and *N. glutinosa* (Antignus & Cohen, 1987), yet none of these hosts were infected by the Brazilian CPMMV isolates, except *N. glutinosa* that showed necrotic local lesions. Additionally, some Brazilian CPMMV isolates caused chlorotic local lesions in *N. benthamiana* and in *N. debneyi*. This symptom has not previously been described for infections caused by CPMMV.

Overall, the host range study indicated that the Brazilian CPMMV isolates are distinct from Israeli and Ghanaian CPMMV isolates previously described (Brunt & Kenten, 1973; Antignus & Cohen, 1987).

The genome organizations and functional domains of viral proteins of Brazilian and Ghanaian isolates are similar. However, some functional domains typically found in the carlavirus RdRp (alkB, otu-like peptidase and papain-like protease) were not detected in the RdRp of Brazilian CPMMV isolates characterized (Martelli *et al.*, 2007; Menzel *et al.*, 2010; Adams *et al.*, 2012). Among Brazilian CPMMV isolates the most divergent was CPMMV:BR:GO:01:1. The possible overlap between ORF1 and ORF2 observed for CPMMV:BR:GO:01:1 isolate, generated by the initiation codon at position 5,621-5,623, is not a common characteristic among carlaviruses, only *Potato virus S* (PVS) isolates have shown this overlap (GenBank access #: AJ863509, AJ863510, FJ813513, FJ813512 and HF571059). This, added to the fact that the second possible initiation codon, at position 5,681-5,683, generates a protein of the same size (234 aa) as the Ghanaian CPMMV isolate and of similar size as the other Brazilian CPMMV isolates (231 aa), suggests that the protein does not originate from a sequence overlap.

When comparing sequence identities among Brazilian isolates and with the Ghanaian isolate, we found considerably greater differences than we had expected, particularly for the polymerase (ORF1). According to the International Committee on Taxonomy of Viruses (ICTV), carlavirus isolates are considered to belong to the same species if they have greater than 72% nt identity (or 80% aa identity) in their polymerase or CP genes (Adams *et al.*, 2012). From our comparisons of Brazilian and Ghanaian CPMMV isolates, the identity values for polymerase were considerably below the threshold, at 60-61% nt and 58-61% aa, but identity values for the CP satisfy this criterion. A possible explanation for the low identity found in the polymerase would be recombination. This was observed for carlavirus *Chrysanthemum virus B* (CVB) (Singh *et al.*, 2012). The isolate CVB-S possesses identity values for the polymerase of 66-67% nt and 57-58% aa with other CVB isolates, and it was shown that the region encompassing nucleotides 538 to 4260 of the CVB-S polymerase was recombinant (Singh *et al.*, 2012). For *Lily symptomless virus* (LSV), recombination was detected in almost the entire RdRp or its C-terminal region, in the TGB and in almost the entire CP region (Singh *et al.*, 2008). We therefore examined the possibility of recombination among our CPMMV isolates.

Recombination events were indeed found in the polymerase (as well as one event encompassing TGB1, TGB2, TGB3 and CP). Only event 1 involved the Ghanaian isolate. An interesting fact was observed in the phylogenetic tree of ORF1 (RdRp) in which CPMMV:BR:GO:01:1 isolate showed a shorter branch length inside of the Brazilian isolates monophyletic group. This may reflect recombination event 4 in CPMMV:BR:GO:01:1, whose recombinant region, supposedly donated by CPMMV:BR:MG:09:3, encompasses almost the entire RdRp. For all other trees the CPMMV:BR:GO:01:1 isolate forms a separate branch from the other Brazilian isolates. Therefore, CPMMV:BR:GO:01:1 is likely a recombinant between a divergent, unidentified major parent (from which the region encompassing ORFs 2, 3, 4, 5 and 6 is derived), and CPMMV:BR:MG:09:03 (or a close relative), from which its RdRp is derived. This explains the phylogeny and the lower identity values between CPMMV:BR:GO:01:1 and the other Brazilian isolates for ORFs 2, 3, 4, 5 and 6. However, it does not explain the low identity values between the six Brazilian isolates and the Ghanaian isolate for the RdRp.

Although the RdRp of Brazilian CPMMV isolates showed low identity with the Ghanaian isolate the high CP identities support the classification of Brazilian isolates as CPMMV, the taxonomic criterion was satisfied. Thus, given the biological and molecular differences with the Ghanaian CPMMV isolate and the current species demarcation criteria for the genus *Carlavirus*, the Brazilian CPMMV isolates should be classified as a new CPMMV strain, denominated CPMMV-BR. The clear divergence of the CPMMV:BR:GO:01:1 isolate compared with other Brazilian isolates suggests subdivisions in the Brazilian strain. The taxonomic criterion clearly showed that RdRp or CP identity should be used for species demarcation in the genus *Carlavirus*. The results presented showed that the correct classification of Brazilian isolates as CPMMV species was only possible because the complete genomes were sequenced; if only RdRp had been sequenced, a new carlavirus species would have been proposed. The current taxonomic criterion can be risky in cases where only partial sequences have been determined, especially RdRp. Only one of these two genes should be considered and CP seems to be the most appropriate. Or, maybe, the time has come to use whole genomes sequences for species demarcation in the genus *Carlavirus*.

We have determined the complete sequences of the genomes of the six Brazilian isolates and biological characteristics. The results demonstrated significant differences between isolates infecting soybean and cowpea, and among the Brazilian isolates. The genetic and biological variability of Brazilian CPMMV isolates in addition with the transmission by the whitefly *B. tabaci* makes the causal agent of stem necrosis a potential threat to the soybean crop. This study increases our knowledge on the biological differences and genetic variability of Brazilian CPMMV isolates, and provides important information for the improved viral detection and disease management.

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Figure Legends:

Figure 1: Symptoms induced in different hosts infected with the six Brazilian CPMMV isolates. **A.** Chlorotic local lesions in *C. amaranticolor;* **B.** Chlorotic local lesions in *C. quinoa*; **C.** Crinkled leaves, mosaic and vein clearing in soybean CD206 inoculated with CPMMV:BR:BA:02; **D.** Crinkled leaves in soybean CD206 inoculated with CPMMV:BR:GO:01:1; **E.** Mosaic and vein clearing in soybean CD206 inoculated with CPMMV:BR:MT:02:1; **F.** Leaf necrosis in soybean CD206 inoculated with CPMMV:BR:MG:09:2; **G.** Systemic necrosis and dwarfism in soybean CD206 inoculated with CPMMV:BR:MG:09:2; **H.** Leaf necrosis in soybean CD206 inoculated with CPMMV:BR:MG:09:3; **I.** Bud blight and stem necrosis in soybean CD206 inoculated with CPMMV:BR:MG:09:3; **J.** Leaf and stem necrosis in soybean CD206 inoculated with CPMMV:BR:GO:10:5; **K.** Bud blight, dwarfism, leaf and stem necrosis in soybean CD206 inoculated with CPMMV:BR:GO:10:5 **L.** Mosaic in common bean cv. Jalo; **M.** Mosaic in cowpea cv. B7 Gurguéia; **N.** Chlorotic local lesions in *N. benthamiana*; **O.** Necrotic local lesions in *N. glutinosa*; **P.** Chlorotic local lesions in *N. debneyi*. The symptoms described in A, B, L, M, N, O and P were induced by all Brazilian CPMMV isolates.

Figure 2: Two-dimensional plot representing the percent sequence identities between the Brazilian CPMMV isolates and the Ghanaian CPMMV isolate (HQ184471) for all open reading frames (ORFs). Percent nucleotide sequence identities are shown above the diagonal and percent amino acid sequence identities below. ORF1, RNA-dependent RNA polymerase (RdRp); ORFs 2-4, triple gene block (TGB1, TGB2 and TGB3, respectively); ORF 5, coat protein (CP); ORF6, nucleic acid binding protein (NABP).

Figure 3: Phylogenetic relationships, based on complete genome sequences, of Brazilian CPMMV isolates and other carlaviruses using Bayesian inference (implemented in MrBayes v. 3.1, with model GTR+I+G and 10 million generations). *Indian citrus ringspot virus* (ICRSV, genus *Mandarivirus*, family *Alphaflexiviridae*) was used as the outgroup. The 5’ UTR, 3’ UTR and intergenic regions were removed from the alignment and the overlapping coding regions were maintained. Support for the nodes is presented as filled circles (posterior probabilities from 0.95 to 1.0) or open circles (posterior probabilities from 0.85 to 0.94). The six Brazilian isolates are indicated in bold. The accession numbers of the sequences are shown next to their acronym.

Figure 4: A. Schematic representation of the recombination events identified among CPMMV isolates by the RDP3 program. Each box represents a viral isolate with the recombination events identified by numbers. **B.** Details of recombination events in the genomes of CPMMV isolates. Recombination detection methods are represented by letters: R=rdp; G=Genecov; B=Bootscan; M= Maximum χ^2 ; C= Chimaera; S= Sister scan; 3=3Seq. Only the lowest p-value is indicated for the underlined method. The CPMMV genome is shown at the top of the figure. The Ghanaian CPMMV isolate (HQ184471) is termed CPMMV HQ184471.

Supplementary figure S1: Two-dimensional plot representing the percent sequence identities between the Brazilian CPMMV isolates and the most closely related carlaviruses. Nucleotide sequence identities are shown above the diagonal and amino acid sequence identities below. The six Brazilian isolates are indicated in red. **A.** ORF1, RNA-dependent RNA polymerase (RdRp); **B, C** and **D.** ORFs 2-4, triple gene block (TGB1, TGB2 and TGB3, respectively); **E.** ORF5, coat protein (CP); **F.** ORF6, nucleic acid binding protein (NABP)**.**

Supplementary figure S2: Phylogenetic relationships, based on the sequences of individual ORFs, of the Brazilian CPMMV isolates with different carlaviruses, determined using Bayesian inference (implemented in MrBayes 3.1, with selection of models GTR+I+G for all ORFs and 10 million generations). *Indian citrus ringspot virus* (ICRSV, genus *Mandarivirus*, family *Alphaflexiviridae*) was used as the outgroup. Bayesian posterior probability values are given between nodes. The six Brazilian isolates are indicated in red. The accession numbers of the sequences are showed next to their acronym. **A.** ORF1, RNA-dependent RNA polymerase (RdRp); **B, C** and **D.** ORFs 2-4, triple gene block (TGB1, TGB2 and TGB3, respectively); **E.** ORF5, coat protein (CP); **F.** ORF6, nucleic acid binding protein (NABP).

Table 1: Symptoms induced in different host plants by the six CPMMV isolates described in this study.

Family	**Species**	**Symptoms* (Plants infected/inoculated)** $^{\text{no of experiments}}$					
		CPMMV:BR:BA:02	CPMMV:BR:GO:01:1	CPMMV:BR:MT:02:1	CPMMV:BR:MG:09:2	CPMMV:BR:MG:09:3	CPMMV:BR:GO:10:5
Amaranthaceae	*Gomphrena globosa*	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
Chenopodiaceae	*Chenopodium amaranticolor*	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}
	Chenopodium quinoa	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}
Cucurbitaceae	*Cucurbita pepo*	- (0/6)^{2}	- (0/6)^{2}	- (0/6)^{2}	- (0/6)^{2}	- (0/6)^{2}	- (0/6)^{2}
Fabaceae	*Glycine max* cv CD 206	**Cl, M, Vc** (9/9)^{3}	**Cl** (9/9)^{3}	**M, Vc** (9/9)^{3}	**Bb, D, Ln, Sn** (9/9)^{3}	**Bb, D, Ln, Sn** (9/9)^{3}	**Bb, D, Ln, Sn** (9/9)^{3}
	G. max cv Pintado	**+** (6/6)^{2}	**+** (6/6)^{2}	**+** (6/6)^{2}	**+** (6/6)^{2}	**+** (6/6)^{2}	**+** (6/6)^{2}
	Phaseolus vulgaris cv Jalo	**M** (7/9)^{3}	**M** (9/9)^{3}	**M** (7/9)^{3}	**M** (7/9)^{3}	**M** (7/9)^{3}	**M** (9/9)^{3}
	P. vulgaris cv Manteigão	- (0/3)^{1}	**+** (2/3)^{1}	**+** (2/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
	P. vulgaris cv Ouro Negro	- (0/6)^{2}	- (0/6)^{2}	- (0/6)^{2}	- (0/6)^{2}	- (0/6)^{2}	- (0/6)^{2}
	Vigna unguiculata cv B7 Gurguéia	**M** (2/6)^{2}	**M** (2/6)^{2}	- (0/6)^{2}	- (0/6)^{2}	- (0/3)^{2}	- (0/3)^{2}
	V. unguiculata cv Pitiúba	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
Solanaceae	*Capsicum annuum*	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
	Datura stramonium	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
	Nicotiana benthamiana	**Cll** (4/6)^{2}	**Cll** (4/6)^{2}	**Cll** (4/6)^{2}	**Cll** (4/6)^{2}	**Cll** (6/6)^{2}	**Cll** (4/6)^{2}
	Nicotiana clevelandii	- (0/9)^{3}	- (0/9)^{3}	- (0/9)^{3}	- (0/9)^{3}	- (0/9)^{3}	- (0/9)^{3}
	Nicotiana glutinosa	**D, Nll** (2/3)^{1}	**D, Nll** (2/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
	Nicotiana Debneyi	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}	**Cll** (6/6)^{2}
	Nicotiana tabacum cv Havana	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
	N. tabacum cv TNN	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
	N. tabacum cv White Burley	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
	N. tabacum cv Xanthi	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}
	Solannum lycopersicum cv Rutgers	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}	- (0/3)^{1}

*Bb= Bud Blight Cl=Crinkled leaves, Cll=Chlorotic local lesions, D=Dwarfism, Ln=Leaf necrosis, M=Mosaic, Nll=Necrotic local lesions, Sn=stem necrosis; Vc=Vein clearing, + Symptomless infection; - no symptoms and negative by ELISA.

Table 2: Genomic organization of the six CPMMV isolates described in this study.

Isolate	Genomic organization*														Genome size (nt)
	5' UTR	ORF1		ORF2		ORF3		ORF4		ORF5		ORF6		3' UTR	
	# nt	nt position	# aa	nt position	# aa	nt position	# aa	nt position	# aa	nt position	# aa	nt position	# aa	# nt	
CPMMV:BR:BA:02	72	73-5,649	1,858	5,678-6,373	231	6,373-6,693	106	6,671-6,877	68	6,893-7,759	288	7,762-8,103	113	88	8,191
CPMMV:BR:GO:01:1	72	73-5,652	1,859	5,681-6,385	234	6,375-6,695	106	6,674-6,880	68	6,895-7,761	288	7,764-8,162	132	36	8,198
CPMMV:BR:MT:02:1	72	73-5,637	1,854	5,666-6,361	231	6,361-6,681	106	6,659-6,865	68	6,881-7,747	288	7,750-8,091	113	89	8,180
CPMMV:BR:MG:09:2	73	74-5,653	1,859	5,682-6,377	231	6,377-6,697	106	6,675-6,881	68	6,897-7,763	288	7,766-8,107	113	89	8,196
CPMMV:BR:MG:09:3	94	95-5,653	1,852	5,682-6,377	231	6,377-6,697	106	6,675-6,881	68	6,897-7,763	288	7,766-8,107	113	89	8,196
CPMMV:BR:GO:10:5	72	73-5,652	1,859	5,681-6,376	231	6,376-6,696	106	6,674-6,880	68	6,896-7,762	288	7,765-8,106	113	88	8,194
CPMMV HQ184471**	73	74-5,584	1,836	5,613-6,217	234	6,308-6,685	125	6,606-6,815	69	6,833-7,699	288	7,702-8,010	102	116	8,127

*ORF1: RNA-dependent RNA polymerase (RdRp); ORFs 2, 3 and 4: triple gene block (TGB1, TGB2 and TGB3, respectively); ORF5: coat protein (CP); ORF6: nucleic acid binding protein (NABP). ** Ghanaian CPMMV isolate (GenBank accession number: HQ184471).

Figure 1:

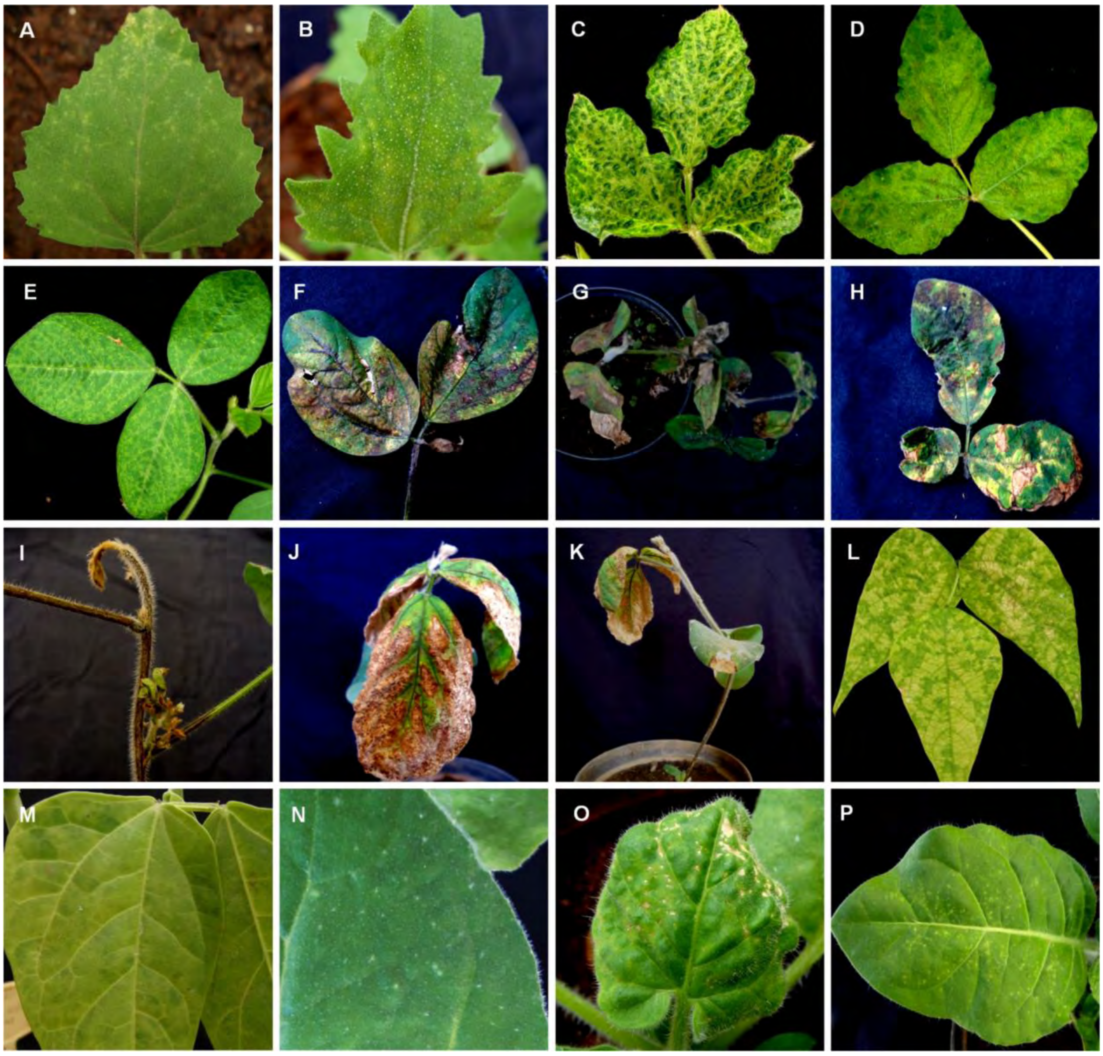
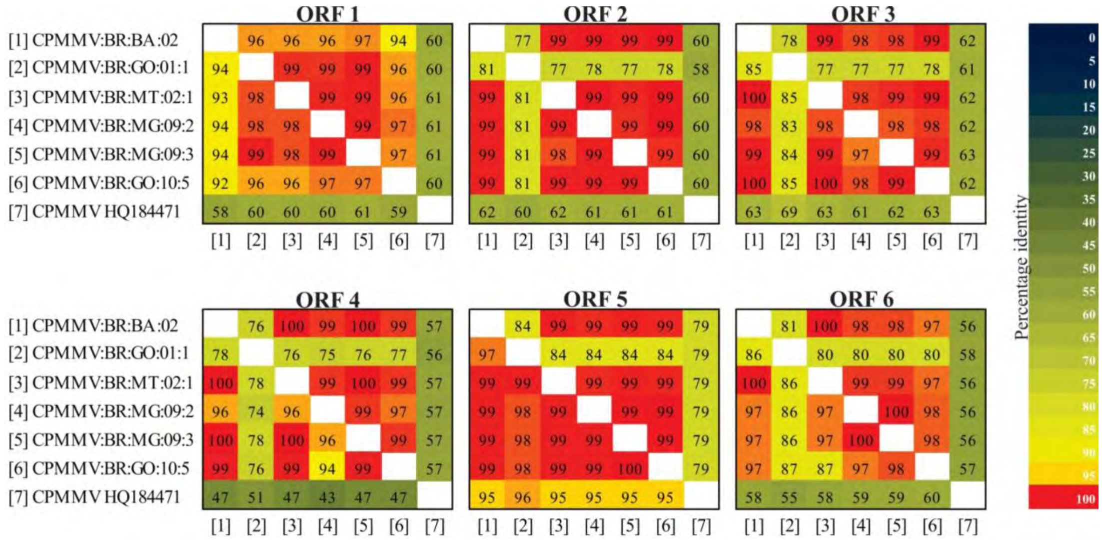


Figure 2



ORF 1

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[3] CPMMV:BR:MT:02:1	93	98		99	99	96	61
[4] CPMMV:BR:MG:09:2	94	98	98		99	97	61
[5] CPMMV:BR:MG:09:3	94	99	98	99		97	61
[6] CPMMV:BR:GO:10:5	92	96	96	97	97		60
[7] CPMMV HQ184471	58	60	60	60	61	59	

ORF 2

	[1]	[2]	[3]	[4]	[5]	[6]	[7]
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[2] CPMMV:BR:GO:01:1	81		77	78	77	78	58
[3] CPMMV:BR:MT:02:1	99	81		99	99	99	60
[4] CPMMV:BR:MG:09:2	99	81	99		99	99	60
[5] CPMMV:BR:MG:09:3	99	81	98	99		99	60
[6] CPMMV:BR:GO:10:5	99	81	99	99	99		60
[7] CPMMV HQ184471	62	60	62	61	61	61	

ORF 3

	[1]	[2]	[3]	[4]	[5]	[6]	[7]
[1] CPMMV:BR:BA:02		78	99	98	98	99	62
[2] CPMMV:BR:GO:01:1	85		77	77	77	78	61
[3] CPMMV:BR:MT:02:1	100	85		98	99	99	62
[4] CPMMV:BR:MG:09:2	98	83	98		98	98	62
[5] CPMMV:BR:MG:09:3	99	84	99	97		99	63
[6] CPMMV:BR:GO:10:5	100	85	100	98	99		62
[7] CPMMV HQ184471	63	69	63	61	62	63	

ORF 4

	[1]	[2]	[3]	[4]	[5]	[6]	[7]
[1] CPMMV:BR:BA:02		76	100	99	100	99	57
[2] CPMMV:BR:GO:01:1	78		76	75	76	77	56
[3] CPMMV:BR:MT:02:1	100	78		99	100	99	57
[4] CPMMV:BR:MG:09:2	96	74	96		99	97	57
[5] CPMMV:BR:MG:09:3	100	78	100	96		99	57
[6] CPMMV:BR:GO:10:5	99	76	99	94	99		57
[7] CPMMV HQ184471	47	51	47	43	47	47	

ORF 5

	[1]	[2]	[3]	[4]	[5]	[6]	[7]
[1] CPMMV:BR:BA:02		84	99	99	99	99	79
[2] CPMMV:BR:GO:01:1	97		84	84	84	84	79
[3] CPMMV:BR:MT:02:1	99	99		99	99	99	79
[4] CPMMV:BR:MG:09:2	99	98	99		99	99	79
[5] CPMMV:BR:MG:09:3	99	98	99	99		99	79
[6] CPMMV:BR:GO:10:5	99	98	99	99	100		79
[7] CPMMV HQ184471	95	96	95	95	95	95	

ORF 6

	[1]	[2]	[3]	[4]	[5]	[6]	[7]
[1] CPMMV:BR:BA:02		81	100	98	98	97	56
[2] CPMMV:BR:GO:01:1	86		80	80	80	80	58
[3] CPMMV:BR:MT:02:1	100	86		99	99	97	56
[4] CPMMV:BR:MG:09:2	97	86	97		100	98	56
[5] CPMMV:BR:MG:09:3	97	86	97	100		98	56
[6] CPMMV:BR:GO:10:5	97	87	87	97	98		57
[7] CPMMV HQ184471	58	55	58	59	59	60	

Figure 3:

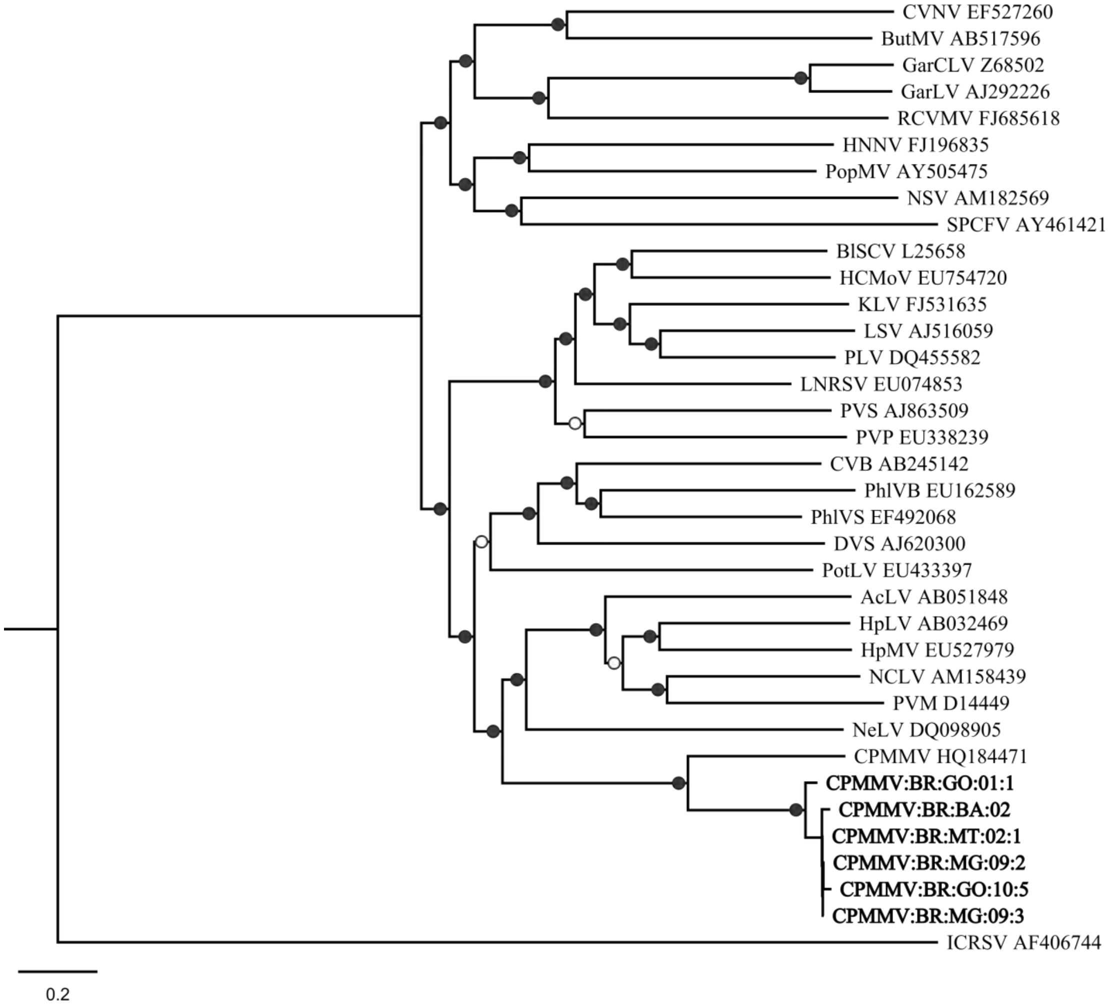

CVNV EF527260
ButMV AB517596
GarCLV Z68502
GarLV AJ292226
RCVMV FJ685618
HNNV FJ196835
PopMV AY505475
NSV AM182569
SPCFV AY461421
BlSCV L25658
HCMoV EU754720
KLV FJ531635
LSV AJ516059
PLV DQ455582
LNRSV EU074853
PVS AJ863509
PVP EU338239
CVB AB245142
PhlVB EU162589
PhlVS EF492068
DVS AJ620300
PotLV EU433397
AcLV AB051848
HpLV AB032469
HpMV EU527979
NCLV AM158439
PVM D14449
NeLV DQ098905
CPMMV HQ184471
CPMMV:BR:GO:01:1
CPMMV:BR:BA:02
CPMMV:BR:MT:02:1
CPMMV:BR:MG:09:2
CPMMV:BR:GO:10:5
CPMMV:BR:MG:09:3
ICRSV AF406744
0.2

Figure 4:

B

Recombinant	Event	Breakpoint	Major Parent	Minor Parent	Site of recombination	Event detected by methods shown	P-value
CPMMV:BR:BA:02	1	91 - 145	CPMMV:BR:GO:01:1	CPMMV HQ184471	ORF1	$\underline{R}$, G, M, C	2.1×10^{-08}
CPMMV:BR:BA:02	2	2965 - 3289	CPMMV:BR:MG:09:2	Unknown	ORF1	R, G, M, $\underline{C}$	2.0×10^{-09}
CPMMV:BR:BA:02	3	285 - 5654	CPMMV:BR:MT:02:1	Unknown	ORF1	R, G, B, M, C, S, $\underline{3}$	2.1×10^{-83}
CPMMV:BR:GO:01:1	4	262 - 5638	Unknown	CPMMV:BR:MG:09:3	ORF1	R, G, B, M, C, S, $\underline{3}$	1.1×10^{-82}
CPMMV:BR:MT:02:1	1	91 - 145	CPMMV:BR:GO:01:1	CPMMV HQ184471	ORF1	$\underline{R}$, G, M, C	2.1×10^{-08}
CPMMV:BR:MG:09:2	1	92 - 146	CPMMV:BR:GO:01:1	CPMMV HQ184471	ORF1	$\underline{R}$, G, M, C	2.1×10^{-08}
CPMMV:BR:MG:09:3	1	89 - 143	CPMMV:BR:GO:01:1	CPMMV HQ184471	ORF1	$\underline{R}$, G, M, C	2.1×10^{-08}
CPMMV:BR:GO:10:5	1	91 - 145	CPMMV:BR:GO:01:1	CPMMV HQ184471	ORF1	$\underline{R}$, G, M, C	2.1×10^{-08}
CPMMV:BR:GO:10:5	5	5961 - 7753	Unknown	CPMMV:BR:MG:09:2	ORF2, 3, 4, 5	B, M, $\underline{C}$	1.8×10^{-06}

Supplementary Table S1: Primers used in RT-PCR and predicted amplicon size for viral detection and cloning.

Fragments	Primer	Primer location**	Primer sequence 5'-3'	Annealing Temperature °C	PCR product size
5' end	Race R	518-539	CCAATGTTGCCCTGTGCCTAC	55	538
ORF1	ORF1 F1	143- 160	TTGCTTCCAAAGCTGCCT	57	1008
	ORF R1	1133-1150	TCTCGTTAGCTGAGGGTT[cD]		
ORF1	ORF1 F2	929-948	AGGTGCTGCCGTCACTTGT	50	1223
	ORF R2	2133-2152	CTGACTTAAGCTCATCTGG		
ORF1	ORF1 F3	1994-2012	TCAGATAAATGAGGGTGG	48	1618
	ORF1 R3	3593-3612	TCCAAGCAAGTCCCTATCT		
ORF1	ORF1 F4	3321-3341	AGGAAAGCCCTACTTGAGGA	50	1209
	ORF1 R4	4510-4530	CTTTACCGCCATAATGAACG		
ORF1	ORF1 F5	4386-4405	GGTTCGATTGTCTCAGATC	50	1347
	ORF1 R5	5713-5733	CTGCCCAGTCGAATGTAATT		
ORF2	ORF2 F	5653-5670	TCCTTTAGGTAGTGAGGC	45	938
	ORF2 R	6572-6590	AAGTTCGTGCCAGTTGAC[cD]		
ORF3	ORF3 F	6301-6321	CTTNATYTGCYTNACNAGGCA	45	552
	ORF3 R	6852-6837	TGTTCTCTNACCAAGT[cD]		
ORF4 - 3' end	ORF 4 F	6523-6540	TAYMRDGAYGGNACHAA*	45	1676
	ORF6 R	8183-8198	TAAAACCAGGAAATAAC[cD]		

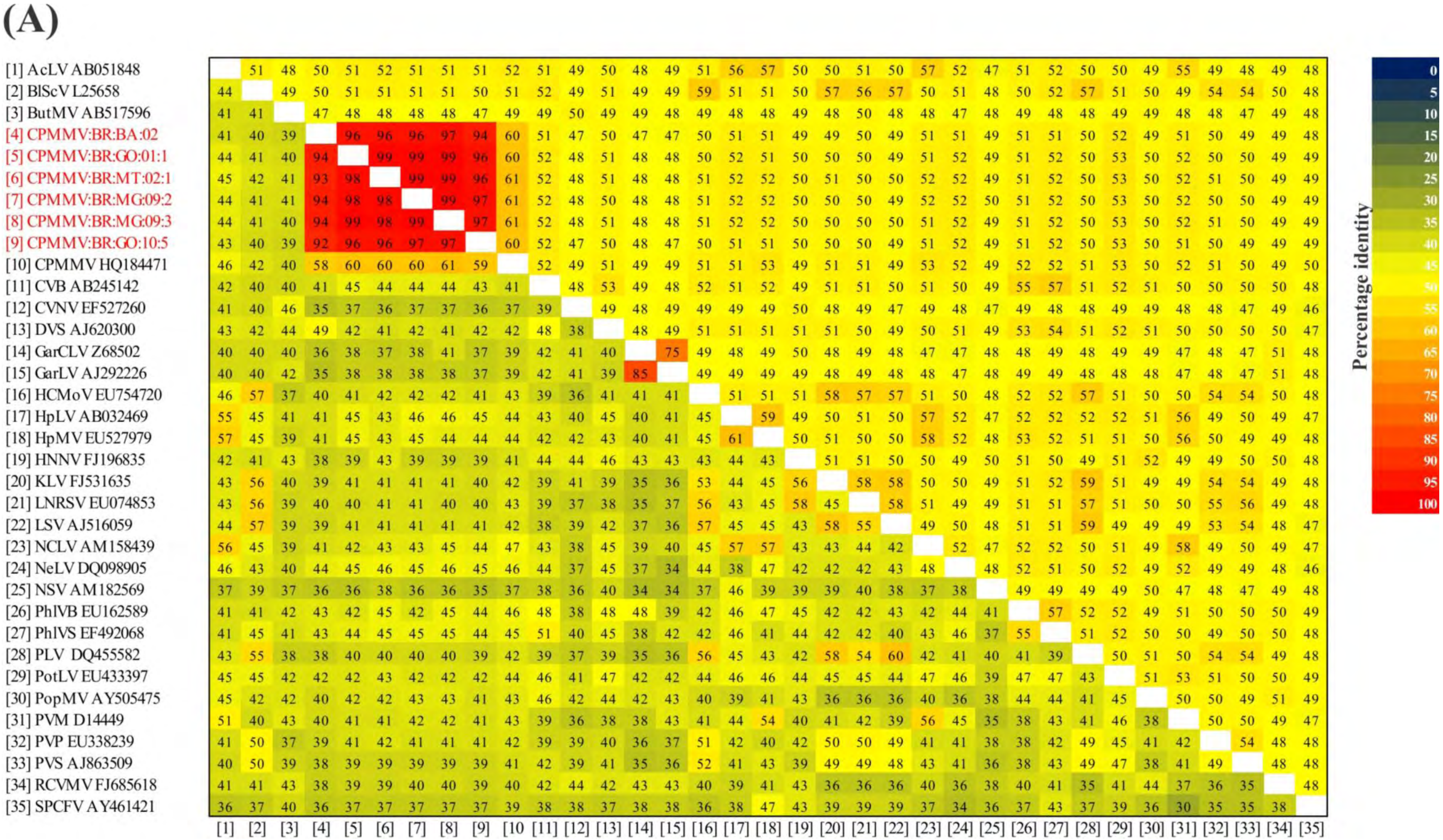
*Described by Nicolaisen and Nielsen (2001). [cD] Used for cDNA synthesis. **Based on the CPMMV:BR:GO:10:5 isolate.

Supplementary Table S2: Carlavirus sequences used for sequences comparisons, phylogenetic and recombination analyses.

Species	Acronym	GenBank accession #
Aconitum latent virus	AcLV	AB051848
Blueberry scorch virus	BlScV	L25658
Butterbur mosaic virus	ButMV	AB517596
Chrysanthemum virus B	CVB	AB245142
Cowpea mild mottle virus	CPMMV	HQ184471 KC884244 KC884245 KC884246 KC884247 KC884248 KC884249
Coleus vein necrosis virus	CVNV	EF527260
*Cucumber vein-clearing virus***	CuVCV	JN591720
Daphne virus S	DVS	AJ620300
Garlic common latent virus	GarCLV	Z68502
Garlic latent virus	GarLV	AJ292226
Helleborus net necrosis virus	HNNV	FJ196835
Hydrangea chlorotic mottle virus	HCMoV	EU754720
Hop latent virus	HpLV	AB032469
Hop mosaic virus	HpMV	EU527979
Kalanchoe latent virus	KLV	FJ531635
Ligustrum necrotic ringspot virus	LNRSV	EU074853
Lily symptomless virus	LSV	AJ516059
Narcissus common latent virus	NCLV	AM158439
Narcissus symptomless virus	NSV	AM182569
Nerine latent virus	NeLV	DQ098905
Passiflora latent carlavirus	PLV	DQ455582
Phlox virus B	PhlVB	EU162589
Phlox virus S	PhlVS	EF492068
Poplar mosaic virus	PopMV	AY505475
Potato latent virus	PotLV	EU433397
Potato virus M	PVM	D14449
Potato virus P	PVP	EU338239
Potato virus S	PVS	AJ863509
Red clover vein mosaic virus	RCVMV	FJ685618
Sweet potato chlorotic fleck virus	SPCFV	AY461421
*Indian citrus ringspot virus**	ICRSV	AF406744

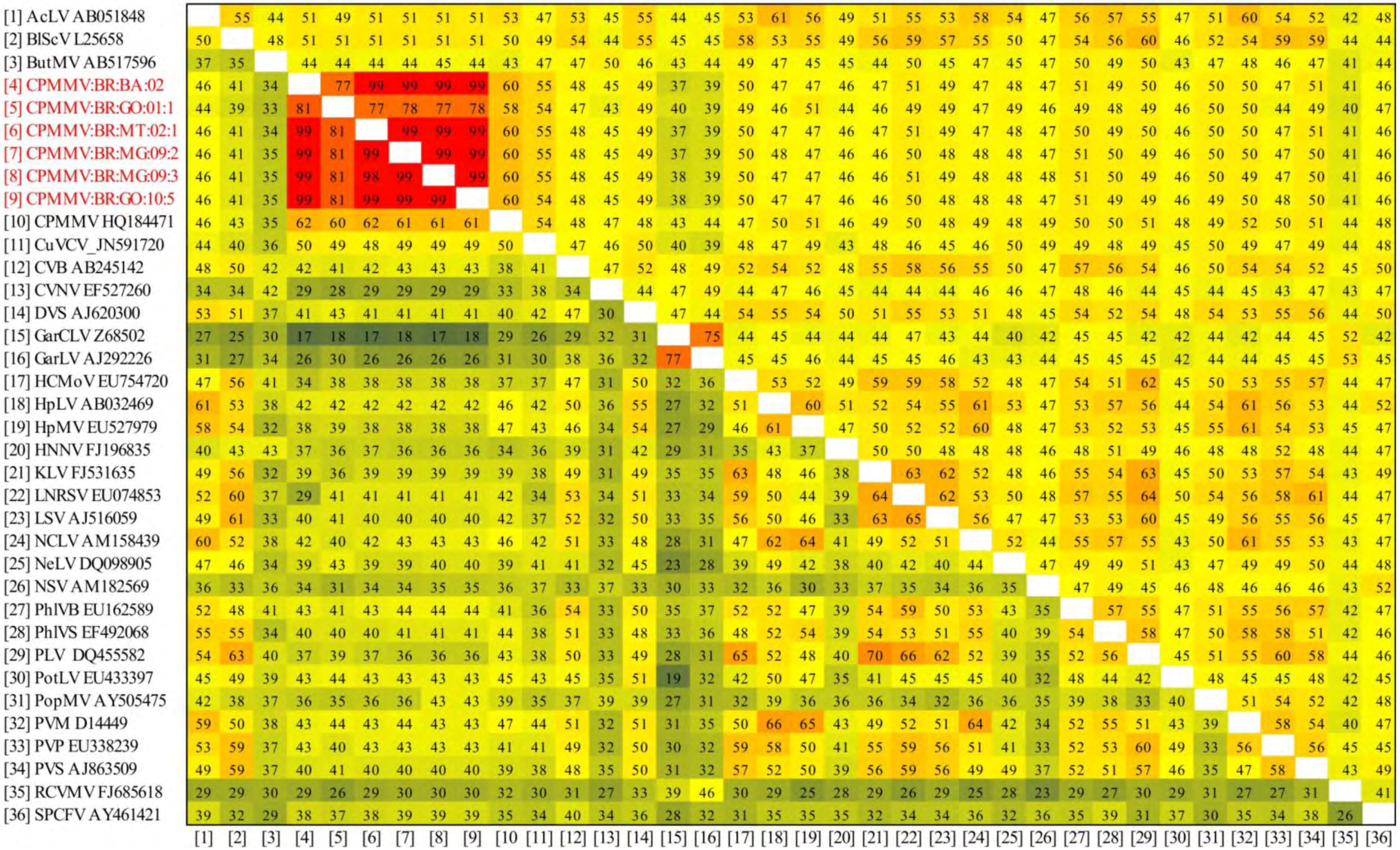
*****Used as outgroup in phylogenetic analysis. **Only carlavirus with partial genome used in the ORF2-6 analyses. The genbank accession # underlined correspond to sequenced isolates in the present study: CPMMV:BR:MG:09:2 (KC884244), CPMMV:BR:MG:09:3 (KC884245), CPMMV:BR:MT:02:1 (KC884246), CPMMV:BR:BA:02 (KC884247), CPMMV:BR:GO:01:1 (KC884248) and CPMMV:BR:GO:10:5 (KC884249). The recombination analysis included only full CPMMV nucleotide sequences.

Figure Supplementary S1:



	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[31]	[32]	[33]	[34]	[35]
[1] AcLV AB051848		51	48	50	51	52	51	51	51	52	51	49	50	48	49	51	56	57	50	50	51	50	57	52	47	51	52	50	50	49	55	49	48	49	48
[2] BlScV L25658	44		49	50	51	51	51	51	50	51	52	49	51	49	49	59	51	51	50	57	56	57	50	51	48	50	52	57	51	50	49	54	54	50	48
[3] ButMV AB517596	41	41		47	48	48	48	48	47	49	49	50	49	49	48	49	48	48	49	48	50	48	48	47	48	49	48	48	49	49	48	48	47	49	48
[4] CPMMV:BR:BA:02	41	40	39		96	96	96	97	94	60	51	47	50	47	47	50	51	51	49	49	50	49	51	51	49	51	51	50	52	49	51	50	49	49	48
[5] CPMMV:BR:GO:01:1	44	41	40	94		99	99	99	96	60	52	48	51	48	48	50	52	51	50	50	50	49	51	52	49	51	52	50	53	50	52	50	50	49	49
[6] CPMMV:BR:MT:02:1	45	42	41	93	98		99	99	96	61	52	48	51	48	48	51	52	52	50	51	50	50	52	52	49	51	52	50	53	50	52	51	50	49	49
[7] CPMMV:BR:MG:09:2	44	41	41	94	98	98		99	97	61	52	48	50	48	48	51	52	52	50	50	50	49	52	52	50	51	52	50	53	50	52	50	50	49	49
[8] CPMMV:BR:MG:09:3	44	41	40	94	99	98	99		97	61	52	48	51	48	48	51	52	52	50	50	50	50	51	52	49	51	52	50	53	50	52	51	50	49	49
[9] CPMMV:BR:GO:10:5	43	40	39	92	96	96	97	97		60	52	47	50	48	47	50	51	51	50	50	50	49	51	52	49	51	52	50	53	50	51	50	49	49	49
[10] CPMMV HQ184471	46	42	40	58	60	60	60	61	59		52	49	51	49	49	51	51	53	49	51	51	49	53	52	49	52	52	51	53	50	52	51	50	49	50
[11] CVB AB245142	42	40	40	41	45	44	44	44	43	41		48	53	49	48	52	51	52	49	51	51	50	51	50	49	55	57	51	52	51	50	50	50	50	48
[12] CVNV EF527260	41	40	46	35	37	36	37	37	36	37	39		49	48	49	49	49	49	50	48	49	47	49	48	47	49	48	48	49	49	48	48	47	49	46
[13] DVS AJ620300	43	42	44	49	42	41	42	41	42	42	48	38		48	49	51	51	51	51	51	50	49	50	51	49	53	54	51	52	51	50	50	50	50	47
[14] GarCLV Z68502	40	40	40	36	38	37	38	41	37	39	42	41	40		75	49	48	49	50	48	49	48	47	47	48	48	49	48	49	49	47	48	47	51	48
[15] GarLV AJ292226	40	40	42	35	38	38	38	38	37	39	42	41	39	85		49	49	49	49	48	49	48	48	47	48	49	49	48	48	48	47	48	47	51	48
[16] HCMoV EU754720	46	57	37	40	41	42	42	42	41	43	39	36	41	41	41		51	51	51	58	57	57	51	50	48	52	52	57	51	50	50	54	54	50	48
[17] HpLV AB032469	55	45	41	41	45	43	46	46	45	44	43	40	45	40	41	45		59	49	50	51	50	57	52	47	52	52	52	52	51	56	49	50	49	47
[18] HpMV EU527979	57	45	39	41	45	43	45	44	44	44	42	42	43	40	41	45	61		50	51	50	50	58	52	48	53	52	51	51	50	56	50	49	49	48
[19] HNNV FJ196835	42	41	43	38	39	43	39	39	39	41	44	44	46	43	43	43	44	43		51	51	50	50	49	50	51	50	49	51	52	49	49	50	50	48
[20] KLV FJ531635	43	56	40	39	41	41	41	41	40	42	39	41	39	35	36	53	44	45	56		58	58	50	50	49	51	52	59	51	49	49	54	54	49	48
[21] LNRSV EU074853	43	56	39	40	40	41	41	40	40	43	39	37	38	35	37	56	43	45	58	45		58	51	49	49	51	51	57	51	50	50	55	56	49	48
[22] LSV AJ516059	44	57	39	39	41	41	41	41	41	42	38	39	42	37	36	57	45	45	43	58	55		49	50	48	51	51	59	49	49	49	53	54	48	47
[23] NCLV AM158439	56	45	39	41	42	43	43	45	44	47	43	38	45	39	40	45	57	57	43	43	44	42		52	47	52	52	50	51	49	58	49	50	49	47
[24] NeLV DQ098905	46	43	40	44	45	46	45	46	45	46	44	37	45	37	34	44	38	47	42	42	42	43	48		48	52	51	50	52	49	52	49	49	48	46
[25] NSV AM182569	37	39	37	36	36	38	36	36	35	37	38	36	40	34	34	37	46	39	39	39	40	38	37	38		49	49	49	49	50	47	48	47	49	48
[26] PhlVB EU162589	41	41	42	43	42	45	42	45	44	46	48	38	48	48	39	42	46	47	45	42	42	43	42	44	41		57	52	52	49	51	50	50	50	49
[27] PhlVS EF492068	41	45	41	43	44	45	45	45	44	45	51	40	45	38	42	42	46	41	44	42	42	40	43	46	37	55		51	52	50	50	49	50	50	48
[28] PLV DQ455582	43	55	38	38	40	40	40	40	39	42	39	37	39	35	36	56	45	43	42	58	54	60	42	41	40	41	39		50	51	50	54	54	49	48
[29] PotLV EU433397	45	45	42	42	42	43	42	42	42	44	46	41	47	42	42	44	46	46	44	45	45	44	47	46	39	47	47	43		51	53	51	50	50	49
[30] PopMV AY505475	45	42	42	40	42	42	43	43	41	43	46	42	44	42	43	40	39	41	43	36	36	36	40	36	38	44	44	41	45		50	50	49	51	49
[31] PVM D14449	51	40	43	40	41	41	42	42	41	43	39	36	38	38	43	41	44	54	40	41	42	39	56	45	35	38	43	41	46	38		50	50	49	47
[32] PVP EU338239	41	50	37	39	41	42	41	41	41	42	39	39	40	36	37	51	42	40	42	50	50	49	41	41	38	38	42	49	45	41	42		54	48	48
[33] PVS AJ863509	40	50	39	38	39	39	39	39	39	41	42	39	41	35	36	52	41	43	39	49	49	48	43	41	36	38	43	49	47	38	41	49		48	48
[34] RCVMV FJ685618	41	41	43	38	39	39	40	40	39	40	42	44	42	43	43	40	39	41	43	36	36	36	40	36	38	40	41	35	41	44	37	36	35		48
[35] SPCFV AY461421	36	37	40	36	37	37	37	37	37	39	38	38	37	38	38	36	38	47	43	39	39	39	37	34	36	37	43	37	39	36	30	35	35	38	

(B)



	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[31]	[32]	[33]	[34]	[35]	[36]
[1] AcLV AB051848		55	44	51	49	51	51	51	51	53	47	53	45	55	44	45	53	61	56	49	51	55	53	58	54	47	56	57	55	47	51	60	54	52	42	48
[2] BlScV L25658	50		48	51	51	51	51	51	51	50	49	54	44	55	45	45	58	53	55	49	56	59	57	55	50	47	54	56	60	46	52	54	59	59	44	44
[3] ButMV AB517596	37	35		44	44	44	44	45	44	43	47	47	50	46	43	44	49	47	45	45	44	50	45	47	45	47	50	49	50	43	47	48	46	47	41	44
[4] CPMMV:BR:BA:02	46	41	34		77	99	99	99	99	60	55	48	45	49	37	39	50	47	47	46	47	51	49	47	48	47	51	49	50	46	50	50	47	51	41	46
[5] CPMMV:BR:GO:01:1	44	39	33	81		77	78	77	78	58	54	47	43	49	40	39	49	46	51	44	46	49	49	47	49	46	49	48	49	46	50	50	44	49	40	47
[6] CPMMV:BR:MT:02:1	46	41	34	99	81		99	99	99	60	55	48	45	49	37	39	50	47	47	46	47	51	49	47	48	47	50	49	50	46	50	50	47	51	41	46
[7] CPMMV:BR:MG:09:2	46	41	35	99	81	99		99	99	60	55	48	45	49	37	39	50	48	47	46	46	50	48	48	48	47	51	50	49	46	50	50	47	50	41	46
[8] CPMMV:BR:MG:09:3	46	41	35	99	81	98	99		99	60	55	48	45	49	38	39	50	47	47	46	46	51	49	48	48	48	51	50	49	46	50	49	47	50	41	46
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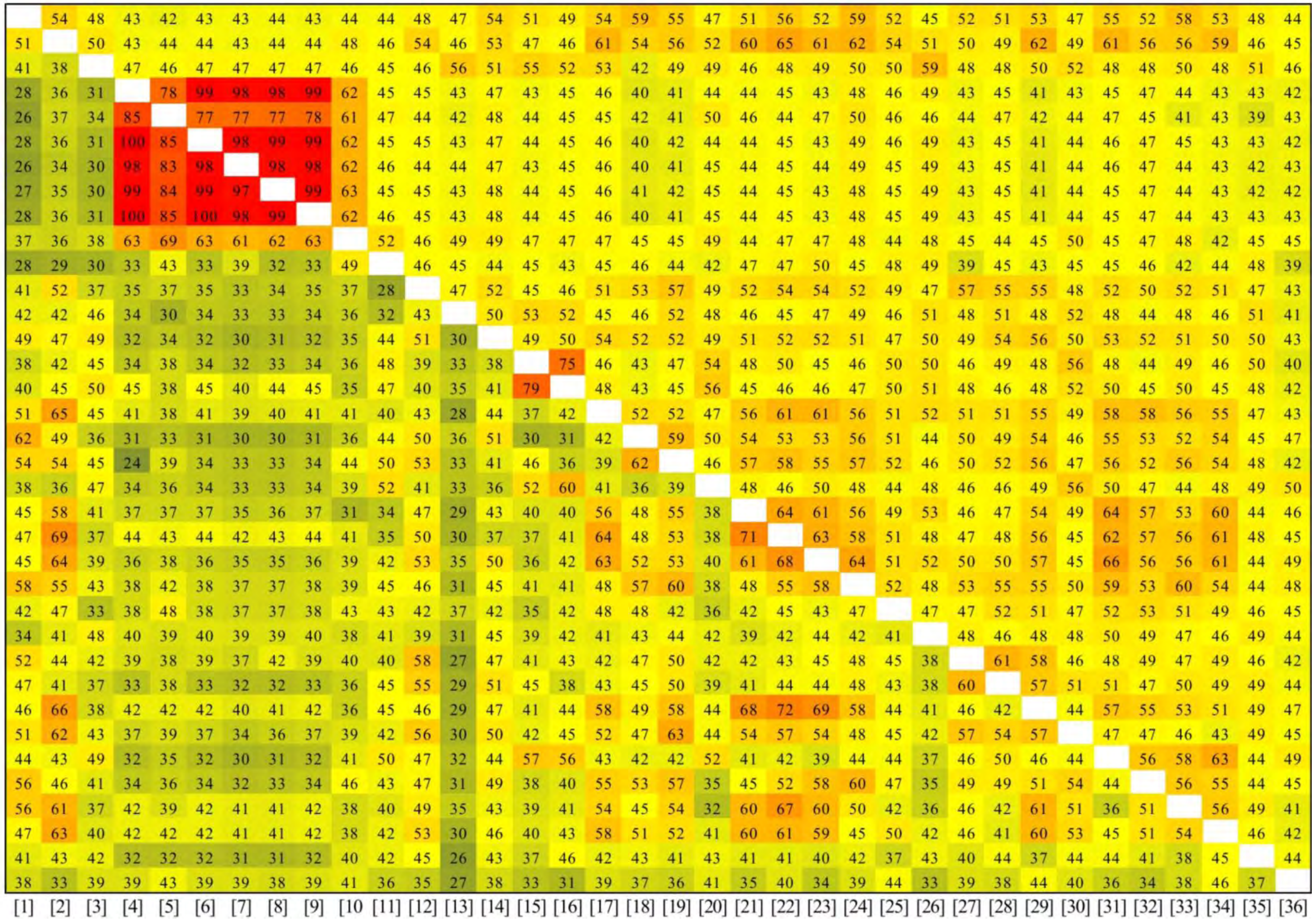

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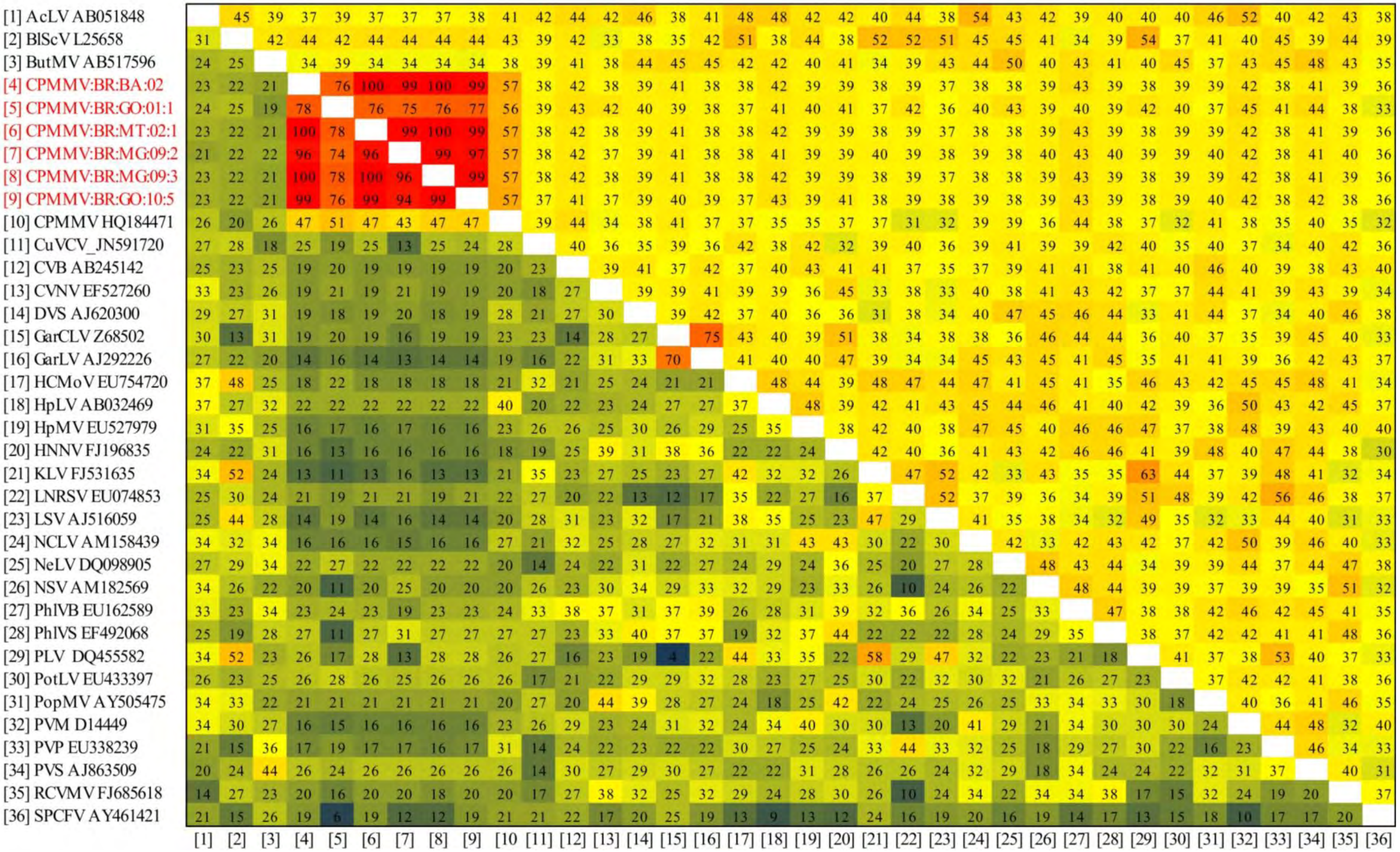

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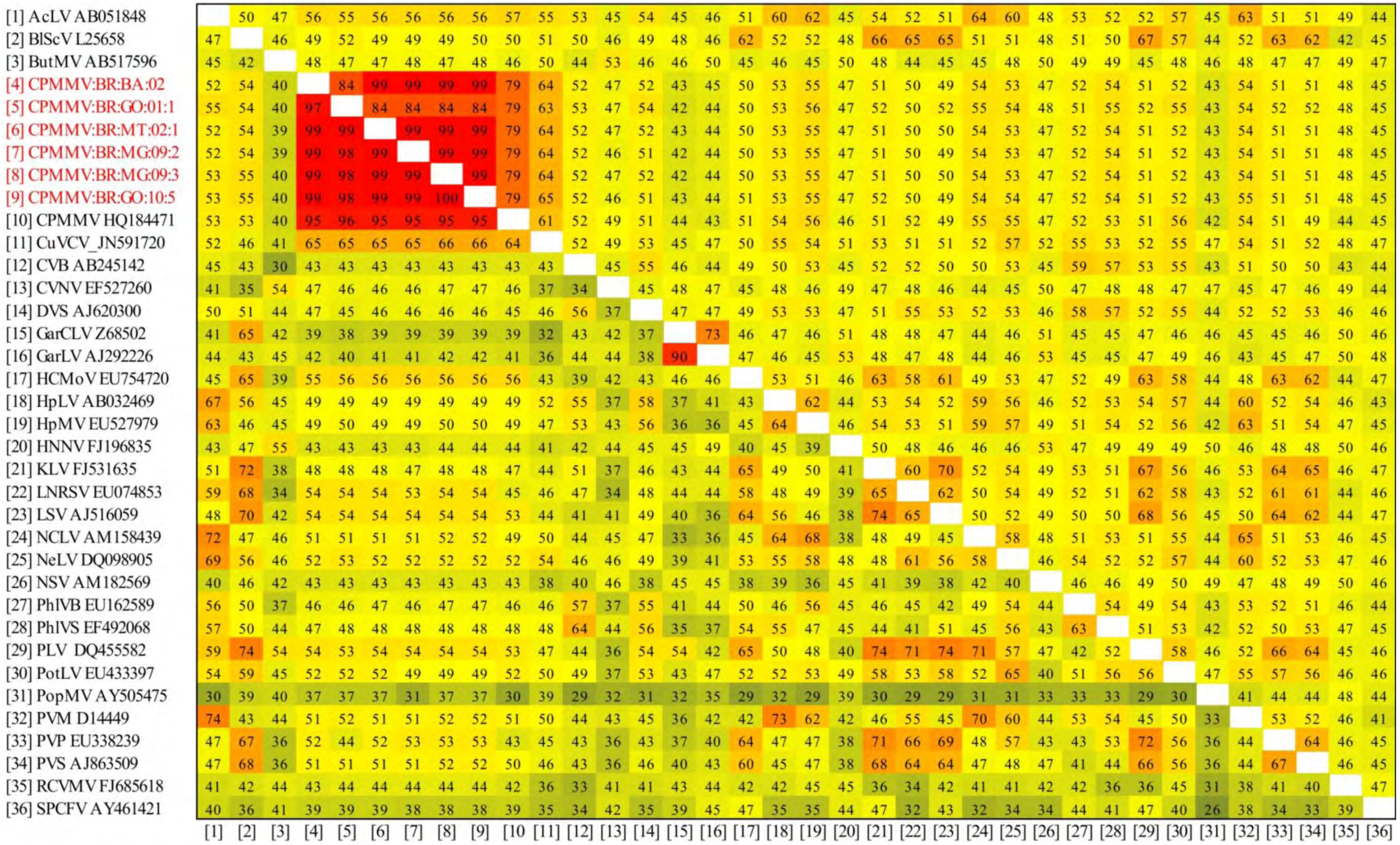

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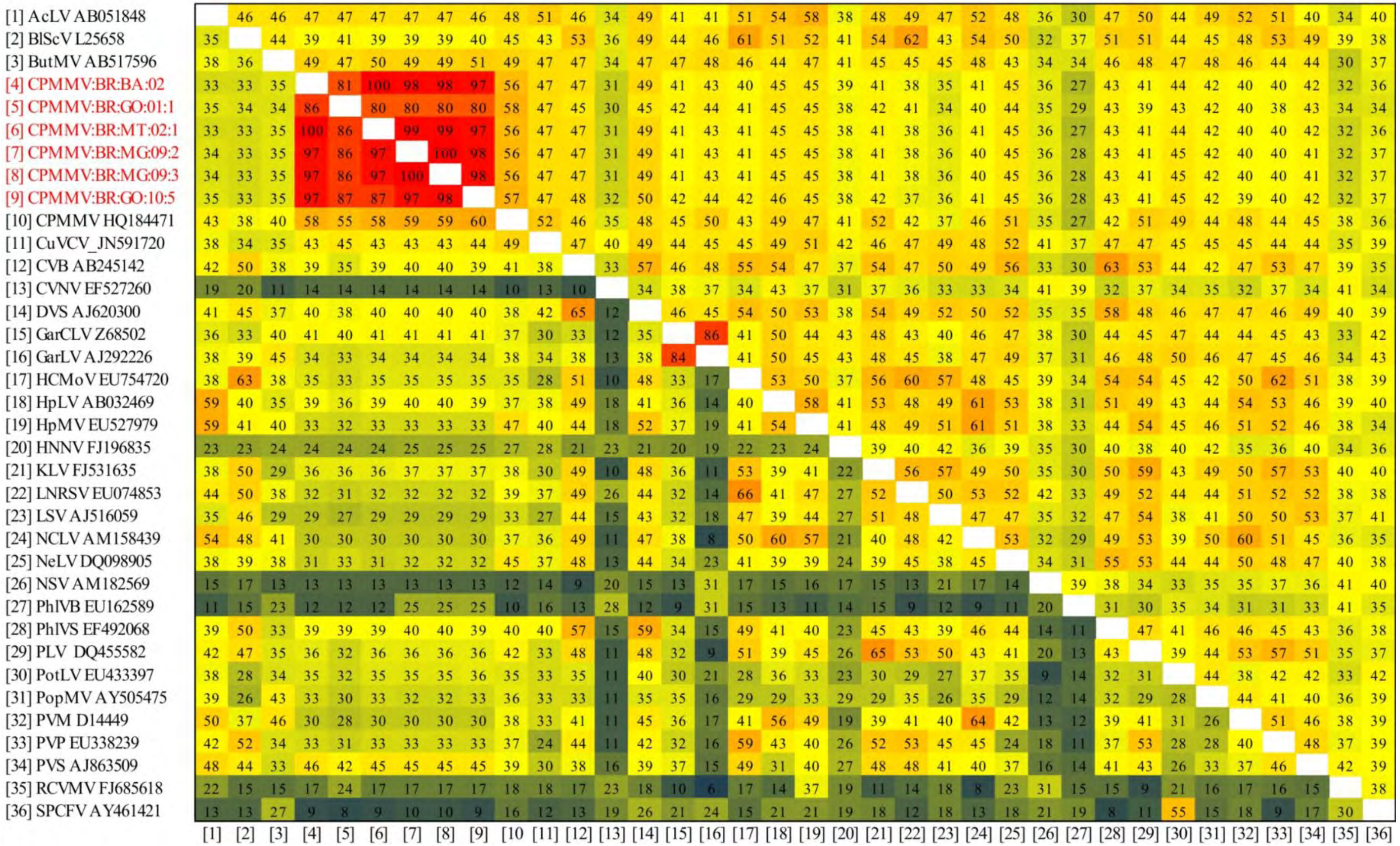

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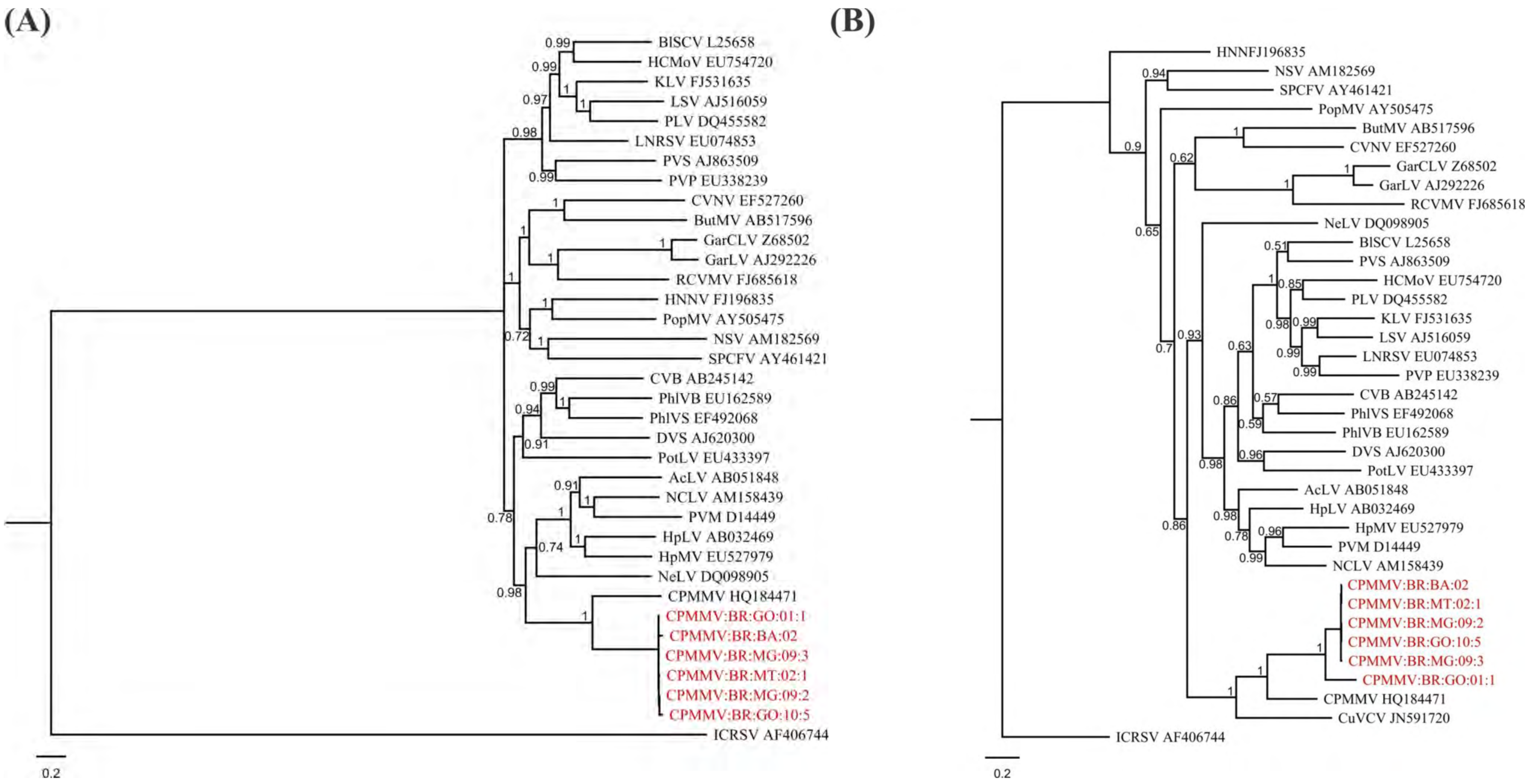
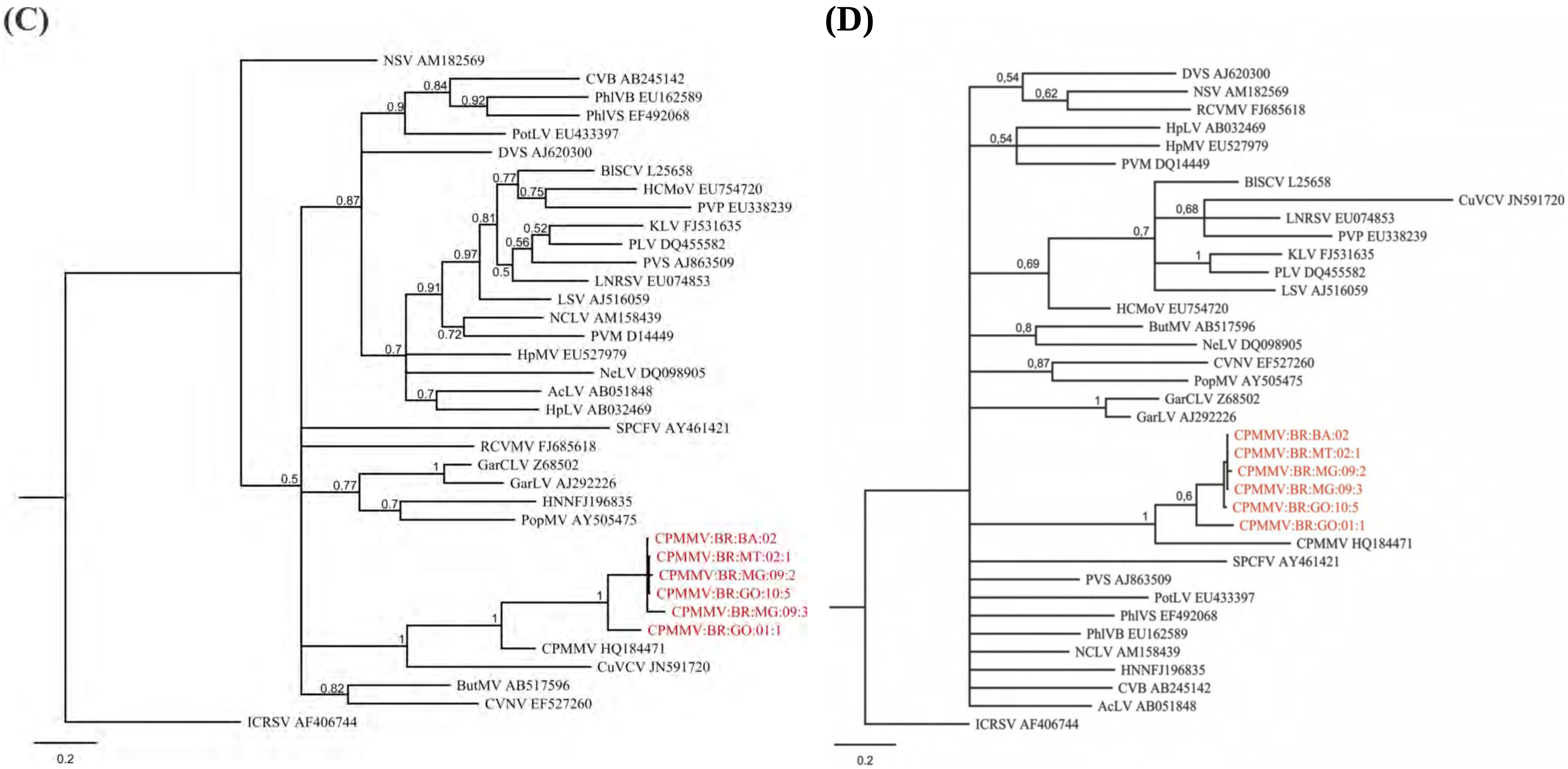
Suplementary Figure S2

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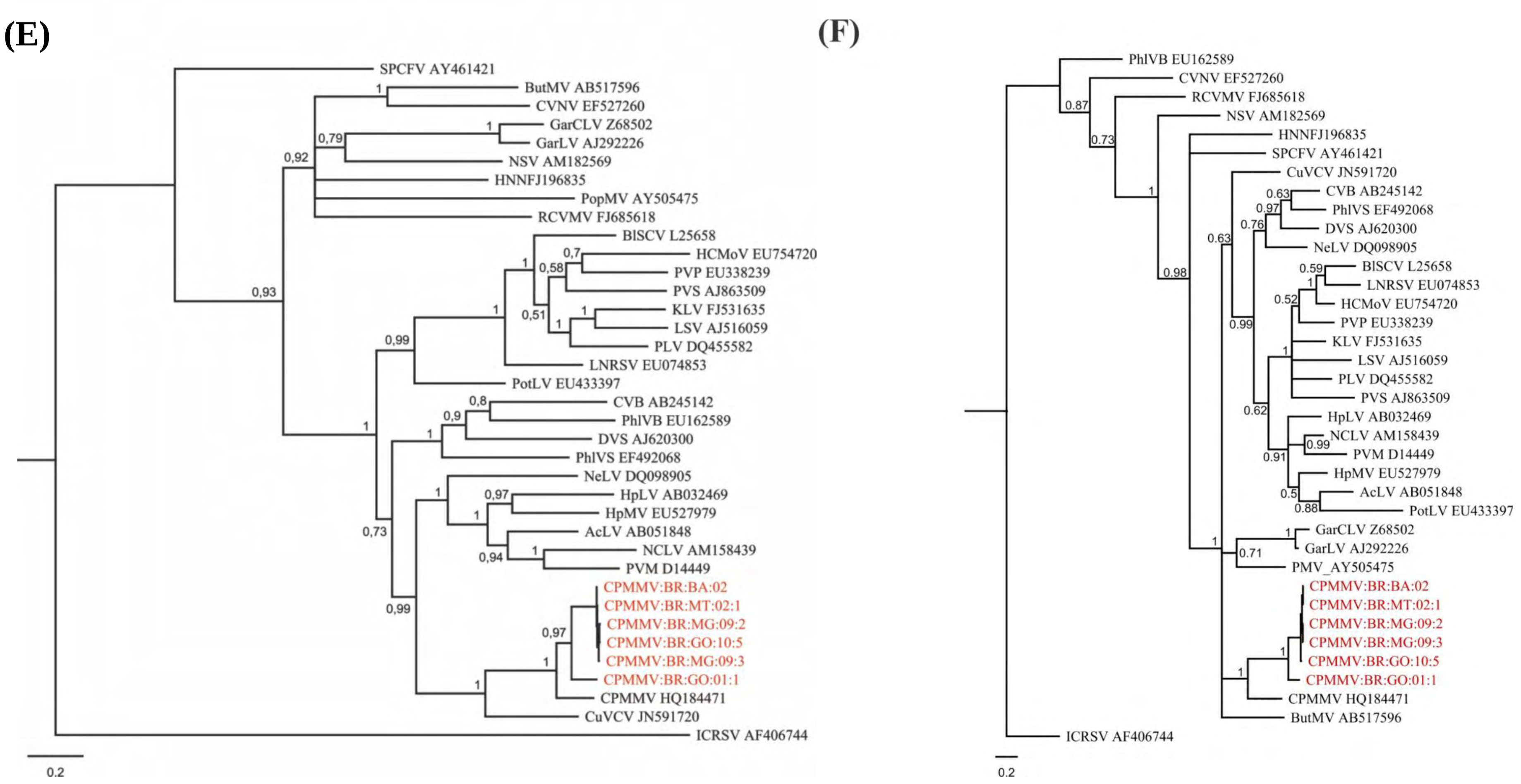
CPMMV:BR:MG:09:2
CPMMV:BR:MG:09:3
CPMMV:BR:GO:10:5
CPMMV:BR:GO:01:1
CPMMV HQ184471
SPCFV AY461421
PVS AJ863509
PotLV EU433397
PhlVS EF492068
PhlVB EU162589
NCLV AM158439
HNNFJ196835
CVB AB245142
AcLV AB051848
ICRSV AF406744
0,54
0,62
0,54
0,68
0,7
1
0,69
0,8
0,87
1
0,6
1
0.2


(E)
SPCFV AY461421
ButMV AB517596
CVNV EF527260
GarCLV Z68502
GarLV AJ292226
NSV AM182569
HNNFJ196835
PopMV AY505475
RCVMV FJ685618
BlSCV L25658
HCMoV EU754720
PVP EU338239
PVS AJ863509
KLV FJ531635
LSV AJ516059
PLV DQ455582
LNRSV EU074853
PotLV EU433397
CVB AB245142
PhlVB EU162589
DVS AJ620300
PhlVS EF492068
NeLV DQ098905
HpLV AB032469
HpMV EU527979
AcLV AB051848
NCLV AM158439
PVM D14449
CPMMV:BR:BA:02
CPMMV:BR:MT:02:1
CPMMV:BR:MG:09:2
CPMMV:BR:GO:10:5
CPMMV:BR:MG:09:3
CPMMV:BR:GO:01:1
CPMMV HQ184471
CuVCV JN591720
ICRSV AF406744
1
0,79
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0,51
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0,94
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1
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(F)
PhlVB EU162589
CVNV EF527260
RCVMV FJ685618
NSV AM182569
HNNFJ196835
SPCFV AY461421
CuVCV JN591720
CVB AB245142
PhlVS EF492068
DVS AJ620300
NeLV DQ098905
BlSCV L25658
LNRSV EU074853
HCMoV EU754720
PVP EU338239
KLV FJ531635
LSV AJ516059
PLV DQ455582
PVS AJ863509
HpLV AB032469
NCLV AM158439
PVM D14449
HpMV EU527979
AcLV AB051848
PotLV EU433397
GarCLV Z68502
GarLV AJ292226
PMV_AY505475
CPMMV:BR:BA:02
CPMMV:BR:MT:02:1
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CPMMV:BR:MG:09:3
CPMMV:BR:GO:10:5
CPMMV:BR:GO:01:1
CPMMV HQ184471
ButMV AB517596
ICRSV AF406744
0.87
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1
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0.76
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0.59
1
0.52
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1
0.62
0.99
0.91
0.5
0.88
1
0.71
1
1
1
0.2

CHAPTER 2

MOLECULAR VARIABILITY OF *Cowpea mild mottle virus* INFECTING SOYBEAN IN BRAZIL

Zanardo, L.G., Silva, F.N., Lima, A.T.M., Castillo-Urquiza, G.P., Milanesi, D.F.M., Almeida, A.M.R., Zerbini, F.M., Carvalho, C.M. Molecular variability of *Cowpea mild mottle virus* infecting soybean in Brazil. Archives of Virology, *Submitted*.

Molecular variability of *Cowpea mild mottle virus* infecting soybean in Brazil

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Keywords: CPMMV, *Carlavirus*, soybean and molecular variability

Abstract

We report the molecular variability of eighteen isolates of *Cowpea mild mottle virus* (CPMMV, *Carlavirus* genus, *Betaflexiviridae* family) infecting soybean fields of different Brazilian states (Bahia, Goiás, Maranhão, Mato Grosso, Minas Gerais and Pará) during the years of 2001 and 2010. The isolates showed a variety of symptoms in soybean cv. CD206, ranging from mild (crinkle/blistering leaves, mosaic and vein clearing) to severe (bud blight, dwarfism, leaves and stem necrosis). Recombination analysis showed that only one CPMMV isolate had a recombinant portion among the eighteen evaluated. Pairwise comparisons and phylogenetic analysis were performed for partial genomes (ORF2-3'terminus) and for each ORF individually (ORF2, 3, 4, 5 and 6), showing the isolates to be distinct. The phylogenetic tree did not show clustering based on the year of collection or geographical origin; some groupings were based on

symptoms. Additionally, the phylogenetic analysis made clear the existence of two distinct strains of the virus, (CPMMV-BR1 and CPMMV-BR2), with molecular variability between these. This is the first study of the molecular variability of CPMMV and is the first time that a large number of CPMMV isolates have been sampled and sequenced.

Introduction

Soybean stem necrosis disease is caused by the carlavirus *Cowpea mild mottle virus* (CPMMV, Family *Betaflexiviridae*, Genus *Carlavirus*). Symptoms of viral infection in soybean plants are variable and include bud blight, necrosis of stem and petiole, dwarfism, mosaic and foliar deformation, with the presence of blisters [3]. The genome of carlaviruses is composed of a single-stranded positive sense RNA molecule (7.8-8.9 Kb) encapsidated in flexuous filamentous particles (10-15 x 650-700 nm) [3, 26, 28]. The non-segmented viral RNA possess a cap structure [m7GpppG] linked to the 5’-terminus region and a poly(A) tail at its 3′ -terminus. It contains usually six Open Reading Frames (ORFs): ORF1 encodes the putative RNA-dependent RNA polymerase (RdRp); ORFs 2, 3 and 4 encode the triple gene block (TGB1-3), essential for virus movement; ORF5 encodes the coat protein (CP); and ORF6 encodes a nucleic acid-binding protein (NABP) containing a zinc finger motif [1, 31, 34]. Recently, a new virus, the sweet potato C6 virus, was described, with genomic organization typical of the genus *Carlavirus*, but without the ORF6 encoding the cysteine-rich protein. Instead a predicted protein was found with no similarity to any known protein [14].

CPMMV is transmitted by the whitefly *Bemisia tabaci* [4, 27, 36, 37] and was originally described infecting cowpea (*Vigna unguiculata*) [7]. In Brazil, although CPMMV was first reported in common bean (*Phaseolus vulgaris*) [13], only in the 2000/01 season was it reported infecting soybean plants, in Goiás state [3]. In addition to the diversity of symptoms caused in soybean, recent studies suggest a rapid spread of this virus in Brazilian soybean fields. Three years after its first identification in soybean, the virus was described infecting soybean fields in the states of Bahia (Barreiras), new localities of Goiás (Acreúna, Luzitania, Porteirão, Quirinópolis and Vianópolis), Mato Grosso (Sorriso), Maranhão (Balsas) and Paraná (Palotina) [2-4]. By 2008, the virus was present in several regions of the states of Paraná, Minas Gerais, Goiás, Mato Grosso, Bahia, Tocantins and Maranhão, infecting soybean plants [2]. The diversity of symptoms indicates the existence of variability among CPMMV isolates.

RNA viruses exhibit a high genetic variability [16, 22, 23, 25]. The evolutionary mechanisms of RNA virus evolution include mutation, recombination and genome reassortment, which act differently in each family of virus [16, 22, 23, 25]. The evolution of RNA viruses is driven by high rates of mutation [25], due to the error-prone replication attributed to the absence of proofreading activity in RNA-dependent RNA polymerases (RdRp) and a short generation time [18, 23, 25].

Recombination and genome reassortment can play an important role in the generation of genetic variability, increasing the potential for evolutionary changes [11]. Recombination most frequently takes place within a viral population in the same host cell, although it also occurs between different viral strains or different viruses [49]. It probably occurs when RdRp ‘jumps’ from the donor RNA templates to the acceptor template during the strand synthesis, remaining bound to the nascent RNA strand, so the hybrid molecule is produced [25, 46]. This process is known as copy-choice replication [25, 46].

So far, no study has been conducted to assess the genetic variability of CPMMV isolates. In fact, only one isolate has had its genome completely sequenced [34], and few isolates have been partially sequenced [4, 5, 24, 37, 51]. Although the virus has been known since 1973 [7], little is known about its variability in Brazil and globally. Considering that Brazil is the second largest producer of soybean in the world, that CPMMV is spread by the whitefly *B. tabaci* and that it has been found in several soybean fields, a greater understanding of the virus is needed. Therefore, this study was performed to assess the genetic variability of Brazilian CPMMV isolates infecting soybean.

Materials and methods

Sampling, detection and characterization of CPMMV isolates

A total of 65 samples of soybean plants showing symptoms of stem necrosis, dwarfism and bud blight were collected between 2009 and 2010 in soybean growing regions of the Brazilian states of Minas Gerais (35 samples collected in January 2009), Mato Grosso (3 samples collected in January 2009), Pará (22 samples collected in March 2009) and Goiás (5 samples collected in January 2010). Additionally, five symptomatic samples (collected between 2001 and 2002 in the Brazilian states of Bahia, Goiás, Maranhão, Mato Grosso and Pará) were provided by one of the authors (AMRA).

All collected samples were stored in a freezer at -80° C, in order to preserve the original samples.

The collected samples were tested by indirect ELISA [12] using a CPMMV polyclonal antiserum [9]. Positive samples were used as inoculum for mechanical inoculation of soybean plants cv. CD206 using 0.1M phosphate buffer, pH 7.2, with 0.1% sodium sulfite. Inoculated plants were maintained in a greenhouse, with average daily temperatures of 26 ± 2^{o}C, for 40 days post-inoculation (dpi) to check the infection and onset of symptoms. CPMMV infection of inoculated plants was again confirmed through indirect ELISA.

Additionally, the presence of begomoviruses and *Soybean mosaic virus* (SMV, genus *Potyvirus*) in mixed infection with CPMMV was checked for all samples. Infection by SMV was tested by indirect ELISA using a specific polyclonal antiserum produced by one of the authors (AMRA), and the presence of begomoviruses was evaluated by total DNA extraction [15] followed by PCR amplification using the universal oligonucleotides PBL1v2040/PCRc1 [44].

RT-PCR and molecular cloning

Leaf tissue of systemically CPMMV-infected soybean plants (positive in biological and serological tests) were submitted to total RNA extraction using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer’s instructions. RT-PCR was performed from 500 ng of total RNA, using Superscript III reverse transcriptase (Invitrogen) and Platinum *Taq* DNA polymerase (Invitrogen), according to the manufacturer’s protocol. The CPMMV primers (40 μM) used in RT-PCR are described in Supplementary Table S1 and amplifying the portion of the genome that includes from ORF2 to the 3 'terminal portion (ORF2-3'end). All amplifications consisted of 35 cycles of the following profile: 94ºC for 1 min, annealing at 45ºC, elongation at 72ºC for 1–2 min (depending on the size of amplicon) and a final extension step at 72ºC for 10 min. The amplicons were gel-purified using the Ilustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), ligated into the pGEM-T Easy Vector (Promega), transformed into *Escherichia coli* DH5α cells and sequenced by Macrogen Inc (Seoul, South Korea).

Pairwise comparisons and recombination analysis

Partial sequences of CPMMV isolates, including ORF2 to 3’-end (fragment of 2514-2519 nt), were assembled using DNA BASER Sequence Assembler v.3.5 (Heracle Biosoft). Pairwise nt comparisons were performed by p-distance using MEGA v. 5 [50], and amino acid sequence comparisons were performed in using the quick alignment option and default settings in DNAMAN v. 7.0 (Lynnon Biosoft).

Multiple sequence alignments were performed using the Muscle module [19] in MEGA v. 5. Detection of potential recombinant sequences was performed using Recombination Detection Program (RDP) v.3.44 [32]. Default settings and a multiple comparison-corrected P-value cutoff of 0.05 were used throughout. Only those recombination events detected by three or more methods cited above were considered. The recombination analysis only involved isolates of this study.

Phylogenetic analysis

Phylogenetic trees were constructed in two ways: (i) for the complete amplified fragment (ORF2-3'-end); or (ii) for each of the five ORFs individually (ORF2 to ORF6). Phylogenetic relationships were inferred using Bayesian inference (BI) in MrBayes v. 3.0 (Ronquist & Huelsenbeck, 2003), with the evolution models selected by MrModeltest v. 2.2 [39] using the Akaike Information Criterion (AIC). The MCMC simulation was run for 20 million generations and sampled once in every 1000 generations. Burn-in was set at 4 million generations resulting in 16000 saved trees. The visualization of the trees was performed on the FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Description of the CPMMV molecular variability

Descriptors of molecular variability were estimated using the DnaSP software v.5.10 [45]. The following descriptors estimated: (i) total number of segregating sites (*s*); (ii) average number of nucleotide differences between sequences (*k*); (iii) nucleotide diversity (π); (iv) number of haplotypes (h); (v) haplotype diversity (Hd) and (vi) Watterson's estimate of the population mutation rate based on the total number of segregating sites (θ-w). The π statistic was also calculated using a sliding window of 100 bases, with a step size of 10 bases for the purpose of estimate the nucleotide diversity throughout the length of ORF2 to ORF6.

Site-specific selection analysis

The gene- and site-specific selection pressures were measured for each of five ORFs evaluated (ORF2-6). The detection of sites under negative and positive selection in the genes was determined using four different maximum-likelihood-based algorithms, Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Random Effects Likelihood (REL) and Partitioning for Robust Inference of Selection (PARRIS) within the HyPhy software package (http://www.hyphy.org/) implemented in the Datamonkey server (www.datamonkey.org) with default conditions. The SLAC algorithm was also used to estimate the mean non-synonymous to synonymous substitutions ratio (dN/dS). The nucleotide substitution model incorporated was the Hasegawa-Kishino-Yano (HKY), only ORF6 incorporated the General Reversible substitution (REV) model in the analysis. Phylogenetic trees corrected for recombination were inferred by GARD (available at the Datamonkey server) and used as input for the selection analysis.

Results

Assessment of symptoms and mixed infections

We evaluated a total of seventy soybean samples collected in different Brazilian states and different years (2001-2010). CPMMV infection was confirmed in thirty of these plants by serological test (data not shown). Of the thirty positive samples, only eighteen induced symptoms in soybean cv. CD206 inoculated in greenhouse and were used in study. Six of them have been characterized previously (Table 1) [52]. Soybean samples did not show mixed infection with begomoviruses and SMV (data not shown).

The symptoms caused by eighteen CPMMV isolates in soybean plants cv. CD206 were highly variable in the greenhouse. The first symptoms were observed from 14 to 28 days post inoculation (dpi). Eight CPMMV isolates caused severe symptoms (bud blight, dwarfism, leaves and stem necrosis), and ten CPMMV isolates caused mild symptoms (crinkled/blistering leaves, mosaic and vein clearing) (Table 1 and Figure 1). Mosaic symptoms accompanied by vein clearing showed variable intensity (Figure 1A-C).

Sequence comparisons and recombination analysis

Viral RNA sequences were obtained from eighteen symptomatic soybean cv. CD206 samples. Nucleotide (nt) and amino acid (aa) sequence comparisons were performed for the ORF5 (coat protein - CP) amongst all seventeen viral isolates and other representative carlaviruses (data not shown), confirming the taxonomic classification of these isolates as a single viral species (CPMMV). All identity values were higher than 72% for nt and 80% for aa. The eighteen CPMMV isolates showed high nt and aa sequence identity in the different ORFs analyzed (ORF2 to ORF6) (Supple. Figure S1). Based on the pairwise comparisons of five ORFs, among all eighteen CPMMV, two distinct groups were evident: the first one composed of three isolates, CPMMV:BR:GO:01:1, CPMMV:BR:PA:02 and CPMMV:BR:GO:10:4 (group 1), that were very similar to each other, and the second composed of the other fifteen isolates (group 2) (Supple. Figure S1).

Sequence comparisons among the isolates of group 1 and isolates of group 2 showed that the identity values were smaller than the values obtained for each group

(Supple. Figure S1). ORF5 showed the highest sequence identity between the two groups of isolates CPMMV (Supple. Figure S1). The identity values among the isolates within each group were higher than 91% (nt and aa) for all ORFs evaluated (Supple. Figure S1), except for ORF3.

Additionally, we verify that the isolates sequenced in this study showed some variations in the number of aa for some predicted proteins based on nt sequences. ORF2 of CPMMV:BR:GO:01:1 and CPMMV:BR:PA:02 isolates supposedly encodes a protein of 234 aa, while for the other isolates a protein of 231 aa was predicted. ORF3 of CPMMV:BR:GO:10:4 isolate encodes a putative protein of 107 aa, for the other isolates 106 aa. Curiously, ORF6 of isolate CPMMV:BR:GO:10:4 showed a putative protein with 103aa, the CPMMV:BR:GO:01:1 and CPMMV:BR:PA:02 isolates supposedly encodes a protein of 132 aa and the other isolates a protein with 113 aa. These, associated with lower levels of identity with isolates found in group 2, suggest that the three isolates of group 1 are apparently distinct.

Partial genome sequences of CPMMV Brazilian isolates (encompassing five ORFs: 2, 3, 4, 5, 6 and the 3 '-end, were used in the recombination analysis. A single putative recombination event was identified among all eighteen partial CPMMV sequences evaluated. This event was identified in a small portion of ORF3 in the CPMMV:BR:MG:09:4 isolate (Breakpoints position: 787-847), and was supported by four analysis methods (*P*-values: RDP = 4.962 x 10^{-16}, Genecov = 6.088 x 10^{-08}, Max chi = 1.793 x 10^{-03}, Chimaera = 8.642 x 10^{-04}). The recombinant CPMMV:BR:MG:09:4 isolate had an unknown virus as a possible minor parent and CPMMV:BR:MG:09:05 isolate as putative major parent.

Phylogenetic analysis

We performed phylogenetic analysis to determine the relationships amongst seventeen CPMMV isolates (the recombinant CPMMV:BR:MG:09:4 isolate was excluded from this analysis). Phylogenetic relationships were reconstructed by BI based on different portions of the genome. This included a partial genome (ORF2 to 3’-end) and each one of the five ORFs individually (ORF 2, 3, 4, 5 and 6) (Figure 2).

The analyses of all resulting trees showed that two clades were formed [posterior probability (pp) of 1]. This was evidenced by a significant genetic distance separating the two groups (Figure 2). Clade 1 includes isolates CPMMV:BR:GO:01:1, CPMMV:BR:PA:02 and CPMMV:BR:GO:10:4, and clade 2 the other fourteen CPMMV isolates of this study (Figure 2).

Although the seventeen CPMMV isolates were collected in different years and regions, several thousands of kilometers apart within Brazil, they did not group in any significant way by these parameters in the phylogenetic trees. The phylogenetic tree analysis showed that isolates collected in Goiatuba and Cristalina, Goiás state, in 2001 and 2010, respectively, and the isolate collected in Paragominas (PA) in 2002 were clustered in clade 1 in all trees (Figure 2). Similarly, in clade 2 of all trees, the isolates collected in Barreiras (BA), Balsas (MA) and Sorriso (MT) in 2002 clustered with isolates collected in different regions of Minas Gerais state in 2009 and in Cristalina (GO) in 2010 (Figure 2, Table 2). We also observed that isolates collected in the same region (Cristalina, GO) clustered in different clades.

The clustering in term of the phylogenetic trees indicated a relation with the symptoms developed in soybean plants cv. CD206. Within clade 1, the two isolates that induce crinkle leaves and blistering (CPMMV:BR:GO:01:1 and CPMMV:BR:PA:02) clustered together in all trees, with pp greater than 0.75, except in the ORF6 tree (Figure 2). Within clade 2, the trees constructed using partial genomes (ORF2-3'-end), ORF5 and ORF6 showed the clustering of severe CPMMV isolates (inducing necrosis, bud blight and dwarfism) (Figure 2A, E and F), and it was well supported in the trees of partial genome and ORF6 (with pp equal to 0.92 and 0.99, respectively) (Figure 2A and 2F). For the ORF2 tree the grouping occurred among the mild CPMMV isolates that induce mosaic and vein clearing with pp equal to 0.79 (Figure 2B). The trees of ORFs 3 and 4 did not show any separation based on symptoms among the isolates from clade 2 (Figure 2C-D).

Additionally, we performed a second clustering analysis with the program STRUCTURE v.2.3.1 [43]. Although this program is used for inferring population structure using genotype data it implements a model-based clustering method. Confirming our results, the analysis showed that the clusters obtained by STRUCTURE v.2.3.1 recovered the same grouping of the phylogeny (data not shown).

Genetic variability of CPMMV isolates

To evaluate the molecular variability of CPMMV isolates we considered two data sets and analyzed the five ORFs individually (ORFs 2 to 6). The first dataset comprised seventeen CPMMV isolates and the second one fourteen, excluding the most distant CPMMV:BR:GO:01:1, CPMMV:BR:PA:02 and CPMMV:BR:GO:10:4 isolates. Additionally, the recombinant CPMMV:BR:MG:09:4 isolate was also excluded from both datasets.

The descriptors for the dataset including seventeen CPMMV isolates indicated higher genetic variability than the dataset with fourteen CPMMV isolates, represented by a higher number of segregating sites (*S*), nucleotide diversity (π), haplotype number (h) and haplotype diversity (Hd) (Table 2). This was verified for all CPMMV ORFs analyzed. The higher values obtained for all indexes were probably a consequence of the inclusion of the three divergent isolates (CPMMV:BR:GO:01:1, CPMMV:BR:PA:02 and CPMMV:BR:GO:10:4). The mutation rate (θ-W) estimated for all datasets was in the order of 10^{-2} (Table 2). In general the nucleotide diversity (π) was lower than 0.09 for all the ORFs of both datasets (Table 2). ORF5 was the region with lowest π value (0.05616 ± 0.01806), and ORF3 the region with highest π value (0.07802 ± 0.02789) in the dataset with seventeen isolates. However, in the dataset with fourteen isolates, ORF4 was the gene with the lowest π value (0.00621 ± 0.00248) and ORF6 the region with the highest (0.01806 ± 0.00287).

Additionally, we evaluated π values throughout the length of ORF2, ORF3, ORF4, ORF5 and ORF6 using the two datasets. We observed that the tendency for π values observed in the graphs is similar for all ORFs, independent of the dataset analyzed (Figure 3) although, as expected, higher π values were found along the lengths of the ORFs in the dataset with sixteen isolates (Figure 3). Within ORF2, the central region seems to be more variable than other regions (Figure 3). In ORF3 the 3’ region of the genes seems to be more variable than the 5’ region, the opposite of that observed for ORF6. In ORF4 there is little variability among CPMMV isolates analyzed, especially for the dataset with thirteen CPMMV isolates (Figure 3). ORF5 is variable along the sequence, and the portion that extends from the 5’ region to the nucleotide position 500 of gene seems to be the most diverse.

Analysis of site-specific selection

Initially the recombinant CPMMV:BR:MG:09:4 isolate was used in the analysis of selection, since the trees phylogenetic corrected for recombination were inferred by GARD and used as input in the analysis. However, the recombination event identified by RDP was not detected by GARD. In the presence of the recombinant isolate, some sites under positive selection were found in ORF3, located exactly in the recombinant portion (data not shown). Thus, we opted to eliminate the recombinant isolate of dataset before proceeding with the selection analysis.

We evaluated the effect of positive and negative selection at each site of ORFs 2 to 6 of the seventeen isolates. All ORFs evaluated showed dN/dS ratios (ϖ) lower than 1, indicating purifying selection (Table 3). The dN/dS ratios for the ORF5 (ϖ=0.1057) was the lowest of the viral ORFs analyzed (Table 3), showing this to be the most constricted region. The highest dN/dS ratios was for ORF6 (ϖ=0.2116).

Selection analyses showed that most of the sites are under negative selection (Table 3). In TGB using the SLAC method, five sites under negative selection were identified in ORF2 (positions 3, 150, 191, 201 and 210), three sites in ORF3 (positions 45, 84 and 96), and no site under negative or positive selection was found in ORF4 (Table 3). The SLAC method identified seven sites under negative selection in ORF5 (positions 68, 80, 97, 120, 140, 180 and 187) and two sites in ORF6 (positions 48 and 65). The FEL method only found sites under negative selection in all ORFs evaluated (Table 3). The REL method showed that all sites in ORFs 2 to 6 were under negative selection. The PARRIS method did not identify any site under positive selection in any of the ORFs analyzed (Table 3).

Discussion

The first identification of CPMMV was about 40 years ago [7] and it was first reported in Brazil 30 years ago [13]. However, our knowledge of the molecular variability of CPMMV and evolutionary aspects is limited. There are few genomic sequences of CPMMV available in public databases (thirteen nucleotide sequences in GenBank), and only one corresponds to a complete genome of an isolate infecting cowpea in Ghana. Additionally, six complete CPMMV sequences were obtained recently [52]. So far, no study has attempted to assess the molecular variability of CPMMV.

The symptom diversity observed in soybean fields drew our attention to CPMMV. Initially, we could not exclude the possibility of mixed infections with other viruses that infect soybean in Brazil, or having distinct species of carlaviruses infecting soybean plants. We did not detect begomoviruses or SMV in the analyzed samples, and comparisons of the nt and aa CP sequences showed that the isolates belong to the CPMMV species. Thus, the different symptoms that have been observed in the soybean fields are caused by different CPMMV isolates. In fact, we demonstrated that the eighteen CPMMV isolates analyzed in this study caused assorted symptoms in soybean cv. CD206 in greenhouse.

To verify molecular variability we analyzed eighteen partial sequences of CPMMV, spanning the ORFs 2, 3, 4, 5, 6 and the 3'- end, of isolates sampled from soybean plants in Brazil. Pairwise comparisons and phylogenetic analysis clearly showed the existence of two groups of isolates, with variations in the sequences of some ORFs. Within each group, CPMMV isolates causing varied symptoms were collected from different Brazilian states in different years, but none of these factors seems to define the clustering of phylogenetic trees, indicating that both groups are widespread and are established in the field. Thus, we can only affirm at the moment that we identified two strains of CPMMV infecting Brazilian soybean fields, denominated CPMMV-BR1 (isolates from group 1, in pairwise comparisons, and clade 1, in phylogenetic trees) and CPMMV-BR2 (isolates from group 2 and clade 2). These strains exhibit different biological characteristics based on soybean cv. CD206. In phylogenetic analysis of ORF 2, 5, 6 and a partial genome a second grouping in clades based on the symptoms is evident, thus asserting that variations among isolates of the same strain also exist.

Two hypotheses are proposed to explain the presence of the same viral strain in geographically distant locations. The first involves the high dissemination efficiency of CPMMV by *B. tabaci* and its flight capacity. It has been demonstrated that CPMMV transmission from soybean to soybean plants by *B. tabaci* occurs in a relatively short time at 15 minutes and a single insect vector is able to transmit CPMMV with transmission rate of 16.7% [33, 36]. Additionally, Byrne (1999) [8] affirmed that the largest long distance flight by *B. tabaci* was 7 km, passively in a stream of air. This type of flying facilitates migration of whitefly populations to distant sites and the consequent colonization of other crops and fields [20], allowing the virus to spread across regions. The insect vector can also be transported easily long distances along transport routes by vehicles.

Flying long distances also allows gene flow among whitefly populations [20]. Currently, there are reports of more than 24 biotypes in the world, and this variability suggests that *B. tabaci* is a complex of species or biotypes [6, 21, 41]. In Brazil, it has been demonstrated that intra- and interpopulation variability exists in *B. tabaci* B-biotype [20, 30]. Additionally, Lima et al. (2012) [30] demonstrated that population differentiation of whitefly occurred mainly according to the plant host, rather than geographical region. Thus, a species or a different biotype or even a different variant of whitefly can be found at a higher frequency in a given region. It may be that these vector biotypes are associated with different strains of the virus, which would strongly influence their dissemination.

The second possibility to explain why isolates geographically and temporally distant are so close molecularly and phylogenetically is the occurrence of seed transmission. However, the transmission by seeds is still controversial and depends on the viral isolate. The Ghanaian CPMMV isolate described by Brunt and Kenten (1973) [7] was transmitted by seeds in soybean, cowpea and with lower frequency in common bean and in Venezuela was demonstrated that CPMMV can be transmitted by yardlong bean seeds [5]. In contrast, Almeida et al. (2005) [4] showed that a Brazilian CPMMV isolate (CPMMV:BR:BA:02) was not transmitted by soybean seeds. If CPMMV transmission by seeds is confirmed for other Brazilian CPMMV isolates, this could explain the distribution of the virus in Brazil. It cannot be excluded that the two situations described above (transmission by whitefly and seeds) may be occurring simultaneously.

Recombination can be an important factor in viral evolution and can result in genetic exchange. Sequence analyses of various RNA and DNA plant viruses provide evidence that recombination may be a major source of variation [22, 23, 29, 35, 46, 49]. In this study we found a single recombinant isolate (CPMMV:BR:MG:09:4) with breakpoints located in ORF3 (TGB2). The recombinant isolate was eliminated from our analysis, because recombination can interfere with phylogeny [42]. In fact, we verified that besides phylogeny, recombination affects the assessment of variability and selection analysis. When the recombinant isolate was used, sites under positive selection were found in recombinant region of ORF3, in the absence of recombinant isolate no site under positive selection was found in this ORF.

Recombination events have previously been reported in different carlavirus species. Analysis involving six isolates of *Chrysanthemum virus B* (CVB) showed sixteen recombination events, thirteen of them involving the RdRp and the other three events involving TGB, CP and NABP [48]. In a study of isolates of *Lily symptomless virus* (LSV) two recombination events were confirmed, and in both events the RdRp had recombinant portions [47]. For *Potato virus S* (PVS) the recombination analyses showed a portion of ORF2 of an isolate as recombinant [17]. In study involving six CPMMV Brazilian isolates, five recombination events were detected, four of these located in the RdRp (ORF1) and one involving others regions of the genome (ORF2-5) [52]. These facts suggest that recombination events in the portion that extends from ORF2 to 3' end are not found frequently.

Analysis of variability descriptors performed for the two datasets showed different results. The dataset with seventeen CPMMV isolates was more variable than the one with fourteen CPMMV isolates. This probably occurred because isolates belonging to different strains were analyzed together. The values of nucleotide diversity (π) were less than 0.09 for all regions of the genome evaluated in the two datasets, in concordance with some π values found by García-Arenal et al. (2001) [22]: the CP of dataset with seventeen isolates showed had a nucleotide diversity (0.05616) similar to the CP of a global population of *Citrus tristeza virus* (CTV, *Closterovirus* genus) (π=0.068). For the dataset with fourteen CPMMV isolates the nucleotide diversity of the CP (π=0.01101) showed a value close to that found for the CP of African populations of *Groundnut rosette assistor virus* (GRAV, *Luteoviridae* family) (π=0.018). Pagan et al. [40] showed for *Pepino mosaic virus* (PepMV, genus *potexvirus*) a π value for TGB (0.0075) similar to the ones we found for the genes that comprising the CPMMV TGB (TGB1: 0.00689; TGB2: 0.00876; and TGB3: 0.00621) of the dataset with fourteen CPMMV isolates. ORF6 could not be compared due to lack of data. Our dataset was also obtained from different geographical regions and with a smaller number of isolates than the studies cited above, but the values were very close to those found by different authors.

The analysis of selection showed that the different regions of the CPMMV genome are under negative selection. Sequence analysis of other viruses shows that in most instances selection is negative, purifying [22]. Selection is associated with every factor in the life cycle of the virus, including the maintenance of structural features and functional activity of proteins [22]. ORF5 (CP) was the region under strongest negative selection of the viral ORFs analyzed, showing it to be the most constricted region. This situation is typical for arthropod-vectored viruses [10].

The results presented here advance our understanding of the molecular variability of CPMMV Brazilian isolates infecting soybean. We have shown that CPMMV isolates collected from different regions of Brazil in different years and causing different symptoms belong to two different strains of CPMMV. This is the first study of the molecular variability of CPMMV, genus *Carlavirus*. Future studies are necessary to clarify the mode of transmission and spread of Brazilian CPMMV isolates.

Such additional information can help in the development and adoption of preventive control measures.

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Figure legends:

Fig. 1 Symptoms observed in soybean cv. CD206 inoculated with different CPMMV isolates. **A.** Weak mosaic and vein clearing in soybean plants inoculated with CPMMV:BR:MG:09:5 isolate; **B.** Intermediate mosaic and vein clearing in soybean plants inoculated with CPMMV:BR:MG:09:11; **C.** Strong mosaic, vein clearing and crinkled leaves in soybean plants inoculated with CPMMV:BR:BA:02; **D.** Stem necrosis and bud blight in soybean plants inoculated with CPMMV:BR:MG:09:6; **E.** Stem and leaf necrosis in soybean plants inoculated with CPMMV:BR:MG:09:5 and **F.** Blistering and crinkled leaves in soybean plants inoculated with CPMMV:BR:GO:01:1. Similar symptoms have been observed for other isolates from the study.

Fig. 2 Phylogenetic relationships, based on the sequences of partial genome (ORFs2-3'end) and individual ORFs, of the seventeen Brazilian CPMMV isolates using Bayesian inference (implemented in MrBayes 3.1, with 20 million generations). Bayesian posterior probability values are given between nodes. **A.** Partial genome (ORFs 2-3'end) with selection of model GTR+G; **B.** ORF 2, triple gene block (TGB1) with selection of model GTR+G; **C-D.** ORF3-4 (TGB2-3) with selection of model HKY+G; **E.** ORF5, coat protein (CP) with selection model HKY+I; **F.** ORF6, nucleic acid binding protein (NABP) with selection model GTR+G**.** The blue bars show the grouping of isolates causing severe or mild symptoms in soybean plants in clade 2.

Fig. 3 Average pairwise number of nucleotide differences per site (nucleotide diversity, π) calculated on a sliding window across the ORF2, 3, 4, 5 and 6. Sequences from data set with seventeen CPMMV isolates (blue line), and data set with fourteen CPMMV isolates (red line). **A.** ORF 2, triple gene block (TGB 1); **B.** ORF 3 (TGB 2); **C.** ORF4 (TGB 3); **D.**ORF5, coat protein (CP); **E.** ORF6, nucleic acid binding protein (NABP).

Supplementary Figure S1 Two-dimensional plot representing the percent sequence identities between the seventeen Brazilian CPMMV isolates for five ORFs [ORF 2, triple block genes (TGB 1); ORF 3 (TGB 2); ORF 4 (TGB 3); ORF5, coat protein (CP) and ORF 6, nucleic acid binding protein (NABP)] evaluated. Nucleotide sequence identities are above the diagonal and amino acid sequence identities below.

Table 1 Isolates of CPMMV obtained from samples of soybean collected in different Brazilian states, and the symptoms induced in soybean plants cv. CD206 inoculated in greenhouse.

Isolates of CPMMV	Location	Coordinates	Year	Symptoms in Soybean CD206[a]	Genbank accession #
CPMMV:BR:BA:02[b]	Barreiras, BA	12°8’54” S, 44°59’33” W	2002	Cl, Ms, Vc	KC884247
CPMMV:BR:GO:01:1[b]	Goiatuba, GO	18°0’40” S, 49°22’10” W	2001	Cl, B	KC884248
CPMMV:BR:GO:10:4	Cristalina, GO	16°46’4” S, 47°36’47” W	2010	Bb, D, Ln, Sn	-
CPMMV:BR:GO:10:5[b]	Cristalina, GO	16°46’4” S, 47°36’47” W	2010	Bb, D, Ln, Sn	KC884249
CPMMV:BR:MA:02	Balsas, MA	07º31’57” S, 46º02’08” W	2002	Cl, Mw, Vc	-
CPMMV:BR:MG:09:1	Tupaciguara, MG	18°36’12” S, 48°41’25” W	2009	Bb, D, Ln, Sn	-
CPMMV:BR:MG:09:2[b]	Capinópolis, MG	18°40’48” S, 49°33’58” W	2009	Bb, D, Ln, Sn	KC884244
CPMMV:BR:MG:09:3[b]	Tupaciguara, MG	18°36’12” S, 48°41’25” W	2009	Bb, D, Ln, Sn	KC884245
CPMMV:BR:MG:09:4	Tupaciguara, MG	18°36’12” S, 48°41’25” W	2009	Mw, Vc	-
CPMMV:BR:MG:09:5	Tupaciguara, MG	18°36’12” S, 48°41’25” W	2009	Bb, D, Ln, Sn	-
CPMMV:BR:MG:09:6	Tupaciguara, MG	18°36’12” S, 48°41’25” W	2009	Bb, D, Ln, Sn	-
CPMMV:BR:MG:09:7	Capinópolis, MG	18°40’48” S, 49°33’58” W	2009	Cl, Mi, Vc	-
CPMMV:BR:MG:09:11	Pontal do Triângulo, MG	19°41’19”S, 50°41’45” W-	2009	Mi, Vc	-
CPMMV:BR:MG:09:12	Pontal do Triângulo, MG	19º31’47”S, 45º57’59” W	2009	Bb, D, Ln, Sn	-
CPMMV:BR:MG:09:15	Uberaba, MG	19º44’54” S, 47º55’55” W	2009	Ms, Vc	-
CPMMV:BR:MG:09:16	Uberaba, MG	19º44’54” S, 47º55’55” W	2009	Mi, Vc	-
CPMMV:BR:MT:02:1[b]	Sorriso, MT	12°33’31” S, 55°42’51” W	2002	Mw, Vc	KC884246
CPMMV:BR:PA:02	Paragominas, PA	02°59’51” S, 47°21’13” W	2002	Cl, B	-

[a] B=Blistering, Bb= Bud Blight, Cl=Crinkled leaves, D=Dwarfism, Ln=Leaf necrosis, Mw=Weak mosaic, Mi=Intermediate mosaic, Ms=Strong mosaic, Nll=Necrotic local lesions, Sn=Stem necrosis; Vc=Vein clearing. [b] Previously sequenced isolates [52]

Table 2 Descriptors of variability for *Cowpea mild mottle virus* (CPMMV) populations obtained from soybean plans in different states of Brazil.

Genome region	Number of isolates*	Region lengh (nt)	S^a	K^b	π^c	H^d	Hd^e	θ-W^f
ORF2 (TGB1)	17	697	190	52.978	0.07623 ± 0.02778	13	0.949	0.08086
	14		29	4.791	0.00689 ± 0.00145	11	0.934	0.01312
ORF3 (TGB2)	17	321	88	25.044	0.07802 ± 0.02789	14	0.971	0.08109
	14		18	2.813	0.00876 ± 0.00168	12	0.967	0.01763
ORF4 (TGB3)	17	207	55	15.787	0.07626 ± 0.02817	7	0.662	0.07859
	14		9	1.286	0.00621 ± 0.00248	5	0.505	0.01367
ORF5 (CP)	17	867	184	48.632	0.05616 ± 0.01806	17	1.0	0.06285
	14		55	9.549	0.01101 ± 0.00116	14	1.0	0.01995
ORF6 (NABP)	17	342	81	21.949	0.07035 ± 0.02104	13	0.949	0.07679
	14		26	6.176	0.01806 ± 0.00287	10	0.923	0.02391

[a] Total number of segregating sites.
[b] Average number of nucleotide differences between sequences.
[c] Nucleotide diversity.
[d] Haplotype number.
[e] Haplotype diversity.
[f] Watterson’s estimate of the population mutation rate based on the total number of segregating sites.
* The CPMMV:BR:MG:09:4 isolate recombinant was excluded from the analysis.

Table 3 Analysis of selection for ORF2, ORF3, ORF4, ORF5 and ORF6 of CPMMV Brazilian isolates implemented in Datamonkey server.

Genome region	Number of Isolates	dN/dS	SLAC[a]		FEL[b]		REL[c]		PARRIS[d]
			PS	NS	PS	NS	PS	NS	PS
ORF2	17	0.1552	-	5	-	59	-	+	-
ORF3	17	0.1668	-	3	-	27	-	+	-
ORF4	17	0.1905	-	-	-	11	-	+	-
ORF5	17	0.1057	-	7	-	89	-	+	-
ORF6	17	0.2116	-	2	-	27	-	+	-

(PS) Sites under positive selection; (NS) sites under negative selection; (-) no site under selection; (+) all sites under selection. [a,b,c,d]Codon-based maximum-likelihood algorithms. [a]Single Likelihood Ancestor Counting (SLAC); [b]Fixed Effects Likelihood; (FEL); [c]Random Effects Likelihood (REL) and [d]Robust Inference of Selection (PARRIS).

Figure 1

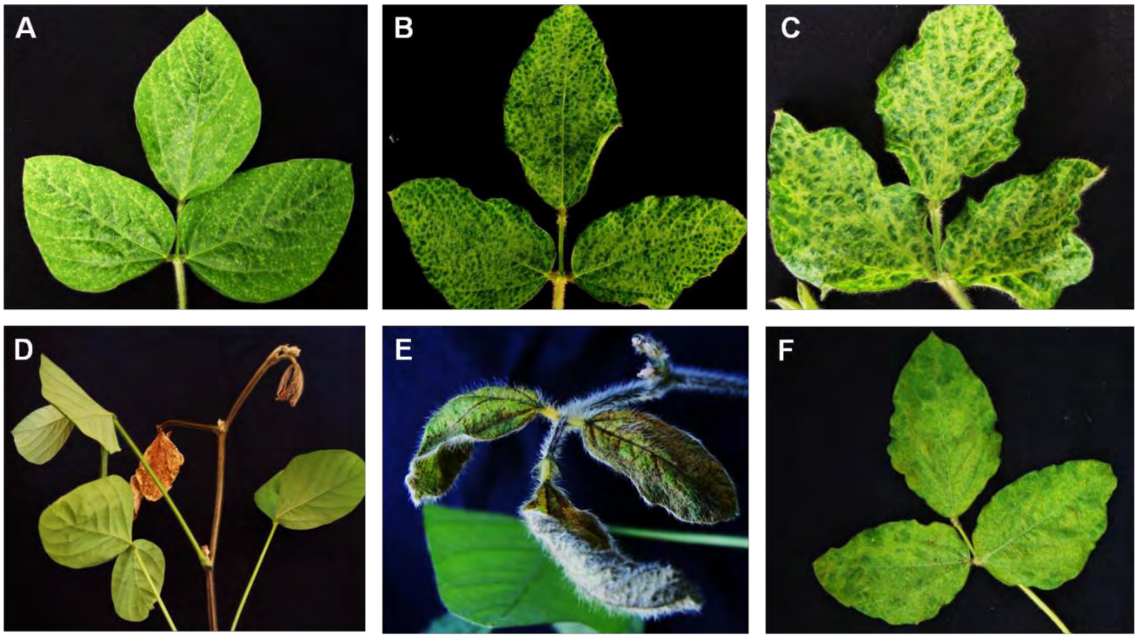


Figure 2

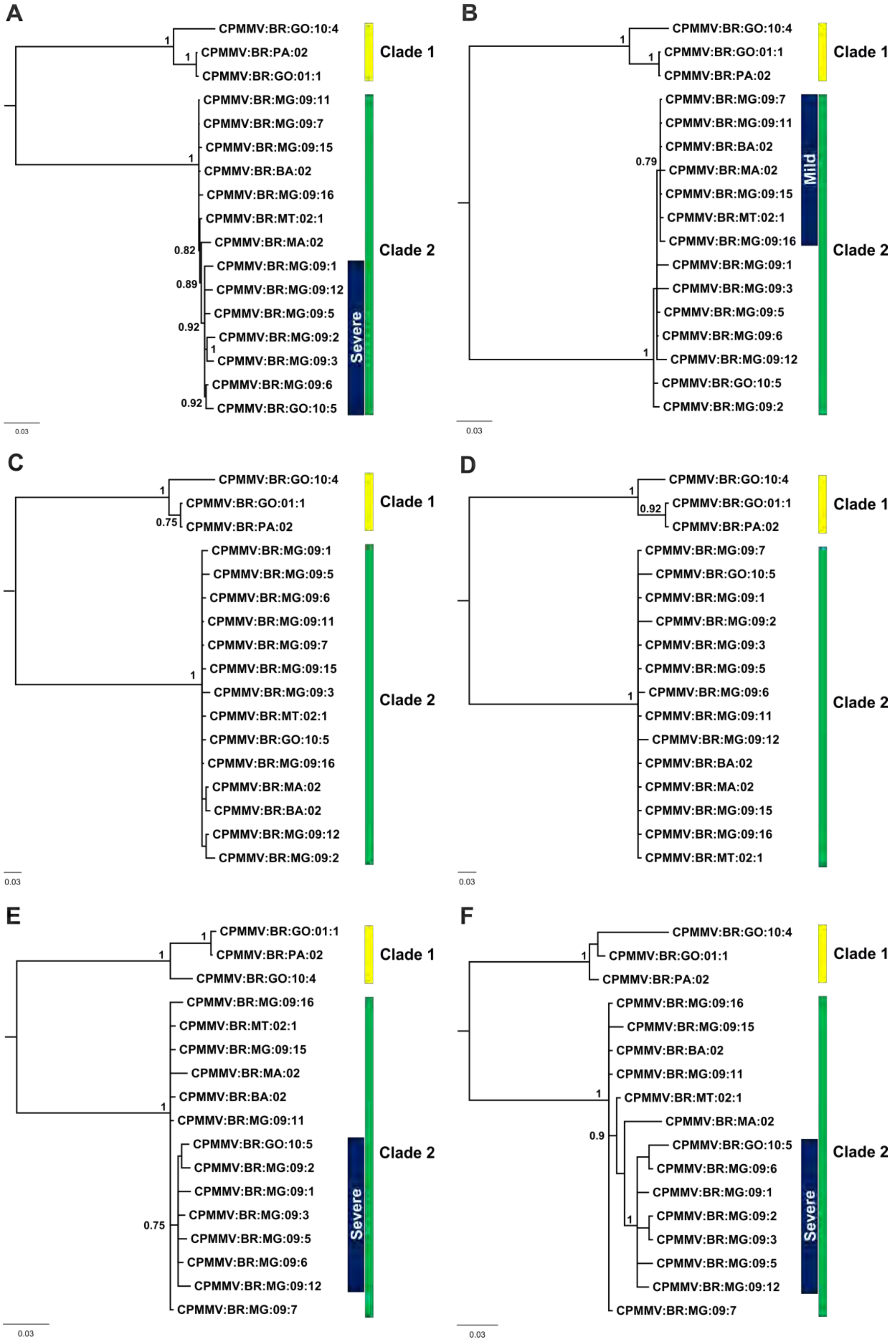
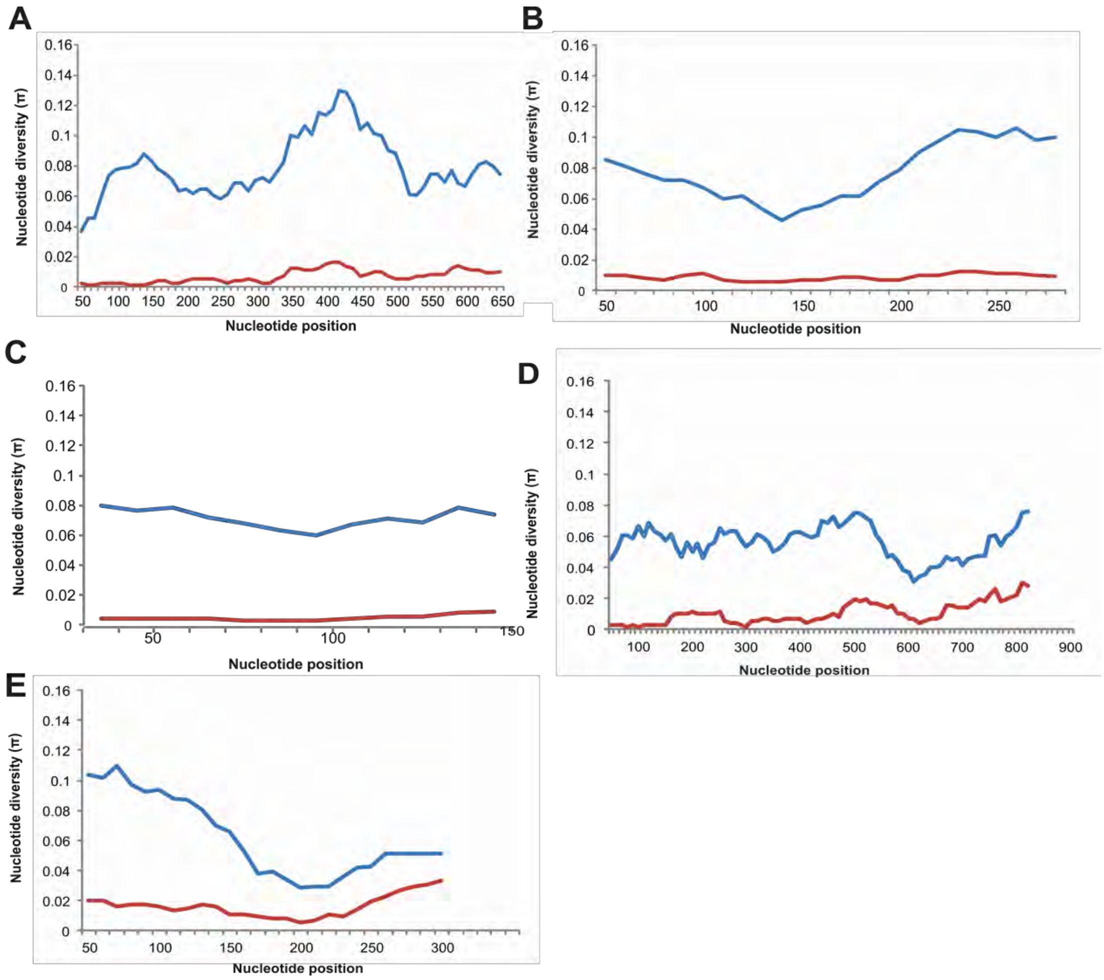

A
CPMMV:BR:GO:10:4
CPMMV:BR:PA:02
CPMMV:BR:GO:01:1
CPMMV:BR:MG:09:11
CPMMV:BR:MG:09:7
CPMMV:BR:MG:09:15
CPMMV:BR:BA:02
CPMMV:BR:MG:09:16
CPMMV:BR:MT:02:1
CPMMV:BR:MA:02
CPMMV:BR:MG:09:1
CPMMV:BR:MG:09:12
CPMMV:BR:MG:09:5
CPMMV:BR:MG:09:2
CPMMV:BR:MG:09:3
CPMMV:BR:MG:09:6
CPMMV:BR:GO:10:5
1
1
1
0.82
0.89
0.92
1
0.92
Clade 1
Clade 2
Severe
0.03
B
CPMMV:BR:GO:10:4
CPMMV:BR:GO:01:1
CPMMV:BR:PA:02
CPMMV:BR:MG:09:7
CPMMV:BR:MG:09:11
CPMMV:BR:BA:02
CPMMV:BR:MA:02
CPMMV:BR:MG:09:15
CPMMV:BR:MT:02:1
CPMMV:BR:MG:09:16
CPMMV:BR:MG:09:1
CPMMV:BR:MG:09:3
CPMMV:BR:MG:09:5
CPMMV:BR:MG:09:6
CPMMV:BR:MG:09:12
CPMMV:BR:GO:10:5
CPMMV:BR:MG:09:2
1
1
0.79
1
Clade 1
Mild
Clade 2
0.03
C
CPMMV:BR:GO:10:4
CPMMV:BR:GO:01:1
CPMMV:BR:PA:02
CPMMV:BR:MG:09:1
CPMMV:BR:MG:09:5
CPMMV:BR:MG:09:6
CPMMV:BR:MG:09:11
CPMMV:BR:MG:09:7
CPMMV:BR:MG:09:15
CPMMV:BR:MG:09:3
CPMMV:BR:MT:02:1
CPMMV:BR:GO:10:5
CPMMV:BR:MG:09:16
CPMMV:BR:MA:02
CPMMV:BR:BA:02
CPMMV:BR:MG:09:12
CPMMV:BR:MG:09:2
1
0.75
1
Clade 1
Clade 2
0.03
D
CPMMV:BR:GO:10:4
CPMMV:BR:GO:01:1
CPMMV:BR:PA:02
CPMMV:BR:MG:09:7
CPMMV:BR:GO:10:5
CPMMV:BR:MG:09:1
CPMMV:BR:MG:09:2
CPMMV:BR:MG:09:3
CPMMV:BR:MG:09:5
CPMMV:BR:MG:09:6
CPMMV:BR:MG:09:11
CPMMV:BR:MG:09:12
CPMMV:BR:BA:02
CPMMV:BR:MA:02
CPMMV:BR:MG:09:15
CPMMV:BR:MG:09:16
CPMMV:BR:MT:02:1
1
0.92
1
Clade 1
Clade 2
0.03
E
CPMMV:BR:GO:01:1
CPMMV:BR:PA:02
CPMMV:BR:GO:10:4
CPMMV:BR:MG:09:16
CPMMV:BR:MT:02:1
CPMMV:BR:MG:09:15
CPMMV:BR:MA:02
CPMMV:BR:BA:02
CPMMV:BR:MG:09:11
CPMMV:BR:GO:10:5
CPMMV:BR:MG:09:2
CPMMV:BR:MG:09:1
CPMMV:BR:MG:09:3
CPMMV:BR:MG:09:5
CPMMV:BR:MG:09:6
CPMMV:BR:MG:09:12
CPMMV:BR:MG:09:7
1
1
1
0.75
Clade 1
Clade 2
Severe
0.03
F
CPMMV:BR:GO:10:4
CPMMV:BR:GO:01:1
CPMMV:BR:PA:02
CPMMV:BR:MG:09:16
CPMMV:BR:MG:09:15
CPMMV:BR:BA:02
CPMMV:BR:MG:09:11
CPMMV:BR:MT:02:1
CPMMV:BR:MA:02
CPMMV:BR:GO:10:5
CPMMV:BR:MG:09:6
CPMMV:BR:MG:09:1
CPMMV:BR:MG:09:2
CPMMV:BR:MG:09:3
CPMMV:BR:MG:09:5
CPMMV:BR:MG:09:12
CPMMV:BR:MG:09:7
1
1
0.9
1
Clade 1
Clade 2
Severe
0.03

Figure 3

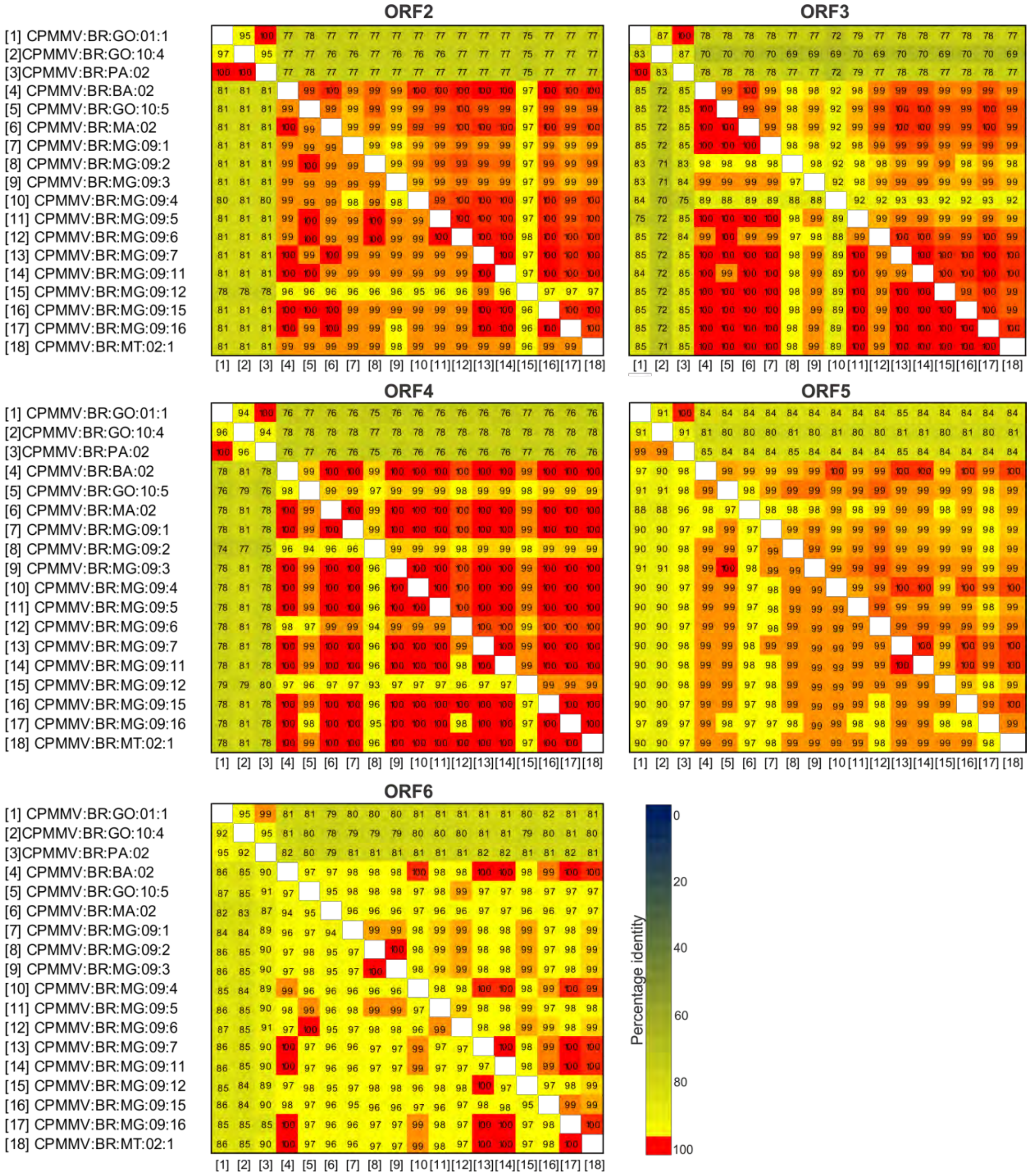

A
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Nucleotide diversity (π)
50 100 150 200 250 300 350 400 450 500 550 600 650
Nucleotide position
B
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Nucleotide diversity (π)
50 100 150 200 250
Nucleotide position
C
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Nucleotide diversity (π)
50 100 150
Nucleotide position
D
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Nucleotide diversity (π)
100 200 300 400 500 600 700 800 900
Nucleotide position
E
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Nucleotide diversity (π)
50 100 150 200 250 300
Nucleotide position

Supplementary Table S1: Primers used in RT-PCR and predicted amplicon size for viral detection and cloning.

Fragments	**Primer**	**Primer sequence 5'-3'**	**PCR product size**
ORF2	ORF2 F	TCCTTTAGGTAGTGAGGC	938
	ORF2 R	AAGTTCGTGCCAGTTGAC^{cD}	
ORF3	ORF3 F	CTTNATYTGCYTNACNAGGCA	552
	ORF3 R	TGTTCTCTNACCAAGT^{cD}	
ORF4 - 3' end	ORF 4 F	TAYMRDGAYGGNACHAA*	1676
	ORF6 R	TAAAACCAGGAAATAAC^{cD}	

*Described by Nicolaisen and Nielsen [38]. The other oligonucleotides were described by Zanardo et al. (2013) [52]. ^{cD} Used for cDNA Synthesis.

Supplementary Figure S1



CONCLUSÕES GERAIS

- Foram sequenciados completamente 6 isolados de CPMMV e outros 12 isolados parcialmente.
- Foi a primeira vez que a sequencia completa de isolados brasileiros de *Cowpea mild mottle virus* (CPMMV) oriundos de soja foi obtida.
- Os isolados virais coletados em soja no Brasil apresentaram características moleculares e biológicas variadas entre si e com o único isolado de CPMMV sequenciado completamente e caracterizado, oriundo de feijão caupi em Gana na África, evidenciando que os isolados brasileiros são de uma estirpe diferente.
- Eventos de recombinação foram encontrados ao longo do genoma viral especialmente na região correspondente à polimerase viral.
- O estudo deixou claro que os sintomas relacionados à doença da necrose da haste da soja eram causados pelo CPMMV e que os isolados virais causam sintomas variados em soja cv. CD206.
- A análise filogenética para os isolados sequenciados parcialmente mostrou que os agrupamentos não se baseavam na origem geográfica ou ano de coleta dos isolados, de fato evidencias de agrupamentos com base nos sintomas causados em soja cv. CD206 foram observados**.**
- Foi demostrada a existência de duas estirpes de CPMMV infectando os campos de soja brasileiros, isso foi evidenciado pela comparação par-à-par de sequencias e através das análises filogenéticas. Os isolados pertencentes a cada estirpe ocasionaram sintomas variados em soja cv. CD206.