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Virome bioprospection in ornamental plants of *Bromeliaceae* and *Orchidaceae* families in Brazilian highlands

JULIANA GABRIELLE ISIDORIO DA SILVA

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Master thesis presented to the University of Brasília as a requirement for obtaining the title of Master in Phytopathology by the Post-Graduate Program in Phytopathology.

Supervisor

Rita de Cássia Pereira Carvalho, Ph.D.

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Dedicated to

The One that the straps of the sandal I am not worthy to untie. Jesus Christ.

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Virome bioprospection in ornamental plants of *Bromeliaceae* and *Orchidaceae* families in Brazilian highlands

Juliana Gabrielle Isidorio da Silva

Master thesis approved on __/__/____ by:

_____

Maurício Rossato (Intern member)

_____

Marilia Santos Silva Patriota (External member)

_____

Prof. PhD. Rita de Cássia Pereira Carvalho (Supervisor/moderator)

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General abstract: Virome Bioprospection in ornamental plants of *Bromeliaceae* and *Orchidaceae* Families in Brazilian Highliands

Viruses are widespread; however, little is understood about the diversity of these biological entities. Estimates suggest that less than 1% of the total virosphere infecting eukaryotes is known, yet information about viruses is constrained to organisms that cause disease and economic losses. The understanding of diversity, evolutionary forces, and their impact is dynamically being determined. In this context, the High Throughput Sequencing (HTS) advent and the development of new tools of bioinformatics have enabled the large-scale detection of new viruses occurring across a wide range of plant hosts. The SARS-CoV-2 pandemic, and the possible rising of new viral diseases in humans and other economically essential eukaryotes have demonstrated that it is necessary to explore the virosphere, including plant virome.

The *Orchidaceae* family is the second-largest family of flowering plants in terms of number of species, being the first-largest epiphytic botanical family. As *Orchidaceae*, that naturally grow in tropical regions, *Bromeliaceae* family species also grow in tropical and subtropical areas. Likewise, the information about viral diversity in both families is scarce. In this context, the virome bioprospection research was conducted with 54 samples, being 18 of Bromeliaceae and 36 of Orchidaceae specimens collected in Central Brazil. For this, DNA and RNA samples were extracted and after viral enriched. Two pools (one DNA pool, and one RNA pool) were submitted to HTS using the Illumina NovaSeq 6000 and HiSeq 2000 platforms, respectively. Bioinformatics programs, CLC Genomic Workbench v. 20.0, and Geneious R11.1 were used to analyze the results. Two new DNA and seven RNA viruses were discovered and described here, in association to orchids and bromeliads.

In the **first chapter**, a literature review on ornamental plant market and the incidence of viruses affecting ornamental plants was organized aiming to explore the *status quo* of virological knowledge in the area.

In the **second chapter**, it is described symptomatic leaf collections of orchids and bromeliads, sample cataloging and subsequent the HTS approach. HTS of the orchid and bromeliad viral RNA pool resulted in 54,088,166 reads and 16,560 contigs, further analyzed to associate the presence of putative RNA viruses in the composed sample. For that purpose, the obtained contigs were submitted to BLASTn analysis confronted against the NCBI virus sequence database. After this, 15 previously undetected viral RNA contig sequences were identified. One sequence displayed phylogenetic relationship to members of the genus *Alphaendornavirus* (family *Endornaviridae*),

two to the genus *Totivirus* (family *Totiviridae*), three to genus *Polerovirus* (family *Solemoviridae*), two RNA 1 and two RNA 2 sequences displayed relationships to members of the genus *Dichorhavirus* (family *Rhabdoviridae*) and one RNA 1 and one RNA 2 displayed identity to members of the genus *Nepovirus* (family *Secoviridae*). Three RNA 1 sequences were identified with genomic features related to members of the genus *Sadwavirus* (family *Secoviridae*), but no RNA 2 was found. Therefore, according to the classification criteria adopted by the International Committee on Taxonomy of Viruses (ICTV), the information found on these previous sequences is insufficient to establish new species. Beside this, a total of seven new viral species were molecularly characterized in the RNA pool. The seven new species were tentatively named **1.** *Alphaendornavirus monocotyledonae* (OQ866133); **2.** *Dichorhavirus monocotyledonae* (OQ866129, OQ866130, OQ866131, OQ866132); **3.** *Nepovirus monocotyledonae* (OQ866134, OQ866135); **4.** *Polerovirus monocotyledonae* 1 (OQ866138); **5.** *Polerovirus monocotyledonae* 2 (OQ866139, OQ866140); **6.** *Totivirus monocotyledonae* 1 (OQ866136); and **7.** *Totivirus monocotyledonae* 2 (OQ866137).

The **third chapter** describes the DNA virome bioprospection strategy using the orchid and bromeliad samples mentioned above. DNA extraction samples from orchids and bromeliads were used to perform Rolling Circle Amplification (RCA) to generate closed DNA viral amplicons. The RCA samples were mixed to form a DNA pool that was sequenced either by Sanger Dideoxy platform or by HTS Illumina NovaSeq 6000 platform. Bioinformatic treatment of the DNA pool sequencing resulted in 11,060,510 reads and 163,808 contigs generated by HTS and Sanger analysis. The details of the results will be discussed in the fourth and fifth chapters.

The **fourth chapter** reports a mixed infection of two known begomoviruses, tomato severe rugose virus (ToSRV) and tomato chlorotic mottle virus (ToCMoV), in orchids of the genus *Dendrobium*. This is the first report of begomoviruses infection in specimens of *Orchidaceae* family.

The **fifth chapter** is a characterization of two new highly divergent begomovirus-like pathogens infecting orchids of the genus *Spathoglottis*. The two putative new viruses were named *Spathoglottis-associated mottle virus* 1 (OQ791967) and *Spathoglottis-associated mottle virus* 2 (OQ791968). PCR primers were designed for both sequences and confirmed the infection in the orchids sample of the genus *Spathoglottis*.

Keywords: Virome, High-Throughput Sequencing (HTS), Monocots, Bioprospection, Ornamental Plants

Resumo Geral: Bioprospecção de viromas de plantas ornamentais das famílias *Bromeliaceae* e *Orchidaceae* no Planalto Central Brasileiro

Os vírus são entidades biológicas comuns em todos os ambientes terrestres, mas pouco se sabe sobre. A avaliação é de que o conhecimento sobre a virosfera eucariótica total seja inferior a 1%. A maior parte da informação está restrita a vírus que causam doenças e perdas econômicas. Outras relações ecológicas e descrição de espécies crípticas de vírus continuam praticamente inexploradas. O segmento de estudo sobre vírus de plantas não é distinto. A compreensão da diversidade, forças evolutivas e seu impacto é desconhecida. No entanto, com o advento do High throughput sequencing (HTS), o estudo do viroma tornou-se tangível e de rápido crescimento. Da mesma forma, as ferramentas de bioinformática melhoraram a compreensão da diversidade viral e dos padrões de evolução. Com a pandemia de SARS-CoV 2 e o possível surgimento de novas doenças virais em humanos e em outros eucariotos, é notório a necessidade da exploração e a geração de novas informações sobre a virosfera terrestre.

A família *Orchidaceae* é a segunda maior família de plantas que florescem. Eles crescem predominantemente em áreas tropicais, assim como as espécies da família *Bromeliaceae*. Os estudos sobre diversidade viral de ambas as famílias são escassos, exceto para as plantas de interesse econômico. Ainda assim, diante de outras espécies economicamente importantes, o volume de pesquisa é incipiente. Neste trabalho, a bioprospecção de viromas foi realizada com espécimes da família *Bromeliaceae* e *Orchidaceae* encontradas em cultivos e reservas ecológicas privadas no planalto central. O HTS de DNA e de RNA dessas plantas foram feitos utilizando a plataforma Illumina NovaSeq 6000 e HiSeq 2000, respectivamente. Programas de bioinformática, CLC Genomic Workbench v. 20.0 e Geneious R11.1, foram utilizados para estudar os resultados encontrados no viroma. Dois novos vírus de DNA e sete de RNA foram descritos infectando orquídeas e bromélias.

No **primeiro capítulo**, uma revisão bibliográfica sobre o mercado de plantas ornamentais e sobre a incidência de viroses sobre nelas foi desenvolvida com o intuito de explorar o *status quo* das

pesquisas na área.

No **segundo capítulo**, a exploração dos resultados de HTS de RNA de 54.088.166 reads e 16.560 contigs foi feita com o intuito de caracterizar os possíveis vírus de RNA presentes. Para isso, os contigs obtidos foram confrontados contra um banco de dados de sequências virais via BLASTn. 15 sequências virais de RNA, até então não caracterizadas foram identificadas. Ao total sete novas espécies virais foram caracterizadas. Sendo uma sequência pertencente ao gênero *Alphaendornavirus* (família *Endornaviridae*), duas pertencentes ao gênero *Totivirus* (família *Totiviridae*), três pertencentes ao gênero *Polerovirus* (família *Solemoviridae*), dois RNA 1 e dois RNA 2 pertencentes ao gênero *Dichorhavirus* (família *Rhabdoviridae*) e um RNA 1 e um RNA 2 pertencente ao gênero *Nepovirus* (família *Secoviridae*). Três sequencias de RNA 1 relacionados ao gênero *Sadwavirus*, família *Secoviridae*, foram identificados. Entretanto, nenhum RNA 2 foi encontrado. De acordo com os critérios de classificação de gênero adotados pelo International Committee on Taxonomy of Viruses (ICTV), as informações encontradas dessas três sequências são insuficientes para estabelecer novas espécies. As sete novas espécies foram nomeadas de *Alphaendornavirus monocotyledonae* (OQ866133); *Dichorhavirus monocotyledonae* (OQ866129, OQ866130, OQ866131, OQ866132); *Nepovirus monocotyledonae* (OQ866134, OQ866135); *Polerovirus monocotyledonae* 1 (OQ866138); *Polerovirus monocotyledonae* 2 (OQ866139, OQ866140); *Totivirus monocotyledonae* 1 (OQ866136); *Totivirus monocotyledonae* 2 (OQ866137).

No **terceiro capítulo**, encontra-se o prefácio dos locais de coleta, da catalogação de amostra e das metodologias empregada nos capítulos subsequentes, o quarto e o quinto capítulo, em que os 11.060.510 reads e 163.808 contigs de HTS são trabalhados.

O **quarto capítulo** é uma Disease note de infecção mista de dois begomovírus conhecidos, o tomato severe rugose virus (ToSRV) e o tomato chlorotic mottle virus (ToCMoV), em orquídeas do gênero *Dendrobium*.

O **quinto capítulo** é uma caracterização de dois patógenos altamente divergentes, pertencentes ao gênero *Geminiviridae*, infectando orquídeas do gênero *Spathoglottis*. As duas novas

sequências virais, denominadas *Spathoglottis-associated mottle virus* 1 (OQ791967) e *Spathoglottis-associated mottle* virus 2 (OQ791968), devem ser consideradas novas espécies. Primers foram desenhados para ambas as sequências e confirmaram a infecção em amostras de orquídeas do gênero *Spathoglottis*.

Palavras-chave: Viroma, High-throughput sequencing (HTS), Monocotiledoneas, Bioprospecção, Plantas Ornamentais

CHAPTER 1

Review | To be submitted as a Review

Review: Economical aspects of ornamental plants market and impacts of viral diseases on ornamental plant production

Review: Economical aspect of ornamental plants market and impacts of viral diseases on ornamental plant production

Juliana Gabrielle Isidorio da Silva[1], Maria Esther de Noronha Fonseca[2], Leonardo Silva Boiteux[2], Tatsuya Nagata[1], Rita de Cassia Pereira Carvalho[1]

[1] Departamento de Fitopatologia, Universidade de Brasília (UnB), Brasília–DF, Brazil.

[2] National Center for Vegetable Crops Research (CNPH), Embrapa Vegetable Crops (Hortaliças), Brasília–DF, Brazil.

Abstract

The market for ornamental plants is diverse and dynamic. The global prospect of the market is to surpass US$ 97.79 billion by 2032. Furthermore, the ornamental plants sector contributes positively to other economic segments, such as indoor/outdoor architectural landscaping and decoration. With the continuous growth of this market worldwide, threats also rise in the same proportion, such as virus-induced diseases. These biological entities constitute a permanent threat to profitability and sustainability of this sector. Estimated losses due to viral infections are around 50%. Yet, few studies exploring the diversity of viruses and their ecological and economic impacts are available in this area. The data organized herein comprises mainly the countries with more research efforts in this field. In tropical countries, such as Brazil, bromeliads and orchids are the market leaders in the ornamental plant sector. Scarce information about virus-induced diseases in members of the families Bromeliaceae and Orchidaceae is also found in the main databanks. The overall lack of information about the management of virus diseases conveys instability to the ornamental horticulture market and it may result in potentially catastrophic economic effects.

Keywords: *Orchidaceae*, *Bromeliaceae*, Brazil, Plant disease

1. Ornamental plant and the ornamental market

Ornamental plants are recognized for their aesthetics and their potential to decorate places [1].

Throughout history, the cultivation of ornamental plants turned into a dynamic global market [2]. The ornamental products encompass plants (potted or not); cut flowers; cut foliage; and propagation material [3]. Its use is versatile, and it is fast growing worldwide. That exponential growth is noticed in both developed and developing countries [4]. However, in developing countries, like Brazil, it is necessary to overcome some obstacles to solidify the market [4].

The overall impact of the ornamental plant market is due to the plasticity of uses and the diversity of the cultivated plant families [2, 3]. Between 2014 and 2021, the worldwide growth rate was 5.9%, with a capital of US$ 49.8 billion [3]. The profitability forecast for this market surpasses US$ 97.79 billion by 2032 [3]. The annual *per capita* consumption in countries with consolidated markets is around US$ 81 [3].

Furthermore, the ornamental plant sector generates indirect revenue in other markets and economic niches, as for example the sector of indoor/outdoor architectural landscaping and decoration [5]. In the United Kingdom, the estimated profit in 2017 was US$ 3.8 billion when considering gardens, parks, and greenhouses tours [5]. It also generated more than 60,000 jobs in the tourism segment [5].

Although the COVID-19 pandemic harmed the profits of some ornamental plant segments, the overall growth was yet significant in the potted plant sector [2, 3]. The change in the profile of the customers was the foremost factor [2]. The awareness of ornamental plants as an element capable of promoting human welfare and environmental benefits was the main reason reported by the customers [2]. That behavior was also observed in the Brazilian market, where the potted plant sector represents 58% of the total production [6].

1.1.The ornamental plant market in Brazil

In emergent nations such as Brazil, an economic expansion was observed in the ornamental market across recent years, although with a modest and seasonal consumption pattern. For instance, in 2021, the profits of the Brazilian ornamental plant market exhibited a growth of 15% with an equivalent capital greater than 2.1 billion dollars [6]. It also accounted for an increase of around 209,000 employments in this sector within the Brazilian territory [6]. Considering Brazil national

context, São Paulo State is the leading ornamental producer, holding almost 48.9% of the production [6]. Furthermore, São Paulo has the highest *per capita* annual consumption (= US$ 17.33) in comparison with the national average of US$ 12.52 [6].

The Federal District (Distrito Federal–DF) is also an important ornamental plant market in Brazilian territory. In 2016, the annual *per capita* consumption was over US$ 8.47, whereas the national average was US$ 5.06 [4]. The sector generated annual profitability of US$ 38.5 million, with an increase of 15% in 2018 [7]. The regional producers cover the demands of about 20% of the local market [7]. This production consists mainly of tropical cut flowers and leaves, such as lobster-claws, torch ginger, and prairie gentian [7]. The local government is carrying out institutional efforts to consolidate the tropical plant production of the DF as competitive sector in the Brazilian market [7]. However, biotic problems (diseases and pests) as well as the implementation of adequate management techniques of the ornamental plants are relevant obstacles to surpass [8].

2. Plant diseases in ornamental plant species

Ornamental production on a global scale has changed dramatically in the past 20 years with the incorporation of new areas of production, including South America [9]. In the new scenario, sustainability is yet a major objective for economically viable ornamental production [9]. The emergence of plant diseases represents a major threat to crops and native flora [10, 11]. Due to its relevance, plant health was the theme of the annual Food and Agriculture Organization (FAO) campaign of 2020 [12]. Viroids and viruses are among the most relevant biological entities that compromise plant health. The estimated rate of yield losses due to virus-induced diseases worldwide is 50%, with a corresponding annual economic loss of US$ 30 billion [13]. However, studies about some species and families, such as ornamental plants species, are scarce with an overall lack of information about viral diseases and viral agents [14]. Another bottleneck is obtaining disease-free products in the ornamental plant industry since continuous regional transit, as well as offshore imports of vegetatively propagated species, can introduce pathogens (including viruses) into new production areas [15].

2.1.Viruses and viroids in ornamental plants

Tulip mania, an event which took place in the sixteenth century, was a historical fact linked to the first record of a viral infection in ornamental plants. On that occasion, tulips that displayed variegated flowers, such as 'Semper Augustus,' had a high market value [16, 17]. However, the quality of those plant bulbs was precarious, and often lost their vigor [16, 17]. That situation was accountable for the formation of the economic bubble [16, 17].

Nonetheless, the origin of this variegated characteristic in tulips was unknown at that time, much less that viruses existed [17]. The discovery of a plant virus and a virus-induced disease was done by the scientist Beijerink in 1898 with *Tobacco mosaic virus* [17, 18]. After this breakthrough, the field of virology, especially plant virology, began to expand [17]. The infectious agent that induced the Rembrandt tulip phenotype, the Tulip Breaking Virus (TBV), was only discovered in 1930 [17], illustrating the relationship between viruses and some ornamental patterns in plants [19]. Like TBV, many other viruses cause symptoms that can increase the market value of an ornamental host plant [19]. Though, like TBV, virus infection in plants may also produce undesirable symptoms, resulting in the degeneration of the host plants over time [19].

Therefore, the selection of aesthetic improvements in ornamental plants must be carried out carefully. Investigating the genetic origin of a given phenotype avoids the selection of plants infected by viruses and viroids [19]. This was the solution for the variegated tulips, in which a plant gene was found to produce a similar phenotype [19]. This contemporary variety is currently sold as Rembrandt tulips [20].

Furthermore, generalist viruses affect several important ornamental crops. For example, the tomato spotted wilt virus (TSWV) is one of the biological entities responsible for severe stunting in tomato crops [19, 21]. This virus is known to infect a wide range of host plants, including ornamental species [21, 22]. It can even cause disease in high-economic-value items of the floriculture industry, such as orchids [22, 23]. Like TSWV, there are other viruses that are responsible for inducing diseases in several crops [23]. Given their economic importance, some

authors such as Rybicki in 2015 [24], and Mitrofanova et al in 2018 [25] generated lists of viral species and genera with the greatest impact on important fields, vegetables, and ornamental crops.

Given this, an extensive data survey was carried out on viruses and viroids that infect ornamental plants. Plant families were chosen based on data released by IBRAFLOR in 2022 [6]. Ornamental plant species from the *Alstromeriaceae*, *Asteraceae*, *Bromeliaceae*, *Cactaceae*, *Liliaceae*, *Orchidaceae*, and *Rosaceae* families were initially chosen. Data on viral and viroid species occurring in these hosts were obtained from the National Center for Biotechnology Information (NCBI) using the whole genome database (https://www.ncbi.nlm.nih.gov/). The encyclopedia of plant viruses and viroids, of Sastri et al of 2019 [23], and the Kitajima list of viruses of 2020 [26] were also used to verify serological test, and electron microscopy viral particles reports in ornamental plants and compare the previous obtained results.

Regarding results of the GenBank database, and bibliographic research, the genus *Emaravirus* was the most dominant viral sequence recorded among the analyzed botanic families (**Figure 1**). However, *Begomovirus* should be considered as the most prevalent viral genus infecting a wide diversity of ornamental plants, because *Emaravirus* genus was associated to infections on roses species only. Considering viroids, the genus *Pospiviroid* is the most found among the analyzed ornamental plants botanic families, comprising 433 record hits (**Figure 1**).

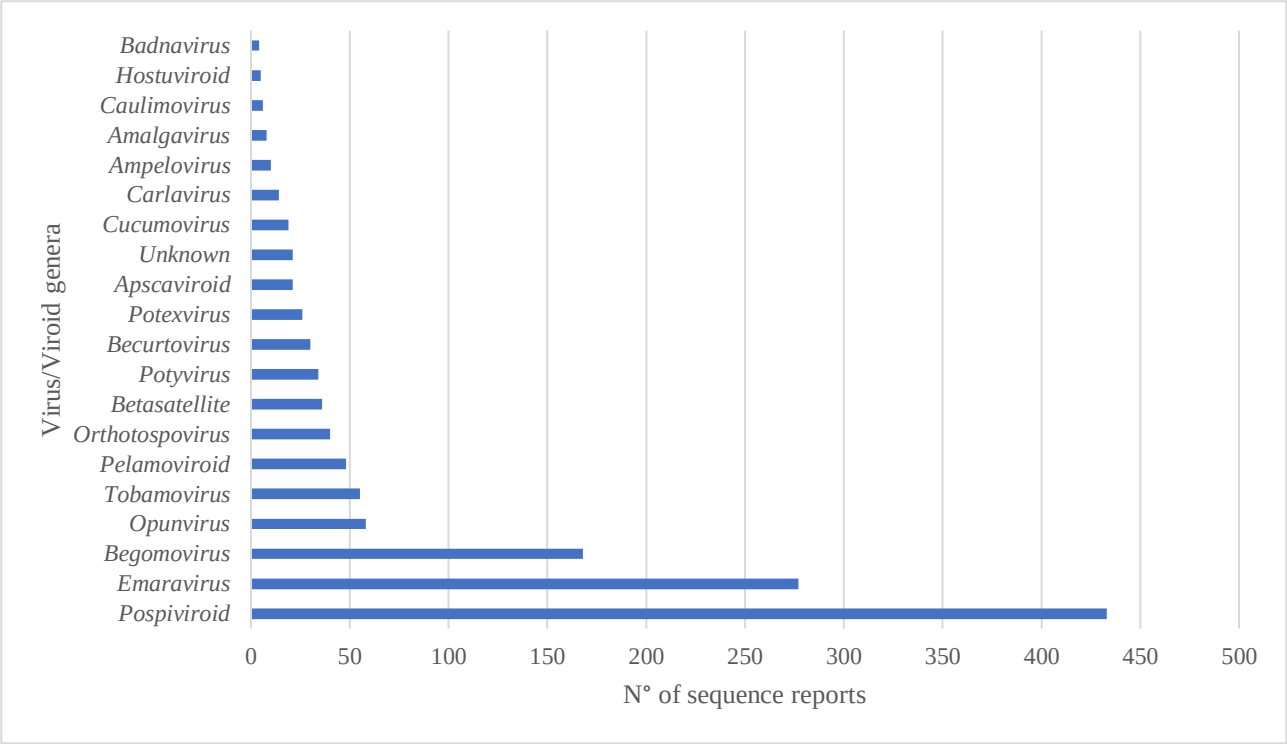

Figure 1. Number of nucleotide complete sequence records from the NCBI GenBank associated to virus/viroid genera infecting ornamental plants from the *Alstromeriaceae*, *Asteraceae*, *Bromeliaceae*, *Cactaceae*, *Liliaceae*, *Orchidaceae*, and *Rosaceae* botany families. The NCBI Virus databank (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) was accessed for consultancy in March 2023.

When analyzing the viral species GenBank record associated with the query ornamental plant families, *Emaravirus rosae* species is the most common, with 277 records found (**Figure 2**). *Emaravirus rosae*, which infect Rosaceae plants, belongs to the negative-strand RNA virus genus named *Emaravirus*. *Emaravirus rosae* commonly causes symptoms of plant twisted growth, short internodes, and reddish mass growth, among other deleterious symptoms [27]. Regarding viroids, *Chrysanthemum stunt viroid*, belonging the *Pospiviroid* genus, was the viroid species most found among the queried ornamental plant record, with 400 records (**Figure 2**). *Chrysanthemum stunt viroid* causes deformation in chrysanthemum flowers and loss of vigor in the infected plant [28]. Therefore, plant infection by emaravirus rosae and chrysanthemum stunt viroid may be very impacting to the ornamental plant production chain, as both roses and chrysanthemums are the most commercialized cut flowers worldwide [6].

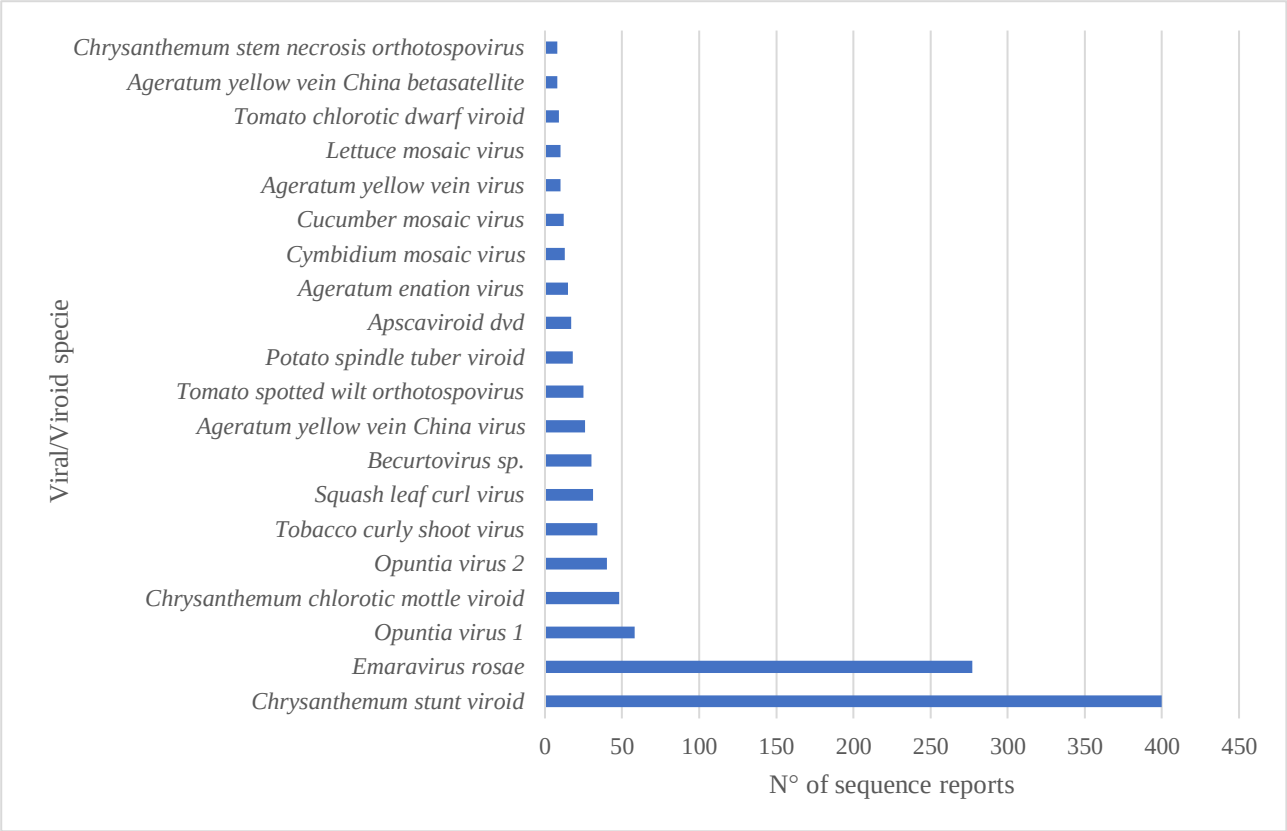

Figure 2. Number of nucleotide complete sequence records from the NCBI GenBank associated to virus/viroid species infecting ornamental plants from the *Alstromeriaceae*, *Asteraceae*, *Bromeliaceae*, *Cactaceae*, *Liliaceae*, *Orchidaceae*, and *Rosaceae* botany families. The NCBI Virus databank (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) was accessed for consultancy in March 2023.

Concerning the geographic distribution, most the virus/viroids GenBank sequence records were described in the United States, South Korea, China, India, Japan, and Brazil (**Figure 3**). The importance of agriculture in most of these countries and the consumption profile of ornamental plants found in these localities justify the observed scenario.

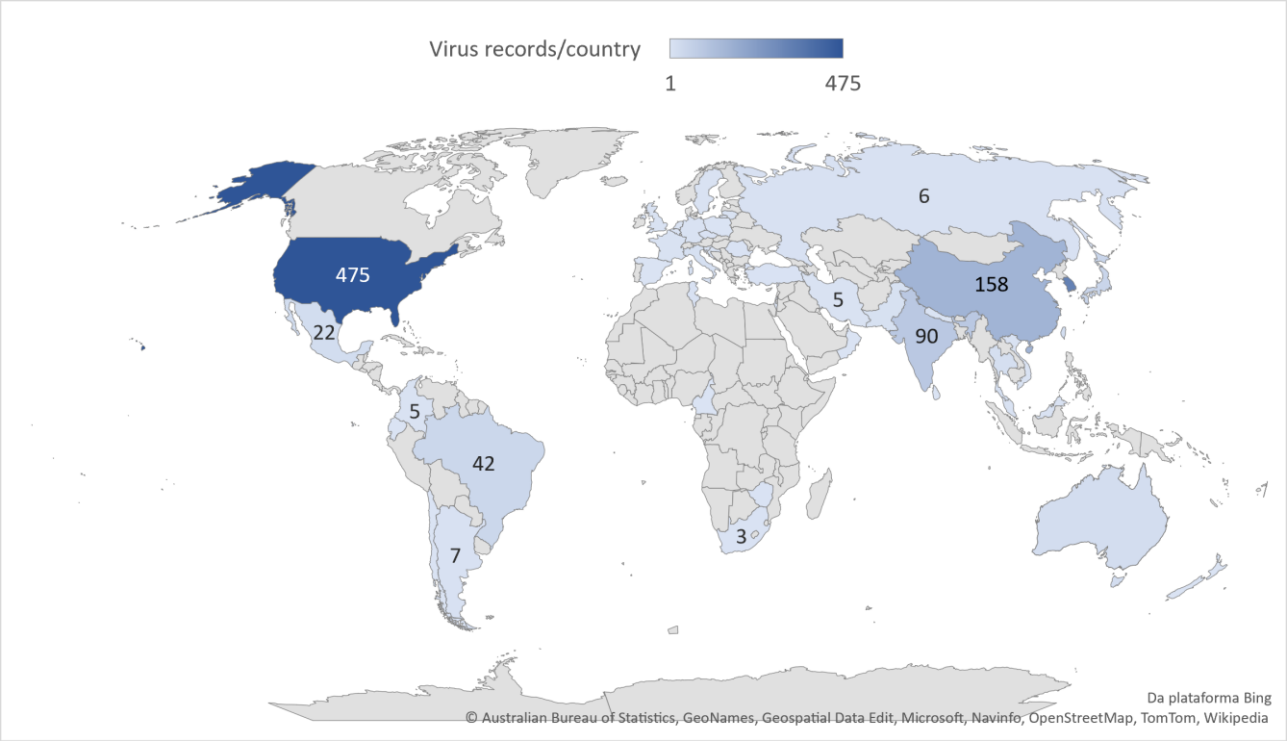

Figure 3. Geographic distribution and density of nucleotide complete sequence records from the NCBI GenBank associated to virus/viroid species infecting ornamental plants from the *Alstromeriaceae*, *Asteraceae*, *Bromeliaceae*, *Cactaceae*, *Liliaceae*, *Orchidaceae*, and *Rosaceae* botany families. The NCBI Virus databank (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) was accessed for consultancy in March 2023.

Besides the GenBank database, the diversity of plant viruses present within the Brazilian territory is comprehensively described in the list elaborated by Kitajima in 2020 [26]. Nevertheless, omic scientific approaches to describe ornamental plant virome occurring in Brazil are still scarce, despite the records found in both GenBank database and Kitajima`s list of viruses occurring in the country. This information gap on viruses that affect ornamental plants makes crop handling extra challenging task within Brazil [14]. Furthermore, as most ornamental plants are exotic to Brazil, the usual importation of virus-infected vegetative propagation material, such as bulbs and seedlings, may introduce impacting exotic quarantine viral pests into the national territory [6]. Therefore, quarantine strategies/services/legislation/scientific research contributing to avoid exotic plant virus introduction is essential to preserve relevant the ornamental market in Brazil and worldwide [12].

Considering the Brazilian scientific sphere, research ornamental plants viruses is mostly performed with plant sample collected in the Southeastern Macroregion, which is the national main producer area of ornamental plants [6, 26, 29]. In the Brazil Midwestern Macroregion, there are few reports of viruses occurring in ornamental plants, despite the growing corresponding market. In this

respect, the present work aggregates relevant information for plant protection in the country, since the samples of symptomatic ornamental plants for virus prospection were collected in the Midwestern localities.

Alarming report of exotic viruses present in South America, which represent risk to the Brazilian ornamental plant production upon viral introduction in the national territory, are listed as quarantine viruses [30, 31]. Banana bract mosaic virus, Pelargonium zonate spot virus, and Tulip breaking virus have been reported infecting ornamental plants in this geographic region [23]. The reports came, respectively, from Colombia, Argentina, and Brazil [26]. Besides this, TBV was described as infecting lilies in a home garden in Brasilia-DF [32]. In conclusion, bioprospection of both exotic and local viromes is a relevant strategy for plant protection.

2.2.Viruses associated with bromeliads and orchids

Species of Bromeliads and orchids are relevant players of the ornamental plant market in the potted plant sector (Moraes et al. 2017; [3, 6, 29, 33]. The pineapple bromeliad and vanilla orchid are as well part of the food, phytopharmaceutical, and cosmetic markets [34, 35]. The use of bromeliads and orchids is versatile and profitable [34, 35]. Bromeliads and orchids usually grow in tropical areas, with a few exceptions of orchids growing in subtropical areas. The *Orchidaceae* family is the second largest flowering family, comprising over 800 genera and 35,000 species, only losing in number of species members to the *Asteraceae* family [29, 35]. *Bromeliaceae* is a botanic family of neo-tropical plants that have the second largest number of epiphytic species, only losing to the *Orchidaceae* family [35, 36, 37].

The data encompassing viruses and viroids in *Bromeliaceae* and *Orchidaceae* species are explored in this section of the present review. Information on viruses infecting ornamental orchids plants is scarce, but bromeliad virome data is even more scarce than orchid virome. Notably, the orchid genera with most virome information available are those well-established in horticulture and the food market, such as *Cymbidium*, *Phalaenopsis*, *Vanilla*, *Pterostylis*, and *Dendrobium* genera, which, altogether, concentrate around half of orchid viral sequence inputs present in the GenBank data (**Figure 4**).

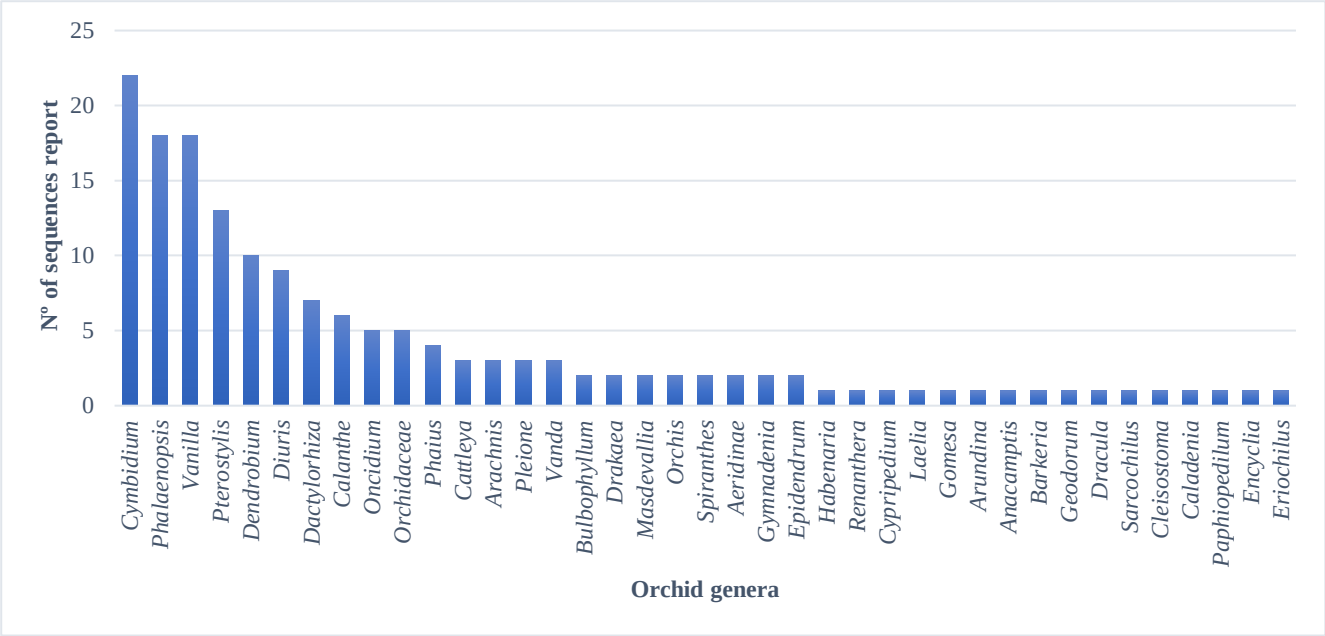

Figure 4. Number of nucleotide complete sequence records from the NCBI GenBank associated to viruses infecting various orchid genera. The NCBI Virus databank (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) associated to *Orchidaceae* family was accessed for consultancy in March 2023.

The number of GenBank viral sequence records associated with bromeliads is even more restricted than those associated with orchids. The bromeliad species with most associated GenBank viral sequences (~97.6% of records found among bromeliad) is *Anana comosus* (pineapple) (**Figure 5**). A single GenBank viral sequence record associated to *Pineapple mealybug wilt-associated virus* 3 (*Ampelovirus* genus) was reported occurring in Cuban infecting the wild pineapple bromeliad [38], whereas only two records associated to *Pineapple mealybug wilt-associated virus* 2, and *Pineapple mealybug wilt-associated virus* 3 (both belonging to the *Ampelovirus* genus) were reported occurring in Brazil infecting ornamental bromeliad, *Bromelia laciniosa* [39].

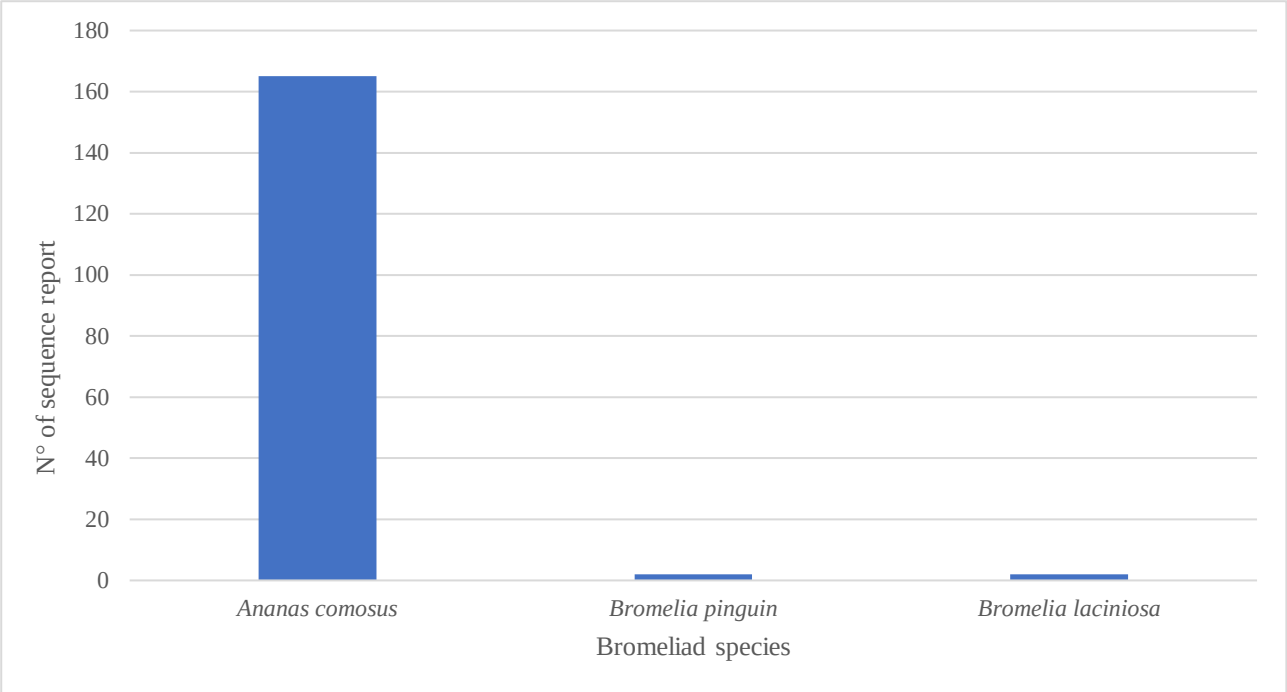

Figure 5. Number of nucleotide complete sequence records from the NCBI GenBank associated to viruses infecting various bromeliad genera. The NCBI Virus databank (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) associated to *Bromeliaceae* family was accessed for consultancy in March 2023.

Regarding the distribution and density of the GenBank viral sequence records in orchids, China holds most of them (**Figure 6**), probably due to the relevance of the *Orchidaceae* family for the Chinese culture [33]. Moreover, orchids are used in horticulture, Chinese traditional medicine, as well as food components [33]. The second country holding numerous GenBank viral sequence records associated to orchids are the United States of America (**Figure 6**).

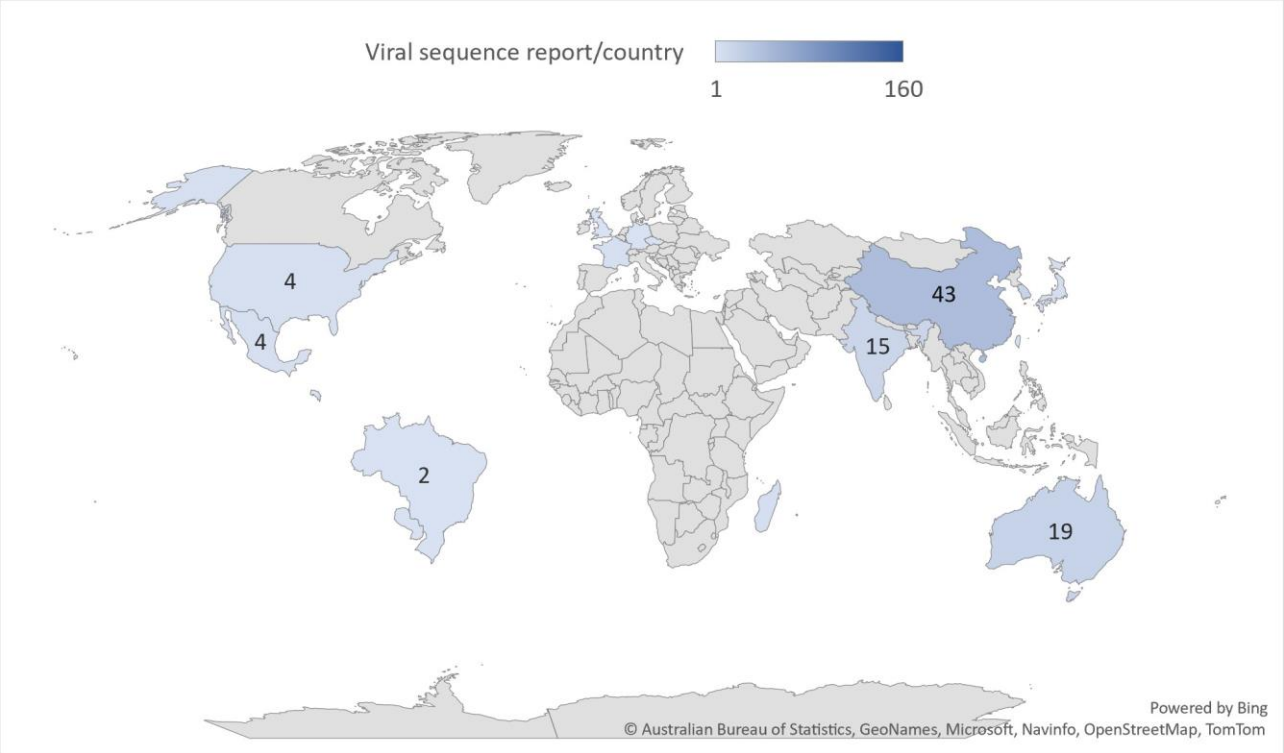

Figure 6. Geographic distribution and density of nucleotide complete sequence records from the NCBI GenBank associated to virus species infecting orchids. The NCBI Virus databank (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) associated to *Orchidaceae* family was accessed for consultancy in March 2023.

Considering distribution and density of GenBank viral sequence records associated with Bromeliads, it is noticeable the prominence of USA, Australia, and Brazil (**Figure 7**), coherent with the relevance of the pineapple crop Brazil, which produces approximately 2253.90 metric tons of pineapple per year, being the third producer worldwide [39].

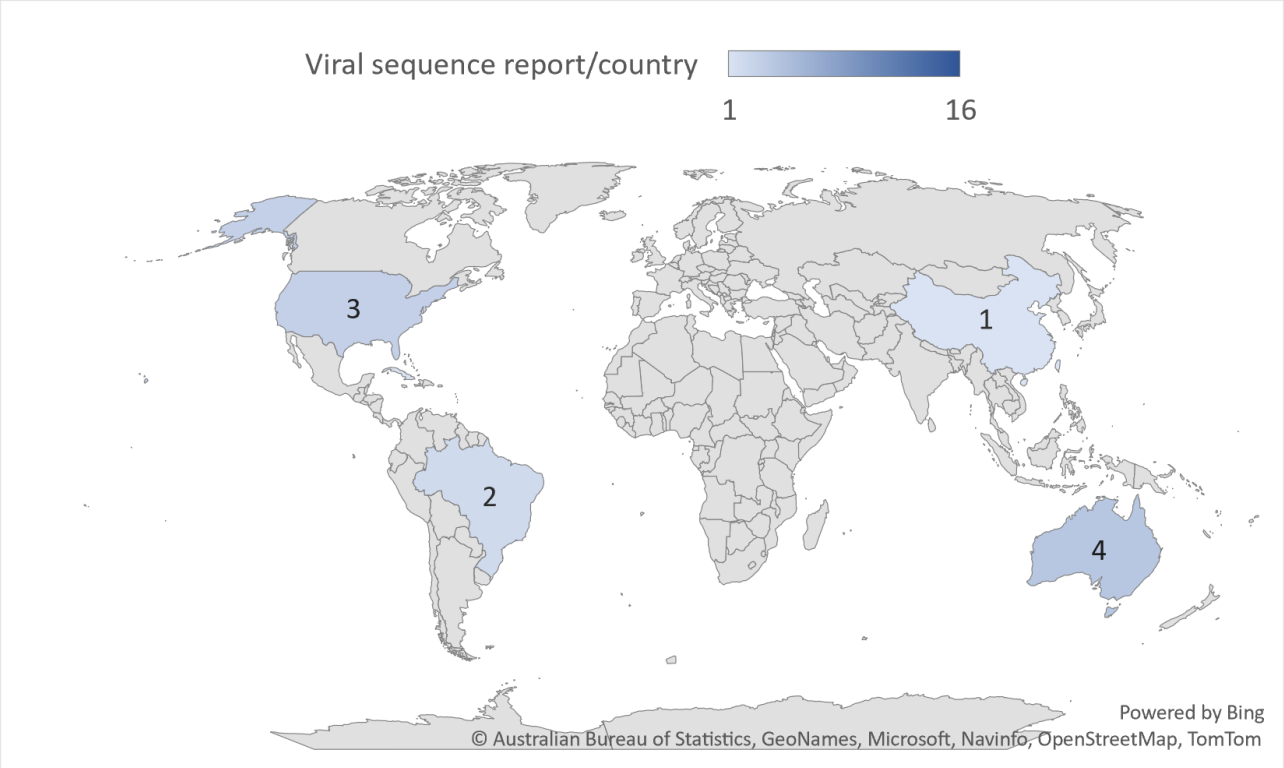

Figure 7. Geographic distribution and density of nucleotide complete sequence records from the NCBI GenBank associated to virus species infecting Bromeliad. The NCBI Virus databank (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) associated to *Bromeliaceae* family was accessed for consultancy in March 2023.

In conclusion, it is necessary to enlarge knowledge on the diversity of ornamental plants viruses. Gap knowledge on ornamental plant virome represents potential risk inefficient diagnostic/detection systems allowing the dissemination across countries of potentially hazardous viruses. In Brazil, the introduction of novel viral pathogens is a real and significant risk since the ornamental plant sector in the country is yet highly dependent on the importation of exotic seeds and bulbs. In this scenario, investments in infrastructure and human resources aiming to establish and improve virus detection systems as well as the financial support (public and private) to virome projects should be recommended strategies. This type of technological advance will be facilitated by the advent of High Throughput Sequencing (HTS) platforms and bioinformatic tools. In the present dissertation, practical applications of these technologies will illustrate these viewpoints.

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CHAPTER 2

Article | To be submitted as an article to *Viruses*

RNA virome of *Orchidaceae* and *Bromeliaceae* ornamental species from production and preservation areas within Central Brazil

RNA virome of *Orchidaceae* and *Bromeliaceae* ornamental species from production and preservation areas within Central Brazil

Juliana Gabrielle Isidorio da Silva[1], Leonardo Silva Boiteux[2], Maria Esther de Noronha Fonseca[2], Robert Miller[1], Tatsuya Nagata[1], Felipe Fochat da Silva Melo[1], Rita de Cassia Pereira-Carvalho[1]

[1] Departamento de Fitopatologia, Universidade de Brasília (UnB), Brasília–DF, Brazil.

[2] National Center for Vegetable Crops Research (CNPH), Embrapa Vegetable Crops (Hortaliças), Brasília–DF, Brazil.

Abstract

High Throughput Sequencing (HTS) platforms and bioinformatics tools have enabled the large-scale detection of new viruses occurring across a wide range of plant hosts. However, viromes of ornamental plants, including members of the *Orchidaceae* and *Bromeliaceae* families, are yet scarce. Herein, HTS of virus enriched pooled sample, extracted from orchid and bromeliad plants collected in three localities in Central Brazil, either production or preservation areas, resulted in the identification of 15 contig sequences associated to 7 new putative virus members of the families *Endornaviridae*, *Rhabdoviridae*, *Secoviridade*, *Solemoviridae*, and *Totiviridae*. The results highlight the importance of virome prospection via HTS, given the economic importance of orchids and bromeliads in the ornamental market in Brazil and worldwide.

Keywords: *Riboviria*, Ornamental Plant, RNA Viruses, Neotropics. High-Throughput Sequencing

Introduction

The *Orchidaceae* is one of the most diverse families of flowering plants, with over 800 genera and nearly 35,000 species described worldwide [1,2]. In Brazilian territory, more than 2,300 *Orchidaceae* species have been catalogued, being distributed over 200 genera [2]. The *Bromeliaceae* is a family of neotropical plants that has the second largest number of epiphytic

species, just behind orchids [3, 4]. The Brazilian Atlantic Forest, the Andean mountains, forests of Central America, and the Guiana Highlands are the major centers of bromeliad diversity and endemism [5]. Important members of the families Orchidaceae and Bromeliaceae are adapted to tropical and subtropical areas and have versatile and profitable usages, including fruit market, ornamental horticulture as well as providing raw materials for food, phytopharmaceutical, and cosmetic industries [1, 2, 3, 4, 6].

Viruses are considered the most abundant biological entities in nature [7, 8, 9]. Studies on diversity provide estimates that only 1% of the viral species is currently known [9]. The advent of High Throughput Sequencing (HTS) and bioinformatic tools has facilitated the detection of new viruses occurring across a wide range of environments, ecological niches, and hosts, including vascular plants [8, 9, 10]. However, viromes of ornamental plants, including members of the Orchidaceae and Bromeliaceae families, comprise a yet largely unexplored area of study [8, 9].

In the present work, HTS analysis were carried out with a pool of foliar samples from bromeliads and orchids cultivated in highland areas of Central Brazil, aiming to investigate their virospheres. In our assessment, seven new viruses were detected and characterized within the families *Endornaviridae*, *Rhabdoviridae*, *Secoviridae*, *Solemoviridae*, and *Totiviridae*, revealing a yet unexplored viral diversity in these important ornamental hosts.

The family *Endornaviridae* encompasses single-stranded, positive sense RNA (ssRNA+) viruses with genomes sizes ranging from 9.7 to 17.6 kb [11]. 24 recognized species have been reported infecting plants, fungi, and oomycetes [11]. The family comprises two genera (*Alphaendornavirus* and *Betaendornavirus*) characterized by their unique genomic domains, sizes, and host ranges [11]. Alphaendornaviruses are plant-associated viruses with large (more than 11.9 kb) genomes [11]. The typical member of this genus is *Oryza sativa alphaendornavirus* [11]. The genome contains a single Open Reading Frame (ORF) encoding a polyprotein [12].

The family *Rhabdoviridae* is composed of viruses with single-stranded, negative sense RNA (ssRNA-) genomes, which currently contains 275 species distributed in 45 genera and three

subfamilies [13]. Members of this family have been reported infecting invertebrates, vertebrates, and plants. Phytoviruses have six genera, which include a subgroup of four genera with unsegmented genomes (viz. *Cytorhabdovirus*, *Alphanucleorhabdovirus*, *Betanucleorhabdovirus*, and *Gammanucleorhabdovirus*) and two genera with bi-segmented genomes (viz. *Dichorhavirus* and *Varicosavirus*) [13, 14]. The genus *Dichorhavirus* is a monophyletic group, which members are transmitted by the false mite of the genus *Brevipalpus* [15, 16, 17]. *Dichorhavirus orchidaceae* is one of the species of this genus previously reported infecting orchids and either causing necrotic or chlorotic ringspots and fleck symptoms [16].

The ssRNA (+) viruses of the family *Secoviridae* are plant-infecting viruses with mono- or bipartite genomes with combined sizes ranging from 9 to 13.7 kb [18, 19, 20]. Members of the family *Secoviridae* infect, in general, dicotyledonous species, inducing economically important diseases [18]. This family has currently 106 members distributed in one subfamily, nine genera, and three subgenera [18]. Two genera (*Sequivirus* and *Waikavirus*) have viruses with monopartite genomes. The remaining seven genera (*Cheravirus*, *Comovirus*, *Fabavirus*, *Nepovirus*, *Sadwavirus*, *Stralavirus*, and *Torradovirus*) are composed of viruses with bipartite genomes [18]. The genomic organization of bipartite viruses comprises the RNA1 (that carries all the information required for replication) and the RNA2 polyprotein (that contains movement protein and coat protein) [18]. The viral replication in individual cells requires the RNA1, although no virus particles are produced [18, 20].

The ssRNA (+) plant viruses of the family *Solemoviridae* have a relatively small (4–6 kb), monopartite polycistronic RNA [21]. The four genera of the family (*Enamovirus*, *Polemovirus*, *Polerovirus*, and *Sobemovirus*) comprise 60 species [21]. The *Polerovirus* genome consists of non-coding 5'- and 3'-regions and seven ORFs, four of them (ORF3a, ORF4, ORF6, and ORF7) are unique to poleroviruses [21]. The infections are phloem-specific, and these viruses are transmitted by aphids [21]. Coinfections with other viruses are common features for members of the genus *Polerovirus* [22].

The dsRNA viruses of the family *Totiviridae* are known as mycoviruses. This family comprises 28 species distributed in five genera: *Giardiavirus*, *Leishmaniavirus*, *Totivirus*, *Trichomonasvirus*, and *Victorivirus* [23]. The RNA length is around 4.6 to 7.0 kpb and the genome has usually two overlapping ORFs [23].

Hereafter, it is described the generation of the 15 contigs corresponding to seven new virus species associated to the RNA pool HTS of the orchid and bromeliad samples collected in Central Brazil.

Materials and methods

Forty-eight symptomatic foliar samples of bromeliads and orchids were collected between January and March of 2022 in three highland areas localities within Central Brazil. The collected leaves displayed typical virus-like symptoms such as chlorosis, mosaic, mottle, and necrotic ringspots (**Supplementary figure 1**). The collection sites were: ***(i)*** "La Bromélia", nursery of bromeliads ornamental plants in Brasília–DF Federative Unity; ***(ii)*** "Orquidário Colorado", ochidarium in Brasília–DF Federative Unity; and ***(iii)*** "Vagafogo" private natural heritage reserve in Pirenópolis–GO Municipality.

The foliar samples were catalogued and photographed (**Supplementary table 1**). Subsequently, the leaves were cut, weighted (1 g each in triplicate samples), and stored in a deep freezer (-80 °C). A pool from symptomatic bromeliads and orchids was assembled by combining 1 gram of each individual foliar sample. The viral particle semi-purification was performed using a modified sucrose (20%) cushion protocol in combination, in the last purification steps, with the Direct-zol RNA Miniprep kit (Zymo Research) and then stored at -80 °C [24]. An aliquot was used to verify the quality of the purification in NanoDrop™ One/One^{C}. Afterward, a fraction of the pool was dried in RNAstable® reagent (Sigma-Aldrich). A dried sample aliquot was sent for sequencing in a HTS platform (Illumina HiSeq 2000 at Macrogen Inc., South Korea).

The HTS results were submitted to GenBank databank to generate the bioproject (PRJNA954071) and SRA (SRR24144734) ID. The SRA was processed according to the following

workflow: ***(i)*** pretreatment and the initial *de novo* assembly of reads using the CLC Genomics Workbench 20.0 program; ***(ii)*** the contigs were submitted to BLASTn data search for viral hits by using Geneious R 11.1 program; ***(iii)*** contigs with viral hits were assembled by using the 'Map to reference' tool available in the Geneious R 11.1 program; ***(iv)*** the assembled contigs were then submitted to the BLASTn tool to search against the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for viral hits; ***(v)*** putative new viral contigs were submitted to NCBI VecScreen tool to check for the presence of any residual vector sequence; ***(vi)*** subsequently, these contigs were analyzed, and annotated; ***(vii)*** then the virus contig ORFs were analyzed using the NCBI Conserved domain tool and with the MAFT alignment tool assay on Geneious R 11.1 program; ***(viii)*** the sequence diversity SDT analysis was performed by using MAFT alignment [25], and ***(ix)*** the corresponding phylogenetic tree was generated by using IQtree web service [26]. Phylogenetic relationships were inferred using Maximum Likelihood (ML) and the single branch support was calculated using approximate Bayes test [26]. The phylogenetic substitution model was determined by the program using the setting "Find best and apply" [26]. Ultrafast bootstrap (UFBoot) was performed using 1,000 replicates, 0,5 of perturbation strength, and 100 unsuccessful iterations to stop [27]. ***(x)*** In the last step, the Newick output file was later uploaded on iTol v.6 to visualize and to generate the image [28]. The virus recombination RDP 4 analyses were performed for RNA 1 and RNA 2 sequences of the bipartite *Secoviridae* family viruses, as a prerequisite for species demarcation criteria [29].

Results

The SRA SRR24144734 of an orchid-bromeliad HTS composed RNA pool generated 15 contig sequences corresponding to new virus species belonging to *Endornaviridae*, *Rhabdoviridae*, *Secoviridade*, *Solemoviridae*, and *Totiviridae* families (**Table 1**). The BLASTn, and BLASTp against the GenBank database revealed that the referred contig sequences as new species of viruses based upon identity levels and query covers (**Table 1**).

Table 1: Sequences of new viruses discovered using High Throughput Sequencing. The NCBI sequence ID, the contig, the sequence length, the given name of the new viruses, the identity, the query cover, the family, and genus are displayed.

Sequence ID (NCBI)	**Contig**	**Length**	**Suggested name**	**Identity/Query cover (%)**		**Genus**
				BLASTn	BLASTp	
			Endornaviridae			
OQ866133	16	15,758	*Alphaendornavirus monocotyledonae*	66.63/8	47/92	*Alphaendornavirus*
			Rhabdoviridae			
OQ866131	7	5,940	*Dichorhavirus monocotyledonae* (RNA 2)	70.42/67	62.67/92	*Dichorhavirus*
OQ866132	8	7,241	*Dichorhavirus monocotyledonae* (RNA 2)	99.56/7	67.27/98	
OQ866129	11	6,487	*Dichorhavirus monocotyledonae* (RNA 1)	99.55/6	63.75/95	
OQ866130	18	3,900	*Dichorhavirus monocotyledonae* (RNA 1)	66.99/37	54/34	
			Secoviridae			
OQ866134	9	6,511	*Nepovirus monocotyledonae* (RNA1)	69.76/26	54.06/82	*Nepovirus*
OQ866135	19	5,952	*Nepovirus monocotyledonae* (RNA 2)	0/0	43/72	
*****	166	5,839	*Sadwavirus-like* (RNA 1)	67.70/34		*Sadwavirus*
*****	314	7 035	*Sadwavirus-like* (RNA 1)	99.51/17	54.41/99	
*****	897	2304	*Sadwavirus-like* (RNA 1)	66.67/48	57.87/93	
			Solemoviridae			
OQ866138	1	5,763	*Polerovirus monocotyledonae* 1	76.54/70	75.83/52	*Polerovirus*

OQ866139	313	5,566	*Polerovirus monocotyledonae* 2	78.65/83	76.15/55	
OQ866140	565	3,335	*Polerovirus monocotyledonae* 2	79.74/80	66.61/49	
			Totiviridae			
OQ866136	322	5,193	*Totivirus monocotyledonae1*	66.50/37	51.68/96	Unclassified
OQ866137	1405	4,345	*Totivirus monocotyledonae 2*	99.03/11	57.01/87	*Totivirus*

* The sequence was not submitted to NCBI nucleotide data bank.

The lengths of the recovered viral contig sequences agree to the genomic information available for each viral family description on the ICTV 2022 Release. Detailed description of the contig sequence association with specific virus families is presented as follows.

Four generated contigs corresponded to novel negative sense RNA virus members of the *Rhabdoviridae* family (**Figure 1**). A phylogenetic tree was constructed using GTR+F+R3 model (**Figure 1**) using a sequence belonging to *Betanucleorhabdovirus* and another to *Gammanucleorhabdovirus* as outgroups in the phylogenetic tree.

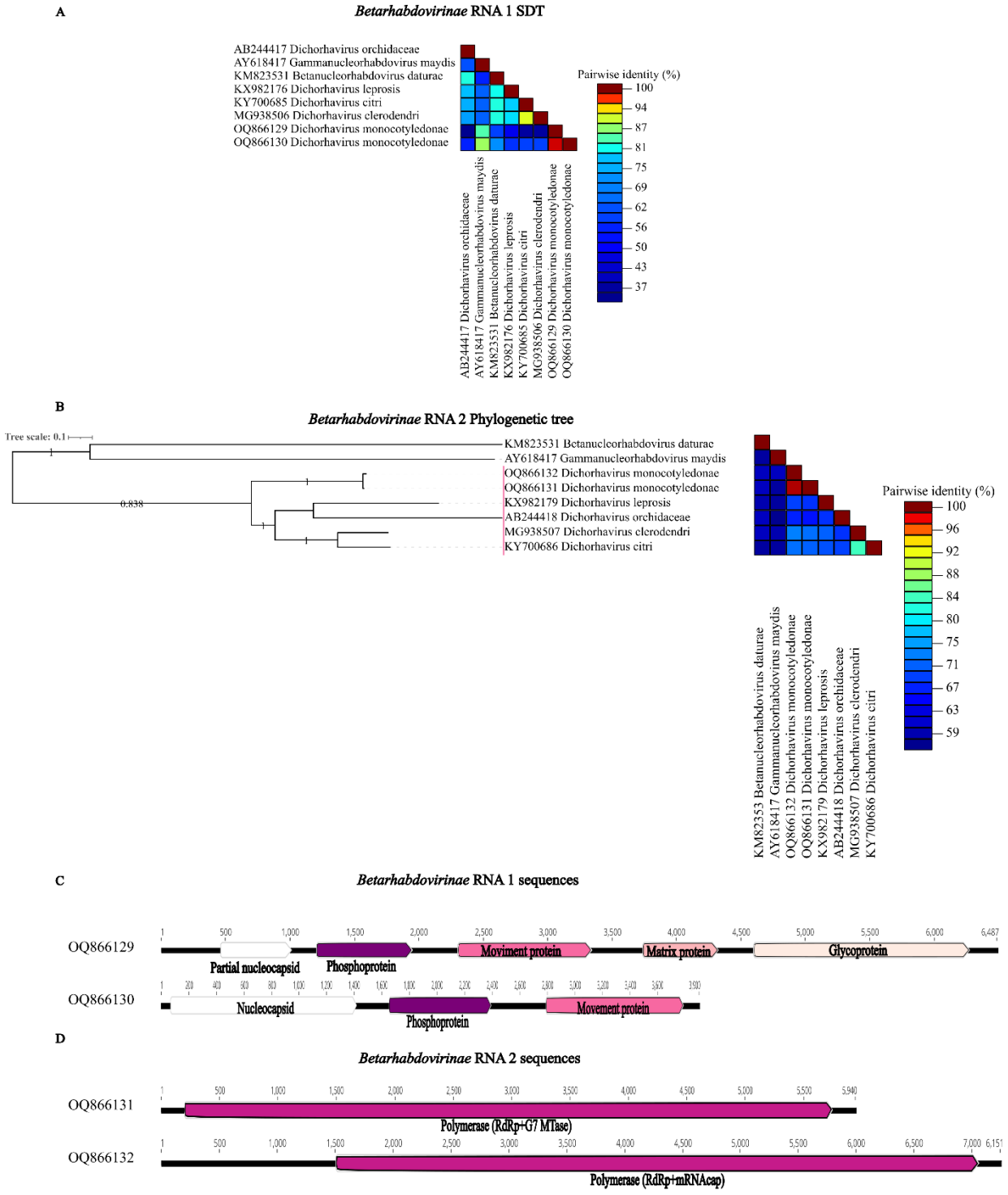

Figure 1. A-D. (**A**) SDT RNA 1 segment sequences comparison matrix. (**B**) Phylogenetic tree, SDT RNA 2 segment sequences comparison matrix. *Betanucleorhabdovirus daturae* (KM82353), and *Gammanucleorhabdovirus maydis* (AY618417) represent the outgroup belonging to the *Betanucleorhabdovirus*, and *Gammanucleorhabdovirus* genera. (**C**) The RNA 1 segment sequences OQ866129, OQ866130. OQ866130 is incomplete. (**D**) The RNA 2 segment sequences OQ866131, OQ866132 of *Dichorhavirus monocotyledonae*.

Nine generated contigs corresponded to new positive sense RNA virus, one was related *Endornaviridae* family (**Figure 2**); five were associated to members of the *Secoviridae* family

(**Figure 3**); the other three viral-like contigs were associated to members of the family *Solemoviridae* (**Figure 4**).

A phylogenetic tree encompassing members of the family *Endornaviridae* was constructed by using the TIM3+F+R3 model (**Figure 2**). All the 12 selected *Endornaviridae* sequences clustered either within the genera *Alphaendornavirus* or *Betaendornavirus*. A *Betaendonavirus* sequence was employed as an outgroup.

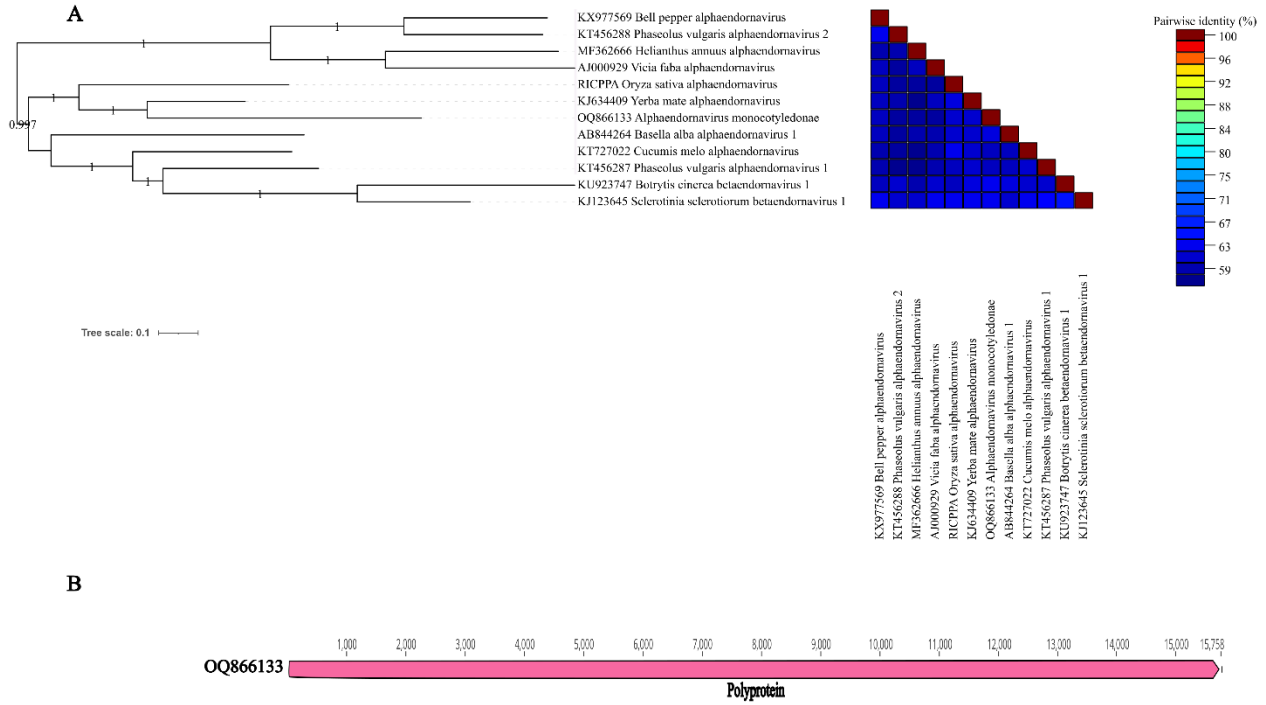

Figure 2. A-B. (**A**) Phylogenetic tree, SDT sequences comparison matrix. *Botrytis cinerea betaendornavirus* (KU923747), and *Sclerotinia sclerotium betaendornavirus* (KJ123645) represent the outgroup belonging to the *Betaendornavirus* genus. (**B**) Sequence and polyprotein of *Alphaendornavirus monocotyledonae* (OQ866133).

A phylogenetic tree involving members of the *Secoviridae* family was constructed by using the TVM+F+R4 model, for the *Secoviridae* RNA 1 genome component, and model TPM3u+F+G4 for the RNA 2 genome component (**Figure 3**). The chosen *Secoviridae* sequences belong to the genera *Fabavirus*, *Nepovirus*, *Sadwavirus*, and *Torradovirus*. The *Fabavirus and Torradovirus* sequences were chosen to compose the outgroups in the phylogenetic tree.

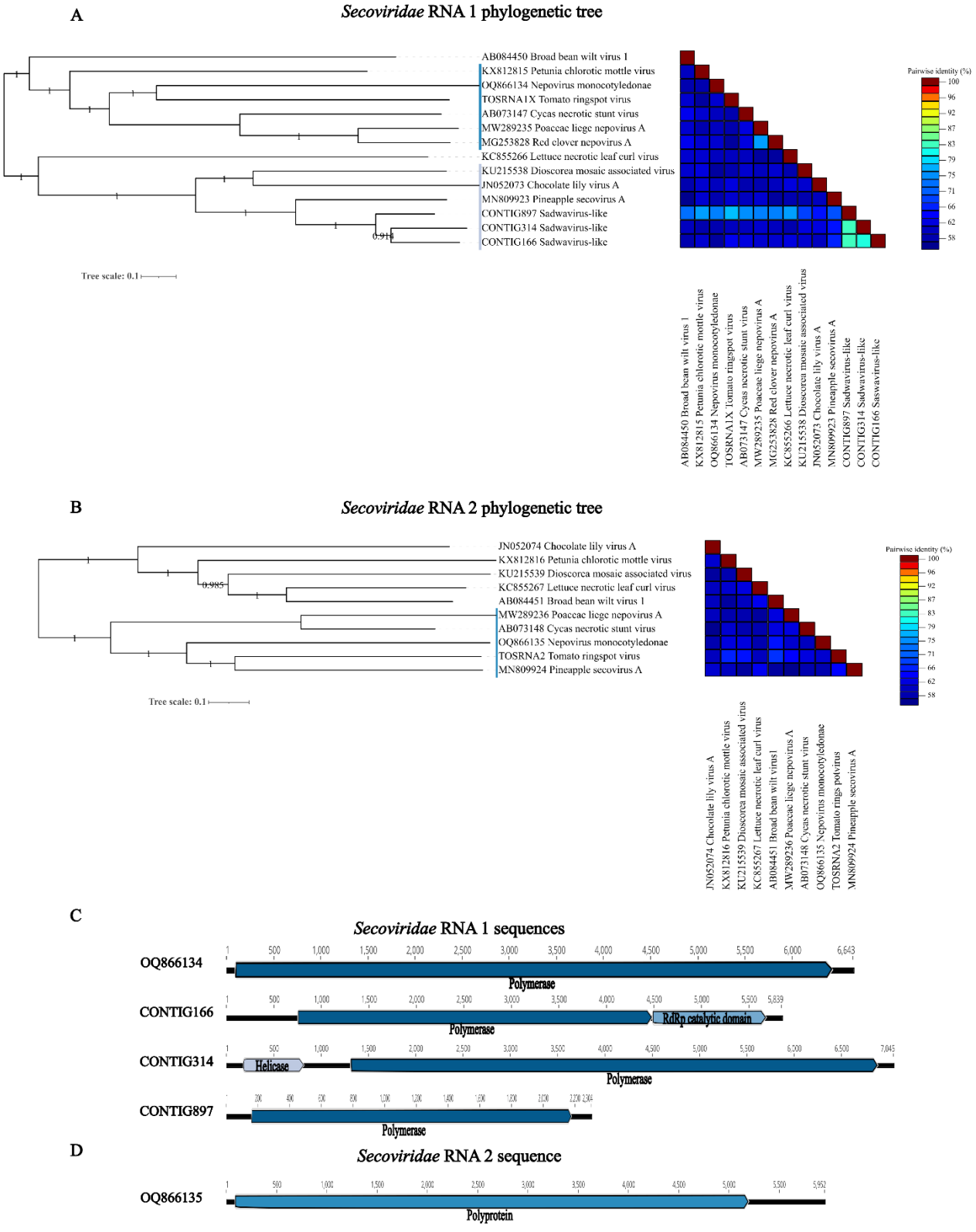

Figure 3. A-D. (**A**) Phylogenetic tree, SDT sequences comparison matrix of the RNA1 segment. (**B**) Phylogenetic tree, SDTsequences comparison matrix of the RNA2 segment. *Broad bean virus* (AB08445), belonging to *Fabavirus* genus, and Lettuce necrotic leaf curl virus (KC855267), belonging to *Torradovirus*, composes the outgroup of both phylogenetic trees. (**C**) Sequence of the new secoviruses RNA1 segment. (**D**) Sequence of the new secovirus RNA2 segment.

A phylogenetic tree of the *Solemoviridae* family was constructed by using the TVMe+I+G4

model (**Figure 4**). The chosen *Solemoviridae* sequences belong to the genera *Enamovirus*, and *Polerovirus*. The *Enamovirus* sequence was employed as the outgroup in the phylogenetic tree.

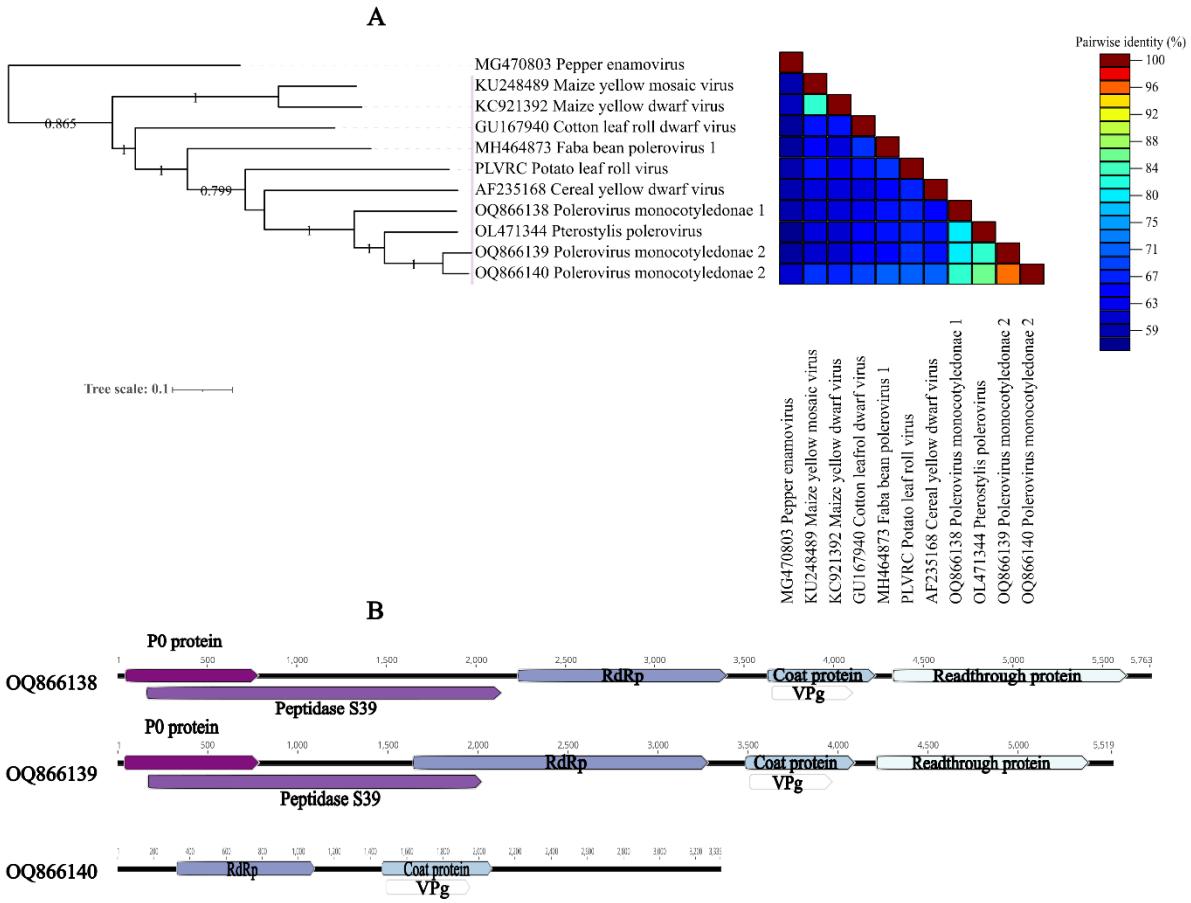

Figure 4. A-B. (A) Phylogenetic tree, SDT sequences comparison. *Pepper enamovirus* (MG470803) represents the outgroup belonging to the *Enamovirus* genus. (B) Sequences of the new poleroviruses: *Polerovirus monocotyledonae* 1 (OQ866138), Polerovirus *monocotyledonae* 2 (OQ866139), and *Polerovirus monocotyledonae* 2 (OQ866140).

Two contigs corresponded to new double-stranded RNA virus of members of the *Totiviridae* family (**Figure 5**). The phylogenetic tree was constructed by using the TIM2+F+G4 model (**Figure 5**). The chosen *Totiviridae* sequences belong to the genera *Giardiavirus*, and *Totivirus*. The *Giardiavirus* sequence was used as the outgroup of this phylogenetic tree.

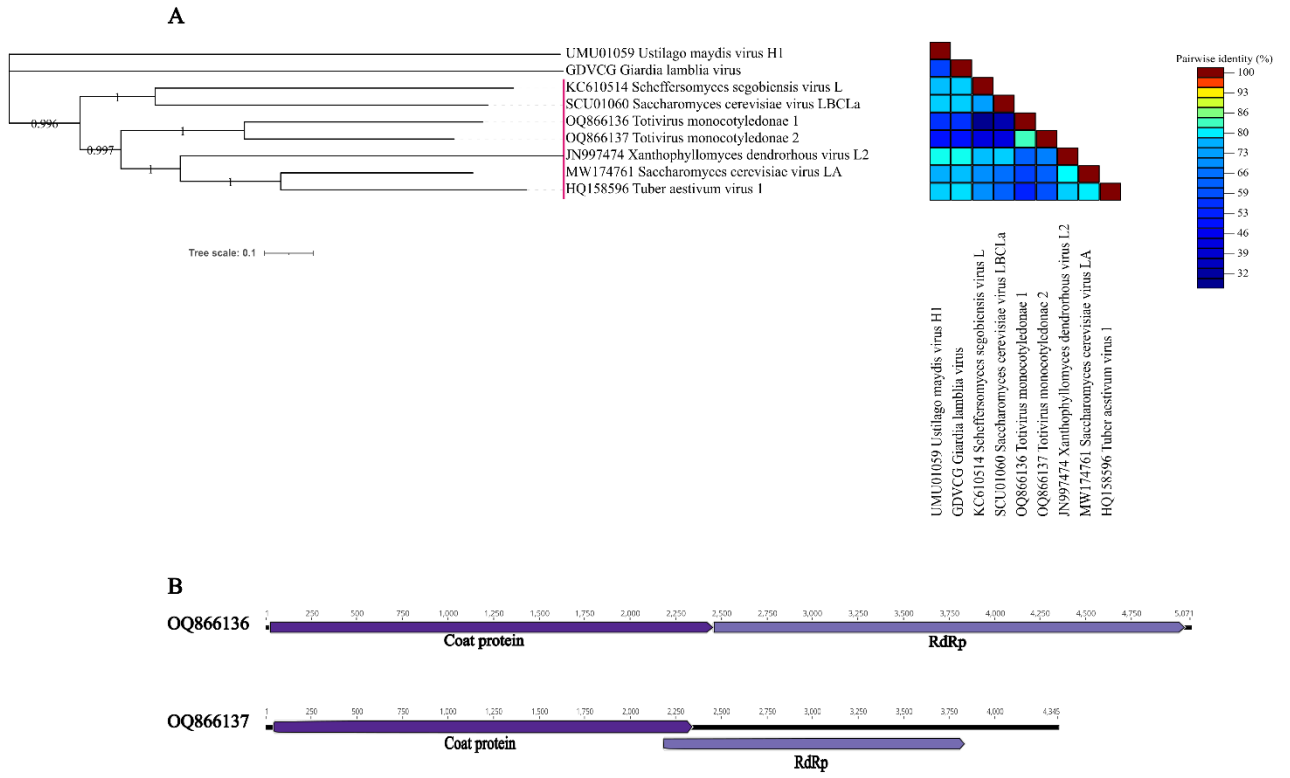

Figure 5. A-B. (**A**) Phylogenetic tree, and SDT sequences comparison matrix. *Giardia lamblia virus* (GDVCF) represents the outgroup belonging to the *Giardiavirus* genus. (**B**) Sequences *Totivirus monocotyledonae* 1 (OQ866136), and *Totivirus monocotyledonae* 2 (OQ866137).

Discussion

After processing the SRA SRR24144734 and in agreement with the criteria established by ICTV, 15 contigs corresponding to novel virus species belonging to the *Endornaviridae*, *Rhabdoviridae*, *Secoviridade*, *Solemoviridae*, and *Totiviridae* families were generated. 12 of the 15 contigs consistently corresponded to seven proposed new virus species, namely, *Alphaendornavirus monocotyledonae* (*Alphaendornavirus*); *Dichorhavirus monocotyledonae* (*Dichorhavirus*); *Nepovirus monocotyledonae* (*Nepovirus*); *Polerovirus monocotyledonae* 1, and *Polerovirus monocotyledonae* (*Polerovirus*); *Totivirus monocotyledonae* 1, and *Totivirus monocotyledonae* 2 (*Totivirus*).

According to the currently established taxonomic criteria to *Alphaendornavirus*, the new genus-related sequence discovered herein (**Figure 2**) should be considered a putative new species since its overall nucleotide sequence identity was below 75% [11]. The suggested name for this

virus is *Alphaendornavirus monocotyledonae*.

According to the established criteria to the *Dichorhavirus* genus, the four new sequences should be considering as novel species. It is noticed that the sequences in the phylogenetic tree are localized inside the *Dichorhavirus* genus. (**Figure 1**). The recognized species demarcation criteria says that the sequences require to share at least two of the following conditions: ***(i)*** minimum nucleotide sequence divergence of 20% in L genes; ***(ii)*** minimum nucleotide sequence divergence of 20% in RNA 1; ***(iii)*** can be distinguished in serological tests; and ***(iv)*** occupy different ecological niches as evidenced by differences in plant hosts and/or arthropod vectors [13, 14]. The requirements ***(i)*** and ***(ii)*** were fulfilled in those four sequences. However, the similarities among the sequences are suggesting that they encompass a single species, with the presence of the RNA 1 and RNA 2 [13, 14]. The suggested name is *Dichorhavirus monocotyledonae*. Furthermore, it is recommended further studies to investigate the biological correlation between the RNA 1 and the RNA 2.

According to the established species criteria to the family *Secoviridae* our results are inconclusive. It was recovered four new RNA 1 sequence and just one RNA 2 (**Figure 3**). In the RNA 1 phylogenetic tree it is noticed that the sequence OQ866134 is localized inside the *Nepovirus* genus, while the contigs 166, 314, and 897 are gathered with the *Sadwavirus* genus (**Figure 3**). The RNA 2 sequence, OQ866135, gathered with the other sequences of *Nepovirus* genus.

The current established criteria by ICTV for *Secoviridae* are: ***(i)*** amino acid sequence of the coat protein (CP) with less than 75% identity; ***(ii)*** conserved Pro-Pol region aa sequence with less than 80% identity; ***(iii)*** differences in antigenic reactions; ***(iv)*** distinct host range; ***(v)*** distinct vector specificity; ***(vi)*** absence of cross-protection; or ***(vii)*** viruses with a bipartite genome, absence of re-assortment between RNA 1 and RNA 2 [18]. It is important to highlight that there is no need to fulfill all the criteria to define a new species [18]. Further analysis with RDP 4 to verify reassortments among the sequences was carried out, for RNA 1 and RNA 2. However, no recombination was detected. Therefore, it was not possible to conclude that the three viral-like

sequences are bona fide new species of the genus *Sadwavirus*, given the absence of the RNA 2. Notwithstanding, we could confirm the identification of a new species of the genus *Nepovirus* for which we propose the tentative name of *Nepovirus monocotyledonae*. It is suggested to conduct additional biological tests to fulfill more criteria of the genus *Sadwavirus*.

The species demarcation criteria of genus *Polerovirus* include: ***(i)*** differences in host range; ***(ii)*** absence of cross-protection relationships; ***(iii)*** differences in serological specificity; and ***(iv)*** differences in amino acid sequence identity of any gene product superior to 10% [21]. Two sequences characterized herein satisfied at least two of these prerequisites. Therefore, according to these established criteria to the *Polerovirus* genus, we detected three new sequences that should be classified as two putative new species: *Polerovirus monocotyledonae* 1 and *Polerovirus monocotyledonae* 2 (**Figure 4**). Biological tests need to be performed to provide information of the other criteria.

According to the established criteria to the genus *Totivirus* genus, the two viral-like sequences should be considering two putative new species. The only criterium required for species demarcation in the genus is the detection in a distinct host species. Also, the results of SDT analysis and phylogenetic tree revealed that the sequences are divergent and should be considered distinct species. The suggested names for the viruses are *Totivirus monocotyledonae* 1 and *Totivirus monocotyledonae* 2.

Conclusion

In conclusion, 15 new contig sequences of viruses with phylogenetic relationships to members of the families *Endornaviridae*, *Rhabdoviridae*, *Secoviridade*, *Solemoviridae*, and *Totiviridae* were detected in the metatranscriptomic data of orchids and bromeliads with 12 of them agreeing with the criteria established by ICTV. Seven new viruses were discovered with HTS analysis. The *Alphaendornavirus monocotyledonae* (*Alphaendornavirus*); *Dichorhavirus monocotyledonae* (*Dichorhavirus*); *Nepovirus monocotyledonae* (*Nepovirus*); *Polerovirus monocotyledonae* 1, and *Polerovirus monocotyledonae* (*Polerovirus*); *Totivirus monocotyledonae* 1,

and *Totivirus monocotyledonae* 2 (*Totivirus*) are the new species proposed in this work. As a next step, it is suggested the primer design, identification of individual sample, and the biological tests of infection. The biological tests are important for establishing the species in the genus *Sadwavirus*. With the given results will be possible measure the impact of those new viruses in the horticulture of bromeliads and orchids.

Author Contributions: Conceptualization.; methodology; writing; funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The manuscript is an original work and has not been published or is under consideration for publication in another journal. The study complies with current ethical considerations. The authors confirm that all the listed authors have participated actively in the study and have seen and approved the submitted manuscript. The authors do not have any possible conflict of interest.

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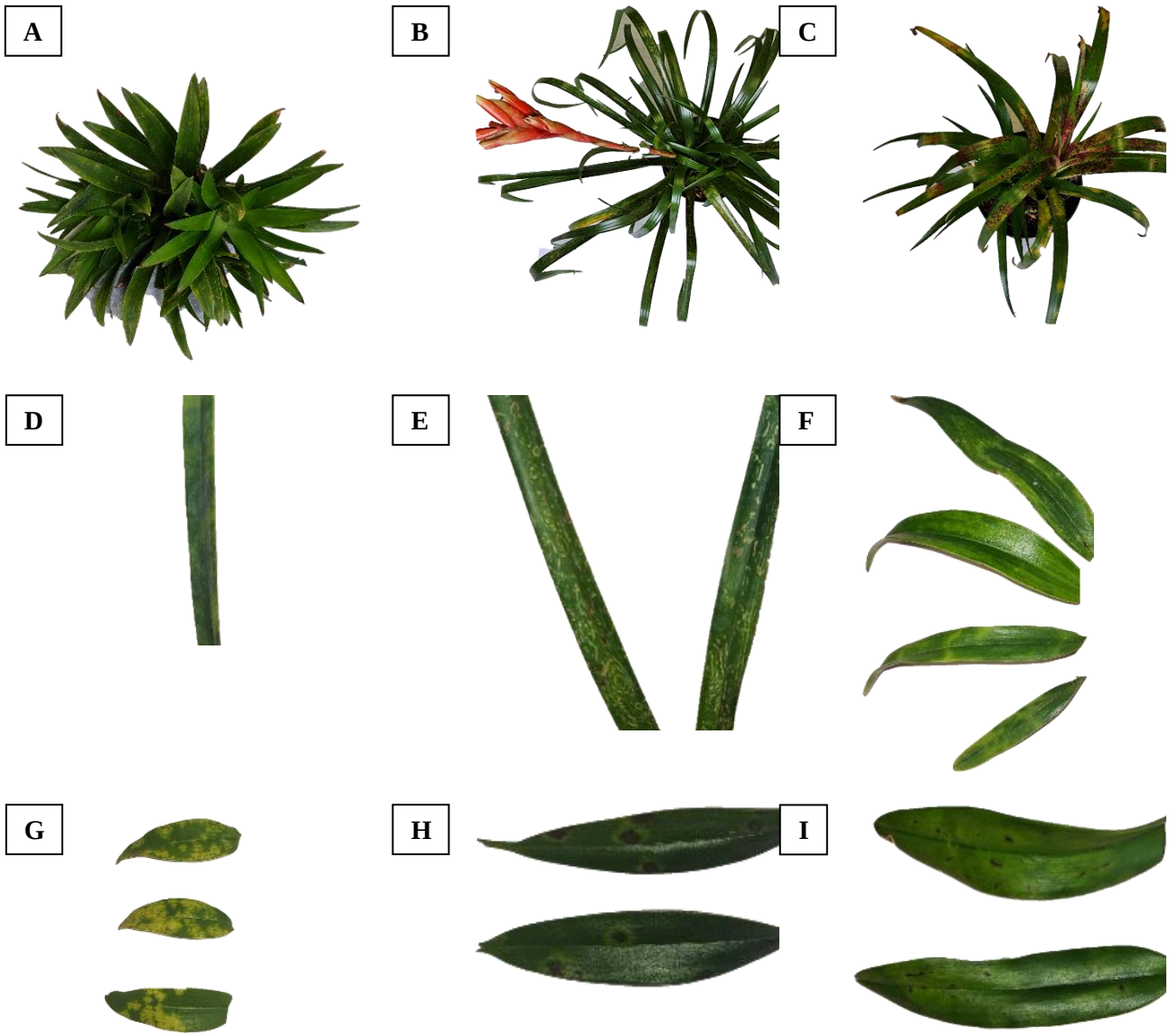

Supplementary Figure 1. A-I. Foliar samples with virus-like symptoms. A-C. Samples from La Bromélia (Núcleo Rural Taquara, Brasília–DF/Brazil). (**A**) Chlorotic spot in *Guzmania* x *Theresa*. (**B**) Chlorotic spot, and mosaic in *Vriesea correia-araujoi*. (**C**) Chlorotic spot, and ringspot in *Vriesea correia-araujoi*. D-F. Samples from Orquidário Colorado (Lago Oeste, Brasília–Df/Brazil). (**D**) Mottle, and yellowing in *Encyclia* sp. (**E**) Ringspot, mottle, yellowing, and necrotic spot in *Encyclia* sp. (**F**) Chlorotic mottle in *Epidendrum* sp. G-I. Samples from Private natural heritage reserve Vagafogo (Pirenópolis–GO/Brazil). (**G**) Chlorosis, mosaic, and black ringspot in *Dendrobium* sp. (**H**) Chlorotic mottle, black ringspot, and necrosis in *Dendrobium* sp. (**I**) Chlorotic mottle, and necrotic spot in *Cattleya* sp.

Supplementary Table 1. Date of samples collected and used for High throughput sequencing (HTS) with identification of local, sample ID, host by genus or species, observed symptoms in the leaves.

Local	Sample ID	Host (Genus or species)	Symptons
La Bromélia (Núcleo Rural Taquara, Brasília–DF/Brazil)	LB01	*Guzmania minor*	Mottle, and chlorotic spot
	LB03	*Guzmania* x *Theresa*	Chlorotic spot
	LB04	*Lemeltonia* sp.	Chlorotic spot, and mottle
	LB05	*Vriesea correia-araujoi*	Chlorotic spot, and mosaic
	LB06	*Vriesea correia-araujoi*	Chlorotic spot, and ringspot
	LB07	*Vriesea correia-araujoi*	Chlorotic filiform spot, and ringspot
	LB09	*Vriesea taritubensis*	Necrotic spot, yellowing
	LB08	*Tillandsia duratii*	Leaf curl and tuning
	LB11	*Guzmania lingulata*	Chlorosis, mottle, and ringspot
	LB12	*Guzmania lingulata*	Chlorosis, mottle, and ringspot
	LB14	*Vriesea taritubensis*	Mottle and yellowing
	LB15	*Vriesea taritubensis*	Chlorotic spot, mottle
	LB16	*Tillandsia flabellata*	Chlorotic spot, mottle, and necrotic spot
	LB17	*Wallisia cyanea*	Coalescent chlorotic spot, and mottle
Orquidário Colorado (Lago Oeste, Brasília–Df/Brazil)	OC01	*Cattleya labiata*	Necrotic spot
	OC02	*Cattleya* sp. (hybrid)	Necrotic spot
	OC03	*Encyclia* sp.	Mottle, yellowing, and necrotic spot
	OC04	*Encyclia* sp.	Ringspot, mottle, yellowing, and necrotic spot
	OC05	*Cattleya* sp.	Necrotic spot, and leaf curling
	OC06	*Dinema* sp.	Tanning, and yellowing
	OC07	*Epidendrum* sp.	Mottle
	OC08	*Epidendrum* sp.	Mottle, and yellowing
	OC09	*Phalaenopsis* sp. (hybrid)	Leaf curling, yellowing, and necrotic spot
	OC10	*Laelia* sp.	Leaf curling, and necrotic spot
	OC11	*Epidendrum* sp.	Chlorotic mottle
	OC12	*Phalaenopsis* sp. (hybrid)	Necrotic spot, and rugose texture
	OC13	*Dendrobium* sp.	Chlorotic mottle, and yellowing

Private natural heritage reserve Vagafogo (Pirenópolis–GO/Brazil)	OC14	*Epidendrum* sp.	Ringspot
	OC15	*Trichoglottis* sp.	Chlorotic spot
	OC16	*Dendrobium* sp.	Mottle, and rugose texture
	OC17	*Cattleya* sp.	Necrotic spot
	OC18	*Dendrobium* sp.	Chlorotic spot
	OC19	*Coelogyne* sp.	Mottle
	OC20	*Encyclia* sp.	Chlorotic mottle
	OC21	*Encyclia* sp.	Mottle
	OP01	*Coelogyne* sp.	Black ringspot
	OP02	*Dendrobium* sp.	Chlorotic mottle, and necrotic spot
	OP03	*Dendrobium* sp.	Chlorosis, mosaic, and black ringspot
	OP04	*Coelogyne* sp.	Necrotic spot with chlorotic halo
	OP05	*Oncidium* sp.	Chlorotic ringspot and yellowing
	OP06	*Phalaenopsis* sp. (hybrid)	Chlorotic spot, and necrotic spot with chlorotic halo
	OP07	*Dendrobium* sp.	Chlorotic mottle, black ringspot
	OP08	*Dendrochilum* sp.	Black ringspot
	OP09	*Spathoglottis* sp.	Chlorotic mottle
	OP10	*Vanda* sp. (hybrid)	Chlorotic mottle, and necrotic spot
	OP11	*Phalaenopsis* sp. (hybrid)	Chlorotic mottle, leaf curling and necrotic spot
	OP12	*Oncidium* sp.	Chlorotic ringspot, mottle, and necrotic spot
	OP13	*Cattleya* sp.	Chlorotic mottle, and necrotic spot
	OP14	*Sophronitis cernua*	Chlorotic mottle
	OP15	*Oncidium* sp.	Chlorotic mottle

CHAPTER 3

Article

Deciphering ssDNA-virus diversity associated to ornamental plants via High-throughput sequencing (HTS).

Deciphering ssDNA-virus diversity associated to ornamental plants via High-throughput sequencing (HTS).

Juliana Gabrielle Isidorio da Silva[1], Maria Esther de Noronha Fonseca[2], Leonardo Silva Boiteux[2], Tatsuya Nagata[1], Rita de Cassia Pereira Carvalho[1]

[1]Departamento de Fitopatologia, Universidade de Brasília (UnB), Brasília–DF, Brazil.

[2]Embrapa Vegetable Crops, National Center for Vegetable Crops Research (CNPH), Brasília–DF, Brazil.

Abstract

The ornamental plants classified in Bromeliaceae and Orchidaceae families are economically important for landscaping in Brazil. Viruses occurring in both families can cause significant yield and quality losses. Herein, high throughput sequencing (HTS) was employed to generate one DNA virome of bromeliads and orchids exhibiting virus-like symptoms in nurseries and natural areas in Brasília–DF. Two previously characterized DNA-A components of begomoviruses and two new begomovirus-like isolates were detected. Despite these new viruses in *Geminiviridae* family being recovered, our study revealed a relatively low diversity of single-stranded DNA viruses associated with these botanic families. Specific primers for two putative novel isolates were designed and used in PCR assays. The partial sequences of the corresponding amplicons were obtained by Sanger dideoxy sequencing.

Keywords: Metagenome, Orchids, Bromeliads, Virome, Begomoviruses

Introduction

A wide array of members of the families Orchidaceae and Bromeliaceae are native to tropical and subtropical areas [1, 2, 3]. Orchidaceae, comprising 800 genera and nearly 35,000 species described worldwide, is considered the second largest family of flowering plants, just behind Asteraceae [2, 3]. The Bromeliaceae family has the second largest number of epiphytic

species [1]. Plants and plant products of both families are widely used as ornamentals, medicine, food, essential oils, and essences [1, 2, 3]. Compounds extracted from bromeliads and orchids plants are used in pharmaceutical industry [1, 2, 3]. Some compounds, as ethanolic extract of *Bromelia balansae*, present potential for use in biopharmaceutical sector [4]. Although their economic importance, there are few virome studies in these plant species.

Viruses are considered the most abundant biological entities in nature [5, 6, 7]. With the development of High Throughput Sequencing (HTS) platforms and bioinformatic analysis, the exploration of new viruses occurring in different environments and ecological niches was facilitated [5, 7, 8]. It is estimated that only 1% of viral diversity worldwide is currently known [5]. Virome of ornamental plants comprises one of the largest unexplored areas of study [5, 7]. This lack of information also includes members of the Orchidaceae and Bromeliaceae families.

Viruses with single-stranded circular DNA (ssDNA) of the family *Geminiviridae* are classified into the order *Geplafuvirales*. The *Geminiviridae* is one of the most abundant families of plant viruses [9]. More than 520 species were characterized in this family, being distributed in 14 genera currently recognized by the International Committee on Taxonomy Viruses (ICTV): *Becurtovirus*, *Begomovirus*, *Capulavirus*, *Citlodavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Maldovirus, Mastrevirus, Mulcrilevirus, Opunvirus, Topilevirus*, *Topocuvirus,* and *Turnucurtovirus* [10]. The genus with the largest number of species is *Begomovirus,* currently composed of 445 species [10].

Herein, high throughput sequencing (HTS) was employed to generate one DNA virome of bromeliads and orchids exhibiting virus-like symptoms in nurseries and natural areas in Brasília–DF. This is a pioneering study on viral diversity in bromeliads and orchids commercialized in Brazilian central plateau.

Material and Methods

Fifty leaf samples corresponding to 16 bromeliads and 34 orchids were collected in three areas in Central Brazil. The samples were collected from plants displayed virus-like symptoms such

as chlorosis, mosaic, mottle, necrotic spots, and ring spots (**Figures 1**, **2**, and **3**). The collection sites were in Brasília–DF and Pirenópolis–GO. The Brasília–DF places were "La Bromelia", a bromeliad glasshouse in Núcleo Rural Taquara, and "Orquidário Colorado", an orchidarium in Lago Oeste. The samples were collected between January and February 2022. The collection site in Pirenópolis–GO was the Private Natural Heritage Reserve "Vagafogo". The collection on this site was performed in March 2022. The symptoms were catalogued and photographed (**Table 1**, **Figures 1**, **2**, and **3**). Later, they were separately cleaned, cut, weighted (1 g each in triplicate subdivisions of the samples), and stored at - 40°C for DNA extraction.

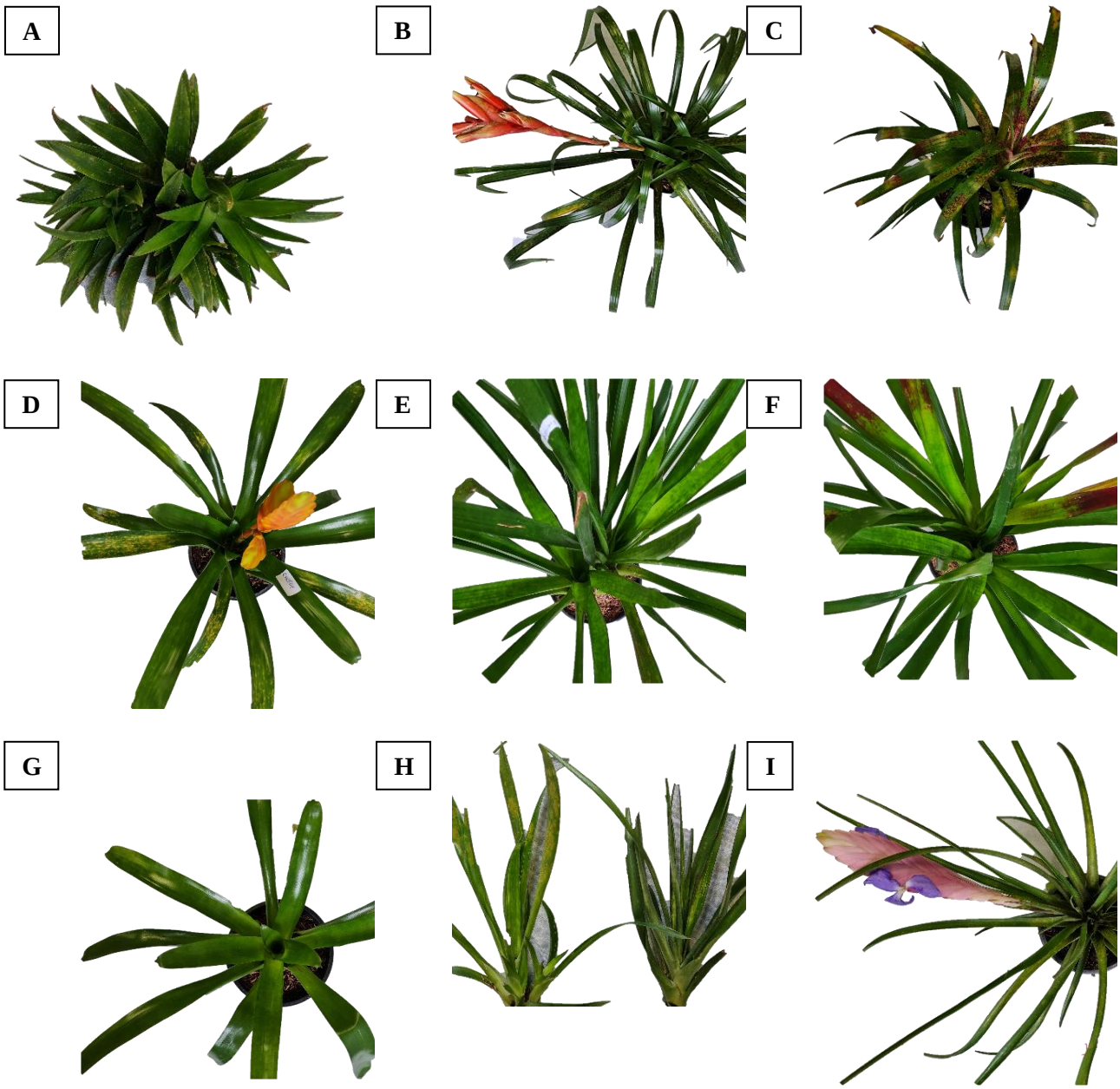

Figure 1. A-I. Leaves displayed virus-like symptoms from plants collected in La Bromélia (Núcleo Rural Taquara, Brasília–DF/Brazil). (**A**) Chlorotic spot in *Guzmania* x *Theresa*. (**B**) Chlorotic spot, and mosaic in *Vriesea correia-araujoi*. (**C**) Chlorotic spot, and ringspot in *Vriesea correia-araujoi*. (**D**) Necrotic spot, yellowing in *Vriesea taritubensis*. (**E**) Chlorosis, mottle, and ringspot in *Guzmania lingulata*. (**F**) Chlorosis, mottle, and ringspot in *Guzmania lingulata*. (**G**) Chlorotic spot, mottle in *Vriesea taritubensis*. (**H**) Chlorotic spot, mottle, and necrotic spot in *Tillandsia flabellata*. (**I**) Coalescent chlorotic spot, and mottle in *Wallisia cyanea*.

Figure 2. A-H. Leaves displayed virus-like symptoms from plants collected in Orquidário Colorado (Lago Oeste, Brasília–Df/Brazil). (**A**) Mottle, and yellowing in *Encyclia* sp. (**B**) Ringspot, mottle, yellowing, and necrotic spot in *Encyclia* sp. (**C**) Mottle in *Epidendrum* sp. (**D**) Chlorotic mottle in *Epidendrum* sp. (**E**) Chlorotic mottle, and yellowing in *Dendrobium* sp. (**F**) Mottle in *Trichoglottis* sp. (**G**) Mottle and yellowing in *Encyclia* sp. (**H**) Mottle in *Encyclia* sp.

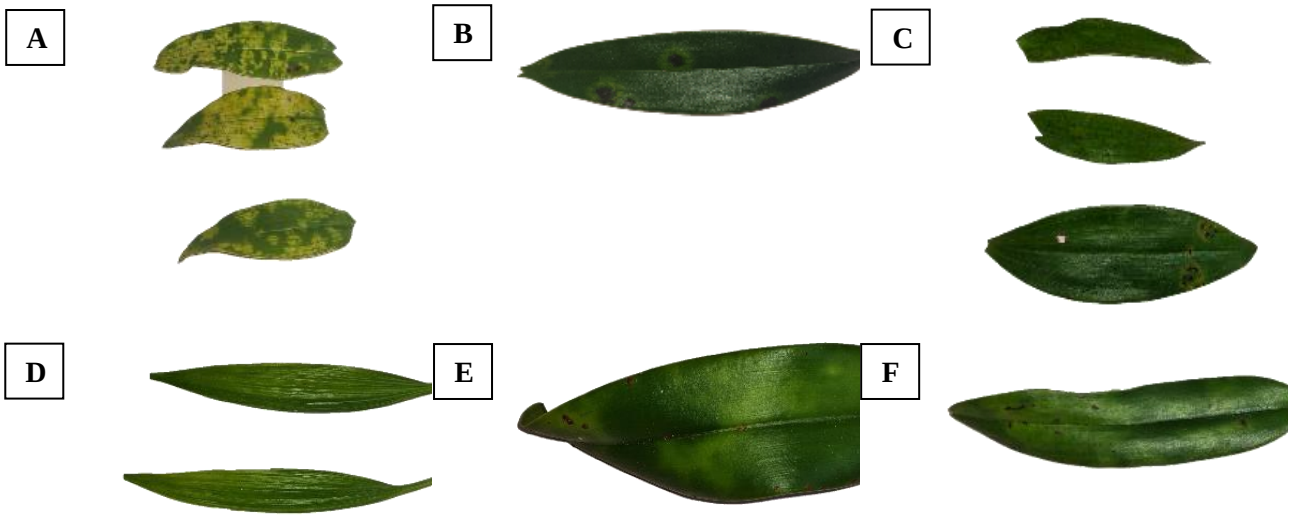

Figure 3. A-F. Leaves displayed virus-like symptoms from plants collected in Private natural heritage reserve Vagafogo (Pirenópolis–GO/Brazil). (**A**) Chlorosis, mosaic, and black ringspot in *Dendrobium* sp. (**B**) Necrotic spot with chlorotic halo in *Coelogyne* sp. (**C**) Chlorotic mottle, black ringspot, and necrosis in *Dendrobium* sp. (**D**) Chlorotic mottle in *Spathoglottis* sp. (**E**) Chlorotic mottle, leaf curling, and necrotic spot in *Phalaenopsis* sp. (hybrid). (**F**) Chlorotic mottle, and necrotic spot in *Cattleya* sp.

Table 1: Date of samples collected and used for High throughput sequencing (HTS) with identification of local, sample ID, host by genus or species, observed symptoms in the leaves, and positive (+) or negative (-) results for viral infection.

Local	Sample ID	Host (Genus or species)	Symptons	Results
La Bromélia (Núcleo Rural Taquara, Brasília–DF/Brazil)	LB01	*Guzmania minor*	Mottle, and chlorotic spot	-
	LB02	*Vriesea garlipiana*	Mosaic	-
	LB03	*Guzmania* x *Theresa*	Chlorotic spot	-
	LB04	*Lemeltonia* sp.	Chlorotic spot, and mottle	-
	LB05	*Vriesea correia-araujoi*	Chlorotic spot, and mosaic	-
	LB06	*Vriesea correia-araujoi*	Chlorotic spot, and ringspot	-
	LB07	*Vriesea correia-araujoi*	Chlorotic filiform spot, and ringspot	-
	LB09	*Vriesea taritubensis*	Necrotic spot, yellowing	-
	LB10	*Vriesea erythrodactylon*	Chlorotic spot	-
	LB11	*Guzmania lingulata*	Chlorosis, mottle, and ringspot	-
	LB12	*Guzmania lingulata*	Chlorosis, mottle, and ringspot	-
	LB13	*Vriesea erythrodactylon*	Mottle, and yellowing	-
	LB15	*Vriesea taritubensis*	Chlorotic spot, mottle	-
	LB16	*Tillandsia flabellata*	Chlorotic spot, mottle, and necrotic spot	-
	LB17	*Wallisia cyanea*	Coalescent chlorotic spot, and mottle	-
	LB18	*Tillandsia usneoides*	Necrosis	-
Orquidário Colorado (Lago Oeste, Brasília–Df/Brazil)	OC01	*Cattleya labiata*	Necrotic spot	-
	OC02	*Cattleya* sp. (hybrid)	Necrotic spot	-
	OC03	*Encyclia* sp.	Mottle, yellowing, and necrotic spot	-
	OC04	*Encyclia* sp.	Ringspot, mottle, yellowing, and necrotic spot	-
	OC05	*Cattleya* sp.	Necrotic spot, and leaf curling	-
	OC06	*Dinema* sp.	Tanning, and yellowing	-
	OC07	*Epidendrum* sp.	Mottle	-
	OC08	*Epidendrum* sp.	Mottle, and yellowing	-
	OC09	*Phalaenopsis* sp. (hybrid)	Leaf curling, yellowing, and necrotic spot	-
	OC10	*Laelia* sp.	Leaf curling, and necrotic spot	-
	OC11	*Epidendrum* sp.	Chlorotic mottle	-
	OC12	*Phalaenopsis* sp. (hybrid)	Necrotic spot, and rugose texture	-

	OC13	*Dendrobium* sp.	Chlorotic mottle, and yellowing	-
	OC14	*Epidendrum* sp.	Ringspot	-
	OC15	*Trichoglottis* sp.	Chlorotic spot	-
	OC16	*Dendrobium* sp.	Mottle, and rugose texture	-
	OC17	*Cattleya* sp.	Necrotic spot	-
	OC20	*Encyclia* sp.	Chlorotic mottle	-
	OC21	*Encyclia* sp.	Mottle	-
	OP01	*Coelogyne* sp.	Black ringspot	-
	OP02	*Dendrobium* sp.	Chlorotic mottle, and necrotic spot	-
	OP03	*Dendrobium* sp.	Chlorosis, mosaic, and black ringspot	-
	OP04	*Coelogyne* sp.	Necrotic spot with chlorotic halo	-
Private	OP05	*Oncidium* sp.	Chlorotic ringspot and yellowing	-
natural	OP06	*Phalaenopsis* sp. (hybrid)	Chlorotic spot, and necrotic spot with chlorotic halo	-
heritage	OP07	*Dendrobium* sp.	Chlorotic mottle, black ringspot	+
reserve	OP08	*Dendrochilum* sp.	Black ringspot	-
Vagafogo	OP09	*Spathoglottis* sp.	Chlorotic mottle	+
(Pirenópolis–	OP10	*Vanda* sp. (hybrid)	Chlorotic mottle, and necrotic spot	-
GO/Brazil)	OP11	*Phalaenopsis* sp. (hybrid)	Chlorotic mottle, leaf curling and necrotic spot	-
	OP12	*Oncidium* sp.	Chlorotic ringspot, mottle, and necrotic spot	-
	OP13	*Cattleya* sp.	Chlorotic mottle, and necrotic spot	-
	OP14	*Sophronitis cernua*	Chlorotic mottle	-
	OP15	*Oncidium* sp.	Chlorotic mottle	-

Table 2: Two sets of specific designed primers for Spathoglottis-associated mottle virus 1 and Spathoglottis-associated mottle virus 2. The primer code, NCBI seq ID, contig number, primer sense, viruses' name, primer sequence, primer length (n), and temperature of melting (in °C) are detailed in the table below.

Code	Seq ID	Contig	Sense	Name	Sequence	n	TM (°C)
SOV1F	OQ79197	6377	Forward	Spathoglottis-associated mottle virus 1	CATTCATATTGATTGCCGTTGGTCC	25	59.5
SOV1R			Reverse		GTAATCATGTTCAAATACGAGGCAAC	25	
SOV2F	OQ79198	7686	Forward	Spathoglottis-associated mottle virus 2	CCCGTCACGTTTGCTCCAGTT	21	61.2
SOV2R			Reverse		GGTAACGCCGAACATTCTCAA	21	

The total DNA was extracted in the individual samples using the modified CTAB protocol with organic solvents [11] and stored at -20 °C. Aliquots of the purified DNA were used as templates for Rolling Circle Amplification (RCA) assays aiming to enrich the samples with circular DNAs [12]. Two aliquot was used to verify the quality of the purification in NanoDrop™ One/One^{C} and other in electrophoresis gel.

The RCA-generated DNAs of 50 samples were pooled and sent for sequencing in a high throughput sequencing (HTS) platform (Illumina NovaSeq 6000) at Agrega. The raw data of the pool was analyzed following a previously validated workflow [13]: **(*i*)** *de novo* assembly of the reads using the CLC Genomics Workbench 20.0 program; **(*ii*)** analysis of the contigs using the BLASTn algorithm, aiming to verify viral hits; **(*iii*)** contigs with viral hits were assembled using the 'Map to reference' tool in Geneious R 11.1 program; **(*iv*)** contigs were later submitted to the BLASTn tool to confirm their identity to viruses, ***(v)*** primers were designed for new viruses contig sequences.

A set of specific primers was designed (using Geneious R 11.1 program) to target genomic segments of putative new viruses found on HTS. A pair of specific primers ('SOV1F' 5'–CAT TCA TAT TGA TTG CCG TTG GTC C–3'/ 'SOV1R' 5'–GTA ATC ATG TTC AAA TAC GAG GCA AC–3') was designed to generate an amplicon with expected size of 2929 pb (GenBank accession OQ79197). A pair of specific primers ('SOV2F' 5'–CCC GTC ACG TTT GCT CCA GTT–3'/ 'SOV2R' 5'–GGT AAC GCC GAA CAT TCT CAA–3') was designed to generate an amplicon with the expected size of 2929 pb (GenBank accession OQ79198) (**Table 2**). The results and further analysis are fully described in **Chapter 5.**

All the individual RCA samples were also submitted to PCR assays targeting the Begomovirus DNA–A component with the degenerated primer pairs 'PALv1978'/ 'PARc496' [14] and 'AF'/'AR' [15] to verify potential infections by members of this group of pathogens. Amplicons of the positive samples were subsequently subjected to Sanger dideoxy sequencing and

the derived sequences were trimmed employing the Geneious program. The results and further analysis are fully described in **Chapter 4**.

Results and Discussion

As result of the Illumina NovaSeq 6000 sequencing, reads were obtained from a pool of 50 ornamental (16 bromeliads and 34 orchids) leaf samples with begomovirus–like symptoms. After assembly, in the CLC Genomics Workbench 11 program, 163,808 contigs were obtained. Most of these contigs were composed of previously characterized begomoviruses such as: tomato severe rugose virus (ToSRV), and tomato chlorotic mottle virus (ToCMoV). However, two full–length DNA–A contigs displayed identity levels of less than 91%, consistent with these viruses are representing new species under the current taxonomic criterion of the genus Begomovirus [16]. The DNA–A component of the putative new species OQ79197, Spathoglottis-associated mottle virus 1, (578 reads) had the highest identity of 77.69% with *Lisianthus enation leaf curl virus*. Whereas the new species OQ79198, Spathoglottis-associated mottle virus 2, (300 reads) had highest identity of 77.77% with *Passionfruit leaf curl virus*. Boths new viruses were detected in the RCA pool.

To detect begomoviruses DNA–B, PCR assays were carried out using degenerate primers targeting the DNA–B component [17]. The results were positive in the sample infected with the DNA–A component. The Illumina results also detected the DNA–B component of ToSRV (99% identity). Only the ToSRV components were recovered, with no detection of ToCMoV.

The complete DNA–A sequences of Spathoglottis-associated mottle virus 1 and Spathoglottis-associated mottle virus 2 were confirmed via Sanger dideoxy sequencing employing primer pair specific for each virus. Sequencing reactions were carried out at the Genomic Analysis Lab (National Center for Vegetable Crops Research – CNPH) using the BigDye® Terminator Cycle Sequencing Ready Reaction Kit version 3.1 protocol (Applied Biosystems, São Paulo–SP, Brazil).

It is recommended further investigation with biological tests in the host and possible vectors. The results are novel for Orchidaceae family and should explore the impact in the cultivation and other host plants.

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Compliance with ethical standards

Conflicts of interest: The authors declare no conflict of interest.

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

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CHAPTER 4

Disease Note | To be submitted as a Disease Note to *Plant Disease*

First worldwide report of a mixed natural infection of *Dendrobium* orchids by tomato severe rugose virus (ToSRV) and tomato chlorotic mottle virus (ToCMoV).

First worldwide report of a mixed natural infection of *Dendrobium* orchids by *Tomato severe rugose* virus (ToSRV) and *Tomato chlorotic mottle virus* (ToCMoV)

Juliana Gabrielle Isidorio da Silva[1], Felipe Fochat da Silva Melo[1], Luciane de Nazaré Almeida dos Reis[1], Maria Esther de Noronha Fonseca[2], Leonardo Silva Boiteux[2], Tatsuya Nagata[1], Rita de Cassia Pereira-Carvalho[1]

[1] Departamento de Fitopatologia, Universidade de Brasília (UnB), Brasília–DF, Brazil.

[2] National Center for Vegetable Crops Research (CNPH), Embrapa Vegetable Crops (Hortaliças), Brasília–DF, Brazil.

Dendrobium is one of the largest genera inside the *Orchidaceae* family, with more than 1547 species (Wang 2021). *Dendrobium* species are largely used as ornamental plants and in the pharma industry (Wang 2021). Seven foliar *Dendrobium* samples with begomovirus-like symptoms were collected in the 'Vagafogo' Private Natural Heritage Reserve in Central Brazil, during field surveys of orchid viruses in March of 2022. Mottle patterns and necrotic spots were observed in the foliar tissue (Figure 1). Total DNA was purified (Boiteux et al. 1999) and employed as the template in rolling circle amplification (RCA) assays for the enrichment of circular DNAs of viral origin (Inoue-Nagata et al. 2004). PCR assays targeting the DNA–A component with the degenerated primer pairs 'PALv1978'/ 'PARc496' (Rojas et al. 1993) and 'AF'/'AR' (Ha et al. 2006) were initially employed to confirm the infection by begomovirus(es). The amplicons were subsequently subjected to Sanger dideoxy sequencing and the derived sequences were trimmed employing the Geneious program. RCA samples were also sent to Illumina NovaSeq 6000 sequencing. *De novo* assembly was performed with the Illumina NovaSeq 6000-derived raw data using CLC Genomics Workbench v. 11. Whole viral genomes were assembled into separate contigs with the Geneious software. Identity levels of the contigs were analyzed via the BLAST algorithm. The results of both Sanger and Illumina sequencing suggested a mixed infection of tomato severe rugose virus (ToSRV) and tomato chlorotic mottle virus (ToCMoV) with an identity of 99% for both

begomoviruses. These results were also confirmed by PCR assays with DNA–A component-specific primers for ToSRV and ToCMoV. PCR assays were also positive with degenerate primers targeting the DNA–B component (Patel et al. 1993) of both begomoviruses. The Illumina results also detected the DNA–B component of ToSRV (99% identity) in a *Dendrobium* sample. This is the first formal report of infection of *Dendrobium* species by begomoviruses. It is also the first report of tomato-infecting begomoviruses in members of the *Orchidaceae* family. It will be of epidemiological interest to elucidate how these begomoviruses were able to infect *Dendrobium* plants since the colonization of orchids by whiteflies was not observed in our surveys.

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Figure 1. Chlorotic mottle and black ringspot symptoms identified in foliar samples of *Dendrobium* species with a mixed infection of tomato severe rugose virus (ToSRV) and tomato chlorotic mottle virus (ToCMoV).

CHAPTER 5

Brief Report | To be submitted as a Brief Report to *Achieves of Virology*

Discovery via high-throughput sequencing (HTS) of two novel and highly divergent begomovirus-like pathogens infecting *Spathoglottis* orchid.

Discovery via high-throughput sequencing (HTS) of two novel and highly divergent begomovirus-like pathogens infecting orchids of genus *Spathoglottis*.

Juliana Gabrielle Isidorio da Silva[1], Felipe Fochat da Silva Melo[1], Luciane de Nazaré Almeida dos Reis[1], Maria Esther de Noronha Fonseca[2], Leonardo Silva Boiteux[2], Tatsuya Nagata[1], Rita de Cassia Pereira Carvalho[1]

[1] Departamento de Fitopatologia, Universidade de Brasília (UnB), Brasília–DF, Brazil.

[2] National Center for Vegetable Crops Research (CNPH), Embrapa Vegetable Crops (Hortaliças), Brasília–DF, Brazil.

Abstract

The terrestrial orchids of the genus *Spathoglottis* are native from Malaysia and New Guinea, being attractive items to the horticultural market. Despite their significance, no surveys of viruses infecting *Spathoglottis* are available. The proper knowledge about viral pathogens enables the establishment of improved management practices. Herein, we carried out pioneer studies on the *Spathoglottis* DNA virome via high-throughput sequencing (HTS). Foliar samples were collected in highland areas of Central Brazil displaying typical virus-like symptoms, including mottle sectors. Two new begomovirus-like sequences (OQ79197 and OQ79198) were recovered after bioinformatic analysis. BLASTn searches indicated identity levels of 77.69% and 77.77% with the begomoviruses *Lisianthus enation leaf curl virus* and *Passionfruit leaf curl virus*, respectively. However, the query cover for the sequences remained below the rate of 40%, indicating putative structural novelties of these begomovirus-like sequences. Virus-specific primers were designed for further investigation in the original sample. The new viruses are provisionally named (Spathoglottis-associated mottle virus 1 and 2). The global dispersion of these viruses should be evaluated since the commercial *Spathoglottis* plants present in Brazil are of exotic origin.

Keywords: Virome, *Geminiviridae*, New viruses, ssDNA viruses, *Orchidaceae*.

Spathoglottis is a genus of terrestrial plants of the Orchidaceae family [1]. *Spathoglottis* accessions are widely used as ornamental plants and are recognized as a fast-growing market niche of flowering plants [1]. *Spathoglottis* species occur naturally in sub-tropical and tropical areas of Asia [1, 2]. There are no reports of *Spathoglottis* species in local flora of Brazil and all commercial plants employed in Brazilian horticulture are imported from Malaysia and New Guinea [2].

The family *Geminiviridae* is composed of viruses with single-stranded circular DNA (ssDNA) genomes, and it is classified within the order *Geplafuvirales*. This family encompasses 520 species distributed in 14 genera recognized by the International Committee on taxonomy viruses (ICTV). Formally recognized genera are *Becurtovirus*, *Begomovirus*, *Capulavirus*, *Citlodavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Maldovirus*, *Mastrevirus*, *Mulcrilevirus*, *Opunvirus*, *Topilevirus*, *Topocuvirus*, and *Turnucurtovirus* [3]. The most expressive genus is *Begomovirus* with currently 445 accepted species [3]. Taxonomic demarcation criteria for the family *Geminiviridae* are dynamic [4]. With new genomic information, most derived from HTS approaches, it is expected to refine the species demarcation criteria [4]. Standards for genera determination in the family comprise host range, vector species (when it is known), genomic organization, and phylogenetic relationships [3, 5, 6, 7].

Despite their economic significance, no surveys of viruses infecting species of *Spathoglottis* are available. The proper knowledge about viral pathogens enables the establishment of improved management practices. Herein, we carried out pioneer studies on the *Spathoglottis* ssDNA virome via high-throughput sequencing (HTS). Fifty-two leaf samples of Orchidaceae and Bromeliaceae species were collected with virus-like symptoms as chlorosis, mosaic, mottle, necrotic spots, and ringspots. The collection places occurred in the cities of Brasília–DF, and Pirenópolis–GO. The collection sites at Brasília–DF were the shops ‘La Bromelia,’ in Núcleo Rural Taquara, and ‘Orquidário Colorado,’ in Lago Oeste. The collection site at Pirenópolis–GO was the Private Natural Heritage Reserve ‘Vagafogo.’ The total DNA was extracted from the samples using the modified CTAB protocol with organic solvents and stored at -20 °C [8]. Rolling circle amplification

(RCA) assays were performed in the DNA samples to enrich circular DNAs [9]. An RCA pool was sent to sequencing in an Illumina NovaSeq 6000 platform at Agrega. The HTS-derived raw data was analyzed following a previously validated workflow [10]: **(*i*)** pre-treatment and de novo assembly of reads using the CLC Genomics Workbench 20.0 program; **(*ii*)** The obtained contigs were submitted to BLASTn search for viral hits using Geneious R 11.1 program; **(*iii*)** Contigs with viral hits were assembled using the 'Map to reference' tool available Geneious R 11.1 program; **(*iv*)** The assembled contigs were later submitted to the BLASTn tool to search for the viruses, and **(*v*)** New virus contigs were later analyzed and sequence annotated (**Figure 1**).

Two contig sequences OQ79197, and OQ79198 (NCBI nucleotide ID) were identified as putative new viruses genetically related to members of the genus *Begomovirus* (**Figure 1**). A list of 30 *Geminiviridae* species was then used to align with the two sequences. The MUSCLE multiple alignment program was performed using Geneious software package with automatic settings. Sequence Demarcation Tool v.1.2 (SDT) program was used to determine the distance between the two sequences and the other available sequences (**Figure 2**). The alignment was also used to generate the phylogenetic tree in IQtree web service [13]. The tree was estimated by maximum likelihood using Bayesian criteria and TPM2u+F+R5 model [14]. Ultrafast bootstrap (UFBoot) was performed using 1,000 replicates, 0.5 of perturbation strength, and 100 unsuccessful iterations to stop [15]. The Newick output file was later uploaded on iTol v.6 to visualize and to generate the image [16]. The RDP 4 assay was performed in the two new viral sequences aiming to identify potential recombination sites [17]. However, no considerable recombination was found in the examined sequences.

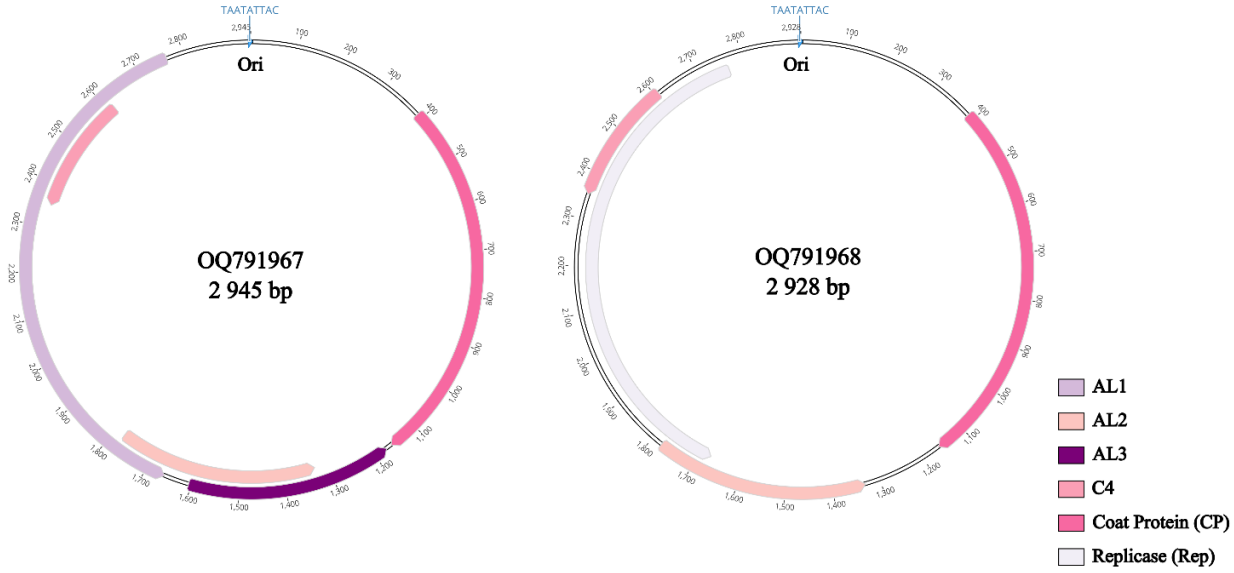

Figure 1. Genomic organization of the putative viruses corresponding to the sequences OQ791967 and OQ791968 recovered from the Spathoglottis ssDNA virome using high-throughput sequencing (HTS) analysis. The coding regions (CDS) found in the circular ssDNA are Coat protein (CP), the factor AL1, that interacts with pRb [11], the transactivate factor AL2 [12], the initiation protein AL3 [12], the multifunctional protein C4 [13], and the replicase.

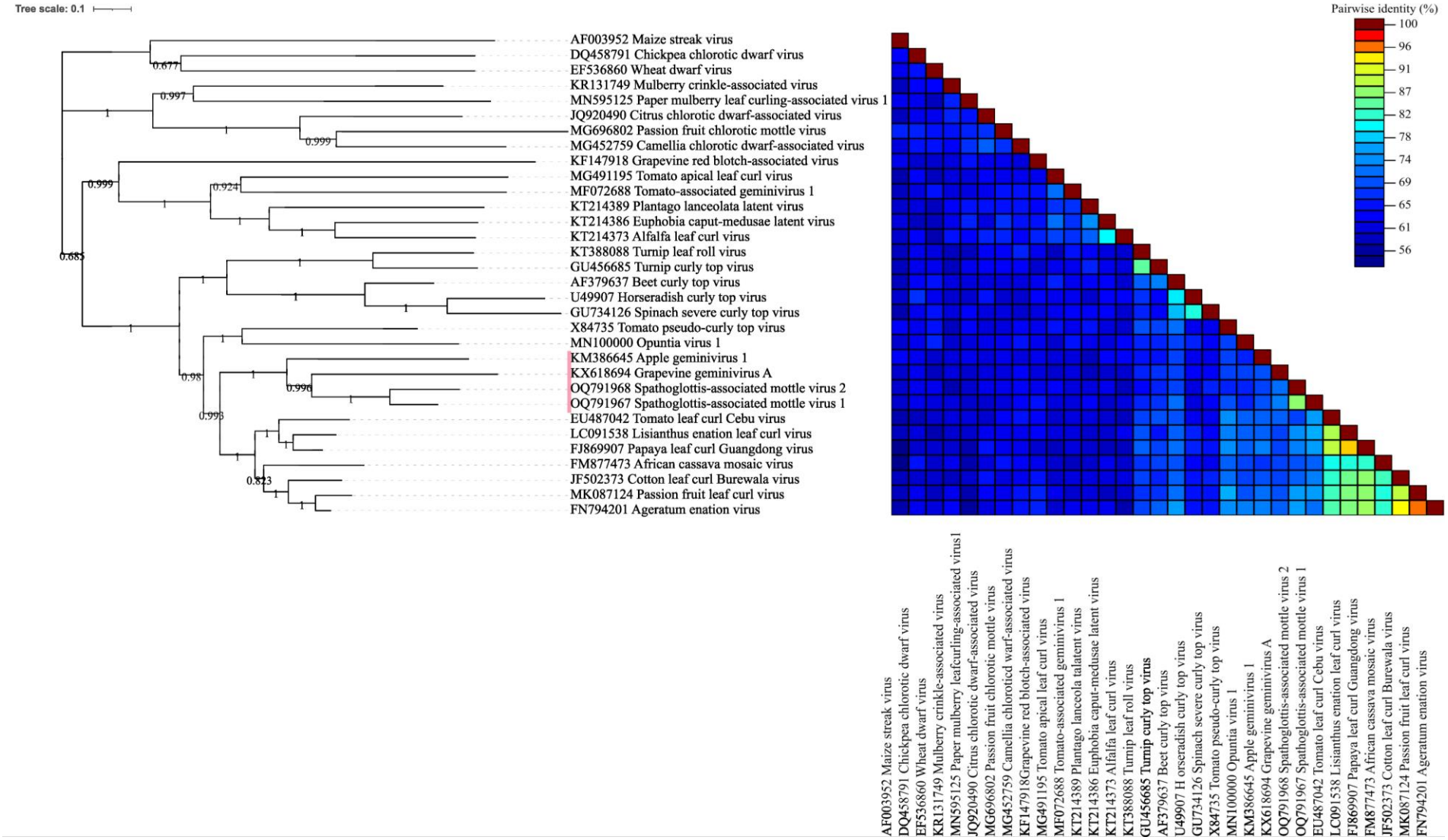

Figure 2. Phylogenetic trees were constructed by using TPM2u+F+R5 model and SDT analysis using muscle alignment.

A set of specific primers was designed to amplify genomic segments of these putative new viruses. A pair of seq OQ79197-specific primers (‘SOV1F’ 5’–CAT TCA TAT TGA TTG CCG TTG GTC C–3’/ ‘SOV1R’ 5’–GTA ATC ATG TTC AAA TAC GAG GCA AC–3’) was designed and the expected amplicon size was 2929 pb. A pair of seq OQ79198-specific primers was also designed (‘SOV2F’ 5’–CCC GTC ACG TTT GCT CCA GTT–3’/ ‘SOV2R’ 5’–GGT AAC GCC GAA CAT TCT CAA–3’) and the expected amplicon size was 2929 pb. PCR tests were performed in all 52 samples using these primers. The sample *Spathoglottis* ‘OP09’ was positive for both viruses, indicating a mixed infection. The sample was collected in the Private Natural Heritage Reserve Vagafogo, Pirenópolis–GO. The symptoms observed in the sample were characterized by chlorotic mottle in the leaves. The sequences of the contigs OQ791967 and OQ791968 have 2945 and 2928 nucleotides, respectively. The TAATATTAC motif was annotated in both sequences. The lengths of the DNA molecules as well as the nonanucleotide motifs of the origin of replication agree to the members of the family *Geminiviridae* [4]. Their genomic organization comprise the coat protein (CP), AL2, and C4 protein domains. The replicase (Rep) domain is absent in the sequence OQ79197. The AL1 and AL3 genes were found only in OQ79197. Others predicted coding regions were found, but no significant matches were detected in the NCBI data of conserved domains. Therefore, it is currently impossible to elucidate their function based upon the sequence information alone. It is suggested further analysis to probe their role in the viral infection pathway. The absence of replicase in the OQ791967 sequence is not expected and remains elusive. Since it is found in mixed infection with the sequence OQ79196, it is possible that this putative virus is using the Rep of the companion virus. The identity between the sequences is 76.8%, indicating that they are different viruses. Therefore, further analysis with single infections of the individual contigs is recommended to prove their interdependence.

The genomic organization of the putative viruses corresponding to the sequences OQ791967 and OQ79196 indicated that they are most likely new circular ssDNA sequences which are different from the species of the *Begomovirus* genus (**Figure 1**). However, the query covered of the

alignment was short, with rate of 37% and 35%. The highest nucleotide identities were 77.69% and 77.77% using BLASTn analysis. According to the established criteria for new species, the sequences should be considered as new viruses, which were provisionally named (*Spathoglottis-associated mottle virus* 1 and *Spathoglottis-associated mottle virus* 2). The terrestrial orchids of the genus *Spathoglottis* are native from Malaysia and New Guinea. Therefore, global dispersion of these viruses should be evaluated since the commercial *Spathoglottis* plants present in Brazil are of exotic origin.

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Declarations

Conflict of interest: The authors declare that they have no conflicts of interest.

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

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