*Tropical Plant Pathology*, vol. 39(1):104-108, 2014

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**SHORT COMMUNICATION**

# Genetic diversity studies of Papaya meleira virus

**Cleidiane B. Daltro[1], Emanuel Felipe Medeiros Abreu[2], Francisco Jose Lima Aragão[3] & Eduardo C. Andrade[2]**

[1]Centro de Ciências Agrárias, Ambientais e Biológicas, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, BA, 44380-000, Brazil; [2]Embrapa Mandioca e Fruticultura, Cruz das Almas, BA, 44380-000, Brazil; [3]Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, 70770-917, Brazil

Author for correspondence: Eduardo C. Andrade, e-mail: eduardo.andrade@embrapa.br

**ABSTRACT**

Papaya (*Carica papaya*) is a fruit crop of great economic and social importance for Brazil and other papaya-producing countries. Brazil is the second largest producer in the world. The papaya sticky disease, caused by Papaya meleira virus (PMeV), has caused great losses in the major Brazilian papaya-producing states. In order to estimate the genetic diversity of PMeV, latex samples were collected from papaya plants in the states of Bahia, Espírito Santo, Pernambuco, Ceará and Rio Grande do Norte, and total RNA was extracted. Specific primer for the replicase region allowed the amplification, by RT-PCR, of a fragment of approximately 560 bp from 31 isolates. The sequence analysis indicated a level of conservation greater than 88% among isolates. Furthermore, comparative analyzes indicated that PMeV has similarity with mycoviruses of the family *Totiviridae*. This phylogenetic relationship was reinforced by the presence of conserved motifs within in the RdRp regions from mycoviruses.
**Key words**: *Carica papaya, Totiviridae*, sticky disease, variability, viruses.

Papaya (*Carica papaya* L.), is a fruit crop that originated and was domesticated in southern Mexico, and now is globally distributed and cultivated in many countries around the world. According to data from the Food and Agriculture Organization (FAO), the global production of papaya in 2010 was about 8 million tons, representing 10% of all tropical fruits produced worldwide (faostat.fao.org). The world's leading producers are India (42%) and Brazil (23.4%). Papaya production in Brazil was about 1,87 million tons on 35,000 hectares, with a value estimated at US$ 650 million (IBGE, 2011). The major papaya producing states are Bahia (928,000 tons), followed by Espírito Santo (560,000 tons), Ceará (112,000 tons) and Rio Grande do Norte (69,000 tons).

The main factors that threaten papaya production are insect pests and diseases, especially those caused by viruses. The papaya sticky disease, caused by Papaya meleira virus (PMeV) was reported in the 1980's affecting papaya plants in the south of Bahia and north of Espírito Santo (Nakagawa et al., 1987; Rodrigues et al., 1989). Soon afterwards, the disease was detected in the states of Pernambuco, Ceara and Rio Grande do Norte (Barbosa et al., 1998a,b). Outside Brazil, Mexico is the only other country where the disease has been reported (Perez-Brito et al., 2012).

The disease's typical symptoms are: excessive exudation of watery latex on the fruit surface, which oxidizes and gives the fruit a stained and sticky aspect; fruit malformation, uneven ripening and changes in the organolepctic properties, rendering it unmarketable.

The PMeV genome composition and size are uncommon among plant viruses. PMeV has a double strand RNA (dsRNA) genome, approximately 12 Kb in length, packed into an isometric particle with *ca.* 50 nm diam (Kitajima et al., 1993; Maciel-Zambolin et al., 2003). The virus can be transmitted by latex injection into the stem apex, and previous work suggests that the virus could be transmitted by *Bemisia tabaci* (Vidal et al., 2005).

In spite of this virus being considered the main viral disease of papaya in Brazil, knowledge on the sequence and genomic organization of the virus is poor. Nucleotide sequence information that is available indicates similarities of PMeV with dsRNA viruses of the family *Totiviridae* (Araujo et al., 2007). Studies on the genetic diversity of PMeV are lacking. To generate genetic information, we carried out a comparative and diversity analysis of a genomic fragment of PMeV isolates from papaya plants collected in the main producer's states of Brazil.

Latex samples from *C. papaya* plants displaying symptoms of sticky disease were collected in production areas in the states of Bahia (Eunápolis, Juazeiro, Porto Seguro and Teixeira de Freitas), Ceará (Acaraú and Quixeré), Espírito Santo (Sooretama and Linhares), Pernambuco (Petrolina) and Rio Grande do Norte (Baraúna and São José de Mipidu). Samples were obtained by injuring the fruit's peel and collecting the latex exuded from such fruits in 2mL microtubes which were then stored in an ultra freezer (-80ºC). Samples were collected between the years 2008 and 2011, except for isolate PMeV-47, which was collected

in 2000. The presence of PMeV in the latex was assessed by extraction of viral dsRNA, treated with DNAse I and visualized in 1% agarose gels after electrophoresis, as described previously (Tavares et al., 2004).

The samples that were proven positive for the presence of PMeV were selected, diluted in 0,1M ammonium citrate buffer, pH 6,0 and the viral dsRNA extracted using Brazol reagent (LGC Biotecnologia), according to the manufacturer's instructions. The final dsRNA pellet was dissolved in 20 µl of nuclease-free water, qualitatively analyzed by electrophoresis on 1% agarose gels and stored at -80°C. Viral cDNA was transcribed from 5 µg of dsRNA with SuperScript III (Invitrogen) according to the manufacturer's instructions, using random hexamers. The genomic region was amplified by PCR with *Taq* DNA polymerase (Invitrogen) using the primer PMeV 5'-ACCACAATGGGTATTTAAAG-3' [acting as a forward and reverse primer (Araujo et al., 2007)]. The amplification process was programmed for initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturing at 94°C for 1 min, primer annealing at 44°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified fragments were cloned using the pGEM-T Easy kit (Promega), according to the manufacturer's instructions. Cloning was confirmed by digestion of plasmid DNA with *Eco*R I and analysis by agarose gel electrophoresis. Viral fragments were sequenced in both directions and the consensus sequence was obtained using DNAMan v. 4 (Lynnon Biosoft).

A total of 184 latex samples were collected from papaya plants showing typical sticky disease symptoms. All samples tested positive for the presence of PMeV based on the presence of viral dsRNA of approximately 12 kbp (*data not shown*). To the diversity study, 31 representative samples were selected (table 1), and a fragment with 560 bp was amplified via RT-PCR, cloned and completely sequenced.

Aiming to obtain some information regarding the PMeV taxonomic relationship, the sequence of isolated PMeV-41 was chosen to be used to search for similarities within the GenBank database (www.ncbi.nlm.nih.gov/genBank). Analysis using tBLASTX (Altschul et al., 1997) indicated a similarity with RdRp from dsRNA mycoviruses members of the family *Totiviridae*. The closest mycovirus was *Phleobiopsis gigantea* virus-2 (PgV-2), a dsRNA virus

**TABLE 1 -** Isolates of Papaya meleira virus obtained and analyzed in this study and respective region of origin and year of collection.

| Isolates | Place of collection | Year of collection |
|---|---|---|
| **1. P03** | Eunápolis, BA | 2011 |
| **2. P10** | Sooretama, ES | 2009 |
| **3.P20** | Quixeré (Itaitinga), CE | 2008 |
| **4. P21** | Quixeré (Itaitinga), CE | 2008 |
| **5. P22** | Acaraú, CE | 2008 |
| **6. P23** | Sooretama, ES | 2009 |
| **7. P27** | Quixere (Oiticica do Miranda), CE | 2008 |
| **8. P36** | Sooretama, ES | 2009 |
| **9. P38** | Sooretama, ES | 2009 |
| **10. P40** | Sooretama, ES | 2009 |
| **11. P41** | Linhares, ES | 2009 |
| **12. P47** | Teixeira de Freitas, BA | 2000 |
| **13. P107** | Linhares, ES | 2009 |
| **14. P122** | Linhares, ES | 2009 |
| **15. P131** | Linhares, ES | 2009 |
| **16. P135** | Linhares, ES | 2009 |
| **17. P136** | Sao Jose de Mipidu, RN | 2009 |
| **18. P137** | Petrolina (projeto Nilo Coelho, N10, lote 1405), PE | 2008 |
| **19. P140** | Juazeiro (Projeto Mandacaru I, lote 44), BA | 2008 |
| **20. P 145** | Baraunas, RN | 2009 |
| **21. P148** | Juazeiro (Projeto Mandacaru I, lote 52), BA | 2008 |
| **22. P151** | Baraúnas, RN | 2008 |
| **23. P153** | Juazeiro (Projeto Mandacaru I, lote 52), BA | 2009 |
| **24. P154** | Juazeiro (Projeto Mandacaru I, lote 42), BA | 2008 |
| **25. P166** | Porto Seguro, BA | 2010 |
| **26. P170** | Juazeiro (Projeto Mandacaru I, lote 44), BA | 2008 |
| **27. P172** | Porto Seguro, BA | 2010 |
| **28. P173** | Porto Seguro, BA | 2010 |
| **29. P174** | Porto Seguro, BA | 2010 |
| **30. P 175** | Juazeiro (Projeto Mandacaru I, lote 51), BA | 2008 |
| **31. P200** | Petrolina (projeto Nilo Coelho, N9 lote 1114), PE | 2008 |
| **32. P000*** | ES | N/A |

with a genome of approximately 10-12kbp (Kozlakidis et al., 2009). Further analysis using the ORF finder tool (www.ncbi.nlm.nih.gov/projects/gorf) yielded a partial ORF with 153 amino acids (aa). A BlastX search confirmed the relationship between PMeV-41 sequence and the RdRp ORF of mycoviruses.

Amino acid sequence comparisons (DNAMan v. 4) with other *Totiviridae* species revealed identities that ranged between 26 to 32% (Table 2). Although these values are not high, they are similar to those observed among the species shown in Table 2 (24 to 37%), with exception of Grapevine associated totivirus-2 (GrAV-2) that share identities of 83 and 63% with *Fusarium graminearum* mycovirus-3 (FgV-3) and *Scleorinia sclerotiorum* mycovirus-L (SsV-L), respectively and between FgV-3 and SsV-L that share 63% of identity. Similar results were observed when the nucleotide sequence was compared. The level of identity between PMeV-41 and the mycoviruses ranged from 47-55%, whereas among mycoviruses was from 53 to 72%.

Moreover, a sequence alignment using Clustal W (Thompson et al., 1994) including PMeV isolates representative of each region and PGV-2 identified six conserved regions between PMeV and PgV-2 (Figure 1). Two of these conserved sequences (WSASGG and GKNRVIWNT) represent conserved domains of RdRp from dsRNA mycoviruses (Bruenn, 1993; Roouthier & Bruenn, 1998; Spear et al., 2010).

**TABLE 2 -** Sequence identity (%) in the RNA-dependent RNA polymerase region between PMeV-41 and other virus from *Totiviridae*. Values above of the diagonal correspond to the partial deduced amino acids and values below correspond to nucleotides.

| | PgV-2 | FgV-3 | DsRV-1 | GraTV-2 | SsV-L | PiRV-3 | PMeV-41 |
|---|---|---|---|---|---|---|---|
| **PgV-2**[1] | | 34 | 37 | 37 | 37 | 28 | 32 |
| **FgV-3** | 57 | | 36 | 63 | 63 | 24 | 27 |
| **DsRV-1** | 55 | 53 | | 39 | 37 | 29 | 30 |
| **GrAV-2** | 54 | 59 | 54 | | 83 | 28 | 28 |
| **SsV-L** | 56 | 57 | 54 | 72 | | 26 | 26 |
| **PiRV-3** | 53 | 53 | 53 | 55 | 57 | | 26 |
| **PMeV-41** | 52 | 49 | 47 | 55 | 53 | 50 | |

[1]PgV-2*: Phleobiopsis gigantea* Mycovirus 2 (AM111097.2); GrAV-2: Grapevine associated Totivirus 2 (GU108594.1); DsRV-1: *Diplodia scrobiculata* RNA virus 1 (EU547739.1); FgV-3 : *Fusarium graminearum* Mycovirus 3 (GQ140626.1); SsV-L: *Sclerotinia sclerotiorum* Mycovirus L (JQ513382.1); PiRV-3: *Phytophthora infestans* RNA virus 3 (JN603241.1).

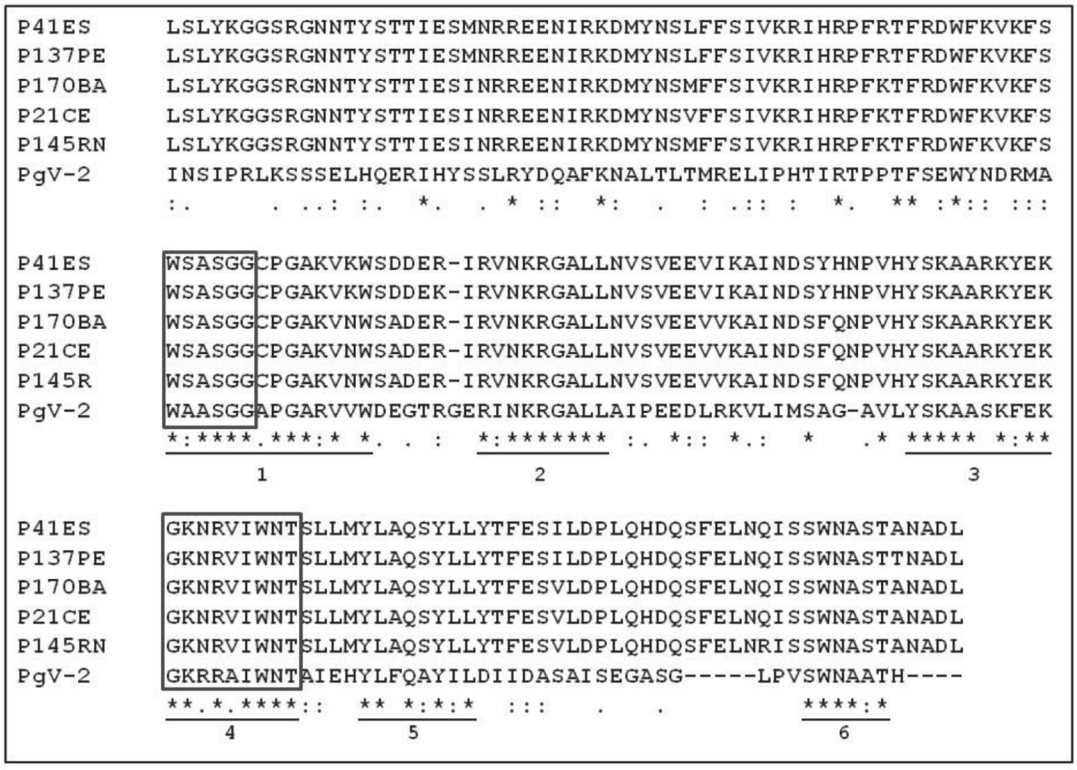


**FIGURE 1 -** Comparison of deduced amino acid sequences of the RdRp of representative isolates of PMeV and *Phleobiopsis gigantea* Mycovirus 2 (PgV-2). Numbers 1-6 refer to conserved regions among PMeV isolates and PgV-2. The rectangles indicate conserved motifs present in RdRps of dsRNA viruses. Asterisks, colons and dots indicate identical amino acid residues, conserved amino acid residues and semi-conserved amino acid residues, respectively.

These results reinforce a probable phylogenetic relationship between PMeV and mycoviruses of the family *Totiviridae*, previously reported (Araujo et al., 2007).

In the genetic diversity analysis, in addition to the 31 PMeV isolates, the sequence of an isolate from the Espírito Santo was included (Araujo et al., 2007), here called P000-ES (sequence not deposited in GenBank). Comparative analysis shows that the PMeV isolates share <88% identity in the nucleotide sequence and <90% in the aa levels among themselves. These identities values indicate that the region is conserved, even if we consider that some isolates were collected in regions far away from each other (e.g:, isolate PMeV-27 collected in CE shared 100% of identity with isolates 107, 122, 131, 135 collected in ES, almost 2000 Km distant) or in nearby regions, but with intervals of more than ten years (e.g.: PMeV-47 shared 99-100% of aa identity with isolates 3, 172, 173).

The identity values among PMeV isolates are close to those observed in a similar study comprising RdRp from nineteen isolates belonging to four possible species of *Trichomonas vaginalis* virus (TvV-1 to 4), which determined that the level of identity ranged from 82 to 94%, depending on the virus specie (Goodman et al., 2011). In another study with TvV isolates from Cuba, it was found that the conservation in the aa sequence of RdRp was of 81 to 85% (Fraga et al., 2012). Furthermore, the level of amino acid identity in the RdRp was similar to the level of identity observed for the complete genome.

Phylogenetic analysis based on alignments of the nucleotide and amino acid sequences of RdRp generated trees with similar topologies and for this reason, only the tree based on the amino acid sequence is shown (Figure 2). The lack of tendency for isolates from the same region to group together was likely due to repeated introduction

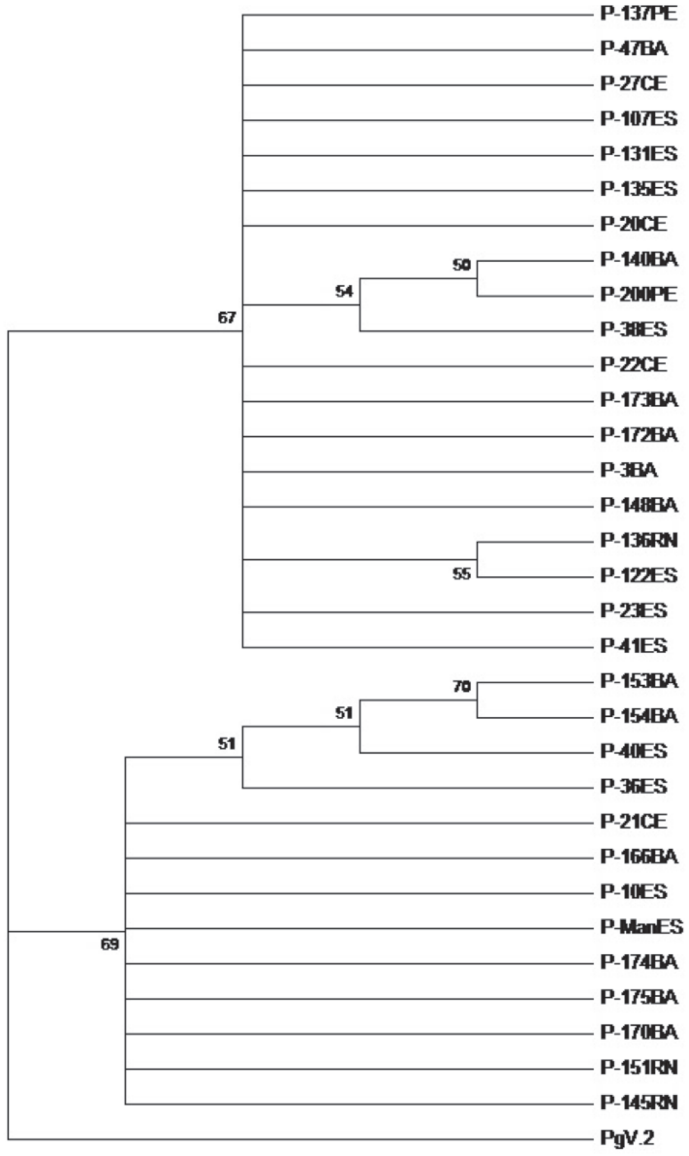


**FIGURE 2 -** Phylogenetic tree based on the deduced amino acid sequence of the putative RdRp from 32 isolates of papaya meleira virus (PMeV) from Brazil and a member of the family *Totiviridae, Phebiopsis gigantea* virus 2 (PgV-2, AM111097.2). The tree was constructed with MEGA 5.0 (Tamura et al., 2011), using the neighbor-joining method and was bootstrapped with 1000 replications.

of multiple virus strains over the years, or due to constant movement of virus isolates through the movement of infested/infected seeds, seedlings or by the putative vector. Although the possibility of virus transmission by seed has never been properly investigated the recent detection of the virus in Mexico (Perez-Brito et al., 2012) suggests that PMeV can be transmitted by seed.

The results presented here, associated with genome characteristics (size and composition) indicates a close relationship of PMeV with mycoviruses of the family *Totiviridae*, reinforcing previous indications that PMeV may represent a novel group of plant viruses (Maciel-Zambolin et al., 2003). Moreover, the genome region analyzed here, even representing just 5% of the genome, is highly conserved among isolates. However, a conclusive taxonomic placement, as well as whether the genomic region used for the analysis of variability reflects the entire genome, will only be possible when the full genome is sequenced.

## ACKNOWLEDGEMENTS

This work was partially funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (grant 474137/2007-4) and Embrapa (grant 03.08.06.056.00) to ECA.

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*TPP-2013-0064*
*Submitted: 1 May 2013*
*Revisions requested: 21 May 2013*
*Accepted: 29 August 2013*
*Section Editor: Jorge Alberto Marques Rezende*