

KÉSSIA DE FÁTIMA DA CUNHA PANTOJA

**IDENTIFICAÇÃO DE VIRUS EM AMENDOIM FORRAGEIRO E PIMENTA POR
SEQUENCIAMENTO DE NOVA GERAÇÃO**

Botucatu

2020

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Tese apresentada à Faculdade de Ciências Agronômicas da Unesp Câmpus de Botucatu, para obtenção do título Doutor em Agronomia (Proteção de Plantas).

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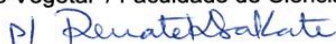
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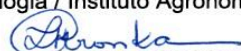
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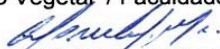
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*Aos meus pais Maria de Fátima e Isac Pantoja, pelo
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irmão Jorge Luiz e sobrinhos Bianca e Pedro,
por todo amor e companheirismo
A Deus, por sua infinita bondade e misericórdia*

Dedico

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“As mais belas flores nascem após os rigorosos invernos. Não tenha medo das dificuldades da vida, pois podem lhe trazer grandes jardins.”

Augusto Cury

RESUMO

A espécie *Arachis pintoï*, conhecida como amendoim forrageiro, é uma leguminosa nativa do Brasil. Muitas são as utilidades atribuídas ao amendoim forrageiro, sendo seu uso mais comum como espécie forrageira, fornecendo alimento em grande quantidade e qualidade aos animais, em plantios puros ou consórcio com gramíneas. A ocorrência de sintomas de viroses em genótipos e cultivos de amendoim forrageiro tem sido observada por pesquisadores em diferentes estados brasileiros. No Brasil, apenas duas espécies virais já foram relatadas: o *Peanut mottle virus* - PeMoV e o *Cowpea mild mottle virus* - CpMMV. O objetivo deste trabalho foi estudar os vírus de plantas de amendoim forrageiro do Banco de Germoplasma da Embrapa Acre e detectar viroses em plantas de pimenta Cumari-do-Pará. Para detectar possíveis vírus nesses genótipos, uma análise de sequenciamento de nova geração foi realizada. A extração total de RNA dos 22 acessos foi realizada com o kit RNA Viral PureLink (Invitrogen) seguida de preparação da biblioteca e sequenciamento do transcriptoma utilizando a plataforma Illumina HiSeq2500. A montagem *de novo* das leituras de 24.659.442 foi realizada usando o software CLC Genomics Workbench v7.0.3. Os 9.709 contigs obtidos foram submetidos a uma pesquisa BLASTn usando o software Geneious v.9.1.5. A análise metagenômica permitiu a identificação de sete espécies de vírus: *Peanut mottle virus* - PeMoV (Potyvirus), *Cucumber mosaic virus* sub-grupo IB (Cucumovirus), *Cowpea chlorotic mottle virus* - CCMV (Bromovirus) e um provável novo membro da família *Potyviridae*, e também novas espécies dos gêneros Emaravirus, Polerovirus e Ampelovirus em amendoim forrageiro. A análise de NGS permitiu obter a sequência completa do genoma do *Peanut mottle virus* (PeMoV) com 9.680pb (GenBank MK396065), que foi confirmada em dois genótipos de *A. pintoï* (BRA cv. BRS Mandobi, BRA042269) por RT-PCR, seguido de sequenciamento de Sanger, usando os primers específicos desenhados para PeMoV. O CCMV foi identificado baseado na sequência dos três componentes do genoma viral, tratando-se da primeira identificação deste vírus em amendoim forrageiro. A sequência nucleotídica completa de um novo membro da família *Potyviridae* foi relatada em plantas de amendoim forrageiro (*Arachis pintoï*) no Brasil exibindo sintomas típicos de vírus, que propomos nomear como *Arachis pintoï mottle virus* (ArPMV). O genoma do RNA de sentido positivo ArPMV possui 9.213

nucleotídeos. A ORF única completa do ArPMV compartilha 47% e 34% de identidade de nucleotídeo e aminoácido, respectivamente, com o isolado mais próximo relacionado, o soybean yellow shoot virus. As análises de sequenciamento de nova geração plataforma Illumina HiSeq2500 também foram realizadas para uma amostra de pimenta cv. Cumari-do-Pará (*Capsicum chinense*) coletada em 2017 em uma fazenda próxima ao município de Altamira, no estado do Pará, mostrando sintomas de amarelecimento internerval, redução de lâmina foliar e encurtamento dos entrenós. Embora seja cultivada em todos os estados brasileiros, a produção é fortemente focada nos estados do norte do país. Os contigs foram submetidos à análise por BLASTX com o software Geneious v9.1.5. no banco de dados viral do NCBI, que revelou identidades de nucleotídeos superiores a 90% com a espécie *Pepper vein yellows virus*- PeVYV (gênero *Polerovirus*, família *Luteoviridae*) e também sequências relacionadas aos gêneros *Ampelovirus* e *Cucumovirus*. Posteriormente, através de montagem “*de novo*”, foi possível obter o genoma completo de uma possível nova espécie pertencente ao complexo de *Polerovirus* da pimenta, cujo nome proposto foi *Pepper vein yellows virus* 8 (PeVYV-8). Este é o primeiro relato de um polerovírus infectando a pimenta Cumari-do-Pará no Brasil e a distribuição do vírus em outras regiões do país precisa ser melhor avaliada.

Palavras-chave: *Arachis pintoi*. *Capsicum*. NGS. *Polerovirus*. *Virus*

ABSTRACT

The species *Arachis pintoï*, known as forage peanuts is a native legume from Brazil. There are many uses attributed to forage peanut, being its most common use as a forage species, providing food in large quantity and quality to the animals, in single crops or consortium with grasses. The occurrence of virus symptoms in forage peanut genotypes and crops has been observed by researchers in different Brazilian states. In Brazil, only two viral species have been reported: *Peanut mottle virus*-PeMoV and *Cowpea mild mottle virus*-CpMMV. The objective of this work was to study the forage peanut plant viruses of the Embrapa Acre Germplasm Bank and to detect viruses in Cumari-do-Pará pepper plants. To detect possible viruses in these genotypes, a new generation sequencing analysis was performed. Total RNA extraction from the twenty-two accessions was performed with the PureLink Viral RNA Kit (Invitrogen) followed by library preparation and transcriptome sequencing using the Illumina HiSeq2500 platform. Re-assembly of the 24,659,442 readings was performed using the CLC Genomics Workbench v7.0.3 software. The 9,709 contigs obtained were subjected to a BLASTn search using the Geneious v.9.1.5 software. Metagenomic analysis allowed the identification of seven virus species: *Peanut mottle virus* - PeMoV (Potyvirus), *Cucumber mosaic virus* subgroup IB (Cucumovirus), *Cowpea chlorotic mottle virus* - CCMV (Bromovirus) and a probable new member of the *Potyviridae* family, and also new species of the genera Emaravirus, Polerovirus and Ampelovirus in forage peanuts. NGS analysis yielded the complete 9,680 bp *Peanut mottle virus* (PeMoV) genome sequence (GenBank MK396065), which was confirmed in two *A. pintoï* (BRA cv. BRS Mandobi, BRA042269) genotypes by RT-PCR, followed by Sanger, sequencing using PeMoV-specific primers. The CCMV was identified based on the sequence of the three components of the viral genome, being the first identification of this virus in forage peanut. The complete nucleotide sequence of a new member of the family Potyviridae was reported in forage peanut plants (*Arachis pintoï*) in Brazil exhibiting typical virus symptoms, which we propose to name as *Arachis pintoï mottle virus* (ArPMV). The ArPMV positive-sense RNA genome is 9,213 nucleotides. The complete ArPMV single ORF shares 47% and 34% nucleotide and amino acid identity, respectively, with the closest related isolate, soybean yellow shot virus.

Sequencing analysis of the new generation Illumina HiSeq2500 platform were also performed for a cv pepper sample. Cumari-do-Pará (*Capsicum chinense*) collected in 2017 on a farm near the municipality of Altamira, state of Pará, showing symptoms of internerval yellowing, leaf blade reduction and internode shortening. The contigs were subjected to BLASTX analysis with Geneious v9.1.5 software. In the NCBI viral database, which revealed nucleotide identities greater than 90% with the Pepper vein yellows virus-PeVYV species (genus Polerovirus, family Luteoviridae) and also sequences related to the genera *Ampelovirus* and *Cucumovirus*. Subsequently, by "de novo" assembly it was possible to obtain the complete genome of a possible new species belonging to the pepper polerovirus complex, whose proposed name was *Pepper vein yellows virus 8* (PeVYV-8). This is the first report of a polerovirus infecting Cumari-do-Pará pepper in Brazil and the distribution of the virus in other regions of the country needs to be better evaluated.

Keywords: *Arachis pinto*. *Capsicum*. NGS. Polerovirus. Virus

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INTRODUÇÃO GERAL

A espécie *Arachis pintoi* (Krap. & Greg), conhecida como amendoim forrageiro é uma leguminosa nativa do Brasil (PURCINO et al., 2004). O gênero *Arachis* pertence à família Fabaceae, subfamília Papilionoideae, tribo Stylosanthinae e subtribo Aeschynomeneae (GREGORY et al., 1980; KRAPOVICKAS E GREGORY, 1994). O primeiro acesso da espécie *A. pintoi* foi obtido pela coleta realizada por Geraldo Pinto, em 1954, junto à foz do Rio Jequitinhonha, em Belmonte, no Estado da Bahia (VALLS, 1992; BARCELOS et al., 2000). A distribuição geográfica desta espécie se expande por uma área que abrange parte dos estados de Goiás, Bahia e Minas Gerais, estendendo-se por toda a costa Atlântica do Brasil (CARVALHO, 2004). No Brasil, as atividades desenvolvidas pelo Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia - EMBRAPA têm contribuído consideravelmente para ampliação das coleções de genótipos de amendoim forrageiro. De acordo com VALLS (2001), mais de 150 acessos de *A. pintoi* encontram-se catalogados.

O amendoim forrageiro é uma leguminosa herbácea perene, de crescimento rasteiro, hábito estolonífero e prostrado que lança estolões horizontalmente em todas as direções, em quantidade significativa, cujos pontos de crescimento são bem protegidos do pastejo realizado pelos animais. Possui o ponto de crescimento protegido, florescimento indeterminado, hábito de crescimento prostrado, habilidade de enraizar nos estolhos e reserva de sementes no solo (JONES, 1993).

É uma forrageira de porte baixo, dificilmente ultrapassando 30-40 cm de altura, possui raiz pivotante, podendo alcançar 1,60 m de profundidade (CALEGARI et al., 1995). O *A. pintoi* é indicado tanto para formação de pastagens quanto para cobertura do solo em culturas perenes, podendo ainda ser utilizado como planta ornamental (PEREIRA; ANDRADE; KARIA, 1996). Pode ser utilizado em consórcios ou como fonte de proteína, visto que apresenta grande resistência ao pastejo e pisoteio expressado pela maior participação na composição botânica do relvado, independentemente da pressão de pastejo imposta (BARCELLOS et al., 1996).

No Brasil, há relato de apenas dois vírus nesta cultura, o potyvirus, Peanut mottle virus (PeMoV) que foi detectado em plantas de amendoim forrageiro do campo experimental da Embrapa Cerrados, no Distrito Federal (DF), que mostravam sintomas de manchas anelares cloróticas (ANJOS et al., 1998), e o carlavirus,

Cowpea mild mottle virus (CpMMV) em plantas de amendoim forrageiro de jardins localizados na área urbana de Londrina, PR e em áreas rurais, apresentavam folhas reduzidas e com sintomas de mosaico (MITUTI et al., 2005).

De acordo com levantamento realizado no Banco ativo de germoplasma de amendoim forrageiro da Embrapa Acre, alguns genótipos apresentam sintomas de viroses como mosqueado, mosaico, anéis cloróticos e necróticos, dentre outros. A ausência de informações a respeito dos vírus que ocorrem nesta cultura dificulta as ações de manejo da virose e programas de melhoramento visando à resistência.

Também foi objeto de estudo nesta tese, vírus infectando a pimenta (*Capsicum* spp.) que no estado do Pará é muito apreciada e cultivada pela agricultura familiar (CARVALHO; BOARI, 2013). As pimentas do gênero *Capsicum* (Solanaceae) são originárias do continente americano. Dentre as 25 espécies descritas, cinco são domesticadas e amplamente utilizadas (*Capsicum annum* var. *annum*, *C. baccatum* var. *pendulum*, *C. chinense*, *C. frutescens* e *C. pubescens*) e três são semi-domesticadas (*C. annum* var. *glabriusculum*, *C. baccatum* var. *praetermissum*, *C. baccatum* var. *baccatum*) (CARVALHO e BIANCHETTI, 2004; REIFSCHNEIDER, 2000).

Entretanto, uma das limitações para *Capsicum* spp. é o aparecimento de doenças de origem viral. Entre os principais vírus do gênero *Potyvirus* pode-se citar *Potato virus Y* (PVY), *Pepper mottle virus* (PepMoV), *Tobacco etch virus* (TEV), *Pepper veinal mottle virus* (PVMV) e *Chilli veinal mottle virus* (ChiVMV) e *Pepper yellow mosaic virus* (PepYMV) (INOUE-NAGATA, 2002), sendo que até o momento seguinte o PVY e PepYMV são descritas no Brasil. O PVY no Brasil foi observado em pimentão pela primeira vez na década de 50 (COSTA e ALVES, 1950) e o PepYMV em 2002 (INOUE-NAGATA et al. 2002). Ambos os vírus são sorologicamente relacionados (CUNHA et al. 2004), e causam sintomas de mosaico indistinguíveis, tornando-os de difícil identificação visual. No Brasil, outros gêneros de vírus já foram relatados em *Capsicum* spp., como o Cucumovirus (*Cucumber mosaic virus* – CMV), Tobamovirus (*Pepper mild mottle virus* – PMMoV, *Tomato mosaic virus* – ToMV), Begomovirus (*Tomato severe rugose virus*) e Luteovirus, Tospovirus (*Tomato spotted wilt virus* -TSWV, *Tomato chlorotic spot virus* - TCSV e *Groundnut ringspot virus* – GRSV (BRIOSIO, 1996).

Em junho de 2017, cerca de 100% das plantas de pimenta das cultivares Cumari-do-Pará mostrando sintomas de amarelecimento internerval, grande redução

de lâmina foliar e encurtamento dos entrenós foram observadas em uma lavoura localizada no município de Altamira, no estado do Pará. O Sequenciamento de Nova Geração (*Next-generation sequencing* - NGS) é uma metodologia inovadora, que possibilita gerar milhões de leituras de uma única vez, de modo rápido e eficaz, sem requerer prévio conhecimento do patógeno, auxiliando na descoberta de novos vírus (ADAMS et al., 2009; MARDIS, 2008; RADFORD et al., 2012).

Com o objetivo de caracterizar e identificar os principais vírus, o sequenciamento de alto desempenho foi utilizado para detectar vírus em amendoim forrageiro do banco ativo da Embrapa Acre e em pimentas do plantio de Altamira-PA, apresentando sintomas característicos de viroses. O trabalho foi dividido em quatro capítulos em formato de artigos científicos. O primeiro capítulo foi intitulado: First report of a putative new pepper vein yellow virus species associated with a vein yellows disease of bonnet pepper plants in Brazil, segundo capítulo: Detection and characterization of the *Cowpea chlorotic mottle virus* in forage peanut (*Arachis pintoï*) in Brazil; terceiro capítulo: Characterization and complete genome sequence of the *Peanut mottle virus* and *Cucumber mosaic virus* in *Arachis pintoï* Krapov. & W.C. Greg. (Fabaceae) in Brazil e quarto capítulo: Identification and characterization of a new member of the *Potyviridae* family infecting *Arachis pintoï* in Brazil

CAPÍTULO 1

Detection and characterization of the *Cowpea chlorotic mottle virus* in forage peanut (*Arachis pintoï*) in Brazil

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Abstract

The *Cowpea chlorotic mottle virus* (CCMV, *Bromovirus*, *Bromoviridae*) was detected in forage peanut plants (*Arachis pintoï*) showing mosaic, yellowing and interveinal chlorosis. Negatively stained partially purified preparations contained isometric particles ca. 30 nm in size, which were also present in the cytoplasm and vacuole of leaf parenchymal cells from CCMV-infected *A. pintoï* leaves. Mechanical transmission of the CCMV leaf extracts from symptomatic *A. pintoï* plants resulted in systemic mosaic in *A. pintoï* cv. BRS Mandobi, *Vigna unguiculata* L. cv Pretinha and *V. unguiculata* sg. *sesquipedalis* cv. De Metro plants; chlorotic patches on leaves of inoculated *Nicotiana occidentalis* and *N. benthamiana*, while inoculated *Chenopodium quinoa* and *C. amaranticolor* reacted with local chlorotic lesions. The causal virus was identified as CCMV based on the complete nucleotide sequence of the three genome components obtained by next-generation sequencing (NGS) made from leaf samples from infected *A. pintoï*. Further analysis revealed nucleotide identities of 88% for RNA1, 84% for RNA2 and 89% for RNA3 (GenBank AF325739, M28817 and HQ611268, respectively). Primer designed based on the obtained genome sequence of CCMV efficiently detected this virus in infected *A. pintoï*

samples. To the best of our knowledge, this is the first report of the *Cowpea chlorotic mottle virus* (CCMV) infecting forage peanut.

Keywords

Next-Generation Sequencing, *Bromovirus*, CCMV, virus

The tropical forage legume *Arachis pinto* Krapov. & W.C. Gregory, also known as forage peanut, belongs to the Fabaceae family. It is a perennial herb native to Brazil, mainly occurring in its Central region, and is also grown in many tropical and subtropical countries around the world, such as Australia and Colombia [1]. *Arachis pinto* is used as a permanent pasture species for intensive grazing systems mainly due to its high protein content and high potential for atmospheric nitrogen fixation [2]. It can also be used as ground cover in open areas or under trees as an ornamental plant [3].

There are several reports of viral diseases affecting *A. pinto* and causing reductions in leaf mass [4]. Among them, three plant viruses have been reported in Colombia, the *Peanut mottle virus* (PeMoV), *Turnip yellows virus* (TuYC) and *Garlic virus A* (GarAV) [5]. The PeMoV was also reported in Brazil [6], as well as the *Cowpea mild mottle virus* (CMMV) [7].

The Active Germplasm Bank of Forage Peanut located at Embrapa, State of Acre, Brazil, contains several genotypes of *A. pinto*, *A. repens* Handro and hybrids (*A. pinto* x *A. repens*) [8]. Some genotypes have been found showing symptoms resembling viral infection, such as leaves with mosaic, crinkle, and yellowing in addition to plant stunting. As an attempt to detect the possible virus(es) present in these accessions, a next-generation sequence analysis was performed followed by bioinformatics analysis. First, total RNA was extracted from symptomatic plant leaves of 22 different genotypes of *A. pinto* using the PureLink Viral RNA/DNA kit (Invitrogen™) and sent for library preparation and transcriptome sequencing using the Illumina HiSeq2500 platform at Centro de Genômica Funcional, Departamento de Zootecnia (LZT) – ESALQ, Brazil. De novo assembly of the 24,659,442 reads was carried out using the CLC Genomics Workbench software v7.0.3. The 594 contigs obtained were submitted to a BLASTn search using Geneious software v9.1.5. The BLAST results revealed the presence of three contigs showing high identities with the *Cowpea chlorotic mottle virus* (CCMV, *Bromovirus*) RNA1 (88% identity - AF325739),

RNA2 (84% identity - M28817) and RNA3 (89% - HQ611268) [9]. Additionally, the data allowed us to determine the complete nucleotide sequence of RNAs 1, 2 and 3, which consisted of 3,116 (MK046914), 2,607 (MK046915) and 2,126 (MK046913) nt, respectively. Subsequently, RT-PCR was carried out using the total RNA extracted from the 22 different genotypes of *A. pinto* with newly designed primers, CCMV 1749F (5' TGTCTACAGTCGGAGCAGGT 3') and CCMV 2263R (5' GTGAACAACCACATCGCCAC 3'). The reaction consisted of 94°C/2 minutes, followed by 40 cycles of 94°C/30 seconds, 54°C/30 seconds and 72°C/30 seconds, and 72°C/5 minutes of final extension. The results confirmed the presence of the CCMV in *A. pinto* with a fragment of the expected size (515 nt) from plants of three different genotypes. Amplicons from two samples (genotype BRA 016357 and genotype BRA 042269) were sent for Sanger sequencing, which confirmed the presence of the CCMV in the tested genotypes (88% nucleotide sequence identity with CCMV isolate accession number HQ611270). These plants exhibited severe mosaic and vein chlorosis (Fig. 1).



Fig. 1 Symptoms caused by the Cowpea chlorotic mottle virus – CCMV from *Arachis pinto*

Furthermore, phylogenetic analyses were performed to compare the complete genome sequences of the three CCMV RNA segments obtained in this study to other complete CCMV RNA sequences from GenBank (Fig. 2). The forage peanut CCMV isolate was placed into a distinct clade from all the CCMV genomes available in GenBank (NC_002028; HQ611268; HQ611271; M28818.1; AF325741; AF428096; AF325738) for all three RNA analyzed, indicating that the CCMV strain from Brazil is genetically distinct from the CCMV strains found in other countries.

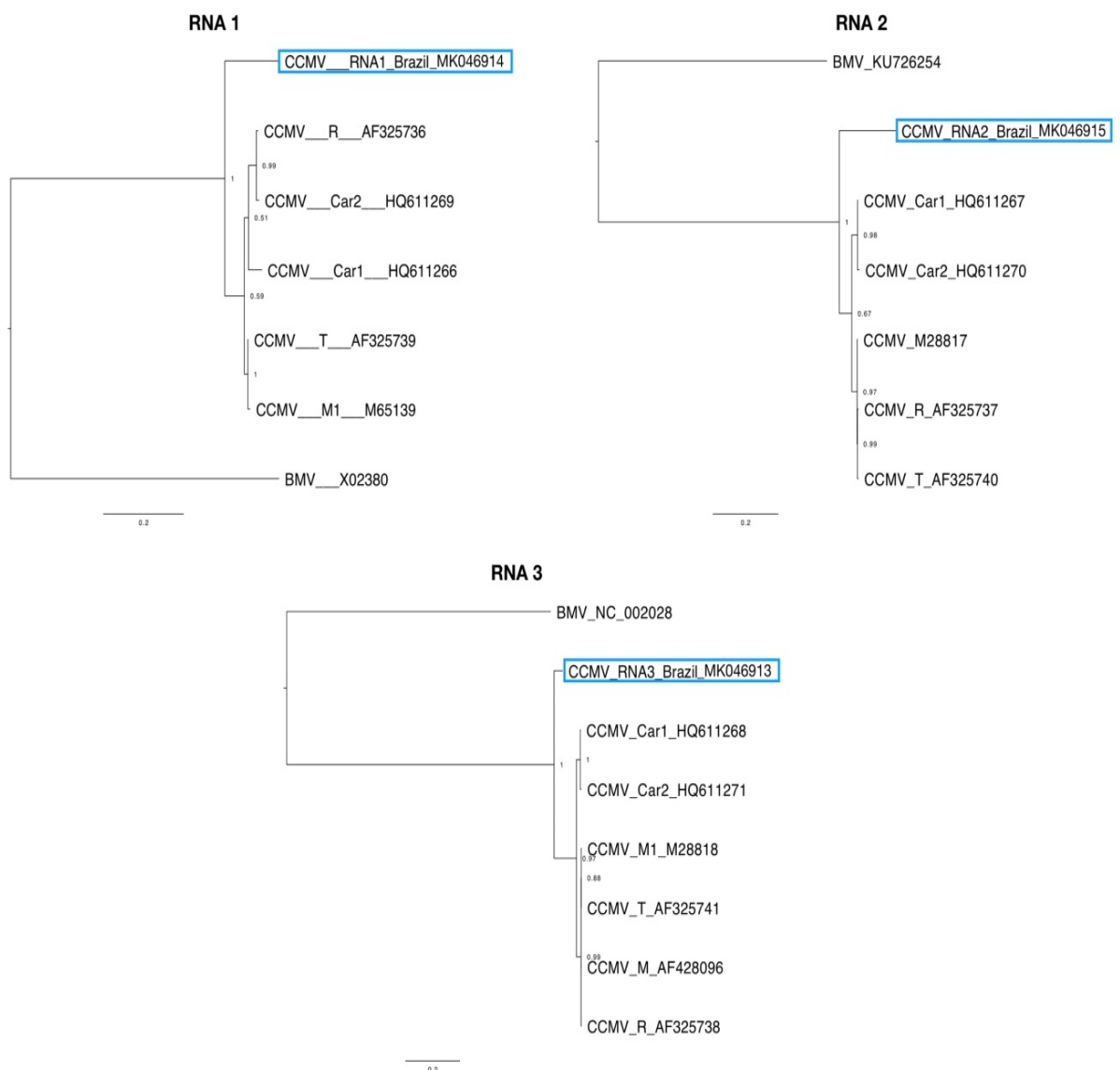


Fig. 2 Bayesian phylogenetic tree of the three complete RNA's genomes from the *Cowpea chlorotic mottle virus* isolate found in Brazil. The Brazilian CCMV isolate from *A. pintoi* clustered into a different sub-clade from all the others CCMV isolates available on GenBank for all the three RNA's;

A partial purification of the virus was performed using leaf samples from the two CCMV-infected genotypes according to Cali and Moyer [10]. The viral RNA was extracted, followed by visualization on agarose gel (1%), and three genomic RNA bands of approximately 3.1 kb, 2.7 kb and 2.1 kb, typical of the genus *Bromovirus*, could be verified [9] (data not shown). The partially purified viral particles were also prepared for transmission electron microscopy after being negatively stained with uranyl acetate according to Kitajima and Nome [11]. For the visualization of cytopathic effects on CCMV-infected *A. pinto* plants, small fragments of symptomatic leaves (1 mm x 3 mm) were fixed in modified Karnovsky fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2), postfixed with 1% OsO₄, dehydrated in acetone, infiltrated and embedded in Spurr low-viscosity resin. Sections were obtained with a Leica Ultracut U6 ultramicrotome equipped with a Diatome diamond knife, stained with uranyl acetate and Reynold's lead citrate, and examined with a JEOL JEM 1011 transmission electron microscope. The images were recorded digitally [11]. Isometric particles ca. 30 nm, some of them empty, were present in the negatively stained partially purified preparation of CCMV. Infected parenchymal cells showed chloroplasts with a lamellar system in different degrees of disorganization and the presence of starch grains. Putative isometric CCMV virions, ca. 25 nm in diameter, could be visualized at high concentrations in the cytoplasm and vacuole (Fig. 3), but filamentous inclusions as described in CCMV infected bean leaf cells [12] could not be seen.

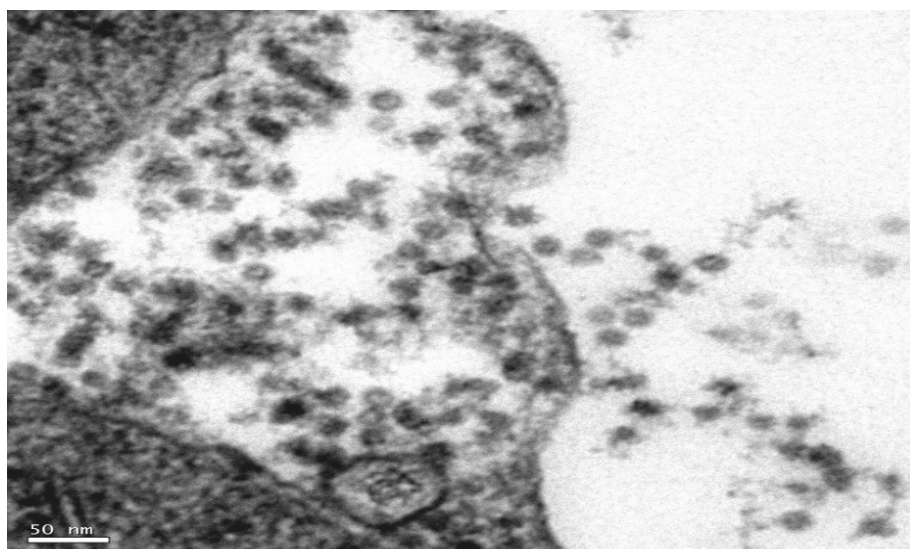


Fig. 3 Transmission electron microscopy of thin section of a parenchymal cell from symptomatic leaf of *Arachis pinto*. Putative *Cowpea chlorotic mottle virus* particles are present in high concentration in the vacuole and cytoplasm

To determine the partial host range of the virus, extracts of CCMV-infected *A. pinto* leaves were first used as inoculum source for sap transmission of the virus to *Chenopodium quinoa* plants, that developed local chlorotic lesions. Monolesional lesions were then used to inoculate *Vigna unguiculata* cv. 'Pretinha'. Leaf extracts were prepared in 0.01 M potassium phosphate buffer, pH 7.0, and 0.1% disodium sulfite at a ratio of 1:10 (w / v). Prior to inoculation, the leaves of the test-plants were sprinkled with a small amount of the 600 mesh abrasive carborundum. Extracts of CCMV-infected leaves of *V. unguiculata* cv. Pretinha were used as an inoculum source to inoculate plants of *C. quinoa*, *C. amaranticolor*, *Vigna unguiculata* sg. *sesquipedalis* cv. De Metro, *Nicotiana occidentalis*, *N. benthamiana* and *A. pinto* cv. BRS Mandobi. Plants of each species had five inoculated replicates and negative controls. Subsequently, plants were kept in a greenhouse for symptom observation. Different types of symptoms could be observed in all thirty plants inoculated. Systemic mosaic was observed in *V. unguiculata* L. cv 'Pretinha' and *V. unguiculata* sg. *sesquipedalis* cv. 'De Metro' plants. In *N. occidentalis* and *N. benthamiana*, chlorotic patches were observed on the leaves; local chlorotic lesions appeared on *C. quinoa* and *C. amaranticolor* (Fig 4). The *A. pinto* cv. 'BRS Mandobi' showed mosaic symptoms. According to Annamalai et al. [12], CCMV causes severe mosaic symptoms and interveinal chlorosis in common peanut, which were also observed in inoculated plants of *A. pinto* cv. 'BRS Mandobi' in this study.



Fig. 4 Symptoms caused by the Cowpea chlorotic mottle virus – CCMV from *Arachis pinto* in mechanically inoculated: 1- *Chenopodium quinoa*; 2- *Nicotiana occidentalis*; 3,4- *Vigna unguiculata* cv. Pretinha

To the best of our knowledge, this is the first report of the *Cowpea chlorotic mottle virus* (CCMV) infecting forage peanut globally and the first report of this virus species in Brazil. Further studies are in progress to determine the possible chrysomelid beetle vector(s) under Brazilian conditions.

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Author contributions

Study conception and design: KFC. Pantoja. Acquisition of data: KFC. Pantoja, AJ. Boari, BR. De Marchi, EW. Kitajima, RC. Gonçalves, GML. Assis, R. Krause-Sakate. Analysis and interpretation of data: KFC. Pantoja, AJ. Boari, BR. De Marchi, T. Mituti, EW. Kitajima, JAM. Rezende, R. Krause-Sakate. Drafting of manuscript: KFC. Pantoja, AJ. Boari, BR. De Marchi, T. Mituti, EW. Kitajima, JAM. Rezende, R. Krause-Sakate, GML. Assis, RC. Gonçalves. Critical revision: KFC. Pantoja, AJ. Boari, BR. De Marchi, T. Mituti, EW. Kitajima, JAM. Rezende, R. Krause-Sakate, GML. Assis, RC. Gonçalves.

Compliance with ethical standards

Conflict of Interest

All authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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CAPÍTULO 2

Characterization and complete genome sequence of the *Peanut mottle virus* and *Cucumber mosaic virus* in forage peanut (*Arachis pintoï*) in Brazil

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Abstract

The forage peanut (*Arachis pintoï*) is mainly used as soil coverage crop. A Next Generation Sequencing analysis was performed in *A. pintoï* accessions and revealed the presence of *Peanut mottle virus* and *Cucumber mosaic virus*. To our knowledge, this is the first report of CMV in *A. pintoï* and the complete genome sequence of a PeMoV isolate in *A.pintoï* from Brazil.

Keywords Forage peanut, virus, NGS

Arachis pintoï (Krapov & W.C. Gregory), commonly known as forage peanut, is a tropical legume that belongs to the Fabaceae family and is used for distinct purposes such as forage, soil coverage in fruit orchards, forests and no-tillage system, erosion control and as an ornamental plant (Carvalho e Quesenberry 2009). The forage peanut has been used as a forage crop in Brazil, Australia, Colombia and Bolivia due its high nutritive value and excellent animal production in grazing management (Palmieri et al., 2012). It is a vegetative propagated plant that particularly allows viruses to accumulate over successive generations. In Brazil, the species *Peanut mottle virus* - PeMoV (Anjos et al. 1998) and *Cowpea mild mottle virus* were previous reported infecting *A. pintoï* (Mituti et al., 2005).

The Active Forage Peanut Germplasm Bank located in Embrapa Acre contains several genotypes of *A. pintoï*, some of them with typical virus symptoms. In order to detect new potential plant viruses in these accessions, a Next Generation

Sequencing analysis was carried out. The total RNA extraction of twenty-two genotypes was performed with the RNA Viral PureLink Kit (Invitrogen™) followed by library preparation and transcriptome sequencing using the Illumina HiSeq2500 platform. Subsequently, the 27,674,874 reads were trimmed and *de novo* assembled using the CLC Genomics Workbench v.7.0.3 software. The contigs obtained were submitted to a BLASTn search using the Geneious v.9.1.5 software. The BLASTn results revealed contigs showing high identities with the Cucumber mosaic virus - CMV, RNA 1 (96% identity - KT921314), RNA2 (90% - JN054637) and RNA3 (97% - JN054635) as well as a contig with high identity with PeMoV (99.2% – MH270528).

We obtained in the analyses the complete genome sequence of the PeMoV with a 9,680bp sequence length (GenBank MK396065) and the draft genome of the CMV was obtained with a 3,339bp, 2,918bp and 2,207bp sequence length for fragments 1 (GenBank - MK387172), 2 (GenBank - MK387173) and 3 (GenBank - MK387174), respectively. Subsequently, a phylogenetic analysis of the PeMoV complete genome (Figure 1) was performed, using the software Mr. Bayes (v.3.2.2), with other complete genomes and revealed little diversity among the PeMoV isolates from different localities in the world. The pairwise identity among the complete genome sequence of different isolates ranged between 95.7 and 99.2%. The Brazilian isolate obtained in this study was closely related to a Chinese isolate (MH270528), presenting 99.2% of identity. In Brazil, the complete genomic sequence of PeMoV was only reported before in a different host, *Arachis hipogaea* L. (KY350138) (Reis, 2016). The pairwise identity between both Brazilian isolates was also high (97.4%).

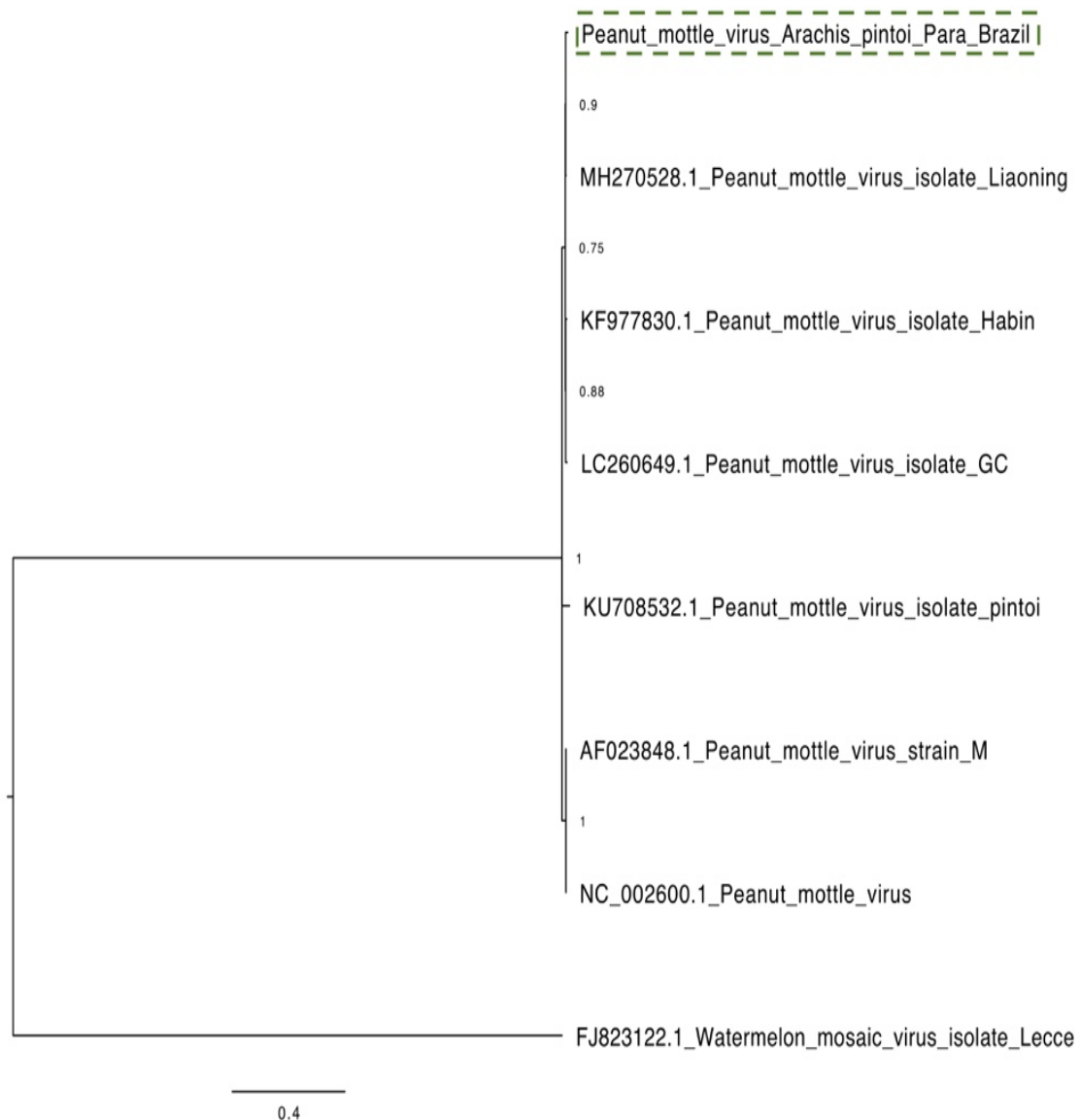


Fig.1 Phylogenetic tree from the complete nucleotide sequence for the Brazilian isolate of *Peanut mottle virus* (PeMoV) compared with other PeMoV isolates from different localities in the world. The *Potyvirus*, Watermelon mosaic virus was used as an outgroup.

Regarding the CMV, based on the phylogenetic analysis and the comparison between the region encoding the coat protein and the draft genome obtained in this study, the Brazilian CMV isolate was classified as belonging to the IB subgroup. Source of sequences are shown Table 1.

Table 1. Accession numbers and country of origin of the sequence data used in the phylogenetic analysis of the *Cucumber mosaic virus* (CMV).

Strain	Gen Bank Accessions			Origin (State)
	RNA1	RNA 2	RNA 3	
Fny	D00356	D00355	D10538	United States (N.Y.)
IA	AB042292	AB042293	AB042294	Indonesia
Ix	U20220	U20218	U20219	Philippines
Leg	D16403	D16406	D16405	Japan
LS	AF416899	AF416900	AF127976	United States (N.Y.)
Ly	AF198101	AF198102	AF198103	Australia
Mf	AJ276479	AJ276480	AJ276481	South Korea
Nt9	D28778	D28779	D28780	Taiwan
O	-	D10209	D00385	Japan
Q	X02733	X00985	M21464	Australia
S	Y10884	Y10885	U37227/AF063610	South Africa
SD	AF071551	D86330	AB008777	China
Tfn	Y16924	Y16925	Y16926	Italy
Trk7	AJ007933	AJ007934	L15336	Hungary
Y	D12537	D12538	D12499	Japan
ER-PSV	U15728	U15729	U15730	United States (Ky.)

The draft nucleotide sequence of the segments RNA1, RNA2 and RNA3 of the CMV revealed a 99% nucleotide sequence identity with an CMV African isolated from cowpea beans (GenBank – FJ8961591) (Figure 2, 3 ,4). There is a prevalence of the CMV subgroup I in Brazil and most isolates are closely related to the subgroup IA. CMV subgroup IB was only detected before in Brazil once, in a pepper plant at Minas Gerais State (Eiras et al., 2004).

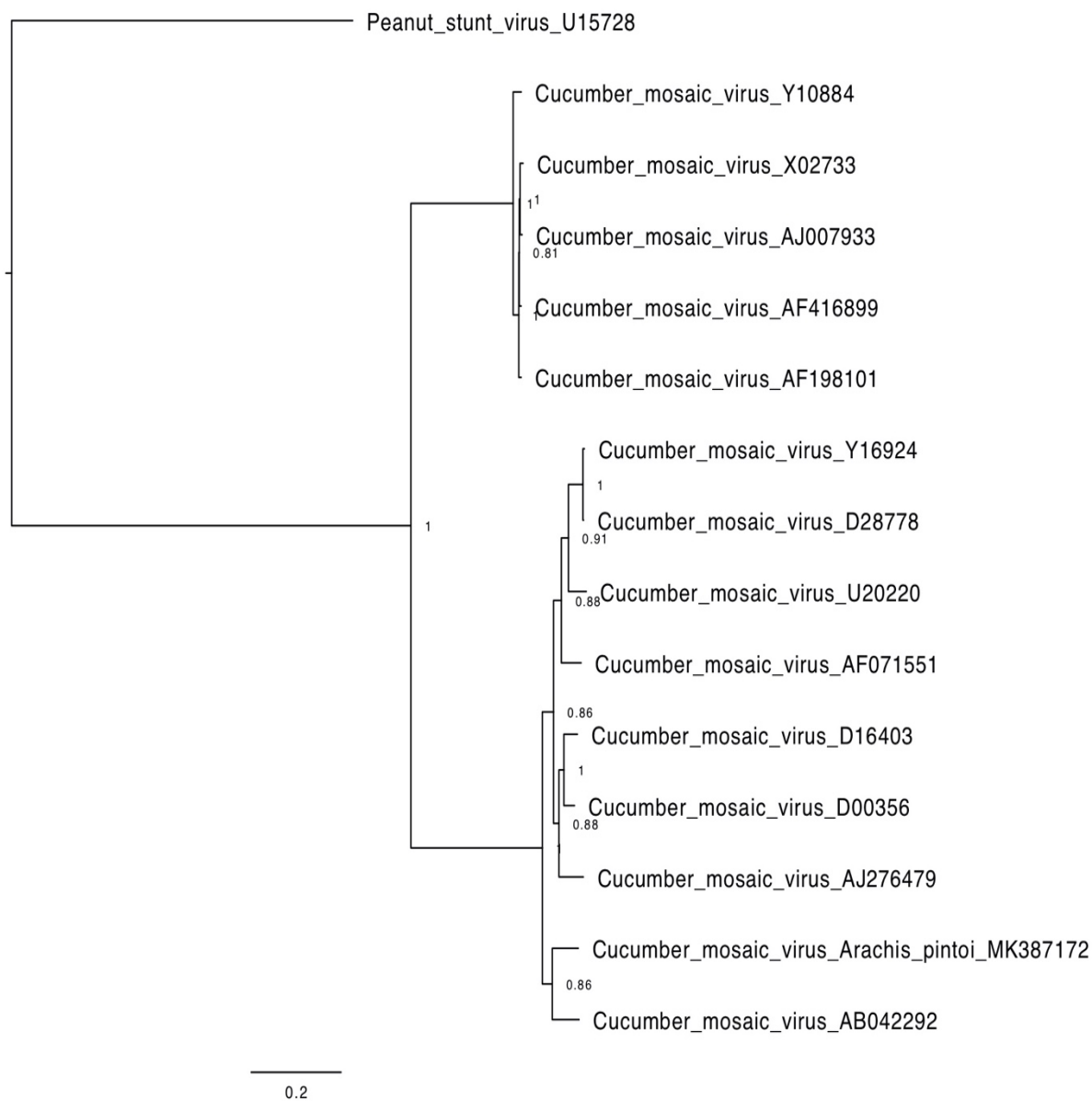


Fig.2 Phylogenetic tree of the RNA1 partial genome nucleotide sequences of the *Cucumber mosaic virus* (CMV) subgroup IB isolate found in forage peanut in Brazil.

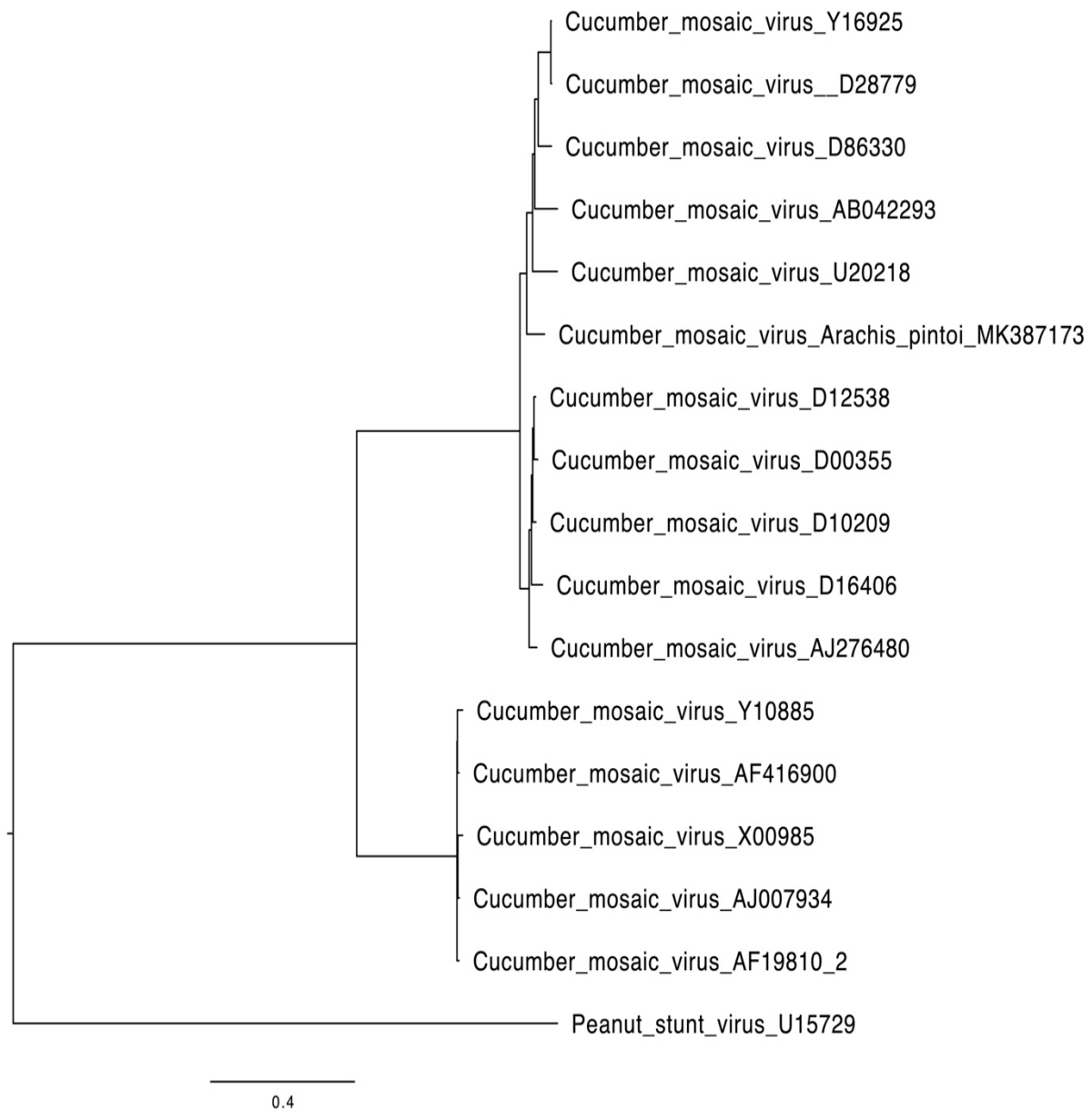


Fig.3 Phylogenetic tree of the RNA2 partial genome nucleotide sequences of the *Cucumber mosaic virus* (CMV) subgroup IB isolate found in forage peanut in Brazil.

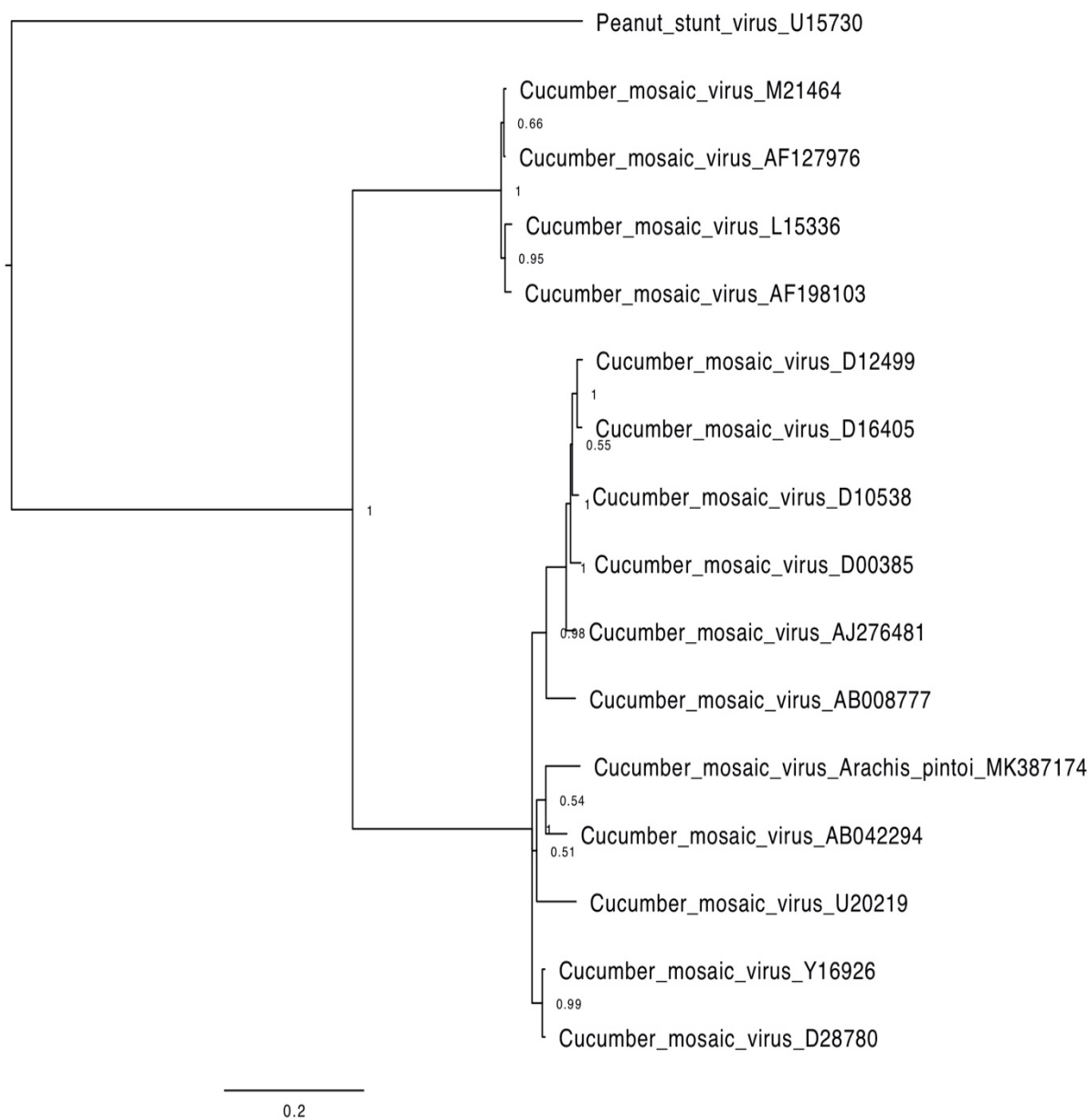


Fig.4 Phylogenetic tree of the RNA 3 partial genome nucleotide sequences of the *Cucurbit mosaic virus* (CMV) subgroup IB isolate found in forage peanut in Brazil.

Afterward, to detect the presence of the viruses in the genotypes, a RT-PCR followed by Sanger sequencing was carried out using specific primers for PeMoV: 8607F (5' GTT GAG ATT GAC GCA GCA GC 3') and V9342R (5' GGG TTC CGA CGT TAC CAT CA 3'); and for CMV: CMVF (5' AAG AAG CTT GTT TCG CGC AT 3') and CMVR (5' CAG ATG TGG GAA TGC GTT GG 3'). It was confirmed the presence of PeMoV in two genotypes of *A. pintoi* (BRA cv. BRS Mandobi and BRA042269). The CMV was also confirmed another two *A. pintoi* genotypes: Crossing *A. pintoi* x *A. pintoi* (C15) and *A. pintoi* x *A. pintoi* (E3).

Both isolates were then submitted to an electron microscopy analysis. Small leaf fragments and the partially purified viral particles were negatively stained with uranyl acetate according to Kitajima and Nome (1999). Flexuous filamentous particles (approximately 740 × 13 nm) typical of potyviruses were observed by electron microscopy on leaves of *A. pintoi* genotype BRA cv. BRS Mandobi (Figure 5). In addition, partially purified viral particles from Hybrid H1 genotype leaves presented typical CMV isometric particles (Figure 6).

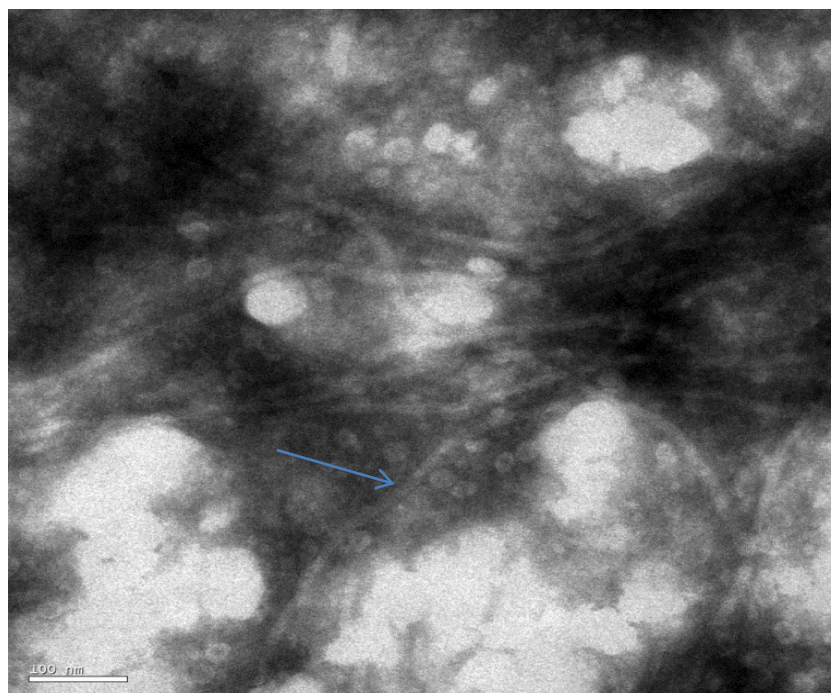


Fig. 5 - Electron micrograph of partially purified forage peanut virus infected with *Peanut mottle virus* (PeMoV).

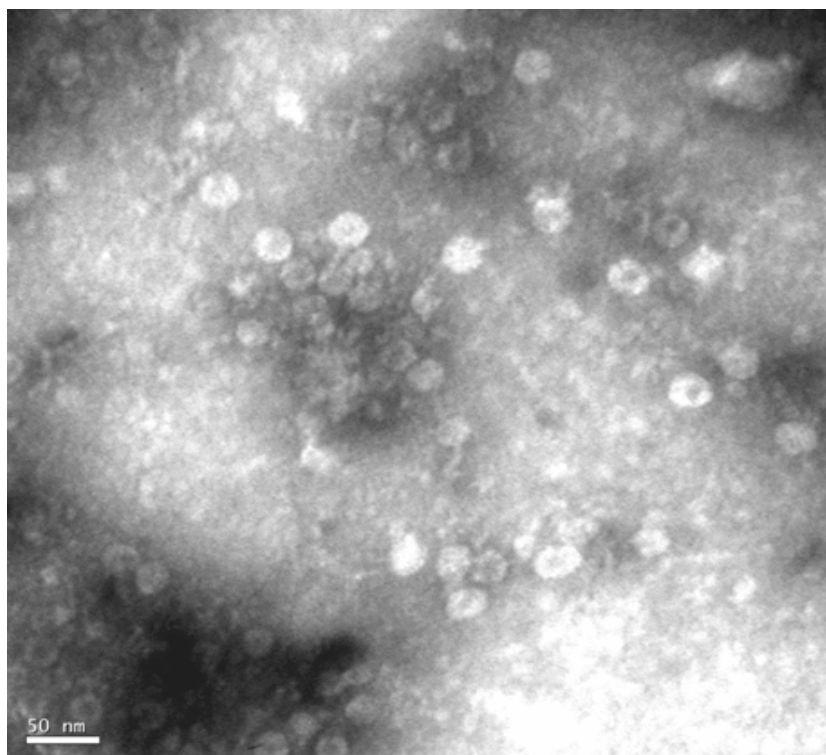


Fig. 6 Transmission electron micrograph of forage peanut leaf cell sections infected with *Cucumber mosaic virus*

Finally, we determined the partial host range of the virus by mechanical transmission to different indicator plants. Firstly, monolesional lesions were obtained using extracts of PeMoV infected *A. pintoi* leaves (separate plants) as inoculum source for sap transmission to *Chenopodium quinoa* plants. PeMoV caused local damage in *C. quinoa* and was purified separately. The lesions were then used for a sap transmission to *Vigna unguiculata* cv. 'Pretinha' plants. Subsequently, extracts of PeMoV infected leaves from *V. unguiculata* cv. Pretinha were separately used as inoculum source to inoculate leaves of the following host plants: *C. quinoa*, *Nicotiana tabacum*, *N. occidentalis*, *N. benthamiana*, *V. unguiculata* cv "pretinha" and *A. pintoi* cv. BRS Mandobi.

For the CMV transmission test, *A. pintoi* x *A. pintoi* (E3) leaf extracts were used as a source of inoculum for sap transmission to *N. tabacum*, *N. occidentalis*, *N. benthamiana*, *Curcubita pepo* L., *V. unguiculata* cv "pretinha" and *A. pintoi* cv. BRS Mandobi. Five of each plant species were inoculated and kept in the greenhouse for symptoms observation. The presence of the virus was confirmed by RT-PCR only in *Curcubita pepo* L.

The PeMoV isolate from *A. pintoi* was successfully sap-transmitted. *Chenopodium quinoa* presented symptoms of local lesion; *Nicotiana tabacum*, *N.*

occidentalis, *N. benthamiana* and *V. unguiculata* cv pretinha showed mottling symptoms, in *A. pinto* cv mandobi chlorotic spot symptoms were observed (Figure 7). The CMV subgroup IB, the plants inoculated with CMV subgroup IB isolate showed symptoms of mosaic in *C. pepo* L plants, and chlorosis in *A. pinto* cv mandobi plants (Figure 8).

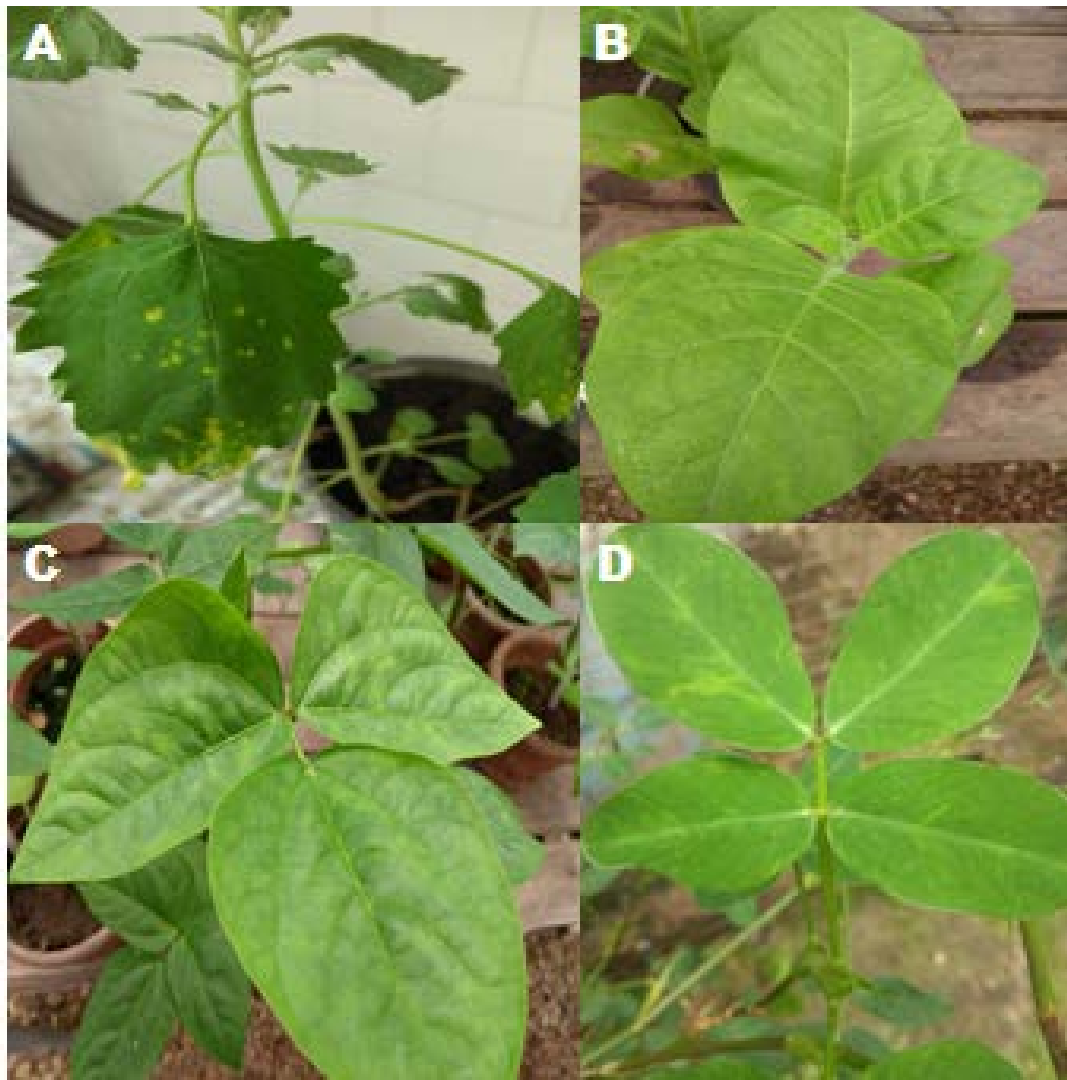


Fig.7 Symptoms caused by the *Peanut mottle virus*: A-Local lesion in *Chenopodium quinoa*; B- mottling in *Nicotiana tabacum* and C- *Vigna unguiculata* cv. 'pretinha' and D- chlorotic spot in *Arachis pinto* cv. Mandobi plants

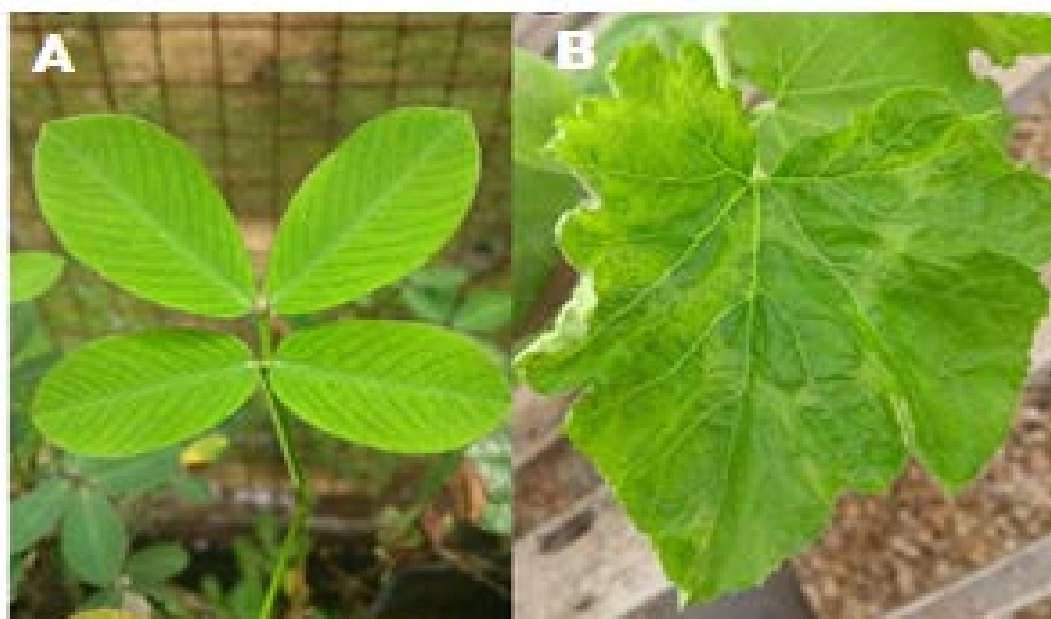


Fig.8 Symptoms caused by the *Cucumber mosaic virus* subgroup IB: A- Chlorosis *Arachis pinto* cv. Mandobi, B- Mosaic in *Curcubita pepo* L. plants

The complete genome sequence of PeMoV in *A. pinto* from Brazil was obtained and can serve as a valuable reference sequence for further studies of this virus. Additionally, this is the first report of infection of *A. pinto* with CMV, which might bring new concerns for the forage peanut crop, as the impacts of this virus are still unknown. More studies are necessary to evaluate the incidence of these viruses in forage peanut cultivation.

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CAPÍTULO 3

Identification and characterization of a new member of the *Potyviridae* family infecting *Arachis pintoi*

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Abstract

The complete nucleotide sequence of a new member of the family *Potyviridae* was reported in forage peanut plants (*Arachis pintoi*), in Brazil, exhibiting typical virus symptoms, which we propose to name as *Arachis pintoi* mottle virus (ArPMV). The ArPMV positive-sense RNA genome is 9,213 nucleotides long and encodes a polyprotein with 2,947 amino acids that is predicted to be cleaved into 11 mature proteins. The complete ArPMV single ORF shares 47% and 34% nucleotide and amino acid identity, respectively, with the closest related isolate, Soybean yellow shot virus. The microscopic analysis revealed elongated viral particles typical of those found in plant cells infected with virus of the *Potyviridae* family.

Keywords: Forage peanut, NGS, *Arachis pinto* mottle virus, ArPMV

The forage peanut, *Arachis pinto* (Krapov. & W.C. Gregory) Fabaceae, is a native legume from Brazil, whose main dispersal area is the central region of the country (PURCINO et al., 2004). It is indicated for pasture formation, as it has high grazing resistance, high protein content and high potential to fix atmospheric nitrogen (MIRANDA et al., 2003). Some diseases, such as viruses, may occur in forage peanut and cause a reduction in leaf mass production (GONÇALVES et al., 2014). So far, few viruses have been reported in the world in the forage peanut crop. Peanut mottle virus (ANJOS et al., 1998) and cowpea mild mottle virus (MITUTI et al., 2005) have been reported in Brazil. In Colombia, peanut mottle virus, turnip yellows virus, *Arachis pinto* virus A and *Arachis pinto* virus B have been reported (SÁNCHEZ et al., 2016). The *Potyviridae* family is one of the most important families of plant viruses, with a wide range of hosts encompassing dicotyledonous and monocotyledonous plant species. The family is, composed of 12 genera and 228 species (ICTV, 2020). The 12 genera are differentiated by biological criteria, mainly transmission by specific vectors, and by molecular data (ADAMS et al., 2005).

The Active Germplasm Bank (BGA) of forage peanut at Embrapa Acre (Rio Branco, – AC, Brazil) includes several accessions of *A. pinto*. Characteristic virus symptoms such as mosaic, chlorosis, leaf mottle and leaf necrosis have been observed in numerous accessions of the BAG (GONÇALVES et al., 2014).

A total of 22 symptomatic and asymptomatic *A. pinto* accessions, including intra and interspecific hybrids (*A. pinto* x *A. repens*), was analysed in this study. Firstly, symptomatic leaves were examined by electron microscopy. To visualize the viral particles, electron microscopy was performed, using the purified virus. A drop of the

purified virus suspension was placed on a piece of "parafilm". A transmission electron microscopy screen was placed on the drop. After a period of 3 to 5 minutes, the screen was removed and washed with distilled water, three times in a row. And later, the contrast was made with a solution of 1% uranyl acetate, for 2 to 3 minutes.

Biological purification of the virus was carried out by three consecutive single lesion passages in *Chenopodium quinoa*, followed by sap-inoculation into plants of *Vigna unguiculata* cv. Pretinha for viral multiplication. The plant extract was inoculated into differentiating hosts including *A. pinto* cv Mandobi, *Arachis hypogaea* cv Runner, *V. unguiculata* cv 'Black', *Nicotiana occidentalis*, *N. benthamiana*, *N. tabacum*, *N. glutinosa*, *C. quinoa* and *C. amaranticolor*. Five plants of each indicator species were used and kept under greenhouse conditions to observe symptoms for up to 30 days. As a control, healthy plants of each tested species were kept for comparison.

Total nucleic acid from leaves of the 22 forage peanut genotypes was extracted using the Purelink Viral RNA/DNA mini kit (Invitrogen). The total RNA was sent, as a pooled sample, to the laboratory of the ESALQ-USP Department of Functional Genomics Center (LZT), Piracicaba-SP, Brazil for sequencing using the Illumina HiSeq2500 platform. The reads obtained were first trimmed, for removal of adapters and low-quality reads, and *de novo* assembled using the CLC Genomics Workbench v7.0.3 software. The obtained contigs were subjected to a tBLASTx (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) search using Geneious v9.1.5. For discovery of new viruses, the contigs were extended and *de novo* assembled repeatedly until all reads of the library were mapped, following the pipeline described by (BLAWID et al., 2017). The complete genome of a putative new virus species, which we proposed to name as *Arachis pinto* mottle virus (ArPMV), was obtained. The ArPMV complete ORF shares 47% and 34% nucleotide and amino acid identity, respectively, with the

closest related virus, the recently described potyvirus soybean yellow shoot virus (SoyYSV, MH890547) (FIGUEIRA et al., 2019).

To identify which forage peanut genotypes were infected with ArPMV, RT-PCR was performed with the oligonucleotides pairs ArPMV-8142For (5'-ACAACGGAATCAGAGGCAACA-3') and ArPMV-8841Rev (5'-TACTTCGCAAGCGTCTGGTT-3'). The primers were designed with the Primer3 software (UNTERGASSER et al., 2012) using the NGS-derived viral genome as a reference. In addition, the complete genome was amplified by RT-PCR, using nine newly designed pairs of oligonucleotides (Table 1) and completely sequenced using the Sanger method.

Table 1. Oligonucleotides used for Sanger sequencing to obtain the complete genome of the Arachis pintoi mottle virus.

Pair #	Oligonucleotides	Sequence	Fragment Length	Melting Temperature (°C)
1	145 F	ACTTCAGGAATGGCAGCTGT	1108 bp	
	1252 R	CGCCAACAACGTGTGGATTT		
2	1139 F	TACCTTACACCAAAGAGTTACATGA	956 bp	
	2094 R	GTAGTCAAGCATACTTCCTGTTGT		
3	1995 F	TGGAAGTAGAACAGTTGGACTACA	956 bp	
	2950 R	TCCTATGTGCTTTAAATGCAGTTAGA		
4	2856 F	TCCAAATCCAATGAGTGCTTACA	966 bp	
	3822 R	TCTTAATCCCAGTGTATGACTAGTT		
5	3728 F	CGTATCTTGTTAACAACCCAGC	975 bp	
	4702 R	GAACATTATTGTCTAAATGCCTGTTT		
6	4601 F	TCAAGCTCAAAGAGGCAACCT	998 bp	
	5599 R	TTTCACCTGTTAATTCGTTTATCACT		
7	5516 F	AGAAACAACCATTTCATGACTTTTTATG	1003 bp	
	6518 R	CGTTGAAATTTTGTACAGAACCA		
8	6443 F	CCATTGATGATGAACTGCCAAA	958 bp	
	7400 R	TCAGGTTTTTGATAGTGTAACCTTATG		
9	7199 F	ATCACCAGTTTTATACACTAAATTGCA	1009 bp	
	8207 R	ACCTCAATTCTTTCGTTCTTTTCA		
10	8682 F	CTCGAGTGCAGCTATGTCGT		
11	OligodT17M10	TCTGCCTTGATAACACTGCTTTTTTTTTTTTTTTTTT		

The PCR cycle consisted of 40 cycles of 94°C for 3 min, 50°C to 54°C (according to each primer) for 30 seg and 72°C for 30 seg plus a final extension at 72°C for 3 min. The amplification products were observed and photographed under UV light after the electrophoretic run on agarose gel (1.0%) and GelRed (Biotium) staining purified using the Wizard Genomic DNA Purification Kit (Promega) and sent to Myleus Biotecnologia Ltda for Sanger sequencing. Additionally, the 5' end was amplified and sequenced using the 5' RACE System for Rapid Amplification of cDNA Ends , v. 2.0 (Invitrogen), according to the manufacturer's instructions. The sequence were assembled and mapped using Geneious v. 9.1.5. Phylogenetic analysis was carried out including the genome obtained in this study and 23 virus sequences of the family *Potyviridae* retrieved from GenBank. The tree was generated using the software MEGA 7, with a 2000 repetition bootstrap.

Microscopic analysis revealed elongated viral particles typical of those found in plant cells infected with viruses of the *Potyviridae* family (Figure 1). The presence of the ArPMV was confirmed by RT-PCR in seven forage peanut genotypes (cv. Alqueire-1, BRA 039187, BRA 014982, BRA 030601, cv. BRS Mandobi, BRA 032972, BRA 042269). The ArPMV induced symptoms of mottled leaves in *A. pinto* cv. Alqueire-1 (Figure 2).

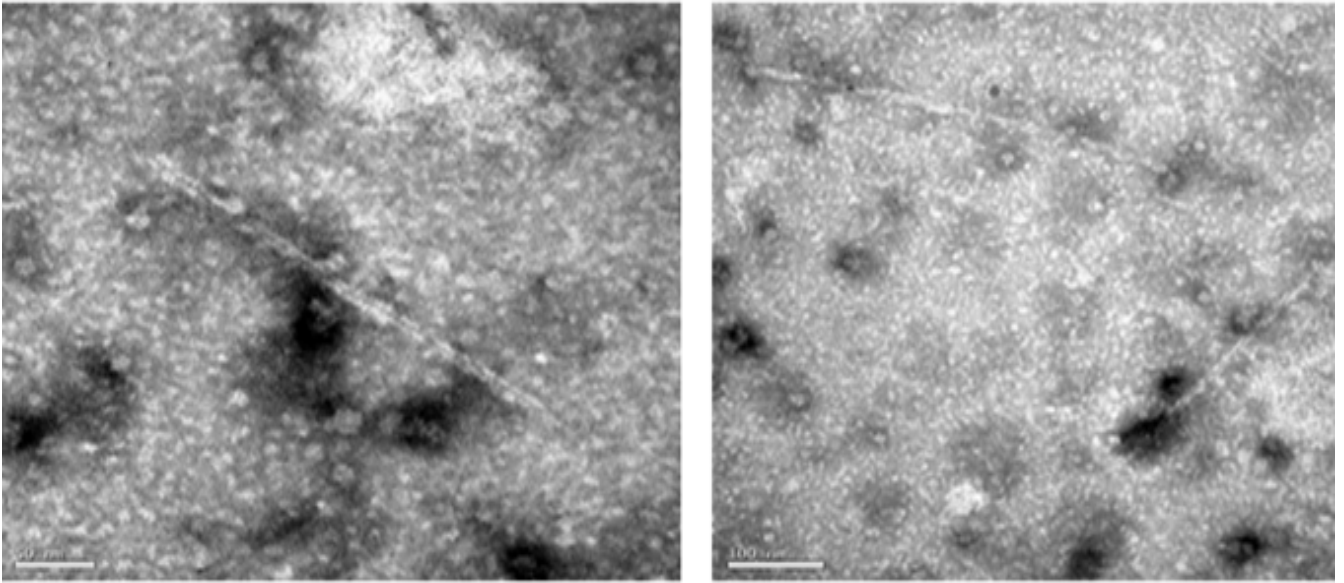


Fig. 1- Electron micrograph of partially purified forage peanut virus with elongated enveloped particles typical of the *Potyviridae* family. Bar scale: 100nm



Fig. 2- Leaf mottle caused by the putative new member of the family *Potyviridae* in its natural host, the forage peanut *Arachis pintoi* cv. Alqueire-1.

Additionally, different symptoms were observed for each plant host among the tested host range. In *C. quinoa*, the virus induced the appearance of local chlorotic lesions which later became necrotic; in *C. amaranticolor*, local chlorotic lesions were observed; *N. occidentalis* and *N. benthamiana* were mottled and *V. unguiculata* L. cv. “Pretinha” spotted and necrotic local lesions were observed (Figure 3).

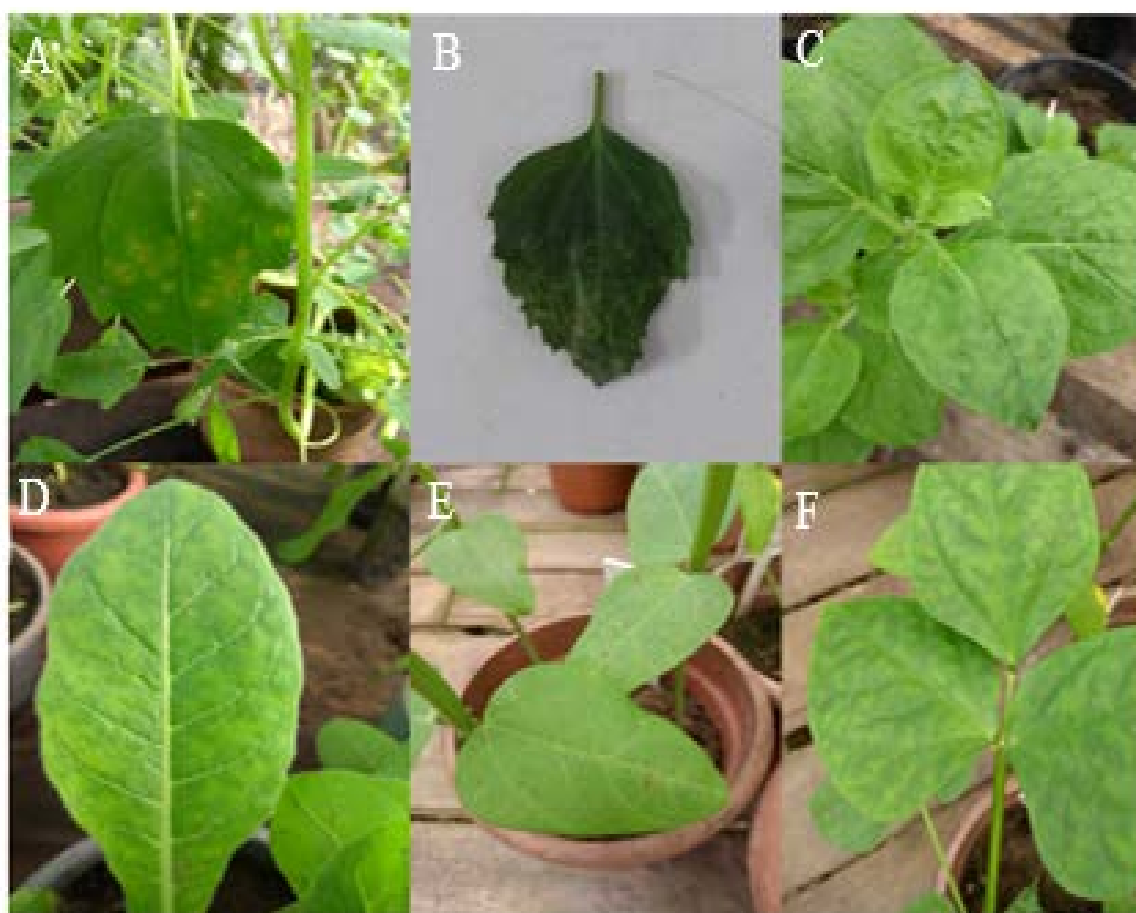


Fig. 3- Symptoms caused by the putative new member of the family *Potyviridae* in the indicator plants: A- *Chenopodium quinoa*; B- *Chenopodium amaranticolor*; C- *Nicotiana benthamiana*; D- *Nicotiana occidentalis*; E and F- *Vigna unguiculata* cv. Pretinha.

The RT-PCR detected the presence of the virus in the indicator plants. The complete *de novo* sequence of the viral genome was obtained by Sanger sequencing using oligonucleotides designed based on the sequence derived from the NGS. There was a 97.7% nucleotide identity between the nucleotide sequences of the NGS-derived

and the Sanger-derived genomes. The complete genome sequence of ArPMV is 9,213-nt in length, excluding the poly (A) tail at its 3' end (Figure 4).

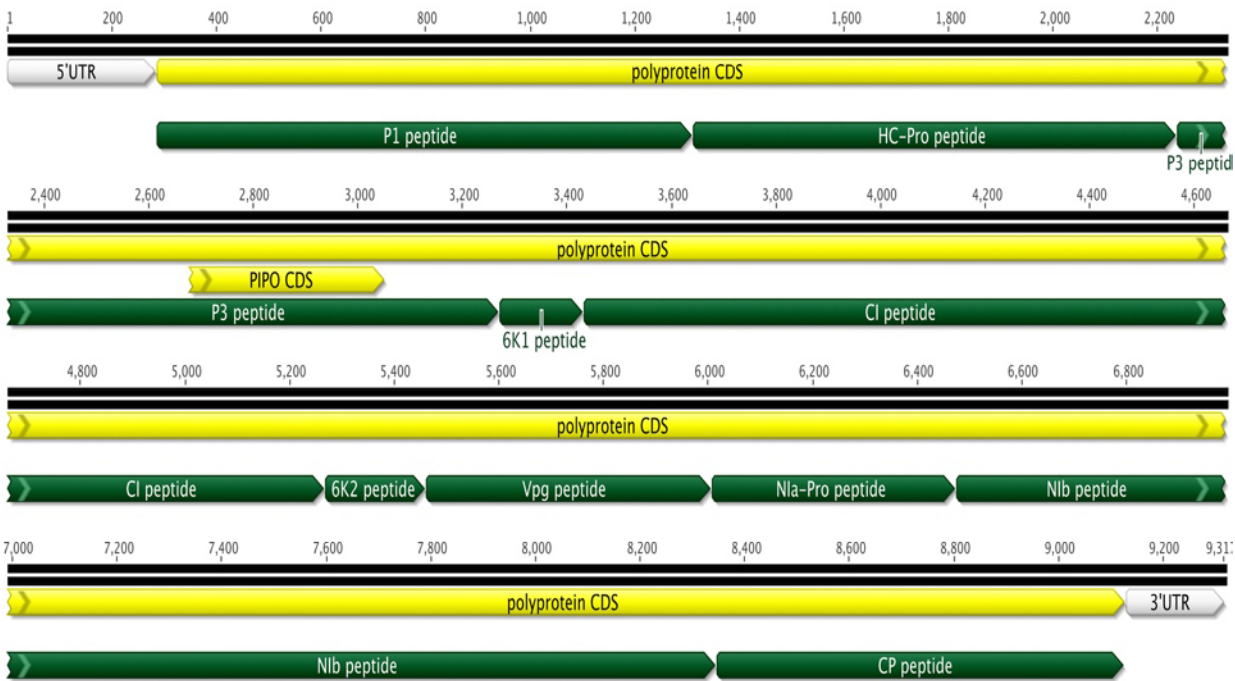


Fig. 4- Schematic representation of the genomic organization of the new member of the Potyviridae family in forage peanut. Geneious Software v. 9.1

The genome was submitted to the GenBank database under the accession number (MN764361). The 5' and 3' UTRs are 286 and 83 nt in length, respectively, and have an open reading frame (ORF) of 8,844 nt encoding a putative 2,947 amino acid polyprotein. The polyprotein is cleaved into 11 mature proteins, namely P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-pro, Nib, PIPO and CP (Table 2). In total, nine cleavage sites and three proteinases were identified: P1 Proteinase (P1 / HCPro), HC Proteinase (HC-Pro / P3) and NIa Proteinase (P3 / 6K1, 6K1 / CI, CI / 6K2, 6K2 / VPg, NIa-Pro / Nib and Nib / CP).

Table 2. Location of the putative cleavage sites within the *Arachis pintoi* mottle virus polyprotein.

Protein	Size kDa	pI	Polyprotein cleavage sites 5' -3'	
			aa sequence (-4/+1)	Amino Acid Position
P1 peptide	38	8.84	CDYY/S	1 – 342
HC-Pro peptide	34	6.74	YTVG/G	343 – 650
P3 peptide	39	8.86	NFQS/R	651 – 995
6K1 peptide	6	5.71	SDEE/G	996 – 1,049
CI peptide	68	7.90	MISS/S	1,050 – 1,661
6K2 peptide	7	5.6	QILD/F	1,662 – 1,725
Vpg peptide	21	7.87	FSFE/A	1,726 – 1,907
Nia-Pro peptide	17	9.53	LIAI/T	1,908 – 2,063
NIb peptide	71	6.60	EVPE/F	2,064 – 2,687
CP peptide	29	7.27	-	2,688 – 2,947
PIPO	14	10.11	ELFE/K	798 - 922
Full ORF	334		-	1 - 2,947

The phylogenetic tree grouped ArPMV in the same sub-clade of the Brazilian unassigned member of the family *Potyviridae*, Soybean yellow shoot virus (SoyYSV) (MH890547) (FIGUEIRA et al., 2019). The brambyvirus, Blackberry virus Y (BVY) (SUSAIMUTHU et al., 2008) was also grouped into a close clade (Figure 5). The brambyvirus, BVY has been identified in several blackberry plants in the southern and south-eastern United States and is assumed to be transmitted by an unidentified air vector (SUSAIMUTHU et al., 2008). SoyYSV also infects species of the *Amaranthaceae*, *Caricaceae*, *Fabaceae* and *Solanaceae* families and is transmitted by *Myzus persicae* and *Aphis gossypii* (FIGUEIRA et al., 2019). The genome analysis revealed more similarities between ArPMV and SoyYSV; and differences with the brambyvirus BVY. The ArPMV complete ORF shares only 41.1% nucleotide identity and 33.4% amino acid identity with BVY (NC 008558). BVY encodes a P1 more than 740- amino acid long in contrast to ArPMV (342- amino acid long) and SoySV (300- amino acid long). In addition, ArPMV as well as SoyYSV lack the alkB motif that is present in BVY (FIGUEIRA et al.2019). Other motifs, such as PTK and KMTC, which are associated with aphid transmission (REVERS et al. 2015) were not found in ArPMV.

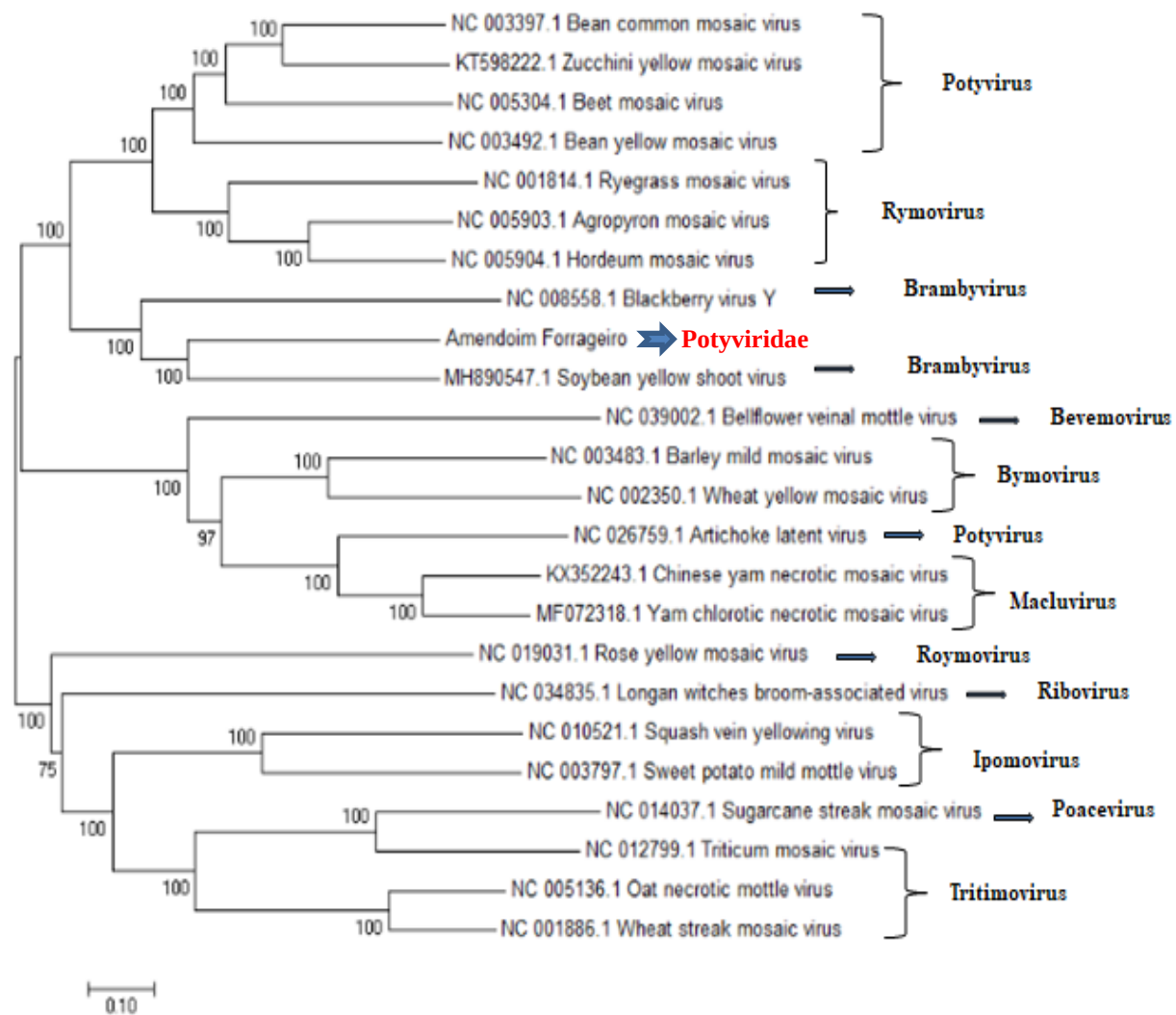


Fig. 5- Phylogenetic tree using the complete nucleotide sequence of species from each genus of the Potyviridae family and the *Arachis pinto* mottle virus from forage peanut obtained in this study. Genera; Accession number and identification of GenBank isolates. Unassigned: *Soybean yellow shoot virus* (SoyYSV) MH890547, *Brambyvirus*: *Blackberry virus Y* (BVY) - NC_008558; *Bymovirus*: *Barley mild mosaic virus* (BaMMV) - NC_003483, *Wheat yellow mosaic virus* (WYMV) - NC_002350, *Ipomovirus*: *Squash vein yellowing virus* (SqVYV) - NC_010521, *Sweet potato mild mottle virus* (SPMMV) - NC_003797; *Macluravirus*: *Artichoke latent virus isolate* (ArLV) - NC_026759, *Chinese yam necrotic mosaic virus* (ChYNMV) - KX352243, *Yam chlorotic necrotic mosaic virus* (YCNMV) - MF072318; *Poacevirus*: *Sugarcane streak mosaic virus* (SCSMV*) - NC_014037, *Triticum mosaic virus* (TriMV*) - NC_012799; *Potyvirus*: *Bean common mosaic virus* (BCMV) - NC_004047, *Bean yellow mosaic virus* (BYMV) - NC_003492, *Beet mosaic virus* (BtMV) - NC004035, *Zucchini yellow mosaic virus* (ZYMV); *Rymovirus*: *Agropyron mosaic virus* (AgMV) - NC_005903, *Hordeum mosaic virus* (HoMV) - NC_005904, *Ryegrass mosaic virus* (RgMV) - NC_001814; *Tritimovirus*: *Oat necrotic mottle virus* (ONMV) - NC_005136, *Wheat streak mosaic virus* (WSMV) - NC_001886; *Roymovirus*: *Rose yellow mosaic virus* (RoYMV*) - NC019031; *Bevemovirus*: *Bellflower veinal mottle virus* (BVMoV*) - NC_039002, Associated: *Longan witches' broom-associated virus* (LWBaV) - NC_034835. The tree was generated using the software MEGA 7 with a 2000 bootstrap repetition

The current genus demarcation criteria within the *Potyviridae* family is <46% nucleotide identity for the entire open reading frame (ADAMS et al., 2005). According to that, ArPMV should not be assigned as a member of any existing genus and is currently considered an unassigned member of the *Potyviridae* family. The possible presence of ArPMV in forage peanuts and other plant hosts from other areas, and its impacts on crop production, will require further research.

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CAPÍTULO 4

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First report of a putative new pepper vein yellows virus species associated with a vein yellows disease of bonnet pepper plants in Brazil

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The bonnet pepper (*Capsicum chinense*) is among the most important vegetables in Brazil. Although it is grown in all Brazilian States, the production is more intense in the northern states of the country. In June of 2017, around 80 pepper plants cv. Cumari-do-Pará showing symptoms of interveinal yellowing, leaf blade size reduction of 80%, and shortening of the internodes (Figure 1-A) were observed in a small farm (1.0 ha) nearby Altamira county, Pará State. Suspecting that the symptoms might be due to a viral disease, two plants were collected and analysed for virus detection by Next Generation Sequencing (NGS). Initially, symptomatic leaves collected from two infected pepper plants cv. Cumari-do-Pará were used as a pooled sample for partial virus purification (Cali and Moyer 1981) followed by total RNA extraction with the RNeasy Plant Mini Kit (Qiagen). Then, a complementary DNA library was constructed from the RNA using the Complete ScriptSeq Kit (Epicenter, Illumina, Inc.) and submitted for a transcriptome sequencing with the Illumina HiSeq2500 platform. The 27,290,912 raw reads (average length 101 bp) were trimmed, to remove low quality bases and adapters, followed by *de novo* assembly using the CLC Genomics

Workbench software v7.0.3. Subsequently, the 1,693 contigs (ranging from 503 to 12,470 bp) obtained were submitted to a BLASTX search with the Geneious software v9.1.5. against NCBI's viral database, which revealed contig identities higher than 89% with *Pepper vein yellows virus* - PeVYV (*Polerovirus*, *Luteoviridae*). No other viruses were detected in this analysis. The trimmed reads, as well as the contigs, were mapped to a PeVYV reference sequence (AB594828) and the consensus sequence (mean coverage of 53.6x) was extracted to generate a 6,203 bp draft genome of PeVYV – isolate Altamira (MK184554). In addition, total RNA was extracted from leaves collected from two symptomatic pepper plants using the Total RNA Purification Kit (Norgen Biotek Corp.) followed by RT-PCR with the newly designed primers PeVYV 3682F (5' GGT GGA TCA CGT AAC ACC CG 3') and PeVYV 4261R (5' ATT TCG GGT TGT GCA GTT GC 3'), which amplifies a 580 bp amplicon of the ORF 3/4. The expected size amplicon was obtained for one sample and directly sequenced (GenBank Accession MK883752), showing 100% nucleotide identity with the NGS-derived sequence. Afterward, a Bayesian phylogenetic analysis revealed a clustering of the draft genome of PeVYV – Altamira with other four complete genome sequences of other species of the pepper vein yellows virus complex (Figure 1-B). Presently, six species of *Pepper vein yellows virus* (PeVYV 1 - 6) are recognised (ICTV 2018b release) with recent description of PeVYV-7 from Saudi Arabia (Kamran et al. 2017). Moreover, the recent report of Pepper whitefly borne vein yellows virus (PeWBYV), a whitefly transmitted recombinant polerovirus with PeVYVs and African eggplant yellows virus as its parents (Ghosh et al. 2019) makes this complex much interesting. The draft genome of the PeVYV – isolate Altamira was most closely related to PeVYV-1 isolate from Japan (89.72% - AB594828) (Murakami et al. 2011), PeVYV-3 from China (89.54% - KP326573) (Liu

et al. 2016), PeVYV-4 isolate from Australia (89.42% - KU999109) (Maina et al. 2016) and PeVYV-5 from Spain (84.4% - KY523072) (Fiallo-Olive et al. 2018), respectively. Additionally, a comparison among the open reading frames of the Brazilian isolate with other poleroviruses was performed (Figure 1-C) and revealed identities of 89.53% in the amino acid sequence identity of the ORF-1 with the closest relatives PeVYV-3 and PeVYV-7. This data supports the assignment of the PeVYV – isolate Altamira as a new species in the pepper vein yellows virus complex, which we propose to name as *Pepper vein yellows virus 8* (PeVYV-8). This is the first report of a polerovirus infecting bonnet pepper in Brazil. The distribution, transmission and impacts of PeVYV-8 in Brazil is still unknown and needs to be further evaluated.

Key-words: *Capsicum chinense*, PeVYV, Polerovirus

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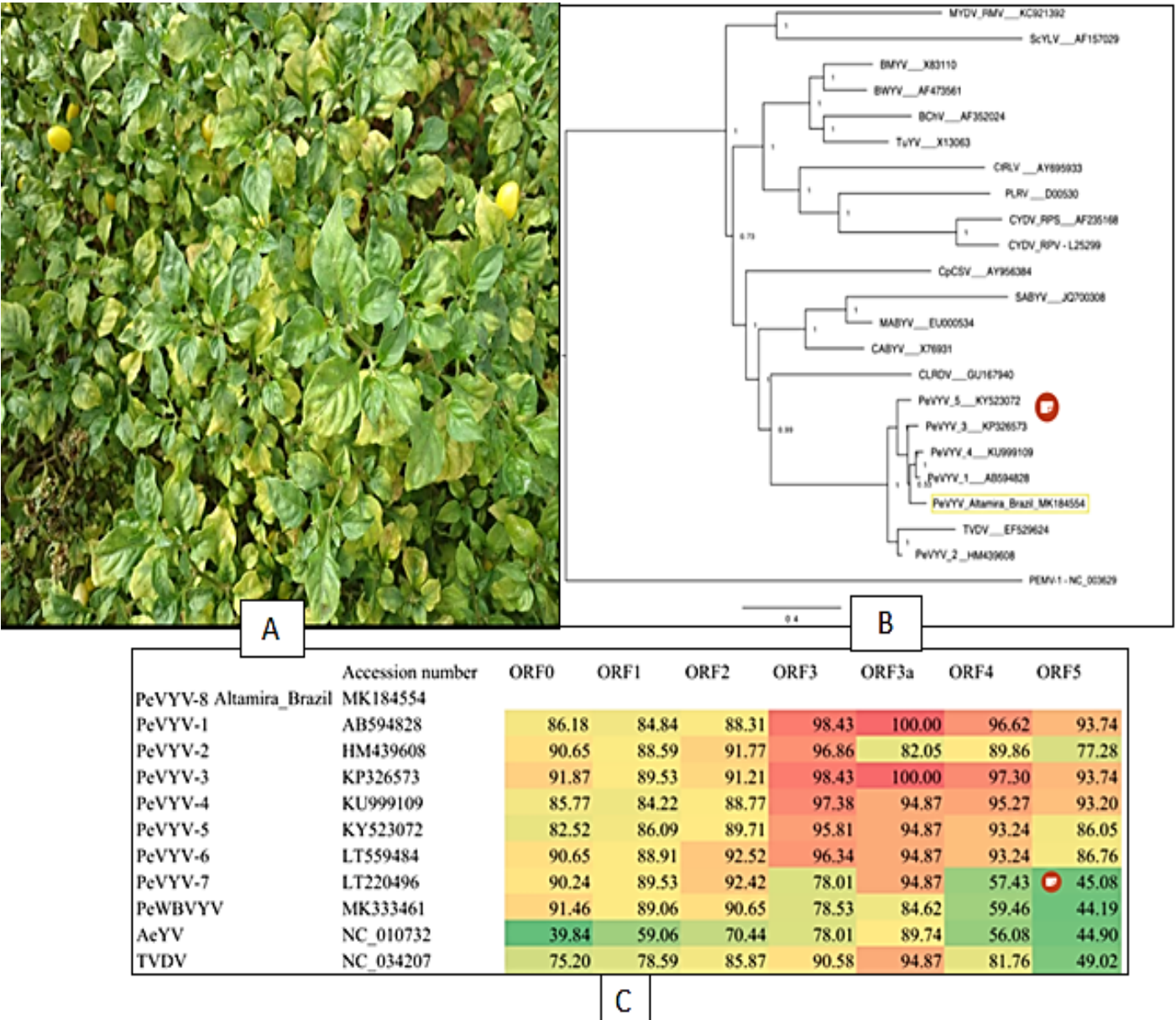


Figure 1. A) Pepper plant, *Capsicum chinense* cv. Cumari-do-Pará, showing symptoms of interveinal yellowing, leaf blade size reduction of 80%, and shortening of the internodes. **B)** Bayesian phylogenetic tree of the draft genome of *Pepper vein yellow virus* isolate Altamira. The sequence obtained in this study is highlighted in yellow. **C)** Amino acid sequence identity comparison among the open reading frames (ORF0 to ORF5 and ORF3a) of the Brazilian isolate with other known poleroviruses retrieved from GenBank. Higher saturation of red and green colour indicate higher and lower amino acid identity, respectively. *A partial sequence of ORF5 from PeVYV-7 was used for comparison.

CONSIDERAÇÕES FINAIS

O sequenciamento de alto desempenho é uma ferramenta de grande potencial para identificação de vírus infectando plantas. Através da análise metagenômica foi possível identificar sete espécies de vírus: *Peanut mottle virus* - PeMoV (Potyvirus), *Cucumber mosaic virus* sub-grupo IB (Cucumovirus), *Cowpea chlorotic mottle virus* - CCMV (Bromovirus), um provável novo membro da família *Potyviridae*, e também novas espécies dos gêneros Emaravirus, Polerovirus e Ampelovirus em amendoim forrageiro.

Em pimenta Cumari-do Pará, foi possível obter o genoma completo de uma possível nova espécie pertencente ao complexo de Polerovirus da pimenta, cujo nome proposto foi *Pepper vein yellows virus* 8 (PeVYV-8) e também sequências relacionadas aos gêneros Ampelovirus e Cucumovirus. Foi possível identificar vírus até então não relatados em amendoim forrageiro, bem como em pimenta Cumari-do-Pará no Brasil.

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Apêndice A: Tabela dos vírus detectados em 22 genótipos de *Arachis pintoi* e híbridos

Nº.	Nº. da parcela no BAG (acessos)	Cultivar / BRA	Tipo	Sintomas	Potyvirus (novo membro)	Potyvirus (PeMoV)	Cucumovirus (CMV subgrupo IB)	Bromovirus (CCMV)	Polerovirus	Emaravirus	Ampelovirus
1	2	cv. Alqueire-1	Não genitor	Mosaico, deformação foliar	+						
2	7	BRA 039985	Genitor	Anéis necróticos, deformação foliar						+	
3	8	BRA 039799	Genitor	Clorose							
4	19	BRA 039187	Genitor	Mosaico	+						+
5	39	BRA 040223	Genitor	mosqueado							
6	53	BRA 014982	Genitor	Mosaico	+						
7	55	BRA 039195	Genitor	Anéis necróticos, deformação foliar, mosaico						+	
8	56	BRA 030601	Genitor	Mosaico	+						
9	57	BRA 030635	Genitor	Mosaico, mosqueado							
10	61	cv. Belmonte	Genitor	Sem sintomas							
11	67	BRA	Genitor	mosaico							
12	77	cv. BRS Mandobi	Genitor	Mosqueado, anéis cloróticos	+	+					
13	80	cv. Amarillo (MG-100)	Não genitor	Mosqueado, clorose							
14	83	BRA 040193	Genitor	Mosaico							
15	90	BRA 016357	Genitor	Mosaico sistêmico				+			
16	E3*	BRA do cruzamento?	Híbrido F1	Sem sintomas			+				
17	E5*	BRA do cruzamento?	Híbrido F1	Mosaico, anéis cloróticos	+						
18	E7*	BRA do cruzamento?	Híbrido F1	Mosaico, mosqueado							
19	C15(98)*	BRA do cruzamento?	Híbrido F1	Mosqueado, anéis cloróticos		+	+		+		+
20	V1(59)*	BRA do cruzamento?	Híbrido F1	Sem sintomas							
21	116	BRA 042269		Mosaico, anéis cloróticos		+		+			+
22	V14953	-		Mosaico, anéis cloróticos							

*C15(98), E3, E7, V1(59) - *Arachis pintoi* x *Arachis pintoi*; E5- *Arachis pintoi* x *Arachis repens*