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RESEARCH ARTICLE / *ARTIGO*

Genetic variability of papaya lethal yellowing virus isolates from Ceará and Rio Grande do Norte states, Brazil

Cleidiane B. Daltro[1], Álvaro J. Pereira[2]*, Renan S. Cascardo[2], Poliane Alfenas-Zerbini[3], José Evando A. Bezerra-Junior[4#], José Albérsio A. Lima[4], Francisco Murilo Zerbini[2] & Eduardo C. Andrade[5]

[1]Centro de Ciências Agrárias, Ambientais e Biológicas, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, BA, 44380-000, Brazil; [2]Departamento de Fitopatologia/BIOAGRO, Universidade Federal de Viçosa, Viçosa, MG, 36570-000, Brazil; [3]Departamento de Microbiologia/BIOAGRO, Universidade Federal de Viçosa, Viçosa, MG, 36570-000, Brazil; [4]Departamento de Fitotecnia, Universidade Federal do Ceará, Fortaleza, CE, 60451-970, Brazil; [5]Embrapa Mandioca e Fruticultura, Cruz das Almas, BA, 44380-000, Brazil

Author for correspondence: Francisco Murilo Zerbini, e-mail: zerbini@ufv.br

ABSTRACT

The papaya (*Carica papaya*) is a fruit crop of great economic importance throughout the Brazilian northeast, which is responsible for 60% of the national output. Papayas in the states of Ceará and Rio Grande do Norte are affected by lethal yellowing disease, caused by papaya lethal yellowing virus (PLYV). Previous work suggested that PLYV is a putative sobemovirus. To assess the genetic variability of PLYV, foliar samples were collected in October 2008 and October 2009 in commercial fields from Ceará and Rio Grande do Norte states, and total RNA was extracted. Specific primers based on the sequence of a previously characterized PLYV isolate were used for the RT-PCR-based amplification of a 900 bp fragment corresponding to the central region of the viral genome. Fragments from 21 viral isolates were cloned and sequenced. Sequence analyses indicated >97% nucleotide sequence identity among the isolates, 94-100% identity with the previously sequenced PLYV isolate, and a lower but significant identity with sobemoviruses (43-48.5%). These results suggest a low genetic variability among PLYV isolates, and are in agreement with the provisional placement of PLYV in the genus *Sobemovirus*. Definitive taxonomic conclusions, however, can only be drawn after the determination of the full-length genomic sequence.
Key words: *Carica papaya*, PLYV, sobemovirus.

RESUMO

Variabilidade genética de isolados do papaya lethal yellowing virus dos estados do Ceará e Rio Grande do Norte, Brasil

O mamão (*Carica papaya*) é uma fruta de grande importância econômica em todo o Nordeste brasileiro, região responsável por 60% da produção nacional. Mamoeiros nos estados do Ceará e Rio Grande do Norte são afetados pela doença denominada amarelo letal do mamoeiro, causada pelo papaya lethal yellowing virus (PLYV). Trabalhos anteriores indicaram que o PLYV é um possível sobemovírus. A fim de estimar a variabilidade genética do PLYV, amostras foliares de mamoeiro foram coletadas em outubro de 2008 e de 2009 em regiões produtoras do Ceará, e Rio Grande do Norte. Oligonucleotídeos específicos baseados na sequência de um isolado de PLYV previamente caracterizado foram utilizados para a amplificação via RT-PCR de um fragmento com 900 pb, correspondente à região central do genoma viral. Fragmentos de 21 isolados virais foram clonados e sequenciados. A análise das sequências de nucleotídeos indicou >97% de identidade entre os isolados, entre 94-100% com o isolado de PLYV previamente sequenciado, e uma identidade baixa, porém significativa, com sobemovírus (43-48,5%). Estes resultados sugerem um baixo grau de variabilidade genética entre isolados de PLYV, e estão de acordo com a classificação provisória do PLYV como membro do gênero *Sobemovirus*. Conclusões de ordem taxonômica, no entanto, só podem ser tiradas após a determinação da sequência genômica completa.
Palavras-chave: *Carica papaya*, PLYV, sobemovírus.

INTRODUCTION

The papaya (*Carica papaya* L.) is a fruit crop of great economic importance in tropical and subtropical countries of the world. Papaya cultivation is widely distributed throughout the tropical regions of the world, extending to 32° latitude North and South, with the possible introduction into Brazil in 1587 (Serrano & Cataneo, 2010). According to the Food and Agriculture Organization of the United Nations (FAO), the world production of papaya represents 10% of all tropical fruits, yielding around 8 million tons, of which 39% are produced in Latin America and the Caribbean (faostat.fao.org). The world's leading producers are India, Brazil, Nigeria, Indonesia and Mexico. In 2008, Brazil produced 1.89 million tons on 36,500 hectares with a production value estimated at U$ 1 billion. The major papaya producer states are Bahia (902,000 tons), Espírito

*Present address: Universidade Estadual do Ceará, Itapipoca CE, 62500-000, Brazil.
Present address: Departamento de Agronomia, Universidade Estadual do Piauí, Picos PI, 64600-000, Brazil.

Santo (630,000 tons), Rio Grande do Norte (106,000 tons) and Ceará (100,000 tons) (Quintino, 2007; Cruz, 2008).

In most states of northeastern Brazil, papaya plants are affected by lethal yellowing disease, caused by papaya lethal yellowing virus (PLYV). This virus was first described in the early 1980's in the state of Pernambuco (Loreto et al., 1983). The disease was later detected in the states of Bahia (Vega et al., 1988), Rio Grande do Norte (Kitajima et al., 1989), Ceará (Lima & Santos, 1991) and Paraíba (Camarço et al., 1996). Initial infection with the virus manifests as yellowing of the younger leaves, which later progresses to more severe symptoms of curled leaves, wilting and senescence. Green spots are commonly found on immature fruit and they turn yellow as the fruit reaches maturity (Lima et al., 2001). PLYV is mechanically transmitted and can be found in the soil (Camarço et al., 1998). The virus has never been reported anywhere else other than northeastern Brazil. PLYV has isometric particles with *ca.* 30 nm in diameter, and an estimated single-stranded positive sense RNA genome of 4.8 kb. The single coat protein (CP) has a molecular mass of 36 kDa. Analysis of the open reading frames (ORFs) encoding the putative RNA-dependent RNA polymerase (RdRp) and coat protein (CP) indicated that PLYV shares sequence similarity to viruses in the genus *Sobemovirus* (Kitajima et al., 1992a; Kitajima et al., 1992b; Silva et al., 1997; Nascimento et al., 2010). However, the complete viral genome has not yet been sequenced and therefore the taxonomical classification of PLYV is provisional. Besides, very few studies have been carried out on the genetic variability of PLYV isolates. To generate information on the variability of PLYV, we performed the cloning, sequencing and genetic analysis of genomic fragments of PLYV isolates from infected papaya plants in the states of Ceará and Rio Grande do Norte.

MATERIAL AND METHODS

Sampling of papaya fields and preliminary viral detection

A total of 27 *C. papaya* foliar samples displaying symptoms of lethal yellowing disease were collected in production regions around the cities of Acaraú (2°53'09" S, 40°07' 12" W), Paraipaba (3°26'20" S, 39°08'52" W) and Quixeré (5°04'26" S, 37°59'20" W) in the state of Ceará, during October 2008, and the city of Baraúna (5°04'48" S, 37°37'00" W) in the state of Rio Grande do Norte, during October 2009 (Table 1). Papaya samples from Acaraú were from cultivars from the Formosa group. All other samples were from the Solo group. Although samples were collected from plants with different ages, they were all at least one year old and in production stage. The presence of PLYV in the samples was assessed by indirect ELISA (Converse & Martin, 1990) using a polyclonal antiserum produced at the Universidade Federal do Ceará. The samples were not tested for the presence of other papaya-infecting viruses.

RNA extraction

Total RNA was extracted from 2 g of infected leaf tissues by grinding in liquid nitrogen and extraction with

TABLE 1 - Isolates of papaya lethal yellowing virus (PLYV) obtained and analyzed in this study

Isolate	Place of collection	Date of collection	GenBank accession number
1	Paraipaba, CE	Oct. 2008	JQ394906
2	Paraipaba, CE	Oct. 2008	JQ394907
3	Paraipaba, CE	Oct. 2008	JQ394908
4	Paraipaba, CE	Oct. 2008	JQ394909
5	Paraipaba, CE	Oct. 2008	JQ394910
6	Paraipaba, CE	Oct. 2008	JQ394911
7	Paraipaba, CE	Oct. 2008	JQ394912
8	Quixeré (Itatinga District), CE	Oct. 2008	JQ394913
9	Quixeré (Boa Esperança District), CE	Oct. 2008	JQ394914
10	Quixeré (Boa Esperança District), CE	Oct. 2008	JQ394915
11	Quixeré (Boa Esperança District), CE	Oct. 2008	JQ394916
14	Quixeré (Oiticica dos Mirandas District), CE	Oct. 2008	JQ394917
18	Acaraú (Irrigated Perimeter, lot C136/3C2), CE	Oct. 2008	JQ394918
19	Acaraú (Irrigated Perimeter, lot C136/3C2), CE	Oct. 2008	JQ394919
20	Acaraú (Irrigated Perimeter, lot C136/3C2), CE	Oct. 2008	JQ394920
21	Quixeré (Itatinga District), CE	Oct. 2008	JQ394921
23	Baraúnas (Velame Farm), RN	Oct. 2009	JQ394922
24	Baraúnas (Velame Farm), RN	Oct. 2009	JQ394923
25	Baraúnas (Velame Farm), RN	Oct. 2009	JQ394924
26	Baraúnas (Velame Farm), RN	Oct. 2009	JQ394925
141	Quixeré (Oiticica dos Mirandas District), CE	Oct. 2008	JQ394926

the Brazol reagent (LGC Biotecnologia), according to the manufacturer's instructions. The final RNA pellet was resuspended in 20 mL of nuclease-free water and stored at -80°C.

RT-PCR, cloning and sequencing

Viral cDNA was transcribed from 5 µl of total RNA (approx. 5 µg) using a PLYV-specific reverse primer (5'-GTG TAT GGC ATA CAG TTA TC-3') which anneals at the 3'-end of the CP ORF based on the sequence of the Marco2 isolate (GU066876) (Amaral et al., 2006). Initially, the RNA, the primer (20 pmol) and nuclease-free water (to complete the volume to 12 µl) were incubated for 5 min at 65°C, and quickly transferred to ice. The reaction was then completed with 4 µl of 5× reaction buffer, 1 µl of 10 mM dNTP mix, 2 µl of 0.1 M dithiothreitol (DTT) and 1 µl (200 units) of SuperScript III reverse transcriptase (Invitrogen) and incubated at 37°C for 1 h followed by 95°C for 5 min. PCR was performed in a total volume of 50 µl, using 2.5 µl of the cDNA, 5 µl 10× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 3 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (2.5 mM each), 20 pmol of each primer [the same reverse primer used in the reverse transcription reaction plus a forward primer, 5' TGA AGC GGA TAT TTC TGG 3', which anneals at the 3'-end of the RdRp ORF (Amaral et al., 2006)] and 1 unit of *Taq* DNA polymerase. The thermocycler (PTC-100, MJ Research Inc.) was programmed for 35 cycles of denaturing at 94°C for 1 minute, primer annealing at 55°C for 2 minutes and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes.

PCR products were analyzed by electrophoresis on 1% agarose gels and were purified from the gels using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions. The amplified fragments were cloned using the pGEM-T-Easy kit (Promega), using the methodology recommended by the manufacturer. Cloning was confirmed by digestion of plasmid DNA with *Eco*R I and analysis by agarose gel electrophoresis. Viral fragments were sequenced in their entirety at Macrogen (Seoul, South Korea).

The nucleotide (nt) sequences obtained from the PLYV isolates were aligned and compared with those from 11 sobemoviruses available in GenBank (Table 2). Partial amino acid (aa) sequences for the RdRp and CP ORFs were deduced using the ORFinder tool (www.ncbi.nlm.nih.gov/projects/gorf). Pairwise sequence comparisons were performed with DNAMan v. 4 (Lynnon Biosoft). The program DnaSP v5 (Librado & Rozas, 2009) was used to determine nucleotide sequence diversity and dN/dS ratios. Multiple sequence alignments were obtained with Clustal W (Thompson et al., 1994). Phylogenetic trees were constructed with MEGA 5.0 (Tamura et al., 2011), using the neighbour-joining algorithm with 2,000 bootstrap replications.

RESULTS AND DISCUSSION

Cloning of PLYV genomic fragments

Out of 27 papaya samples collected in Rio Grande do Norte and Ceará states, 21 were positive for the presence of PLYV based on results of indirect ELISA using a PLYV-specific polyclonal antiserum (*data not shown*). An approximately 900 bp DNA fragment was successfully amplified via RT-PCR from total RNA extracted from these 21 samples, cloned and completely sequenced (Table 1).

Sequence comparisons

A preliminary analysis of the sequences using BLAST*n* (Altschul et al., 1990) indicated the PLYV Marco2 isolate (GenBank access number GU066876) as the closest related virus, with >94% nt sequence identity with all isolates (*data not shown*). Further analysis using the

TABLE 2 - Viruses used for nucleotide and amino acid sequence alignments, comparisons and construction of phylogenetic trees. All viruses belong to the genus *Sobemovirus*, except where indicated

Virus	GenBank accession number
Papaya lethal yellowing virus (PLYV) - Marco2[a]	GU066876
Southern bean mosaic virus (SBMV)	AF055887
Sesbania mosaic virus (SeMV)	AY004291
Southern cowpea mosaic virus (SCPMV)	M23021
Cocksfoot mottle virus (CfMV)	Z48630
Lucerne transient streak virus (LTSV)	U31286
Rice yellow mottle virus (RYMV)	L20893
Subterranean clover mottle virus (SCMoV)	AF208001
Turnip rosette virus (TRoV)	AY177608
Ryegrass mottle virus (RGMoV)	AB040446
Rubus chlorotic mottle virus (RuCMV)	AM940437
Imperata yellow mottle virus (IYMV)	AM990928
Potato leafroll virus (PLRV)[b]	D00530

[a] Possible sobemovirus, partial sequence.
[b] Gen. *Polerovirus*, Fam. *Luteoviridae*; used as an outgroup.

ORF finder tool indicated that the 900 bp fragment included two partial ORFs in the same sense, one corresponding to a putative viral RNA dependent RNA polymerase (RdRp) and the other corresponding to the viral coat protein (CP). Nucleotide sequence identities for the RdRp ORF of all isolates were >93% with PLYV Marco2, and for the CP ORF were >94% (Table 3). Together, these results confirmed the identification of all 21 isolates as PLYV.

Pairwise comparisons indicated that the 900 bp fragments amplified from the 21 viral isolates shared >97% nt sequence identity amongst themselves (*data not shown*). Separate comparisons for the RdRp and CP ORFs indicated nt identities >94% and >95%, respectively, amongst the 21 isolates (Table 3). These results are equivalent to those observed for the sobemovirus *Rice yellow mottle virus* (RYMV), for which the average nt diversity for the full genomic sequences of 16 isolates was 7%, and the maximum diversity between any two isolates was 10% (Fargette et al., 2004). Considering that some of the locations where the samples were collected are approximately 370 km apart, that samples were collected within a one-year interval (Table 1), and that the dN/dS ratios for the RdRp and CP ORFs were 0.108 and 0.185, respectively, the genetic variability of the virus can be considered to be low.

PLYV was first recognized in the 1980's (Loreto et al., 1983), and has always been restricted to a relatively small geographical area (*ie*, it has never been reported anywhere else). Therefore, the odds of the virus having been introduced from another region are low. Most likely the virus was present in wild hosts and jumped to payaya plants once this crop became widely cultivated in that area. If that is the case (but it should be clear that no evidence is available to support this hypothesis), then it is reasonable to assume that multiple "host jumping" events could have occurred, and the genetic base of the viral population in papaya plants would not necessarily be narrow. Interestingly, a vector has not yet been found for PLYV, and it has been suggested that the virus may not have one (Camarço et al., 1998). Vector transmission is a notorious genetic bottleneck. Should a mode of transmission which does not impose a genetic bottleneck (*eg*, through soil) be demonstrated, this would provide further evidence against a founder effect acting upon the viral population in papaya plants. The low genetic variability detected here could therefore be a feature of this virus.

Sequence comparisons with known sobemoviruses indicated that the putative RdRp of PLYV is much more similar to the corresponding protein of sobemoviruses than the CP (Table 4). In addition, motifs in nucleotide and deduced amino acid sequences of sobemovirus proteins are also present in the PLYV sequences (*data not shown*), reinforcing the relationship between PLYV and other members of the genus *Sobemovirus*.

The deduced amino acid sequences of the RdRp showed the greatest identity with the sobemoviruses *Subterranean clover mottle virus* (SCMoV; 84%), *Turnip rosette virus* (TRoV) and *Lucerne transient streak virus* (LTSV) (83% in both cases), and *Ryegrass mottle virus* (RGMoV; 81%) (Table 4). Identity values for the deduced amino acid sequence of the CP were 35% with *Southern cowpea mosaic virus* (SCPMV) and 33% with *Southern bean mosaic virus* (SBMV) (Table 4). However, a closer examination of Table 4 indicates that the CP of sobemoviruses is much more variable than the RdRp. Except for the CPs of SBMV, SCPMV and *Sesbania mosaic virus* (SeMV), which share 65-77% identity, those of other sobemoviruses share only 8-40% identity (Table 4). Identity values for the RdRps vary from 36 to 82% (Table 4). Therefore, the identity values observed between the PLYV CP and those from other sobemoviruses is within the normal range for this group of viruses.

As it had been previously suggested that PLYV could be a member of the genus *Tombusvirus*, we also performed sequence comparisons with tombusviruses as well as viruses in other genera of the family *Tombusviridae*. The greatest identity values observed were 13% for the RdRp and 17% for the CP of *Tomato bushy stunt virus* (TBSV) (*data not shown*). The identity values for the RdRp are much lower than those observed with sobemoviruses. While the identity values observed for the CP are within the same range observed among sobemoviruses, it must be noted that the CPs of sobemoviruses and necroviruses (genus *Necrovirus*, family *Tombusviridae*) are known to be distantly related, with an early recombination event being suggested to be involved in the evolution of these genera (Truve & Fargette, 2011).

Phylogenetic analysis

Results of phylogenetic analysis corroborate those of sequence comparisons. A phylogenetic tree based on the nucleotide sequence of the 900 bp fragment shows that all 21 isolates obtained from Ceará and Rio Grande do Norte states are closely related to each other as well as to the Marco2 isolate, with RGMoV being the closest sobemovirus (Figure 1). Trees based on the amino acid sequences of the CP and RdRp had similar topologies (*data not shown*).

Together, the results of sequence comparisons and phylogenetic analyses indicate that PLYV is much more closely related to sobemoviruses than to tombusviruses, and are consistent with the classification of PLYV as a species in the genus *Sobemovirus*. However, the sequenced fragment represents only aproximately 25% of the viral genome. Furthermore, the number of complete sobemovirus sequences is low, and therefore it is difficult to state which region of the genome (if any) could be representative of the entire genome (Truve & Fargette, 2011). The study by Fargette et al. (2004) indicates that the CP is quite variable (our data shows the same) but at the same time, phylogenetic trees based on the entire genome or the CP sequence have very similar topologies. Therefore, at least for RYMV, the CP sequence can be used to type isolates.

TABLE 3 - Percent nucleotide sequence identities among papaya lethal yellowing virus (PLYV) isolates from Rio Grande do Norte and Ceará states. Values above the diagonal correspond to the coat protein (CP) coding region. Values below the diagonal correspond to the RNA-dependent RNA polymerase (RdRp) coding region. Marco2 is a previously sequenced isolate from Ceará state (GenBank accession number GU066876)

	Marco2	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**14**	**18**	**19**	**20**	**21**	**23**	**24**	**25**	**26**	**141**
Marco2	---	96	99	96	97	100	99	95	96	95	99	95	94	96	96	100	96	97	98	97	98	96
1	94	---	98	99	96	96	96	98	99	99	97	99	99	99	99	97	99	98	98	97	98	99
2	98	99	---	98	98	99	98	98	98	97	99	98	97	99	98	98	98	96	96	98	99	99
3	94	100	99	---	98	97	96	99	99	99	97	99	99	99	99	97	99	98	98	98	98	99
4	99	97	98	97	---	99	98	96	97	96	99	96	95	97	98	99	97	95	95	96	98	96
5	99	97	98	97	99	---	99	97	97	96	99	97	96	97	97	99	97	98	98	97	98	96
6	99	98	98	97	99	99	---	96	98	96	99	96	95	97	96	98	96	97	97	96	97	96
7	94	98	99	98	97	97	97	---	99	99	97	99	99	99	99	97	99	98	98	96	97	99
8	94	100	99	100	97	97	98	98	---	99	97	99	99	99	99	97	100	98	98	97	98	99
9	93	98	99	98	96	97	97	97	98	---	97	99	99	99	99	97	99	98	98	96	97	99
10	99	97	98	97	99	99	99	97	96	97	---	97	96	98	97	99	97	95	95	97	98	96
11	100	98	100	98	98	98	98	97	98	97	98	---	99	99	99	97	99	98	98	96	97	99
14	93	97	98	97	96	97	98	96	97	98	97	96	---	99	99	97	99	97	97	95	96	99
18	93	100	98	100	96	96	97	98	100	98	96	98	97	---	99	97	99	98	98	97	98	99
19	93	100	99	100	97	97	98	98	100	98	97	98	97	100	----	97	99	98	97	97	98	99
20	97	96	98	96	99	99	99	95	96	95	99	95	94	96	96	---	97	97	97	96	97	97
21	94	100	99	100	97	97	98	98	100	98	97	98	97	100	100	96	---	98	98	97	98	99
23	98	98	98	98	97	96	97	97	98	97	97	97	96	98	98	97	98	---	100	98	99	98
24	98	98	99	98	96	96	98	97	98	97	97	97	96	98	98	97	98	100	---	98	99	98
25	94	97	98	97	96	96	96	96	97	96	96	96	95	97	97	96	97	98	98	---	98	98
26	100	98	98	98	97	97	98	97	98	97	97	97	96	98	98	97	98	100	100	98	---	97
141	93	98	99	98	96	97	97	97	98	100	97	97	98	98	98	95	98	97	97	96	97	---

TABLE 4 - Percent amino acid sequence identities between papaya lethal yellowing virus (PLYV) proteins and those of known sobemoviruses. For simplicity, only isolate 8 from Rio Grande do Norte state is shown. Values above the diagonal refer to the coat protein (CP) and values below the diagonal refer to the RNA-dependent RNA polymerase (RdRp). See Table 2 for full virus names and GenBank accession numbers

	PLYV 8	**SBMV**	**SeMV**	**SCPMV**	**CfMV**	**LTSV**	**RYMV**	**SCMoV**	**TRoV**	**RGMoV**	**RuCMV**	**IYMV**
PLYV 8	--	33	25	35	18	20	16	13	28	20	19	17
SBMV	56	--	77	70	22	11	31	24	34	28	24	20
SeMV	57	77	--	65	22	28	21	29	28	27	31	19
SCPMV	50	57	60	--		19	23	31	37	19	24	20
CfMV	64	71	52	66	--	14	29	22	19	12	25	30
LTSV	83	46	45	36	46	--	12	25	8	11	14	15
RYMV	53	81	45	49	55	76	--	18	26	17	20	40
SCMoV	84	66	62	64	55	77	57	--	27	34	24	22
TRoV	83	65	65	63	46	66	57	54	--	12	25	17
RGMoV	81	77	77	70	44	57	71	55	64	--	26	18
RuCMV	60	47	46	63	44	39	60	57	44	41	--	33
IYMV	59	82	45	48	54	39	56	68	50	44	48	--

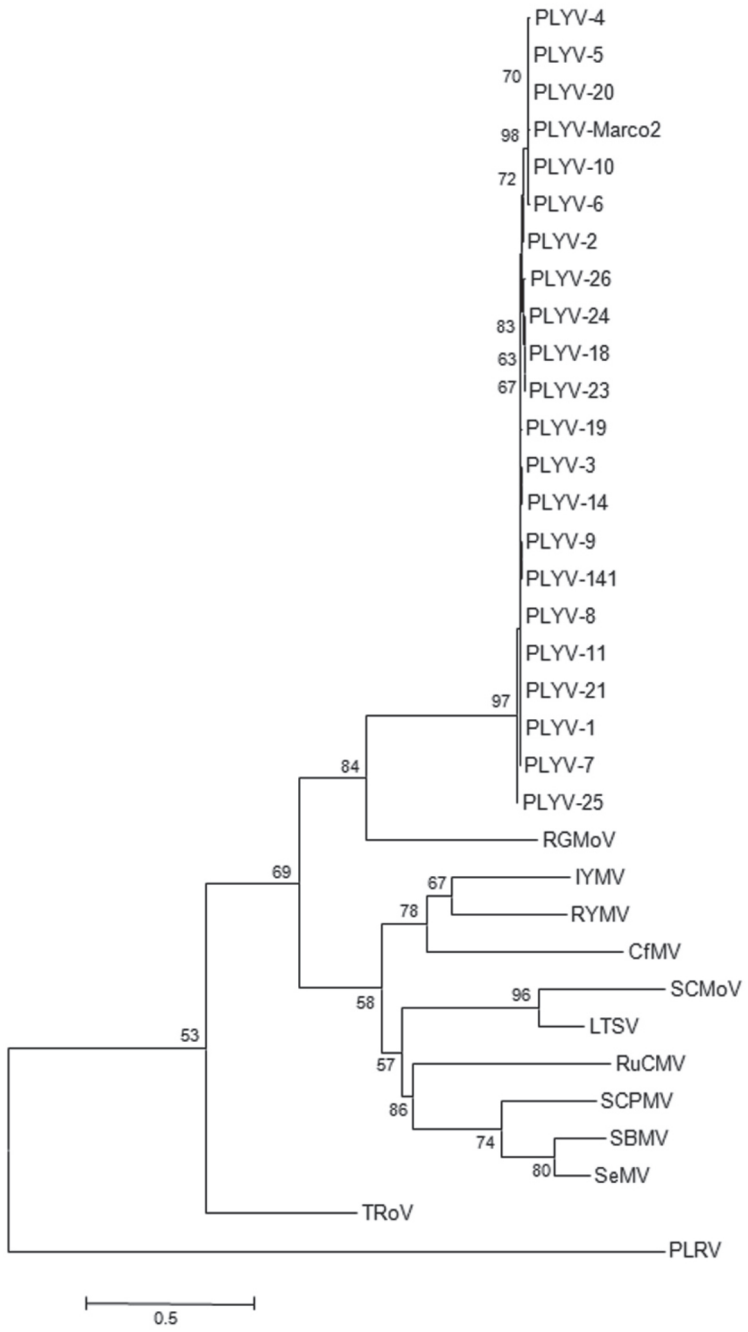

FIGURE 1 - Phylogenetic tree based on the nucleotide sequence of a 900 bp fragment, encompassing part of the putative RdRp and CP open reading frames, from 21 isolates of papaya lethal yellowing virus (PLYV) from Ceará and Rio Grande do Norte states, Brazil, and viruses in the genus *Sobemovirus*. *Potato leafroll virus* (genus *Polerovirus*, family *Luteoviridae*) was included as an outgroup. The tree was constructed using the neighbor-joining method and the K2+I+G nucleotide substitution model, and was bootstrapped with 2000 replications. See Table 2 for full virus names and GenBank accession numbers.

In any event, a definitive taxonomic placement for PLYV will require the determination of its complete genomic sequence, since it is not unreasonable to assume that the remaining part of the genome could be divergent from known sobemoviruses.

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