Virus Genes
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# Discovery and molecular characterization of a novel enamovirus, Grapevine enamovirus-1

**João Marcos Fagundes Silva[1] · Maher Al Rwahnih[2] · Rosana Blawid[1] · Tatsuya Nagata[1] · Thor Vinícius Martins Fajardo[3]**

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**Abstract** In this study, we describe a novel putative *Enamovirus* member, Grapevine enamovirus-1 (GEV-1), discovered by high-throughput sequencing (HTS). A limited survey using HTS of 17 grapevines (*Vitis* spp.) from the south, southeast, and northeast regions of Brazil led to the detection of GEV-1 exclusively on southern plants, infecting four grapevine cultivars (Cabernet Sauvignon, Semillon, CG 90450, and Cabernet franc) with a remarkable identity of around 99% at the nucleotide level. This novel virus was only detected in multiple-virus infected plants exhibiting viral-like symptoms. GEV-1 was also detected on a cv. Malvasia Longa by RT-PCR. We performed graft-transmissibility assays on GEV-1. The organization, products, and cis-acting regulatory elements of GEV-1 genome are also discussed here. The near complete genome sequence of GEV-1 was obtained during the course of this study, lacking only part of the 3′ untranslated terminal region. This is the first report of a virus in the family *Luteoviridae* infecting grapevines. Based on its genomic properties and phylogenetic analyses, GEV-1 should be classified as a new member of the genus *Enamovirus*.

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✉ Thor Vinícius Martins Fajardo
thor.fajardo@embrapa.br

[1] University of Brasília, Brasília, DF, Brazil

[2] Department of Plant Pathology, University of California, Davis, CA, USA

[3] Embrapa Grape & Wine, Bento Gonçalves, RS, Brazil

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## Text

A limited survey was performed on 17 grapevine samples subjected to high-throughput sequencing (HTS). These plants were collected from three different grapevine collections from the south (11), northeast (2), and southeast (4) regions of Brazil, and the symptoms in the *V. vinifera* hosts were downward rolling of leaves and reddening or yellowing, whereas other genotypes were asymptomatic [1, 2]. Following a typical metagenomic pipeline using HTS, we were able to identify a new putative *Enamovirus* member, tentatively named Grapevine enamovirus-1 (GEV-1), infecting distinct grapevine cultivars in Brazil. The family *Luteoviridae* comprises three genera, *Luteovirus*, *Polerovirus,* and *Enamovirus*. These viruses have a positive-sense RNA genome of around 5.2–6.3 kb [3]. The genus *Enamovirus* has only one recognized viral species, *Pea enation mosaic virus-1* (PEMV-1), and two putative members, Citrus vein enation virus (CVEV) and Alfalfa enamovirus-1 (AEV-1). The systemic movement of PEMV-1, type species of the genus *Enamovirus*, is provided by an umbravirus [4], although this has not been reported for the remaining putative enamoviruses. Viruses in the family *Luteoviridae* are transmitted by aphids in a circulative non-persistent manner [3].

To characterize the viromes of these plants, double-stranded RNA (dsRNA) extracts were subjected to HTS on the Illumina HiSeq 2000 platform. Briefly, reads were trimmed, *de novo* assembled (CLC bio, Qiagen, USA), and subjected to a BLASTX search against the NCBI viral

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RefSeq database. This led to the identification of a novel luteovirid in four grapevine samples of different cultivars: Cabernet Sauvignon (S1M-CS), CG 90450 (S12-CG), Semillon (S16-SE), and Cabernet franc (S19-CF). All vines exhibiting symptoms of viral infection were collected from the Rio Grande do Sul (RS) state, south region of Brazil. Seven sets of primers (Table 1) were designed to confirm the infection of GEV-1 on the S1M-CS vine, and amplicons corresponding to sets 1, 6, and I were sequenced, which verified an identity of 99% with the contigs built by *de novo* assembly. Contigs with *Luteoviridae* hits in the samples S1M-CS (1), S12-CG (5), S16-SE (1), and S19-CF (5) covered, respectively, 99, 93, 99, and 95% of the near complete GEV-1 sequence, obtained after rapid amplification of the cDNA ends (RACE) in the 5′ extremity by the Terminal deoxynucleotidyl transferase (TdT) method. After scaffolding of the fragmented S12-CG and S19-CF contigs, an identity of around 99% at the nucleotide level was verified for GEV-1 among these four samples. Mixed infections were present in all GEV-1 positive samples [1, 2] and the virus communities in these samples included 11 pathogens: Grapevine Cabernet Sauvignon reovirus (GCSV), *Grapevine vein clearing virus* (GVCV), *Grapevine Red Globe virus* (GRGV), *Grapevine leafroll-associated virus 2* and 3 (GLRaV-2, -3), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine fleck virus* (GFkV), *Grapevine rupestris vein feathering virus* (GRVFV), and *Grapevine yellow speckle viroid 1* (GYSVd-1). Additionally, GEV-1 was detected on a Malvasia Longa vine by RT-PCR during the virus indexing of this cultivar. The near complete genome of GEV-1 isolate CS-BR (6227 bp), lacking only part of the 3′ untranslated sequence, was deposited in GenBank under accession KX645875. The isolate SE-BR near complete sequence (6176 bp), obtained only by *de novo* assembly, was also deposited in GenBank under accession KY820716.

To assess the graft transmissibility of the novel virus, cv. Cabernet Sauvignon (S1M-CS) was grafted onto 16 healthy cv. 1103P plants (*V. berlandieri* x *V. rupestris*). Graft-transmissibility of GEV-1 was confirmed in 13 out of 16 positive samples by performing RT-PCR 5 months after grafting.

Luteovirids are known to harbor five to ten open reading frames (ORFs) usually displayed as two gene blocks separated by a non-coding intergenic region [5–7]. The 5′-proximal block contains the two partially overlapping ORFs 1 and 2, plus an additional ORF encoding a silencing suppressor protein (ORF 0) in the genera *Enamovirus* and *Polerovirus*. The 3′-proximal gene block contains the ORFs corresponding to the coat protein (ORF 3), an extension of the coat protein translated by an in-frame stop codon readthrough (ORF 5) and a movement protein (ORF 4) located within ORF 3 in the genera *Luteovirus* and *Polerovirus* that is absent in the genus *Enamovirus*. ORFs 3, 4, and 5 are translated from a subgenomic RNA (sgRNA). GEV-1 genomic features (Fig. 1a) are discussed below.

ORF 0 (nt 335–1279) overlaps ORF 1 and potentially encodes a 34.9 kDa protein (P0), presumably a suppressor of host RNAi machinery. The F-box-like domain (LPxxI/L(x10–13)P) found in the P0 of polero- and enamoviruses is necessary for its silencing suppressor activity [8, 9]. Interestingly, it was verified that only the first leucine is conserved on GEV-1. ORF 1 (nt 471–2912) potentially encodes an 89.8 kDa protein (P1), it contains a conserved 3C-like serine peptidase followed by the genome-linked viral protein (VPg). The conserved domain H(x25)D(x70–80)GxSG of the S39 serine protease is positioned between nt 1350 and 1841 [10]. Alignments

**Table 1** Primers used for detection of Grapevine enamovirus-1 (GEV-1)

| Name | Sequence | 5′ nt position | Length (bp) |
|---|---|---|---|
| Set1F | CACACTTGCTTCTCTTCTCG | 50 | 749 |
| Set1R | CCAACGTAAGCGAATAGTCG | 798 | |
| Set2F | GTTGGAGAGAGGAAAGAATCGG | 1323 | 684 |
| Set2R | GGGTTTGTCTGTGACCTCATAGTC | 2006 | |
| Set3F | AGGCCAAGAGGGGCAAGAAATTGT | 2434 | 530 |
| Set3R | CGGCAGATTTTGATCTAGCAGCTC | 2963 | |
| Set4F | ACAAGCAGGAGTTGAGGATG | 3423 | 600 |
| Set4R | CGACGAGCATTTTACCCACA | 4022 | |
| Set5F | GGACAGAGGTTGCATTGCGTAT | 4622 | 505 |
| Set5R | TTGAAACCGAGCCAAGTGAGTGTC | 5126 | |
| Set6F | TTCCCTTGGGAGACTCGGTTCTAT | 5263 | 735 |
| Set6R | AAACATGACCACCCGTCTCATAGC | 5997 | |
| SetIF | AAAGTGGTGTGTCGCTATGG | 3850 | 638 |
| SetIR | GGCAAACGAATTTACCAAGAACG | 4487 | |

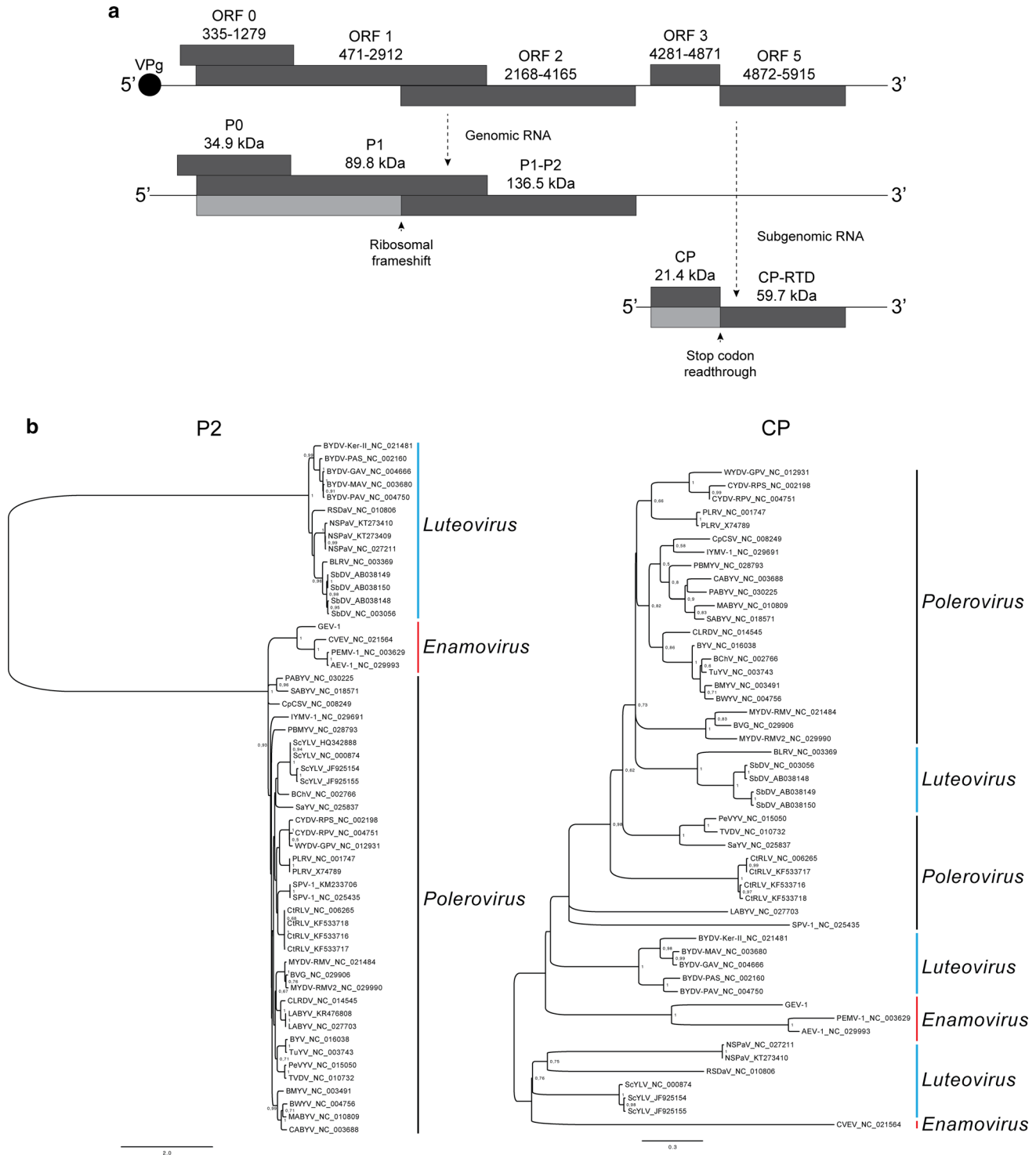


**Fig. 1** **a** GEV-1 genome organization and **b** Maximum likelihood trees (JTT + G(4) + I; Bootstrap = 1000 replications) for the family *Luteoviridae* using the P2 and CP amino acid sequences. Trees were inferred with MEGA 7 [28]. Alignments were performed with MUSCLE. Trees were midpoint rooted

with PEMV-1 suggest that the first VPg cleavage site (E/S) at the GEV-1 genome is positioned at nt 1938 [11, 12], but we were unable to deduce the second proteolysis site. The W(A, G)D motif followed by a DE-rich region [13] is located between nt 2079 and 2111. ORF 2 (nt 2168–4165) is translated by a -1 frameshift from ORF 1, originating a fusion protein (P1–P2) containing the RNA-dependent RNA polymerase (RdRp), of a predicted molecular mass of

136.55 kDa. The highly conserved GDD box motif [14] is located between nt 3869 and 3877. ORF 3 (nt 4281–4871) potentially encodes a 21.4 kDa protein, which corresponds to the coat protein (CP). ORF 5 (nt 4872–5915) encodes the readthrough domain (RTD) of the CP-RTD fusion protein, predicted to have a total molecular mass of 59.7 kDa. This protein is needed for efficient aphid transmission [15]. GEV-1 lacks the C-terminal portion of the CP-RTD protein that is responsible for limiting the infection of luteo- and poleroviruses to the phloem [4]. The amino acid identity between GEV-1 and others enamoviruses is below 44% for all ORFs.

Viruses in the family *Luteoviridae* employ a wide range of translational mechanisms which are regulated by cis-acting RNA elements (CRE) embedded in the virus genome [6, 16]. GEV-1 ORF 0 possesses a leaky start codon UAU**AUG**U, allowing the translation of ORF 1 [17, 18]. Two signals are required for the -1 ribosomal frameshift at ORF 1, the heptanucleotide sequence XXXYYYZ and a downstream pseudoknot or very stable RNA secondary structure located six to eight nucleotides from the frameshift site [19]. We found the TTTAAAC sequence located at nt 2168 and a pseudoknot seven nt downstream of this site, predicted with the RNAPKplex program [20]. The CCNNNN tandem repeat motif associated with ORF 3 stop codon readthrough [21] is located between nt 4887 and 4935, 15 nucleotides downstream from the termination site. Remarkably, GEV-1 readthrough site at ORF 3 (UUG**UGA**UAU) is not similar to any previously reported *Luteoviridae* [18].

Maximum likelihood trees for the family *Luteoviridae* were estimated based on the P2- and CP-translated sequences (Fig. 1b). The ORF 2 is separated from ORF 3 by an intergenic region which is a probable hot spot for recombination among luteovirids [22, 23] so incongruences in the trees when considering these two distinct regions are expected. In both trees, GEV-1 clusters together with PEMV-1 and AEV-1, indicating that GEV-1 is more closely related to the genus *Enamovirus*.

Using HTS of dsRNA extracts from 17 samples, some of them exhibiting viral-like symptoms, we identified in four samples, within a virus community, a new putative *Enamovirus* member, provisionally named Grapevine enamovirus-1 (GEV-1), infecting distinct grapevine cultivars from the south region of Brazil. The distinguishing feature of the genus *Enamovirus* is the lack of a movement protein [3]. No ORF corresponding to this protein could be identified on GEV-1. Bioassays confirmed infection and graft-transmissibility of GEV-1. Due to the lack of a movement protein, it is possible that GEV-1 needs co-infection with another virus for its cell-to-cell movement and graft-transmissibility. Phylogenetic analyses revealed that GEV-1 is more closely related to the genus *Enamovirus*. Based on these data, GEV-1 should be classified as a new member of the genus *Enamovirus*. Due to its sensitivity, HTS have been proposed as a diagnostic tool for biosecurity and quarantine surveillance [24–26]. Despite being a valuable tool for discovering novel viruses in metagenomic samples, information regarding the biological significance of a newly discovered virus such as pathogenicity, transmission, host range, and epidemiology often cannot be obtained by these means [27]. In addition, grapevines often present complex pathosystems, and further studies are needed to understand the interaction between these pathogens and their effect on the vines health, development, and quality of the grapes.

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**Author's contributions** TVMF designed and supervised the study, and performed initial confirmation of infection and graft-transmissibility assays. MAR performed *de novo* assemblies and BLASTX searches, as well as primer design. Further bioinformatics analysis and 5′ RACE were performed by JMFS and RB. TN supervised the study. JMFS wrote the manuscript, and all authors contributed to the review.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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

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