Universidade de Brasília
Instituto de Ciências Biológicas
Departamento de Biologia Celular
Programa de Pós-Graduação em Biologia Molecular

Diversidade de vírus de genoma de RNA em maracujazeiros do Brasil

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Brasília, DF

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Molecular da Universidade de Brasília, como parte dos requisitos necessários à obtenção do título de Doutor em Biologia Molecular.

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Resumo geral

O maracujá é uma planta tropical (gênero *Passiflora*, família Passifloraceae) cultivada em diversas regiões do mundo. Atualmente o Brasil é destaque por ser o maior produtor do fruto, por ter um dos maiores centros de origem e de diversidade de espécies, bem como, abrigar um dos maiores bancos de germoplasma de *Passiflora* destinados a conservação e preservação da espécie, e o uso dos recursos genéticos que destas plantas dispõe em programas de melhoramento genético. Diversos sintomas de etiologia viral têm sido relatados na cultura no país, entretanto estudos sobre esses vírus ainda são escassos. Em etapas anteriores deste trabalho, foram identificados vírus ainda não descritos infectando maracujazeiros nos estados do Mato Grosso do Sul e Bahia. A fim de expandir ainda mais o conhecimento sobre a diversidade viral nesta importante cultura, este estudo objetivou investigar a diversidade de vírus em espécies de *Passiflora* do Brasil e caracterizar novos vírus identificados. Para isso, diversos maracujazeiros cultivados em campos comerciais foram amostrados em várias regiões do país, bem como, acessos de *Passiflora* spp. mantidas no Banco de Germoplasma "Flor da Paixão", situado no Distrito Federal. Neste estudo usamos e combinamos várias ferramentas e metodologias a fim de caracterizar os vírus presentes nestas plantas. Isto inclui, tecnologia de sequenciamento de alto rendimento (do inglês, High-Throughput Sequencing- HTS), ferramentas de bioinformática, RT-PCR, PCR, clonagem molecular, RACE (do inglês, 5'- and 3'-rapid amplification of cDNA ends), hibridização de ácidos nucleicos por Southern blot, e sequenciamento de Sanger. Nossos resultados identificaram a ocorrência inédita de vários vírus (prováveis novas espécies, e vírus descritos em outras culturas) infectando diferentes espécies cultivadas e não cultivadas de passiflora. Nossos achados incluem o cowpea aphid-borne mosaic virus (CABMV, gênero *Potyvirus*, família *Potyviridae*), lettuce chlorosis virus (LCV, gênero *Crinivirus*, família *Bromoviridae*), cowpea mild mottle virus (CPMMV, gênero *Carlavirus*, família *Betaflexiviridae*), bean associated cytorhabdovirus (BaCV, espécie *Cytorhabdovirus caricae*, gênero *Cytorhabdovirus*, família *Rhabdoviridae*), curcubit aphid-borne yellow virus (CABYV, gênero *Polerovirus*, família *Solemoviridae*), e candidatos a novas espécies na família *Rhabdoviridae*. Esses vírus foram identificados em sua grande maioria em infecção mista com pelo menos um vírus, principalmente com o CABMV, que é o principal vírus que afeta a cultura no Brasil. Nossos dados também revelam que esses vírus não estão restritos apenas a uma região/estado. Ainda caracterizamos isolados CABYV de maracujazeiros, comparando-os com cepas da mesma espécie, o que revelou um complexo de dez distintos vírus que foram caracterizados anteriormente como pertencentes a mesma espécie. Nossos resultados expandem a diversidade viral conhecida em maracujazeiros do Brasil e no mundo. Estas informações serão extremamente úteis para entender a epidemiologia destes vírus no país, para o desenvolvimento de estratégias de controle mais eficazes dessas viroses, e ainda servirão como suporte em programas de melhoramento genético envolvendo a cultura.

Palavras-chave: Sequenciamento HTS, novos vírus, infecção mista, *Passiflora*.

General abstract

Passion fruit is a tropical plant (genus *Passiflora*, family Passifloraceae) cultivated in different regions of the world. Currently, Brazil is the largest producer of passionfruit, has one of the largest origin and diversity centers of species, and harbors one of the largest *Passiflora* germplasm banks focusing on the conservation, preservation, and in use of the genetic resources of these plants in genetic improvement programs. Diverse symptoms of viral etiology have been reported in passionfruit in the country. However, these viruses are poorly studied. In previous stages of this work were identified viruses infecting passion fruit that were not yet reported in Brazil. To expand the knowledge about the viral diversity in this important crop, this study aimed to investigate virus diversity in *Passiflora* species in Brazil and to characterize newly identified viruses. For this, several passion fruit trees cultivated in commercial fields were sampled in several regions of the country, as well *Passiflora* spp. accessions from the Germplasm Bank "Flor da Paixão" situated in Distrito Federal. In this study, we used a combination of several tools and methodologies to characterize the viruses present in these plants, including High-Throughput Sequencing (HTS), bioinformatics tools, RT-PCR, PCR, molecular cloning, RACE (5'/3'-rapid amplification of cDNA ends), Southern blot hybridization, and Sanger sequencing. Our results revealed several unprecedented viruses (putative new species and viruses described in other crops) infecting different cultivated and non-cultivated species of passionflower. Cowpea aphid-borne mosaic virus (CABMV, genus *Potyvirus*, family *Potyviridae*), lettuce chlorosis virus (LCV, genus *Crinivirus*, family *Bromoviridae*), cowpea mild mottle virus (CPMMV, genus *Carlavirus*, family *Betaflexiviridae*), bean-associated cytorhabdovirus (BaCV, species *Cytorhabdovirus caricae*, genus *Cytorhabdovirus*, family *Rhabdoviridae*), and curcubit aphid-borne yellow virus (CABYV, genus *Polerovirus*, family *Solemoviridae*), and novel candidate species in the family *Rhabdoviridae* were detected. Most viruses were identified in mixed infections with at least one other virus, mainly CABMV, the predominant virus affecting passion fruit in Brazil. Our data also revealed that these viruses are not restricted to one region/state. CABYV isolates from passion fruit were also characterized and compared with other isolates, revealing a complex of ten distinct species in the genus *Polerovirus*. Our results expand the viral diversity of passion fruit in Brazil and worldwide. This information will be valuable to understand the epidemiology of these viruses in the country for the development of more effective control strategies. It will serve as support in the genetic improvement programs involving the crop.

Keywords: HTS sequencing, novel viruses, mixed infection, *Passiflora*

Introdução geral

O maracujá é uma planta tropical (gênero *Passiflora*, família Passifloraceae) que se originou principalmente na América do Sul e é cultivada em diversas regiões do mundo. O Brasil abriga um dos mais extensos centros de diversidade e origem de espécies de *Passiflora* (Faleiro et al., 2019). O maracujá tem muitos usos nas indústrias cosmética, medicinal e ornamental. No entanto, seu principal uso é como alimento, consumido *in natura* ou produzido comercialmente em forma de suco. O maracujá é uma das principais frutas cultivadas no Brasil e com significativa importância econômica e social (Cerqueira-Silva, Jesus, et al., 2014). O país é o maior produtor de maracujá, responsavel por cerca de 50% a 60% da produção mundial. O Brasil também é o principal consumidor de maracujá *in natura* e processado (Wijeratnam, 2016).

Em 2021, o Brasil teve uma produção girando em torno de 683 mil toneladas (IBGE, 2021), sendo a Bahia (207.488 mil toneladas), Ceará (177.291 mil toneladas) e Santa Catarina (47.857 mil toneladas) os principais estados produtores. No entanto, a produção nacional diminuiu nos ultimos anos, desde 2010-2011 quando o país atingiu uma produção de cerca de 922-923 mil toneladas (IBGE, 2021). Um dos fatores que está associado a esse declínio na produtividade inclui o aumento da incidência de patógenos que afetam a cultura, bem como insetos, ácaros, nematoides, fungos, bacterias, e vírus (Fischer e Rezende, 2008; Machado et al. 2017; Santos-Jiménez et al., 2022).

A infecção por vírus é um fator limitante na produção de diversas culturas de importância econômica. Portanto, estratégias de manejo e controle de viroses em uma cultura, principalmente aquelas baseadas na geração de genótipos resistentes, dependem diretamente de um amplo conhecimento da diversidade viral na cultura.

As tecnologias baseadas em sequenciamento de alto rendimento (do inglês, High-Throughput Sequencing- HTS) tem sido amplamente usada na detecção de patógenos, incluindo vírus de plantas ao redor do mundo. Essas tecnologias associadas a diversas ferramentas de bioinformática têm possibilitado a identificação da diversidade de vírus em muitas culturas agrícolas, sem a necessidade de conhecimento prévio do patógeno viral (Bester, Cook, Breytenbach, et al., 2021; Bester, Cook e Maree, 2021; Fitzpatrick et al., 2021; Fontenele, Alves-Freitas, et al., 2018; Fontenele et al., 2021; Fontenele et al., 2020; Fontenele, Abreu, et al., 2018; Koloniuk et al., 2022; Maachi et al., 2021; Rivarez et al., 2021; Vazquez-Iglesias et al., 2022). Dessa forma, é possível identificar vírus já descritos e novos vírus ainda não relatados na literatura.

Essa tecnologia tem sido também utilizada para caracterização do viroma de maracujazeiros no mundo e no Brasil. Algumas dessas descobertas incluem o passion fruit chlorotic mottle virus (gênero *Citlodavirus*, família *Geminiviridae*) (Fontenele, Abreu, et al., 2018), cucurbit aphid-

borne yellows virus (gênero *Polerovirus*, família *Solemoviridae*) (Vidal et al., 2018), passiflora mitovirus-like (gênero *Mitovirus*, família *Narnaviridae*) (Santos et al., 2020), plant-associated genomovirus7 (gênero *Gemykolovirus*, família *Genomoviridae*), gulupa bacilliform virus A (espécie *Badnavirus passiflorae*, gênero *Badnavirus*, família *Caulimoviridae*) (Cardona, García, et al., 2022; Cardona, Higuita, et al., 2022; Sepúlveda et al., 2022), citrus leprosis virus, hibiscus strain C2 (gênero *Cilevirus*, família *Kitaviridae*) (Olmedo-Velarde et al., 2022), citrus-associated rhabdovirus (espécie *Cytorhabdovirus caricae*, gênero *Cytorhabdovirus*, família *Rhabdoviridae*) (Zhang, Huang, et al., 2020). Todos os relatos indicam que a diversidade viral em maracujá parece ser muito maior do que se conhece atualmente.

Hipótese

Existe uma diversidade desconhecida de vírus infectando espécies de *Passiflora* do Brasil.

Objetivo geral

O objetivo desta tese é investigar a diversidade de vírus de genoma de RNA em espécies de *Passiflora* do Brasil e caracterizar novos isolados com potencial de ser novas espécies de vírus identificadas.

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Chapter 1

List of viruses infecting or associated with passion fruit

This chapter is a preliminary version of a review intended to be published as "List of viruses infecting or associated with passion fruit". Andreza Henrique Vidal and Simone Graça Ribeiro.

List of viruses infecting or associated with passion fruit

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1.1. Introduction

Passion fruit is a tropical plant of the genus *Passiflora* (family Passifloracea) grown in tropical and subtropical regions around the world (Wijeratnam, 2016). The passion fruit name in portuguese (maracuja) originates from the Tupi-Guarani language and means “food in the form of a gourd” (Faleiro et al., 2016). The passion fruit is also known as passionflower, which comes from the association of the flower structures of passion fruit (three pistils represent the Holy Trinity, the three stylets the nails used in the crucifixion, and the five anthers representing the wounds of Christ) with the "Passion of the Christ" (Souza and Meletti, 1997). These plants have different applications and are widely used for food, mainly for juice production, for herbal medicines, and ornamental purposes (Castillo et al., 2020; Cerqueira-Silva, Jesus, et al., 2014).

Many different pathogens affect the passion fruit causing significant losses in yield and fruit quality across the world, one of the most significant are the viruses (Ocampo et al., 2021). Fifty-six viruses belonging to at least fifteen plant virus families and twenty genera (classified and unclassified) have been reported infecting or associated with passion fruit crops (Figure 1, Table 1). These viruses are constituted of DNA or RNA genomes, have a variety of genome organization and particle morphology, and are transmitted by different insect vectors and by different modes of transmission.

Most of the viruses identified in *Passiflora* spp. plants are constituted of RNA genome (*n*= 39) and are mainly members of the genus *Potyvirus*, family *Potyviridae* (Figure 1). The DNA viruses (*n*= 17) are mostly of the genus *Begomovirus*, family *Geminiviridae* (Figure 1).

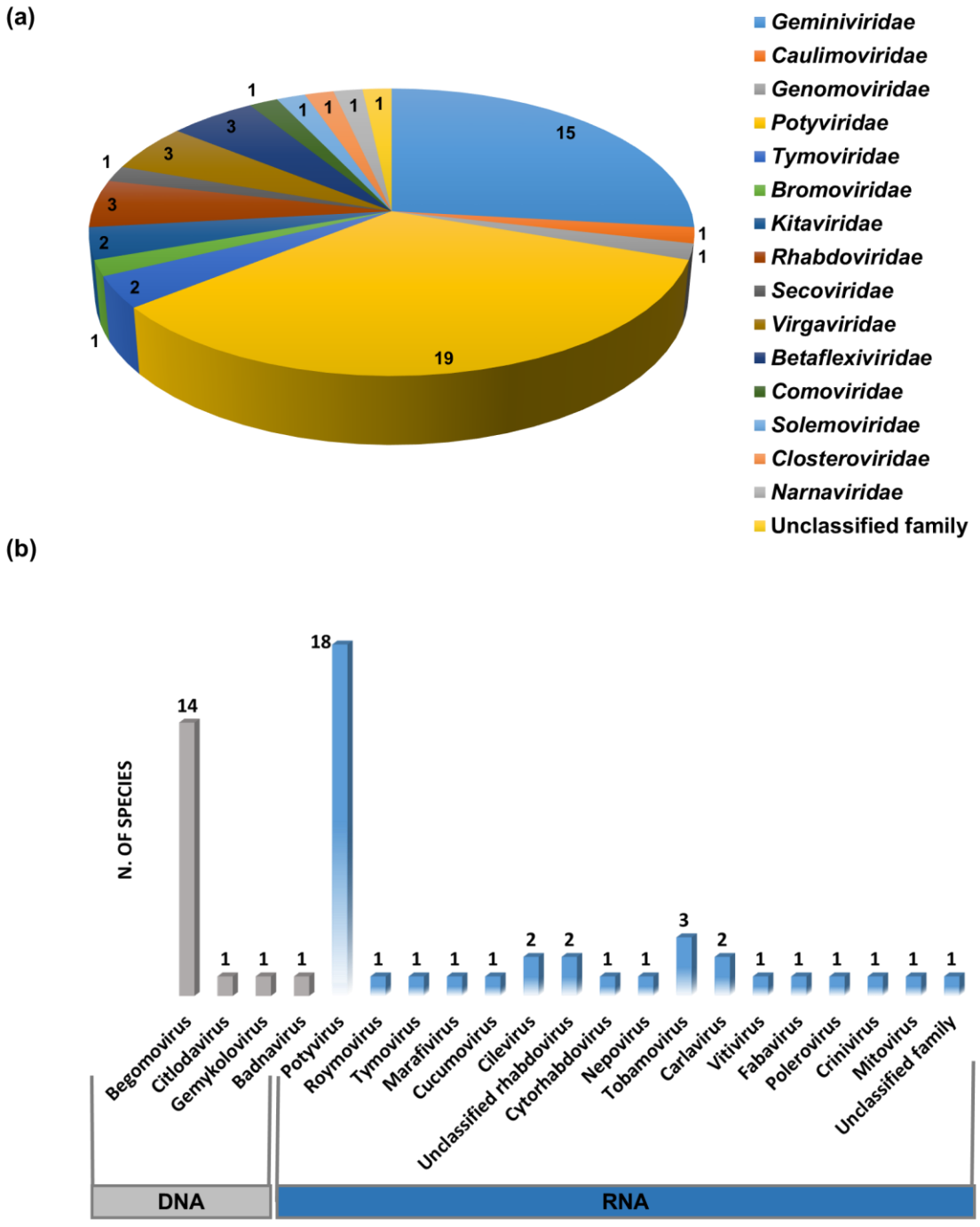

Figure 1. Family (a) and genus (b) of viruses reported in passion fruit.

Despite diverse viruses were documented infecting passion fruit plants around the world, only a small number of these viruses are considered of major economic concern to the crop. Among those, we can mention the viruses responsible for the passion fruit woodiness disease (PWD), members of the genus *Potyvirus* (family *Potyviridae*). PWD can be caused by infection with cowpea aphid-borne mosaic virus (McKern et al., 1994), East Asian passiflora virus (Iwai et al., 1996), Ugandan passiflora virus (Ochwo-Ssemakula et al., 2012), passion fruit woodiness virus (McKnight, 1953), telosma mosaic virus (Chiemsombat et al., 2014), and passiflora mottle virus (Do et al., 2021; Xie et al., 2019). The disease is considered a limiting factor for passion fruit

production, once potyvirus-infected plants show reduced plant size, deformed leaf and fruits, fruit woodiness, and eventually can lead to the death of the plant, due to the attack of opportunistic pathogens on plants with PWD (Cerqueira-Silva, Conceição, et al., 2014; Nascimento et al., 2006; Rezende, 2006). Below we list the viruses reported in *Passiflora* species (Table 1).

Table 1. First report of viruses infecting or associated with passion fruit plants.

Taxonomy: Family/Genus **Virus name**	**Acronym**	**Reference**
Geminiviridae*/*Begomovirus		
Euphorbia leaf curl virus	EuLCV	(Cheng et al., 2014)
Papaya leaf curl Guangdong virus	PalCuGDV	(Cheng et al., 2014)
Sida micrantha mosaic virus	SiMMV	(Alves, 2012)
Sida mottle virus	SiMoV	(Alves, 2012)
Passionfruit severe leaf distortion virus	PSLDV	(Ferreira et al., 2010)
Passion flower little leaf mosaic virus	PLLMV	(Novaes et al., 2002; Novaes et al., 2003)
Passion fruit leaf distortion virus	PLDV	(Vaca-Vaca et al., 2017; Vaca-Vaca et al., 2016)
Melochia yellow mosaic virus	MelYMV	(Spadotti et al., 2019)
Sida mottle Alagoas virus	SiMAV	(Mituti et al., 2019)
Euphorbia mosaic virus	EuMV	(Polston et al., 2017)
Cotton leaf curl Multan virus	CLCuMuV	(Tang et al., 2020)
Papaya leaf curl China virus	PaLCuCNV	(Huang et al., 2020)
Passion fruit leaf curl virus	PFLCuV	(Venkataravanappa et al., 2022)
Ramie mosaic Yunnan virus= Ramie mosaic virus	RMYnV= RamMV	(Chen et al., 2020)
Geminiviridae/Citlodavirus		
Passion fruit chlorotic mottle virus	PCMoV	(Fontenele, Abreu, et al., 2018)
Genomoviridae/Gemykolovirus		
Plant-associated genomovirus 7	PaGmV7	(Fontenele et al., 2020)
Caulimoviridae/Badnavirus		
Gulupa bacilliform virus A (species *Badnavirus passiflorae*)	GBVA	(Cardona, García, et al., 2022; Cardona, Higuita, et al., 2022; Sepúlveda et al., 2022)
Potyviridae/Potyvirus		
Cowpea aphid-borne mosaic virus= South african passiflora virus	CABMV= SAPV	(Brand et al., 1993; McKern et al., 1994)
Passiflora ringspot virus	PFRSV	(Wijs, 1974)
Bean yellow mosaic virus	BYMV	(Parrella and Castellano, 2002; Parrella and Lanave, 2009)
Passion fruit mottle virus	PaMV	(Chang, 1992)
Passiflora mosaic virus	PfMoV	(Sokhandan et al., 1997; Webster et al., 2007)

Soybean mosaic virus	SMV	(Benscher et al., 1996)
Passiflora foetida virus Y= Passiflora virus Y	PfVY= PaVY	(Parry et al., 2004)
East Asian Passiflora virus	EAPV	(Iwai et al., 1996; Iwai, Terahara, et al., 2006; Iwai, Yamashita, et al., 2006)
Passion fruit crinkle virus	PCV	(Chang et al., 1996)
Passiflora chlorosis virus	PaCV	(Baker and Jones, 2007)
Sri Lankan passion fruit mottle virus	SLPMV	(Dassanayake and Hicks, 1992)
Ugandan Passiflora virus	UPV	(Ochwo-Ssemakula et al., 2012)
Passion fruit woodiness virus	PWV	(McKnight, 1953)
Telosma mosaic virus	TeMV	(Chiemsombat et al., 2014)
East asian passiflora distortion virus	EAPDV	(Riska et al., 2019)
Passiflora mottle virus= Passion fruit severe mottle-associated virus= Passionfruit Vietnam potyvirus= Passionfruit Vietnam virus	PaMoV= PFSMoAV= PVNV= PVV	(Do et al., 2021; Xie et al., 2019)
Uraria mosaic virus	UMV	(Nakasato et al., 2020)
Turnip mosaic virus	TuMV	(Chen et al., 2021)
Thailand Passiflora potyvirus	ThPV	(Benscher et al., 1993; Fisher and Kyle, 1994)
Potyviridae/ Roymovirus		
Passifora edulis symptomless virus	PeSV	(Jover-Gil et al., 2018)
Tymoviridae/Tymovirus		
Passion fruit yellow mosaic virus	PFYMV	(Crestani et al., 1986)
Tymoviridae/Marafivirus		
Purple passionfruit leaf deformation virus	PpLDV	(Cardona, Restrepo, et al., 2022)
Bromoviridae/Cucumovirus		
Cucumber mosaic virus	CMV	(Magee, 1948)
Kitaviridae/Cilevirus		
Passion fruit green spot virus	PFSGV	(Kitajima et al., 1997)
Citrus leprosis virus (Hibiscus strain C2)	CiLV-C2H	(Olmedo-Velarde et al., 2022)
Rhabdoviridae/Cytorhabdovirus		
Bean-associated cytorhabdovirus; Citrus-associated rhabdovirus strains (species *Cytorhabdovirus caricae*)	BaCV; CiaRV	(Zhang, Huang et al. 2020; Vidal, Felix et al. 2023)
***Rhabdoviridae*/ unclassified genus**		
Passion fruit vein clearing virus	PVCV	(Chagas et al., 1987; Kitajima and Crestani, 1985)
Passionfruit rhabdovirus	PFRSV	(Pares et al., 1997; Pares et al., 1983)
Secoviridae/Nepovirus		

Tomato ringspot virus	ToRSV	(Koenig and Fribourg, 1986)
Virgaviridae/Tobamovirus		
Maracuja mosaic virus	MarMV	(Fribourg et al., 1987; Koenig and Fribourg, 1986)
Passion fruit mosaic virus= maracuja mosaic virus Florida strain	PFMV= MarMV-FL	(Song and Ryu, 2011; St Hill et al., 1992)
Hibiscus latent Fort Pierce virus	HLFPV	(Xie et al., 2022)
Betaflexiviridae/Carlavirus		
Passiflora latent virus= Passiflora latent carlavirus	PLV	(Brandes and Wetter, 1963)
Cowpea mild mottle virus	CPMMV	(Vidal, Felix et al. 2023)
Betaflexiviridae/Vitivirus		
Grapevine virus A	GVA	(Galleti et al., 2006; Lombardi and Galleti, 2007)
Comoviridae/Fabavirus		
Broad bean wilt virus 2	BBWV2	(Kobayashi, 2004; Yonaha et al., 1993)
Solemoviridae/Polerovirus		
Cucurbit aphid-borne yellows virus	CABYV	(Vidal et al., 2018)
Closteroviridae/Crinivirus		
Lettuce chlorosis virus	LCV	(Vidal et al., 2021)
Narnaviridae/Mitovirus		
Passiflora mitovirus-like	PMV	(Santos et al., 2020)
Unclassified family/Unclassified genus		
Purple granadilla mosaic virus= Purple passion fruit mosaic virus	PGMV	(Chagas et al., 1984)

1.2. List of DNA virus infecting passion fruit plants

Currently, seventeen DNA viruses have been identified infecting passion fruit plants (Figure 1). These include viruses belonging mainly to the genus *Begomovirus* and *Citlodavirus*, family *Geminiviridae*, viruses belonging to the genus *Badnavirus*, family *Caulimoviridae*, and genus *Gemykolovirus*, family *Genomoviridae*.

Geminiviridae/Begomovirus

Euphorbia leaf curl virus (EuLCV): EuLCV was first reported on passion fruit in Taiwan (Cheng et al., 2014) in the variety 'Tainung No. 1' (*P. edulis* × *P. edulis* f. flavicarpa). It was also reported in *P. edulis* in South Korea (Kil et al., 2016; Kim et al., 2018) and China (Huang et al., 2021; Ye et al., 2022). Passion fruit plants with EuLCV showed symptoms of systemic mottle, mosaic, yellowing or yellow spotting, stunting, chlorotic or chlorotic ringspot, curling of leaves, and malformation.

Papaya leaf curl Guangdong virus (PalCuGDV): PalCuGDV was identified in mixed infection with EuLCV in the variety 'Tainung No. 1' from Taiwan (Cheng et al., 2014), in *P. edulis* in South Korea (Kim et al., 2018) and China (Huang et al., 2021).

Sida micrantha mosaic virus (SiMMV): SimMV was found infecting *P. edulis* plants in orchards in Minas Gerais state, Brazil, and was not transmitted by whitefly *Bemisia tabaci*. (Alves, 2012).

Sida mottle virus (SiMoV): SiMoV was identified infecting *P. edulis* plants in orchards in states of Para and Rio de Janeiro in Brazil (Alves, 2012). SiMoV passion fruit isolate was transmitted by bioballistics to *P. morifolia* and *Sida rhombifolia* that showed light mosaic and severe mosaic, respectively. It was also transmitted mechanically to *Nicotiana benthamiana*, but not transmitted by *B. tabaci* (Alves, 2012).

Passionfruit severe leaf distortion virus (PSLDV): PSLDV was identified in yellow passionfruit (*P. edulis* f. flavicarpa) plants in several regions of Bahia state, Brazil (Ferreira et al., 2010; Rodrigues et al., 2019). It is transmitted by *B. tabaci* MEAM1 (Chinelato, 2022) and infected plants display severe symptoms of dwarfing, mosaic, yellowing, yellow spots, leaf distortion, and malformation and cracking in the fruit. (Ferreira et al., 2010; Rodrigues et al., 2019).

Passion flower little leaf mosaic virus (PLLMV): PLLMV was reported in yellow passion fruit (*P. edulis* f. flavicarpa) in the Bahia state, Brazil (Novaes et al., 2002). PLLMV is vectored by whiteflies and infected plants showed symptoms like yellow mosaic, reduction in leaf lamina and in the plant growth. Fruits are small and malformed and there is a decrease in their production (Novaes et al., 2003).

Passion fruit leaf distortion virus (PLDV): Reported in Colombia, PLDV was found infecting passion fruit (*P. edulis*) plants that showed yellow mosaic and leaf deformation (Vaca-Vaca et al., 2017).

Melochia yellow mosaic virus (MelYMV): The natural occurrence of MelYMV was reported in *P. edulis* (variety Sol do Cerrado) in an orchard in Mato Grosso do Sul state, Brazil (Spadotti et al., 2019). Plants infected by this begomovirus exhibit symptoms of mosaic, yellow spots, leaf curling and malformation (Spadotti et al., 2019). The virus was transmitted to passion fruit plants by biolistics, but not by *B. tabaci* MEAM1 or MED (Spadotti et al., 2019).

Sida mottle Alagoas virus (SiMAV): This begomovirus was identified in *P. edulis*, cultivar Flora Brasil-200, that exhibited symptoms of severe mosaic, yellow spots, leaf deformation, and blisters, collected in Rio Grande do Norte state, Brazil (Mituti et al., 2019).

Euphorbia mosaic virus (EuMV): Passion fruit plant (*P. edulis*) showing symptoms of leaf distortion and necrotic spots sampled in Florida, USA were identified with EuMV (Polston et al., 2017). Passion fruit 'Lilikoi' was inoculated with EuMV and infected plants developed symptoms like mild mottling followed by necrotic spots, leaf deformation, and flower abortion.

Cotton leaf curl Multan virus (CLCuMuV): In China, CLCuMuV was detected in *P. edulis* plants that showed symptoms of leaf curling and dark green, swollen veins (Polston et al., 2017).

Papaya leaf curl China virus (PaLCuCNV): Purple (*P. edulis* Sims.) and yellow (*P. edulis* f. flavicarpa) passion fruit plants that showed leaf curl, malformation on leaves, maintained in an orchard in China were detected with PaLCuCNV (Huang et al., 2020).

Passion fruit leaf curl virus (PFLCuV): PFLCuV is a novel begomovirus found recently associated with leaf curl disease of passion fruit in India (Venkataravanappa et al., 2022).

Ramie mosaic Yunnan virus (RMYnV)= Ramie mosaic virus (RamMV): The begomovirus was renamed by ICTV as RMYnV (Fiallo-Olivé et al., 2021) and also known as RamMV was reported for the first time in infected rami (*Boehmeria leiophylla*) plants in China (Li et al., 2010; Zhao et al., 2018). Later, the RamMV was identified in cultivated purple passion fruit plants also in China (Chen et al., 2020). These plants displayed virus-like symptoms of stunting, mosaic, yellow or necrotic spots (Chen et al., 2020).

Geminiviridae/Citlodavirus

Passion fruit chlorotic mottle Virus (PCMoV): The citlodavirus PCMoV was found in a passion fruit (*P. edulis*) plant from Mato Grosso do Sul state, Brazil in mixed infection with CABMV (Fontenele, Abreu, et al., 2018). PCMoV infectious clone was able to infect *N. benthamiana, P. edulis, P. alata,* and *Arabdopsis thaliana* by biobalistics (Fontenele, Abreu, et al., 2018). Infected *N. benthamiana* plants displayed symptoms of chlorotic spots, mottle and a growth impairment, while in other plants no symptoms were visualized (Fontenele, Abreu, et al., 2018).

Genomoviridae/Gemykolovirus

Plant-associated genomovirus 7 (PaGmV7): PaGmV7 was found associated with *P. edulis* in Bahia state, Brazil (Fontenele et al., 2020). However, there is still lack of evidence that genomoviruses associated with plants are in fact plant-infecting viruses or if they are infecting microorganisms associated with the plant.

Caulimoviridae/Badnavirus

Gulupa bacilliform virus A (GBVA) (species *Badnavirus passiflorae*): GBVA is strain belonging to the species *Badnavirus passiflorae* and was identified in asymptomatic purple

passion fruit (*P. edulis* f. *edulis*) in Colombia (Cardona, Higuita, et al., 2022; Sepúlveda et al., 2022). GBVA was found in high incidence with other RNA viruses in adult plants, plantlets, and seeds buds newly germinated (Cardona, García, et al., 2022; Cardona, Higuita, et al., 2022; Sepúlveda et al., 2022).

1.3. List of RNA viruses infecting passion fruit

The greatest diversity of viruses identified in passion fruit plants is of viruses with RNAgenome. There are at least thirty-nine viruses described in the crop, belonging to at least sixteen genera (classified and unclassified) (Figure 1). This includes viruses belonging to the genera *Potyvirus* and *Roymovirus* (family *Potyviridae*), *Tymovirus* and *Marafivirus* (family *Tymoviridae*), *Cucumovirus* (family *Bromoviridae*), *Cilevirus* (family *Kitaviridae*), Unclassified genus and *Cytorhabdovirus* (family *Rhabdoviridae*), *Nepovirus* (family *Secoviridae*), *Tobamovirus* (family *Virgaviridae*), *Carlavirus* and *Vitivirus* (family *Betaflexiviridae*), *Fabavirus* (family *Comoviridae*), *Polerovirus* (family *Solemoviridae*), *Crinivirus* (family *Closteroviridae*), and *Mitovirus* (family *Narnaviridae*). RNA viruses are presently the main viruses associated with the most serious problem of viral etiology that affects the passion fruit cultivation, the passion fruit woodiness disease (PWD).

Potyviridae*/*Potyvirus

Cowpea aphid-borne mosaic virus (CABMV)= South African passiflora virus (SAPV): The potyvirus CABMV is the main virus associated with passion fruit crop, and is associated with the PWD in Africa and Brazil (McKern et al., 1994; Nascimento et al., 2006). CABMV was reported in passion fruit orchards in several states in Brazil (Cerqueira-Silva, Conceição, et al., 2014). Another strain denominated SAPV was identified in South Africa, and later was re-classified as CABMV (Brand et al. 1993), McKern et al. 1994). CABMV is aphid-transmitted (*Aphis gossypii, A. fabae*, and *A. craccivora*; Hemiptera: Aphididae) or mechanically transmitted during pruning (Santos-Jiménez et al., 2022). Plants infected by CABMV show reduced size, reduction in the leaf size, different levels of wrinkling, mosaic, bubbles, blisters on the leaf, and woodiness and deformation of the fruits (Mendes et al., 2022; Nascimento et al., 2006; Rezende, 2006).

Passiflora ringspot virus (PFRSV): PFRSV is a virus identified causing ringspot in *P. edulis* in the Ivory Coast (Wijs, 1974). The virus was able to infect Passifloraceae and Leguminosae species, and is transmitted mechanically or by insect vectors like aphids, but seed transmission was not detected (Wijs, 1974).

Bean yellow mosaic virus (BYMV): BYMV was identified for the first time in the ornamental *P. caerulea*, also known as blue passionflower, in Croatia (Pleše and Wrischer, 1984). Later, BYMV was reported in southern Italy infecting a blue passionflower plant growing in a

private garden (Parrella and Castellano, 2002; Parrella and Lanave, 2009). Passiflora infected by BYMV from Croatia showed spotting, vein clearing and deformation of leaflets (Pleše and Wrischer, 1984), while *Passiflora* from Italy showed chlorotic spots and light mottling (Parrella and Castellano 2002).

Passion fruit mottle virus (PaMV): PaMV was identified in purple passion fruit plants (*P. edulis* Sims X *P. edulis* f. flavicarpa, variety Tainung No. 1 [TN-1]) showing mild mottling in Taiwan (Chang, 1992).

Passiflora mosaic virus (PfMoV): The PfMoV isolates were originally described as passion fruit woodiness virus (PWV) infecting *P. subpeltata*, *P. edulis*, and one passion fruit genotype in New South Wales, Australia (Sokhandan et al., 1997; Webster et al., 2007). Later, the coat protein (CP) sequences of these isolates were compared, and indicated that they belong to a distinct group which was named passiflora mosaic virus (Webster et al., 2007).

Soybean Mosaic Virus (SMV): SMV was found infecting *P. edulis* f. flavicarpa (yellow passion fruit), *P. ligularis* (granadilla), and *P. quadrangularis* (badea) that displayed severe mosaic, epinasty, defoliation, and premature death in Colombia (Benscher et al., 1996). Later, SMV was found to infect purple *P. edulis* f. edulis (gulupa) also in Colombia (Camelo García, 2010; Gordillo Alarcón, 2011; Jaramillo Mesa et al., 2018). Symptoms associated to these plants include green spots on unripe fruits and ring spots on ripe fruits, deformation on fruits, and mottling, vein banding, mosaics, chlorosis, deformation on leaves, and deformation at the terminal tips of the branches (Camelo García, 2010; Jaramillo Mesa et al., 2018). Aphid (*Aphis gossypii* and *Toxoptera citricida*) (Benscher et al., 1996) and seed transmission were confirmed (Jaramillo Mesa et al., 2018).

Passiflora foetida virus Y (PfVY)= passiflora virus Y (PaVY): PfVY was originally identified in Australia and Indonesia infecting *P. foetida*, and in commercial *P. edulis* hybrids (Parry et al., 2004). Other PfVY strain was found infecting *Macroptilium atropurpureum* (siratro plants) in Taiwan, and named passiflora virus Y (PaVY) (Chiang et al., 2012). Later, the virus also was identified in passion fruit plants in the USA (Baker et al., 2014). Infected plants showed symptoms like yellow or green mosaic, sometimes ringspots and chlorotic spots (Parry et al., 2004). PfVY is vectored by *Aphis gossypii* (Parry et al., 2004).

East Asian Passiflora virus (EAPV): The potyvirus EAPV induces PWD and was originally described as passionfruit woodiness virus, but was later characterized as East Asian passiflora virus, a new virus species (Iwai et al., 1996; Iwai, Terahara, et al., 2006; Iwai, Yamashita, et al., 2006). EAPV was firstly found in Japan infecting a purple passion fruit (*P. edulis* ×*P. edulis* f. flavicarpa, variety "Summer queen") hybrid that showed deformed and woodiness fruits (Iwai et al., 1996). Later, the virus was identified in Taiwan (Chong et al., 2018), Vietnam, and Japan

infecting purple passion fruit (*P. edulis* Sims x *P. edulis* f. flavicarpa, variety Tainung No. 1 [TN-1]) (Chong et al., 2018; Do et al., 2021; Fukumoto et al., 2012). The virus was able to infect mechanically the wild species *P. foetida*, purple passion fruit (*P. edulis*) TN-1 cultivar, and alternative host plants, inducing symptoms like local lesions, mosaic, leaf curl, chlorotic mottling, yellowing, latent infection, and systemic necrosis, leaf and fruit deformation (Chong et al., 2018; Do et al., 2021; Iwai, Yamashita, et al., 2006; Riska et al., 2020; Riska et al., 2019).

Passion fruit crinkle virus (PCV): PCV was reported in China infecting purple passion fruit (*P. edulis* Sims× *P. edulis* f. flavicarpa, variety 'Tainung No. 1) that showed crinkle leaves (Chang et al., 1996). PCV was able to infect golden passion fruit via the aphid *Myzus persicae*, and mechanically transferred to different species of Passifloraceae and experimental hosts, which displayed systemic foliar mottling and crinkling (Chang et al., 1996).

Passiflora chlorosis virus (PaCV): A PaCV isolate was originally identified in *P. incence* plants with chlorotic symptoms in Florida, USA (Baker et al., 2014; Baker and Jones, 2007). Later, additional isolates were identified in *P. edulis* plants in Germany (Ochwo-Ssemakula et al., 2012) and Israel (Fresnillo et al., 2022).

Sri Lankan passion fruit mottle virus (SLPMV): SLPFMV is a sap-transmissible virus isolated from golden passion fruits (*P. edulis* f. flavicarpa) in Sri Lanka (Dassanayake and Hicks, 1992). The virus was able to infect mechanically 23 species in 5 plant families, inducing systemic infection. Later, additional isolates were identified in different *Passiflora spp*. (Dassanayake and Hicks, 1992). SLPFMV was transmitted by aphids like *M. persicae*, *A. spiraecola*, *A. gossypii*, and *A. craccivora* and mechanically to passion fruit plants that displayed symptoms of mosaic, chlorotic mottle, chlorotic lesions, chlorotic flecking, vein yellow, and leaf distortion (Dassanayake and Hicks, 1992).

Ugandan Passiflora virus (UPV): UPV is another potyvirus reported to cause PWD in passion fruit (*P. edulis* Sims) in Uganda and Rwanda (Mbeyagala et al., 2019; Ochwo-Ssemakula et al., 2012; Waweru et al., 2019). PWD like symptoms associated with the UPV infection include chlorotic spots, malformation, crinkle, mottle, mosaic, and distortion on leaf, and malformation and woodiness of fruits (Ochwo-Ssemakula et al., 2012; Waweru et al., 2019). UPV isolates were able to infect mechanically experimental species of the families Solanaceae and Chenopodiaceae (Ochwo-Ssemakula et al., 2012).

Passion fruit woodiness virus (PWV): The potyvirus PWV occurs only in Australia, it is associated to PWD disease in the country (Fukumoto et al., 2013; McKnight, 1953), infecting different *Passiflora* spp. causing mild to severe symptoms (Sokhandan et al., 1997; Wylie and Jones, 2011). PWV symptoms include chlorotic local lesions, necrotic local lesions, chlorotic spotting, chlorotic, stunting, wilting, systemic mottle, vein banding, vein clearing, vein clearing,

die-back, leaf distortion, mosaic, bunching, wooden and distorted fruits (Parry et al., 2004; Sokhandan et al., 1997; Wylie and Jones, 2011). The virus could be transmitted mechanically, leaf graft, and aphid (McKnight, 1953; Omatsu et al., 2004).

Telosma mosaic virus (TeMV): TeMV is an important potyvirus reported in high incidence in passion fruit in recent years, and one of the accountable causes of PWD in the crop (Fu et al., 2021; Yu et al., 2021). TeMV was first reported on passion fruit in Thailand (Chiemsombat et al., 2014), in different provinces of mainland China (Chen et al., 2018; Xie et al., 2020; Xie et al., 2017; Yang et al., 2018, 2021; Yu et al., 2021), and in Vietnam (Do et al., 2021; Ha et al., 2008). In Thailand, the virus was reported naturally infecting purple passion fruit plants, and was transmitted mechanically to *P. edulis* variety Purple No.2, which showed severe mosaic, yellow spot mottling, distortion on the leaf, mosaic skin on green fruit, and decreased fruit size (Chiemsombat et al., 2014). In China, TelMV is pervasive in purple and yellow passionfruit plants, and has been identified in symptomatic and symptomless plants (Yu et al., 2021). Symptomatic *P. edulis* plants from China displayed mosaic, crinkle, and shrinkage on the leaves, small foliage, and uneven fruit coloring (Chen et al., 2018; Xie et al., 2020; Xie et al., 2017; Yang et al., 2018). In Vietnam, *P. edulis* plants displayed mosaic, shrinkage, leaf distortion, and woodiness and deformation on fruits (Do et al., 2021). TeMV from Vietnam was transmitted mechanically to other hosts, and in yellow passion fruit (*P. edulis* f. flavicarpa) inducing foliar symptoms like severe leaf mosaic, distortion, and slight yellowing on systemic leaves one month after inoculation. Six months after inoculation, plants developed symptoms like size reduction, malformation, and woodiness of the fruits (Do et al., 2021). Besides affecting adversely the development the fruits, TeMV infection also modulates the phytochemical components of *P. edulis* fruits (Chen et al., 2018).

East Asian Passiflora distortion virus (EAPDV): EAPDV is a potyvirus identified causing the deformation of passion fruits (*P. edulis*) in Japan (Riska et al., 2020; Riska et al., 2019). EAPDV was able to infect mechanically *P. foetida* (wild maracuja, bush passionfruit, or marya–marya) causing leaf curl and mosaic symptoms on the upper trifoliate leaves. It was also transmitted to purple passionfruit (*P. edulis*) by grafting from an infected *P. foetida* stem (Riska et al., 2019). Cultivars of French bean was systemically infected with induced mosaic symptoms (Riska et al., 2019). Overall mosaic, leaf curl, and fruit malformation were associated with the infection by EAPDV in purple passion fruit both in natural conditions and in greenhouse experiments (Riska et al., 2019). Later, the coinfection of EAPDV and the potyvirus East Asian Passiflora virus was found in passion fruit in Japan (Riska et al., 2020). The effects of coinfection tested in *P. foetida* revealed symptoms more severe in the leaf and differential viral titers (Riska et al., 2020).

Passiflora mottle virus (PaMoV) = passionfruit Vietnam virus (PVV) = passionfruit Vietnam potyvirus (PVNV) = passion fruit severe mottle-associated virus (PFSMoAV): PVV, PVNV, and PFSMoAV are members of the species *Passiflora mottle virus* (Inoue-Nagata et al., 2022). PaMoV was identified in *P. edulis* in Vietnam (Do et al., 2021). In China, other strain related to PVNV tentatively named PFSMoAV was identified infecting *P. edulis* plants that exhibiting severe mottle symptoms (Xie et al., 2019). Later, three strains identified in the Vietnam were classified as PaMoV and identified as one of the viruses responsible for PWD in the country (Do et al., 2021).

Uraria mosaic virus (UMV): UMV was found to infect newly bred passion fruit cultivars that showed mild ringspot on fruits and mild mosaic on leaves growing in Japan (Nakasato et al., 2020). Nakasato, Fujioka et al. 2020 demonstrated that UMV was transmitted mechanically to different experimental host causing latent infection in inoculated plants (Nakasato et al., 2020).

Turnip mosaic virus (TuMV): TuMV was found in yellow passion fruit (*P. edulis*) in China (Chen et al., 2021). Passion fruit plants infected with TuMV sampled from Guangdong Province displayed symptoms of stunting, green-yellow mosaic, or necrotic spots (Chen et al., 2021), while plants from Fujian Province showed mosaic, crinkle, and yellow spots (Li et al., 2023).

Thailand passiflora potyvirus (ThPV): A virus called ThPV was mentioned by Fisher and Kyle (Fisher and Kyle, 1994, 1996), but no additional registry was found about this virus.

Potyviridae/Roymovirus

Passifora edulis symptomless virus (PeSV): PeSV was identified in asymptomatic passion fruit plants (*P. edulis*, cultivar 'Passion Dream' and genotype anthesis all year) grown in Israel (Jover-Gil et al., 2018).

Tymoviridae*/*Tymovirus

Passion fruit yellow mosaic virus (PFYMV): PFYMV was originally identified from golden passion fruit (*P. edulis* f. flavicarpa) plants in several regions in Rio de Janeiro state, Brazil (Crestani et al., 1986). The plants displayed virus symptoms of yellow net, yellow mosaic, and leaf crinkle. Later, the tymovirus was found in Colombia infecting passion fruit (*P. edulis* Sims.) plants that showed yellow mosaic leaf symptoms (Morales et al., 2002), and in purple passion fruit (*P. edulis* f. edulis) with symptoms such as mosaic, mottling along the veins, enation and severe leaflet deformation (Jaramillo Mesa et al., 2019). The virus was transmitted by grafting and mechanically to different *Passiflora* spp. that developed systemic symptoms (Crestani et al., 1986; Morales et al., 2002).

Tymoviridae*/*Marafivirus

Purple passionfruit leaf deformation virus (PpLDV): PpLDV was characterized in purple passion fruit (*P. edulis*) in Colombia (Cardona, Restrepo, et al., 2022).

Bromoviridae*/*Cucumovirus

Cucumber mosaic virus (CMV): CMV was originally reported associated with PWD in yellow passion fruit (*P. edulis* Sims) plants growing in Australia (Magee, 1948; Taylor and Kimble, 1964). Other reports of CMV in *Passiflora* species were from the USA (Teakle et al., 1963), Japan (Yonaha et al., 1979), Brazil (Gioria et al., 2002) (Colariccio et al., 1987), Italy (Parrella and Sorrentino, 2009), Ecuador (Yeturu et al., 2017), and Colombia (Cardona, García, et al., 2022).

Kitaviridae*/*Cilevirus

Passion fruit green spot virus (PFSGV): PFSGV was originally reported in high incidence causing passion fruit green spot disease (PfGS) in orchards in several Brazilian states (Antonioli-Luizon et al., 2009; Jesus et al., 2017; Kitajima et al., 2003; Kitajima et al., 1997; Luizon, 2010; Moraes et al., 2006; Pires, 2022; Ramos-González et al., 2020; Tassi, 2018). Recently, PFSGV was found in *P. edulis* that displayed leaf mottling, rugose mosaic, and leaf distortion, chlorotic spots, yellowing, green spots in senescent leaves, and green vein banding growing in Colombia (Roy et al., 2022). *P. edulis* plants infected with this cilevirus showed necrotic lesions on leaves, stems, and fruits, green spots in mature fruits, senescent leaves, and death of the plants (Kitajima et al., 1997; Ramos-González et al., 2020). The virus is transmitted by the mites *Brevipalpus phoenicis* (Kitajima et al., 1997) and *Brevipalpus yothersi* (Pires, 2022; Tassi, 2018).

Citrus leprosis virus (CiLV-C2H, Hibiscus strain C2): CiLV-C2H hibiscus strain was found infecting *P. edulis* grown in residential properties and community gardens in Honolulu, Hawaii (Olmedo-Velarde et al., 2022). The passion fruit plants with CiLV-C2H showed symptoms of chlorotic spots on young leaves and green spots in senescing leaves, and were found be colonized by the mites *Brevipalpus yothersi*, identified as vector of CiLV-C2H (Olmedo-Velarde et al., 2022).

Rhabdoviridae/Cytorhabdovirus

Bean-associated cytorhabdovirus (BaCV) and citrus-associated rhabdovirus (CiARV) (species *Cytorhabdovirus caricae*): CiaRV was identified infecting yellow (*P. edulis* Sims) (Zhang, Huang, et al., 2020) and purple passion fruit (*P. edulis* × *P. edulis* f. favicarpa) (Huang et al., 2022) in China, while BaCV was found infecting different *Passiflora* spp. from the Germoplasm Bank "Flor da Paixão" and commercial fields in Distrito Federal, Brazil (Chapter 3) (Vidal, Felix et al. 2023). Symptomatic passion fruit plants from China exhibited yellow spots, while passion fruit plants from Brazil exhibited mosaic, yellow spots, blisters, vein banding, and

leaf deformation (Chapter 3) (Zhang, Huang et al. 2020; Vidal, Felix et al. 2023). BaCV is whitefly-transmitted to bean, soybean, and cowpea plants by the whitefly *Bemisia tabaci* (Pinheiro-Lima et al., 2020), but for passion fruit plants this event is still not documented.

***Rhabdoviridae*/ unclassified genus**

Passion fruit vein clearing virus (PVCV): PVCV is an unclassified rhabdovirus identified in passion fruit (*P. edulis* f. flavicarpa) in Brazil (Chagas et al., 1987; Kitajima and Crestani, 1985). Symptomatic plants showed short internodes, brittle leaves with a typical vein clearing, stunting, fruit deformation, and low yield production. (Kitajima and Crestani, 1985).

Passionfruit rhabdovirus (PRV**):** PRV was identified as a putative nucleorhabdovirus infecting passionfruit (*P. edulis*) in Australia (Pares et al., 1983). Passion fruit plants with PRV were also infected with PWV and symptoms apparently were similar to those induced by PWV infection.

Secoviridae*/*Nepovirus

Tomato ringspot virus (ToRSV): ToRSV was found in passion fruit plants (*P. edulis*) that showed mosaic and ring spot symptoms in Peru (Koenig and Fribourg, 1986). The virus was able to infect mechanically plants belonging to several families including Passifloracea, inducing mainly chlorotic or necrotic spots or rings symptoms (Koenig and Fribourg, 1986).

Virgaviridae*/*Tobamovirus

Maracuja mosaic virus (MarMV): The tobamovirus MarMVA was characterized in *P. edulis* Sims 'Flavicarpa' in Peru (Fribourg et al., 1987; Koenig and Fribourg, 1986; Song et al., 2006). The virus induced systemic infection causing mosaic and incomplete necrotic rings in *P. edulis* and *Nicotiana benthamiana*, respectively, and necrotic local lesions or leaf rugosity in plants of different families (Fribourg et al., 1987).

Passion fruit mosaic virus (PFMV)= maracuja mosaic virus Florida strain (MarMV-FL): PFMV was found in *P. incarnata* plants in a germplasm collection in Florida, USA (Song and Ryu, 2011; St Hill et al., 1992), and designated as MarMV-FL, a distinct strain of MarMV from Peru (Fribourg et al., 1987). This virus induced systemic mosaic and local lesions in passiflora plants and other alternative plant hosts inoculated mechanically (Song and Ryu, 2011; St Hill et al., 1992).

Hibiscus latent Fort Pierce virus (HLFPV): HLFPV is a putative tobamovirus found *P. edulis* without obvious symptoms of viral disease growing in a passion fruit orchard in China (Xie et al., 2022).

Betaflexiviridae/Carlavirus

Passiflora latent virus (PLV)= passiflora latent carlavirus: PLV is a virus associated with passion fruit tip necrosis (PTN) disease (Pares et al., 1997), which has been reported in different *Passiflora* spp. in Germany (Brandes and Wetter, 1963), United States (St Hill et al., 1992), Australia (Pares et al., 1997), Israel (Spiegel et al., 2007), New Zealand (Tang et al., 2008), South Korea (Choi and Ju, 2023), and China (Bao et al., 2023). The virus was also detected in *P. coerulea*, *P. suberosa*, *Passiflora* × Incense, *P. edulis* f. edulis (purple passion fruit), *P. edulis* f. flavicarpa (yellow passion fruit), *P. subpeltata*, and *P. tarminiana* (banana passion fruit) (Brandes and Wetter, 1963; Pares et al., 1997; Spiegel et al., 2007; St Hill et al., 1992; Tang et al., 2008). *P. tarminiana* from New Zealand showed chlorotic lesions on leaves, while purple passion fruit cv. 'Passion Dream' displayed foliar and fruit mosaic symptoms (Spiegel et al., 2007; Tang et al., 2008). The PLV was transmitted mechanically to *Chenopodium spp*. inducing local chlorotic lesions, systemic leaf chlorosis and necrosis, and mottling on upper leaves (Brandes and Wetter, 1963; Pares et al., 1997; Spiegel et al., 2007; St Hill et al., 1992; Tang et al., 2008). In *P. edulis f. edulis, P. edulis f. flavicarpa, P. foetida, and P. incarnata*, the virus induced and discreet systemic foliar mosaic symptoms (Spiegel et al., 2007; St Hill et al., 1992).

Cowpea mild mottle virus (CPMMV): CPPMV was found infecting different *Passiflora* spp. from the Germoplasm Bank "Flor da Paixão" in Distrito Federal, Brazil (Chapter 3). *Passiflora* plants with CPPMV exhibited mosaic, chlorotic mosaic, yellow spots, blisters, vein banding, yellow vein, and leaf deformation (Chapter 3) (Vidal et al., 2023). Co-infection with the cytorhabdovirus BaCV were identified in some these plants (Chapter 3) (Vidal et al., 2023). Both viruses are vectored by whitefly *Bemisia tabaci* in other crops (Pinheiro-Lima et al., 2020), but for passion fruit there is no data for the transmission.

Betaflexiviridae/Vitivirus

Grapevine virus A (GVA): The vitivirus GVA was identified in Brazil and induced ultrastructural changes mesophyll cells in asymptomatic *P. alata* (Galleti et al., 2006; Lombardi and Galleti, 2007).

Comoviridae*/*Fabavirus

Broad bean wilt virus 2 (BBWV2): BBWV2 is a fabavirus that has been detected in passionfruit (*P. edulis* Sims) in Japan (Kobayashi, 2004; Nakasato et al., 2020; Yonaha et al., 1993)

Solemoviridae/Polerovirus

Cucurbit aphid-borne yellows virus (CABYV): The first occurrence of CABYV in passion fruit was reported in Northeastern Brazil (Vidal, Sanches et al. 2018; Vidal, Lacorte et al. 2023). Later, the virus was found in passion fruit from China (Zhang, Zhao, et al., 2020). In Brazil,

passion fruit plants evaluated showed severe symptoms of mosaic, blisters, and leaf deformation, and plants were identified in mixed infection with CABMV (Vidal et al., 2018), while in China, passion fruit plants showed mottling and mosaic (Zhang, Zhao, et al., 2020).

Closteroviridae/Crinivirus

Lettuce chlorosis virus (LCV): The crinivirus LCV was reported to infect *Passiflora* spp. plants in Brazil (Vidal et al., 2021). The virus was found in *P. auriculata* and *P. alata* plants in the Germplasm Bank "Flor da Paixão" in Distrito Federal, and in *P. edulis* in a commercial field in Bahia state. Plants were found in mixed infection with CABYV and showed symptoms like slight yellowing, yellow dots, mosaic, blistering, and leaf deformation (Vidal et al., 2021). Recently, the virus was found in mixed infection with the rhabdovirus BaCV, and the potyvirus CABMV in passion fruit plants from a commercial field in Distrito Federal (Chapter 3) (Vidal et al., 2023).

Narnaviridae*/*Mitovirus

Passiflora mitovirus-like (PMV): PMV was identified in different species of *Passiflora* from Germplasm Bank "Flor da Paixão" in Distrito Federal, Brazil (Santos et al., 2020).

Unclassified family/Unclassified genus

Purple granadilla mosaic virus (PGMV) =Purple passion fruit mosaic virus: PGMV or purple passion fruit mosaic virus is an unclassified virus identified in purple granadilla (*P. edulis*) in São Paulo, Brazil (Chagas et al., 1984). Infected plants exhibit mild or line pattern mosaic on the leaves, vein clearing, irregular chlorotic bands on the veins, and fruits from infected plants are smaller, deformed, and woody (Fischer and Rezende, 2008). PGMV was sap transmitted to purple granadilla and yellow passion fruit, and vectored by chrysomelid beetle *Diabrotica speciosa* (Fischer and Rezende, 2008; Kitajima, 2020).

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Chapter 2

Occurrence of lettuce chlorosis virus in *Passiflora* spp. in Brazil

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SHORT COMMUNICATION

Occurrence of lettuce chlorosis virus in *Passiflora* spp. in Brazil

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Lettuce chlorosis virus (LCV, *Crinivirus*, *Closteroviridae*) is a single-stranded, positive-sense RNA virus with a bipartite genome encapsidated in filamentous particles of about 750 to 800 nm, and is transmitted by the whitefly *Bemisia tabaci* (Liu et al. 2000; McLain et al. 1998).

LCV was first identified and characterized infecting lettuce (*Lactuca sativa* L.) in California (USA) (Duffus et al. 1996; McLain et al. 1998) and since then has been identified causing disease in diverse crops including vegetables, fruit trees, ornamental, and medicinal plants in several countries. Natural infections of LCV have been identified in lettuce, beets, papaya, and cilantro in the USA (Alabi et al. 2017, 2019; Duffus et al. 1996; Wisler et al. 1997), in papaya in Mexico (Alcalá-Briseño et al. 2020), beans in Spain (Ruiz et al. 2013), cannabis in Israel (Hadad et al. 2019), and tomato, tobacco, and periwinkle in China (Tian et al. 2017; Zhang et al. 2017; Zhao et al. 2018). LCV was also recently reported infecting periwinkle in Brazil (Favara et al. 2019).

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Brazil is the leading global producer of passion fruit and one of the most important diversity centers of *Passiflora* species. The *Passiflora* germplasm bank "Flor da Paixão" (BAG-FP) is maintained at Embrapa Cerrados, in Brasília, DF, Brazil, and is intended for the conservation, characterization, and research on *Passiflora* genetic resources, including passionflower breeding programs. In recent years, efforts have been carried out to evaluate the viral diversity in cultivated passion fruit in Brazil (Ferreira et al. 2010; Fontenele et al. 2018; Mituti et al. 2019; Ramos-González et al. 2020; Rodrigues et al. 2019; Spadotti et al. 2019; Vidal et al. 2018). Currently, the main disease of viral etiology associated with passion fruit globally is the passionfruit woodiness disease (PWD). In Brazil, the high incidence of PWD is a serious problem that has caused production losses of passion fruit and is associated with cowpea aphid-borne mosaic virus (CABMV) (Cerqueira-Silva et al. 2014; Nascimento et al. 2006). However, scant information is available on other viruses infecting cultivated and native *Passiflora* species in Brazil and worldwide.

Diverse virus-like symptoms, including mosaic, yellowing, yellow spots, blisters, and leaf deformation, were observed in *Passiflora* accessions maintained at BAG-FP. In the present study, we analyzed symptomatic leaf samples of 21 passionflowers collected from BAG-FP in 2017 by high throughput sequencing (HTS) to identify possible viral infections in these plants.

The double-strand RNA (dsRNA) fraction was isolated from total RNA of the samples using a protocol that combines TRIzol™ Reagent (Invitrogen, USA) and a modified method described by Alves-Freitas et al. (2019), Castillo et al. (2011), and Valverde et al. (1990) for dsRNA extraction. About 100 mg of leaf tissue was macerated in liquid nitrogen, homogenized with TRIzol® Reagent (1 mL), and

Occurrence of lettuce chlorosis virus in *Passiflora* spp. in Brazil

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2.1. Abstract

Virus-like symptoms including mosaic, yellowing, yellow spots, blisters, and leaf deformation were observed in *Passiflora* accessions from the germplasm bank "Flor da Paixão" (BAG-FP) maintained at Embrapa Cerrados, Distrito Federal, Brazil. High throughput sequencing analysis of dsRNA extracted from symptomatic leaves of *Passiflora* spp. collected from BAG-FP revealed

the presence of lettuce chlorosis virus (LCV, *Crinivirus*, *Closteroviridae*) and cowpea aphid-borne mosaic virus (CABMV, *Potyvirus*, *Potyviridae*). LCV was detected in *P. auriculata* and *P. alata*, in both cases in mixed infection with CABMV. The occurrence of LCV was also investigated in samples collected from passion fruit commercial fields in Bahia state, Brazil. LCV was detected in a *P. edulis* plant from Dom Basílio, also in mixed infection with CABMV. To our knowledge, this is the first report of the natural occurrence of LCV in *Passiflora* species and the first report of CABMV infection in *P. auriculata*.

Keywords: HTS, LCV, passion fruit, CABMV

2.2. Main text

Lettuce chlorosis virus (LCV, *Crinivirus*, *Closteroviridae*) is a single-stranded, positive-sense RNA virus with a bipartite genome encapsidated in filamentous particles of about 750 to 800 nm, and is transmitted by the whitefly *Bemisia tabaci* (McLain, Castle et al. 1998; Liu, Wisler et al. 2000).

LCV was first identified and characterized infecting lettuce (Lactuca sativa L.) in California (USA) (Duffus, Liu et al. 1996; McLain, Castle et al. 1998) and since then has been identified causing disease in diverse crops including vegetables, fruit trees, ornamental, and medicinal plants in several countries. Natural infections of LCV have been identified in lettuce, beets, papaya, and cilantro in the USA (Duffus, Liu et al. 1996; Wisler, Duffus et al. 1997; Alabi, Al Rwahnih et al. 2017; Alabi, Gaytán et al. 2019), in papaya in Mexico (Alcalá-Briseño, Casarrubias-Castillo et al. 2020), beans in Spain (Ruiz, Simón et al. 2013), cannabis in Israel (Hadad, Luria et al. 2019), and tomato, tobacco, and periwinkle in China (Tian, Tian et al. 2017; Zhang, Zhang et al. 2017; Zhao, Zhu et al. 2018). LCV was also recently reported infecting periwinkle in Brazil (Favara, Camelo-García et al. 2019).

Brazil is the leading global producer of passion fruit and one of the most important diversity centers of *Passiflora* species. The *Passiflora* germplasm bank "Flor da Paixão" (BAG-FP) is maintained at Embrapa Cerrados, in Brasília, DF, Brazil, and is intended for the conservation, characterization, and research on *Passiflora* genetic resources, including passionflower breeding programs. In recent years, efforts have been carried out to evaluate the viral diversity in cultivated passion fruit in Brazil (Ferreira, Barros et al. 2010; Fontenele, Abreu et al. 2018; Vidal, Sanches et al. 2018; Mituti, Spadotti et al. 2019; Rodrigues, Rocha Sobrinho et al. 2019; Spadotti, Bello et al. 2019; Ramos-González, Santos et al. 2020). Currently, the main disease of viral etiology associated with passion fruit globally is the passionfruit woodiness disease (PWD). In Brazil, the high incidence of PWD is a serious problem that has caused ~~constant~~ production losses of passion fruit and is associated with cowpea aphid-borne mosaic virus potyvirus (CABMV) (Nascimento, Santana et al. 2006; Cerqueira-Silva, Conceição et al. 2014). However, scant information is

available on other viruses infecting cultivated and native *Passiflora* species in Brazil and worldwide.

Diverse virus-like symptoms, including mosaic, yellowing, yellow spots, blisters, and leaf deformation, were observed in *Passiflora* accessions maintained at BAG-FP. In the present study, we analyzed symptomatic leaf samples of 21 passionflowers collected from BAG-FP in 2017 by high throughput sequencing (HTS) to identify possible viral infections in these plants.

The double-strand RNA (dsRNA) fraction was isolated from total RNA of the samples using a protocol that combines TRIzol™ Reagent (Invitrogen, USA) and a modified method described by Alves-Freitas et al. (2019), Castillo et al. (2011), and Valverde et al. (1990) for dsRNA extraction. About 100 mg of leaf tissue was macerated in liquid nitrogen, homogenized with TRIzol® Reagent (1 mL), and incubated at room temperature (5 min). Next, chloroform (0.2 ml) was added, mixed, and samples were centrifuged at 12,000 × g at 4°C for 15 min. The aqueous phase (0.5 ml) was transferred to a microtube, and the chloroform step was repeated. The aqueous phase was transferred to a microtube, and absolute ethanol was added to a final concentration of 16%.

The dsRNA was isolated from total cellular RNA by cellulose affinity chromatography using a custom assembled micro-spin column. The micro-spin column was assembled by piercing a small hole in the bottom of a capless 0.6 ml microtube, adding 30 mg of glass wool (medium fiber, CAS 65997173 - Dinâmica) and 120 mg of cellulose (cellulose fibers, medium, C6288-SIGMA) at the bottom of the tube. The micro-column was placed in a 2 ml microtube and centrifuged at 5,000 × g (3 s). The micro-column was equilibrated with wash buffer [0.5 ml of 1×STE (0.05 M Tris-base; 0.1 M NaCl; 0.001 M EDTA)] containing 16% ethanol and centrifuged at 5,000 × g (3 s). The sample extract was transferred to the micro-column and centrifuged at 5,000 × g (3 s).

The flow-through extract was used for RNA isolation according to TRIzol® Reagent guide for the steps precipitation, wash, solubilization, and RNA elution.

The micro-spin column was transferred to a 2 ml microtube, washed three times with wash buffer (0.5 ml), and centrifuged at 5,000 × g (3 s). The dsRNA was eluted with 1×STE buffer (0.5 ml) by centrifuging at 10,000×g (6 s). Then, the dsRNA was precipitated with isopropanol (0.6 ml), incubated at -20°C (20 min), and centrifuged at 13,000 × g at 4°C (20 min). The dsRNA pellet was air-dried, resuspended with Milli-Q RNase-free water (50 µl), and stored at -20°C. The quality and quantity of the RNA samples were assessed by spectrophotometry (Nanodrop ND-1000, Thermo Fisher Scientific, USA).

The extracted dsRNA from the 21 samples was pooled (4 µl from each sample) into a single tube. To achieve the necessary amount of RNA for sequencing (100 µg in total), 2 µl of total

RNA from ten samples (samples 1621, 1613, 1598, 1632, 1633, 1605, 1617, 1602, 1595, 1638) was added for library preparation. The pooled RNA sample was preserved in RNAstable® (Biomatrica, USA). The sequencing library (BAG1-PF) was prepared using Illumina TruSeq Stranded Total RNA kit, and plant ribosomal RNA was depleted using the Ribo-Zero Plant kit. The library was sequenced on an Illumina HiSeq 2500 sequencer (Macrogen Inc., South Korea).

A total of 10,741,018 pair-end reads (100 bp) were obtained for BAG1-PF. The raw reads were checked for quality using FastQC (Andrews 2010), trimmed using Trimmomatic (Bolger, Lohse et al. 2014), and *de novo* assembled using SPAdes assembler (Bankevich, Nurk et al. 2012) with k-mer = 66. Contigs [>200 nucleotides (nt)] were analyzed using BLASTn and BLASTx against the NCBI virus database. Five contigs with identities to LCV and 46 contigs that corresponded to cowpea aphid-borne mosaic virus (CABMV, *Potyvirus*, *Potyviridae*) were identified (Table 1). Two contigs of 306 and 403 nts were related to LCV-RNA1 (ORF3/p23). Contig 403 and 306 had the highest sequence identity of 99.41% and 98.94%, respectively, with LVC-BR from periwinkle from Brazil (MN216391). The other three contigs of 426, 367, and 240 nt were related to LCV-RNA2. Contig 426 (ORF8, coat protein minor) had a sequence identity of 99.76% to isolates LCV-BR (periwinkle MN216392), LCV-PTX (papaya, KY271956), and LCV-California (lettuce, FJ380119). Contig 367 (ORF9/p27) had the highest sequence identity of 98.78% with equivalent sequences of LCV-BR, LCV-PTX, and LCV-California. Contig 240 (ORF3/HSP70h) is identical (100%) to isolates LCV-BR, LCV-California, and LCV-PTX.

Table 1. Summary of HTS de novo assembled contigs with similarity to lettuce chlorosis virus (LCV) and cowpea aphid-borne yellow virus (CABMV) and results of mapping the short reads to reference sequences.

Virus	No. of contigs	Length (nt)	BLASTn		Map to reference		
			Coverage (%)	ID (%)	Coverage (%)	ID (%)	No. of mapped reads
LCV-RNA1	2	306-403	92-97	98-99	61.6	92.8	2.279[a]
LCV-RNA2	3	240-426	90-97	98-100	70.6	93.3	4.529[b]
CABMV	46	200- 1443	90-99	86-95	97	94.2	91.147[c]

Reference sequences MN216391[a], MN216392[b], and HQ880243[d].ID: identity.

Mapping of the short reads to reference sequences for LCV (MN216391, MN216392) and CABMV (HQ880243) was done with Geneious Prime® 2019.2.3 using default parameters (Table 1). 2.279 reads mapped to LCV-BR-RNA1, encompassing 61.6% of its sequence, whereas 4.529 short reads aligned to LCV-BR-RNA2, covering 70.6% of the sequence. The LCV-BR genome covered by the short reads mapping had >92.8% sequence identity for both RNA1 and RNA2. 91.147 reads from BAG1-PF mapped to CABMV- MG-Avr covering 97% of the genome with sequence identity >94.2%.

To validate the HTS results and verify the presence of LCV in individual *Passiflora* spp. samples, virus-specific primers based on the contigs were designed. RT-PCR was done using total RNA from the samples and SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, USA) with primers Bag1226lt317LCV16F (5'GACGGGAGATTCCAATTGTGTAAA3)/Bag1226lt317LCV260R (5'TGAACCATTTTGAAGCAATTCTCCA3) that amplify the partial RNA1 p8/p23 genes (244 nt), and LCVRNA22793F (5'AAGGTTCAGATCCGTTCATCTTGTA3')/ LCVRNA23997R (5'CTTCCACGCATTCTCTGAATAAGTC3') that amplifies the partial RNA2-HSP70h/p6.4/p60 genes (1.199 nt). All RT-PCR products were visualized by electrophoresis in agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, USA) (Figure. 1).

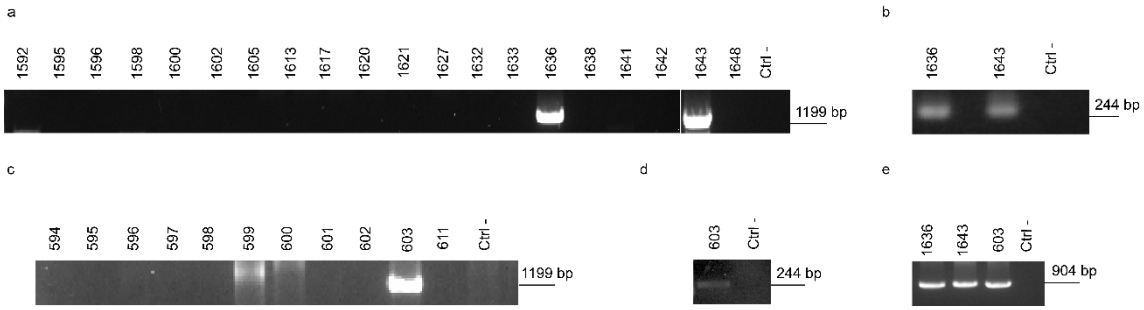

Figure 1. Detection of LCV and CABMV in Passiflora spp. plants. Agarose gel electrophoresis of RT-PCR LCV-RNA2 amplicons in all Passiflora spp. samples (a) BAG1-PF and (c) Dom Basílio, LCV-RNA1 for the positive samples to LCV-RNA2 of (b) BAG1-PF and (d) Dom Basílio, and CABMV RT-PCR detection on the positive samples to LCV (e). Ctrl - = negative control

LCV was detected in one sample of *P. auriculata* (sample 1636) and one of *P. alata* sample (sample 1643) from the 21 examined BAG1-PF passionflower samples (9.5% incidence) (Figure 1a and b). The LCV-RNA1 and RNA2 RT-PCR amplicons for both samples were recovered, purified, and Sanger sequenced.

The pairwise identities of the sequences were calculated using SDT v1.2 (Muhire, Varsani et al. 2014) with MUSCLE alignment (Edgar 2004) using analogous sequences of other LCV isolates. Analysis of genome-partial pairwise identities revealed that the LCV-RNA1 and LCV-RNA2 from *P. auriculata* (MN564796, MN564795) and *P. alata* (MN564797-MN564798) share >96% nucleotide identity for both RNAs with LCV isolates of lettuce (FJ380118, FJ380119) and papaya (KY271955, KY271956) from the USA, and the isolate of periwinkle (MN216391, MN216392) from Brazil. The LCV isolates described to date display 81-99% (RNA1) and 90-100% (RNA2) nucleotide identities in the genome regions used to characterize LCV from passionflower.

The occurrence of LCV was also investigated in 11 samples collected in 2015 from passion fruit commercial fields (three months after planting) in Bahia state, the current largest producer of the fruit in the country. LCV was detected in one *P. edulis* (sample 603) plant from Dom Basílio

(Figure 1 d). RT-PCR products for LCV were obtained and sequenced. SDT analysis between the sequence of the isolate from *P. edulis* from Dom Basílio and isolates from *P. auriculata* (MN564795, MN564796) and *P. alata* (MN564797, MN564798) from BAG-PF showed an identity of >98.5% for LCV-RNA1 and >97.9% for LCV-RNA2, indicating that there are very similar isolates of LCV occurring in passionflower plants from different regions of the country.

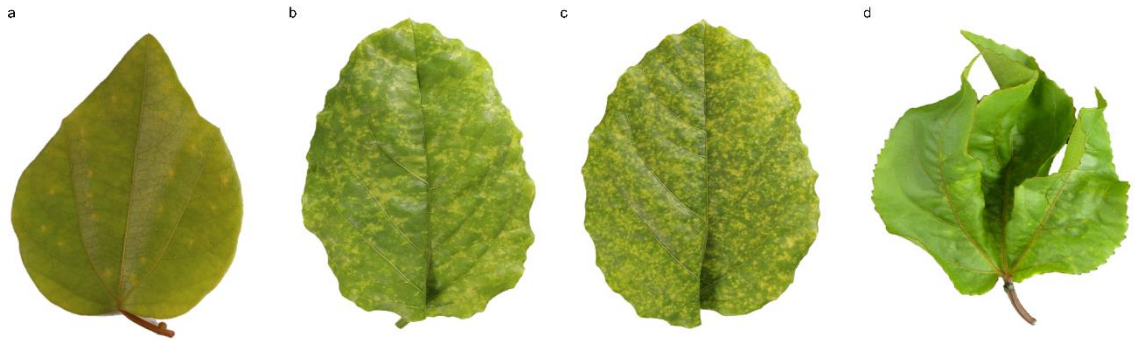

Figure 2. Symptoms observed in *Passiflora* spp. infected by LCV and CABMV. Samples from Germplasm bank "Flor da Paixão": (a) *P. auriculata*: slight yellowing; (b) *P. alata*: mosaic and leaf deformation; (c) *P. alata*: mosaic, yellow dots. Samples from a commercial field in Dom Basílio: (d) *P. edulis*: blistering and leaf deformation.

LCV has a wide host range and has been identified, causing disease in many crops, including vegetables, fruit trees, ornamental, and medicinal plants in several countries. Our results expand LCV hosts to the Passifloraceae plant family, including the wild *P. auriculata* species and the cultivated passionfruit *P. edulis* and *P. alata*.

Considering that 46 contigs corresponding to CABMV were identified in BAG1-PF library, the presence of this potyvirus was also evaluated in the samples infected by LCV. Primers CABMVLNJP2492F (5'GGTTCGTGATGTTTTGGTGCC3')/ CABMVLNJP3373R (5'CAAAAAGCACGCACTCACAAATC3') targeting the partial HC-Pro/p3 genes were designed based in the contigs from HTS BAG1-PF and the sequence from CABMV isolate MG-Avr from passionfruit (HQ880243), and used in RT-PCR to detect CABMV. Mixed infections of LCV and CABMV in the samples from BAG-PF and Dom Basílio were confirmed (Figure 1e). All CABMV RT-PCR products were Sanger sequenced. CABMV isolates from *P. auriculata* (sample 1636), *P. alata* (sample 1643), and *P. edulis* (sample 603) showed a range of 80-90% sequence identity to CABMV isolates already characterized.

Virus-like symptoms such as slight yellowing, mosaic, leaf deformation, and yellow spots were visualized in the samples positive for LCV and CABMV (Figure 2). Passion fruit woodiness disease (PWD) is the main viral disease that affects passion fruit worldwide, and in Brazil, it is induced by CABMV (Nascimento, Santana et al. 2006; Cerqueira-Silva, Conceição et al. 2014). To minimize the losses caused by PWD, the passion fruit fields that were cultivated for 4-5 years, are now planted yearly, turning passion fruit to an annual crop (Sampaio, Scudeller et al. 2008). The Dom Basílio commercial field sample was three months old when sampled and already

exhibited virus-like symptoms (Figure 2). Mixed infections of viruses in plants can generate more severe symptoms of a disease caused by the synergistic interaction between the viruses present (Moreno and López-Moya 2020). The effects induced by LCV in passionfruit are unknown and maybe being neglected by the presence of CABMV.

LCV is likely present in other regions of Brazil, infecting *Passiflora* spp. as well as other hosts. Prior to this report of LCV in commercial passion fruit (*P. edulis*) field in Bahia state, Northeast of Brazil, and in the Germplasm bank "Flor da Paixão", BAG-FP (*P. alata* and *P. auriculata*) situated in in the Midwest of the country, LCV had been reported infecting periwinkle from São Paulo state, Southeast of Brazil (Favara, Camelo-García et al. 2019).

To our knowledge, this is the first report of LCV infecting *Passiflora* spp. in the world and the first report of CABMV in *P. auriculata*. The epidemiology and actual impact of the crinivirus LCV in the passion fruit production in Brazil needs to be evaluated in additional studies.

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Chapter 3

Occurrence of bean-associated cytorhabdovirus and cowpea mild mottle virus infecting cultivated and wild *Passiflora* spp. in Brazil

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ABSTRACT

Bean-associated cytorhabdovirus (BaCV) is a strain of *Cytorhabdovirus caricae* (genus *Cytorhabdovirus*, family *Rhabdoviridae*) identified in Brazil, in bean and soybean plants, in mixed infection with cowpea mild mottle virus (CPMMV, genus *Carlavirus*, family *Betaflexiviridae*). Ensuing the report of citrus-associated rhabdovirus (CiaRV — a citrus strain of *Cytorhabdovirus caricae*) infecting passion fruit in China, we speculated that BaCV, and possibly CPMMV, could be present in passion fruit crops in Brazil. This was tested by screening cultivated and wild *Passiflora* spp., and hybrid by RT-PCR and amplicon sequencing, which confirmed the presence of BaCV and CPMMV in several *Passiflora* spp. A total of 114 surveyed plants were collected in multiple locations from 2016 to 2021 and BaCV was identified in 4 of 19 cultivated *P. edulis* plants from a commercial field in Distrito Federal, Central Brazil. Moreover, while BaCV was detected in 4 of the 55 *Passiflora* accessions sampled at the Germplasm Bank "Flor da Paixão" (BAG-FP), CPMMV was identified in 14 of these 55 *Passiflora* spp. and hybrid accessions. This is the first record of BaCV in *P. edulis* in Brazil, and CPMMV and BaCV in other wild *Passiflora* species in the world. To investigate the potential mixed infection of BaCV and CPMMV-infected plants and other viruses previously identified infecting *Passiflora* spp. in Brazil, RT-PCR was conducted with specific primers for lettuce chlorosis virus (LCV, genus *Crinivirus*, family *Closteroviridae*) and cowpea aphid-borne mosaic virus (CABMV, genus *Potyvirus*, family *Potyviridae*). Most of the inspected plants had a mixed infection with these viruses. Our results warrant further studies to evaluate these viruses' epidemiology, impacts, and interactions in the passion fruit crop.

Passion fruit (genus *Passiflora*, family Passifloraceae) is a tropical plant cultivated in many countries. The major center of diversity for the *Passiflora* genus is Brazil, which is also the main producer of passion fruit. The pulp is consumed fresh or used in the food, cosmetic, and phytotherapy industries (Faleiro et al., 2019, 2020).

In recent years, virus infection has reduced the yield and longevity of passion fruit orchards in Brazil. The most important viral disease that affects passion fruit in Brazil is passionfruit woodiness disease (PWD), which is associated exclusively with the potyvirus cowpea aphid-borne mosaic virus (CABMV, genus *Potyvirus*, family *Potyviridae*) (Cerqueira-Silva et al., 2014). However, several other viruses have emerged in passion fruit crops around the world and in Brazil, including passiflora

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3.1. Abstract

Bean-associated cytorhabdovirus (BaCV) is a strain of *Cytorhabdovirus caricae* (genus *Cytorhabdovirus*, family *Rhabdoviridae*) identified in Brazil, in bean and soybean plants, in mixed infection with cowpea mild mottle virus (CPMMV, genus *Carlavirus*, family *Betaflexiviridae*). Ensuing the report of citrus-associated rhabdovirus (CiaRV — a citrus strain of *Cytorhabdovirus caricae*) infecting passion fruit in China, we speculated that BaCV, and possibly CPMMV, could be present in passion fruit crops in Brazil. This was tested by screening cultivated and wild *Passiflora* spp., and hybrid by RT-PCR and amplicon sequencing, which confirmed the presence of BaCV and CPMMV in several *Passiflora* spp. A total of 114 surveyed plants were

collected in multiple locations from 2016 to 2021 and BaCV was identified in 4 of 19 cultivated *P. edulis* plants from a commercial field in Distrito Federal, Central Brazil. Moreover, while BaCV was detected in 4 of the 55 *Passiflora* accessions sampled at the Germplasm Bank "Flor da Paixão" (BAG-FP), CPMMV was identified in 14 of these 55 *Passiflora* spp. and hybrid accessions. This is the first record of BaCV in *P. edulis* in Brazil, and CPMMV and BaCV in other wild *Passiflora* species in the world. To investigate the potential mixed infection of BaCV and CPMMV-infected plants and other viruses previously identified infecting *Passiflora* spp. in Brazil, RT-PCR was conducted with specific primers for lettuce chlorosis virus (LCV, genus *Crinivirus*, family *Closteroviridae*) and cowpea aphid-borne mosaic virus (CABMV, genus *Potyvirus*, family *Potyviridae*). Most of the inspected plants had a mixed infection with these viruses. Our results warrant further studies to evaluate these viruses' epidemiology, impacts, and interactions in the passion fruit crop.

Keywords: BaCV, CPMMV, LCV, CABMV, passion fruit

3.2. Main text

Passion fruit (genus *Passiflora*, family *Passifloraceae*) is a tropical plant cultivated in many countries. The major center of diversity for the *Passiflora* genus is Brazil, which is also the main producer of passion fruit. The pulp is consumed fresh or used in the food, cosmetic, and phytotherapy industries (Faleiro et al. 2019, 2020).

In recent years, virus infection has reduced the yield and longevity of passion fruit orchards in Brazil. The most important viral disease that affects passion fruit in Brazil is passionfruit woodiness disease (PWD), which is associated exclusively with the potyvirus cowpea aphid-borne mosaic virus (CABMV, genus *Potyvirus*, family *Potyviridae*) (Cerqueira-Silva et al. 2014). However, several other viruses have emerged in passion fruit crops around the world and in Brazil, including passiflora edulis symptomless virus (PeSV, genus *Roymovirus*, family *Potyviridae*) (Jover-Gil et al. 2018), passion fruit chlorotic mottle virus (PCMoV, genus *Citlodavirus*, family *Geminiviridae*) (Fontenele et al. 2018), sida mottle Alagoas virus (SiMAV, genus *Begomovirus*, family *Geminiviridae*) (Mituti et al. 2019), *Badnavirus passiflorae* (genus *Badnavirus*, Family *Caulimoviridae*) (Sepúlveda et al. 2021), melochia yellow mosaic virus (MelYMV, genus *Begomovirus*, family *Geminiviridae*) (Spadotti et al. 2019), lettuce chlorosis virus (LCV, genus *Crinivirus*, family *Closteroviridae*) (Vidal et al. 2021), cucurbit aphid-borne yellows virus (CABYV, genus *Polerovirus*, family *Solemoviridae*) (Vidal et al. 2018), hibiscus latent fort pierce virus (HLFPV, genus *Tobamovirus*, family *Virgaviridae*) (Xie et al. 2022), citrus-associated rhabdovirus (CiaRV, genus *Cytorhabdovirus*, family *Rhabdoviridae*) (Zhang et al. 2020), and passion fruit green spot virus (PfGSV, genus *Cilevirus*, family *Kitaviridae*) (Ramos-González et al. 2020).

Bean-associated cytorhabdovirus (BaCV) is a strain of *Cytorhabdovirus caricae* (genus *Cytorhabdovirus*, family *Rhabdoviridae*), a negative single-strand RNA virus. BaCV is transmitted by the whitefly *Bemisia tabaci* MEAM1 to bean and soybean plants in Brazil, and was identified frequently in mixed infection with the positive single-strand RNA virus cowpea mild mottle virus (CPMMV, genus *Carlavirus*, family *Betaflexiviridae*) (Alves-Freitas et al. 2019; Pinheiro-Lima et al. 2020, 2023). BaCV has also been reported in common beans in Mexico (Chiquito-Almanza et al., 2021). Other strains of *C. caricae* include papaya virus E (PpVE), reported in papaya (Medina-Salguero et al. 2019) and three wild Fabaceae in Ecuador (Cornejo-Franco et al. 2021), and citrus-associated rhabdovirus (CiaRV) described in citrus, paper bush, and passion fruit in China (Zhang et al. 2020). Some of the symptoms we previously observed in passion fruit plants in Brazil resembled those described for CiaRV-infected passion fruit in China (Zhang et al. 2020). Hence, in this research, we investigated the occurrence of BaCV and its frequent cohort CPMMV in cultivated passion fruit and in wild *Passiflora* spp. and hybrid plants in Brazil.

Samples analyzed in this study were collected during field surveys conducted between 2016 and 2021 on commercial orchards from different locations in Brazil. A total of 114 samples of symptomatic and asymptomatic passion fruit plants were collected in the years of 2016, in Cuité (*n*=24), Paraíba; 2017, in Planaltina (*n*=19) and 2018, in Brazlândia (*n*=20), Distrito Federal; and 2021, in Seropédica (*n*=51), Rio de Janeiro. In addition, *Passiflora* spp. and hybrid accessions from the Germplasm Bank "Flor da Paixão" — BAG-FP (Planaltina, Distrito Federal) collected in 2017 (*n*=37) and 2019 (*n*=18) were also analyzed. Virus-like symptoms including mosaic, blisters, yellow spots, yellow vein, and leaf deformation were observed on the passion fruit plants (Figure 1).

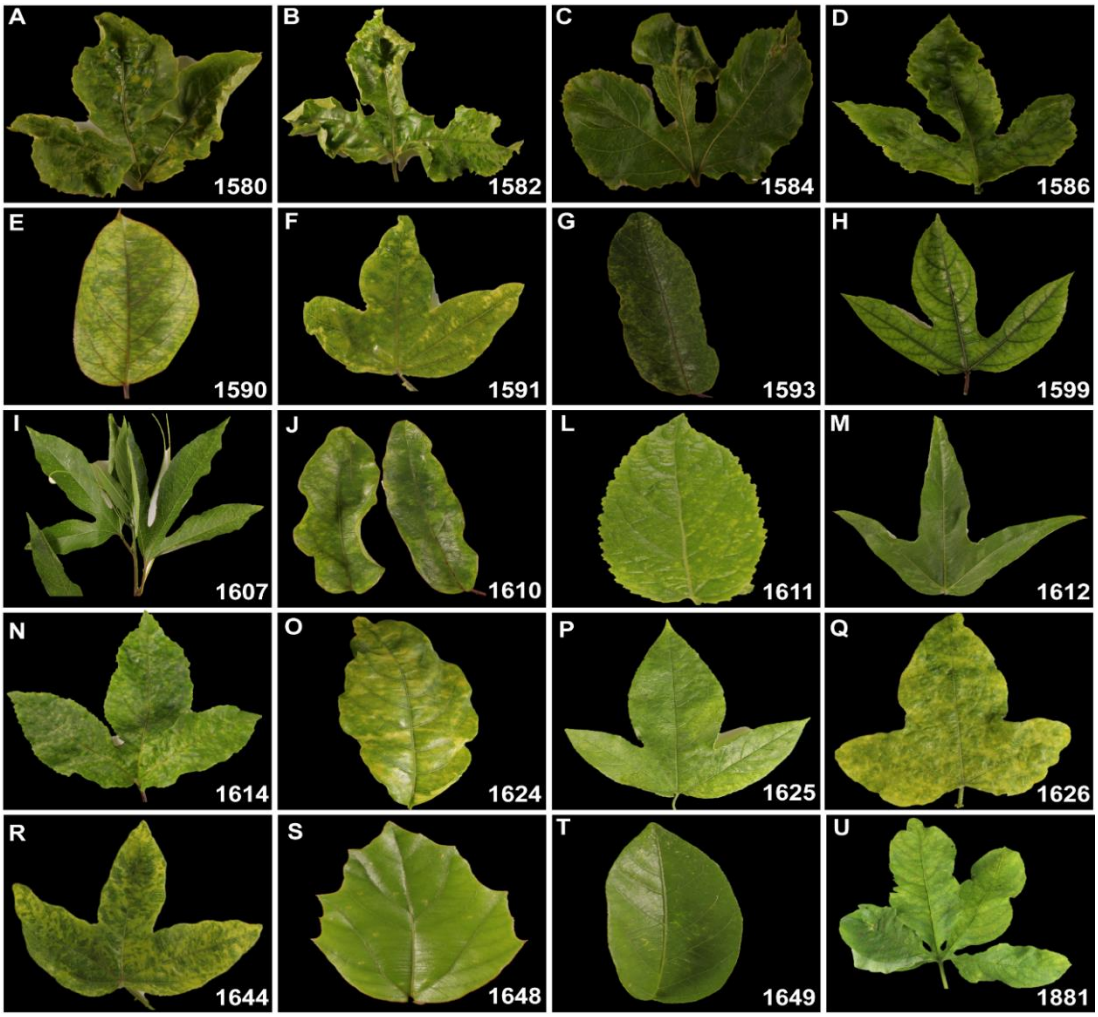

Figure 1. Symptoms displayed by *Passiflora* spp. and hybrid plants analyzed in this research. Plants collected at Planaltina commercial field: (A) 1580- *P. edulis*: mosaic, blisters, leaf deformation; (B) 1582- *P. edulis*: mosaic, blisters, leaf deformation; (C) 1584- *P. edulis*: yellow spots, leaf deformation; (D) 1586- *P. edulis*: yellow spots, blisters, leaf deformation. Plants collected at the BAG-Flor da Paixão: (E) 1590- *P. maliformis*: mosaic; (F) 1591- *P. eichleriana*: chlorotic mosaic, leaf deformation; (G) 1593- *P. galbana*: mosaic; (H) 1599- *P. cacao*: mosaic, vein banding; (I) 1607- *P. incarnata*: mosaic; (J)1610- *P. galbana*: mosaic, leaf deformation; (L) 1611- *P. malacophylla*: yellow spots; (M) 1612- *P. suberosa*: mosaic; (N) 1614- *P. edulis*:, mosaic, blisters, leaf deformation; (O) 1624- *P. riparia*: mosaic, leaf deformation; (P) 1625- *P. hatschbachii*: mosaic; (Q) 1626- *P. gardneri*: mosaic, blisters, leaf deformation; (R) 1644- *P. eichleriana* x *P. gibertii*: mosaic, leaf deformation; (S) 1648- *P. rufa*: asymptomatic; (T) 1649- *Passiflora* spp.: yellow vein; (U) 1881- *P. cincinnata*: mosaic.

Leaf tissue (100 mg) was pulverized with liquid nitrogen, and total RNA was extracted using TRIzol™ Reagent (Invitrogen, USA), following the manufacturer's recommendations. For the detection of virus-specific amplicons using two-step RT-PCR assay, total RNA was used in reverse transcription (RT) assays with oligo-DT and random primers and M-MLV Reverse Transcriptase (Promega, USA). In the second step, the cDNA was amplified by PCR with Taq DNA Polymerase, recombinant (5 U/µL) (Thermo Fisher Scientific, USA) according to the manufacturers' instructions. For the amplification of BaCV, the set of primers BaCV-6491F/BaCV-7178R that amplify a partial G/L gene (678 nt) (Alves-Freitas et al. 2019) was used

in the PCR assays. To obtain CPMMV-specific amplicons, the primers CPMMVB22095F (5'-AATCCTGGCTTTGAACTCGGA-3')/CPMMVB22535R (5'-ATCGATCAGAGTATTTGAAGCCC-3') that amplify the partial CP gene (457 nt) were designed based on CPMMV:BR:GO:10:4 (GenBank KF554101) sequence and used for the PCR. All RT-PCR products were visualized by electrophoresis in agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, USA).

BaCV was identified in 7.2% (4/55) of the plants from BAG-FP, three *Passiflora* spp. and one hybrid plant (*P. cacao*, *P. gardneri*, *P. edulis*, and *P. eichleriana* x *P. gibertii);* and in 21% (4/19) of *P. edulis* plants from Planaltina commercial field (Table 1). CPMMV was detected in 25.4% (14/55) of the *Passiflora* accessions from the BAG-FP (*P. eichleriana* x *P. gibertii* [*n* = 2], P. galbana [*n* = 2], *P. gardneri*, *P. hatschbachii*, *P. incarnata*, *P. malacophylla*, *P. maliformis*, *P. riparia*, *P. rufa*, *Passiflora* spp., *P. suberosa*, and *P. cincinnata*). BaCV and CPMMV co-infection was detected in *P. gardneri* and one *P. eichleriana* x *P. gibertii* hybrid (Table 1).

Table 1. Samples of *Passiflora* spp. and hybrid tested positive for bean-associated cytorhabdovirus (BaCV), cowpea mild mottle virus (CPMMV), lettuce chlorosis virus (LCV), and cowpea aphid-borne mosaic virus (CABMV) in this study and GenBank accession of virus-specific products sequenced.

Sample number	Species	Virus (GenBank accession)
1580[a]	*P. edulis*	BaCV (OL780853), LCV (ON469824 -RNA1; ON46982-RNA2)
1582[a]	*P. edulis*	BaCV, CABMV
1584[a]	*P. edulis*	BaCV (OL780854)
1586[a]	*P. edulis*	BaCV (OL780855), LCV, CABMV
1588[a]	*P. edulis*	LCV, CABMV
1590[b]	*P. maliformis*	CPMMV, CABMV
1591[b]	*P. eichleriana x P. gibertii*	BaCV (OL780849), CPMMV (OL780856), CABMV
1593[b]	*P. galbana*	CPMMV, CABMV
1599[b]	*P. cacao*	BaCV (OL780851), CABMV
1607[b]	*P. incarnata*	CPMMV (OL780859), CABMV
1610[b]	*P. galbana*	CPMMV (OL780860), CABMV
1611[b]	*P. malacophylla*	CPMMV, CABMV
1612[b]	*P. suberosa*	CPMMV (OL780858), CABMV
1614[b]	*P. edulis*	BaCV (OL780852), CABMV
1624[b]	*P. riparia*	CPMMV, CABMV
1625[b]	*P. hatschbachii*	CPMMV, CABMV
1626[b]	*P. gardneri*	BaCV (OL780850), CPMMV, CABMV
1644[b]	*P. eichleriana* x *P. gibertii*	CPMMV (OL780857), CABMV (ON469826)
1648[b]	*P. rufa*	CPMMV, CABMV
1649[b]	*Passiflora* spp.	CPMMV, CABMV
1881[b]	*P. cincinnata*	CPMMV, CABMV

[a] Sampled in Planaltina commercial field [b] Sampled in BAG-FP

To confirm the identity of the viruses recovered from the *Passiflora* spp. And hybrid plants, we selected some representative samples, and the virus-specific PCR products of the expected size were excised from the gel, purified, and sequenced bi-directionally by the Sanger method (Macrogen, South Korea). Then, the sequences were analyzed and assembled in Geneious Prime® 2019.2.3 (Kearse et al. 2012) and subjected to an online BLASTn search analysis.

Sanger sequencing of the amplicons and BLASTn search confirmed the identity of these viruses. BaCV isolates from BAG-FP plants (OL780849, OL780850, OL780851, and OL780852) and Planaltina field samples (OL7808453, OL780854, and OL7808455) share 98.97% to 99.85% identity with BaCV (MK202584, MT811775), 94.69% to 95.28% identity with PpVE (MH282832), and 68.06% to 78.71% identity with CiaRV (MT302541 to MT302547). In addition, pairwise alignment of nucleotide sequences of all Brazilian Passiflora BaCV isolates using SDT program (Muhire et al. 2014) showed that they share an identity of >99%.

Likewise, sequences of *Passiflora*-infecting CPMMV isolates (OL7808456, OL7808457, OL7808458, OL7808459, OL7808460, and OL7808461) have an identity of 99.4% and share identities ranging from 94.53% to 99.13% with Brazilian CPMMV isolates from *Glycine max* (KF554101, KC884248, DQ885940) and *Phaseolus vulgaris* (MK20258).

Recently, the lettuce chlorosis virus (LCV, genus Crinivirus, family Closteroviridae) was identified in *P. alata* and *P. auricultada* from BAG-FP, and in *P. edulis* from Dom Basílio, Bahia state (Vidal et al. 2021). Therefore, we inspected the passion fruit samples collected in the field for LCV infection.

LCV was detected according to Vidal et al. (2021). The PCR assays used two sets of primers. The first set, Bag1226lt317LCV16F/Bag1226lt317LCV260R, amplifies a 244 nt fragment of RNA1, corresponding to the partial p8/p23 genes. The second set, LCVRNA22793F/LCVRNA23997R, amplifies a 1,199 nt portion of RNA2, corresponding to the partial HSP70h/p6.4/p60 genes.

Of the *Passiflora* plants investigated in this study, LCV was identified in three *P. edulis* plants from the Planaltina commercial field (Table 1). Moreover, two of the plants were also infected with BaCV. RT-PCR products of LCV from sample 1580 was Sanger-sequenced and had its identity confirmed. BLASTn search revealed that LCV-RNA1 (ON469824) has 99.18% identity and LCV-RNA2 (ON469825) shares 99.50% identity with the LCV isolates (MN564795 to MN564798) previously identified in samples from the BAG-FP (Vidal et al. 2021).

The majority of the positive samples for BaCV, CPMMV, and LCV exhibited strong viral symptoms of mosaic, blisters, yellow spots, yellow vein, and/or leaf deformation, as shown in Figure 1. The predominant symptoms visualized in these samples are typical of CABMV infection. Plants infected by CABMV can display different levels of symptoms severity, ranging

from woodiness and deformation of the fruits, blisters, wrinkling, and mosaic on the leaf lamina and leaf distortion (Gonçalves et al. 2021; Nascimento et al. 2006). The first report of LCV in passion fruit was in mixed infection with CABMV (Vidal et al. 2021). Therefore, we also evaluated the presence of this potyvirus in the samples infected by BaCV, CPMMV, and LCV (Table 1).

RT-PCR was performed according to Vidal et al. (2021) using the set of primers CABMVLNJP2492F/ CABMVLNJP3373R that amplify the partial HC-Pro/p3 genes (904 nt). CABMV was detected in all samples from BAG-FP (Table 1). Three samples from the Planaltina commercial field were infected with CABMV. Only samples 1580 and 1584 were negative, although the plants exhibited leaf deformation similar to CABMV infection (Figure 1). CABMV RT-PCR amplicon sequenced (ON469826) showed a range of 80–88% sequence identity to CABMV isolates available in GenBank.

The phylogenetic relationships of *Passiflora* spp. and hybrid isolates of BaCV, CPMMV, LCV, and CABMV with other isolates available in GenBank were determined. To generate the phylogenetic trees, partial sequences obtained in this study and analogous nucleotide sequences extracted from complete virus sequences from GenBank were used. The sequences were aligned using MUSCLE (Edgar 2004) implemented in Geneious Prime® 2019.2.3. The alignments were used in the phylogenetic analysis by the Maximum Likelihood method in the IQ-TREE multicore version 2.1.4-beta COVID-edition using the best nucleotide substitution model GTR+I+G (Minh et al. 2020).

Phylogenetic analysis (Figure 2) showed that the BaCV isolates from *Passiflora* are more related to BaCV-LUZ (MT811775) and BaCV-BR-GO (MK202584) isolates, identified in *Phaseolus vulgaris* collected in Brazil. The CPMMV isolates from *Passiflora* grouped with CPMMV:BR:GO:01:1 Brazilian isolate (KC884248) from *Glycine max*. The CABMV ON469826 was more related to CABMV/BJL1 Brazilian isolate (MN124782) from *Passiflora* spp. For LCV, both RNA1 and RNA2 from *Passiflora* isolates were more related to LCV-California (FJ380118, FJ380119), LCV-PTX (KY271955, KY271956), and LCV-BR (MN216391, MN216392), identified in lettuce and papaya in the USA, and periwinkle in Brazil, respectively.

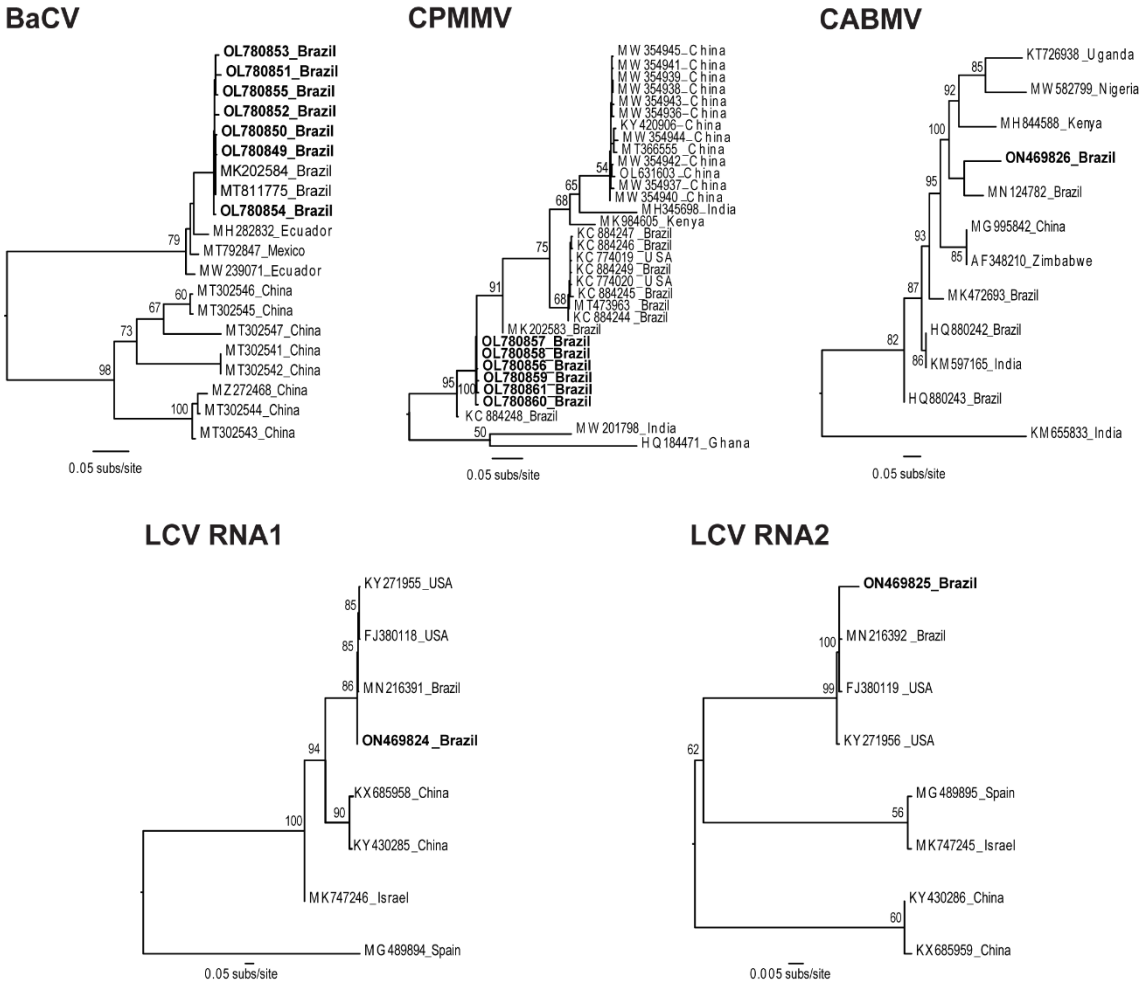

Figure 2. Phylogenetic trees based on the partial nucleotide sequence of bean-associated cytorhabdovirus (BaCV, G/L genes), cowpea mild mottle virus (CPMMV, CP gene), cowpea aphid-borne mosaic virus (CABMV, HC-Pro/p3 genes), and lettuce chlorosis virus (LCV, RNA1 p8/p23 genes and RNA2 HSP70 h/p6.4/p60 genes) obtained this study (highlighted in bold) and analogous nucleotide sequences extracted from complete genome sequences available in GenBank. All phylogenetic analysis were inferred by the maximum-likelihood method based on the GTR + I + G model with 1000 bootstrap replicates in IQ-TREE. Bootstrap support values > 50% are displayed.

We were unable to distinguish BaCV, CPMMV, or LCV infection based on symptoms, to a certain extent, because most plants presented infection with multiple viruses. Previously, strong symptoms of yellow spots were visualized in passion fruit (*P. edulis*) infected by CiaRV in China (Zhang et al. 2020). We could visualize yellow spots on the leaves in some of the P. edulis samples infected by BaCV. For example, samples 1580 and 1584 (Figure 1A and C) exhibit yellow spots scattered on the leaf, while in sample 1586 (Figure 1D), the yellow spots are distributed on the tip of the leaf. These symptoms are present in much lower intensity than in the plants infected by CiaRV. Moreover, these symptoms are most frequently accompanied by leaf deformation and mosaic (Figure 1). Even in BaCV single infection, as in sample 1584 (Figure 1C), symptoms can be misinterpreted as induced by CABMV infection.

In this investigation, we identified the frequent occurrence of mixed infections of BaCV, CPMMV, LCV, and CABMV. Some plants were infected with up to three of these viruses (Table 1). For example, sample 1586 is infected by BaCV, LCV, and CABMV, and sample 1591 by BaCV, CPMMV, and CABMV. Mixed infections of plant viruses are common and frequently reported, especially in perennial and semi-perennial crops (Moreno and López-Moya 2019). As in the present study, mixed infections of BaCV and CPMMV were also identified in bean and soybean plants in Brazil (Alves-Freitas et al. 2019; Pinheiro-Lima et al. 2020, 2023). Mixed infection of viruses may trigger synergistic effects generating more severe symptoms on the plant, hindering the identification of the viral etiological agent, consequently making it more challenging to manage and control these diseases.

Vectors are an essential factor for the establishment of viruses in crops. BaCV, CPMMV, and LCV infect hosts belonging to diverse plant families and are transmitted by the whitefly B. tabaci (Duffus et al. 1996; Lamas et al. 2017; Marubayashi et al. 2010; Pinheiro-Lima et al. 2020). *B. tabaci* are polyphagous insects that may transmit viruses between different host plants and contribute to the establishment of these viruses in passion fruit crops. Although in this work we did not observe whiteflies feeding or colonizing the passion fruit plants during sampling, high populations of whiteflies have been reported in passion fruit fields in Brazil and were associated with outbreaks of begomoviruses in the crop (Alves 2008, 2012; Ferreira et al. 2010; Mituti et al. 2019; Novaes et al. 2003; Rodrigues et al. 2019).

Our findings expand the host range of BaCV and CPMMV to different species of *Passiflora*. We also highlight a diversity of viruses still unknown in an important fruit crop and native wild species in Brazil. Additional studies are needed to evaluate these viruses' epidemiology, impacts, and interactions in passion fruit.

3.3. References

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Chapter 4

Characterization of cucurbit aphid-borne yellows virus (CABYV) from passion fruit in Brazil: evidence of a complex of species within CABYV isolates

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Characterization of cucurbit aphid-borne yellows virus (CABYV) from passion fruit in Brazil: evidence of a complex of species within CABYV isolates

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Abstract: High-throughput sequencing (HTS) has been an important tool for the discovery of plant viruses and their surveillance. In 2015, several virus-like symptoms were observed in passion fruit (PF) plants in Bahia state, Brazil. Using HTS technology, bioinformatics tools, RT-PCR, and Sanger sequencing, we identified the cucurbit aphid-borne yellows virus (CABYV, *Polerovirus, Solemoviridae*) in co-infection with cowpea aphid-borne mosaic virus (CABMV, *Potyvirus, Potyviridae*) in PF, in green manure, and spontaneous plants in several localities in Bahia. Complete genomes of CABYV-PF isolates were determined and analyzed with other CABYV isolates available in GenBank that were identified in various countries. Phylogenetic analysis and pairwise identity comparison with CABYV isolates showed that CABYV-PFs are more closely related to French and Spanish isolates. Overall analyses of all the CABYV genomes revealed that these could represent ten distinct species and thus proposed reclassifying these CABYV as isolates into ten species, tentatively named "*Polerovirus cucurbitae1*" to "*Polerovirus crcurbitae10*". CABYV-PF is a member of "*Polerovirus cucurbitae1*".

Keywords: polerovirus; CABYV; CABMV; mixed infection; *Passiflora*

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1. Introduction

Passion fruit is a tropical plant (genus *Passiflora*, family Passifloraceae) cultivated in several regions of the world, Brazil being the largest producer of yellow passion fruit (*Passiflora edulis* Sims). Virus diseases are a common problem in passion fruit crops and are responsible for significant reductions in the yield and quality of the fruits.

Characterization of cucurbit aphid-borne yellows virus (CABYV) from passion fruit in Brazil: evidence of a complex of species within CABYV isolates

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4.1. Abstract

High-throughput sequencing (HTS) has been an important tool for the discovery of plant viruses and their surveillance. In 2015, several virus-like symptoms were observed in passion fruit (PF) plants in Bahia state, Brazil. Using HTS technology, bioinformatics tools, RT-PCR, and Sanger sequencing, we identified the cucurbit aphid-borne yellows virus (CABYV, *Polerovirus*, *Solemoviridae*) in co-infection with cowpea aphid-borne mosaic virus (CABMV, *Potyvirus*, Potyviridae) in PF, in green manure, and spontaneous plants in several localities in Bahia. Complete genomes of CABYV-PF isolates were determined and analyzed with other CABYV isolates available in GenBank that were identified in various countries. Phylogenetic analysis and pairwise identity comparison with CABYV isolates showed that CABYVPFs are more closely related to French and Spanish isolates. Overall analyses of all the CABYV genomes revealed that these could represent ten distinct species and thus proposed reclassifying these CABYV as isolates into ten species, tentatively named "*Polerovirus curcubitaeprimum* to *Polerovirus curcubitaenonum*", and "*Polerovirus melo*". CABYV-PF is a member of "*Polerovirus curcubitaeprimum*".

Keywords: polerovirus; CABYV; CABMV; mixed infection; *Passiflora*

4.2. Introduction

Passion fruit is a tropical plant (genus *Passiflora*, family Passifloraceae) cultivated in several regions of the world, Brazil being the largest producer of yellow passion fruit (*Passiflora edulis* Sims). Virus diseases are a common problem in passion fruit crops and are responsible for significant reductions in the yield and quality of the fruits.

Several viruses have been identified in passion fruit plants in Brazil. Outbreaks of various begomoviruses (genus *Begomovirus*, family *Geminiviridae*) were reported in pas-sion fruit fields in different regions of the country [1-4]. Other viruses, such as grapevine virus A (GVA, genus *Vitivirus*, family *Betaflexiviridae*) [5], passion fruit yellow mosaic virus (PFYMV, genus *Tymovirus*, family *Tymoviridae*) [6], cucumber mosaic virus (CMV, genus *Cucumovirus*, family *Bromoviridae*) [7], passion fruit green spot virus (PfGSV, genus *Cilevirus*, family *Kitaviridae*) [8,9], purple granadilla mosaic virus (PGrMV, unclassified) [10], passion fruit chlorotic mottle virus (PCMoV, genus *Citlodavirus*, family *Geminiviridae*) [11,12], lettuce chlorosis virus (LCV, genus *Crinivirus*, family *Closteoviridae*) [13] were also identified in this crop.

Despite the emergence of these viruses, the most serious outbreaks of virus diseases in passion fruit in the country have been associated with cowpea aphid-borne mosaic virus (CABMV, genus *Potyvirus*, family *Potyviridae*). CABMV is transmitted by diverse aphid species, including *Aphis gossypii* and *Myzus persicae*, and causes passion fruit woodiness disease (PWD) in Brazil [14,15]. Nonetheless, in 2015, a high incidence of severe virus-like symptoms (mosaic, yellowing, blisters, leaf, and fruit deformations) was observed in several passion fruit fields in Bahia, Northeast

Brazil. Considering these symptoms, the pos-sibility of infection by viruses other than CABMV was raised. Hence, we explored the viruses present in these plants by using high throughput sequencing (HTS) technology as a detection method.

This analysis revealed the occurrence of cucurbit aphid-borne yellows virus (CABYV, genus *Polerovirus*, family *Solemoviridae*), hitherto unknown to infect passion fruit, in mixed infection with CABMV in passion fruit plants collected in Lençóis and Jussiape, Bahia state [16]. CABYV was first identified and characterized infecting melon and cucumber in France [17]. Since then, it has been detected in diverse cucurbit crops, broad bean, chick-pea, and passion fruit in many regions in Europe, Asia, Africa, South and North America, and Oceania [18-27]. CABYV is transmitted by aphids such as *A. gossypii* and *M. persicae*, but not mechanically [17]. Recently, a recombinant CABYV isolate was characterized in melon plants in Brazil, which was shown to be vectored by the whitefly *Bemisia tabaci* [28].

In this study, we determined the full genome sequences of CABYV isolates from pas-sion fruit plants from different regions in the state of Bahia in Brazil. The molecular char-acterization of these CABYV isolates and the comparison to other CABYV sequences available in GenBank unveiled a complex of different species within CABYV, leading to a proposed regrouping of CABYV sequences within the *Polerovirus* genus.

4.3. Materials and Methods

4.3.1. Plant material

Fifty-nine passion fruit samples (*Passiflora* spp.) were collected in June and July 2015 in different passion fruit fields in Bahia (Figure 1). Passion fruit plants exhibited severe virus-like symptoms (blisters, mosaic, yellowing, and leaf deformation) and were sampled in Marcionílio de Souza (*n*= 6 *P. edulis*), Seabra (*n*= 8 *P. edulis*), Morro do Chapéu (*n*= 2 *P. edulis*), Brumado (*n*=2 *P. edulis*), Dom Basílio (*n*=11 *P. edulis*), Lençóis (*n*=21; *n*=3 *P. cincinnata*, *n*=3 *P. alata*, *n*=15 *P. edulis*), and Jussiape (*n*= 9 *P. edulis*). In the same regions, several plants of different species used as green manure or spontaneous non-cultivated plants were also collected in Lençóis (*n*=104), Dom Basílio *(n*=25), Jussiape (*n*=8), Seabra (*n*=11), Marcionílio Souza (*n*=12), Brumado (*n*=38), Morro do Chapéu (*n*=5). All leaf samples were stored at -80 °C until analyzed. Preliminary results from plants collected in Lençóis and Jussiape have been reported [16].

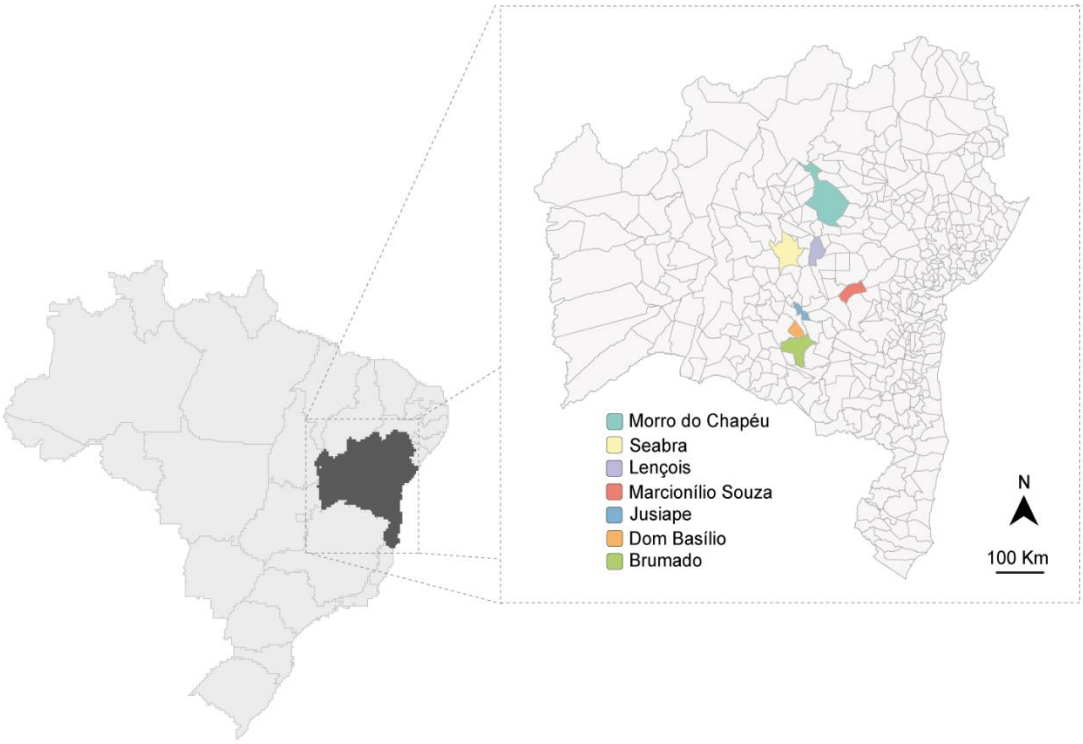

Figure 1. Map of Bahia state in Brazil showing the regions (in color) where plant material was collected.

4.3.2. Nucleic acid extraction, high-throughput sequencing, and data analysis

Double-stranded RNA (dsRNA) and total RNA were extracted from passion fruit plants (*n*= 59), according to Vidal et al. [13].

DsRNA aliquots (4 µl from each sample) were used to create two pools for the se-quencing, according to Vidal et al. [13]. Pool PM1BA (*n*= 29) is composed of samples from Marcionílio de Souza, Seabra, Morro do Chapéu, Brumado, and Dom Basílio, while sam-ples from Lençóis and Jussiape constitute pool PM2BA (*n*= 30). To achieve the necessary amount of RNA for sequencing (100 µg in total), 10 µl of total RNA of samples 502 and 594 were added in PM1BA, and 10 µl of total RNA of samples 724 and 581 in PM2Ba.

The libraries were prepared using Illumina TruSeq Stranded Total RNA and Ribo-Zero Plant kits and sequenced on an Illumina HiSeq 2500 platform (Macrogen Inc., South Korea). Paired-end reads (100 bp) generated in the Illumina HiSeq were checked for quali-ty using FastQC [29]. The sequencing adapters were removed and the low-quality reads were checked using Trimmomatic [30]. The paired-end reads were then submitted to de novo assembly to obtain contigs using SPAdes assembler [31] with *k-mer*= 64. Contigs [>200 nucleotides] were compared against the NCBI virus database using BLAST search tool.

4.3.3 Virus detection and Sanger sequencing

The detection of CABYV and CABMV in the individual samples was conducted by RT-PCR essays using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase kit (Invitrogen, USA), aliquots of total RNA, and virus-specific primers described in the literature. For CABYV detection, we used the set of primers Modified-CE-9F (a primer modified from CE-9F)/CE-10R [32] that amplify ~600 nt corresponding to the complete CP (coat protein) and partial MP (movement protein) genes. For CABMV, the primers CABMVLNJP2492F/CABMVLNJP3373R [13] that amplify ~900 nt corresponding partial genes HC-Pro/p3 were used. Characteristics and sequence of primers used in this report are summarized in Table S1.

All amplicons were visualized by electrophoresis in agarose gel stained with ethidi-um bromide (Invitrogen, USA). Amplicons of selected samples with the expected size were excised, gel-purified, cloned in the pCR™2.1-TOPO™ vector following the manufacturer's instructions (Invitrogen, USA), and Sanger sequenced (Macrogen, South Korea). All sequences obtained were analyzed and assembled in Geneious Prime® 2022.1.1 software (Biomatters Ltd, New Zealand).

4.3.4 Southern blot of CABYV RT-PCR

To confirm the identity of RT-PCR products, we used Southern blot hybridization with a CABYV-specific probe. After electrophoresis, the amplicons were transferred onto a nylon membrane Hybond™-XL (GE Healthcare, UK) with denaturation buffer (0.5 N NaOH, 1.5 M NaCl) following the manufacture´s protocol. Blots were UV crosslinked using a UV Stratalinker 1800 (Stratagene). The probe consisted of a CABYV- CP/MP-derived fragment labeled with radioactive [α32P] dCTP using an Amersham™ Rediprime™ II DNA Labeling System kit (GE Healthcare, UK). Hybridizations were carried out overnight at 65°C. Signals were detected by exposing the blots for 24h in Fujifilm Imaging Plate BAS-IP MS (Fujifilm, JA), and the images were generated on a Fujifilm FLA-3000 Scanner.

4.3.5. Classification of spontaneous plants positive to CABYV and CABMV

Classification of virus-positive spontaneous plants was based on plant morphology and DNA barcode rbcL and matK genes, according to Fazekas et al. [33]. First, DNA ex-traction was done using CTAB method [34]. Then, the rbcL and matK genes were ampli-fied with Taq DNA Polymerase, recombinant (Invitrogen, USA) using the sets of primers SI_For/SI_Rev and KIM 3F/KIM 1R [35] (Table S1).

All amplicons were visualized by electrophoresis in agarose gel stained with ethidi-um bromide (Invitrogen, USA). PCR products were excised from the gel, purified and Sanger sequenced at Macrogen Inc. (South Korea). Sequences were assembled in Geneious Prime® 2022.1.1. and analyzed in BOLD Identification System website [36].

4.3.6. 5'and 3'end method for rapid amplification of cDNA ends (RACE)

Based on CABYV PF-M2BA (MH257573) sequence obtained from HTS data of pool PM2Ba [16], CABYV-specific primers (Table S1) were designed and used in the RACE method to determine the 5' and 3' ends of CABYV isolates from passion fruit. The RACE method was performed according to Alves-Freitas et al. [37], Schuster et al. [38], and Nico-lini et al. [39].

For the 5' RACE, cDNA was synthesized with SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, USA) using 5µg of RNA and primer CABYVRACE581R (10 mM). cDNA obtained was treated with RNase H (USB, USA) and RNase A (Thermo Fisher Scientific, USA), and purified with PureLink™ Quick Gel Extraction Kit (Thermo Fisher Scientific, USA). A homopolymeric tail of deoxycytidine (dCTP) was added to the cDNA 3' end using Terminal Deoxynucleotidyl Transferase, Recombinant (Promega, USA). The cDNA was dialyzed with an MF-Millipore™ Membrane Filter, 0.025 µm pore size (Merck, USA). To obtain 5'end fragments, two PCR assays were performed. The first PCR reaction was done using a cDNA prepared with gene-specific primer 1- GSP1 (CABYVRACE581R), and the forward anchor primer AAP (Table S1). For the second PCR, reactions were done using aliquots of the first PCR as a template and primers GSP2 (CABYVRACE581R) and anchor forward primer AUAP (Table S1).

For the 3' RACE, first, a poly-A tail was added to the RNA (5µg) using the *Escherichia coli* poly (A) polymerase (New England Biolabs, USA). Then, the cDNA was synthesized with SuperScript™ IV Reverse Transcriptase (Invitrogen, USA) and anchored primer M10PacIT50VN (Table S1). Finally, the cDNA was treated with RNase H and RNase A and used in the PCR reactions. To obtain 3'end fragments, two PCR assays were made. The first PCR was done using the cDNA, and the primers GSP1 CABYVRACE5063F and M10 (Table S1). In the second PCR, aliquots of the first PCR were used as a template with primers GSP2 CABYVRACE5365F and M10 (Table S1).

All PCRs assays described above were performed with LongAmp® Taq DNA Poly-merase (New England Biolabs, USA). The final PCR products of ~400 bp (5'RACE) and ~350 bp (3'RACE) were gel-purified, cloned into pCR™2.1-TOPO® vector (Life Technologies, USA), and sequenced by Sanger method (Macrogen, South Korea). Sequences were analyzed in Geneious Prime® 2022.1.1. and used to design primers required to amplify the complete CABYV sequences.

4.3.7 Complete sequence of CABYV from passion fruit

The complete genome of CABYV isolates from passion fruit was determined by Sanger sequencing of overlapping RT-PCR products covering the entire genomes, as shown in Figure 2a. Passion fruit plants from Seabra (samples 558 and 564), Morro do Chapéu (sample 799), and Lençóis (samples 724, 726, and 729) were selected for full-length CABYV genome sequencing.

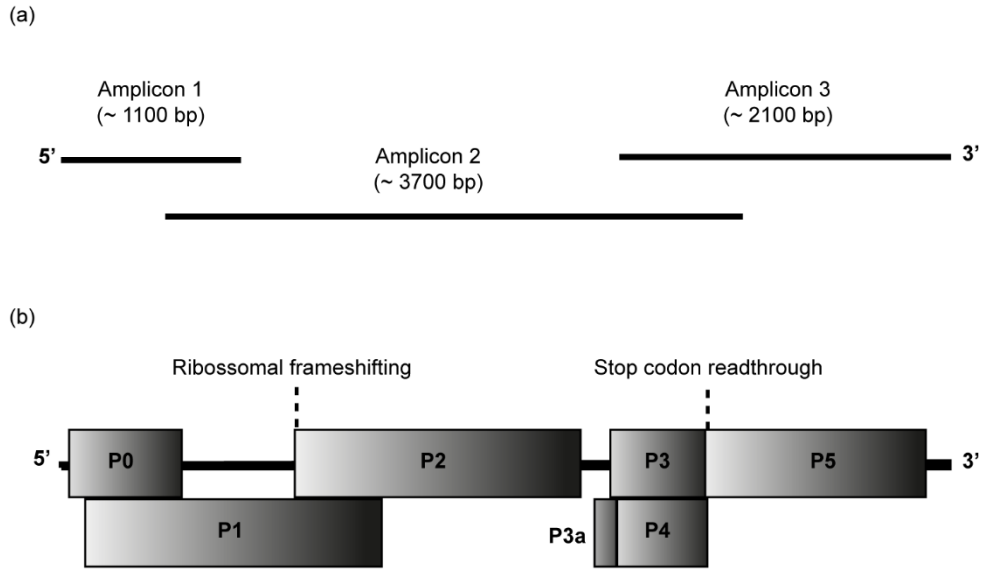

Figure 2. Passion fruit cucurbit aphid-borne yellows virus (CABYV) genome. (a) Amplicons 1 to 3 were cloned and sequenced to obtain the complete CABYV genome sequences from passion fruit. (b) Genome organization of the Brazilian CABYV from passion fruit showing the location of the predicted ORFs P0-P5 and P3a. ORF0 encodes the P0 protein, a putative RNA silencing suppressor; ORF1 and ORF2 overlap and encode the P1-P2 protein which functions as RNA-dependent RNA polymerase; ORF3a encodes P3a protein, a systemic movement protein; ORF3 encodes P3 protein, the coat protein; ORF4 encodes the P4 protein, a long-distance movement protein; ORF5 is expressed by the suppression of the ORF3 stop codon to produce a CP-read-through domain (CP-RTD, P3-P5 protein) which is involved in the transmission by aphids.

cDNA was synthesized with SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, USA), total RNA, and reverse primer CABYV3R (Table S1). Three sets of primers (for amplicons 1, 2 and 3) were used to recover the full-length genome of CABYV in PCRs assays performed with KAPA HiFi Hotstart DNA polymerase (Roche Molecular Systems, USA). All sets of primers used to amplify the amplicons 1, 2, and 3 are summarized in Table S1. CABYV5F and CABYV3R primers were designed based on sequences obtained by the RACE recovery of 5' and 3' ends. CABYV377F and CABYV1186R are primers derived from MH257573 sequence [16]. Additional specific primers are from a previous report [32]. Amplicon 1 of ~1.100 bp, amplicon 2 of ~3.700 bp, and amplicon 3 of ~2.100 bp were purified, cloned into the pJET 1.2/blunt vector (ThermoFisher Scientific, USA), and Sanger sequenced by primer walking (Macrogen, South Korea). Sequences were assembled in Geneious Prime® 2022.1.1. BLASTn search was used to check the identities among sequences obtained in this research and other CABYV sequences in GenBank. Open reading frames (ORFs) were annotated using ORF Finder (accessible at https://www.ncbi.nlm.nih.gov/orffinder/).

4.3.8 Phylogenetic analysis

A search in the GenBank database of the National Center for Biotechnology Infor-mation (NCBI) (accessible at https://www.ncbi.nlm.nih.gov/) accessed on November 15th 2022 for

complete CABYV isolates sequences and viruses belonging to the family *Solemoviridae* was performed, and the sequences retrieved. Pairwise nucleotide and amino ac-id identity scores were calculated with Sequence Demarcation Tool (SDT) v1.2 [40]. MUSCLE [41] alignments generated in Geneious Prime® 2022.1.1. were used to infer the maximum-likelihood (ML) phylogenetic trees using RAxML-NG v. 1.0.3 software [42]. The best-fit model TIM2+I+G4 was found for ML phylogenetic tree using ModelTest-NG v0.1.7 [43]. The ML trees were calculated with 1000 bootstrap replicates and final trees were edited and visualized using FigTree v1.4.4 (accessible at http://tree.bio.ed.ac.uk/software/figtree/).

4.3.9 Recombination analysis

Recombination analyses were performed with RDP4 v.4.100 software [44] with de-fault settings, and a Bonferroni corrected *p-value* cut-off of 0.01. Analyses were conducted using RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq methods, and only recombination events supported by at least three methods were considered.

4.4. Results and Discussion

4.4.1. HTS data and identification of CABYV and CABMV

High-throughput sequencing technology (HTS) has been an important tool for ex-ploring the diversity of viruses in plants. In this research, a HTS approach allowed the identification of the RNA-viruses CABYV (genus *Polerovirus*, family *Solemoviridae*) and CABMV (genus *Potyvirus*, family *Potyviridae*) in passion fruit library PM1Ba and confirmed the results for PM2Ba [16].

The two dsRNA passion fruit libraries sequenced by Illumina Hiseq 2500 resulted in 12,273,995 (PM1Ba) and 11,755,714 (PM2Ba) raw paired-end 100-bp reads. After trimming and processing, the reads were assembled into 104,931 contigs (72 to 12,062 nt in size) for PM1BA and 87,231 contigs (87 to 15,812 nt of length) for PM2BA.

All contigs (> 200 nt) were analyzed by BLASTn against the viral NCBI database. Thirteen contigs of 203 to 485 nt and one of 5663 nt sharing >90% nt identities with CABYV were identified in PMIBA and PM2BA libraries, respectively. Other fifteen contigs in PM1BA and thirty-nine in PM2BA were also identified, showing similarity with a potyvirus. These contigs ranged from 203 nt to 8458 nt in size, with 87% to 93% nt identity with CABMV.

Mapping all raw paired-end reads was performed on NCBI RefSeq genomes of CABYV (NC_003688) and CABMV (NC_004013) (Table 1). A total of 396 reads in PM1BA and 33,291 reads in PM2BA with >91% identity with CABYV reference sequence were identified in the mapping. For CABMV, we identified 722,828 reads in PM1BA and 2,641,2942 reads in PM2BA, with an identity of >97% regarding the reference genome.

4.3.2. Detection of CABYV and CABMV in passion fruit plants

In this study we identified a high incidence of CABYV and CABMV in the passion fruit samples. The presence of CABYV in passion fruit samples was evaluated by RT-PCR, followed by Southern hybridization with a CABYV-derived probe. Amplicons (~600 nt) of the expected size were visualized in the RT-PCR gel electrophoresis for several passion fruit samples. The amplicon identity was confirmed by positive hybridization with a CABYV-specific probe in the Southern blot (Figure S1).

In total, 21 (~36%) of the 59 tested passion fruit samples were identified to be infected with CABYV. CABYV was identified in five regions in Bahia (Figure S1; Table S2; Table S3). A high incidence of CABYV in the tested samples was recorded with 62.5% (5/8) of the plants in Seabra, 50% (1/2) in Morro do Chapéu, 27.2% (3/11) in Dom Basílio, and 11.1% (1/9) in Jussiape. In Lençóis, 52.3% (11/21) of the passion fruit plants were positive for CABYV, which was identified in at least one sample of three *Passiflora* species sampled in this region (01/03 of *P. cincinnata*; 02/03 of *P. alata*; 8/15 of *P. edulis*) [16]. On the other hand, plants from Marcionílio de Souza and Brumado were negative for CABYV.

Since CABMV is the most common virus infecting passion fruit, we tested the plants for its presence by RT-PCR (Figure S2). Initially, CABMV detection with CABMVM1MX_3726F/CABMVM1MX_5039R primers [11] was negative for some plants from Lençóis and Jussiape [16], despite these plants exhibited typical symptoms induced by this potyvirus. This pair of primers are based on sequences of CABMV from Mato Grosso do Sul [11] and apparently were not suitable to screen for CABMV from Bahia (Northeastern Brazil). Indeed, RT-PCR using another set of primers, CABMVLNJP2492F/CABMVLNJP3373R, described by Vidal et al. [13] confirmed the CABMV infection in the majority of the plants previously identified as negative, tested by Vidal et al. [16] with CABMVM1MX_3726F/CABMVM1MX_5039R primers.

As a result of this new round of analysis, CABMV was identified in 83.3% (5/6) of plants from Marcionílio de Souza, in 87.5% (7/8) from Seabra, 50% (1/2) from Brumado, in 72.7% (8/11) of the samples from Dom Basílio, in 71.4% (15/21) from Lençóis, and in all plants sampled in Morro do Chapéu (2/2) and Jussiape (9/9) (Figure S2a; Table S2; Table S3).

Mixed infections of CABYV and CABMV were also often identified in the passion fruit plants surveyed (Table S2). Both viruses were found in co-infection in 62.5% (5/8) of the samples from Seabra, in 38% (8/21) from Lençóis, 50% (1/2) from Morro do Chapéu, in 22% (3/11) from Dom Basílio, and in 11.1% (1/9) from Jussiape.

CABYV and CABMV cause several types of symptoms in their hosts. For example, in-fection by CABYV in cucurbits induces yellowing, thickening of older leaves, decrease in the number of

fruits by plant, and depending upon cultivar and other biotic and abiotic factors, the intensity of the symptoms can vary [17]. Infection by CABMV in passion fruit induces different levels of wrinkling, blisters, mosaic, deformation, and anatomical changes in the leaf, while the fruits can display woodiness and deformation [99,100].

In this study, we were unable to associate CABYV with a particular symptom type. The CABYV-positive passion fruit plants exhibited similar symptoms, like as crinkling, mosaic, leaf and fruit deformation, blistering, yellow spot, chlorosis, yellowing, vein band-ing, green spot, vein whitening, and purplish leaf (Table S3). Most of these symptoms resemble those induced by CABMV. The major symptom usually associated with CABYV is leaf yellowing. Three *P. edulis* plants (samples 739, 732, and 716) from Lençóis were detected with CABYV in single infection, and showed symptoms of blistering, leaf deformation, mosaic, and vein banding (Table S3). In addition to the mixed infection of CABYV and CABMV in these plants, we have recently reported in sample 603 (*P. edulis*, Table S3) collected in Dom Basílio, a mixed infection of the crinivirus lettuce chlorosis virus and CABMV [13]. It is worth mentioning that in addition to RNA viruses, it is possible that these plants are also infected with DNA viruses.

CABYV seems to be disseminated in passion fruit producing areas in Bahia, Brazil. New screenings should be conducted in other producing areas to verify the dispersal of CABYV in Brazil. So far, preliminary results indicate the occurrence of mixed infection of CABYV and CABMV in passion fruit plants from experimental fields in Rio de Janeiro, Southeast of the country [45].

4.4..3. Detection of CABYV and CABMV in spontaneous and green manure plants and classification of positive plants

Of all spontaneous and green manure plants evaluated in this study, CABYV was detected only in plants from Lençóis. Of 104 spontaneous and green manure plants tested from Lençóis, seven were positive for CABYV infection (Figure S3). CABMV was evaluated only in the CABYV-positive plants (Figure S2b). The potyvirus was detected in three of the seven plants infected with CABYV.

These virus-positive plants were classified at the genus level. DNA barcoding with matK and rbcL genes was adopted to aid in classifying these plants (Table S4). Three PCR products of rbcL (544 nt) and matK (821 nt to 823 nt) sequenced from samples 540, 543, and 717 showed >99% similarity with *Macroptilium* spp. (genus *Macroptilium*, family Fabaceae). From sample 757, only the rbcL gene (544 nt) was sequenced and found 99.63% similar to the rbcL gene from *Stylosanthes* spp. (genus *Stylosanthes*, family Fabaceae). For samples 541, 760, and 761, the PCR products sequenced for rbcL (543 to 544 nt) and matK (811 to 826 nt) had 98.05% to 100%

identity with *Cucumis* spp. (genus *Cucumis*, family Cucurbitaceae), *Sida* spp. (genus *Sida*, family Malvaceae), and *Bignonia* spp. (genus *Bignonia*, family Bignoniaceae), respectively.

It is known that viruses can spill over into cultivated plants from spontaneous plants or from cultivated plants to spontaneous plant populations, which could lead to the emergence of new viruses or increase the host range of a virus [86,87]. We identified green manure and spontaneous plants as new hosts for CABYV and CABMV. Both viruses have a wide range of host plants and can infect cultivated and non-cultivated plants of diverse plant families. CABMV can infect plants of the families Passifloraceae, Fabaceae, Cucurbi-taceae, Solanaceae, Chenopodiaceae, Amaranthaceae, and Poaceae [90-93]. CABYV has a wide range of Cucurbitacea plants as hosts and can infect other several plants families, such as Brassicaceae, Asteraceae, Malvaceae, Fabaceae, Amaranthaceae, Chenopodiaceae, Papaveraceae, Lamiaceae, Portulacaceae, Solanaceae, and Passifloraceae [16,17,20,27,32,46-50]. However, CABYV infection in *Macroptilium* spp., *Stylosanthes* spp., *Sida* spp., and *Bignonia* spp., as well as, CABMV infection in *Sida* spp. and *Bignonia* spp. have not yet been reported. It is possible that these alternative host plants are natural reservoirs of CABYV and CABMV in Brazil.

4.3.4. Sanger sequencing and confirmation of CABYV and CABMV

Sanger sequencing of selected RT-PCR amplicons confirmed the identity of CABYV and CABMV in passion fruit, green manure and spontaneous plants. CABYV-derived CP/MP amplicons sequences (600 nt) obtained from passion fruit plants from Seabra (sample 564; OP909788), Morro do Chapéu (sample 799; OP909789), Dom Basílio (sample 611; OP909791), Lençóis (samples 724, 726, 729, 738, 739; OP909790, and OP909792 to OP909795), Jussiape (sample 580; OP909796) as well as isolates from spontaneous and green manure plants (samples 540, 541, 543, 717, 757, 761, 760; OP909797 to OP909803) from Lençóis have a nt identity of >99% amongst them. BLASTn search also revealed that these CABYV isolates share highest nt identity of 98% to 100% with sequence CABYV-PF-M2Ba (MH257573) [16]. In addition, CABYV isolates showed >94% nt identity with di-verse sequences of CABYV available in GenBank.

CABMV HC-Pro/p3 fragments (904 nt) obtained from passion fruit from Lençóis (samples 524, 724; OP909781 and OP909783), Marcionilio de Souza (sample 500; OP909780), Brumado (sample 629; OP909782), and Morro do Chapéu (sample 799; OP909784), and of spontaneous plants (samples 717, 760, 761; OP909785 to OP909787) showed a nt sequence identity >93% among them. These isolates also share 88% to 92.24% nt identity with the Brazilian CABMV MN124782 isolate from *Passiflora* spp. from Brazil.

4.4.5. Genome characterization of CABYV from passion fruit

CABYV isolates from six passion fruit plants (Seabra: samples 558 and 564; Morro do Chapéu: sample 799; and Lençóis: samples 724, 726, and 729) were selected for further molecular characterization. The complete genome of the isolates, herein referred to as CABYV-PF, was determined by Sanger sequencing of three RT-PCR amplicons (amplicons 1 to 3), as schematized in Figure 2a. At least two clones of each amplicon were sequenced. In the complete genome assembly, the consensus sequence extracted from at least two clones of each amplicon was used. In the end, consensus sequences of amplicons 1 to 3 were considered for the final assembly.

In the sequence analysis of clones from samples 724 and 726, we observed that am-plicons 1 and 2 (corresponding regions of ORF0 to ORF1) had several polymorphisms in the nucleotide sequence. These fragments with polymorphisms likely represent variants of CABYV in the same plant and did not interfere with P0 and P1 ORF prediction. For amplicon 3, few polymorphisms were observed for samples 724 and 726, and the sequences obtained from the other samples. Thus, based on the polymorphic sequences, we considered three sequences from sample 724 and two from sample 726 as variants of CABYV in these samples.

Nine full sequences of CABYV-PF isolates were obtained and characterized. The se-quences from plants collected in Seabra (CABYV-PF-558 and CABYV-PF-564; OP909804 and OP909805), Morro do Chapeu (CABYV-PF-799; OP909806), and Lençóis (CABYV-PF-724-1, CABYV-PF-724-2, CABYV-PF-724-1, CABYV-PF-726-1, CABYV-PF-726-2, and CABYV-PF-729; OP909807 to OP909812) were deposited in the GenBank.

These isolate´s sequences ranged from 5672 nt to 5677 nt and showed a typical pol-erovirus genome organization (Figure 2b). The genomes have seven open reading frames (ORFs), a 5' untranslated region (UTR) of 20 nt, a 3' UTR that ranges from 161 to 163 nt, and between the ORF2 and ORF3a, there is a non-coding internal region (IR) of 81nt. All CABYV-PF isolates showed the same genomic organization, with minor differences only in P3-P5 ORFs, which encode P3-P5 gene/fusion protein CP-RTD (coat protein-read-through).

ORFs 0, 1, and 2 are situated closer to the 5'UTR. The ORF0 (P0 protein) overlaps the ORF1 (P1 protein) in 596 nts, and ORF1 overlaps the ORF2 in 547 nts. The ORF0 is 717 nt long and encodes the P0 protein of 239 aa containing the conserved domains of the Lu-teo_P0 super-family and a F-box [51-54]. The ORF1 has 1893 nt and encodes the P1 protein of 631 aa. The P1 protein presents an aa sequence similar to the serine protease do-main of the Peptidase_S39 family and virus protein genome-linked (VPg) typical of poleroviruses [55]. The ORF1 (1893 nt) and ORF2 (1275 nt) encode the P1-P2 protein (RNA-directed RNA polymerase- RdRP) of 1056 aa through -1 ribosomal frameshift mechanism due to by a slippery sequence 5'-GGGAAAC-3'/5'-UUUCCC-3' [55,56]. The P1-P2 protein displays the motifs typical of RdRps [52].

Closer to the 3'UTR are localized the ORFs 3a, 3, 4 and 5. Located upstream of ORF3 (P3 protein), the ORF3a (P3a protein) has the translation initiated by an ATA codon driven by a Met-tRNA, resulting in an N-terminal methionine instead of an isoleucine [57,58]. The ORF3a has 138 nt and codes for a putative P3a protein of 46 aa. The ORF3 (P3 protein) codes for the coat protein (CP) that overlaps almost entirely with the movement protein (ORF4/P4 protein). The ORF3 of 600 nt encodes the P3 protein of 200 aa that presents the aa sequence of Luteo-coat super-family [59]. P3 protein (CP) of all CABYV-PF isolates have the aa sequence GILKAYHE typical of poleroviruses, which share the 5'-G[I/M]LK[A/S]YHE-3' motif sequence [52]. The ORF4/P4 protein has 576 nt and encodes a putative movement protein of 192 aa. Located downstream of ORF3, the ORF5 is a translational in-frame read-through of the ORF3 stop codon to produce the P3-P5 protein, also known as fusion protein CP-RTD [52,55]. The P3-P5 was the sole protein that showed a difference in size among the CABYV-PF isolates, ranging in size from 2004 nt to 2013 nt, coding for a protein of 668 aa to 671 aa. The conserved proline-rich sequence 5'-PPPPGPSPT[P/-]P[P/S]PPPP-3' typical of CABYV and other poleroviruses [52] was identified in P5 and was located just downstream of the CP stop codon. Two minor changes were observed in the proline-rich sequence in CABYV-PF799 (5'-PPPPGPSPT[-]PPPPPP-3') and CABYV-PF729 (5'-PPPPGPSPTPP[S]PPPP-3') isolates.

4.4.6. Phylogenetic relation of the CABYV-PF isolates

CABYV-PF isolates showed high similarities with CABYV isolates available in Gen-Bank. BLASTn search showed that CABYV-PF (OP909804 to OP909812), as expected, shared the highest nucleotide identities of 94.39% to 99.50% with CABYV PF-M2BA (MH257573), a partial genome sequence determined from passion fruit and reported by our group [16]. High nt identities of 90.4% to 94.26% were also observed with CABYV sequences from France (MT027103, X76931, MZ202344) and Spain (JF939812-14, MW051362, MW051363).

As of November 2022, 56 complete CABYV sequences were available in GenBank. These sequences were determined from Cucurbitaceae and Solanaceae infected plants in localities of South Korea (*n*=31), Spain (*n*=5), China (*n*=6), France (*n*=3), Brazil (*n*=3), Taiwan (*n*=2), India (*n*=2), Papua New Guinea (*n*=1), Japan (*n*=1), United States (*n*=1), Indonesia (*n*=1), and Timor-Leste (*n*=1) (Table 1). CABYV complete sequences were retrieved from GenBank and compared with CABYV-PF isolates.

CABYV sequences retrieved from GenBank have been characterized as belonging to the genus *Polerovirus* into the family *Luteoviridae*. Recently, a new taxonomy was pro-posed, including *Polerovirus*, *Enamovirus*, *Polemovirus*, and *Sobemovirus* as genera belonging to the family *Solemoviridae* [55]. To better understand the relationship of the CABYV isolates to other solemoviruses, a phylogenetic tree was inferred based on the full-length sequences of CABYV

isolates deposited in GenBank, CABYV-PF isolates from this study, and members of the *Solemoviridae*. In the phylogenetic tree (data not shown), all CABYV isolates were grouped with members of the genus *Polerovirus*.

A phylogenetic tree comprising CABYV-PF (OP909804 to OP909812) and all other CABYV sequences showed that the passion fruit isolates were most closely related to the Brazilian, French, and Spanish isolates (Figure 3).

Based on the evolutionary distance observed in this tree, there is a diversification for the other CABYV strains. Different clades were formed by sequences from South Korea, China, Japan, the United States, India, Taiwan, Indonesia, and Timor-Leste. These groups were similar to the phylogroups described by Khanal et al. [18]. The distances were more evident for Spanish (JF939813) [32], Indian (MN688219 MN688220) [60], Chinese (HQ439023) [61], and Taiwanese (JQ700306) [62] isolates that were characterized as recombinants. The Brazilian isolates from melon (LC217993, LC217994, and LC516688), which are more related to CABYV-PF were also identified as recombinants [22, 28].

Recombination increases the genetic diversity of viruses [63]. Therefore, as an alternative view of the phylogenetic relationships of the CABYV isolates, we repeated the phylogenetic analyses excluding the recombinant sequences. Different groups were observed with isolates from different geographical regions, showing high genetic distances. CABYV-PF isolates still grouped with the French and Spanish isolates (Figure 4).

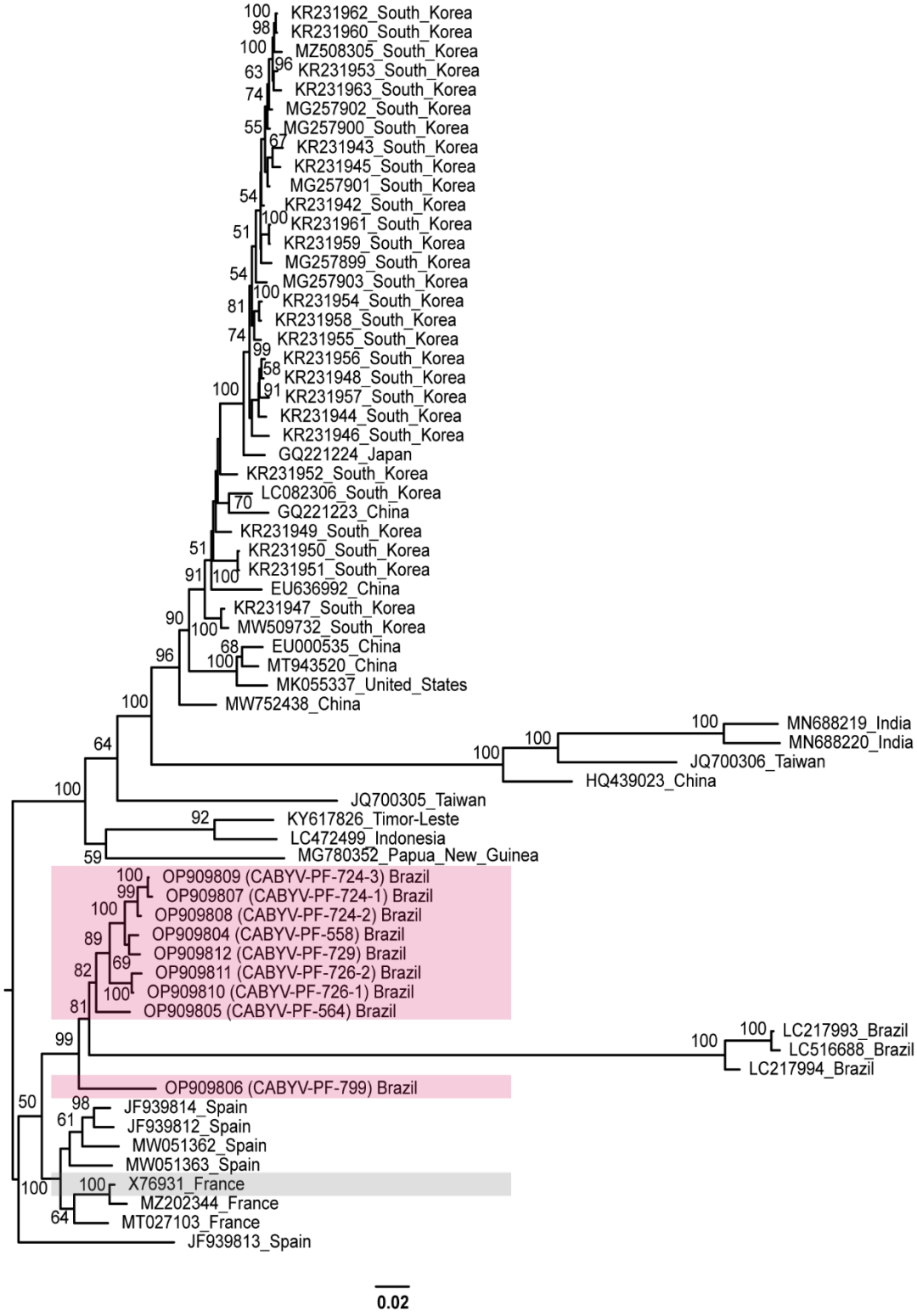

Figure 3. Midpoint rooted phylogenetic tree of the complete nucleotide sequences of the CABYV sequences obtained in this study and of other isolates available in GenBank. Pink and gray boxes highlight CABYV-PF isolates and CABYV reference sequence, respectively. Phylogenetic trees were reconstructed using maximum likelihood in RAxML-NG v. 1.0.3 software using a MUSCLE alignment generated in the Geneious Prime® 2022.1.1. Bootstrap support values >50% for 1000 replicates are displayed on or near the branches. The bar indicates 0.02 nucleotide substitutions per site.

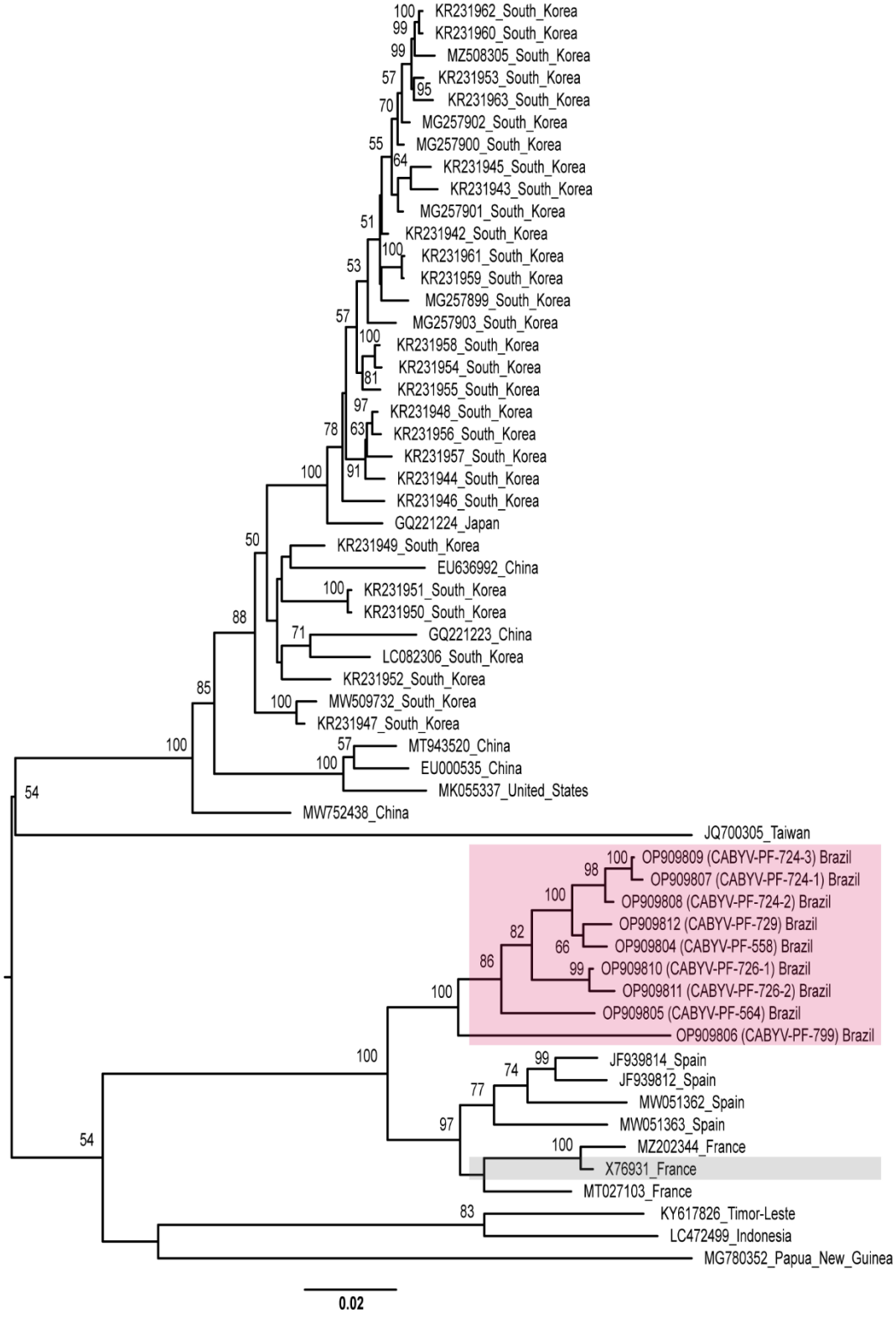

Figure 4. Midpoint rooted phylogenetic tree of the complete genome sequences of CABYV sequences excluding CABYV recombinant sequences. Pink and gray boxes highlight CABYV-PF isolates and CABYV reference sequence, respectively. Phylogenetic trees were reconstructed using maximum likelihood in RAxMLNG v. 1.0.3 software using the MUSCLE alignment generated in Geneious Prime® 2022.1.1. Bootstrap support values >50% for 1000 replicates are displayed on or near the branches. The bar indicates 0.02 nucleotide substitutions per site.

4.4.7. Relation between CABYV-PF isolates and Brazilian recombinant isolates

Interestingly, CABYV isolates from melon from Brazil were more related to the passion fruit isolates. These isolates were characterized as recombinants between CABYV-N from France (X76931) and an unknown virus [22,28].

Analysis of the full-genome sequences revealed that CABYV-PF isolates were highly similar (94.3% to 96.7% nt identity) to the Brazilian melon isolates in the region that corresponds to 5'UTR, P0, P1, P1-P2 (positions 1 to 3361 nt), up to the intergenic region, while the remainder of their genomes differed substantially.

Brazilian CABYV melon isolates were previously characterized as recombinants of CABYV-N from France as the major parent and an unknown minor parent [22]. Since we sequenced seven new genomes from Brazilian CABYV passion fruit isolates, we reassessed the recombination analysis, revealing that the CABYV melon isolates (LC217993, LC217994, LC516688), originated from a recombination event involving CABYV-PF-726-2, as the putative major parent, and a minor unknown parent (Figure S4). Our results support that the recombination event probably occurred in Brazil between CABYV common-type isolates, as those from passion fruit, and another unknown polerovirus, as hypothesized by Costa et al. [28].

We have investigated the possible presence of the CABYV recombinant type in the passion fruit plants evaluated in this study. The HTS data was reanalyzed, searching for the recombinant type, but no evidence was found. Mapping of all the reads using the Brazilian sequences from melon as references covered only ~60% of the genome (the non-recombinant portion). On the other hand, no read matched the recombinant region, represented by ~ 40% of the genome. Thus, we discarded the possibility of the recombinant type infecting these passion fruit plants. Likewise, Costa et al. [28] evaluated the occurrence of CABYV common-type in melon plants infected with the recombinant CABYV by RT-PCR with specific primers based on the CABYV-N (X76931) and CABYV-PF (MH257573) sequences with negative results.

We also ruled out that the CABYV isolates detected in green manure and spontaneous plants evaluated in this study are the Brazilian recombinant type. The portion of the CABYV genome (complete P3 [CP] and partial P4 [MP] genes) sequenced share a nt identity of about 64% with the recombinant type and >99% with the passion fruit isolates, i.e. common-type, and related to X76931. One of the alternative hosts we identified for the CABYV-common type is *Cucumis* spp. (Table S4). Since the Brazilian recombinant type also infects different *Cucumis* species [28], the recombination event that originated the Brazilian recombinant type likely occurred in a cucurbit plant, possibly a *Cucumis* species. Despite the negative results reported by Costa et al [28], it is possible that CABYV-common type is infecting *Cucumis* or other cucurbits in producing fields, alone or in mixed infections with the recombinant type. Future surveys in cucurbits should address this issue.

Recombination analysis also revealed events of intraspecific recombination in CABYV-PF (Figure S4). However, as pointed out by Kassem et al. [32], these results should be taken with caution, considering that, apparently, this plant has a mixed infection with CABYV variants.

4.4.8. Amino acid pairwise identity and classification of CABYV isolates

Phylogenetic relation (Figure 3 and 4) based on the complete genome sequence of CABYV isolates from this study and those reported in previous studies revealed a diversification of CABYV. Currently, one of the species demarcation criteria for the genus *Polerovirus* is based on differences in amino acid sequence identity of any gene product of greater than 10% [55]. This criterion has been used to propose novel member species in the genus *Polerovirus* [64-71]. Accordingly, CABYV isolates that present an amino acid identity <90% in at least one protein regarding CABYV-N (RefSeq: NC_003688, GenBank: X76931), the first isolate identified in melon plants in France [17], would be considered as members of a different virus species. Therefore, based on the phylogenetic studies and the species demarcation criterion for the genus *Polerovirus*, we raised the question: Do the CABYV viruses reported in the literature fit this criterion?

Initially, we considered the non-recombinant sequences in the comparisons with CABYV-N from France (X76931). Pairwise identity analysis of the complete nucleotide sequence by Sequence Demarcation Tool (SDT) showed that CABYV-PF isolates share 77% to 94% identity with CABYV isolates previously reported. Analysis of individual deduced protein sequences (P0 to P5, and P3a) revealed that CABYV-PF isolates share an aa identity of 88.7% to 91.2% in P0; of 90% to 92.7% in P1; of 95.4% to 97% in P2; of 95.6% to 100% in P3a; of 97% to 99% in P3; of 95.3% to 96.9% in P4, and 93.6% to 95.1% in P5 compared to CABYV-N from France (X76931). Similar percentages between Spanish (JF939812, JF939814, MW051363, and MW051362) and French (MT027103 and MZ202344) isolates were also observed for all genes. All pairwise identities can be accessed in the supplementary file Table S5.

Among all CABYV isolates analyzed, P0 was the most divergent protein (Table S5), in agreement with LaTourrette et al. [72], which identified this genome region as hypervariable with high nucleotide diversity. P0 of CABYV-PF-799 (89.5%), CABYV-PF-724-1 (88.7%), and CABYV-PF-723-3 (89.1%) showed amino acid identity <90% regarding the French CABYV-N isolate (sequence X76931). However, these isolates share >90% amino acid identity with other CABYV-PF isolates (OP909804 to OP909812), which share >90% aa identity with the French isolate (sequence X76931). Thus, all CABYV-PF isolates should be considered strains of the same virus species.

Except for the Spanish (JF939814, JF939812, MW051363, MW051362) and French isolates sequences (MT027103, MZ202344), in the other CABYV isolates' sequences, it was observed in

the pairwise identity a difference >10% amino acid identity in at least two proteins regarding the French CABYV-N isolate (X76931) (Table S5). Overall, this difference could be perceived by taking into account P0 and P1. However, besides P0 and P1, other proteins also showed an identity difference of > 10% in the aa sequence for some isolates. Considering the sequence divergence among all CABYV isolates (Table S5) and according to the mentioned species demarcation criterion for the genus *Polerovirus*, the CABYV isolates were grouped into different species. Isolates CABYV-PF, Spanish (JF939814, JF939812, MW051363, MW051362), and French (MT027103, MZ202344, and X76931) would be classified as members of the same species since they share aa identities >90% for all proteins. Other non-recombinant CABYV isolates would be grouped into at least four other species. The suggested classification for the cucurbit aphid-borne yellows virus can be accessed in Table 1.

Table 1. Proposed classification for the cucurbit aphid-borne yellows virus.

Specie name; Virus name-acronym **GenBank accession number**	**Country (state; city)**	**Host**	**Reference**
Polerovirus curcubitaeprimum Cucurbit aphid-borne yellows virus 1- CABYV1			
X76931	France	*Cucumis melo*	[17]
MT027103	France	*Cucumis melo*	Unpublished
MZ202344	France	*Physalis floridana*	Unpublished
JF939812	Spain	*Cucurbita pepo*	[32]
JF939814	Spain	*Cucurbita pepo*	
MW051363	Spain	*Citrullus lanatus*	[73]
MW051362	Spain	*Cucumis melo*	
OP909804 (CABYV-PF-558)	Brazil (Bahia;Seabra)	*Passiflora edulis*	This study
OP909805 (CABYV-PF-564)	Brazil (Bahia;Seabra)	*Passiflora edulis*	
OP909806 (CABYV-PF-799)	Brazil (Bahia; Morro do Chapéu)	*Passiflora edulis*	
OP909807 (CABYV-PF-724-1)	Brazil (Bahia; Lençóis)	*Passiflora edulis*	
OP909808 (CABYV-PF-724-2)	Brazil (Bahia; Lençóis)	*Passiflora edulis*	

OP909809 (CABYV-PF-724-3)	Brazil (Bahia; Lençóis)	*Passiflora edulis*	
OP909810 (CABYV-PF-726-1)	Brazil (Bahia; Lençóis)	*Passiflora edulis*	
OP909811 (CABYV-PF-726-2)	Brazil (Bahia; Lençóis)	*Passiflora edulis*	
OP909812 (CABYV-PF-729)	Brazil (Bahia; Lençóis)	*Passiflora edulis*	
Polerovirus curcubitaesecundum			
Cucurbit aphid-borne yellows virus 2- CABYV2			
MG257899	South Korea	*Cucumis sativus*	Unpublished
KR231957	South Korea	*Cucumis melo*	[61]
KR231942	South Korea	*Cucumis melo*	
KR231943	South Korea	*Cucumis melo*	
KR231944	South Korea	*Cucumis melo*	
KR231945	South Korea	*Cucumis melo*	
KR231946	South Korea	*Cucumis melo*	
KR231947	South Korea	*Cucumis melo*	
KR231948	South Korea	*Cucumis melo*	
KR231949	South Korea	*Cucumis melo*	
KR231950	South Korea	*Cucumis melo*	
KR231951	South Korea	*Cucumis melo*	
KR231952	South Korea	*Cucumis melo*	
KR231953	South Korea	*Cucumis melo*	
KR231954	South Korea	*Cucumis melo*	
KR231955	South Korea	*Cucumis melo*	
KR231956	South Korea	*Cucumis melo*	
KR231958	South Korea	*Cucumis melo*	
KR231959	South Korea	*Cucumis melo*	
KR231960	South Korea	*Cucumis melo*	
KR231961	South Korea	*Cucumis melo*	
KR231962	South Korea	*Cucumis melo*	
KR231963	South Korea	*Cucumis melo*	

LC082306	South Korea	*Cucumis melo*	Unpublished
MG257900	South Korea	*Cucumis sativus*	
MG257901	South Korea	*Cucumis melo*	
MG257902	South Korea	*Cucumis melo*	
MG257903	South Korea	*Citrullus lanatus*	
MW509732	South Korea	*Cucumis melo*	
MZ508305	South Korea	*Cucumis sativus*	
EU636992	China	*Cucumis melo*	
GQ221223	China	*Cucurbita pepo*	
MT943520	China	*Luffa cylindrica*	
MW752438	China	*Cucumis melo*	
GQ221224	Japan	*Cucumis sativus*	
EU000535	China	*Cucurbita moschata*	[74]
MK055337	United States	*Cucurbita máxima*	[75]
Polerovirus curcubitaetertium			
Cucurbit aphid-borne yellows virus 3- CABYV3			
LC472499	Indonesia	*Cucumis sativus*	Unpublished
KY617826	Timor-Leste	*Cucumis sativus*	[76]
Polerovirus curcubitaequartum			
Cucurbit aphid-borne yellows virus 4- CABYV4			
MG780352	Papua New Guinea	*Cucumis sativus*	[24]
Polerovirus curcubitaequintum			
Cucurbit aphid-borne yellows virus 5- CABYV5			
JQ700305	Taiwan	*Momordica charantia*	[62]
Polerovirus curcubitaesextum			
Cucurbit aphid-borne yellows virus 6- CABYV6			

JF939813	Spain	*Cucumis melo*	[32]
Polerovirus curcubitaeseptimum Cucurbit aphid-borne yellows virus 7- CABYV7			
JQ700306	Taiwan	*Luffa aegyptiaca*	[62]
Polerovirus curcubitaeoctavum Cucurbit aphid-borne yellows virus 8- CABYV8			
MN688219	India	*Cucurbita pepo*	[60]
MN688220	India	*Citrullus lanatus*	
Polerovirus curcubitaenonum Cucurbit aphid-borne yellows virus 9- CABYV9			
HQ439023	China	*Cucurbita pepo*	Unpublished
Polerovirus melo; cucurbit whitefly-borne yellows virus – CWBYV			
LC217993	Brazil (Rio Grande do Norte; Mossoro)	*Cucumis melo*	[22,28]
LC217994	Brazil (Bahia; Juazeiro)	*Cucumis melo*	
LC516688	Brazil (Rio Grande do Norte; Mossoro)	*Cucumis melo*	

The distinct species were renamed according to the new binomial nomenclature for virus species [77]. The species were tentatively named "*Polerovirus curcubitaeprimum*, *Polerovirus curcubitaesecundum*, *Polerovirus curcubitaetertium*, *Polerovirus curcubitaequartum*, and *Polerovirus curcubitaequintum*", and the correspondent virus member names would be cucurbit aphid-borne yellows virus 1 to cucurbit aphid-borne yellows virus 5 (CABYV1 to CABYV5) (Table 1).

Based on the amino acid identity, all CABYV-PF from Brazil and isolates from France and Spain were included in the proposed "*Polerovirus curcubitaeprimum*" species. Thirty-eight isolates from South Korea, China, Japan, and United States were included in the proposed "*Polerovirus curcubitaesecundum*" species. Isolates from Papua New Guinea (MG780352) and Taiwan (JQ700305) would be included as members of the species "*Polerovirus curcubitaequartum*, and *Polerovirus curcubitaequintum*", respectively. The isolates from Indonesia and Timor-Leste were included as members of the "*Polerovirus curcubitaetertium*" species, with the addendum that these isolates two isolates differ only in the P0 (89.5% aa

identity), while for the other proteins, these isolates share >90% aminoacid identity as seen in Table S5.

The recombinant isolates also were included in analyses, and thus five additional species were considered. Hence, the isolates from Taiwan (JQ700306), from India (MN688219 and MN688220), and from China (HQ439023) would be separated according to differences in P0, P1, and P2, while isolate from Spain (JF939813) diverges in the P0 and P1 (Table S5). Indian isolates (MN688219 and MN688220) were designated as strains of the same species but diverged only in P0, sharing 89.2% amino acid identity, similar to the case of Indonesian and Timorese isolates. The Brazilian recombinants (LC217994, LC217993, and LC516688) could be joined to compose a species according to differences seen in the P3a, P3, P4, and P5.

The Spanish, Taiwanese, Indian, Chinese, and Brazilian recombinant isolates were placed in the species named "*Polerovirus curcubitaesextum*, *Polerovirus curcubitaeseptimum*, *Polerovirus curcubitaeoctavum*, *Polerovirus curcubitaenonum*, and *Polerovirus melo*" (Table 1). The virus names proposed for the virus members of these species are cucurbit aphid-borne yellows virus 6 to cucurbit aphid-borne yellows virus 9 (CABYV6 to CABYV9). For the Brazilian isolates from melon, the name cucurbit whitefly-borne yellows virus (CWBYV) was suggested by Costa et al. [28].

According to our findings, the 56 complete sequences described as CABYV strains belong to a complex of polerovirus species and, along with CABYV-PF identified in this study, would be classified in at least ten distinct species that infect mainly Cucurbitaceae, Solanaceae, and Passifloraceae plants (Table 1).

4.5. Conclusions

In this study, we obtained the complete genome of CABYV isolates from passion fruit and simultaneously categorized several strains previously identified as CABYV around the world as belonging to a complex of 10 different species in the genus *Polerovirus*. Members of these species infect mainly hosts in the families Cucurbitaceae, Solanaceae, and Passifloraceae. The passion fruit isolates were classified as members of the "*Polerovirus curcubitaeprimum*" species. We detected a high incidence of CABYV1 in mixed infection with CABMV in passionfruit plants, which can be an emergent problem in this fruit crop in Brazil. Further studies are needed to evaluate the epidemiology of CABYV1, the impacts of the interaction of mixed CABYV1/CABMV in crop productivity, and the identification of the insect vectors responsible for the transmission/establishment of these viruses. This information can provide a better understanding of the biology of CABYV and the establishment of measures to control the spread of CABYV in passion fruit crops in the country.

4.6. References

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4.7. Supplementary tables

Table S1. Primers used in this study.

Target	Primer name	Sequence 5'- 3'	Gene (bp)	Reference
CABYV	Modified-CE-9F	ATGAATACGGTCGCGGCTAGAAATC	CP/MP 600	[32]
	CE-10R	CTATTTCGGGTTCTGGACCTGGC		[13]
CABMV	CABMVLNJP2492F	GGTTCGTGATGTTTTGGTGCC	HC-Pro/p3 ~900	[13]
	CABMVLNJP3373R	CAAAAAGCACGCACTCACAAATC		[35]
rbcL	SI_For	ATGTCACCACAAACAGAGACTAAAGC	rbcL ~550	[35]
	SI_Rev	GTAAAATCAAGTCCACCRCG		[35]
matK	KIM 3F	CGTACAGTACTTTTGTGTTTACGAG	matK ~850	[35]
	KIM 1R	ACCCAGTCCATCTGGAAATCTTGGTTC		[39]
5'Race GSP 1	AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	5' end ~700	This study
	CABYVRACE581R	AAAGCGAGGCGAGTGTAAGC		[39]
5'Race GSP 2	AUAP	GGCCACGCGTCGACTAGTAC	5' end ~400	This study
	CABYVRACE300R	GGCGGGCAGTCGTAAATCA		[39]
3'Race GSP 1	M10	AAGCAGTGTTATCAACGCAGA	3' end ~700	This study
	CABYVRACE5063F	GCGTCTACAAGCTCTCGTCTC		[39]
3'Race GSP 2	M10	AAGCAGTGTTATCAACGCAGA	3' end ~350	This study
	CABYVRACE5365F	CGCACAGTCAGAAAAACTCGC		[39]
3'Race	M10PacIT50VN	AAGCAGTGTTATCAACGCAGATTAATTAAT50VN	cDNA	This study
Amplicon1	CABYV5F	ACAAAAGATACGAGCGGGTGATG	~1100	This study
	CABYV1186R	TTGCTTAACACAGATGCATGAGTTT		This study
Amplicon2	CABYV377F	GATACAACGAAACCAGACAAACCTT	~3700	[32]
	CE-10R	CTATTTCGGGTTCTGGACCTGGC		This study
Amplicon3	Modified-CE-9F	ATGAATACGGTCGCGGCTAGAAATC	~2100	This study
	CABYV3R	ACACCGAAACGCCAGGGGG		[32]

GSP: Gene-Specific Primer, AAP: Abridged Anchor Primer, AUAP: Abridged Universal.

Table S2. Number of passion fruit plants positive for CABYV and CABMV.

Sampling location	**Nº of positive plants**		
Plant species	**CABYV**	**CABMV**	**1 CABYV+CABMV**
Marcionílio de Souza			
Passiflora edulis	0/6 (0.0)2	5/6 (83.3%)	0/6 (0.0)
Seabra			
Passiflora edulis	5/8 (62.5%)	7/8 (87.5%)	5/8 (62.5%)
Brumado			
Passiflora edulis	0/2 (0.0)	1/2 (50%)	0/2 (0.0)
Morro do Chapéu			
Passiflora edulis	1/2 (50%)	2/2 (100%)	1/2 (50%)
Dom Basílio			
Passiflora edulis	3/11 (27.2%)	8/11 (72.7%)	3/11 (27.2%)
Lençóis			
Passiflora cincinnata	01/03	02/03	01/03
Passiflora alata	02/03	03/03	02/03
Passiflora edulis	8/15	10/15	05/15
Total	11/21 (52.3%)	15/21 (71.4%)	8/21 (38%)
Jussiape			
Passiflora edulis	1/9 (11.1%)	9/9 (100%)	1/9 (11.1%)

[1] Co -infection.
[2] Percentage of infected plants is in parenthesis

Table S3. List of passion fruit plants evaluated in this study, symptoms observed, and virus identified.

City	Code	Species	Symptoms	Virus
Marcionílio de Souza	500	*Passiflora edulis*	CR, B, M	CABMV
	501	*Passiflora edulis*	M	CABMV
	502	*Passiflora edulis*	M	CABMV
	503	*Passiflora edulis*	M	CABMV
	504	*Passiflora edulis*	M, YS, B, CR, LD	Not detected
	505	*Passiflora edulis*	M, YS, LD	CABMV
Seabra	557	*Passiflora edulis*	M, C, LD	CABYV, CABMV
	558	*Passiflora edulis*	M, C, LD	CABYV, CABMV
	559	*Passiflora edulis*	M, C, LD	CABYV, CABMV
	560	*Passiflora edulis*	M, C, LD	CABYV, CABMV
	561	*Passiflora edulis*	M, C, LD	CABMV
	562	*Passiflora edulis*	M, C, LD	Not detected
	563	*Passiflora edulis*	M, C, LD	CABMV
	564	*Passiflora edulis*	M, C, LD	CABYV, CABMV
Brumado	629	*Passiflora edulis*	M, B, LD	CABMV
	630	*Passiflora edulis*	LD	Not detected
Morro do Chapéu	798	*Passiflora edulis*	M, B	CABMV
	799	*Passiflora edulis*	M	CABYV, CABMV
Dom Basílio	594	*Passiflora edulis*	Y, LD	Not detected
	595	*Passiflora edulis*	M	Not detected
	596	*Passiflora edulis*	M, VB	CABMV
	597	*Passiflora edulis*	M	CABYV, CABMV
	598	*Passiflora edulis*	M	CABMV
	599	*Passiflora edulis*	IS	Not detected
	600	*Passiflora edulis*	LD, IS	CABMV

	601	*Passiflora edulis*	LD, M, Y	CABMV
	602	*Passiflora edulis*	IS, M	CABYV, CABMV
	603	*Passiflora edulis*	YD, LD	CABMV
	611	*Passiflora edulis*	Y, LD, M	CABYV, CABMV
	523	*Passiflora cincinnata*	M, LD	Not detected
	524	*Passiflora cincinnata*	M	CABMV
	525	*Passiflora cincinnata*	M	CABYV, CABMV
	716	*Passiflora edulis*	VB	CABYV
	721	*Passiflora edulis*	GS, M, B	CABYV, CABMV
	723	*Passiflora edulis*	B, LD, M	CABMV
Lençóis	724	*Passiflora edulis*	B, LD, M	CABYV, CABMV

Abbreviation of symptoms: Crinkled (CR), mosaic (M), leaf deformation (LD), fruit deformation (FD), Blistering (B), yellow spot (YS), chlorosis (C), yellowing (Y), insect damage (ID), vein banding (VB), green spot (GS), vein whitening (VW), leaf purplish (LP).

Table S4. List of green manure or spontaneous plants positive for CABYV and CABMV, and symptoms observed.

Code	Family/ Genus	Species	Symptoms	Virus
540	Fabaceae/ *Macroptilium*	*Macroptilium* spp.	M	CABYV
541	Cucurbitaceae/ *Cucumis*	*Cucumis* spp.	Y	CABYV
543	Fabaceae/ *Macroptilium*	*Macroptilium* spp.	VB	CABYV
717	Fabaceae/ *Macroptilium*	*Macroptilium* spp.	UR	CABYV, CABMV
757	Fabaceae/ *Stylosanthes*	*Stylosanthes* spp.	YS	CABYV
760	Malvaceae/ *Sida*	*Sida* spp.	Y	CABYV, CABMV
761	Bignoniaceae/ *Bignonia*	*Bignonia* spp.	AS	CABYV, CABMV

Symptoms: mosaic (M), yellow spot (YS), chlorosis (C), yellowing (Y), vein banding (VB), asymptomatic (AS), and unregistered (UR).

Table S5. Pairwise nucleotide (nt) and amino acid (aa) sequence percentage identities generated with SDT for the complete genome and proteins P0 to P5 and P3a of CABYV-PF and other CABYV sequences from GenBank.

This supplementary table in excel file format can be downloaded at: https://www.mdpi.com/article/10.3390/v15020410/s1

4.8. Supplementary figures

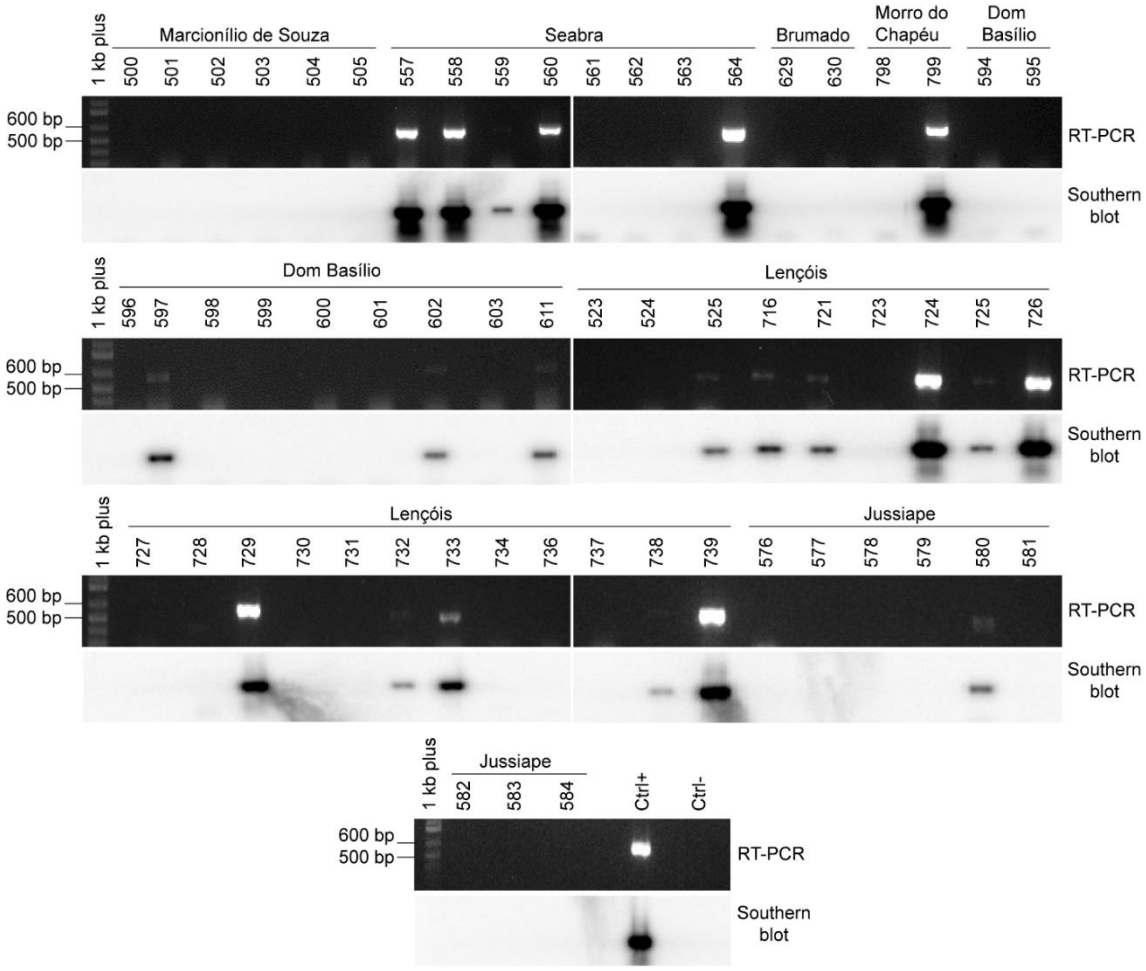


Figure S1. RT-PCR and Southern blot hybridization detection of CABYV in passion fruit plants. RT-PCR positive control: Ctrl+ (RNA from pool PM2Ba). RT-PCR negative control: Ctrl- (H2O).

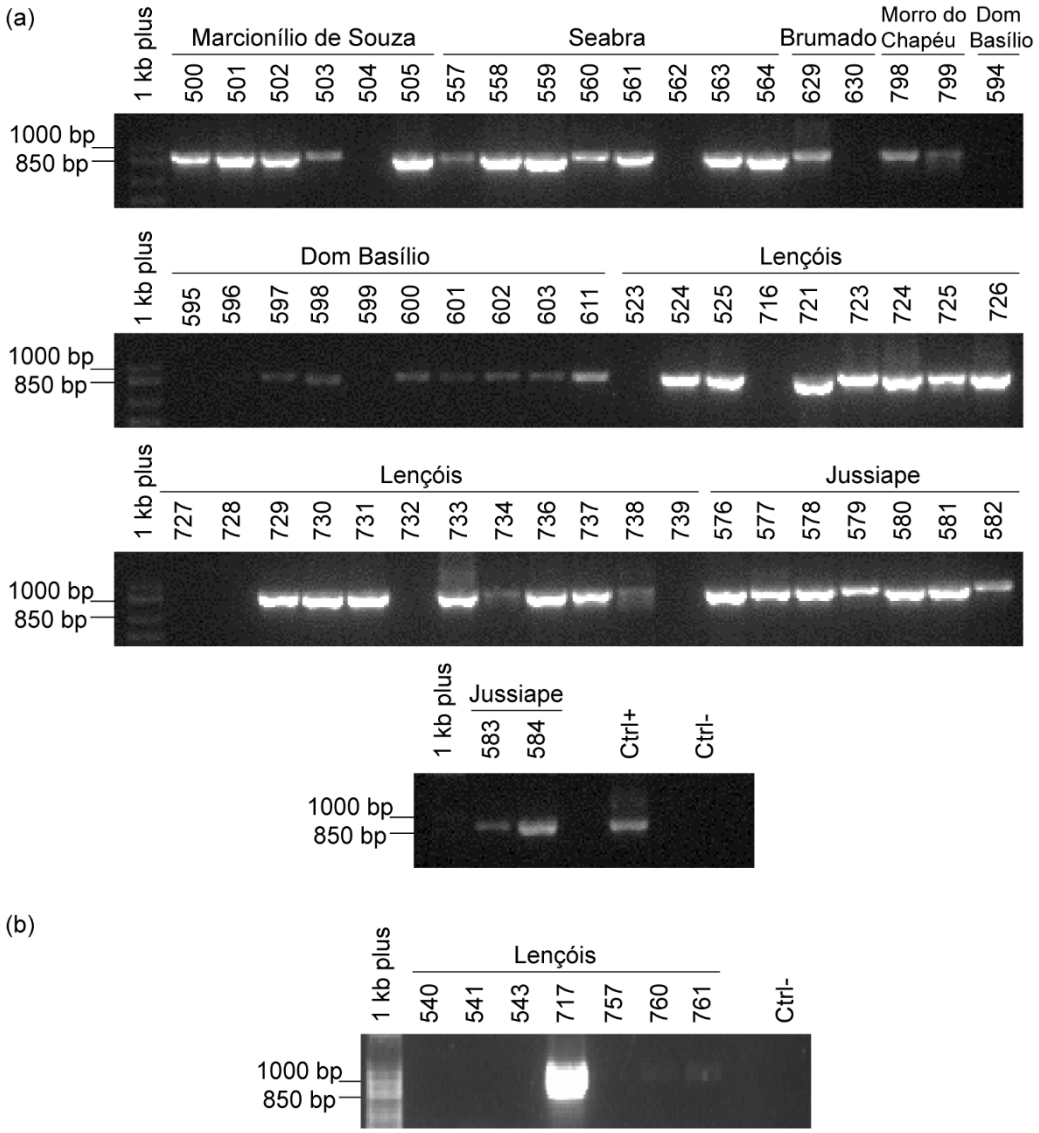


Figure S2. CABMV detection by RT-PCR in all passion fruit plants (a), and in green manure/spontaneous plants CABYV-positives (b). RT-PCR positive control: Ctrl+ (RNA from pool PM2Ba). RT-PCR negative control: Ctrl- (H2O).

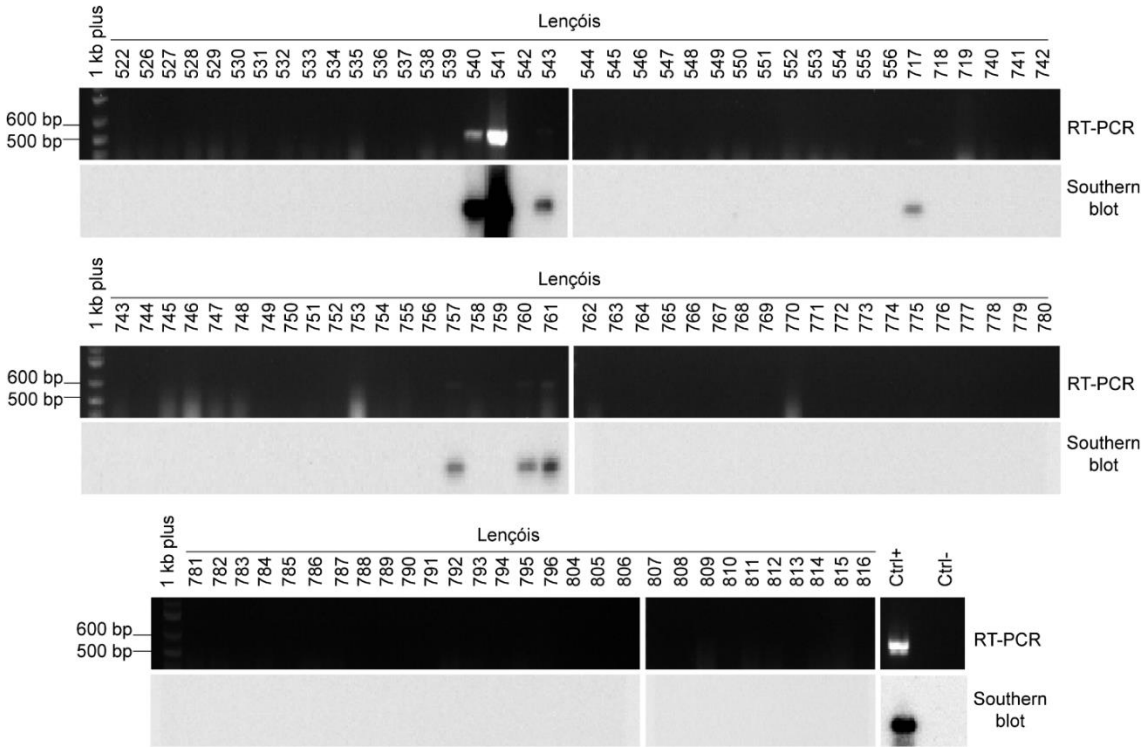
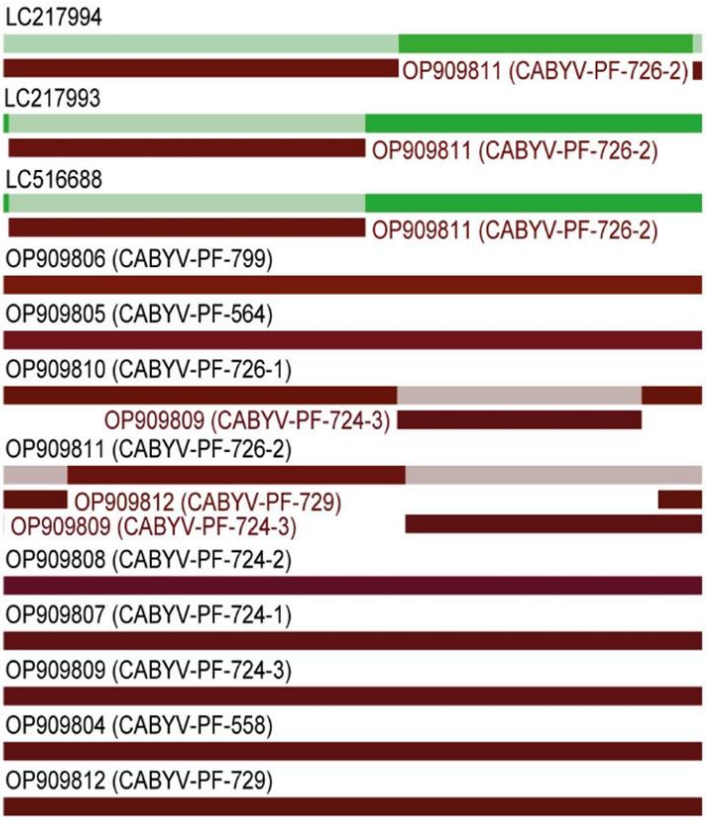


Figure S3. CABYV detection in green manure/spontaneous plants from Lençóis by RT-PCR and Southern blot hybridization. RT-PCR positive control: Ctrl+ (RNA total from pool PM2Ba). RT-PCR negative control: Ctrl- (H2O).

(a)



(b)

Sequence	Recombination breakpoint (nt)	Average p-Value (RDP)	Method of detection						
			R	G	B	M	C	S	T
LC217994	1-3347	1.12 X 10-73	+	+	+	+	+	+	+
	5673-5707	1.12 X 10-73	+	+	+	+	+	+	+
LC217993	50-3064	1.12 X 10-73	+	+	+	+	+	+	+
LC516688	40-3064	1.12 X 10-73	+	+	+	+	+	+	+

Figure S4. Recombination analysis among Brazilian CABYV isolates. (a) Schematic diagram showing putative recombinant fragments of Brazilian recombinant isolates from melon and CABYV-PF detected in RDP4. (b) Details of recombination events detected by RDR4 software in the genomes of CABYV-Brazilian melon recombinant isolates. Methods: R = RDP, G = GENECONV, B =Bootscan, M = MaxChi, C = Chimaera, S = SiScan, T = 3Seq.

Chapter 5

Caracterização de novos rhabdovirus infectando *Passiflora* spp. do Brasil

This chapter is under construction in the format of a paper intended to be published as "Characterization of novel rhabdoviruses infecting *Passiflora* spp. in Brazil". Andreza Henrique Vidal, Ana Clara Rodrigues Abreu, Monique Jacob Xavier Vianna, Cristiano Lacorte, Emanuel Felipe Medeiros Abreu, Gustavo Pereira Felix, Dione Mendes Teixeira Alves-Freitas, Bruna Pinheiro-Lima, Isadora Nogueira, Fabio Gelape Faleiro, Raul Castro Carriello Rosa, Onildo Nunes Jesus, Marcio Martinello Sanches, Rosana Blawind, José Leonardo Santos Jiménez, Maitê Freitas Silva Vaslin, Elliot Watanabe Kitajima, Arvind Varsani, Fernando Lucas Melo, Simone Graça Ribeiro.

Caracterização de novos rhabdovirus infectando *Passiflora* spp. do Brasil

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5.1. Introdução

Recentes descobertas têm aumentado substancialmente o conhecimento sobre a diversidade de vírus que infectam importantes culturas ao redor do mundo. Isso tem sido favorecido particularmente pelo uso massivo das tecnologias de sequenciamento de alto rendimento (do inglês, High-Throughput Sequencing- HTS), uma vez que essa abordagem permite a identificação de vírus em ambientes distintos, em hospedeiros sem sintomas aparentes, e sem o conhecimento prévio do patógeno. Essa abordagem tem sido intensamente aplicada nos últimos anos para identificação de vírus que pertencem a família *Rhabdoviridae* (Alves-Freitas et al., 2019; Bejerman et al., 2021; Ma et al., 2022; Vieira et al., 2022).

O maracujá é um fruto produzido por plantas do gênero *Passiflora* (família Passifloraceae) cultivada em várias regiões do mundo. O Brasil é responsável pela maior produção do fruto no mundo com cerca de 690 toneladas em 2021 (IBGE, 2021). O fruto é altamente valorizado na indústria alimentícia para produção de sucos, ou destinados ao consumo in natura, além ampla utilização na indústria cosmética e fitoterápica, e mais recentemente essas plantas têm sido fortemente exploradas e cultivadas como plantas ornamentais (Faleiro et al., 2020).

Existem pouquíssimos relatos de rhabdovirus em maracujazeiros no mundo. O primeiro registro é datado da década de 80 de um nucleorhabdovirus não classificado, denominado de passionfruit rhabdovirus (PRV) encontrado infectando *Passiflora edulis* crescidas em Sydney, na Austrália (Pares et al., 1983). Mais tarde, outro provável nucleorhabdovirus, denominado passion fruit vein clearing virus (PVCV) foi identificado em maracujazeiros em vários estados brasileiros e associado **a** uma condição de "enfezamento" nestas plantas (Chagas et al., 1987; Kitajima, 2020; Kitajima and Crestani, 1985). Contudo, não há nenhum dado molecular ou caracterização taxonômica associada ao PRV e PVCV. Recentemente, dois vírus membros da espécie *Cytorhabdovirus caricae* (gênero *Cytorhabdovirus*) foram identificados em maracujazeiros. O citrus-associated rhabdovirus (CiaRV) reportado em *P. edulis* na China (Zhang et al., 2020), e o bean associated-cytorhabdovrius (BaCV) identificado em várias espécies de *Passiflora* do Brasil (Chapter3) (Vidal, Lacorte, et al., 2023).

Atualmente a família *Rhabdoviridae* é composta por 246 espécies de vírus dividida em três subfamílias e 40 gêneros, sendo a Subfamilia *Betarhabdovirinae* composta pelos gêneros *Alphnucleorhabdovirus*, *Betanucleorhabdovirus*, *Gammanucleorhabdovirus*, *Cytorhabdovirus*, *Dichorhavirus* e *Varicosavirus* formados por vírus que infectam plantas e vetores artrópodes (Walker et al., 2022).

Os rhabdovirus apresentam genoma de RNA senso negativo fita simples, com genomas variando de 10 a 16 kb, podendo ser monopartido, com alguns poucos membros apresentando genomas bipartidos (Bejerman et al., 2021). Esses vírus apresentam uma organização genômica típica dos membros da família, codificando pelos menos cinco proteínas: 5'- nucleocapsídeo (N), fosfoproteína (P), proteína da matriz (M), glicoproteína (G) e RNA polimerase dependente de RNA (L)- 3', separados por regiões intergênicas flanqueadas por sequências relativamente conservadas ao longo do genoma que atuam no início da transcrição do mRNA (Walker et al., 2022). O genoma ainda pode codificar proteínas acessórias flanqueadas pelas mesmas regiões intergênicas conservadas que antecedem as demais proteínas. Em sua grande maioria, as proteínas acessórias ainda não têm função conhecida (Walker et al., 2015).

Neste estudo, utilizando tecnologia HTS para estudar e caracterizar o viroma de maracujazeiros do Brasil foram identificados sequencias de novos rhabdovirus associados a cultura.

5.2. Materiais e métodos

5.2.1. Material vegetal

Neste estudo foram avaliados maracujazeiros coletados em 2015 no estado da Bahia (Marcionílio de Souza *n*= 6 *P. edulis*; Seabra *n*= 8 *P. edulis*; Morro do Chapéu *n*= 2 *P. edulis*; Brumado n=2 *P. edulis*; Dom Basílio n=11 *P. edulis*; Lençóis *n*= 3 *P. cincinnata*; *n*= 3 *P. alata*, *n*= 15 *P. edulis*; Jussiape *n*= 9 *P. edulis*), no Distrito Federal em 2017 (acessos de *Passiflora* spp. do Banco de Germoplasma "Flor da Paixão"- BAG-FP: Planaltina *n*= 58; Campo: Planaltina *n*= 19), 2018 (Brazlândia *n*= 20), e 2019 (BAG-PF *n*= 18), em 2016 no estado da Paraíba (Cuité *n*= 24), em 2019 no estado de Pernambuco (Vitoria de Santo Antão *n*= 11) e em 2021 no estado do Rio de Janeiro (Seropédica *n*= 51). Essas plantas tiveram folhas amostradas em estudos anteriores (Chapter 2, 3 e 4) (Vidal et al., 2021; Vidal, Felix, et al., 2023; Vidal, Lacorte, et al., 2023; Vidal et al., 2018), e são mantidas em nossa coleção armazenadas a -80 °C.

5.2.2. Extração de dsRNA, sequenciamento HTS, e Bioinformática

dsRNAs foram obtidos de plantas de 37 acessos do BAG-FP, e usados para compor dois pools denominados BAG2-PF (*n*= 17 amostras) e BAG3-PF (*n*= 20 amostras). O preparo dos dsRNAs, a montagem dos pools, e o sequenciamento High-Throughput Sequencing (HTS) foram todos preparados e realizados conforme descrito por Vidal (Vidal et al., 2021).

Neste estudo foram reanalisadas as bibliotecas sequenciadas anteriormente BAG1-PF (*n*= 21 amostras) (Chapter 2) (Vidal et al., 2021), PM1BA (*n*= 29 amostras) e PM2BA (*n*= 30 amostras) (Chapter 4) (Vidal, Lacorte, et al., 2023), bem como as bibliotecas BAG2-FP (*n*= 17 amostras) e BAG3-FP (*n*= 20 amostras) construídas neste estudo.

Os *reads* foram trimados e montados usando CLC Genomics Workbench (Workbench). Os *contigs* foram comparados usando a ferramenta Blastx contra um banco local de dados de sequências virais, e posteriormente confirmadas no banco de sequencias do NCBI. As sequências virais identificadas foram obtidas a partir do consenso gerado pelo *de novo assembly* dos *contigs* no programa Geneious Prime® 2022.1.1 (Kearse et al., 2012). A predição da organização genômica foi inferida no programa Geneious Prime® 2022.1.1 e com auxílio da ferramenta ORFfinder (do inglês, Open Reading Frame Finder acessado em: https://www.ncbi.nlm.nih.gov/orffinder/).

5.2.3. Detecção do vírus identificados nos dados do HTS

Com base nas sequencias virais identificadas a partir dos dados do sequenciamento HTS, primers específicos foram projetados para cada vírus e usados em ensaios de RT-PCR para avaliar a sua presença nas amostras individuais. Cada amostra foi submetida a uma RT-PCR específica para o vírus usando o kit SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen, USA).

Os produtos da RT-PCR obtidos foram visualizados em gel de agarose corados com SYBR® Safe (Invitrogen, USA). Os fragmentos do tamanho esperado foram excisados e purificados com o kit Wizard® SV Gel and PCR Clean-Up System (Promega) conforme as recomendações do fabricante.

Os amplicons purificados foram clonados com auxílio do kit CloneJET PCR Cloning (Thermo Fisher Scientific, USA). O DNA plasmidial foi extraído utilizando o kit Wizard® Plus SV Minipreps DNA Purification Systems (Promega, USA) conforme as instruções do fabricante. Os clones finais foram sequenciados por Sanger (Macrogen- Coreia do Sul). As sequências obtidas foram analisadas e montadas com auxílio do programa Geneious Prime® 2022.1.1.

5.2.4. Obtenção do genoma completo dos novos rhabdovirus

A fim de obter o genoma quase completo dos putativos novos rhabdovirus e baseados nas sequencias preditas do HTS, primers que amplificam fragmentos com overlaping >150 nt foram projetados. As extremidades 5’ e 3’ serão confirmadas em etapas posteriores do trabalho pelo método de RACE conforme Nicolini et al., (2012).

5.2.5. Análise genômica e filogenética

As árvores filogenéticas foram inferidas usando o método de maximum-likelihood (ML) implementado no software RAxML-NG v. 1.0.3 (Kumar et al., 2018), a partir de um alinhamento múltiplo das sequências de aminoácidos da proteína L geradas no Geneious Prime® 2022.1.1 usando o programa MAFFT (Multiple Alignment using Fast Fourier Transform) (Katoh et al., 2002). O modelo LG+I+G4+F foi usado para gerar todas as árvores ML. Os modelos das árvores foram encontrados usando o ModelTest-NG v0.1.7 (Darriba et al., 2020). As árvores ML foram calculadas com 1000 réplicas de bootstrap, e as árvores finais foram editadas e visualizadas usando o FigTree v1.4.4 (acessível em http://tree.bio.ed.ac.uk/software/figtree/).

Os percentuais de identidade de nt e aa foram calculados com a Ferramenta de Demarcação de Sequência (SDT) v1.2 (Muhire et al., 2014).

5.2.6. Microscopia eletrônica de transmissão novos rhabdovirus

Em 2019 uma segunda coleta foi realizada no BAG-FP. Folhas de plantas com resultado positivo para rhabdovirus foram coletadas, o RNA total foi extraído e usado em reações de RT-PCR para confirmação do vírus. As amostras para exame de microscopia eletrônica foram preparadas de acordo com (Pinheiro-Lima et al., 2020), a partir de secções foliares de passiflora testadas positivas para o vírus, seguido da visualização em microscópio eletrônico de transmissão.

5.3. Resultados e discussão

5.3.1. Predição preliminar das sequencias dos novos rhabdovirus infectando maracujazeiros

O HTS permitiu a identificação de sequências com identidade com rhabdovirus nas plantas analisadas. A análise dos dados gerados pelo HTS das bibliotecas BAG1-PF, BAG2-FP, BAG3-FP (Acessos de *Passiflora* spp. do Banco Ativo de Germoplasma "Flor da Paixão"- BAG-FP), PM1BA e PM2BA (maracujazeiros de campos comerciais no estado da Bahia) revelou sequências com baixa identidade (<50%) com rhabdovirus nas bibliotecas BAG2-FP, BAG3-FP e PM1BA.

Com auxílio de ferramentas de bioinformática, sequências genômicas parciais foram preditas (Figura 1). A primeira sequência denominada provisoriamente de passiflora cytorhabdovirus (PFCV-BAG3), e uma segunda denominada passiflora nucleorhabdovirus 2 (PaNV2-BAG3) foram identificadas e preditas a partir dos dados sequenciados da biblioteca BAG3-FP. Duas sequências adicionais foram identificadas e preditas a partir dos dados sequenciados da biblioteca PM1Ba, e denominada provisoriamente de passiflora nucleorhabdovirus 1 (PaNV1-PM1BA).

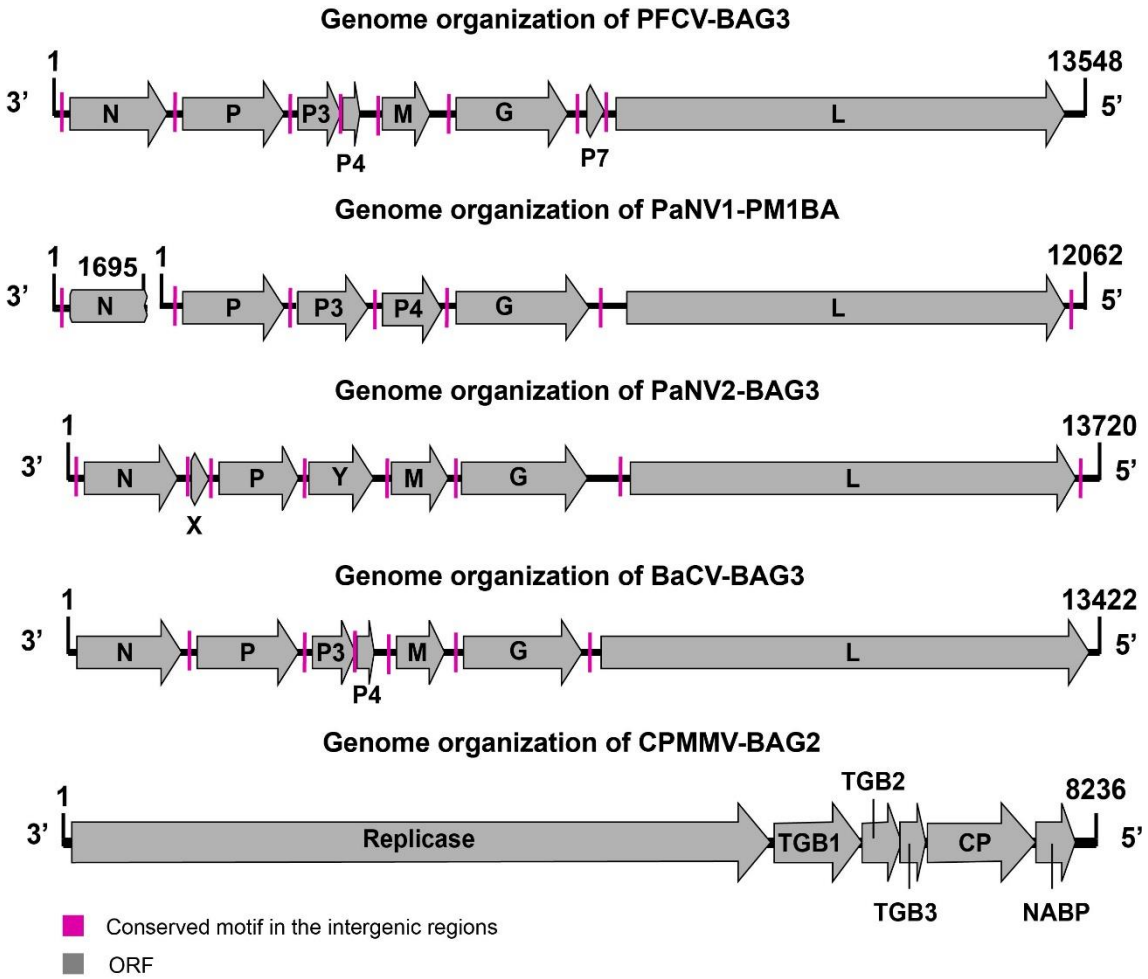

Figure 1. Representação esquemática da organização genômica das sequências sequencias identificadas nas análises do HTS. PFCV-BAG3: passiflora cytorhabdovirus. PaNV1-PM1BA: passiflora nucleorhabdovirus 1. PaNV2-BAG3: passiflora nucleorhabdovirus 2. BaCV-BAG3: bean-associated cytorhabdovirus. CPMMV-BAG2: cowpea mild mottle virus. N (Nucleocapsídeo), P (Fosfoproteina), M (Matrix), G (Glicoproteina) e L (RNA-dependente RNA polymerase) representam ORFs que codificam as proteínas estruturais típicas dos rhabdovirus. ORFs X, Y, P3, P4, e P7 indicam putativas proteínas acessórias. Replicase, TGB1, TGB2, TGB3 (triple gene block 1, 2, 3), CP (coat protein), e NABP (nucleic binding protein) representam ORFs que codificam as proteínas estruturais típicas dos carlavirus.

O PFCV-BAG3 (Figura 1) tem 13548 nt, e possui uma identidade de aa de 50.28% com uma cobertura de 43% com a proteína L do putativo cytorhabdovirus gymnadenia_x_densiflora_virus_1 (DAF42331). A sequência do PFCV-BAG3 apresenta uma organização genômica típica de rhabdovirus (Walker et al., 2022), com cinco ORFs: N (ORF1) (1311 nt; 437 aa), P (ORF2) (942 nt; 314 aa), M (ORF5) (657 nt; 219 aa), G (ORF6) (1767 nt; 589 aa) e L (ORF8) (5994 nt; 1998 aa). Todas as ORFs são precedidas por uma sequência repetitiva consenso bem conservada 5' – WARGAAAAAYHWACCWGA – 3'. Adicionalmente, três outras proteínas hipotéticas foram identificadas e denominadas como P3 (ORF3) (381 nt; 127 aa), P4 (ORF4) (663 nt; 221 aa) situada entre as proteínas P e M, e P7 (ORF7) (174 nt; 58 aa) situada entre as proteínas G e L. Estas proteínas não apresentam similaridade com nenhuma outra proteína do banco de dados, contudo são precedidas pela mesma região repetitiva que antecede

as demais ORFs, portanto estas foram designadas como proteínas acessórias, o que é bem comum entre os rhabdovirus (Walker et al., 2015). Ainda não sabemos se a P3 e a P4 são proteínas distintas, uma vez que ambas são precedidas pela mesma sequência repetitiva, ou se codificam uma única proteína. Similarmente, o bean-associated cytorhabdovirus (BaCV) também apresenta ORFs putativas denominadas P3 e P4, com a mesma disposição genômica do PFCV-BAG3 (Alves-Freitas et al., 2019).

O PaNV2-BAG3 (Figura 1) tem uma sequência composta por 13720 nt, compartilha maior identidade de aa de 45%, com uma cobertura da sequência de 42%, com a proteína L do physostegia chlorotic mottle virus (QDF44105), um membro do gênero *Alphanucleorhabdovirus.* O PaNV2-BAG3 também apresenta uma organização genômica típica de rhabdovirus com cinco ORFs: N (ORF1) (1368 nt; 456 aa), P (ORF3) (951 nt; 317 aa), M (ORF5) (768 nt; 256 aa), G (ORF6) (1824 nt; 608 aa) e L (ORF7) (5859 nt; 1953 aa). Uma sequência repetitiva que precede todas as ORFs ao longo do genoma foi também identificada com consenso 5'-HHTTARWWAAAACCCWWYMY – 3'. Com base nessas regiões repetitivas, duas ORFs adicionais foram preditas. A ORF2/proteína X (321 nt; 107 aa) localizada entre as proteínas N e P, não apresenta similaridade com nenhuma outra proteína viral do banco de dados. A ORF4/proteína Y (867 nt; 289 aa) localizada entre as proteínas P e M, possui 28% de identidade de aa, com uma cobertura de 98%, com uma putativa proteína de movimento (Y) do eggplant mottled dwarf nucleorhabdovirus (CDK85636) (Pappi et al., 2016). Esse resultado sugere a existência de uma provável proteína de movimento no PaNV2-BAG3.

Para o PaNV1-PM1BA não fomos capazes de predizer uma única sequência com a organização genômica de 5'-N-P-M-G-L-3', como foi para o PFCV-BAG3 e PaNV-BAG3 (Figura 1). Entretanto, duas sequencias identificadas (>1000 nt) a partir dos dados do sequenciamento da biblioteca PM1BA apresentam identidades com membros do gênero *Alphanucleorhabdovirus* já caracterizados.

A primeira sequência com 1695 nt (Figura 1) apresenta uma ORF parcial (1347 nt; 449 aa) e compartilha 40.2% de identidade de aa, e uma cobertura de 69%, com a proteína N do putativo alphanucleorhabdovirus artemisia capillaris nucleorhabdovirus 1 (UKL15216). A segunda sequência com 12062 nt (Figura 1) compartilha maior identidade de aa de 52.4% com uma cobertura de 41% com a proteína L do papaya nucleorhabdovirus 1 (QHB15169), e a segunda maior identidade de aa de 39% (cobertura de 48%) com a mesma proteína do artemisia capillaris nucleorhabovirus 1. A sequência de 12061 nt apresenta cinco ORFs. A ORF1 a ORF4 e a ORF5 codificam as proteínas P (981 nt; 327 aa), G (1863 nt; 621aa) e L (5871 nt; 1957 aa), respectivamente. Todas as ORFs preditas em ambas as sequências são antecedidas por uma região repetitiva consenso 5' - NWRWWWWMAAAACCCYMWMYW – 3'. Entre as proteínas P e G foram identificadas duas ORFs adicionais, ORF3/P3 (906 nt; 302 aa) e ORF4/P4 (828 nt; 276 aa) que não apresentam similaridade com nenhuma proteína do banco de dados. Portanto, não foi

possível predizer quais proteínas são codificadas por essas ORFs, mas com base nas características gerais dos rhabdovirus (Walker et al., 2022), é provável que uma codifique a proteína M e outra uma proteína acessória. Acreditamos que essas duas sequências fazem parte do mesmo vírus que nomeamos de PaNV1-PM1Ba (Figura 1), uma vez que que ambas apresentam identidades com o mesmo grupo de vírus, e foram preditas a partir do mesmo conjunto de dados.

O critério vigente de demarcação de espécies para os gêneros *Cytorhabdovirus* e *Alphanucleorhabdovirus* baseia-se na percentagem de identidade de nucleotídeos do genoma completo e sequência de aminoácidos das proteínas. Diferentes espécies devem apresentar identidade de nt dos genomas completos <75%, e identidade da sequência de aa entre genes cognatos (5-N-P-M-G-L-3') <80% (Walker et al., 2022). Com base nos dados apresentados para o PFCV-BAG3, PaNV2-BAG3 e PaNV1-PM1BA, há a indicação da ocorrência isolados candidatos a três novas espécies de rhabdovirus infectando *Passiflora* spp. do Brasil. O PFCV-BAG3 parece ser um candidato ao gênero *Cytorhabdovirus*, e PaNV2-BAG3 e PaNV1-PM1BA candidatos ao gênero *Alphanucleorhabdovirus*.

Essa relação foi confirmada com base na análise filogenética Maximum Likelihood (Figura 2 e 3) gerada a partir de um alinhamento múltiplo das sequências de aa da proteína L de todos os membros que compõe os gêneros *Alphanucleorhabdovirus* e *Cytorhabdovirus* que infectam de plantas, aceitas pelo ICTV.

O PFCV-BAG3 mostrou ser relacionado com membros do gênero *Cytorhabdovirus* (Figura 2), agrupando com *gymnadenia densiflora virus 1* (BK014305), e trachyspermum ammi virus 1 (BK014309) (Bejerman et al., 2021). Além disso a filogenia mostrou que esse grupo de vírus é mais relacionado com alguns *Cytorhabdovirus* que são transmitidos por afídeos. Isso sugere que afídeos são fortes candidatos a serem os vetores de transmissão do PFCV em maracujazeiros. É evidente que essa hipótese precisa ser avaliada em estudos posteriores.

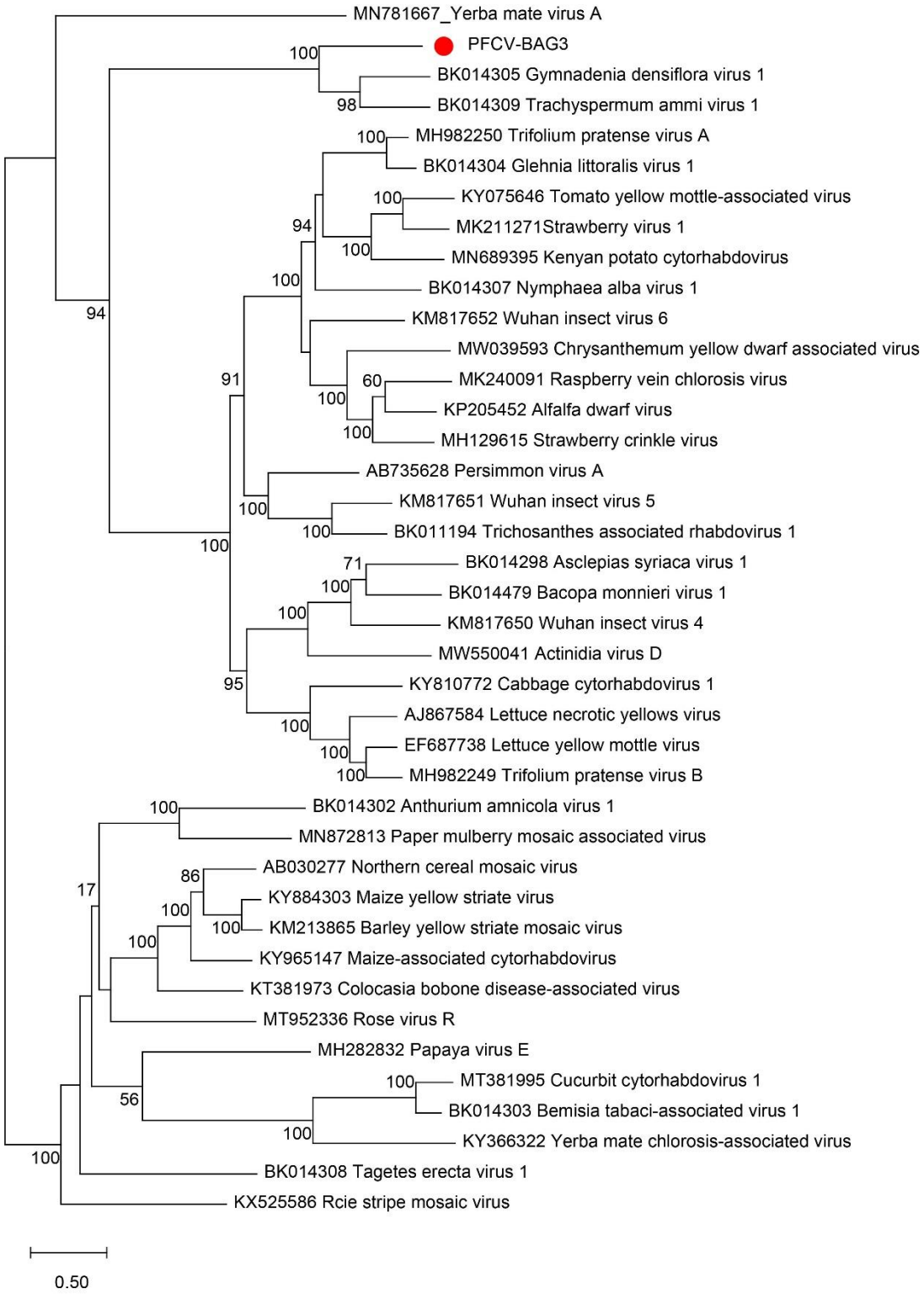

Figure 2. Árvore filogenética da sequência de aminoácidos da proteína L do PFCV-BAG3 obtida neste estudo e membros que compõe o gênero *Cytorhabdovirus* aceitas pelo ICTV. Ponto em vermelho destaca a sequência do PFCV obtida neste estudo. A árvore filogenética foi construída usando o método Maximum Likelihood geradas no RAxML-NG v. 1.0.3 software a partir de um alinhamento múltiplo usando MAFFT e gerado no Geneious Prime® 2022.1.1. Os valores de bootstrap >50% para 1000 réplicas são exibidos próximos às ramificações. A barra indica 0,50 substituições de aminoácidos por sítio.

O PaNV1-PM1Ba e PaNV2-BAG3 clusterizaram com membros do gênero *Alphanucleorhabdovirus* (Figura 3). O PaNV2-BAG3 formou um clado com o potato yellow dwarf virus (GU734660), constricta yellow dwarf virus (KY549567), joa yellow blotch associated virus (MW014292), eggplant mottled dwarf virus (KJ082087), e physostegia chlorotic mottle virus (KX636164). O PaNV1-PM1BA clusterizou com rice yellow stunt virus (AB011257), wheat yellow striate virus (MG604920), e artemisia capillaris nucleorhabdovirus 1 (OM372677). Além disso, quase todos Alphanucleorhabdovirus com qual o PaNV1 e PaNV2 clusterizaram são conhecidos por serem transmitidos por cigarrinhas, o que sugere que esse inseto possa ser um candidato a transmissão dos alphanucleorhabdovirus de maracujá. Essa hipótese precisa ser investigada em estudos futuros.

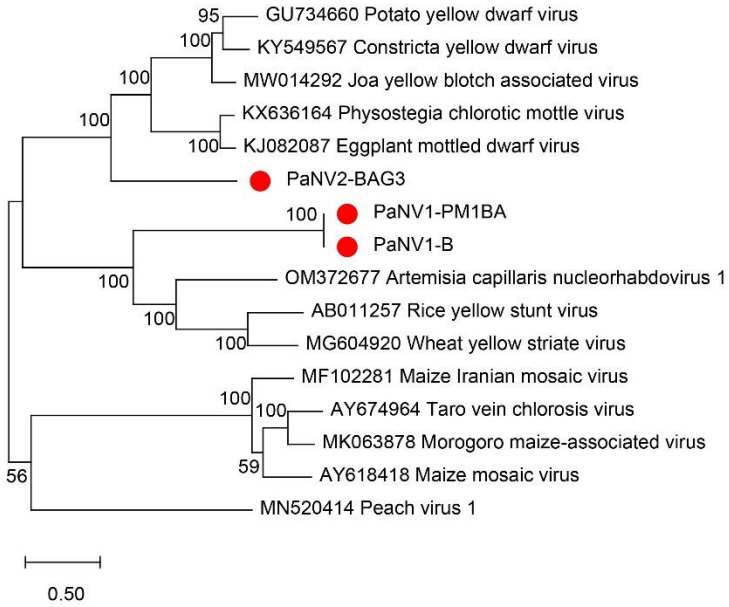

Figure 3. Árvore filogenética da sequência de aminoácidos da proteína L do PaNV1-PM1BA, PaNV1-B, e PaNV2-BAG3, obtidas neste estudo e membros que compõe o gênero *Alphanucleorhabdovirus* aceitas pelo ICTV. Pontos em vermelho destacam as sequências do PaNV1 e PaNV2 obtidas neste estudo. Árvore filogenética foram construídas usando o método Maximum Likelihood geradas no RAxML-NG v. 1.0.3 software a partir de um alinhamento múltiplo usando MAFFT gerado no Geneious Prime® 2022.1.1. Os valores de bootstrap >50% para 1000 réplicas são exibidos próximos às ramificações. A barra indica 0,50 substituições de aminoácidos por sítio.

Nossas análises também revelaram que PFCV-BAG3 não está restrito apenas a biblioteca BAG3. Usando PFCV-BAG3 como sequência de referência e mapeando os reads de todas as bibliotecas sequenciadas (BAG-FP1, BAG2-FP, PM1BA e PM2BA), foi possível identificar sequências adicionais com >90% de identidade com o PFCV-BAG3 nas bibliotecas BAG2-FP e PM1BA. Para os outros rhabdovirus nenhuma evidência foi identificada nas outras bibliotecas.

Baseada relação filogenética, na baixa similaridade de sequência apresentada pelos rhabdovirus de maracujazeiros, e no critério para classificação de novas espécies, o PFCV-BAG3 se enquadraria como uma nova espécie no gênero *Cytorhabdovirus*, enquanto o PaNV2-BAG3, e

PaNV1-PM1BA se enquadrariam como isolados pertencentes a novas espécies dentro do gênero *Alphanucleorhabdovirus*. Ainda, baseado na nova nomenclatura binomial (Zerbini et al., 2022) adotada para classificação de espécies virais, nos propomos que para o PFCV seja adotado o nome da espécie "*Cytorhabdovirus passiflorae",* enquanto para o PaNV1 e PaNV2 sejam adotados "*Alphanucleorhabdovirus passiflorae"* and "*Alphanucleorhabdovirus passionis"*, respectivamente.

5.3.2. Predição preliminar da sequência genômica de isolados de CPMMV e BaCV nas bibliotecas do BAG2-FP e BAG3-FP

Além dos novos rhabdovirus, a análise dos dados gerados pelo HTS das bibliotecas do BAG2-FP e BAG3-FP (acessos de *Passiflora* spp. do Banco Ativo de Germoplasma "Flor da Paixão"-BAG-PF) permitiu a identificação de sequencias com identidade com o bean-associated cytorhabdovirus (BaCV, espécie *Cytorhabdovirus caricae*, gênero *Cytorhabdovirus*, família *Rhabdoviridae*), e o cowpea mild mottle virus (CPMMV, gênero *Carlavirus*, família *Betaflexiviridae*) identificados anteriormente nessas mesmas plantas por PCR e sequenciamento Sanger (Vidal, Felix, et al., 2023).

As plantas do BAG-FP identificadas com BaCV e CPMMV, junto com outras plantas foram usadas para compor as bibliotecas BAG2-FP e BAG3-FP. Em ambas as bibliotecas, sequencias com alta identidade com BaCV (99.51% a 100% de identidade de nt) e CPMMV (84% a 98.89% identidade de nt) foram encontradas. Baseado nessas sequencias, duas sequencias genômicas para ambos os vírus foram preditas.

A partir da montagem *de novo* dos contigs e mapeamento dos reads a um genoma de referência para os dois vírus, genomas quase completos e parciais foram obtidos. Na biblioteca BAG3-FP uma sequência consenso de 13.422 nt para o BaCV foi obtida (Figura 1). A sequência denominada de BaCV-BAG3 compreende o genoma quase completo, faltando apenas as extremidades 5' e 3'. O BaCV-BAG3 tem uma organização genômica típica do rhabdovirus, contendo sete ORFs: 3′-N-P-P3-P4-M-G-L-5' (Figure 1). A sequência codante é flanqueada por 121 e 142 nt na extremidade 3' e 5' respectivamente, e todas as ORFs são precedidas por uma sequência conservada repetitiva: consensus 5 '-AAGAAAAACCGGGAKC-3'. Já na biblioteca BAG2-FP só foi possível a predição de sequencias parciais do BaCV. A pesquisa BLASTn mostrou que o BaCV-BAG3 tem uma identidade de nt entre 78% a 99,77% com os isolados BaCV (MK202584) do Brasil, papaya virus E (PpVE, MH282832) do Equador, e citrus-associated rhabdovirus (CiaRV, MT302541) da China.

Contigs com identidade com CPMMV também foram identificados em ambas as bibliotecas. Entretanto, uma sequência quase completa foi obtida apenas a partir dos dados do BAG2-FP. Foi obtida uma sequência consenso de 8.236 nt (CPMMV-BAG2). O CPMMV-BAG2 apresenta uma organização genômica típica de carlavirus, contendo seis ORFs, e regiões não traduzidas de 3'-

61nt e 169 nt-5'. As ORFs preditas codificam: replicase, triple gene block 1, 2, 3 (TGB1, TGB2, TGB3), proteína do capsídeo (CP) and nucleic binding protein (NABP) (Adams et al., 2012). Pesquisa BLASTn revelaram que a sequência CPMMV-BAG2 apresenta identidades de nt que variam entre 73,27% a 98,81% com vários isolados de CPMMV disponíveis no GenBank.

Além do BaCV e CPMMV, o crinivirus lettuce chlorosis virus (LCV, gênero *Crinivirus*, família *Closteroviridae*) foi identificado nas plantas do BAG-FP (Vidal et al., 2021), entretanto, a reanalise dos dados da biblioteca BAG1-PF (Vidal et al., 2021) (Chapter 2) usando outros montadores não permitiu a predição da sequência genômica do RNA1 e RNA2 que compõe o genoma LCV.

5.3.3. Distribuição dos rhabdovirus PFCV, PaNV1, PaNV2 no BAG-FP e regiões produtoras do Brasil, e em co-infeccao com outros vírus

Baseado nas sequencias preditas anteriormente, primers específicos foram projetados e usados em ensaios de RT-PCR para a detecção dos rhabdovirus nas amostras individuais. Além das amostras que compuseram o as bibliotecas iniciais sequenciadas, foram avaliadas amostras de maracujazeiros oriundos de outras regiões produtoras do país.

O PaNV2-BAG3 foi detectado apenas no BAG-FP, em quatro acessos de *Passiflora* (Tabela 1). Sendo *P. galbana* (amostra 1593), *P. quadrangularis* (amostra 1603), *P. riparia* (amostra 1624) e *P. gardneri* (amostra 1626) identificadas com o vírus.

O PaNV1-PM1BA foi identificado apenas na amostra 564, uma planta de *P. edulis* coletada na região de Seabra, no Estado da Bahia (Tabela 1). Considerando que o PaNV1-PM1BA foi identificado apenas em uma amostra dentre todas que compõe a biblioteca PM1BA, essa seria mais uma evidência de que as duas sequências (Figura 1) encontradas na análise inicial do sequenciamento pertencem ao mesmo vírus.

O PaNV1-PM1BA e PaNV2-BAG3 foram identificados inicialmente por HTS e a sua presença confirmada por RT-PCR nas plantas que compõe o BAG-PF situado no Distrito Federal, e na região de Seabra no estado da Bahia. Nas demais regiões avaliadas, nenhuma evidência desses dois vírus foi identificada (Tabela 1).

Dos três rhabdovirus identificados nesse estudo, o PFCV-BAG3 foi identificado em alta incidência e em mais de uma localidade no país. Com base nas análises do sequenciamento HTS foram encontradas sequencias do PFCV-BAG3 nas amostras que compõem as bibliotecas do BAG2, BAG3 e PM1Ba. O PFCV-BAG3 foi identificado em 17 acessos do BAG-FP, sendo três acessos positivos da biblioteca BAG2 e 14 acessos da biblioteca BAG3 (Tabela 1). Nas 29 amostras da Bahia que compõe a biblioteca PM1BA, o PFCV-BAG3 foi detectado em 11 plantas vindas de diferentes locais. Na região de Marcionílio de Souza 28,5% (2/7) das plantas foram positivas, Seabra 37,5% (3/8), Morro do Chapéu 50% (1/2), e em Dom Basílio 45,4% (5/11) (Tabela 1).

Além das amostras da Bahia (BA, região Nordeste), e do BAG-FP (DF, região Centro-Oeste), o PFCV-BAG3 foi identificado em regiões produtoras do Distrito federal (DF, região Centro-Oeste), Rio de Janeiro (RJ, região Sudeste), Paraíba (PB, região Nordeste), e Pernambuco (PE, região Nordeste), infectando espécies cultivadas, selvagens e híbridos de passiflora (Tabela 1). Nas amostras coletadas em Seropédica (RJ), o PFCV-BAG3 foi identificado em 94,2% (49/52) das plantas, de maracujazeiros do genótipo FB300 (25/25), e em híbridos H09-110/111 (24/27). Esse vírus parece estar amplamente distribuído nessa região, visto que foi detectado em quase todas as plantas avaliadas. Além do RJ, o rhabdovirus foi identificado em 9% (1/11) dos maracujazeiros (*P. edulis*) amostrados na região de Vitória de Santo Antão (PE), e em 16,6% (4/24) dos maracujazeiros (*P. edulis*) coletados em Cuité (PB). No DF, além da ocorrência no BAG-PF, o PFCV-BAG3 foi detectado em 55,5% das plantas coletadas nas regiões de Brazlândia [5 (1 *P. edulis* e 4 *P. alata*)/9] e em 52,6% das plantas coletadas em Planaltina [10 (*P. edulis*)/19)].

De acordo com os nossos resultados, o PFCV-BAG3 parece ser um vírus bem distribuído tendo sido identificado nos acessos mantidos no BAG-PF, localizado no Distrito Federal, e em diferentes regiões produtoras no estado da Bahia, Distrito Federal, Paraíba, Pernambuco e Rio de Janeiro (Tabela 1). Como a incidência do PFCV-BAG3 não está restrita a um único estado brasileiro, é provável que também esteja presente em outras localidades. Esses resultados sugerem a existência de uma rota de dispersão de mudas infectadas ou de algum vetor viral.

Em estudos anteriores foram identificados, os vírus lettuce chlorosis virus (LCV, gênero *Crinivirus*, família *Closteroviridae*), cowpea aphid-borne mosaic virus (CABMV, gênero *Potyvirus*, família *Potyviridae*), bean-associated cytorhabdovirus (BaCV, espécie *Cytorhabdovirus caricae*, gênero *Cytorhabdovirus*, família *Rhabdoviridae*), cowpea mild mottle virus (CPMMV, gênero *Carlavirus*, família *Betaflexiviridae*), e cucurbit aphid-borne yellows virus (CABYV, genus *Polerovirus*, family *Solemoviridae*) (Abreu et al., 2020; Vidal et al., 2021) infectando parte dessas plantas analisadas (Tabela 1). Dessa forma, quase todas as plantas identificadas com os rhabdovirus estão em infecção mista com pelo menos um vírus, principalmente o CABMV. O registro destes virus na tabela 1 corroboram as observações anteriores da presença frequente de infecções mistas em maracujazeriros (Abreu et al., 2020; Vidal et al., 2021).

Tabela 1. Detecção do passiflora cytorhabdovirus (PFCV-BAG3), passiflora nucleorhabdovirus 2 (PaNV2-PM1Ba), passiflora nucleorhabdovirus 1 (PaNV1-PM1Ba), cowpea aphid-borne mosaic virus (CABMV), lettuce chlorosis virus (LCV), cowpea mild mottle virus (CPMMV), bean associated cytorhabdovirus (BaCV), e curcubit aphid-borne yellow virus (CABYV) nas amostras individuais de *Passiflora* spp.

Código	**Espécie**	**Vírus detectados**
Distrito Federal; BAG-FP		
1632	BRS Estrela do Cerrado (*P.coccinea* x *P. setacea*)	CABMV
1621	BRS Rosea Púrpura (*P.incarnata* x *P. quadrifaria* x *P. setacea*)	CABMV
1633	BRS Roseflora (*P. coccinea* x *P. setacea* x *P. setacea*)	CABMV
1649	*Passiflora* spp.	CABMV; CPMMV; PFCV
1592	*P. alata*	CABMV
1614	*P. edulis* flor branca 2	CABMV; BaCV
1600	*P. mucronata*	CABMV
1641	*P. actínia*	CABMV
1596	*P. alata*	CABMV
1597	*P. alata*	CABMV
1643	*P. alata*	CABMV; LCV
1646	*P. ambígua*	CABMV
1636	*P. auriculata*	CABMV; LCV
1642	*P. auriculata*	CABMV
1620	*P. biflora*	CABMV
1630	*P. caerulea*	CABMV; PFCV
1604	*P. capparidifolia*	CABMV
1594	*P. capparidifolia*	CABMV
1645	*P. edulis*	CABMV
1639	*P. edulis*	CABMV
1591	*P. eichleriana x P. giberti*	CABMV; CPMMV; BaCV; PFCV
1644	*P. eichleriana x P. gibertii*	CABMV; CPMMV
1623	*P. elegans*	CABMV
1619	*P. ferrugínea*	CABMV; PFCV
1628	*P. foetida*	CABMV
1593	*P. galbana*	CABMV; CPMMV; PFCV; PaNV2
1610	*P. galbana*	CABMV; CPMMV
1626	*P. gardneri*	CABMV; BaCV; CPMMV; PaNV2
1625	*P. hatschbachii*	CABMV; CPMMV; PFCV
1607	*P. incarnata*	CABMV; CPMMV; PFCV
1590	*P. maliformis*	CABMV; CPMMV; PFCV
1609	*P. mucronata*	CABMV; PFCV
1603	*P. quadrangulares*	CABMV; PFCV; PaNV2

1622	*P. riparia*	CABMV; PFCV
1624	*P. riparia*	CABMV; CPMMV; PFCV; PaNV2
1648	*P. rufa*	CABMV; CPMMV; CPMMV; PFCV
1608	*Passiflora sp.*	CABMV; PFCV
1640	*Passiflora sp.*	CABMV
1612	*P. suberosa*	CABMV; CPMMV
1631	*P. subrotua*	CABMV
1627	*P. tholozanii*	CABMV
1635	*P. triloba*	CABMV
1606	*P. vitifolia*	CABMV
1599	*P. cacao*	CABMV; BaCV; PFCV
1611	*P. malacophylla*	CABMV; CPMMV; PFCV
1615	*P. serratodigitata*	CABMV; PFCV
Bahia; Marcionílio de Souza		
502	*P. edulis*	CABMV; PFCV
504	*P. edulis*	PFCV
Bahia; Seabra		
559	*P. edulis*	CABMV; CABYV; PFCV
560	*P. edulis*	CABMV; CABYV; PFCV
562	*P. edulis*	PFCV
564	*P. edulis*	CABMV; CABYV; PaNV2
Bahia; Morro do Chapéu		
799	*P. edulis*	CABMV; CABYV; PFCV
Bahia; Dom Basílio		
595	*P. edulis*	PFCV
596	*P. edulis*	CABMV; PFCV
598	*P. edulis*	CABMV; PFCV
600	*P. edulis*	CABMV; PFCV
611	*P. edulis*	CABMV; CABYV; PFCV
Rio de Janeiro; Seropédica		
2251	*P. edulis* FB300	CABMV; PFCV
2252	*P. edulis* FB300	CABMV; PFCV
2253	*P. edulis* FB300	CABMV; PFCV
2254	*P. edulis* FB300	CABMV; PFCV
2255	*P. edulis* FB300	CABMV; PFCV
2256	*P. edulis* FB300	CABMV; PFCV
2257	*P. edulis* FB300	CABMV; PFCV
2258	*P. edulis* FB300	CABMV; PFCV
2259	*P. edulis* FB300	CABMV; PFCV
2260	*P. edulis* FB300	CABMV; PFCV
2261	*P. edulis* FB300	CABMV; PFCV
2262	*P. edulis* FB300	CABMV; PFCV

2263	*P. edulis* FB300	CABMV; CABYV; PFCV
2264	*P. edulis* FB300	CABMV; CABYV; PFCV
2265	*P. edulis* FB300	CABMV; PFCV
2266	*P. edulis* FB300	CABMV; CABYV; PFCV
2267	*P. edulis* FB300	CABMV; CABYV; PFCV
2268	*P. edulis* FB300	CABMV; PFCV
2269	*P. edulis* FB300	CABMV; PFCV
2270	*P. edulis* FB300	CABMV; PFCV
2271	*P. edulis* FB300	CABMV; PFCV
2272	*P. edulis* FB300	CABMV; PFCV
2273	*P. edulis* FB300	CABMV; PFCV
2274	*P. edulis* FB300	CABMV; PFCV
2275	*P. edulis* H09-110/111	CABMV; PFCV
2276	*P. edulis* H09-110/111	CABMV; PFCV
2277	*P. edulis* H09-110/111	CABMV; CABYV; PFCV
2278	*P. edulis* H09-110/111	CABMV; CABYV; PFCV
2279	*P. edulis* H09-110/111	CABMV; PFCV
2280	*P. edulis* H09-110/111	CABMV; PFCV
2281	*P. edulis* H09-110/111	CABMV; PFCV
2282	*P. edulis* H09-110/111	CABMV; PFCV
2283	*P. edulis* H09-110/111	CABMV; PFCV
2284	*P. edulis* H09-110/111	CABMV; PFCV
2285	*P. edulis* H09-110/111	CABMV; PFCV
2286	*P. edulis* H09-110/111	CABMV; PFCV
2287	*P. edulis* H09-110/111	CABMV; PFCV
2288	*P. edulis* H09-110/111	CABMV; PFCV
2289	*P. edulis* H09-110/111	CABMV; PFCV
2290	*P. edulis* H09-110/111	CABMV; PFCV
2291	*P. edulis* H09-110/111	CABMV; PFCV
2292	*P. edulis* H09-110/111	CABMV; PFCV
2293	*P. edulis* H09-110/111	CABMV; PFCV
2294	*P. edulis* H09-110/111	CABMV; PFCV
2295	*P. edulis* H09-110/111	CABMV; PFCV
2296	*P. edulis* H09-110/111	CABMV; PFCV
2298	*P. edulis* H09-110/111	CABMV; PFCV
2299	*P. edulis*	CABMV; PFCV
Distrito Federal; Planaltina		
1790	*Passiflora* spp.	CABMV; PFCV
1796	*Passiflora* spp.	CABMV; PFCV
1798	*Passiflora* spp.	CABMV; PFCV
1800	*Passiflora* spp.	CABMV; PFCV
1802	*Passiflora* spp.	CABMV; PFCV
1588	*P. edulis*	CABMV; LCV; PFCV

Distrito Federal; Brazlândia		
1730	*P. alata* "Mel do cerrado"	PFCV
1731	*P. alata* "Mel do cerrado"	PFCV
1734	*P. alata* "Mel do cerrado"	PFCV
1736	*P. alata* "Mel do cerrado"	PFCV
1738	*P. edulis*	BaCV
1740	*P. edulis*	CABMV; PFCV
1743	*P. edulis*	CABMV; PFCV
Paraiba; Cuité		
1481	*P. edulis*	CABMV; PFCV
1487	*P. edulis*	CABMV; PFCV
1490	*P. edulis*	CABMV; PFCV
1492	*P. edulis*	CABMV; PFCV
Pernambuco; Vitória de Santo Antão		
1755	*P. edulis*	CABMV; PFCV

Para confirmação da identidade dos isolados de PFCV, amplicons que correspondem a proteína N complete de 11 plantas foram sequenciados. Sequências obtidas de 1488 nt (proteína N: 436 aa; 1331 nt) mostraram perfis de identidade de nt de >99.4%, e identidade de aa de >98.9% com a sequência PFCV-BAG3.

Para o PaNV2 (amostra 1593), uma sequência de 1718 nt (proteína N: 478 aa; 1437 nt) mostrou identidade de nt 95.3% e identidade de aa de 93.6% em relação a sequência de PFCV-BAG3. Ainda foi observado que a sequência de 1718 nt obtida por Sanger do PaNV2 (amostra 1593) apresentava uma inserção de 69 nt em relação a sequência PaNV2-BAG3 na posição 722 nt. Mais de um clone foi sequenciado e o mesmo padrão foi encontrado em todas sequências. O mapeamento dos *reads* usando a sequência 1718 nt obtida por Sanger, usando-a como Ref-Seq permitiu a identificação dos 69 nt nos dados do HTS. Provavelmente os clones sequenciados e a sequência PaNV2-BAG3 correspondem a diferentes genótipos do mesmo vírus.

Para o PaNV1, uma sequência de 1352 nt que corresponde a região intergênica e cobre parte da proteina L (1215; 405 aa) foi obtida da amostra 564. A sequência apresenta 99.9% e 100% de identidade de nt e aa, respectivamente, com a sequência PaNV1-PM1BA.

5.3.4. Caracterização do genoma dos novos rhabdovirus

Por meio de ensaios de RT-PCR usando primers baseados nas sequencias preditas pelas análises do HTS foi possível obter o genoma quase completo dos três rhabdovirus identificados neste estudo, faltando apenas a extremidades das sequências que serão confirmadas pelo método de RACE.

A sequência genômica PaNV1-PM1BA identificado apenas na amostra 564 (*P. edulis*) de Seabra (Tabela 1) foi determinado neste estudo pelo sequenciamento de Sanger de cinco amplicons, que variam de 2.1 kb a 3.6 kb, e sobreposição de >200 nt (Figura 4A).

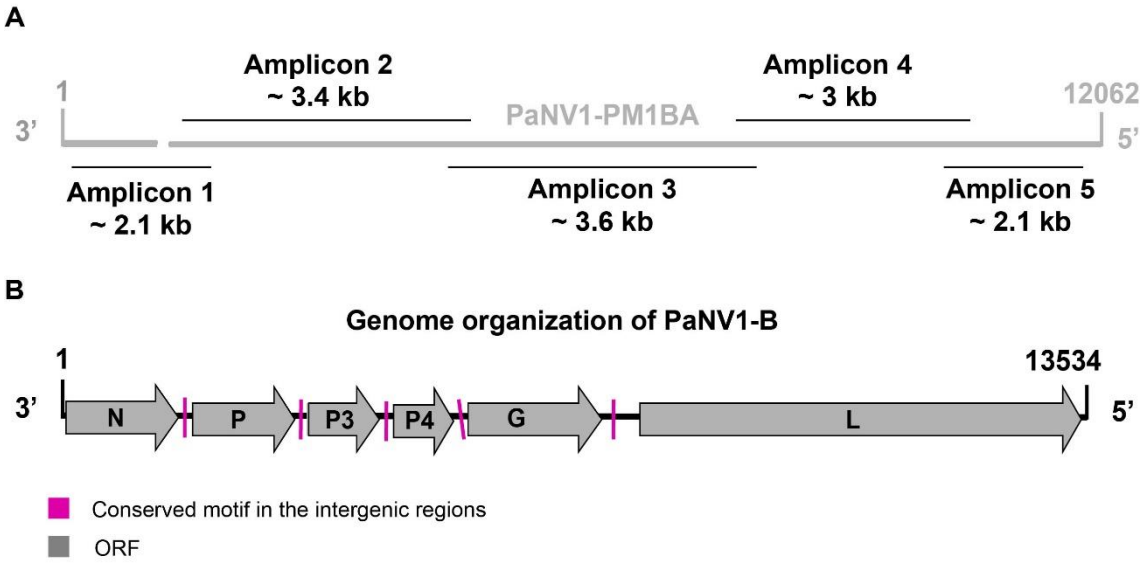

Figure 4. Amplicons e estratégia de sequenciamento para obtenção do genoma completo do PaNV1-B (A) e organização genômica do PaNV1-B (B). 3'-N-P-P3-P4-G-L-5' representam as proteínas codificadas pelo PaNV1-B.

Uma sequência de 13,416 nt (PaNV1-B) foi obtida e apresenta seis ORFs na ordem 5'-N-P-P3-P4-G-L-3' (Figura 4B). Regiões intergênicas consenso conservadas 5'-NWRTWWWMAAAACCCYAACYW-3' separam os genes. Para P3 e P4, assim como visto para o PaNV1-PM1BA, nenhuma similaridade de sequência foi identificada quando comparada com outras sequencias do banco de dados. Da mesma forma, não conseguimos identificar uma proteína M, assim como visto para PNV1-PM1BA (Figura 1) O genoma completo do PaNV1-B apresentou 63.56% a 86% de identidade de nt com coberturas que variaram entre de 6% a 16% com *Aphanucleorhabdovirus* e outros rhabdovirus relacionados.

A identidade de sequência pareada dos genes cognatos de rhabdovirus com membros que compõe as espécies tipos do gênero *Aphanucleorhabdovirus* (Tabela 2) mostram que PaNV1-B tem identidades nt que variam entre 46,5% a 60,5%, e identidade de aa que variam entre 19,6% a 59,6% com membros do *Alphanucleorhabdovirus.* A análise filogenética (Figura 3), mostrou que assim como o PaNV1-PM1BA, o PaNV1-B também está relacionado com os alphanucleorhabdovirus AB011257, MG604920 e OM372677.

Baseado nos critérios de similaridade de sequência para demarcação de espécies do gênero *Aphanucleorhabdovirus*, organização do genoma e posição filogenética, o PaNV1 de fato representa um novo membro do gênero *Aphanucleorhabdovirus*, na família *Rhabdoviridae*. PaNV1 é o primeiro *Aphanucleorhabdovirus* identificado em *P. edulis*.

Para o PFCV-BAG3 e PaNV2-BAG3, ainda não foi possível obter o genoma completo da região codante de isolados individuais. Este trabalho será continuado e as sequencias preditas pelas análises de HTS serão usadas como base amplificar o genoma completo a partir das amostras individuai.

Apesar de ter realizado algumas tentativas a fim de confirmar extremidades 3’ e 5’ do PFCV-BAG3, PaNV1-BAG3 e PaNV2-BAG3 por RACE ainda não logramos obter as sequencias correspondentes às extremidades 3’ e 5’ destes vírus. Este trabalho será continuado e serão realizadas novas tentativas.

Tabela 2. Percentagens de identidade de sequência pareadas de nucleotídeos (nt) e aminoácidos (aa) entre os genes cognatos codificados (3-'N-P-M-G-L-5') pelo genoma PaNV1-B geradas pelo SDT, em comparação com membros do gênero *Alphanucleorhabdovirus* pelo ICTV.

Acesso GenBank _ vírus	**N**		**P**		**M**		**G**		**L**	
	nt	**aa**	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**	**nt**	**aa**
PaNV1-B	100,0	100,0	100,0	100,0	-	-	100,0	100,0	100,0	100,0
AB011257_rice yellow stunt virus	53,0	53,0	48,8	22,4	100,0	100,0	54,2	27,9	53,4	40,9
AY618418_maize mosaic virus	51,4	51,4	50,4	27,6	48,0	31,3	51,0	28,6	50,8	32,6
AY674964_taro vein chlorosis virus	49,8	49,8	51,7	21,8	51,6	19,2	50,5	28,3	52,0	33,8
BK014297_agave tequilana virus 1	51,2	51,2	53,2	24,7	53,3	22,3	50,8	25,7	51,7	33,1
GU734660_potato yellow dwarf virus	50,8	50,8	54,2	24,3	54,7	28,9	50,4	24,7	51,5	34,9
KJ082087_eggplant mottled dwarf virus	50,0	50,0	52,7	23,1	51,8	20,2	51,1	25,0	52,7	36,9
KX636164_physostegia chlorotic mottle virus	51,8	51,8	52,7	23,3	51,5	20,2	51,3	26,1	53,4	36,2
KY549567_constricta yellow dwarf virus	52,6	52,6	52,0	25,7	48,9	25,2	49,0	27,9	52,0	36,2
MF102281_maize Iranian mosaic virus	51,2	51,2	51,2	21,9	50,1	24,0	49,8	27,9	52,0	34,9
MG604920_wheat yellow striate virus	51,4	51,4	51,5	24,0	60,5	59,6	52,1	28,4	54,1	41,2
MK063878_morogoro maize-associated virus	51,9	51,9	46,4	27,2	53,1	23,3	49,5	29,6	51,3	33,7
MN520414_peach virus 1	53,1	53,1	52,9	24,0	53,6	21,2	49,7	26,9	51,7	34,4
MW014292_joa yellow blotch associated virus	51,0	51,0	50,9	23,8	51,7	26,4	49,5	26,6	52,0	36,4
OM372677_artemisia capillaris nucleorhabdovirus 1	51,2	51,2	50,6	21,7	51,8	28,6	51,9	33,6	54,5	41,8

5.3.5. Microscopia eletrônica de transmissão

Uma importante característica dos rhabdovirus que infectam planta é a morfologia da partícula viral, o local de replicação e morfogênese do vírus nas células infectadas. Membros dos gêneros *Alphanucleorhabdovirus* (parte do antigo gênero *Nucleorhabdovirus*) e *Cytorhabdovirus* apresentam vírions envelopados com morfologia da partícula em forma de bala/baciliforme, e foram historicamente estabelecidos com base nos locais de replicação e morfogênese do vírus, tendo os nucleorhabdovirus replicando e maturando nos núcleos, e os cytorhabdovirus no citoplasma (Walker et al., 2022).

Usamos microscopia eletrônica de transmissão (TEM) para visualizar as partículas virais e citopatologia nos tecidos de plantas infectadas dos acessos do BAG-PF. Para isso, uma segunda coleta de tecido foliar foi realizada em 2019, onde as plantas foram confirmadas com o vírus por RT-PCR, e então submetidas as análises por TEM.

Na amostra 1626 foram identificadas partículas baciliformes no núcleo, típicas de membros *Alphanucleorhabdovirus* (Figura 5A-C), inclusões do tipo catavento características da citopatologia induzida por potyvirus (Figura 5D), bem como inclusões citoplasmáticas de agregados de partículas, alongadas e flexuosas, similar a carlavirus (Figura 5E-F). Esses resultados corroboram os resultados da RT-PCR, tendo sido identificados na amostra 1626 o potyvirus CABMV, o carlavirus CPMMV, o cytorhabdovirus BaCV, e o alphanucleorhabdovirus PaNV2-BAG3 (Tabela 1). Apesar do BaCV ter sido identificado nesta planta, nenhuma evidência de cytorhabdovirus foi visualizada nas análises de microscopia eletrônica.

Na amostra 1624, não foram visualizadas partículas, apenas massas compactadas de material filamentoso no citoplasma podendo representar o viroplasma, local da replicação viral (Figura 5G-H). Esses achados são similares ao viroplasma observado em infecções pelo cytorhabdovirus BaCV (Pinheiro-Lima et al., 2020). A amostra 1624 foi testada positiva para o cytorhabdovirus PFCV por RT-PCR (Tabela 1). Embora não tenha sido visualizada nenhuma evidência da presença de outro vírus, nessa amostra foi detectada a presença de CABMV, CPMMV e PaNV2 por RT-PCR.

Devido à escassez das partículas presentes nos tecidos de passiflora e pela dificuldade em localizar as partículas nos tecidos foliares, acreditamos que os cytorhabdovirus e alphanucleorhabdovirus possam ocorrer em baixa concentração nas plantas de maracujá. O mesmo foi observado em plantas de feijão infectadas pelo cytorhabdovirus BaCV (Pinheiro-Lima et al., 2020).

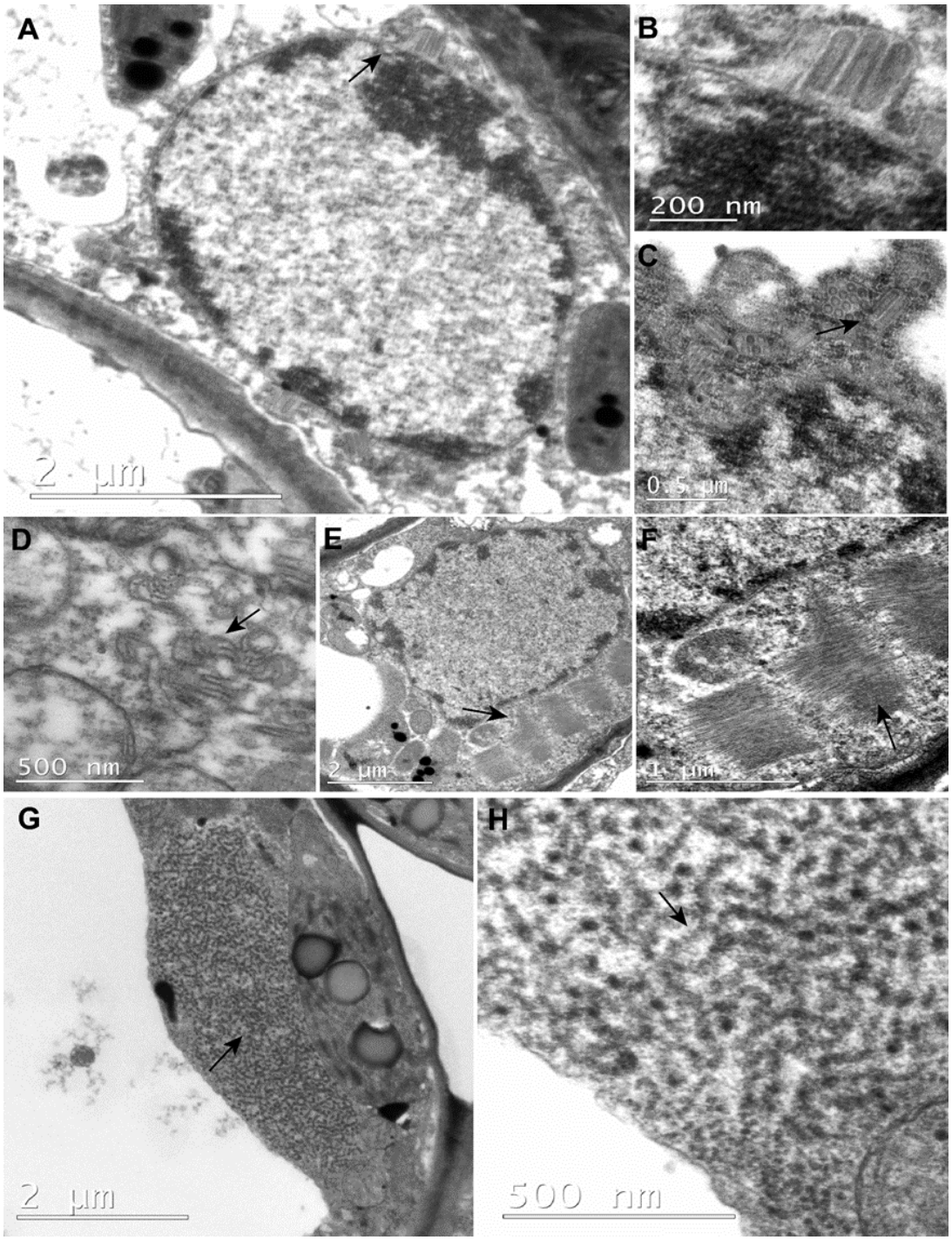

Figure 5. Micrografias eletrônicas de transmissão de folhas de passiflora do BAG-FP. Amostra 1626: partículas baciliformes do alphanucleorhabdovirus PaNV2 (A, B, C), inclusões do tipo "cata-vento" característicos de potyvirus CABMV (D), inclusão citoplasmática de agregado de partículas, alongadas e flexuosas típicas de carlavirus CPMMV (E, F). Amostra 1624: viroplasma característico da infecção por cytorhabdovirus (G-H).

Como mencionado anteriormente, existem pouquíssimos relatos de rhabdovirus em maracujazeiros no mundo. Nossos achados são inéditos e expandem a diversidade rhabdovirus que infectam plantas, bem como a diversidade viral em uma importante cultura no país. Além disso, a caracterização desses prováveis novos rhabdovirus, a disponibilização de método de diagnose molecular por RT-PCR, assim como a identificação das espécies de passiflora

suscetíveis, é fundamental para fornecer informações para a escolha de plantas não infectadas para os cruzamentos em programas de melhoramento.

5.4. Considerações finais

Esse capítulo é uma versão preliminar do que será o artigo final. As sequências de isolados virais descritas neste capítulo correspondem a sequências parciais, faltando confirmar as extremidades por RACE. O genoma quase completo do PaNV1 foi determinado neste estudo, faltando apenas as sequencias da região 3'e 5'. Os genomas completos do PFCV e PaNV2 serão determinados a partir das amostras individuais em etapas posteriores na continuação deste trabalho. Essas informações serão fundamentais para classificação final destes vírus.

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Discussão geral

O maracujá é uma importante cultura para o Brasil, uma vez que o país é um dos maiores produtores de maracujazeiros do mundo, abriga um grande centro de diversidade de espécies, e conta com diversos programas de melhoramento genético destinados a cultura. Este torna-se o cenário perfeito para o desenvolvimento e estabelecimento de doenças, sejam elas, de origem fúngica, bacteriana ou viral. É fato que, a ocorrência desses patógenos tem trazido grandes prejuízos para a cultura e para o produtor, tornando muitas vezes inviável a manutenção e permanência desses pomares, bem como a comercialização dos frutos.

Dentre essas patologias, doenças ocasionadas por vírus tem sido uma das principais causas da perda da produtividade de maracujazeiros no Brasil. A doença do endurecimento dos frutos do maracujazeiro (do inglês, passion fruit woodiness disease- PWD) é a principal doença de etiologia viral que afeta a cultura no país (Cerqueira-Silva et al., 2014). No Brasil, a PWD tem sido associada a infecção pelo potyvirus cowpea aphid-borne mosaic virus (CABMV) (Cerqueira-Silva et al., 2014; Rodrigues et al., 2014). Devido aos danos causados pelo CABMV na cultura, os pomares de maracujá que tinham uma vida útil de quatro anos, agora são renovados anualmente (Pinto, Peixoto, et al., 2008). Essa doença também afeta maracujazeiros em outras regiões do mundo, entretanto é associada a outros vírus. PDW pode ser causada por East Asian Passiflora virus (Iwai et al., 1996), Ugandan Passiflora virus (Ochwo-Ssemakula et al., 2012), passion fruit woodiness virus (McKnight, 1953), telosma mosaic virus (Chiemsombat et al., 2014), e passiflora mottle virus (Do et al., 2021; Xie et al., 2019).

Além dos vírus responsáveis pela PWD, nos últimos anos uma diversidade viral tem sido relatada infectando maracujazeiros ao redor do mundo, e também aqui no Brasil. Estes vírus são constituídos de genomas de RNA e DNA e pertencem a diferentes grupos de vírus (Chapter 1).

No Brasil, os vírus de genoma de DNA identificados na cultura são o passion fruit chlorotic mottle virus (PCMoV, gênero *Citlodavirus*, família *Geminiviridae*) (Fontenele et al., 2018), sida mottle Alagoas virus (SiMAV, gênero *Begomovirus*, família *Geminiviridae*) (Mituti et al., 2019), melochia yellow mosaic virus (MelYMV, gênero *Begomovirus*, família *Geminiviridae*) (Spadotti et al., 2019), plant-associated genomovirus7 (gênero *Gemykolovirus*, família *Genomoviridae*) (Fontenele et al., 2020), sida micrantha mosaic virus (SiMMV, gênero *Begomovirus*, família *Geminiviridae*), sida mottle virus (SiMoV, gênero *Begomovirus*, família *Geminiviridae*) (Alves, 2012), passionfruit severe leaf distortion virus (PSLDV, gênero *Begomovirus*, família *Geminiviridae*) (Ferreira et al., 2010), e o passion flower little leaf mosaic virus (PLLMV, gênero *Begomovirus*, família *Geminiviridae*) (Novaes et al., 2002).

Já os vírus de genoma RNA identificados em maracujazeiros no país, além do potyvirus CABMV, inclui o passion fruit yellow mosaic virus (PFYMV, gênero *Tymovirus*, família *Tymoviridae*) (Crestani et al., 1986), cucumber mosaic virus (CMV, gênero *Cucumovirus*, família

Bromoviridae) (Gioria et al., 2002), passion fruit vein clearing virus (PVCV, gênero não classificado, família *Rhabdoviridae*) (Chagas et al., 1987), grapevine virus A (GVA, gênero *Vitivirus*, família *Betaflexiviridae*) (Galleti et al., 2006), purple granadilla mosaic virus (PGMV) ou purple passion fruit mosaic virus (não classificado) (Chagas et al., 1984), passion fruit green spot virus (PfGSV, gênero *Cilevirus*, família *Kitaviridae*) (Ramos-González et al., 2020), passiflora mitovirus-like (gênero *Mitovirus*, família *Narnaviridae*) (Santos et al., 2020), e o cucurbit aphid-borne yellows virus (CABYV, gênero *Polerovirus*, família *Solemoviridae*) (Vidal et al., 2018).

Nessa tese, nós trazemos dados que ampliam a essa diversidade de virus infectantes de maracujazeiros no país, são eles: o lettuce chlorosis virus (LCV, gênero *Crinivirus*, família *Closteroviridae*) (Chapter 2) (Vidal et al., 2018), o bean-associated cytorhabdovirus (BaCV, espécie *Cytorhabdovirus caricae*, gênero *Cytorhabdovirus*, família *Rhabdoviridae*), e o cowpea mild mottle virus (CPMMV, gênero *Carlavirus*, família *Betaflexiviridae*) (Chapter 3) (Vidal, Felix, et al., 2023). No último capítulo (Chapter 5) descrevemos a identificação e caracterização de novos isolados com potencial de ser novas espécies candidatas a família *Rhabdoviridae*, são eles: o passiflora cytorhabdovirus (PFCV) candidato ao gênero *Cytorhabdovirus*, e o passiflora nucleorhabdovirus 1 (PaNV1) e passiflora nucleorhabdovirus 2 (PaNV2), candidatos ao gênero *Alphanucleorhabdovirus*. Além disso, caracterizamos um polerovirus de maracujazeiros, o CABYV, e concomitantemente, nós propomos uma reclassificação dos isolados de CABYV identificados em outros estudos, em pelo menos dez espécies dentro do gênero *Polerovirus* (Chapter 4) (Vidal, Lacorte, et al., 2023).

Atualmente, estamos passando por uma transição dentro da classificação taxonômica de vírus, para o qual está sendo adotado a nomenclatura binomial (Siddell et al., 2020; Zerbini et al., 2022). Baseada nessa nova nomenclatura todas as proposições das espécies feitas neste estudo (Chapter 4 e 5) seguiram essa nova regra (Vidal, Felix, et al., 2023).

Neste estudo, destacamos a co-infecção entre os vírus identificados nessa tese, juntamente com o potyvirus CABMV em quase 100% das plantas amostradas (Chapter 2,3,4,5). Acreditamos que presença do CABMV em quase em todas as plantas positivas para o LCV, BaCV, CPMMV, CABYV, PFCV, PaNV1 e PaNV2 (Chapter 5) pode estar sobrepondo ou mascarando os efeitos da presença desses vírus, uma vez, que os sintomas mais aparentes são característicos da infecção por esse potyvirus.

A ocorrência de múltiplas infecções pode estar trazendo prejuízos ainda maiores para a cultura, comprometendo a vida útil destes pomares. Por exemplo, o maracujazeiro tornou-se uma cultura anual visando diminuir os impactos induzidos pela PWD, induzida pelo CABMV. Entretanto, as plantas provenientes de Lençóis (Bahia) tinham apenas três meses de plantio, e uma a alta incidência do CABMV e CABYV foi detectada nessa região (Chapter 4) (Vidal, Lacorte, et al., 2023). Além dos vírus de genoma de RNA nas plantas analisadas, que foi o foco do nosso

estudo, também não excluímos a possibilidade da infecção por vírus constituídos de genoma de DNA nestas mesmas plantas.

Os vírus reportados nessa tese, parecem estar bem distribuídos no país. Apesar de termos identificado grande parte dessa diversidade nos acessos de *Passiflora* spp. mantidos no Banco de Germoplasma "Flor da Paixão"- BAG-FP, nossos resultados indicam que eles não estão restritos a uma única localidade, tendo sido identificado em campos comerciais de maracujazeiros em diferentes regiões do país (Chapter 5). Portanto, além das regiões identificadas neste estudo, levantamos a possiblidade da ocorrência destes vírus em outras localidades. O BAG-FP possui cultivares, híbridos e espécies silvestres de passiflora provenientes de diferentes regiões, biomas e agrossistemas brasileiros (Faleiro et al., 2020). Essas plantas são coletadas e vêm de diferentes locais, são multiplicadas em forma de mudas, e em seguida mantidas no BAG-FP, que é uma estrutura composta de casa de vegetação com telado, mas alguns acessos também são mantidos e multiplicados no campo (Faleiro et al., 2020). Dessa forma, os vírus podem ter sido adquiridos no próprio BAG-FP, mas também não descartamos a possibilidade de que esses acessos já tenham sido coletados com infecção viral.

A incidência desses vírus em diferentes localidades pode sugerir a existência de uma rota de dispersão seja por sementes, mudas infectadas, ou transmissão por algum inseto vetor.

A propagação de maracujazeiros é feita principalmente através de sementes, ou por enxertia e estaquia em menor frequência. Vários vírus podem ser transmitidos por sementes (Sastry, 2013), assim como o CPMMV que existem dados da transmissão dele por sementes (Sutrawati et al., 2021; Yadav et al., 2013). Entretanto, para o CABMV, LCV, e BaCV nenhuma transmissão por sementes foi relatada. Para os novos rhabdovirus essa teoria deveria ser investigada. Contudo, o CPMMV e CABMV são vírus transmitidos mecanicamente. Logo, a produção de mudas por enxertia e estaquia poderiam favorecer a dispersão destes vírus, e favorecer a ocorrência deles em outras localidades por transporte/cultivo de mudas possivelmente infectadas. Para os outros vírus, a transmissão por via mecânica ou por enxertia ainda não foi descrita.

Durante as coletas nenhum inseto vetor foi visto colonizando os maracujazeiros avaliados neste estudo. No BAG-FP, observamos a presença de lagartas se alimentando de folhas de algumas plantas, indicando a possível invasão do telado por algum tipo de inseto. Nos últimos anos, uma alta população de mosca branca tem sido encontrada em maracujazeiros no Brasil, e tem sido associado a outbreaks de begomovirus como passionflower little leaf mosaic virus (PLLMV) (Alves, 2008; Novaes et al., 2003), sida mottle alagoas virus (SiMAV) (Mituti et al., 2019), passionfruit severe leaf distortion virus (PSLDV) (Ferreira et al., 2010; Rodrigues et al., 2019), sida mottle virus (SiMoV), e sida micrantha mosaic virus (SiMMV) (Alves, 2012). Afídeos também tem sido encontrado em maracujazeiros e associados a ocorrência do CABMV (Moritz, 2020). Outros insetos como, lagartas, percevejos, besouros, mosca (moscas-das-frutas), vaquinhas, abelhas, tripes, cigarrinhas, cupins, e ácaros também acometem a cultura (Machado et

al., 2017). Entretanto, se considerarmos que alguns dos vírus identificado neste estudo compartilham o mesmo inseto vetor, essa característica poderia estar favorecendo o estabelecimento destes vírus na cultura.

O CABMV e CABYV podem ser transmitidos naturalmente pelos afídeos *Aphis gossypii* e *Myzus persicae* (Garcêz et al., 2015; Lecoq et al., 1992). O BACV, CPMMV e LCV são conhecidos por serem transmitidos por mosca branca *Bemisia tabaci* (Liu et al., 2000; McLain et al., 1998; Pinheiro-Lima et al., 2020). O recombinante do CABYV identificado aqui no Brasil, e para o qual nós propomos que seja considerado uma nova espécie de *Polerovirus* (Chapter 4) (Vidal, Lacorte, et al., 2023), foi caracterizado em plantas de meloeiros como sendo transmitido por mosca branca (Costa et al., 2020).

Para os novos rhabdovirus identificados neste estudo, ainda não temos nenhuma evidência do inseto vetor associado à transmissão. Contudo, acreditamos que o PFCV possa ser transmitido por afídeos, baseado na sua relação filogenética com outros *Cytorhabdovirus* transmitidos por esses insetos (Chapter 5). Além disso, o PFCV foi detectado em alta incidência, e em quase 100% dos casos em infecção mista com o CABMV (Chapter 5) que também é transmitido por afídeos e apresenta sempre alta incidência nas plantas detectadas. Já foi visto que um mesmo inseto pode transmitir diferentes vírus ou diferentes isolados (Mondal et al., 2016; Pinto, Rezende, et al., 2008). Baseado na relação filogenética do PaNV1 e PaNV2 com outros *Alphanucleorhabdovirus* (Chapter 5), há indícios que esses dois rhabdovirus poderiam ser transmitidos por cigarrinhas. É evidente, que essas hipóteses precisam ser testadas experimentalmente em estudos posteriores, bem como a avaliação das possíveis espécies vetoras do CABYV, LCV, BaCV, e CPMMV em maracujazeiros.

A identificação do PFCV, PaNV1, e PaNV2 amplia a diversidade de rhabdovirus dos gêneros *Cytorhabdovirus* e *Alphanucleorhabdovirus* que infectam plantas. Adicionalmente, expande a diversidade de rhabdovirus infectantes de maracujazeiros. Anteriormente, baseado em dados biológicos, o passion fruit vein clearing virus (PVCV) identificado no Brasil, e o passionfruit rhabdovirus (PRV) identificado na Austrália foram caracterizados como rhabdovirus não classificados (Chagas et al., 1987; Kitajima, 2020; Kitajima and Crestani, 1985; Pares et al., 1983). Mais recentemente, cepas da espécie *Cytorhabdovirus cariace*, citrus-associated rhabdovirus (CiaRV) na China (Zhang, Huang et al. 2020), e o bean associated-cytorhabdovrius (BaCV) foram associados a cultura (Chapter 3) (Vidal, Felix, et al., 2023).

A identificação do LCV, BaCV, CPMMV, PFCV, PaNV1, e PaNV2 no BAG-FP mostram-se uma descoberta inédita e uma ferramenta importante para investigar acessos de *Passiflora* spp. como fontes de resistência a esse vírus, bem como o uso desses acessos em programas de melhoramento destinado a cultura na geração de genótipos resistente ou até mesmo livres de vírus.

Neste estudo foram identificados diferentes vírus, incluindo isolados candidatos a serem membros de novas espécies, infectando uma importante cultura no país. Nossos achados ainda

expandem a gama de hospedeiro do LCV, CABMV, BaCV e CPMMV. Baseado nos nossos estudos e em relatos anteriores, acreditamos que o problema ocasionado por vírus na cultura do maracujazeiro no país pode não ser associado apenas ao CABMV, mas sim a um complexo de vírus que podem estar infectando essas plantas. É evidente a necessidade de mais estudos a fim de avaliar o impacto desses vírus na cultura, e elucidação do inseto vetor para auxiliar no manejo e controle desses vírus na cultura.

Conclusão geral

- Nossos resultados mostram uma diversidade de vírus inédita (vírus identificados anteriormente em outras culturas, e isolados candidatos a serem membros de espécies de vírus) associada a maracujazeiros no Brasil. Ademais, acreditamos que os vírus aqui descritos representam apenas uma parte dessa diversidade.
- Os vírus encontrados nas amostras do BAG-FP avaliadas nesse estudo provavelmente não estão restritos a uma única localidade, bem como restritos as amostras do BAG-FP, uma vez que as plantas do BAG-FP são espécimes provenientes de mudas oriundas do campo ou vegetação nativa de outras regiões do Brasil.
- Os maracujazeiros brasileiros têm como principal patógeno viral o CABMV, mas existem outros vírus circulando nessa cultura, e em sua grande maioria em infecção mista com o potyvirus.
- As rhabdovirus descritos nesse trabalho expandem a diversidade de vírus infectantes de plantas na família *Rhabdoviridae.*
- Esses achados serão fundamentais para programas de melhoramento genético que objetivem a geração de genótipos resistentes a esses vírus.
- É imprescindível mais estudos a fim de avaliar os impactos desses vírus na cultura, objetivando entender interação deles com essas plantas, a ocorrência em infecção única e mista, epidemiologia, e inseto vetor.

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Outras publicações

Durante o período de doutorado tive a oportunidade de colaborar com o trabalho de outros colegas que resultaram em 4 publicações adicionais:

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Occurrence of bean-associated cytorhabdovirus in soybean fields in Brazil

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ABSTRACT

Bean-associated cytorhabdovirus (BaCV) is the first described whitefly-transmitted rhabdovirus. BaCV was experimentally transmitted by *Bemisia tabaci* MEAM1 to common bean, cowpea (*Vigna unguiculata*), and soybean (*Glycine max*), but natural infection has been documented only in common bean. Therefore, we investigated the occurrence of BaCV in 123 soybean plants collected in five Brazilian states by RT-PCR and sequencing. BaCV was detected in 11 plants from Distrito Federal and Goiás. Moreover, BGMV and CPMMV were detected in most of the BaCV-positive plants (9 out of 11). The potential impact of BaCV infection or coinfection in soybean production remains to be determined.

Bean-associated cytorhabdovirus (BaCV), an isolate of *Cytorhabdovirus caricae* (genus *Cytorhabdovirus*, family *Rhabdoviridae*), is the first rhabdovirus described as being whitefly-transmitted (Pinheiro-Lima et al., 2020). BaCV was identified in common bean (*Phaseolus vulgaris*) in Brazil (Alves-Freitas et al., 2019) and shares 97% identity with papaya virus E from Ecuador (Medina-Salguero et al., 2019). Other isolates were reported in citrus (*Citrus* spp.), passion fruit (*Passiflora edulis*), and paper bush (*Edgeworthia chrysantha*) in China (Zhang et al., 2021); common bean in Mexico (Chiquito-Almanza et al., 2021), and non-cultivated Fabaceae (*Centrosema plumieri*, *Macroptilium lathyroides*, and *Rhynchosia minima*) in Ecuador (Cornejo-Franco et al., 2022). BaCV was experimentally transmitted by *Bemisia tabaci* MEAM1 to common bean, cowpea (*Vigna unguiculata*), and soybean (*Glycine max*) (Pinheiro-Lima et al., 2020), but natural field infection on soybeans has not been described. Therefore, to verify the occurrence of BaCV in soybean crops in Brazil, samples collected between 2014 and 2020 were analyzed. Leaf samples were collected from plants showing virus-like mottle symptoms and asymptomatic plants. Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) from 123 plants collected in Minas Gerais (n = 37), Goiás - GO (n = 19), Paraná (n = 30), Rio Grande do Sul (n = 8) and Distrito Federal - DF (n = 29). BaCV was detected by conventional RT-PCR with the specific primers BaCV_1F/BaCV_1579R (amplicons size of 1579 bp) (Pinheiro-Lima et al., 2020) in 9% (11/123) of soybeans from areas where the virus had previously been detected in common beans. Seven BaCV-positive plants were collected in DF (2015 and 2017) and four in GO (2016) with similar mottle symptoms as cowpea mild mottle virus (CPMMV, genus *Carlavirus*, family *Betaflexiviridae*) or bean golden mosaic virus (BGMV, genus *Begomovirus*, family *Geminiviridae*) infected plants (Fernandes et al., 2009; Barreto da Silva et al., 2020). In common bean, BaCV was frequently found in mixed infections with the whitefly-transmitted CPMMV and BGMV (Pinheiro-Lima et al., 2020). To determine whether multiple infections also occur in soybean, the 11 BaCV-positive plants were tested by PCR and RT-PCR using specific primers for BGMV (amplicon of size 400 bp) (Bonfim et al., 2007) and for CPMMV (500 bp long amplicon) (Lamas et al., 2017). The three viruses were detected in nine plants, BaCV and CPMMV in one and BaCV single infection in another. The identity of the detected viruses was further confirmed by Sanger sequencing, and their amplicon sequences (OM333242, OM420284, and OM333241) showed 100% nucleotide identity with the isolate BaCV-BR-GO (MK202584), 99.7% with BGMV Ponte Nova-MG (MG334552), and 99.3% with CPMMV Florida_-Whiteflies_2007 (KC774019), respectively. This is the first report of the natural occurrence of BaCV in soybean, one of the most important cash crops in the world. Moreover, as observed in common beans, BaCV

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A fungal glycoprotein mitigates passion fruit woodiness disease caused by *Cowpea aphid-borne mosaic virus* (CABMV) in *Passiflora edulis*

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Abstract Passion fruit woodiness disease is responsible for severe losses in passion fruit production around the world. The disease is caused by *Cowpea aphid-borne mosaic virus* (CABMV), an aphid-transmitted potyvirus. Traditional sanitary measures against the disease, such as vector elimination and cross protection, have not been successful, resulting in elimination and replanting of passion fruit plants each season. To find new alternatives for disease control, we tested the use of a peptidogalactomannan (pGM) extracted from the fungus *Cladosporium herbarum* to activate passion fruit defense mechanisms, enabling plants to tolerate passion fruit woodiness disease (PWD). Passion fruit seedlings were spray-treated with pGM in a greenhouse three days before mechanical inoculation with CABMV. Experiments were set up in a completely randomized design, and disease incidence and severity were compared between water- and 100 μg ml^{-1} pGM-treated plants. Woodiness symptoms and certain developmental parameters of water- and pGM-treated plants were evaluated over five weeks. pGM treatment did not affect plant normal development. Plants that were both treated with pGM and inoculated with the virus showed very mild or no foliar CABMV disease symptoms and had the same growth and developmental patterns as the healthy uninoculated control plants. pGM led to the accumulation of antioxidant enzymes such as peroxidase and superoxide dismutase in the leaf tissues as well as their respective mRNAs. In addition, a ten- and twofold transcription induction of the mRNAs of the defense-related genes such as *chitinase I* (*PR-3*) and *phenylalanine ammonia-lyase* (*PAL*), respectively, were observed in pGM-treated seedlings. These results

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Novel circular DNA virus identified in *Opuntia discolor* (*Cactaceae*) that codes for proteins with similarity to those of geminiviruses

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Abstract

Viral metagenomic studies have enabled the discovery of many unknown viruses and revealed that viral communities are much more diverse and ubiquitous than previously thought. Some viruses have multiple genome components that are encapsidated either in separate virions (multipartite viruses) or in the same virion (segmented viruses). In this study, we identify what is possibly a novel bipartite plant-associated circular single-stranded DNA virus in a wild prickly pear cactus, *Opuntia discolor*, that is endemic to the Chaco ecoregion in South America. Two ~1.8 kb virus-like circular DNA components were recovered, one encoding a replication-associated protein (Rep) and the other a capsid protein (CP). Both of the inferred protein sequences of the Rep and CP are homologous to those encoded by members of the family *Geminiviridae*. These two putatively cognate components each have a nonanucleotide sequence within a likely hairpin structure that is homologous to the origins of rolling-circle replication (RCR), found in diverse circular single-stranded DNA viruses. In addition, the two components share similar putative replication-associated iterative sequences (iterons), which in circular single-stranded DNA viruses are important for Rep binding during the initiation of RCR. Such molecular features provide support for the possible bipartite nature of this virus, which we named utkilio virus (common name of the *Opuntia discolor* in South America) components A and B. In the infectivity assays conducted in *Nicotiana benthamiana* plants, only the A component of utkilio virus, which encodes the Rep protein, was found to move and replicate systemically in *N. benthamiana*. This was not true for component B, for which we did not detect replication, which may have been due to this being a defective molecule or because of the model plants (*N. benthamiana*) used for the infection assays. Future experiments need to be conducted with other plants, including *O. discolor*, to understand more about the biology of these viral components.

INTRODUCTION

The known plant-infecting single-stranded DNA viruses are found in two families – *Geminiviridae* and *Nanoviridae* – both of which have circular genomes. Nanovirus genomes are multipartite, comprising six to eight genome components, while some geminiviruses in the genus *Begomovirus* have bipartite genomes, whereas other geminiviruses have monopartite genomes. In some cases, geminiviruses and nanoviruses can be found in association with sub-viral single-stranded DNA molecules known as satellites. These satellites (alphasatellites, betasatellites and deltasatellites [1–3]) are classified into two families, *Alphasatellitidae* [1]

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Abbreviations: CFDV, coconut foliar decay virus; CP, capsid protein; CRESS, circular replication-associated protein encoding single stranded; HUH, his-hydrophobe-his; RCR, rolling circle replication; Rep, replication associated protein; SF3, superfamily 3.
One supplementary table and one supplementary data file are available with the online version of this article.

viruses

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Article

Transmission of the Bean-Associated Cytorhabdovirus by the Whitefly *Bemisia tabaci* MEAM1

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Abstract: The knowledge of genomic data of new plant viruses is increasing exponentially; however, some aspects of their biology, such as vectors and host range, remain mostly unknown. This information is crucial for the understanding of virus–plant interactions, control strategies, and mechanisms to prevent outbreaks. Typically, rhabdoviruses infect monocot and dicot plants and are vectored in nature by hemipteran sap-sucking insects, including aphids, leafhoppers, and planthoppers. However, several strains of a potentially whitefly-transmitted virus, papaya cytorhabdovirus, were recently described: (i) bean-associated cytorhabdovirus (BaCV) in Brazil, (ii) papaya virus E (PpVE) in Ecuador, and (iii) citrus-associated rhabdovirus (CiaRV) in China. Here, we examine the potential of the *Bemisia tabaci* Middle East-Asia Minor 1 (MEAM1) to transmit BaCV, its morphological and cytopathological characteristics, and assess the incidence of BaCV across bean producing areas in Brazil. Our results show that BaCV is efficiently transmitted, in experimental conditions, by *B. tabaci* MEAM1 to bean cultivars, and with lower efficiency to cowpea and soybean. Moreover, we detected BaCV RNA in viruliferous whiteflies but we were unable to visualize viral particles or viroplasm in the whitefly tissues. BaCV could not be singly isolated for pathogenicity tests, identification of the induced symptoms, and the transmission assay. BaCV was detected in five out of the seven states in Brazil included in our study, suggesting that it is widely distributed throughout bean producing areas in the country. This is the first report of a whitefly-transmitted rhabdovirus.

Keywords: common bean; *Phaseolus vulgaris*; cytorhabdovirus; whitefly; *Bemisia tabaci*; vector; virus transmission; virus evolution