**UNIVERSIDADE FEDERAL DE ALAGOAS**
**REDE NORDESTE DE BIOTECNOLOGIA**

**ADSO LEVI SOARES DE FIGUEIREDO MENDES**

# Molecular characterization of a new badnavirus associated with air yam (*Dioscorea bulbifera* L.) and development of a loop-mediated isothermal amplification (LAMP) assay for rapid detection of *Dioscorea baciliform AL virus*

**MACEIÓ-AL**
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Tese apresentada à Universidade Federal de Alagoas como parte das exigências do Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia (Renorbio) para obtenção do título de *Doctor Scientiae*.

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ADSO LEVI SOARES DE FIGUEIREDO MENDES

**Caracterização molecular de um novo badnavírus associado ao cará-moela (*Dioscorea bulbifera* L.) e desenvolvimento de um protocolo baseado em *loop-mediated isothermal amplification* (LAMP) para detecção rápida de *Dioscorea bacilliform AL virus***

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia – RENORBIO, Ponto Focal Alagoas, Universidade Federal de Alagoas, como requisito parcial para a obtenção do Título de Doutor em Biotecnologia, Área de Concentração: Biotecnologia em Agropecuária.

Aprovada em: 22/06/2023.

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**SUMÁRIO**

## RESUMO

Inhames (*Dioscorea* spp.) são importantes culturas de subsistência cultivadas em todo o mundo, e o cará-moela (*Dioscorea bulbifera*) está entre as 12 espécies plantadas comercialmente. A produtividade e a troca segura de germoplasma de *Dioscorea* spp. são prejudicados por várias pragas e doenças, e os vírus representam uma ameaça significativa principalmente devido à propagação vegetativa do inhame que propicia o acúmulo e disseminação viral. A família *Caulimoviridae* compreende 11 gêneros de vírus de DNA fita dupla circular, dos quais *Badnavirus* é difundido onde quer que o inhame seja plantado, e a transmissão viral ocorre principalmente por propagação vegetativa e diferentes espécies de cochonilhas. Uma nova espécie de badnavírus, *Dioscorea bacilliform BL virus*, foi recentemente relatada em plantas de *D. bulbifera* no Brasil, mas apenas sequências parciais da transcriptase reversa (RT) e ribonuclease H (RNase H) foram caracterizadas. Para recuperar a sequência completa do genoma de isolados de dioscorea bacilliform BL virus (DBBLV), o DNA total obtido de uma planta de inhame infectada por DBBLV foi usado como molde para enriquecimento do DNA viral circular via amplificação por círculo rolante e submetido ao sequenciamento Illumina de alto rendimento. Dois genomas completos de 7.196 (DBBLV-DBH1) e 7.342 (DBBLV-DBH3) pares de bases de comprimento foram montados *de novo* a partir de 1.273.444 e 539.840 *reads* e uma profundidade de cobertura de 177 e 74x, respectivamente. Ambos os isolados pertencem ao novo badnavírus DBBLV, compartilhando ≥88% de identidade de nucleotídeos com pelo menos um isolado de DBBLV. A análise de recombinação mostrou um padrão complexo de recombinação interespécies envolvendo os isolados DBBLV-DBH1 e DBBLV-DBH3 como sequências recombinantes ou parentais em quatro eventos independentes. As árvores filogenéticas reconstruídas com base no genoma completo e nas sequências de nucleotídeos da RT-RNase H mostrou que os novos isolados de DBBLV são mais proximamente relacionados às sequências do Dioscorea bacilliform AL virus (DBALV), mas algumas incongruências topológicas foram observadas entre essas duas árvores. Além disso, um protocolo baseado em *loop-mediated isothermal amplification* (LAMP) foi desenvolvido para detecção de DBALV, o badnavírus prevalente associado ao inhame no Brasil. As reações de LAMP foram relizadas em termociclador em tempo real por 60 minutos a 65 ºC, com medição da fluorescência a cada um minuto. Após a verificação da viabilidade do ensaio de LAMP, o limite de detecção foi testado com diluições seriadas, mostrando que o protocolo desenvolvido foi capaz de detectar o DNA viral na diluição de $12{,}5 \times 10^{-7}$ ng/µL, equivalente a $5.5 \times 10^{3}$ cópias/µL.



**Palavras-chave**: *Dioscorea*, dioscorea baciliform virus, *Caulimoviridae*, Dioscoreaceae.

## ABSTRACT

Yams (*Dioscorea* spp.) are important staple crops cultivated worldwide, and air yam (*Dioscorea bulbifera*) is among the 12 commercially grown species. The productivity and safe germplasm exchange of *Dioscorea* spp. are hindered by several pests and diseases, and viruses pose a significant threat especially due to the vegetative propagation of yams that propitiate viral accumulation and dissemination. At least eight viral families have been reported infecting yams. The family *Caulimoviridae* comprises 11 genera, of which *Badnavirus* is widespread wherever yams are planted, and badnaviral transmission occurs mainly by vegetative propagation and different species of mealybugs. A putative new badnavirus has been recently reported in *D. bulbifera* plants in Brazil, and tentatively named as dioscorea bacilliform BL virus (DBBLV), but only partial sequences of the reverse transcriptase (RT) and ribonuclease H (RNase H) genomic region have been characterized. To recover the complete genome sequence of DBBLV isolates, total DNA obtained from an DBBLV-infected air yam plant was used as template for enrichment of viral circular DNA via rolling circle amplification and subjected to high-throughput Illumina sequencing. Two complete genomes of 7,196 (DBBLV-DBH1) and 7,342 (DBBLV-DBH3) bp in size were *de novo* assembled from 1,273,444 and 539,840 reads and a coverage depth of 177 and 74x, respectively. Both isolates belong to the species novel *Dioscorea bacilliform BL virus*, sharing ≥88% nucleotide identity with at least one isolate of DBBLV. The recombination analysis showed a complex interspecies recombination pattern involving the DBBLV-DBH1 and DBBLV-DBH3 isolates as putative recombinant or parental sequences in four independent events. The Bayesian phylogenetic tree reconstructed based on complete genome and RT-RNase H nucleotide sequences showed the new DBBLV isolates were more closely related to dioscorea bacilliform AL virus (DBALV) sequences, but some topological incongruences were observed between these two trees. Also, a loop-mediated isothermal amplification (LAMP)-based protocol was developed and tested for detection of DBALV, the most prevalent badnavirus species affecting yams in Brazil. LAMP reactions were performed in a real-time thermocycler for 60 minutes at 65 ºC, measuring the fluorescence each one minute. Once the feasibility the proposed LAMP assay was confirmed, the limit of detection or the analytical sensitivity was tested with 10-fold serial dilutions, showing that the proposed protocol was able to detect viral DNA in a concentration of 12,5 x $10^{-7}$ ng/µL, equivalent to 5.5 x $10^{3}$ copies/µL.



**Keywords**: *Dioscorea*, dioscorea bacilliform viruses, *Caulimoviridae*, Dioscoreaceae.

## INTRODUCTION

The genus *Dioscorea* comprises more than 640 wild and domesticated species commonly known as yams. Millions of people from Africa, South America, Asia, and the Pacific tropical countries rely on cultivated yams as staple food. *Dioscorea bulbifera* L, known in Brazil as gizzard yam and air yam, among other common names, is one of the 12 staple yam species (LEBOT, 2019). Besides its high potential as invasive species in places like Florida, United States of America (DEY et al., 2018), air yam is cultivated throughout the tropics being important as non-conventional edible plant, also referred to as NCEP (KINUPP; LORENZI, 2014), and has therapeutical properties used in traditional Chinese, Indian and African medicine (NARULA et al., 2003; GHOSH et al., 2012, IKIRIZA et al., 2019). In consonance with ethnobotanical uses, pharmacological properties of *D. bulbifera* have been assessed through clinical trials (IKIRIZA et al., 2019; KUNDU et al, 2021).

Yams are susceptible to several pests and diseases which can cause important yield losses in the field or in storage, although incidence can vary depending on country, region, species, variety, and the cropping system used (LEBOT, 2019). Those pests and diseases have not only quantitative impact on production, but also affect the quality of yams, turning them less attractive to the consumers (AMUSA et al., 2003; LUO et al., 2022). Among these phytosanitary issues, viruses are those of major concern because they are the most difficult to control, spread easily with planting material and are present in all yam-growing regions around the globe (KENYON et al., 2008; LUO et al., 2022). Generally, small tubers or tuber pieces (setts) are used as planting materials for propagation of cultivated *Dioscorea* spp. resulting in accumulation and perpetuation of viruses in yam germplasm (SEAL & MULLER, 2007; KENYON et al., 2008.; BÖMER et al, 2018), making the development of diagnostic tools and providing virus-free planting materials urgent needs (BÖMER et al, 2018).

Viruses belonging to the families *Alphaflexiviridae*, *Betaflexiviridae*, *Bromoviridae*, *Caulimoviridae*, *Closteroviridae*, *Potyviridae*, *Secoviridae*, and *Tombusviridae* have been reported infecting yams worldwide (LUO et al., 2022). *Badnavirus* and *Dioscovirus* are the representative genera of *Caulimoviridae* reported in different yam species. The former is widespread in cultivated *Dioscorea* spp. plantations around the world (KENYON et al., 2008; LUO et al., 2022). The genus *Badnavirus* is comprised by reverse-transcribing plant viruses with bacilliform non-enveloped virions with 30 nm width and 130 nm in length (modal particle), but variation from 60 nm to 900 nm in length is commonly observed. Inside the single particles, one molecule of double-stranded non-covalently closed circular DNA of 7.2–9.2 kbp is present and each strand has a single discontinuity (TEYCHENEY et al, 2020). Typical genomes consist of three open reading frames (ORFs) coding for proteins P1 (unknown

function), P2 (virion-associated protein) and the polyprotein P3, with movement protein, capsid protein, aspartic protease, reverse transcriptase (RT) and ribonuclease H (RNase H) protein precursors (TEYCHENEY et al., 2020). Leaf symptoms like chlorosis and deformation are usually associated with badnaviruses, but asymptomatic infection is also common (SEAL et al., 2014; LUO et al., 2022). To date, eight species of yam badnaviruses are recognized by International Committee on Taxonomy of Viruses (ICTV, talk.ictvonline.org) and are collectively known as dioscorea bacilliform viruses (DBVs): *Badnavirus alphadioscoreae*, *Badnavirus betadioscoreae*, *Badnavirus gammadioscoreae*, *Badnavirus deltadioscoreae*, *Badnavirus epsilondioscoreae*, *Badnavirus zetadioscoreae*, *Badnavirus etadioscoreae*, and *Badnavirus tetadioscoreae*. Based on partial RT-RNase H sequences, a putative new badnavirus has been recently reported associated with *D. bulbifera* plants in Brazil, and it has been tentatively named as Dioscorea bacilliform BL virus (SANTOS et al., 2022). Diverse groups of endogenous pararetroviral sequences (EPRVs) have been reported in yam plants belonging to the *Dioscorea cayenensis-rotundata* species complex (BOUSALEM et al., 2009; SEAL et al., 2014; UMBER et al., 2014), with dioscorea rotundata endogenous virus 12 (eDBV12) also being reported in *D. bulbifera* (SANTOS et al., 2022).

Here, the complete genome of the putative new badnavirus associated with air yam, Dioscorea baciliform BL virus (DBBLV), was molecularly characterized using high-throughput sequencing. Genome analysis revealed the presence of at least three open reading frames (ORFs 1-3), and other badnavirus hallmark features such as tRNA met primer binding site sequence, polyadenylation signal and TATA box. The Recombination analysis detected four putative recombination events involving DBBLV genomes as recombinant or parental sequence. Phylogenetic trees inferred by Bayesian analyses of complete genomes and RT-RNase H sequences showed close relationship of DBBLV and DBALV isolates, with a slight incongruence related to the isolate DBBLV-DBH1, a putative recombinant. Further, loop-mediated isothermal amplification (LAMP)-based protocol was developed and tested for detection of DBALV, the most prevalent badnavirus species affecting yams in Brazil.

## BACKGROUND

### Yam

The term yam is used to name the more than 640 species into the genus *Dioscorea* L., family Dioscoreaceae (LEBOT, 2019; LUO et al., 2022). Cultivated yams are important staple crops for millions of people throughout tropical and subtropical countries, being one of the most relevant food sources in the sub-Saharan Africa (BHATTACHARJEE et al., 2011; DARKWA et al., 2019). Besides its importance for food security, yams are present in distinct aspects of West African life culture, economy, and religion (ZANNOU et al., 2004). The African continent is the world's largest yam producer, responsible for 97.8% of the annual production, amounting to over 73 million tons. The Americas rank second, accounting for 1.38% of the global production, which is equivalent to more than 1 million tons annually (FAOSTAT, 2021). *Dioscorea* species are widespread in tropical and subtropical countries but also cultivated in a few temperate areas. *Dioscorea alata*, *D. cayenensis*, and *D. rotundata* are the most cultivated species. In Brazil, the world's 13$^{th}$-largest and Americas' second-largest producer with over 250,000 tons (FAOSTAT, 2021), the cultivation of those major yams is concentrated in the Northeast Region with the cultivars of yellow yam known as "inhame-da-Costa" (*D. cayanensis*) and water yam named "inhame São Tomé" (*D. alata*) being the most cultivated (NORONHA et al., 2020; LOVERA et al., 2020; ASSUNÇÃO et al., 2023). *D. bulbifera*, *D. dumetorum*, *D. esculenta*, *D. japonica*, *D. nummularia*, *D. oppositifolia*, *D. pentaphylla*, *D. transversa*, and *D. trifida* are often considered minor yam crops (LEBOT, 2019). However, in times of food scarcity, these crops and several wild species are also of great importance as food sources (BHATTACHARJEE et al., 2011).

### Air yam

*Dioscorea bulbifera* L., also referred to as air yam, is named due to its aerial bulbils that grow at the base of the petioles with an average weight of 300-500g (Figure 1; LEBOT, 2019). Geographic origin of air yam seems to be both Africa and Asia (WILKIN, 2001), but according to LEBOT (2020) it is present in the wild state in Africa, Asia, and Oceania, being now cultivated throughout the tropics. In Brazil, it is considered a non-conventional edible plant, also known as NCEP (KINUPP; LORENZI, 2014), and several studies have also focused on the therapeutical properties of *D. bulbifera* that has been widely used in traditional medicine (NARULA et al., 2003; GHOSH et al., 2012, GUAN et al., 2017; IKIRIZA et al., 2019). Although more than 100 compounds have been characterized from air yam, it is important to note that *D. bulbifera* may also present nephrotoxicity and hepatotoxicity activities (GUAN et al., 2017).

Figure 1. Air yam (*Dioscorea bulbifera*) plants with gizzard-like bulbils also known as aerial tuber formed from the base of petioles. Author: Philippe VERNIER; Bulbil of *D. bulbifera*, Haiti, 2005. Source: https://commons.wikimedia.org/wiki/File:Bulbifera.jpg.

Yam quality and productivity can be affected by several pests and diseases resulting in yield losses in field and storage conditions (MOURA, 2005; LEBOT, 2019). Virus infections are of great concern in vegetatively propagated crops like yams because this process facilitates accumulation, survival, and dissemination of the virus (SEAL & MULLER, 2007; KENYON et al., 2008). Consequently, infected plants become less vigorous, and quality of tubers decrease (AMUSA et al., 2003). Plants with viral diseases show variable symptoms depending on the virus group responsible for the infection, but severe leaf chlorosis, mosaic, shoestring, interveinal chlorosis, dwarfism, and leaf distortion are often observed. These foliar symptoms can result in a reduction of the photosynthetic capacity of the host with negative effects on production, tuber quality, and, in some cases, may cause plant death (THOUVENEL; DUMONT, 1988).

Several viruses from different families have been reported to infect yams: *Alphaflexiviridae* (genus *Potexvirus*), *Betaflexiviridae* (genus *Carlavirus*), *Bromoviridae* (genus *Cucumovirus*), *Caulimoviridae* (genera *Badnavirus* and *Dioscovirus*), *Closteroviridae* (genus *Ampelovirus*), *Potyviridae* (genera *Macluravirus* and *Potyvirus*), *Secoviridae* (genus *Sadwavirus*), and *Tombusviridae* (genus *Aureusvirus*) (SILVA et al., 2019; SUKAL et al., 2020; LUO et al., 2022).

**Family *Caulimoviridae***

*Caulimoviridae* is the only known family of plant-infecting viruses with double-stranded DNA (dsDNA) genomes (NASIM & DEY, 2021). The non-covalently closed circular dsDNA genomes range from 7.1 to 9.8 kbp in size (TEYCHENEY et al., 2020). Viral replication occurs in the cytoplasm by reverse transcription of a pre-genomic RNA through the viral reverse transcriptase without integration into the host genome (HOHN; ROTHNIE 2013; TEYCHENEY et al., 2020). Due to these features, and because the circular dsDNA genome is encapsidated instead of the single-stranded RNA intermediate, the term 'plant-infecting pararetroviruses' is used to refer to this family (TEMIN, 1989; HOHN & RICHERT-PÖGGELER 2006). The genus demarcation criteria are based on virion morphology, genome organization, transmission mode and vector species, and host types. Members of the family are grouped in eleven genera: *Badnavirus* and *Tungrovirus* have bacilliform-shaped particles, while *Caulimovirus*, *Cavemovirus*, *Petuvirus*, *Rosadnavirus*, *Ruflodivirus*, *Solendovirus*, and *Soymovirus* are encapsidated into isometric particles. The genera *Dioscovirus* and *Vaccinivirus* have been recently proposed but no virion morphology is known to date (TEYCHENEY et al., 2020).

Members of *Caulimoviridae* have a wide geographic distribution, with most species of *Tungrovirus*, *Badnavirus*, and *Dioscovirus* being located in tropical and subtropical regions, and some species are also widespread in temperate and sub-Antarctic regions (TEYCHENEY et al., 2020). Members of *Dioscovirus* and *Tungrovirus* infecting exclusively monocotyledonous plants, while *Caulimovirus*, *Cavemovirus*, *Petuvirus*, *Rosadnavirus*, *Ruflodivirus*, *Solendovirus*, *Soymovirus*, and *Vaccinivirus* infect dicotyledonous hosts. Interestingly, *Badnavirus* are associated with both monocotyledonous and dicotyledonous hosts (TEYCHENEY et al., 2020). Natural transmission depends on the genus of the virus and occurs via insect vector, contact with other plants, seeds or pollen, and vegetative propagation. Mechanical inoculation and grafting techniques are also viable ways to transmit caulimoviruses (TEYCHENEY et al., 2020).

Infections by members of *Caulimoviridae* may cause a variety of symptoms depending on the virus and host species involved, and climatic conditions. While *Caulimovirus*, *Cavemovirus*, *Petuvirus*, *Rosadnavirus*, *Solendovirus*, and *Soymovirus* predominantly cause mosaic or vein clearing, *Badnavirus* and *Tungrovirus* are usually associated with interveinal chlorotic mottling, stunting, yellow-orange foliar discoloration and streaking (TEYCHENEY et al., 2020). The sole member of the genus *Ruflodivirus*, Rudbeckia flower distortion virus (RuFDV), induce severe flower deformation and phyllody in *Rudbeckia hirta* L., symptoms usually associated with phytoplasma infections (LOCKHART et al., 2017).

Pararetroviruses lack an integrase-like protein and do not require integration into the host's genome as a step in viral replication, but viral DNA integration may occur for the genera *Badnavirus*, *Caulimovirus*, *Cavemovirus*, *Petuvirus*, and *Solendovirus* through illegitimate recombination, resulting in endogenous pararetroviral sequences (EPRVs), the most abundant class of endogenous viral sequences in different plant species (GAYRAL & ISKRA-CARUANA, 2009; GONG & HAN, 2018; TEYCHENEY et al., 2020). Most EPRVs are incomplete and not functional, with some replication-competent EPRVs occurring in hosts such as *Musa balbisiana*, *Petunia hybrida*, and *Nicotiana edwardsonii* (GAYRAL & ISKRA-CARUANA, 2009; TEYCHENEY & GEERING, 2011)

**Genus *Badnavirus***

*Badnavirus* is by far the largest genus in the family *Caulimoviridae* with 68 species recognized by the ICTV (https://talk.ictvonline.org/). The name of the genus is a derivation from bacilliform DNA viruses (TEYCHENEY et al., 2020). Single molecule, non-covalently closed dsDNA badnaviral genomes range from 7.2 to 9.2 kbp (each strand with a single discontinuity) and is encapsidated into bacilliform-shaped virions (TEYCHENEY et al., 2020). Virion width is uniform (30 nm) in the genus and length is usually 130 nm, although particles ranging from 60 nm to 900 nm in length are often observed (TEYCHENEY et al., 2020).

Badnaviruses encode at least three open reading frames (ORF 1-3; Figure 2a), but the number of ORFs can reach up to seven such as Rubus yellow net virus (KALISCHUK et al. 2013, TEYCHENEY et al., 2020). The ORF1 protein of Commelina yellow mottle virus is virion-associated (CHENG et al., 1996), while ORF2 of cacao swollen shoot virus encodes a nucleic acid-binding protein (JACQUOT et al., 1996). ORF3 is quite similar in members of *Badnavirus*, being the largest ORF and encoding a polyprotein that comprises the movement protein, viral capsid, the aspartate protease, and the reverse transcriptase (RT) and ribonuclease H (RNase H) domains (Figure 2b; BHAT et al., 2016; TEYCHENEY et al., 2020). The species demarcation criteria in the genus *Badnavirus* are based on the host range, vector specificity, and differences of more than 20% in nucleotide sequence of the RT-RNase H genomic region (TEYCHENEY et al., 2020).

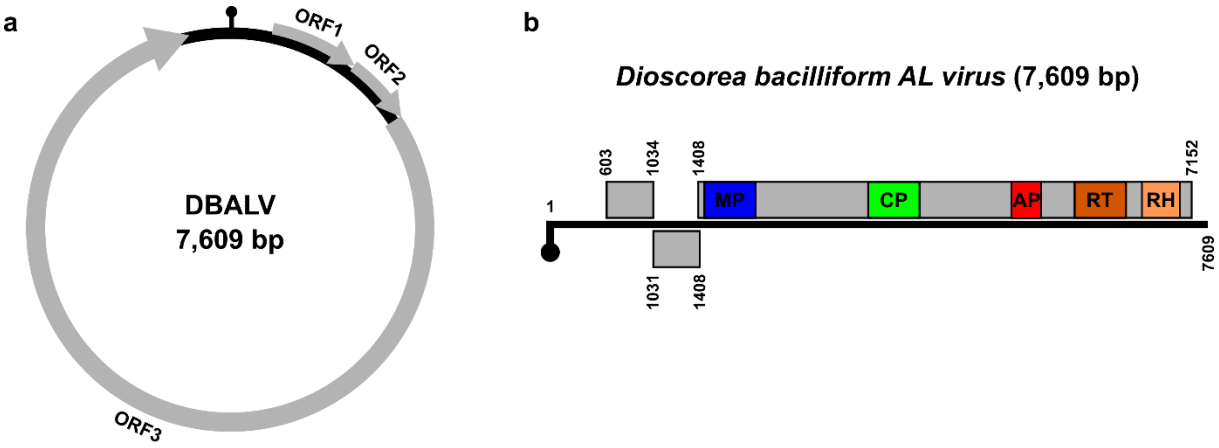


Figure 2. Genome organization of the yam-infecting badnavirus dioscorea bacilliform AL virus (DBALV). Three open reading frames (ORFs 1-3) are encoded by the viral genome (a), and ORF3 comprises important conserved domains such as movement protein (MP), capsid protein (CP), aspartate protease (AP), reverse transcriptase (RT), and ribonuclease H (RNase H). Source: the author (2022).

Species belonging to the genus *Badnavirus* have been reported infecting economically important crops such as rice (*Oryza sativa* L.) (OMURA et al., 1983), sugarcane (*Saccharum officinarum* L.) (LOCKHART et al., 1988), banana (*Musa* spp.) (LOCKHART & OLSZEWKI, 1993), cacao (*Theobroma cacao* L.) (POSNETTE, 1944), *Citrus* spp. (AHLAWAT et al., 1996) *Capsicum* spp. (LOCKHART et al, 1997), yams (*Dioscorea* spp.) (PHILLIPS et al., 1999), and taro (*Colocasia esculenta* (L.) Schott.) (YANG et al., 2003). Dissemination of badnaviruses occurs primarily by vegetative propagation and insect vector. Several mealybug species are responsible for the horizontal transmission of the majority of *Badnavirus*, with a few instances being transmitted by aphids or lace bugs (BHAT et al., 2016; TEYCHENEY et al., 2020). Seed transmission has been reported for Commelina yellow mottle virus, Kalanchoe top-spotting virus, cacao swollen shoot Togo B virus (previously known as cacao swollen shoot virus), Piper yellow mottle virus and taro bacilliform virus (HEARON & LOCKE, 1984; QUAINOO et al., 2008; HAREESH & BHAT, 2010; DEESHMA & BHAT, 2014; BHAT et al., 2016). Experimental transmission by virus-containing crude sap, partially purified and purified preparations or by *Agrobacterium*-mediated inoculation has been achieved for badnaviral species associated with cacao, *Kalanchoe blossfeldiana* Poelln., black pepper, fig, and yams (KEITH et al., 2021; LOCKHART & FERJI, 1988; BHAT et al., 2003; LANEY et al., 2012; PHILLIPS et al., 1999). Transmission of badnaviruses by dodder or wedge-grafting were also reported for citrus and black pepper (AHLAWAT et al., 1996; BHAT et al., 2003).

Badnaviruses are widespread in yam-growing regions throughout the world and are commonly found in mixed infections with other yam-infecting viral groups such as *Potyvirus* (ENI et al., 2008; LUO et al., 2022). Previous studies performed in Africa and South Pacific suggested that the badnavirus diversity in *Dioscorea* spp. is high, with at least twelve different viral species being detected (ENI, et al., 2008). So far, eight ICTV-approved badnaviruses have been reported from yams: Dioscorea bacilliform AL virus, Dioscorea bacilliform SN virus, Dioscorea bacilliform AL virus 2, Dioscorea bacilliform ES virus, Dioscorea bacilliform RT virus 1, Dioscorea bacilliform RT virus 2, Dioscorea bacilliform RT virus 3, and Dioscorea bacilliform TR virus (https://talk.ictvonline.org/ictv-reports). A putative new member of the genus has been recently identified infecting *D. bulbifera* in Brazil which has been tentatively named as Dioscorea bacilliform BL virus (DBBLV). However, only partial RT-RNase H sequences of this new badnavirus have been recovered and further studies are required to fully characterize its genome sequence (SANTOS et al., 2022).

**Diagnosis of yam viruses**

Bacilliform badnaviral-like particles have been firstly detected in yam plants in association with flexuous viruses causing brown spot disease in the 1970's (HARRISON; ROBERTS, 1973; MANTELL; HAQUE, 1978), but the complete characterization of a DBV occurred only in the late 1990's and the name dioscorea alata bacilliform virus (DaBV) was proposed (BRIDDON et al., 1999; PHILLIPS et al., 1999), which was later renamed as Dioscorea bacilliform AL virus (DBALV). A second DBV species was reported in 2007 and named Dioscorea sansibarensis bacilliform virus (DsBV), currently known *Badnavirus betadioscoreae* (SEAL; MULLER, 2007). The detection of badnaviruses has relied on serological tests like protein A sandwich-enzyme-linked immunosorbent assay (PAS-ELISA) and PCR (BRIDDON et al., 1999; PHILLIPS et al., 1999; SEAL; MULLER, 2007; KENYON et al., 2008; BOUSALEM et al., 2009), but the later method is unable to differentiate episomal from integrated DBV sequences (BOUSALEM et al., 2009). To solve this problem, it has been proposed the combination of rolling circle amplification (RCA, for enrichment of circular DNA molecules) and viral PCR detection with specific or degenerated primers (BÖMER et al., 2016). Further, immunocapture-PCR (IC-PCR) has been used to correctly identify episomal and integrated badnaviruses (ÜMBER et al., 2017).

Serological and genetic heterogeneity, presence at low titers, occurrence of EPRVs and asymptomatic infections are factors that make the diagnosis of badnaviruses challenging for yam and other crops (HARPER et al., 2003; SEAL; MULLER, 2007; KENYON et al., 2008, BOUSALEM et al., 2009; SUKAL et al., 2017). Also, the fact that yam badnaviruses are

widespread in all growing areas, including germplasm collections, hinders the safe germplasm exchange (KENYON et al., 2008; BOUSALEM et al., 2009; SEAL et al., 2014; BÖMER et al., 2018; LUO et al., 2022). Therefore, the development of reliable diagnostic tools and obtention of virus-free planting material are urgent as the detection and correct identification of the virus play a vital role in seedling indexing programs and disease management (SEAL et al., 2014; DIOUF et al., 2022).

Loop-mediated isothermal amplification (LAMP) is a method described more than 20 years ago, which is capable of detecting DNA with high sensitivity and specificity under isothermal conditions in less than one hour (NOTOMI et al., 2000). It requires at least four primers (two inner and two outer primers) that recognizes six independent sequences in the target DNA. Further, with the use of two additional loop primers, the amplification time can be considerably decreased (NAGAMINE et al., 2002; BOTELLA et al., 2022). Rapid and specific RT-LAMP protocols have been described for direct detection of yam infecting potyviruses (genus *Potyvirus*, family *Potyviridae*) such as Japanese yam mosaic virus (JYMV) and Yam mosaic virus (YMV), with RT-LAMP detection of YMV being 100 times more sensitive in the viral detection when compared to standard RT-PCR approaches (Fukuta et al., 2003; Nkere et al., 2018).

## LITERATURE CITED


Ahlawat YS, Pant RP, Lockhart BEL, Srivastava M, Chakraborty NK, Varma A (1996) Association of a badnavirus with citrus mosaic disease in India. Plant Dis 80(5):590-592

Amusa NA, Adigbite AA, Muhammed S, Baiyewu RA (2003) Yam diseases and its management in Nigeria. Afr J Biotechnol 2(12):497-502

Assunção MC, De Souza Junior FJC, Dos Santos Neto JC, et al (2023) *Pratylenchus zeae* in yam, *Dioscorea cayenensis* and *D. alata*, in Brazil. Australasian Plant Dis Notes 18:26. https://doi.org/10.1007/s13314-023-00512-3

Bhat AI, Devasahayam S, Sarma YR, Pant RP (2003) Association of a badnavirus in black pepper (*Piper nigrum* L.) transmitted by mealybug (*Ferrisia virgata*) in India. Curr Sci 1547:1550

Bhat AI, Hohn T, Selvarajan R (2016) Badnaviruses: the current global scenario. Viruses 8(6):177

Bhattacharjee R, Gedil M, Sartie A et al (2011) *Dioscorea*. In: Wild Crop Relatives: Genomic and Breeding Resources. Springer, Berlin, Heidelberg, pp 71-96

Bömer M, Turaki A, Silva G, et al (2016) A Sequence-Independent Strategy for Amplification and Characterisation of Episomal Badnavirus Sequences Reveals Three Previously Uncharacterised Yam Badnaviruses. Viruses 8:188. https://doi.org/10.3390/v8070188

Bömer M, Rathnayake AI, Visendi P, et al (2018) Complete genome sequence of a new member of the genus Badnavirus, Dioscorea bacilliform RT virus 3, reveals the first evidence of recombination in yam badnaviruses. Arch Virol 163:533–538. https://doi.org/10.1007/s00705-017-3605-9

Botella JR (2022) Point-of-Care DNA Amplification for Disease Diagnosis and Management. Annu Rev Phytopathol 60:1–20. https://doi.org/10.1146/annurev-phyto-021621-115027

Bousalem M, Durand O, Scarcelli N, et al (2009) Dilemmas caused by endogenous pararetroviruses regarding the taxonomy and diagnosis of yam (*Dioscorea* spp.) badnaviruses: analyses to support safe germplasm movement. Arch Virol 154(2):297-314

Briddon RW, Phillips S, Brunt A, Hull R (1999) Analysis of the sequence of dioscorea Alata bacilliform virus: comparison to others members of the badnavirus group. Virus Genes 18:277–283. https://doi.org/10.1023/A:1008076420783

Cheng CP, Lockhart BE, Olszewski NE (1996) The ORF I and II proteins of *Commelina yellow mottle virus* are virion-associated. Virol 223:263-271

Darkwa K, Olasanmi B, Asiedu R, Asfaw A (2020) Review of empirical and emerging breeding methods and tools for yam (*Dioscorea* spp.) improvement: Status and prospects. Plant Breed 139(3):474-497

Deeshma KP, Bhat AI (2014) Further evidence of true seed transmission of *Piper yellow mottle virus* in black pepper (*Piper nigrum* L.). J Plant Crops 42(3):289-293

Dey KK, Sugikawa J, Kerr C, Melzer, MJ (2019) Air potato (*Dioscorea bulbifera*) plants displaying virus-like symptoms are co-infected with a novel potyvirus and a novel ampelovirus. Virus Genes *55*(1):117-121

Diouf MB, Festus R, Silva G, et al (2022) Viruses of Yams (Dioscorea spp.): Current Gaps in Knowledge and Future Research Directions to Improve Disease Management. Viruses 14:1884. https://doi.org/10.3390/v14091884

Eni AO (2008) Sequence diversity among badnavirus isolates infecting yam (*Dioscorea* spp.) in Ghana, Togo, Benin and Nigeria. Arch Virol 153:2263-2272

FAOSTAT (2021) Food and Agriculture Organization of the United Nations. FAOSTAT Statistical Database. Rome. Available at: https://www.fao.org/faostat/en/#data/QCL [Accessed 15 July 2023]

Fukuta S, Iida T, Mizukami Y, Ishida A, Ueda J, Kanbe M, Ishimoto Y (2003) Detection of Japanese yam mosaic virus by RT-LAMP. Arch Virol 148(9):1713-20. doi: 10.1007/s00705-003-0134-5. PMID: 14505084.

Gayral P, Iskra-Caruana ML (2009) Phylogeny of *Banana streak virus* reveals recent and repetitive endogenization in the genome of its banana host (*Musa* sp.). J Mol Evol 69(1):65-80

Gong Z, Han GZ (2018) Euphyllophyte paleoviruses illuminate hidden diversity and macroevolutionary mode of *Caulimoviridae*. J Virol 92(10): e02043-17.

Ghosh S, Ahire M, Patil S et al (2012) Antidiabetic activity of *Gnidia glauca* and *Dioscorea bulbifera*: potent amylase and glucosidase inhibitors. Evid Based Complement Alternat Med 2012:1-10

Guan XR, Zhu L, Xiao ZG, Zhang YL, Chen HB, Yi T (2017) Bioactivity, toxicity and detoxification assessment of *Dioscorea bulbifera* L.: a comprehensive review. Phytochem Rev 16(3):573-601

Hareesh PS, Bhat AI (2010) Seed transmission of *Piper yellow mottle virus* in black pepper (*Piper nigrum* L). J Plant Crops 38(1):62-65

Harper G, Richert-Pöggeler KR, Hohn T, Hull R (2003) Detection of Petunia vein clearing virus: model for the detection of DNA viruses in plants with homologous endogenous pararetrovirus sequences. J Virol Methods 107:177–184

Harrison BD, Roberts IM (1973) Association of virus-like particles with internal brown spot of yam (Dioscorea alata). Tropical Agriculture

Hearon SS, Locke JC (1984) Graft, pollen, and seed transmission of an agent associated with top spotting in *Kalanchoë blossfeldiana*. Plant Dis 68(4):346-350

Hohn T, Richert-Pöggeler KR (2006) Replication of plant pararetroviruses. In: Hefferon KL (ed) Recent Advances in DNA Virus Replication, Research Signpost, Kerala, India, pp 289-319

Ikiriza H, Ogwang PE, Peter EL et al (2019) *Dioscorea bulbifera*, a highly threatened African medicinal plant, a review. Cogent Biol 5(1):1631561

Jacquot E, Hagen LS, Jacquemond M, Yot P (1996) The open reading frame 2 product of cacao swollen shoot badnavirus is a nucleic acid-binding protein. Virol 225(1):191-195

Kalischuk ML, Fusaro AF, Waterhouse PM, Pappu HR, Kawchuk LM (2013) Complete genomic sequence of a *Rubus yellow net virus* isolate and detection of genome-wide pararetrovirus-derived small RNAs. Virus Res 178:306-313

Keith, CV, Ramos-Sobrinho R, Marelli, JP, Brown JK (2021) Construction of an Infectious Clone of the Badnavirus Cacao Swollen Shoot Ghana M Virus and Infectivity by Gene Gun-and *Agrobacterium*-Mediated Inoculation. Front Agron 3: 774863

Kenyon L, Lebas BSM, Seal SE (2008) Yams (*Dioscorea* spp.) from the South Pacific Islands contain many novel badnaviruses: implications for international movement of yam germplasm. Arch Virol 153(5):877-889

Kinupp VF, Lorenzi H (2014) Plantas Alimentícias Não Convencionais (Panc) No Brasil: Guia De Identificação, Aspectos Nutricionais E Receitas Ilustradas. Instituto Plantarum De Estudos Da Flora, São Paulo

Kundu BB, Vanni K, Farheen A, Jha P, Pandey DK, Kumar, V (2021) *Dioscorea bulbifera* L. (Dioscoreaceae): A review of its ethnobotany, pharmacology and conservation needs. S Afr J Bot 140:365-374

Laney AG, Hassan M, Tzanetakis IE (2012) An integrated badnavirus is prevalent in fig germplasm. Phytopathol 102(12):1182-1189

Lebot, V (2019) Tropical root and tuber crops, 2nd edn. Cabi, Wallingford, Oxfordshire, UK ; Boston, MA

Lockhart B, Mollov D, Olszewski N, Goldsmith N (2017) Identification, transmission and genomic characterization of a new member of the family *Caulimoviridae* causing a flower distortion disease of *Rudbeckia hirta*. Virus Res 241:62-67

Lockhart BEL, Autrey LJC (1988) Occurrence in sugarcane of a bacilliform virus related serologically to banana streak virus. Plant Dis 72(3):230-233

Lockhart BEL, Ferji Z (1988) Purification and mechanical transmission of Kalanchoë topspotting-associated virus. In: VII International Symposium on Virus Diseases of Ornamental Plants 234:73-78

Lockhart BEL, Kiratiya-Angul K, Jones P et al (1997) Identification of *Piper yellow mottle virus*, a mealybug-transmitted badnavirus infecting *Piper* spp. in Southeast Asia. Eur J Plant Pathol 103(4):303-311

Lockhart BEL, Olszewski NE (1993) Serological and genomic heterogeneity of banana streak badnavirus: implications for virus detection in *Musa* germplasm. In International Symposium on Genetic Improvement of Bananas for Resistance to Diseases and Pests, CIRAD-FLHOR, Montpellier (France), 7-9 Sep

Lovera M, de Castro GMC, da Rocha Pires N et al (2020) Pyrodextrinization of yam (*Dioscorea* sp.) starch isolated from tubers grown in Brazil and physicochemical characterization of yellow pyrodextrins. Carbohydr Polym 242:116382. https://doi.org/10.1016/j.carbpol.2020.116382

Luo GF, Podolyan A, Kidanemariam DB, Pilotti C, Houliston, G, & Sukal AC (2022) A Review of Viruses Infecting Yam (*Dioscorea* spp.). Viruses 14(4):662

Mantell SH, Haque SQ (1978) Incidence of Internal Brown Spot Disease in White Lisbon Yams (Dioscorea alata) During Storage. Experimental Agriculture 14:167–172. https://doi.org/10.1017/S0014479700008589

Migliori A, Lastra R (1978) Study of viruses on *Commelina diffusa* Burm. in Guadeloupe. Ann Phytopathol 10(4):467-477)

Moura RM (2005) Doenças do Inhame-Da-Costa. In: Kimati H, Amorim L, Bergamin-Filho A, Camargo LEA, Rezende JAM (eds.) Manual De Fitopatologia V2, 4th edn. Ceres, São Paulo, pp 415-419

Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Molecular and Cellular Probes 16:223–229. https://doi.org/10.1006/mcpr.2002.0415

Narula A, Kumar S, Bansal KC, Srivastava PS (2003) In vitro micropropagation, differentiation of aerial bulbils and tubers and diosgenin content in *Dioscorea* bulbifera. Planta Med 69:778-779. http://dx.doi.org/10.1055/s-2003-42781

Nasim N, Dey N (2021) Pararetroviruses: Plant Infecting dsDNA Viruses. Plant Mol Biol Report 40:106-118

Nkere CK, Oyekanmi JO, Silva G, Bömer M, Atiri GI, Onyeka J, Maroya NG, Seal SE, Kumar PL (2018) Chromogenic detection of yam mosaic virus by closed-tube reverse transcription loop-mediated isothermal amplification (CT-RT-LAMP). Arch Virol. 163(4):1057-1061. doi: 10.1007/s00705-018-3706-0. Epub 2018 Jan 8. PMID: 29308543; PMCID: PMC5854734.

Noronha MA, Assunção MC, Costa MGS, et al (2020) First Report of *Aphelenchoides besseyi* Causing Leaf Spot on Yam (*Dioscorea cayenensis*) in Brazil. Plant Disease 104:3083. https://doi.org/10.1094/PDIS-03-20-0511-PDN

Omura T, Minobe Y, Kimura I, Hibino H, Tsuchizaki T, Saito Y (1983) Improved purification procedure and RNA segments of *Rice ragged stunt virus*. Jpn J Phytopathol 49(5):670-675

Phillips S, Briddon RW, Brunt AA, Hull R (1999) The partial characterization of a badnavirus infecting the Greater Asiatic or water yam (*Dioscorea alata*). J Phytopathol 147(5):265-269

Posnette AF (1944) Viruses of cacao in Trinidad. Trop Agric 21:105–106

Quainoo AK, Wetten AC, Allainguillaume J (2008) Transmission of cocoa swollen shoot virus by seeds. J Virol Methods 150(1-2):45-49

Santos GBL, Ferro MMM, Feijó FM, Ramos-Sobrinho R, Assunção IP, Lima GSA (2022) Badnavirus sequence diversity reveals one previously uncharacterized badnavirus associated with air yam (*Dioscorea bulbifera* L.) in Brazil. Trop Plant Pathol 47:807–813

Seal S, Muller E (2007) Molecular analysis of a full-length sequence of a new yam badnavirus from *Dioscorea sansibarensis*. Arch Virol 152(4):819-825

Seal S, Turaki A, Muller E et al (2014) The prevalence of badnaviruses in West African yams (*Dioscorea cayenensis-rotundata*) and evidence of endogenous pararetrovirus sequences in their genomes. Virus Res 186:14-154

Silva G, Bömer M, Rathnayake AI et al (2019) Molecular characterization of a new virus species identified in yam (*Dioscorea* spp.) by high-throughput sequencing. Plants 8(6):167

Sukal A, Kidanemariam D, Dale J, et al (2017) Characterization of badnaviruses infecting Dioscorea spp. in the Pacific reveals two putative novel species and the first report of dioscorea bacilliform RT virus 2. Virus Research 238:29–34. https://doi.org/10.1016/j.virusres.2017.05.027

Sukal AC, Kidanemariam DB, Dale JL, Harding RM, James AP (2020) Characterization and genetic diversity of Dioscorea bacilliform viruses present in a Pacific yam germplasm collection. Plant Pathol 69(3):576-584

Temin HM (1989) Retrons in bacteria. Nature, 339:254-255

Teycheney PY, Geering AD, Dasgupta I et al (2020) ICTV Virus taxonomy profile: *Caulimoviridae*. J Gen Virol 101(10):1025

Teycheney PY, Geering ADW (2011) Endogenous viral sequences in plant genomes. In: Caranta C, Aranda MA, Tepfer M, Lopez-Moya JJ (eds) Recent Advances in Plant Virology, Caister Academic Press , UK, pp 347-366

Thouvenel JC, Dumont R (1988) An epidemiological approach to the study of yam mosaic virus in the Ivory Coast. Proceedings of the International Society for Tropical Root Crops, Bangkok, Thailand, 643:648

Umber M, Filloux D, Muller E et al (2014) The genome of African yam (*Dioscorea cayenensis-rotundata* complex) hosts endogenous sequences from four distinct badnavirus species. Mol Plant Pathol 15:790-801

Umber M, Gomez R-M, Gélabale S, et al (2017) The genome sequence of Dioscorea bacilliform TR virus, a member of the genus Badnavirus infecting Dioscorea spp., sheds light on the possible function of endogenous Dioscorea bacilliform viruses. Arch Virol 162:517–521. https://doi.org/10.1007/s00705-016-3113-3

Wilkin P (2001) Dioscoreaceae of South-Central Africa. Kew Bull 56(2):361-404

Yang IC, Hafner GJ, Revill PA, Dale JL, Harding RM (2003) Sequence diversity of South Pacific isolates of *Taro bacilliform virus* and the development of a PCR-based diagnostic test. Arch Virol 148(10):1957-1968

Zannou A, Ahanchédé A, Struik PC, Richards P, Zoundjihékpon J, Tossou R, Vodouhè S (2004) Yam and cowpea diversity management by farmers in the Guinea-Sudan transition zone of Benin. NJAS - Wagening J Life Sci 52(3–4):393-420. https://doi.org/10.1016/S1573-5214(04)80023-X

# CHAPTER 1

## COMPLETE GENOME SEQUENCE OF THE NEW BADNAVIRUS Dioscorea bacilliform BL virus INFECTING *Dioscorea bulbifera* L. IN BRAZIL

Adso L.S. Figueiredo, Giancarlo B.L. Santos, Roberto Ramos-Sobrinho, Mayra M.M. Ferro, Iraildes P. Assunção, Gaus S.A. Lima. Complete genome sequence of the new badnavirus Dioscorea bacilliform BL virus infecting *Dioscorea bulbifera* L. in Brazil. Archives of Virology, *submitted*.

**Complete genome sequence of the new badnavirus Dioscorea bacilliform BL virus infecting *Dioscorea bulbifera* in Brazil**

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**Abstract**

A complex of badnavirus species collectively referred to as *Dioscorea* bacilliform viruses (DBVs) is widespread wherever yams (*Dioscorea* spp.) are cultivated. To characterize the complete genome of a putatively new badnavirus associated with air yam (*Dioscorea bulbifera* L.) in Brazil, total DNA from a dioscorea bacilliform BL virus (DBBLV)-infected plant was used as template for badnaviral genome amplification by rolling circle amplification (RCA) and subjected to high-throughput Illumina sequencing. Two badnavirus-like full-length genomes of 7,196 (DBBLV-DBH1) and 7,342 (DBBLV-DBH3) bp in size were recovered from 1,273,444 and 539,840 reads and a coverage depth of 177 and 74x, respectively. Based on pairwise nucleotide sequence identity for the reverse transcriptase (RT) and ribonuclease H (RNase H) genomic region, both isolates belong to the species novel *Dioscorea bacilliform BL virus*, sharing 75-95% identity. The RT-RNase H-inferred Bayesian phylogenetic tree showed that the new isolates grouped with other DBBLV sequences. The isolate DBBLV-DBH1 was predicted as recombinant, with breakpoints located in ORF1 and ORF3, while DBBLV-DBH3 was identified as putative minor parent in two independent recombination events having isolate of *Dioscorea bacilliform AL virus* and *Dioscorea bacilliform RT virus 3* as recombinant sequences.

**Keywords:** *Dioscorea*, dioscorea bacilliform viruses, *Caulimoviridae*, high-throughput sequencing, Dioscoreaceae.

## 1. Introduction

The genus *Dioscorea* (family Dioscoreaceae) is comprised by more than 640 plant species, and cultivated yams (*Dioscorea* spp.) are considered staple crops in several countries from Africa, South America, Asia, and tropical Pacific [1]. Yams are among the most important tuber and root crops worldwide, being surpassed only by potato, sweet potato, and cassava [1, 2]. In Brazil, yams are also of great socioeconomic relevance, especially for low-income communities where these crops play a crucial role for food security [3]. Although *Dioscorea bulbifera*, also known as air yam, is considered an invasive species that may represent a problem to native forests [1, 4], it is one of the 12 most commonly cultivated yam species and has many known ethnobotanical uses and pharmacological properties [1, 5, 6].

Viruses pose a serious threat to commercially grown plants, especially to crops that are vegetatively propagated like yams as the use of contaminated planting materials usually results in viral accumulation and perpetuation, and loss of host vigor [7, 8, 9], therefore, developing reliable diagnostic tools is a major step to providing virus-free propagative materials [9]. Unfortunately, a high diversity of viral species from different families such as *Alphaflexiviridae*, *Betaflexiviridae*, *Bromoviridae*, *Caulimoviridae*, *Closteroviridae*, *Potyviridae*, *Secoviridae*, and *Tombusviridae* have been reported infecting yams, making it difficult the routine deployment of molecular tools for virus diagnostics and identification [2, 10, 11].

The genus *Badnavirus* (family *Caulimoviridae*) is the second largest DNA plant virus group and infect a wide range of economically important crops such as yams [2, 12]. Badnaviruses have non-covalently closed, double-stranded circular DNA genomes with 7.2-9.2 kbp in size that are encapsidated into bacilliform particles and encode at least three open reading frames (ORFs) [13, 14]. The ORF1 protein is virion-associated [15], and ORF2 encodes a nucleic acid-binding protein [16]. ORF3 is the largest, ~80% of the viral genome, and encodes a polyprotein with conserved domains for the movement protein, capsid protein, aspartate protease, reverse transcriptase (RT), and ribonuclease H (RNase H) [13]. The criterion of ≥80% nucleotide identity for RT-RNase H sequences has been established, by the International Committee on Taxonomy of Viruses (ICTV), as the threshold for species demarcation into genus *Badnavirus* [13].

The yam-infecting badnaviruses are collectively referred to as *Dioscorea* bacilliform viruses (DBVs), and eight species are currently accepted by the ICTV: *Badnavirus alphadioscoreae*, *Badnavirus betadioscoreae*, *Badnavirus gammadioscoreae*, *Badnavirus deltadioscoreae*, *Badnavirus epsilondioscoreae*, *Badnavirus zetadioscoreae*, *Badnavirus etadioscoreae*, and *Badnavirus tetadioscoreae* (https://talk.ictvonline.org/ictv-

reports/ictv_online_report/reverse-transcribing-dna-and-rna-viruses/w/caulimoviridae).
Recently, a putative new badnaviral species infecting *D. bulbifera* plants in Brazil has been characterized based on RT-RNase H partial sequences and tentatively named as *Dioscorea bacilliform BL virus* [17].

## 2. Materials and Methods

A *D. bulbifera* plant collected in the state of Alagoas, Brazil, in 2021, showing leaf curling and chlorosis symptoms was previously diagnosed by our research group [17] as being infected by the putative new badnavirus Dioscorea bacilliform BL virus (DBBLV). To characterize the circular DNA virome of this sample, total DNA was extracted from leaf tissue using the method described in Doyle & Doyle [18] and used as template for enrichment of circular DNA viral genomes via rolling circular amplification (RCA) using the Illustra TempliPhi Amplification Kit (GE Healthcare, Buckinghamshire, United Kingdom) following the manufacturer's protocol. Paired-end libraries with 250 bp mean insert size were constructed, from RCA aliquots, using the Illumina DNA Prep kit (Illumina Inc., California, USA) according to the manufacturer's instructions, and subjected to high-throughput sequencing (HTS) using the Illumina MiSeq platform at the Life Sciences Core Facility (LaCTAD) from the State University of Campinas (Campinas, São Paulo, Brazil).

The quality of the Illumina reads was assessed with the program FastQC v.0.11.5 (www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the software Trimmomatic v.0.38 [19] was used to trim low-quality reads (Q score < 20) and adaptor sequences with a sliding window of 4. *De novo* assembly of the trimmed Illumina reads was performed in SPAdes v.3.15.4 [20]. To further evaluate the quality of the circular contigs, Bowtie2 [21] was used to map trimmed reads against the badnavirus-like genome sequences and manual adjustment was performed in IGV v.2.4.13[22]. Then, Illumina-based contigs were subjected to BLASTn analysis [23] to find the most similar sequences in the NCBI-GenBank non-redundant database. Putative viral ORFs were identified with NCBI ORF Finder (www.ncbi.nlm.nih.gov/orffinder).

Complete genomes of yam- and taro (*Colocasia esculenta*)-infecting badnaviruses were downloaded from NCBI-GenBank (accessed on June 05, 2022; Supplementary Table S1) and, together with the new genomes recovered from the *D. bulbifera* sample, comprised the dataset used in the analyses reported in the present study. A second dataset consisted of RT-RNase H (~1,230 bp) sequences retrieved from the full-length genomes. Pairwise nucleotide sequence comparisons were carried out using both complete genome and RT-RNase H datasets in Sequence Demarcation Tool (SDT) v.1.2 [24]. The ICTV-established species demarcation criterion of ≥80% nucleotide identity for the RT-RNase H loci was adopted [13]. Multiple

sequence alignments were constructed using the MUSCLE algorithm [25] implemented in MEGA 7 [26]. The aligned complete genomes were analyzed for putative recombination events using the Recombination Detection Program (RDP) v.4. [27], and only recombination events predicted by at least five different methods implemented in the RDP4 package were considered reliable.

Bayesian phylogenetic trees were inferred from full-length genome and RT-RNase H nucleotide sequences using MrBayes v.3.2.3 [28] on the CIPRES web portal [29]. The general time reversible (GTR) with a gamma (G) rate heterogeneity and invariable (I) sites was determined as the best-fit model of nucleotide substitution, according to the Akaike Information Criterion (AIC), for both datasets using MrModelTest v.2.3 [30]. The Bayesian Inference (BI) analysis was conducted using two replicates of four chains each for 10 million generations with sampling at every 1,000 steps. Five thousand trees (2,500 per run) were discarded as a burn-in. The posterior probability values [31] were determined from the majority-rule consensus trees reconstructed based on the 15,000 remaining trees. The programs FigTree v.1.4 (ztree.bio.ed.ac.uk/software/figtree) and Inkscape v.1.1 (https://inkscape.org/pt/) were used to visualize and edit the phylogenetic trees.

## 3. Results and Discussion

Two complete badnavirus-like genomes of 7,196 (DBBLV-DBH1) and 7,342 (DBBLV-DBH3) bp in size were *de novo* assembled from 1,273,444 and 539,840 Illumina reads, with a coverage depth of 177 and 74x, respectively. Three ORFs (ORFs 1-3) were predicted in each viral genome, and additional badnaviral hallmark features such as $tRNA^{met}$ primer binding site sequence, polyadenylation signal, and TATA box were also found (Fig. 1). The new genome sequences reported here were submitted to the NCBI-GenBank database under accession nos. ON792314-ON792315.

The pairwise nucleotide sequence comparisons showed that the RT-RNase H sequences of the newly characterized *D. bulbifera*-infecting badnaviral isolates shared 78% identity one with another, and ≥88% nucleotide identity with at least one isolate of the putative species novel *Dioscorea bacilliform BL virus* deposited in GenBank (Supplementary Figure S1). According to the ICTV-approved *Badnavirus* species demarcation criterion of ≥80% nucleotide identity for RT-RNase H sequences, the new full-length genome sequences reported here represent isolates of DBBLV [13], herein referred to as DBBLV-DBH1 and DBBLV-DBH3. Further, the complete genomes of DBBLV-DBH1 and DBBLV-DBH3 shared 71% nucleotide identity each other, and 62-76% identity with previously reported yam-infecting badnaviruses (Supplementary Figure S2). The relatively low nucleotide identity shared by the new DBBLV

isolates, at the genome level, suggest these isolates might represent diverging DBBLV lineages probably affected by recurring interspecies recombination (see below).

The recombination analysis performed in the RDP4 package identified at least four independent recombination events having the new DBBLV isolates as predicted recombinant or parental sequences (Supplementary Table S2). The isolate DBBLV-DBH1 was identified as recombinant, with recombination breakpoints located in ORF1 and initial portion of ORF3 (near to ORF2), and dioscorea bacilliform TR virus (DBTRV)- and dioscorea bacilliform AL virus (DBTRV)-like isolates predicted as putative minor and major parents, respectively (event 12; Supplementary Table S2). Further, an independent recombination event having an DBTRV isolate as recombinant sequence and breakpoints in the central region of ORF3 was identified, with dioscorea bacilliform AL virus 2 (DBALV)- MH404158 and DBBLV-DBH1 predicted as minor and major parents, respectively (event 16; Supplementary Table S2). Two additional interspecies recombination events involving the DBBLV-DBH3 isolate as the predicted minor parent were identified in putatively recombinant DBALV and dioscorea bacilliform RT virus 3 (DBRTV3) sequences (events 4 and 7, respectively; Supplementary Table S2).

The Bayesian phylogenetic tree reconstructed based on complete genome sequences showed that the new DBBLV isolates were more closely related to DBALV sequences, but DBBLV-DBH1 and DBBLV-DBH3 did not form a monophyletic group (Fig. 2a). The RT-RNase H tree showed some topological incongruences when compared to the full-length genomes, with DBALV and DBBLV sequences grouping into two well-supported sister clades in this tree (Fig. 2b). Together, these results suggest the clustering observed for the DBBLV genomes is probably affected by the complex recombination pattern observed here.

An increasing number of badnaviral species has been reported infecting *Dioscorea* spp. in the main cultivation regions, and at least eight species are officially recognized by the ICTV (https://talk.ictvonline.org/ictv-reports/ictv_online_report/reverse-transcribing-dna-and-rna-viruses/w/caulimoviridae). Here, complete genome sequences of the *D. bulbifera*-infecting badnavirus *Dioscorea bacilliform BL virus* were characterized for the first time, contributing to the knowledge about the viral species diversity affecting yams. Considering badnaviruses may cause significant yield and quality losses in commercial yam plantations, additional studies aiming to assess the potential damages caused by DBBLV, and other badnaviruses, to the most commonly cultivated *Dioscorea* species are needed to support disease management programs. Further, these results highlight the importance of developing molecular tools that could be used in routine detection and identification of yam-associated viruses.

Recombination seems to strongly affect the genetic variability of badnaviruses infecting cultivated hosts such as *Theobroma cacao* (cacao), *Musa* spp. (banana), *Saccharum* spp.

(sugarcane) and *Dioscorea rotundata* [9, 32-34]. The intergenic region has been reported as hotspot of recombination and most recombination breakpoints are between this region and ORF1 [9, 34], ORF2 [33, 35] or C-terminal of ORF3 [32-33], very similar to what was reported here (Supplementary Table S2). Further, this complex interspecies recombination pattern observed for both DBBLV-DBH1 and DBBLV-DBH3 isolates reinforces that this evolutionary mechanism might be important for molecular diversification of yam-infecting badnaviruses. Also, the characterization of additional DBV complete genomes associated with cultivated and wild yam species will help to better understand the genetic diversity and evolutionary forces shaping the population structuring of these viruses.

## 4. References


1. Lebot, V (2019) Tropical root and tuber crops, 2nd edn. Cabi, Wallingford, Oxfordshire, UK, Boston, MA
2. Luo GF, Podolyan A, Kidanemariam DB, Pilotti C, Houliston, G, & Sukal AC (2022) A Review of Viruses Infecting Yam (*Dioscorea* spp.). Viruses 14(4):662
3. Siqueira MVBM (2011) Yam: a neglected and underutilized crop in Brazil. Hortic Bras, 29, 16-20.
4. Dey KK, Sugikawa J, Kerr C, Melzer, MJ (2019) Air potato (*Dioscorea bulbifera*) plants displaying virus-like symptoms are co-infected with a novel potyvirus and a novel ampelovirus. Virus Genes 55(1):117-121
5. Ikiriza H, Ogwang PE, Peter EL et al (2019) *Dioscorea bulbifera*, a highly threatened African medicinal plant, a review. Cogent Biol 5(1):1631561
6. Kundu BB, Vanni K, Farheen A, Jha P, Pandey DK, Kumar, V (2021) *Dioscorea bulbifera* L. (Dioscoreaceae): A review of its ethnobotany, pharmacology and conservation needs. S Afr J Bot 140:365-374
7. Seal S, Muller E (2007) Molecular analysis of a full-length sequence of a new yam badnavirus from *Dioscorea sansibarensis*. Arch Virol 152(4):819-825
8. Kenyon L, Lebas BSM, Seal SE (2008) Yams (*Dioscorea* spp.) from the South Pacific Islands contain many novel badnaviruses: implications for international movement of yam germplasm. Arch Virol 153(5):877-889
9. Bömer M, Rathnayake AI, Visendi P, Silva G, Seal SE (2018) Complete genome sequence of a new member of the genus *Badnavirus*, *Dioscorea bacilliform RT virus 3*, reveals the first evidence of recombination in yam badnaviruses. Arch Virol 163(2):533-538
10. Silva G, Bömer M, Rathnayake AI et al (2019) Molecular characterization of a new virus species identified in yam (*Dioscorea* spp.) by high-throughput sequencing. Plants 8(6):167

11. Sukal AC, Kidanemariam DB, Dale JL, Harding RM, James AP (2020) Characterization and genetic diversity of Dioscorea bacilliform viruses present in a Pacific yam germplasm collection. Plant Pathol 69(3):576-584
12. Eni AO (2008) Sequence diversity among badnavirus isolates infecting yam (*Dioscorea* spp.) in Ghana, Togo, Benin and Nigeria. Arch Virol 153:2263-2272
13. Teycheney PY, Geering AD, Dasgupta I et al (2020) ICTV Virus taxonomy profile: *Caulimoviridae*. J Gen Virol 101(10):1025
14. Bhat AI, Hohn T, Selvarajan R (2016) Badnaviruses: the current global scenario. Viruses 8(6):177
15. Cheng CP, Lockhart BE, Olszewski NE (1996) The ORF I and II proteins of *Commelina yellow mottle virus* are virion-associated. Virol 223:263-271
16. Jacquot E, Hagen LS, Jacquemond M, Yot P (1996) The open reading frame 2 product of cacao swollen shoot badnavirus is a nucleic acid-binding protein. Virol 225(1):191-195
17. Santos GBL, Ferro MMM, Feijó FM, Ramos-Sobrinho R, Assunção IP, Lima GSA (2022) Badnavirus sequence diversity reveals one previously uncharacterized badnavirus associated with air yam (*Dioscorea bulbifera* L.) in Brazil. Trop Plant Pathol *submitted*
18. Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15
19. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina Sequence Data. Bioinformatics 30(15): 2114–2120.
20. Bankevich A, Nurk S, Antipov D et al (2012) SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J Comput Biol 19(5):455-77
21. Langmead B, Salzberg S (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359
22. Robinson JT, Thorvaldsdóttir H, Wenger AM, Zehir A, Mesirov JP (2017). Variant Review with the Integrative Genomics Viewer (IGV). Cancer Res 77:31-34
23. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
24. Muhire BM, Varsani A, Martin DP (2014) SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. PLoS ONE 9:e108277
25. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797
26. Kumar S, Stecher G, Tamura K (2015) MEGA7: molecular evolutionary genetics analysis version 7. Mol Biol Evol 33:1870–1874
27. Martin DP, Murrell B, Golden M, Khoosal A, Muhire B (2015) RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evol 1:1–5

28. Ronquist F, Teslenko M, van der Mark et al (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Syst Biol 61:539-542
29. Miller MA, Holder MT, Vos R, Midford PE, Liebowit, T, Chan L, Hoover P, Warnow T (2010) The CIPRES Portals. CIPRES. Available online: http://www.phylo.org/sub-sections/portal (accessed on June 14, 2022).
30. Nylander JAA (2004) MrModeltest2. v.2.3 Evolutionary Biology Centre, Uppsala University Available online: https://github.com/nylander/MrModeltest2
31. Rannala B, Yang Z (1996) Probability distributions of molecular evolutionary trees: a new method of phylogenetic inference. J Mol Evol 43:304–311
32. Sharma SK, Kumar PV, Geetanjali AS, Pun KB, Baranwal VK (2015) Subpopulation level variation of banana streak viruses in India and common evolution of banana and sugarcane badnaviruses. Virus Genes 50:450-465
33. Ramos-Sobrinho R, Chingandu N, Gutierrez AO, Marelli J-P, Brown JK (2020) A Complex of Badnavirus Species Infecting Cacao Reveals Mixed Infections, Extensive Genomic Variability, and Interspecific Recombination. Viruses 12:443 doi:10.3390/v12040443
34. Janiga PK, Nithya K, Viswanathan R (2023) Dynamics of Genetic Diversity Among Indian Sugarcane Bacilliform Virus Species and Implications of Associated Recombination Events in the Virus. Sugar Tech 25:705–716. https://doi.org/10.1007/s12355-022-01224-1
35. Ishwara Bhat A, Selvarajan R, Balasubramanian V (2023) Emerging and Re-Emerging Diseases Caused by Badnaviruses. Pathogens 12:245. https://doi.org/10.3390/pathogens12020245

**List of Figures**

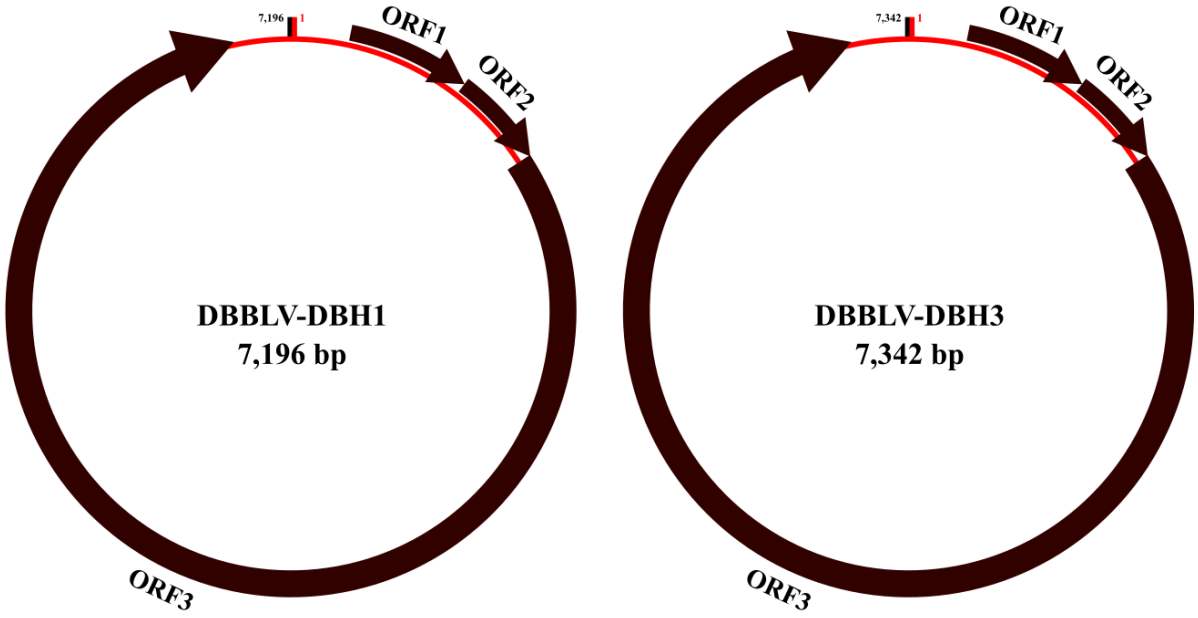



Fig. 1. Genomic organization of the newly discovered badnaviruses dioscorea bacilliform BL virus isolates (DBBLV-DBH1 and DBBLV-DBH3) infecting *Dioscorea bulbifera* in Brazil.

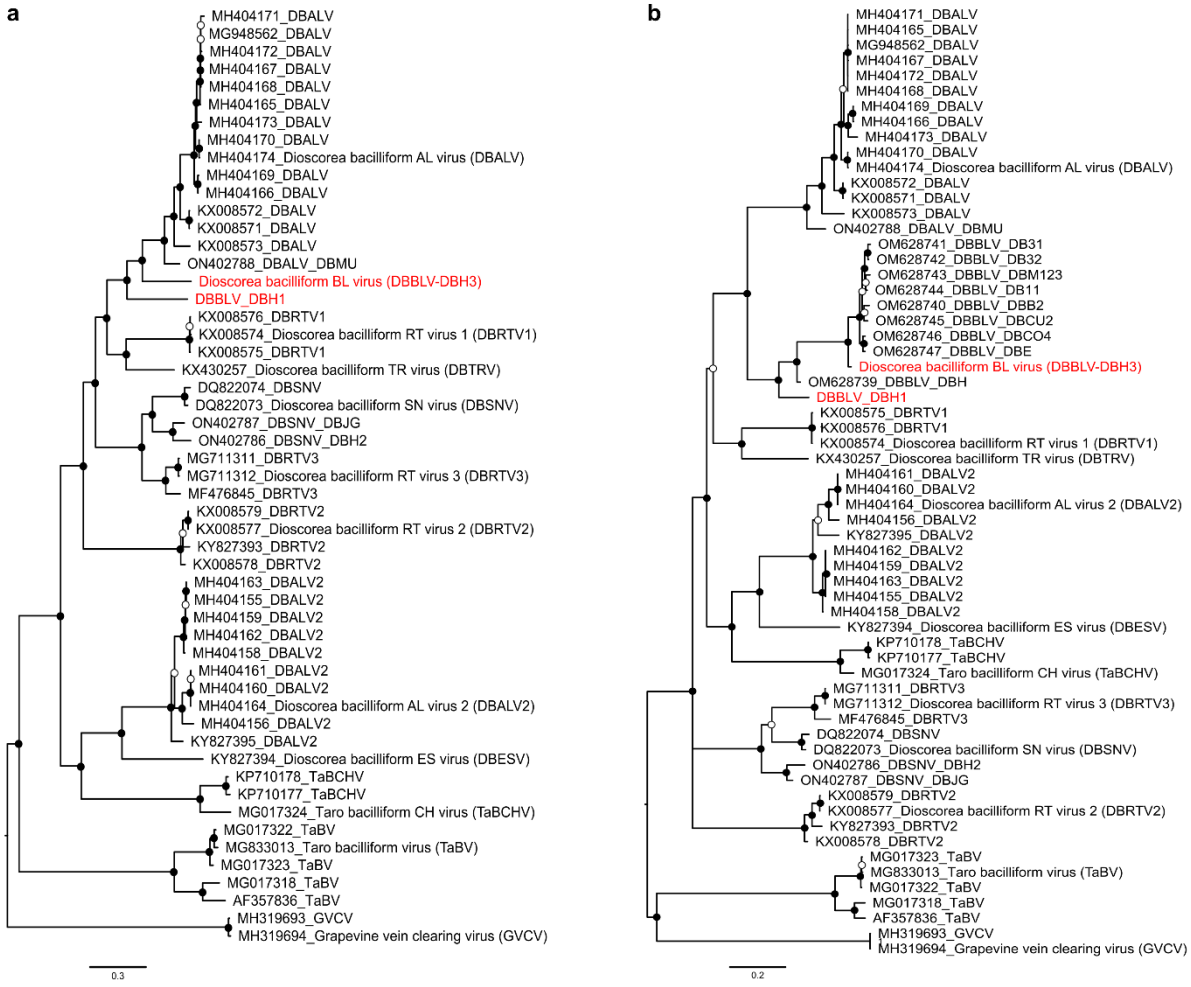



Fig. 2. Bayesian phylogenetic trees based on complete genome (a) and RT-RNase H (b) nucleotide sequences of yam-infecting badnaviruses. Posterior probability values between 0.99-1.0 (filled circles) and 0.50-0.98 (empty circles) are shown near to each branch node. The isolates reported here are indicated in red. Dioscorea bacilliform AL virus (DBALV), dioscorea bacilliform AL virus 2 (DBALV2), Dioscorea bacilliform BL virus (DBBLV), dioscorea bacilliform RT virus 1 (DBRTV1), dioscorea bacilliform RT virus 2 (DBRTV2), dioscorea bacilliform RT virus 3 (DBRTV3), dioscorea bacilliform TR virus (DBTRV), dioscorea bacilliform SN virus (DBSNV), dioscorea bacilliform ES virus (DBESV), taro bacilliform virus (TaBV), taro bacilliform CH virus (TaBCHV), and grapevine vein clearing virus (GVCV).

**Supplementary Material**

Supplementary Table S1. Complete genome sequences of yam-infecting badnaviruses downloaded from NCBI-GenBank (accessed on June 5, 2022).

| Species | Acronym | GenBank Accession Nos. |
|---|---|---|
| *Dioscorea bacilliform AL virus* | DBALV | MG948562, MH404165, MH404166, MH404167, MH404168, MH404169, MH404170, MH404171, MH404172, MH404173, MH404174, KX008571, KX008572, KX008573, ON402788 |
| *Dioscorea bacilliform AL virus 2* | DBALV2 | KY827395, MH404155, MH404156, MH404158, MH404159, MH404160, MH404161, MH404162, MH404163, MH404164 |
| *Dioscorea bacilliform RT virus 1* | DBRTV1 | KX008574, KX008575, KX008576 |
| *Dioscorea bacilliform RT virus 2* | DBRTV2 | KY827393, KX008577, KX008578, KX008579 |
| *Dioscorea bacilliform RT virus 3* | DBRTV3 | MG711311, MG711312, MF476845 |
| *Dioscorea bacilliform TR virus* | DBTRV | KX430257 |
| *Dioscorea bacilliform SN virus* | DBSNV | DQ822073, DQ822074, ON402786, ON402787 |
| *Dioscorea bacilliform ES virus* | DBESV | KY827394 |
| *Taro bacilliform virus* | TaBV | AF357836, MG017322, MG017323, MG833013, MG017318 |
| *Taro bacilliform CH virus* | TaBCHV | KP710178, KP710177, MG017324 |
| *Grapevine vein clearing virus* | GVCV | MH319693, MH319694 |

Supplementary Table S2. Predicted recombination events detected, based on complete genome sequences, within the *Colocasia*- and *Dioscorea*-infecting badnaviruses.

| Events | Breakpoints[1] | | Recombinant[4] | Parents[4] | | Methods[2] | *P* values[3] | |
|---|---|---|---|---|---|---|---|---|
| | Begin | End | | Minor | Major | | Lowest | Highest |
| 1 | 179 | 428 | MH404171_DBALV | Unknown | MG948562_DBALV | RGBMCS3 | 7.8E-126 | 1.5E-11 |
| 2 | 7526 | 687 | ^KX008572_DBALV<br>KX008571_DBALV | MF476845_DBRTV3 | MH404172_DBALV<br>MH404165_DBALV | RGBMCS3 | 4.8E-77 | 3.7E-20 |
| 3 | 36 | 646 | ^KX008573_DBALV | MG711312_DBRTV3 | MH404172_DBALV | RBMCS3 | 1.5E-51 | 1.2E-16 |
| 4 | 7405 | 1060 | ^ON402788_DBALV | **DBBLV_DBH3** | MH404172_DBALV | RGBMCS3 | 6.5E-41 | 7.4E-12 |
| 5 | 74 | 628 | KX430257_DBTRV | MH404168_DBALV | KX008576_DBRTV1 | RGBMCS3 | 1.6E-34 | 2.8E-04 |
| 6 | 474 | 560 | ^MG948562_DBALV | Unknown | MH404172_DBALV | RGBMCS3 | 5.7E-33 | 1.4E-10 |
| 7 | 81 | 666 | ^MF476845_DBRTV3<br>MG711311_DBRTV3<br>MG711312_DBRTV3 | **DBBLV_DBH3** | DQ822073_DBSNV<br>DQ822074_DBSNV | RGBMCS3 | 9.4E-29 | 1.5E-10 |
| 8 | 309* | 550 | MH404169_DBALV | Unknown | MH404170_DBALV | RGBMCS3 | 2.6E-22 | 3.6E-02 |
| 9 | 7231 | 7320 | ^MG711312_DBRTV3 | DQ822073_DBSNV | MG711311_DBRTV3 | RGBM3 | 2.5E-16 | 1.6E-04 |
| 10 | 693* | 923 | MG711311_DBRTV3<br>MG711312_DBRTV3 | KX008573_DBALV | MF476845_DBRTV3 | RGBMCS3 | 1.3E-15 | 1.3E-02 |
| 11 | 1241* | 1276 | MG833013_TaBV | Unknown | MG017323_TaBV | RGBM3 | 5.7E-15 | 1.0E-04 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 12 | 614* | 1341 | **^DBBLV_DBH1** | KX430257_DBTRV | MG948562_DBALV | **R**BMCS3 | **6.5E-15** | **5.4E-06** |
| 13 | 460 | 551 | ^MH404172_DBALV | Unknown | MH404170_DBALV | RGBMCS | **5.2E-19** | **2.7E-05** |
| 14 | 2942 | 3171 | ^ON402788_DBALV | KX008571_DBALV | Unknown | RGBS3 | **8.7E-08** | **1.2E-03** |
| 15 | 1191 | 1234 | ^MG017318_TaBV | Unknown | AF357836_TaBV | RGBMCS3 | **6.0E-18** | **4.9E-02** |
| 16 | 3832 | 4175 | KX430257_DBTRV | MH404158_DBALV2 | **DBBLV_DBH1** | **R**BCS3 | **1.9E-06** | **0.04** |
| 17 | 2634 | 2994 | ^KY827393_DBRTV2 | Unknown | KX008577_DBRTV2 | RGBMCS | **1.6E-05** | **3.5E-02** |

[1]Numbering starts at the 5' end of the minus-strand primer-binding site and increases clockwise.

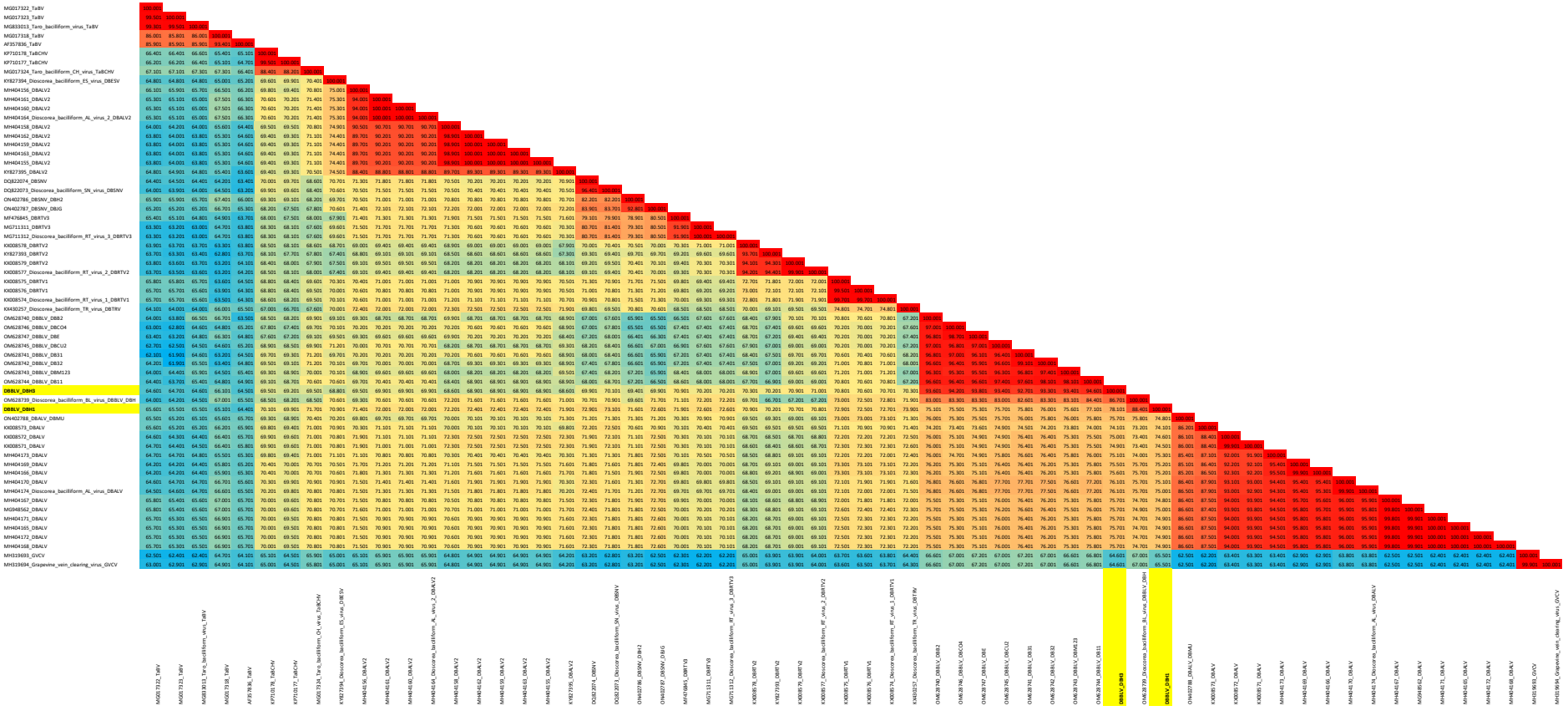
[2]R, RDP; G, GeneConv; B, Bootscan; M, MaxChi; C, Chimera; S, SisScan; 3, 3SEQ.

[3]The *P* values are for the methods indicated in bold (highest) and red (lowest).

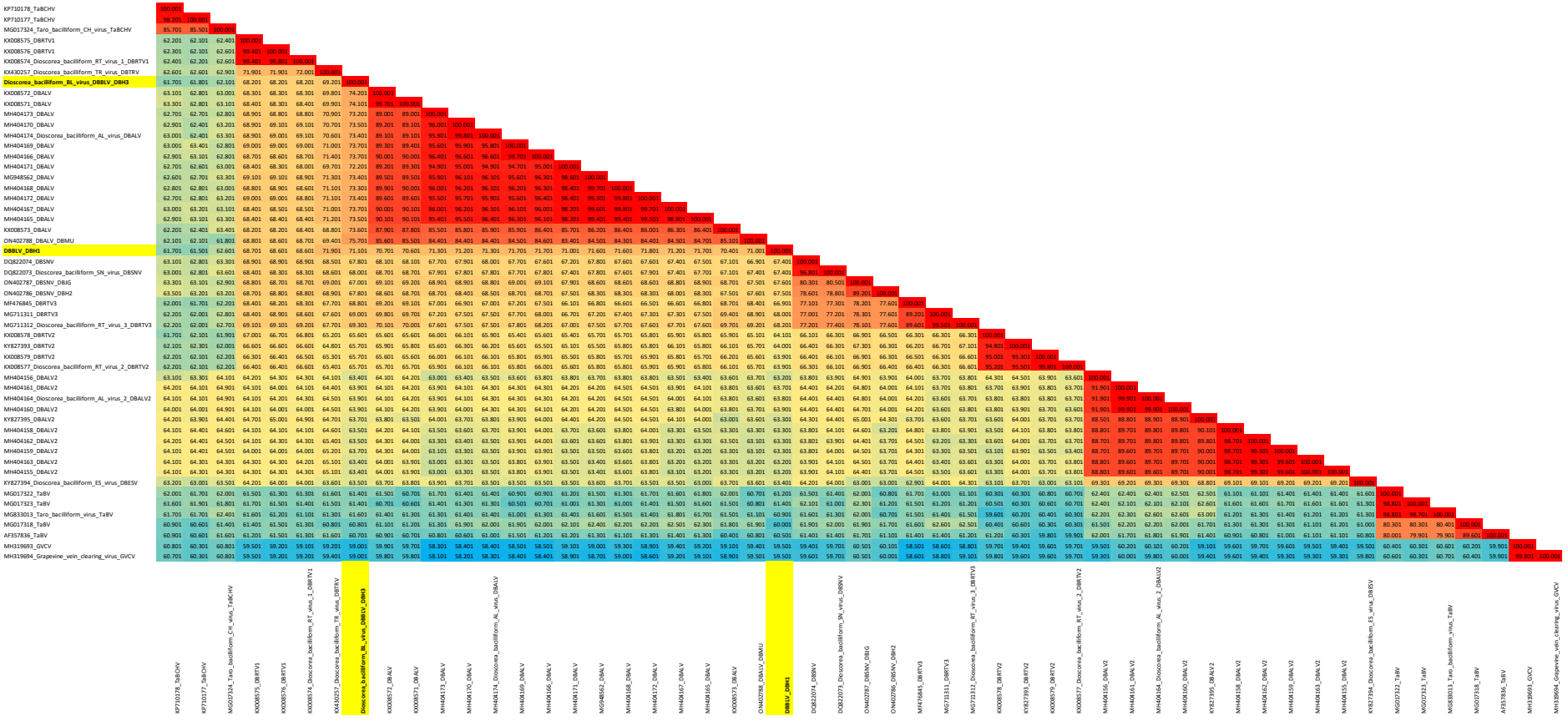
[4]DBALV=dioscorea bacilliform AL virus; DBALV2=dioscorea bacilliform AL virus 2, DBBLV=dioscorea bacilliform BL virus, DBRTV1=dioscorea bacilliform RT virus 1, DBRTV2=dioscorea bacilliform RT virus 2, DBRTV3=dioscorea bacilliform RT virus 3, DBTRV=dioscorea bacilliform TR virus, DBSNV=dioscorea bacilliform SN virus, TaBV=taro bacilliform virus.

^ The recombinant sequence may have been misidentified (one of the identified parents might be the recombinant).

* The recombinant breakpoint may have been misidentified.



Supplementary Figure S1. Pairwise sequence comparisons based on RT-RNase H nucleotide sequences of yam-infecting badnaviruses. Dioscorea bacilliform AL virus (DBALV), dioscorea bacilliform AL virus 2 (DBALV2), Dioscorea bacilliform BL virus (DBBLV), dioscorea bacilliform RT virus 1 (DBRTV1), dioscorea bacilliform RT virus 2 (DBRTV2), dioscorea bacilliform RT virus 3 (DBRTV3), dioscorea bacilliform TR virus (DBTRV), dioscorea bacilliform SN virus (DBSNV), dioscorea bacilliform ES virus (DBESV), taro bacilliform virus (TaBV), taro bacilliform CH virus (TaBCHV), and grapevine vein clearing virus (GVCV).



Supplementary Figure S2. Pairwise sequence comparisons based on complete genome sequences of yam-infecting badnaviruses. Dioscorea bacilliform AL virus (DBALV), dioscorea bacilliform AL virus 2 (DBALV2), Dioscorea bacilliform BL virus (DBBLV), dioscorea bacilliform RT virus 1 (DBRTV1), dioscorea bacilliform RT virus 2 (DBRTV2), dioscorea bacilliform RT virus 3 (DBRTV3), dioscorea bacilliform TR virus (DBTRV), dioscorea bacilliform SN virus (DBSNV), dioscorea bacilliform ES virus (DBESV), taro bacilliform virus (TaBV), taro bacilliform CH virus (TaBCHV), and grapevine vein clearing virus (GVCV).

# CHAPTER 2

## LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR RAPID DETECTION OF Dioscorea bacilliform AL virus (DBALV)

Adso L.S. Figueiredo, Roberto Ramos-Sobrinho, Giancarlo B.L. Santos, Iraildes P. Assunção, Gaus S.A. Lima. Loop-mediated isothermal amplification (LAMP) assay for rapid detection of Dioscorea bacilliform AL virus (DBALV). European Journal of Plant Pathology, *submitted*.

**Loop-mediated isothermal amplification (LAMP) assay for rapid detection of Dioscorea bacilliform AL virus (DBALV)**

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**Abstract**

Yams (*Dioscorea* spp.) are important food crops that feed millions of people in tropical and subtropical regions of the world, especially in the African continent where most of the production is concentrated. Dioscorea bacilliform viruses (DBVs; genus *Badnavirus*, family *Caulimoviridae*) are present wherever yams are planted and impose great challenges to farmers and germplasm bank management. Routine diagnostics based on protein A sandwich (PAS)-enzyme-linked immunosorbent assay (ELISA), PCR and RT-PCR have considerable limitations and there is an urgent need for the development of fast and reliable detection tools for DBVs. In Brazil, Dioscorea bacilliform AL virus (DBALV) is the prevalent DBV reported so far, and its diagnosis is mainly based on the analysis of the RT-RNase H region amplified by PCR. Here, we have developed a loop-mediated isothermal amplification (LAMP) assay to rapidly detect DBALV. The LAMP primers were designed based on aligned complete genome sequences of DBALV retrieved from NCBI-GenBank. Total DNA from DBALV-infected *Dioscorea bulbifera* plants was used as template for LAMP reactions that were carried out for 60 minutes at 65 ºC in a real-time thermocycler and the fluorescence curves were obtained using the green (FAM) channel, acquiring every 60 seconds. The test with 10-fold dilutions of purified PCR products showed good sensitivity of the assay, detecting DBALV in a concentration of 1.25 fg/µL ($10^{-7}$ dilution), representing 5.5 x $10^{3}$ copies of the target viral DNA.

**Keywords:** *Dioscorea* spp., *Badnavirus*, diagnosis, limit of detection, *Caulimoviridae*.

## 1. Introduction

Cultivated yams (*Dioscorea* spp.) are important staple food sources responsible for nutrient intake of millions of people in the tropical and subtropical regions worldwide, also playing a significant role in income generation and cultural aspects, especially in West Africa where it is mainly grown by smallholder farmers (Zannou et al., 2004; Darkwa et al., 2019). Three yam species, *Dioscorea alata*, *D. cayenensis*, and *D. rotundata*, are widely cultivated, but at least nine other species in the genus *Dioscorea* (*D. bulbifera*, *D. dumetorum*, *D. esculenta*, *D. japonica*, *D. nummularia*, *D. oppositifolia*, *D. pentaphylla*, *D. transversa*, and *D. trifida*) are socioeconomically important and considered minor edible yams (Lebot, 2019). In Brazil, *D. alata* and the species complex *D. cayanensis-rotundata* are the most planted materials (Lima et al., 2013; Siqueira et al., 2014).

Yams are vegetatively propagated, which allows the viruses to accumulate and be easily disseminated to geographically distant areas (Seal & Muller, 2007; Kenyon et al., 2008). Viruses belonging to the genus *Badnavirus* (family *Caulimoviridae*) pose a serious treat do these crops as they are widespread in all growing regions and germplasm accessions (Eni et al., 2008; Bousalem et al., 2009; Luo et al., 2022). So far, eight different species of dioscorea bacilliform viruses (DBVs) are officially accepted by the International Committee on Taxonomy of Viruses (ICTV, talk.ictvonline.org). In Brazil, the badnavirus *Dioscorea bacilliform AL virus* seems to be the prevalent species associated with cultivated yams in the country, but dioscorea bacilliform SN virus (DBSNV), dioscorea bacilliform RT virus (DBRTV), and the putative new species *Dioscorea bacilliform BL virus* have been recently reported from *Dioscorea* spp. (Lima et al, 2013; Guimaraes et al., 2015; Nascimento et al., 2020; Santos et al., 2022).

The detection of badnaviruses has been performed through different serological and molecular approaches such as protein A sandwich (PAS)-enzyme-linked immunosorbent assay (ELISA), PCR and RT-PCR (Briddon et al., 1999; Phillips et al., 1999; Seal & Muller, 2007; Kenyon et al., 2008; Bousalem et al., 2009), Further, to differentiate between possible episomal and integrated badnavirus sequences, the use of rolling circle amplification (RCA, to enrich the circular DNA virome), followed by viral PCR detection with specific or degenerated primers, or yet immunocapture-PCR (IC-PCR), have been proposed (Bömer et al., 2016; Ümber et al., 2017). Serological and genetic heterogeneity, presence at low titers, occurrence of endogenous pararetroviruses (EPRVs), and asymptomatic infections are challenging features during implementation of a large-scale badnavirus diagnostic program (Harper et al., 2003; Seal &

Muller, 2007; Kenyon et al., 2008, Bousalem et al., 2009; Sukal et al., 2017). Therefore, the development of fast and reliable viral detection tools may play a crucial role in seedling indexing programs and disease management (Seal et al., 2014; Diouf et al., 2022).

The loop-mediated isothermal amplification (LAMP) is a method described more than 20 years ago, which is capable of detecting DNA and RNA with high sensitivity and specificity under isothermal conditions in less than one hour (Notomi et al., 2000). It requires at least four primers that recognizes six independent sequences in the target DNA. Further, with the use of two additional loop primers, the amplification time can be considerably reduced (Nagamine et al., 2002; Botella et al., 2022). LAMP protocols for detection of badnaviruses such as *Citrus yellow mosaic virus* and *Banana streak virus* have been previously reported (Peng et al., 2012a; Anthony Johnson et al., 2014). Rapid and specific RT-LAMP protocols have also been described for direct detection of yam infecting potyviruses (genus *Potyvirus*, family *Potyviridae*) such as Japanese yam mosaic virus (JYMV) and Yam mosaic virus (YMV), with RT-LAMP detection of YMV being 100 times more sensitive in the viral detection than standard RT-PCR approaches (Fukuta et al., 2003; Nkere et al., 2018).

## 2. Material and methods

To design LAMP primers for detection of the main badnavirus associated with *Dioscorea* spp. in Brazil (Lima et al, 2013; Guimaraes et al., 2015), complete genome sequences of DBALV were downloaded from NCBI-GenBank (accessed on June 05, 2022; Supplementary Table S1). To confirm the viral taxonomy, pairwise nucleotide sequence comparisons were performed using both complete genome and RT-RNase H datasets in Sequence Demarcation Tool (SDT) v.1.2 (Muhire et al., 2014). The ICTV-established species demarcation criterion of ≥80% nucleotide identity for the RT-RNase H loci was adopted (Teycheney et al., 2020). The DBALV full-length sequences were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in Geneious Prime v.2022.2.2 (https://www.geneious.com), and the consensus sequence was used to design the LAMP primers with the New England Biolabs LAMP primer design tool v.1.4.1 (https://lamp.neb.com/) and manually modified.

To test the feasibility of the LAMP assay for detection of DBALV, the primer sets were initially tested, in triplicate, against total DNA extracted from *Dioscorea bulbifera* plants, previously tested by PCR and Sanger sequencing (SANTOS et al., 2022), using the WarmStart LAMP Kit (DNA & RNA) (New England Biolabs, USA) according to the manufacturer´s

protocol. The detection was performed using the Rotor Gene Q (Qiagen, Germany) real-time thermocycler, and the curves were obtained using the green (FAM) channel at 65°C for 60 minutes, acquiring every 1 minute. Nuclease-free water was used as negative or non-template control. The primer set DBALV-LAMP-MP-SET1 (Table 1), which was able to successfully detect the virus without amplification signal in the negative control was additionally tested to determine the limit of detection of the LAMP assay.

Total DNA from *D. bulbifera*, previously reported as being infected by the DBALV-DBJ2 isolate (Santos et al., 2022) was used as template for PCR amplifications with primers DBALV-LAMP-MP-SET1/F3 and LAMP-MP-SET1/B3, which amplifies a fragment of 207 bp in size located in the MP domain of DBALV-DBJ2 ORF3. The PCR amplification was carried out in a final volume of 15.0µL [0.2µL (5U/µL) of Taq DNA polymerase (Ludwig Biotechnology, Brazil), 1.5µL of 10x buffer, 1.2 µL (10 mM) of dNTP mix, 0.5µL (50 mM) of MgCl2, 1.0µL (10 pmol/µL) of each primer, 8.7µL of nuclease-free water and 1.0 µL (25 ng/µL) of template DNA] and the cycling parameters were initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The expected size amplicon was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) following the manufacturer´s instructions. The quality and concentration of the purified PCR product was determined using the Microvolume Spectrophotometer L-Quant (Loccus Biotecnologia, Brazil).

To assess the limit of detection or analytical sensitivity of DBALV-LAMP-MP-SET1, this set of LAMP primers were tested against different concentrations of the purified DBALV-DBJ2 MP PCR amplification product. Briefly, 10-fold serial dilutions were prepared in nuclease-free water, with the following DNA concentrations 12.5, 1.25, 0.125, 0.0125, 0.00125, 0.000125, 0.0000125, 0.00000125, and 0.000000125 ng/µL. The LAMP reactions were performed using the WarmStart LAMP Kit (DNA & RNA) (New England Biolabs, USA) according to the manufacturer´s protocol. The detection was carried out using the Rotor Gene Q (Qiagen, Germany) real-time thermocycler, and the curves were obtained using the green (FAM) channel at 65°C for 60 minutes, acquiring every 1 minute. Nuclease-free water was used as negative or non-template control, and each treatment was comprised by four repetitions.

## 3. Results and Discussion

A total of 15 complete genomes were retrieved form NCBI-GenBank, and pairwise nucleotide sequence comparisons of the RT-RNase H loci showed the isolates shared 85.1-

100% identity (Figure 1). Based on the ≥80% species demarcation criterion for the genus *Badnavirus* (Teycheney et al., 2020), they belong to the same species, *Dioscorea bacilliform AL virus*. Further, the DBALV isolates showed 83.4 to 99.8% nucleotide identity among the full-length genome sequences (Figure 2). Two sets of LAMP primers were designed based on nucleotide sequences of the MP domain located in ORF3 (Figure 3). To increase the possibility of detecting multiple variants, degenerated positions were manually added in the primer design (Table 1).

To initially test the efficiency of the LAMP primers, total DNA from two field samples of DBALV-infected *D. bulbifera* plants were used as template. The real-time analysis showed that the fluorescence curves reached a plateau between 15 to 28 minutes, while no fluorescence signal was observed in the negative controls with the DBALV-LAMP-MP-SET1 primers. Unfortunately, amplification from both DNA samples and negative controls were observed when used DBALV-LAMP-MP-SET2 (data not shown), and these LAMP primers were not considered for further analysis.

Finally, to determine the limit of detection of the DBALV-LAMP-MP-SET1, these primers were tested against several DNA concentrations. Based on the PCR fragment size (207 bp) and DNA concentration, the DNA copy number was determined for each one of the 10-fold serial dilutions, 10=5.5 x $10^{10}$ copies/µL, $10^{-1}$=5.5 x $10^{9}$ copies/µL, $10^{-2}$=5.5 x $10^{8}$ copies/µL, $10^{-3}$=5.5 x $10^{7}$ copies/µL, $10^{-4}$=5.5 x $10^{6}$ copies/µL, $10^{-5}$=5.5 x $10^{5}$ copies/µL, $10^{-6}$=5.5 x $10^{4}$ copies/µL, $10^{-7}$=5.5 x $10^{3}$ copies/µL, and $10^{-8}$=5.5 x $10^{2}$ copies/µL (Figure 4). The DBALV-LAMP-MP-SET1 were able to detect the DBALV-DBJ2 isolate in a concentration of 0.00000125 ng/µL or 1.25 fg/µL ($10^{-7}$ dilution), which represents 5.5 x $10^{3}$ copies, and a mean time of 22 minutes. Together, these results suggest the limit of detection of the LAMP primers is ≤ 5.5 x $10^{3}$ > 5.5 x $10^{2}$ copies of the target viral DNA.

Reverse transcriptase (RT)-LAMP is largely known as an efficient assay for detection of RNA molecules, and sensitivity 100-1000-fold higher than conventional PCR or RT-PCR have been observed (Wei et al., 2012; Peng et al., 2012a; Anthony Johnson et al., 2014; Jeevalatha et al., 2018; Wilisiani et al., 2019; Lu et al., 2021). LAMP assays with similar detection limits (~2 fg/µL) to the one found in our study have been previously reported for tomato leaf curl New Delhi virus-[potato] (ToLCNDV-[potato]), tomato leaf curl New Delhi virus (ToLCNDV), pepper yellow leaf curl Indonesia virus (PepYLCIV), and tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) (genus *Begomovirus*, family *Geminiviridae*) (Jeevalatha et al., 2018; Wilisiani et al., 2019). The primers targeting ToLCNDV, PepYLCIV

and TYLCKaV detected these begomoviruses at the same time, but without being able to differentiate them (Wilisiani et al., 2019). Recently, a LAMP-lateral flow dipstick (LFD) test has been described for detection of the badnavirus *Chinaberry tree badnavirus 1*, where 5 fg of the viral DNA was determined as the limit of detection (Lu et al., 2021). Further, LAMP protocols detecting approximately $10^3$ fg/µL of the target virus have been described for banana streak virus (BSV; genus *Badnavirus*) and cucumber mosaic virus (CMV; genus *Cucumovirus*) (Peng et al., 2012a; Peng et al., 2012b).

LAMP-based assays present several advantages, and versatility, for diagnostic purposes when compared to PCR or RT-PCR protocols. LAMP is an isothermal reaction that requires low complexity equipment and can be easily performed using a water bath or heat block in less than 30 minutes (Notomi et al., 2000; Nagamine et al., 2002). Similar to other approaches, the LAMP amplification products may be analyzed by gel electrophoresis, but less time-consuming methods such as naked eye observation with addition of intercalating dyes like SYBR Green I, lateral flow dipstick (LFD), real-time equipment that measures fluorescence and turbidity can also be applied (Botella, 2022). Indeed, in-field LAMP detection of begomoviruses infecting *Cucurbitaceae* and *Solanaceae* species has been developed, which uses a portable real-time fluorometer (Wilisiani et al., 2019). Similar to previously reported LAMP assays described for viral detection (Peng et al., 2012a; Anthony Johnson et al., 2014; Lu et al., 2021), the protocol reported here showed high sensitivity, being able to detect DBALV titers as low as 1.25 fg/µL (5.5 x $10^3$ copies). Together, these results suggest the suitability of the LAMP approach for diagnosis of the yam-infecting badnavirus DBALV.

## 4. References


Anthony Johnson, A. M., Dasgupta, I., & Sai Gopal, D. V. R. (2014). Development of loop-mediated isothermal amplification and SYBR green real-time PCR methods for the detection of Citrus yellow mosaic badnavirus in citrus species. *Journal of Virological Methods*, *203*, 9–14. https://doi.org/10.1016/j.jviromet.2014.03.013

Bömer, M., Turaki, A., Silva, G., Kumar, P., & Seal, S. (2016). A Sequence-Independent Strategy for Amplification and Characterisation of Episomal Badnavirus Sequences Reveals Three Previously Uncharacterised Yam Badnaviruses. *Viruses*, *8*(7), 188. https://doi.org/10.3390/v8070188

Botella, J. R. (2022). Point-of-Care DNA Amplification for Disease Diagnosis and Management. *Annual Review of Phytopathology*, *60*(1), 1–20.

https://doi.org/10.1146/annurev-phyto-021621-115027

Bousalem, M., Durand, O., Scarcelli, N., Lebas, B. S. M., Kenyon, L., Marchand, J.-L., Lefort, F., & Seal, S. E. (2009). Dilemmas caused by endogenous pararetroviruses regarding the taxonomy and diagnosis of yam (Dioscorea spp.) badnaviruses: Analyses to support safe germplasm movement. *Archives of Virology*, *154*(2), 297–314. https://doi.org/10.1007/s00705-009-0311-2

Briddon, R. W., Phillips, S., Brunt, A., & Hull, R. (1999). Analysis of the sequence of dioscorea Alata bacilliform virus: Comparison to others members of the badnavirus group. *Virus Genes*, *18*(3), 277–283. https://doi.org/10.1023/A:1008076420783

Darkwa, K., Olasanmi, B., Asiedu, R., & Asfaw, A. (2020). Review of empirical and emerging breeding methods and tools for yam (Dioscorea spp.) improvement: Status and prospects. *Plant Breeding*, *139*(3), 474–497. https://doi.org/10.1111/pbr.12783

Diouf, M. B., Festus, R., Silva, G., Guyader, S., Umber, M., Seal, S., & Teycheney, P. Y. (2022). Viruses of Yams (Dioscorea spp.): Current Gaps in Knowledge and Future Research Directions to Improve Disease Management. *Viruses*, *14*(9), 1884. https://doi.org/10.3390/v14091884

Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC bioinformatics*, *5*(1), 1–19.

Eni, A. O., Hughes, J. d'A, Asiedu, R., & Rey, M. E. C. (2008). Sequence diversity among badnavirus isolates infecting yam (*Dioscorea* spp.) in Ghana, Togo, Benin and Nigeria. Archives of Virology, 153(12), 2263–2272. https://doi.org/10.1007/s00705-008-0258-8

Fukuta, S., Iida, T., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., & Ishimoto, Y. (2003). Detection of Japanese yam mosaic virus by RT-LAMP. *Archives of Virology*, *148*(9), 1713–1720. https://doi.org/10.1007/s00705-003-0134-5

Greeshma, M., Bhat, A. I., & Jeevalatha, A. (2023). Rapid onsite detection of piper yellow mottle virus infecting black pepper by recombinase polymerase amplification-lateral flow assay (RPA-LFA). *Journal of Virological Methods*, *315*, 114695. https://doi.org/10.1016/j.jviromet.2023.114695

Guimarães, K. M. C., Silva, S. J. C., Melo, A. M., Ramos-Sobrinho, R., Lima, J. S., Zerbini, F. M., Assunção, I. P., & Lima, G. S. A. (2015). Genetic variability of badnaviruses infecting yam (Dioscorea spp.) in northeastern Brazil. *Tropical Plant Pathology*, *40*(2), 111–118. https://doi.org/10.1007/s40858-015-0023-y

Harper, G., Richert-Pöggeler, K. R., Hohn, T., & Hull, R. (2003). Detection of petunia vein-

clearing virus: Model for the detection of DNA viruses in plants with homologous endogenous pararetrovirus sequences. *Journal of Virological Methods*, *107*(2), 177–184. https://doi.org/10.1016/S0166-0934(02)00231-8

Jeevalatha, A., Kaundal, P., Kumar, R., Raigond, B., Kumar, R., Sharma, S., & Chakrabarti, S. K. (2018). Optimized loop-mediated isothermal amplification assay for Tomato leaf curl New Delhi virus-[potato] detection in potato leaves and tubers. *European Journal of Plant Pathology*, *150*(3), 565–573. https://doi.org/10.1007/s10658-017-1300-z

Keizerweerd, A. T., Chandra, A., & Grisham, M. P. (2015). Development of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of Sugarcane mosaic virus and Sorghum mosaic virus in sugarcane. *Journal of Virological Methods*, *212*, 23–29. https://doi.org/10.1016/j.jviromet.2014.10.013

Kenyon, L., Lebas, B. S. M., & Seal, S. E. (2008). Yams (Dioscorea spp.) from the South Pacific Islands contain many novel badnaviruses: Implications for international movement of yam germplasm. *Archives of Virology*, *153*(5), 877–889. https://doi.org/10.1007/s00705-008-0062-5

Lebot, V. (2019). Tropical root and tuber crops, 2nd edn. Cabi, Wallingford, Oxfordshire, UK, Boston, MA.

Lima, J. S., Lima, A. T. M., Castillo-Urquiza, G. P., Silva, S. J. C., Assunção, I. P., Michereff, S. J., Zerbini, F. M., & Lima, G. S. A. (2013). Variabilidade genética de isolados de badnavírus infectando inhame (Dioscorea spp.) no nordeste do Brasil. *Tropical Plant Pathology*, *38*, 349–353. https://doi.org/10.1590/S1982-56762013005000017

Lu, H., Tang, J., Sun, K., & Yu, X. (2021). Identification of a New Badnavirus in the Chinaberry (Melia azedarach) Tree and Establishment of a LAMP-LFD Assay for Its Rapid and Visual Detection. *Viruses*, *13*(12), Artigo 12. https://doi.org/10.3390/v13122408

Luo, G.-F., Podolyan, A., Kidanemariam, D. B., Pilotti, C., Houliston, G., & Sukal, A. C. (2022). A Review of Viruses Infecting Yam (Dioscorea spp.). *Viruses*, *14*(4), 662. https://doi.org/10.3390/v14040662

Muhire, B. M., Varsani, A., & Martin, D. P. (2014). SDT: A virus classification tool based on pairwise sequence alignment and identity calculation. *PloS One*, *9*(9), e108277. https://doi.org/10.1371/journal.pone.0108277

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, *16*(3), 223–229. https://doi.org/10.1006/mcpr.2002.0415

Nascimento, J. P. M. D., Lima, M. O. D., Peixinho, G. D. S., Ferro, M. M. D. M., Silva, S. J. C. D., Veasey, E. A., Lima, G. S. D. A., & Assunção, I. P. (2020). Incidência e caracterização molecular de badnavírus em bancos de germoplasma de inhame no Brasil. *Summa Phytopathologica*, *46*(3), 242–249. https://doi.org/10.1590/0100-5405/206682

Nkere, C. K., Oyekanmi, J. O., Silva, G., Bömer, M., Atiri, G. I., Onyeka, J., Maroya, N. G., Seal, S. E., & Kumar, P. L. (2018). Chromogenic detection of yam mosaic virus by closed-tube reverse transcription loop-mediated isothermal amplification (CT-RT-LAMP). *Archives of Virology*, *163*(4), 1057–1061. https://doi.org/10.1007/s00705-018-3706-0

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12), e63.

Peng, J., Fan, Z., & Huang, J. (2012). Rapid Detection of Banana Streak Virus by Loop-mediated Isothermal Amplification Assay in South China. *Journal of Phytopathology*, *160*(5), 248–250. https://doi.org/10.1111/j.1439-0434.2012.01885.x

Peng, J., Shi, M., Xia, Z., Huang, J., & Fan, Z. (2012). Detection of cucumber mosaic virus isolates from banana by one-step reverse transcription loop-mediated isothermal amplification. *Archives of Virology*, *157*(11), 2213–2217. https://doi.org/10.1007/s00705-012-1376-x

Phillips, S., Briddon, R. W., Brunt, A. A., & Hull, R. (1999). The Partial Characterization of a Badnavirus Infecting the Greater Asiatic or Water Yam (Dioscorea alata). *Journal of Phytopathology*, *147*(5), 265–269. https://doi.org/10.1111/j.1439-0434.1999.tb03829.x

Santos, G. B. L., Ferro, M. M. M., Feijo, F. M., Ramos-Sobrinho, R., Assunção, I. P., & Lima, G. S. A. (2022). Badnavirus sequence diversity reveals one previously uncharacterized virus associated with air yam (Dioscorea bulbifera L.) in Brazil. *Tropical Plant Pathology*, *47*(6), 807–813. https://doi.org/10.1007/s40858-022-00536-7

Seal, S., & Muller, E. (2007). Molecular analysis of a full-length sequence of a new yam badnavirus from Dioscorea sansibarensis. *Archives of Virology*, *152*(4), 819–825. https://doi.org/10.1007/s00705-006-0888-7

Seal, S., Turaki, A., Muller, E., Kumar, P. L., Kenyon, L., Filloux, D., Galzi, S., Lopez-Montes, A., & Iskra-Caruana, M.-L. (2014). The prevalence of badnaviruses in West African yams (Dioscorea cayenensis-rotundata) and evidence of endogenous pararetrovirus sequences in their genomes. *Virus Research*, *186*, 144–154. https://doi.org/10.1016/j.virusres.2014.01.007

Siqueira, M., Nascimento, W. F., Silva, L. R. G., Ferreira, A. B., Silva, E. F., Ming, L. C., & Veasey, E. A. (2014). Distribution, management and diversity of yam local varieties in Brazil: A study on *Dioscorea alata*L. *Brazilian Journal of Biology*, *74*, 52–61. https://doi.org/10.1590/1519-6984.17112

Sukal, A., Kidanemariam, D., Dale, J., James, A., & Harding, R. (2017). Characterization of badnaviruses infecting Dioscorea spp. In the Pacific reveals two putative novel species and the first report of dioscorea bacilliform RT virus 2. *Virus Research*, *238*, 29–34. https://doi.org/10.1016/j.virusres.2017.05.027

Teycheney, P.-Y., Geering, A. D. W., Dasgupta, I., Hull, R., Kreuze, J. F., Lockhart, B., Muller, E., Olszewski, N., Pappu, H., Pooggin, M. M., Richert-Pöggeler, K. R., Schoelz, J. E., Seal, S., Stavolone, L., Umber, M., & Report Consortium, I. (2020). ICTV Virus Taxonomy Profile: Caulimoviridae. *The Journal of General Virology*, *101*(10), 1025–1026. https://doi.org/10.1099/jgv.0.001497

Umber, M., Gomez, R.-M., Gélabale, S., Bonheur, L., Pavis, C., & Teycheney, P.-Y. (2017). The genome sequence of Dioscorea bacilliform TR virus, a member of the genus Badnavirus infecting Dioscorea spp., sheds light on the possible function of endogenous Dioscorea bacilliform viruses. *Archives of Virology*, *162*(2), 517–521. https://doi.org/10.1007/s00705-016-3113-3

Wei, Q.-W., Yu, C., Zhang, S.-Y., Yang, C.-Y., Miriam, K., Zhang, W.-N., Dou, D.-L., & Tao, X.-R. (2012). One-step detection of Bean pod mottle virusin soybean seeds by the reverse-transcription loop-mediated isothermal amplification. *Virology Journal*, *9*(1), 187. https://doi.org/10.1186/1743-422X-9-187

Wilisiani, F., Tomiyama, A., Katoh, H., Hartono, S., Neriya, Y., Nishigawa, H., & Natsuaki, T. (2019). Development of a LAMP assay with a portable device for real-time detection of begomoviruses under field conditions. *Journal of Virological Methods*, *265*, 71–76. https://doi.org/10.1016/j.jviromet.2018.10.005

Zannou, A., Ahanchédé, A., Struik, P. C., Richards, P., Zoundjihékpon, J., Tossou, R., & Vodouhè, S. (2004). Yam and cowpea diversity management by farmers in the Guinea-Sudan transition zone of Benin. *NJAS - Wageningen Journal of Life Sciences*, *52*(3), 393–420. https://doi.org/10.1016/S1573-5214(04)80023-X

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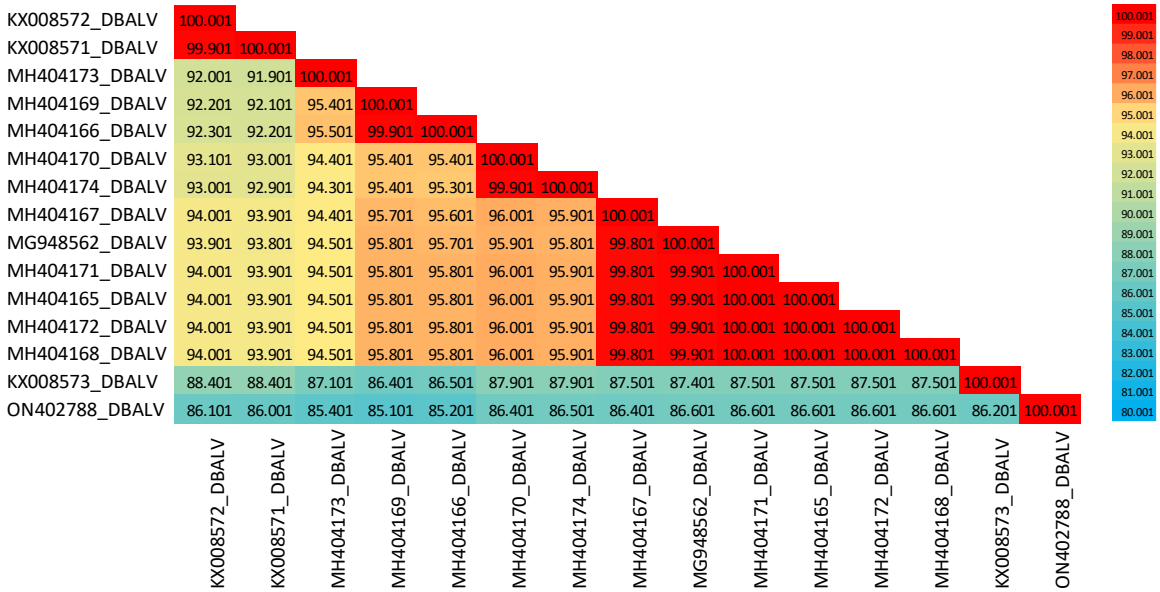


**Figure 1.** Pairwise sequence comparisons based on RT-RNase H nucleotide sequences of Dioscorea bacilliform AL virus (DBALV).

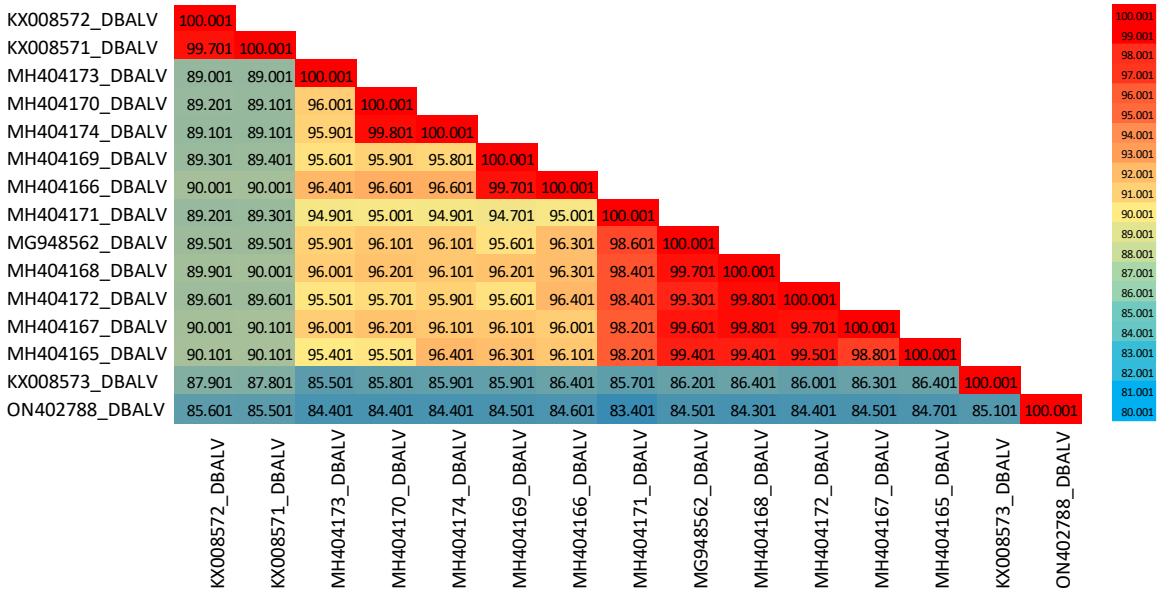


**Figure 2.** Pairwise sequence comparisons based on complete genome sequences of Dioscorea bacilliform AL virus (DBALV).

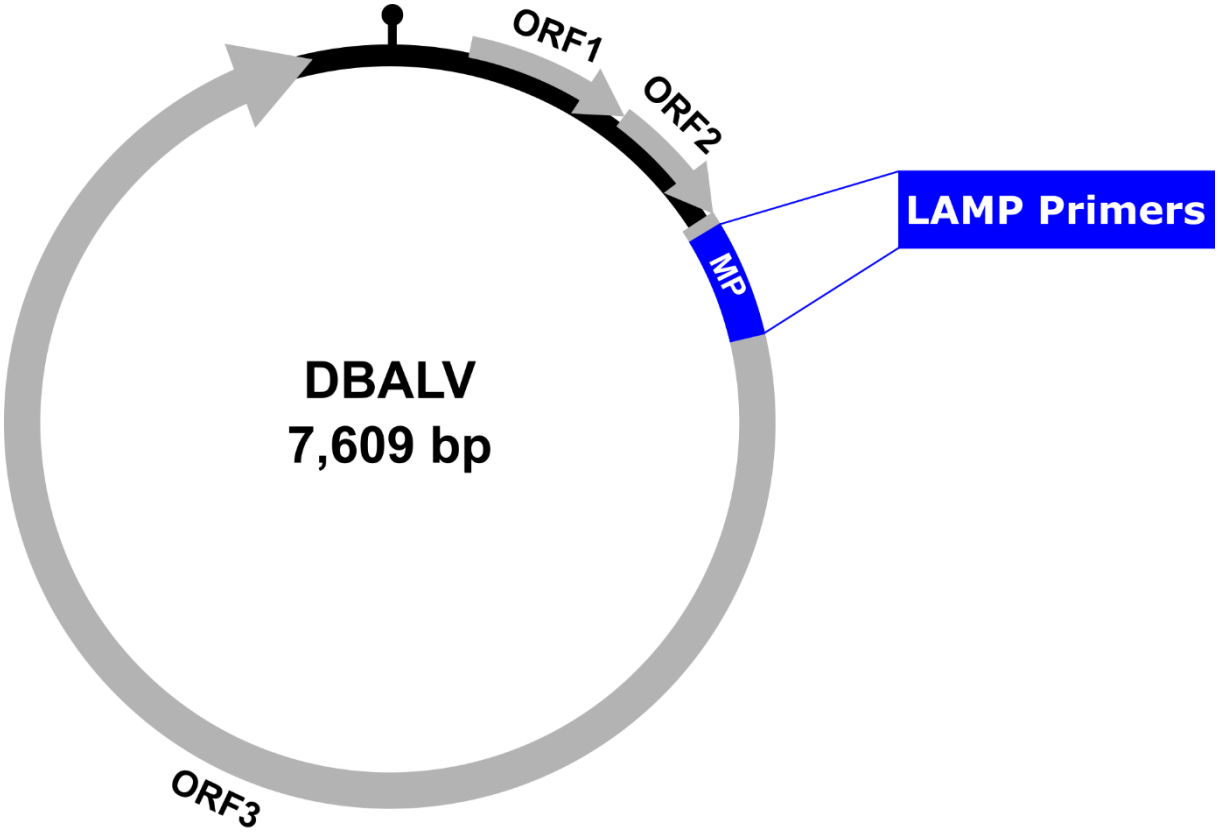


**Figure 3.** Genomic organization of the Dioscorea-infecting badnavirus Dioscorea bacilliform AL virus isolates (DBALV). The Movement Protein (MP) region where the LAMP primer sets were designed are indicated by blue boxes.

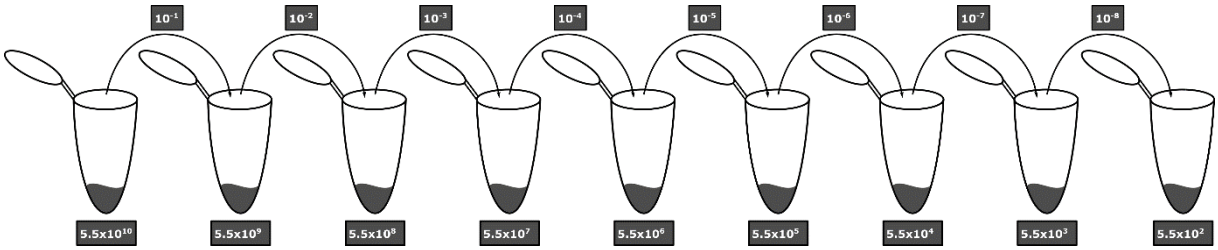


**Figure 4.** Serial dilution (10-fold) of the purified PCR product amplified with primers DBALV-LAMP-MP-SET1/F3 and LAMP-MP-SET1/B3 and used to determine the limit of detection of the LAMP-MP-SET1. The copy number of target DNA is shown at the bottom in grey boxes.

## List of Tables

**Table 1**. List of LAMP primers for rapid detection of DBALV.

| Primer sets | Sequence (5'-3')* |
|---|---|
| ***Set1*** | |
| DBALV-LAMP-MP-SET1/F3 | GCTTCGCATACTCTATTTCAG |
| DBALV-LAMP-MP-SET1/B3 | TG**Y**GGTACTGGTTTGTATCG |
| DBALV-LAMP-MP-SET1/FIP | GAGTTTCCTGGAATTCTTCAGTTGA**R**CATCACCGACTACTTTGTC |
| DBALV-LAMP-MP-SET1/BIP | AATTTGGTTGTGCCAACACAACAATTGAGAT**R**CTCCCGTC |
| DBALV-LAMP-MP-SET1/LF | TGCTTGCACACCATGTGT |
| DBALV-LAMP-MP-SET1/LB | CAACTTTTGCCGAGGAGAGG |
| ***Set2*** | |
| DBALV-LAMP-MP-SET2/F3 | ACCTCCAGTTCTGTAG**Y**A |
| DBALV-LAMP-MP-SET2/B3 | GGACACTCTCGAGCATAG |
| DBALV-LAMP-MP-SET2/FIP | GT**Y**TT**W**GA**Y**TTCCGCACACCATATAAATTCCAAT**Y**CCAGGCT |
| DBALV-LAMP-MP-SET2/BIP | CC**R**CACCAGTCTCATGTCCG**Y**TCCACAGAG**R**AAGCATT |
| DBALV-LAMP-MP-SET2/LF | TCTT**Y**TCAGCTCCTCCATAGT |
| DBALV-LAMP-MP-SET2/LB | CATTGAGAA**RM**GAAAGCA**YY**TGA |

*Degenerated positions are indicated in bold.

**Supplementary Material**

**Supplementary Table S1.** Complete genome sequences of yam-infecting badnaviruses downloaded from NCBI-GenBank (accessed on June 5, 2022).

| Species | Acronym | GenBank Accession# |
|---|---|---|
| *Dioscorea bacilliform AL virus* | DBALV | MG948562 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404165 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404166 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404167 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404168 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404169 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404170 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404171 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404172 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404173 |
| *Dioscorea bacilliform AL virus* | DBALV | MH404174 |
| *Dioscorea bacilliform AL virus* | DBALV | KX008571 |
| *Dioscorea bacilliform AL virus* | DBALV | KX008572 |
| *Dioscorea bacilliform AL virus* | DBALV | KX008573 |
| *Dioscorea bacilliform AL virus* | DBALV | ON402788 |

## GENERAL CONCLUSIONS

Dioscorea bacilliform viruses (DBVs) pose a significant threat to global yam cultivation and the safe exchange of germplasm. Extensive research is essential to comprehend the diversity of these viruses and to establish reliable diagnostic tools.

In Chapter 1, a high-throughput sequencing approach was employed to molecularly characterize two complete genome sequences of a putative novel member of the *Badnavirus* genus, Dioscorea bacilliform BL virus (DBBLV), previously reported naturally infecting *Dioscorea bulbifera* plants. Furthermore, our research unveiled that recombination plays a pivotal role in the molecular diversification of DBBLV and other badnaviruses that infect yams. Chapter 2 proposed a loop-mediated isothermal amplification (LAMP)-based protocol, demonstrating its efficiency as a rapid, reliable, and sensitive method for detecting the most prevalent badnavirus affecting yams in Brazil, Dioscorea bacilliform AL virus (DBALV). These findings contribute to the growing body of knowledge regarding badnaviruses associated with *Dioscorea* spp., providing a valuable tool for accurate virus detection. This tool can be instrumental in disease management and genetic breeding programs while also mitigating the risk of yam-infecting badnavirus transmission through germplasm exchange.

Future studies should explore important aspects of DBBLV, including its genetic variability, population structure, and its potential to infect other cultivated *Dioscorea* species. Additionally, efforts should continue to focus on the development of diagnostic tools for DBBLV and other DBVs.