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## The expanding menagerie of Prunus-infecting luteoviruses

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#### Abstract

Members of the genus Luteovirus are responsible for economically destructive plant diseases worldwide. Over the past few years, three luteoviruses infecting Prunus trees have been characterized. However, the biological properties, prevalence, and genetic diversity of those viruses have not yet been studied. High throughput sequencing of samples of various wild, cultivated, and ornamental Prunus species enabled the identification of four novel species in the genus Luteovirus for which we obtained complete or nearly complete genomes. Besides, we identified another new putative species recovered from Sequence Read Archive data. Furthermore, we conducted a survey on peach-infecting luteoviruses in eight European countries. Analyses of 350 leaf samples collected from germplasm, production orchards, and private gardens showed that peachassociated luteovirus (PaLV), nectarine stem pitting-associated virus (NSPaV), and a novel luteovirus, peach-associated luteovirus 2 (PaLV2), are present in all countries, while the most prevalent virus was NSPaV, followed by PaLV. An analysis of the genetic diversity of these viruses was also conducted. Moreover, the biological indexing on GF305 peach indicator plants demonstrated that PaLV and PaLV2, like NSPaV, are transmitted by graft at relatively low rates. No clear viral symptoms have been observed either in graftinoculated GF305 indicators, or in different peach tree varieties observed in an orchard. The data generated during this study provide a broader overview of the genetic diversity, geographical distribution and prevalence of peach-infecting luteoviruses, and suggest these viruses are likely asymptomatic in peach under most circumstances.


Keywords: HTS, Stone fruit, Luteovirus, geographical distribution, biological indexing

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## Introduction

Almond and other stone fruits, such as plum, peach, sweet and sour cherry, and apricot belong to the genus Prunus in the family Rosaceae. Numerous graft-transmissible pathogens including viruses, viroids, and phytoplasmas have been described in Prunus and are responsible for economically important diseases, affecting the fruit industry worldwide (Hadidi and Barba, 2011). Prunus species host over 60 different viral and viroid species from diverse families including Betaflexiviridae, Bromoviridae, Secoviridae, Botourmiaviridae, Closteroviridae, Tymoviridae, Potyviridae, Tombusviridae, Pospiviroidae and Avsunviroidae (Hou et al. 2020; Maliogka et al. 2018; Rubio et al. 2017; Umer et al. 2019)

Members of the genus Luteovirus are responsible for some of the most economically important viral diseases in cereals (Miller and Rasochová 1997; Walls et al. 2019), and have also been detected in many other crops or ornamental plants including fruit trees (Bag et al. 2015; Igori et al. 2017b; Khalili et al. 2020; Lenz et al. 2017; Liu et al. 2018; Shen et al. 2018; Wu et al. 2017). The genus Luteovirus, formerly belonging to the family Luteoviridae, has recently been re-assigned to the family Tombusviridae (Miller and Lozier 2022). Its members have a single-stranded, messenger-sense RNA genome predicted to encode four to six (potentially eight) proteins, depending on the viral species considered (Bag et al. 2015; Hillman and Esteban, 2011; Lenz et al. 2017; Smirnova et al. 2015). Open reading frame 1 (ORF1) encodes a replication-association protein (P1), while ORF2 encodes the viral RNA-dependent RNA polymerase (RdRp). Following a -1 frameshift, RdRp is expressed as a P1-P2 fusion protein. ORF 3a, 3, 4, and 5 are translated from sub-genomic RNA1 (sgRNA1) (Domier and D'Arcy 2008; Smirnova et al. 2015). ORF3
codes for the coat protein (CP), while ORF5 is expressed as a fusion to the CP following the suppression of the leaky stop codon terminating ORF3. The small ORF3a, which is located upstream of ORF3, is translated from a non-AUG start codon (Smirnova et al. 2015) and its P3a product has been shown to be implicated in viral movement. The ORF4, which completely overlaps with the CP gene, encodes the movement protein (MP), and is translated via leaky scanning of the ORF3 start codon due to its poor context for initiation (Dinesh-Kumar and Miller 1993; Domier and D'Arcy 2008). A second subgenomic RNA, sgRNA2, likely expresses the P6 protein (Kelly et al. 1994). ORF7 encodes the putative P7 protein of unknown function and has been recently described in the genome of cherryassociated luteovirus (ChALV) (Lenz et al. 2017).

Prior to the present study, three Prunus-infecting luteoviruses had been described: nectarine stem pitting-associated virus (NSPaV) is the first luteovirus identified in peach (Prunus persica) by Bag et al in the USA in 2015 (Bag et al. 2015). Since then, NSPaV was reported naturally to infect peach in China, Hungary, South Korea, Australia (Igori et al. 2017a; Jo et al. 2017; Krizbai et al. 2017; Lu et al. 2017), and in P. mume (Japanese apricot) in Japan (Candresse et al. 2017). Furthermore, it has been experimentally shown that NSPaV can infect $P$. avium (sweet cherry) and $P$. tomentosa (Nanking cherry) (Villamor et al. 2016). Later, ChALV was characterized in $P$. avium and $P$. cerasus from the Czech Republic (Lenz et al. 2017). Peach-associated luteovirus (PaLV) was initially described in the USA from peach material imported from Georgia and Spain (Wu et al. 2017) and has since been reported, again from peach, in China, South Korea, Italy, and Hungary (Barath et al. 2022; Igori et al. 2017b; Sorrentino et al. 2018; Zhou et al. 2018).

Luteoviruses generally have aphid vectors (Ali et al. 2014) but this has not yet been verified for Prunus-infecting luteoviruses.

The association between Prunus luteoviruses and symptoms in their hosts is still unclear. Even for NSPaV that was initially isolated from nectarine trees showing extensive pitting on their woody cylinder (Bag et al. 2015), the authors pointed out the difficulty to correlate the symptoms with the virus presence. In addition, in another study (Villamor et al. 2016), NSPaV was detected together with a marafivirus in multiple nectarine and peach trees, suggesting a complex or non-existent relationship between the stem pitting symptoms and the two viruses. The same conclusion can be drawn from two studies on the PaLV pathogenicity (Sorrentino et al. 2018; Wu et al. 2017). Similarly, in the case of ChALV, it was not possible to draw clear conclusions due to the presence of other co-infecting viruses (Lenz et al. 2017).

The discovery of stone fruit tree viruses using high throughput sequencing (HTS) approaches has sped up over the last two decades (Hou et al. 2020; Maliogka et al. 2018; Rubio et al. 2017). But one of the limitations of these studies is that there are plenty of novel viruses discovered for which no or only very limited information is available on their biological properties and prevalence to assess the potential risk they might pose to the trees (Massart et al. 2017).

Using the HTS approach, we identified four new Prunus-infecting luteoviruses in the present study. A fifth one was discovered following a screening approach of publicly available Prunus RNA-Seq Sequence Read Archive (SRA) data. All five novel Prunusinfecting luteovirus species were characterized at the molecular level. Besides, we evaluated the peach-infecting luteoviruses for their graft transmissibility and, as a part of
a European field survey of peach trees, their prevalence, distribution, and genetic variability.

## Material and methods

Plant material origin. Fifty peach tree ( $P$. persica) accessions introduced between 1937 and 2010 from different countries in the Prunus INRAE Biological Resource Center (BRC Toulenne, France) were indexed by HTS. For each accession, five leaves from different parts of the tree were collected in June 2019 and pooled in equal ratios, constituting the sample analyzed by HTS. In addition, a few trees belonging to various Prunus species were also analyzed by HTS. For these trees, leaf samples were collected over the 20132021 period in various countries, regardless of the presence of symptoms (Table 1). Until used, fresh leaf tissues were either desiccated over anhydrous $\mathrm{CaCl}_{2}$ (Sigma Aldrich Chimie, Saint-Quentin-Fallavier, France) and stored at room temperature or at $-80^{\circ} \mathrm{C}$. To evaluate the prevalence of the luteoviruses identified in $P$. persica, samples from peach trees originating from seven European countries (in addition to the 50 French samples cited above) were obtained either from germplasm collections or production orchards. Between 26 and 51 trees were thus sampled depending on the country: Belgium (26), Greece (30), Czech Republic (43), Italy (51), Slovakia, Spain, and Turkey (50 each). These 350 peach trees were analyzed individually for the presence of some of the Prunusinfecting luteoviruses, including NSPaV, PaLV (known luteoviruses) and PaLV2 (a new luteovirus), while MaLV and PhaLV (the novel luteoviruses characterized in this work) where analyzed as pooled samples.

Double-stranded RNA extraction, library preparation, and sequencing. Doublestranded RNAs (dsRNA) were purified from pooled leaves (S1, S3, S4, and S7 samples, Table 1) by batch chromatography on cellulose CC41 (Whatman) as described (Marais et al. 2018), and converted to cDNA using LDF primers (François et al. 2018, Supplementary Table S1) and SuperScript ${ }^{T M} \|$ Reverse Transcriptase according to manufacturer's instructions (Invitrogen/Fisher Scientific, Illkirch, France). Each cDNA preparation was subjected to a random PCR amplification using multiplex identifier (MID) adaptors (François et al. 2018, Supplementary Table S1), allowing to sequence all the samples in a multiplexed format. Five microliters of cDNA were amplified according to Marais et al. (2018) in a $50 \mu$ reaction containing $10 \times$ buffer, 4 mM dNTPs, $1 \mu \mathrm{M}$ primer MID tag, 1.25 U Dream Taq DNA polymerase (Thermo Fisher Scientific). Random PCR amplification was performed for one cycle of $94^{\circ} \mathrm{C}$ for $1 \mathrm{~min} ; 65^{\circ} \mathrm{C}$ for $0 \mathrm{~s} ; 72^{\circ} \mathrm{C}$ for 45 s , and 40 cycles of $94^{\circ} \mathrm{C}$ for $0 \mathrm{~s} ; 45^{\circ} \mathrm{C}$ for $0 \mathrm{~s} ; 72^{\circ} \mathrm{C}$ for 5 min , and 1 final cycle of 5 min at $72^{\circ} \mathrm{C}$ and 5 min at $37^{\circ} \mathrm{C}$. Following the purification of the PCR products using a MinElute PCR Purification Kit (Qiagen SAS France, Courtaboeuf, France), PCR products were pooled equimolarly before being sent for Illumina sequencing on a Hiseq3000 platform ( $2 \times 150 \mathrm{bp}$ ) [outsourced at the GetPlage INRAE platform (Toulouse, France) or Azenta (Leipzig, Germany)].

Alternatively, dsRNAs were extracted from 1 g of leaf tissue ( S 5 and S 6 samples, Table 1) using the CF11 cellulose protocol of De Paulo and Powell (DePaulo and Powell, 1995) and converted into double-stranded cDNA using the Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The sequencing library was prepared using the Illumina compatible MuSeek Library Preparation Kit (Thermo

Scientific) with the double-stranded cDNA as input material and sequenced using a HiSeq2500 system in 1x100 bp mode (SEQme.eu, Dobříš, Czech Republic).

High throughput sequencing of total RNAs. Total RNAs were extracted from desiccated leaves of the $P$. mahaleb sample (S8, Table 1) using a modified CTAB procedure (Chang et al. 1993), reverse-transcribed, ribodepleted, and sequenced (HiSeq3000 $2 \times 150 \mathrm{bp}$ ). Alternatively, total RNAs were isolated from four leaves $(100 \mathrm{mg})$ of the $P$. armeniaca sample (S2, Table 1) using the Plant/Fungi Total RNA purification kit (Norgen Biotek). Purified RNAs were ribodepleted using the QIAseq FastSelect-rRNA Plant Kit (Qiagen) and a library prepared using the NEBNext Ultra II Directional RNA Library Prep Kit before being sequenced in a multiplex run (NovaSeq6000, $2 \times 161 \mathrm{bp}$, Institute of Experimental Botany, CAS, Olomouc, Czech Republic).

HTS data analyses. Sequencing reads were quality-trimmed using CLC Genomic workbench software version 21.0.3 (Qiagen) or Geneious Prime (Biomatters Ltd, Auckland, New Zealand). Following de novo assembly of contigs, a BlastX analysis was performed against the GenBank non-redundant (nr) protein database restricted to viruses, to identify viral contigs. Sequence datasets were also analyzed by mapping trimmed reads on a collection of reference viral genomes (min length fraction=0.9; min similarity fraction $=0.7$ ). The initially identified luteoviral contigs were then scaffolded (if needed) and extended by multiple rounds of mapping using residual reads in CLC Genomics Workbench to generate nearly complete genomic sequences. For isolates of known viruses, no further effort was made to fill small internal gaps or the genome terminal ends, but for newly discovered viruses, the genomic sequences were completed as described below.

Completion of the genome sequence of the identified new viruses. In order to obtain the complete genome sequence of the newly discovered viruses peach-associated luteovirus 2 (PaLV2) and mume-associated luteovirus (MaLV), Rapid Amplification of cDNA Ends (RACE) experiments were carried out for both 5' and 3' ends using the SMARTer® RACE 5'/3'Kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) and heat-denaturated $\left(10 \mathrm{~min}\right.$ at $\left.99^{\circ} \mathrm{C}\right)$ dsRNAs as a template, following the manufacturer's instructions. Alternatively, the cherry luteovirus A (ChLVA) genome termini amplification was done using total RNAs and 5'- and 3'-RACE kits following the manufacturer's recommendations (Invitrogen, Waltham, MA, USA) with the virus-specific primers (Supplementary Table S1). Prior to the 3'-RACE, total RNAs were polyadenylated using ATP and poly(U) polymerase following the manufacturer's recommendations (NEB, Ipswich, MA, USA). Obtained RACE products sequenced (Eurofins Genomics, Ebersberg, Germany) using the virus-specific primers. All specific RACE primers used were designed from the sequence of the identified viral contigs and are listed in Supplementary Table S1. Data mining. To uncover potential new luteoviruses in publicly available RNA-Seq data, we performed an analysis on SRA using Serratus, an open-source cloud computing infrastructure (Edgar et al. 2022) that seeks the closest matched SRA sequences to an input virus using a 102 amino acid (aa) viral RNA-dependent RNA polymerase sequence (RdRp palmprint). The sequence of the contig thus identified from a Prunus humilis SRA from China (SRR12442710) has been deposited in GenBank under the BK061315 accession number.

Phylogenetic, recombination and genetic population analyses. Multiple alignments of nucleotide (nt) or amino acid (aa) sequences were performed using the ClustalW
program (Thompson et al. 1994) implemented in Mega 7 (Kumar et al. 2016). Phylogenetic trees were constructed using the neighbor-joining technique with strict nt or aa distances and randomized bootstrapping to evaluate branching validity. Mean diversities, and genetic distances (p-distances calculated on $n t$ or aa identity) were calculated using Mega 7. The RDP4 program (Martin et al. 2015) was used to search for potential recombination events in the luteovirus genomic sequences obtained in this study.

## Molecular detection of luteoviruses by RT-PCR for HTS validation, prevalence

 determination, and genetic diversity analysis. Total nucleic acids (TNA) were extracted from Prunus leaves according to the procedure 1 described in Foissac et al. (2005). The virus-specific primers were designed using the identified viral contigs sequences (Supplementary Table S1) and used to detect the targeted viruses by two-step RT-PCR assays. Briefly, TNA were first submitted to a reverse transcription initiated by pdN ${ }_{6}$ primers and using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Complementary DNAs were then amplified using specific primers and either the Dream Taq DNA polymerase (Thermo scientific) or the Advantage 2 polymerase mix (Takara Bio Europe). Amplified products were analyzed by agarose gel electrophoresis and Sanger sequenced on both strands (Eurofins). The PCR product sequences have been deposited in the GenBank database under the accession numbers ON637949 to ON638176.Graft transmission to GF305 peach indicator seedlings. Based on their virome composition, 24 peach trees of the INRAE Prunus BRC were selected for biological indexing. New flush twigs were collected in June 2021 and kept at $4^{\circ} \mathrm{C}$ prior to chipbudding on GF305 peach indicator seedlings. The grafting assays were carried out using
two twigs as budwood for every peach accession and 3-10 grafted seedlings per accession depending on twig size. Each grafted seedling was grafted with two bark pieces. In total, 199 GF305 plants were graft-inoculated in addition to five negative controls self-grafted using healthy GF305 plants free of Prunus viruses and viroids. The grafted plants were maintained under controlled greenhouse conditions for six months to monitor the appearance of symptoms. After the first cycle of observation, the plants were stored at $2^{\circ} \mathrm{C}$ to induce artificial dormancy. After 3.5 months of dormancy, the graftinoculated plants were cut back to 30 cm high and placed again in greenhouse for a second cycle of observation.

The presence of the various viruses in the grafted GF305 seedlings was assessed by testing leaves and using specific RT-PCR assays. The identity of the amplicons was confirmed by Sanger sequencing. Graft transmissibility rate was assessed by sampling individually each inoculated GF305 plant for 10 accessions, with 4-10 grafted seedlings per accession. For the other 14 accessions, grafted GF305 seedlings ( $3-10$ grafted plants) were not tested individually but as a pool of leaves from all grafted plants for each accession. A positive reaction would indicate that at least one of the grafted trees had acquired the virus.

## Results

Identification of four novel Luteovirus species and of new Prunus hosts for NSPaV.
As part of a systematic effort to explore the virome of Prunus species, dsRNAs or total RNAs extracted from a wide range of Prunus samples were analyzed by HTS. Following reads quality trimming, de novo assembly and contigs annotation based on BlastX
analysis, several contigs with similarities to Luteovirus genus members were identified in a range of samples. Contigs of interest were then assembled into scaffolds and extended by successive rounds of residual reads mapping to yield finalized contigs spanning in many cases near-complete genomes. A detailed analysis of the assembled genomes (see below) revealed that four of them shared less than $90 \%$ aa identity in at least one of their encoded proteins with known luteoviruses, which is below the molecular demarcation threshold (10\% aa divergence in any gene product) for new species in the genus Luteovirus (Hillman and Esteban, 2011). Overall, four sequences representing potentially four new species were thus identified in samples from $P$. mume (S1), P. persica (S4), P. cerasus (S6), and P. mahaleb (S8) (Tables 1 and 2, Supplementary Table S2), with the proposed names of mume-associated luteovirus (MaLV), peach-associated luteovirus 2 (PaLV2), cherry luteovirus A (ChLVA), and Prunus mahaleb-associated luteovirus (PmaLV), respectively. The genomic sequences of the PaLV2, MaLV, and ChLVA isolates were completed by filling internal gaps by PCR if needed, and by determining 5 ' and $3^{\prime}$ genome ends by RACE. The $5,822 \mathrm{nt}$ contig for PmaLV, lacking only 10 nt and 40 nt at the 5 ' and 3 ' ends respectively, as judged from a comparison with the most closely related luteovirus, ChALV (NC_031800) was not completed. The corresponding genome sequences have been deposited in the GenBank database under the accession numbers ON408234 (PaLV2), ON408236 (MaLV), ON408238 (PmaLV) and ON146357 (ChLVA) (Supplementary Table S2). The number of HTS reads mapped to each genome and the average genome coverage are presented in Supplementary Table 2. In addition to these complete genomic sequences, near-complete genomes were also obtained from other Prunus samples, allowing the identification of divergent variants of MaLV in P. armeniaca (sample S2) and P. incisa (sample S3), of ChLVA in a second P. cerasus from cv Cigany
(S6) and of a variant of NSPaV from P. cerasus (S7) (Table 1, Supplementary Table S2). NSPaV infection was also identified in a P. brigantina sample (S9), but the low viral concentration precluded the assembly of large contigs. The infection status of all samples was in all cases validated using virus-specific RT-PCR assays and sequencing of the amplicons. The near-complete genomic sequences of MaLV and NSPaV isolates have been deposited in GenBank under the following accession numbers: ON408233 (NSPaV, P. cerasus), ON408235 (MaLV, P. incisa) and ON408237 (MaLV, P. armeniaca), (Supplementary Table S2).

## Identification of a novel Luteovirus species from publicly available Prunus RNASeq

 data. To uncover other luteoviruses infecting Prunus, the Serratus tool (Edgar et al. 2022) was used with RdRp sequences of PaLV2 and MaLV, two of the four newly identified viruses in this study, as queries. At the species level, only one RNAseq SRA (P. humilis from China, SRR12442710) was identified, with a contig showing $83 \%$ aa identity in the highly conserved RdRp motif with both queries, indicating that this sequence likely represents a new species in the genus Luteovirus of this tentative agent. The SRA dataset was downloaded and, following de novo assembly using CLC Genomics Workbench, a large contig of $5,202 \mathrm{nt}$ (nearly full-length, in comparison to other Prunus-infecting luteoviral genomes) was identified. This contig shows only $48-73 \% \mathrm{nt}$ identity with any known luteovirus species, suggesting this isolate belongs to a novel species in the genus Luteovirus. The sequence of this contig has been deposited in GenBank (BK061315) and the name Prunus humilis-associated luteovirus (PhaLV) is proposed for the corresponding novel species (Supplementary Table S2).
## Molecular characterization and phylogenetic affinities of the five novel

 luteoviruses. As indicated above, the full-length genomic sequences of PaLV2 (S4), MaLV (S1) and ChLVA (S6) isolates were determined and shown to be respectively 5,780 $\mathrm{nt}, 5,748 \mathrm{nt}$, and 5,726 nt. A near-complete genome of $5,822 \mathrm{nt}$ is also available for PmaLV (S8), together with near-complete genomes of the MaLV isolates from $P$. armeniaca (5,733 nt, S2) and P. incisa (5,705 nt, S3), as well as a near-complete genome for a second ChLVA isolate from $P$. cerasus cv Cigany (5,689 nt, S6). The NSPaV scaffold detected in $P$. cerasus represents very likely the complete genome of this isolate (4,993 $\mathrm{nt}, \mathrm{S} 7$ ). The near-complete genome assembled from SRA data for PhaLV (5,202 nt) could obviously not be completed by the RACE experiment but the available sequence covers completely the virus open reading frames (ORFs).The genomes of ChLVA, MaLV, PaLV2, PhaLV, and PmaLV encode six to eight ORFs and have an organization similar to those of other members of the genus Luteovirus (Table 2 and Fig. 1A). The main variability observed concerns the short P6 and P7 ORFs, which are missing in some viruses or isolates: ORF6 is absent in one isolate of MaLV (S3 from P. incisa) and PaLV2 (Fig. 1A) and ORF7 is absent in most Prunus-infecting luteoviruses with the exception of PaLV, ChALV and ChLVA (Table 2). Surprisingly, unlike the previously reported reference NSPaV isolate from P. persica, the NSPaV isolate reported here from $P$. cerasus has an ORF6. There is thus both between-species and withinspecies presence-absence variability for these two small putative ORFs. The second main divergence from the typical genomic organization for luteoviruses concerns NSPaV, with the $P$. cerasus isolate lacking an ORF4 and an ORF3a and having a shorter ORF5, as previously reported for other NSPaV isolates and for almond luteovirus 1 (AILV1) (Bag et al. 2015; Khalili et al. 2020). ORF3a is also missing in the genome of PmaLV (Table 2).

A phylogram constructed using a whole-genome sequence alignment of all Prunusinfecting luteoviruses divides them into two clades (Fig. 1B). While NSPaV and AILV1 form a distinct clade, the rest of the Prunus-infecting luteoviruses groups together with a high bootstrap support. Interestingly, the ORF encoding the MP is systematically present in luteoviruses belonging to this latter group, whereas it is absent in NSPaV and AILV1. Phylogenetic trees based on the sequences of P1-P2 and P3-P5 fusion proteins were also generated and showed the same clustering pattern (Supplementary Fig. S1).

To precisely determine the phylogenetic affinities between Prunus-infecting luteoviruses, pairwise comparisons for the P1-P2 and P3-P5 proteins were performed (Supplementary Fig. S2). Whatever the luteovirus species and the protein considered, the level of aa identity was less than $90 \%$, with the exception of PmaLV and ChALV which show $95 \%$ aa identity in the P1-P2, but only 88\% in the P3-P5, supporting the notion that they should belong to distinct species. In addition, viral isolates identified as belonging to the same species, i.e NSPaV-P. cerasus, MaLV-P. incisa, MaLV-P. armeniaca, and ChLVA-Cigany, displayed more than $90 \%$ of aa identity in their various proteins with those of their respective reference isolates, thus confirming their taxonomic assignation (Supplementary Fig. S2).

To determine whether recombination has played a role in the evolution of the newly identified luteoviruses, an RDP4 recombination analysis was performed on a full genome multiple alignment. No recombination signature with significant support involving Prunusinfecting luteoviruses was detected (data not shown).

HTS virome characterization of peach accessions in INRAE Prunus BRC. As part of the Prunus virome characterization effort, a total number of 50 P. persica accessions were
individually analyzed by dsRNA-based HTS indexing. Upon demultiplexing and quality trimming steps, an average of 1.5 million reads (range 0.24 to 5 million reads) were obtained per individual sample. Apart from infrequent infections involving well-known peach-infecting viruses such as apple chlorotic leafspot virus (Betaflexiviridae), prunus necrotic ringspot virus (Bromoviridae), little cherry virus 1 (Closteroviridae), plum bark necrosis stem pitting-associated virus (Closteroviridae) and peach latent mosaic viroid (Avsunviroidae), BlastX analysis of the assembled contigs revealed that NSPaV, PaLV, and the newly discovered PaLV2 showed high prevalence in the peach accessions analyzed. The HTS reads datasets were also analyzed by mapping trimmed reads on reference luteovirus genomes and the results were validated by RT-PCR using corresponding virus-specific detection primers. Altogether, the results showed that $96 \%$ of the 50 peach accessions are infected by NSPaV, compared to $38 \%$ for PaLV and $54 \%$ for PaLV2.

Resampling of the luteovirus-infected trees was performed in 2021, two years after the original sampling, as well as observations for any leaf or wood symptoms. No clear symptoms of viral infection could be identified in the field-grown trees and, in particular no symptoms of stem pitting on their bark or woody cylinder. RT-PCR testing of leaf samples showed that viral infection was detected again in $71 \%, 77 \%$, and $87 \%$ of the trees initially found infected in 2019 by PaLV, PaLV2, and NSPaV, respectively, indicating that infection by any of the three viruses could persist over a 2-year period but also that no further spread had apparently occurred. In order to evaluate the distribution of the viruses within individual trees, individual leaves taken from five different parts of the canopy of three trees were separately tested by virus-specific RT-PCR. NSPaV, PaLV and PaLV2 were detected in 9/10, 5/5, 10/10 individual leaves, respectively.

## Geographical distribution, prevalence and genetic diversity of peach infecting

 luteoviruses. As shown above, three luteoviruses (NSPaV, PaLV, and the new PaLV2) had high prevalence in the French peach BRC samples. To study the geographical distribution of luteoviruses in peach in Europe, 350 peach samples originating from seven countries including Belgium, Czech Republic, Greece, Italy, Slovakia, Spain, and Turkey were collected trying to maximize varietal diversity and without taking into consideration the presence of potential viral symptoms. All samples were tested by RT-PCR using virusspecific primers individually as above (Supplementary Table S1). Amplicons from positive samples were subjected to direct Sanger sequencing in order to confirm the specificity of the amplification and assess the genetic diversity of the various viruses (see below). Remarkably, all three viruses (NSPaV, PaLV and PaLV2) were identified in peach samples from all seven countries, their incidences are shown in Table 3. On the contrary, all tested peach samples were found negative for the Prunus-infecting luteoviruses not reported so far in peach, including MaLV, and PhaLV. The most prevalent virus is NSPaV with an average prevalence of $66 \%$ [range $27 \%$ (Italy) to $100 \%$ (Czech Republic)], followed by PaLV with an average prevalence of $40 \%$ [range 6\% (Turkey) to $88 \%$ (Slovakia)] and finally PaLV2 with an average prevalence of 14\% [range 3\% (Greece) to $54 \%$ (France)]. In total, 216 different varieties out of 256 varieties ( 71 samples had no information available on their variety) were found to be infected by either NSPaV or PaLV or PaLV2. A subset of amplicons (up to 15 per virus and per country) were submitted to Sanger sequencing and the nucleotide sequences, together with all available reference sequences, were used to construct a phylogenetic tree for each virus (Fig. 2). A total of 103 amplicon sequences were thus generated for NSPaV, 87 for PaLV, and 38 for PaLV2. The overall mean nt diversities in the short PCR fragments used for detection (3.7\% +/-$0.006 \%$ for NSPaV and PaLV2, $7.1 \%+/-0.009 \%$ for PaLV), as well as the topology of the trees (Fig. 2) show a generally limited genetic variability between isolates originating from different countries.

Graft-transmissibility of peach-infecting luteoviruses. In order to provide some insights into the biology of the peach-infecting luteoviruses, their graft transmissibility to GF305 peach seedling indicators as well as the symptoms induced were evaluated using samples from the INRAE Prunus BRC for which a full HTS viral indexing had been confirmed by specific RT-PCR assays. This included accessions with single or multiple luteoviral infections, with or without mixed infections with other well-known Prunus viruses or viroids (see above). A 100\% transmission rate was observed for other co-infecting viruses and viroids, including PNRSV, ACLSV and PLMVd, confirming the efficiency of the transmission assay (Table 4). On the other hand, for 10 accessions based on the individual testing of inoculated GF305, the overall rate of transmission of NSPaV was estimated at $55.4 \%$, while that of PaLV was $30 \%$ and that for PaLV2 at $8.3 \%$ (Table 4). The rates of transmission from individual accessions were also quite variable but could not be easily correlated with the infection status (single or multiple infections) of the original peach accession. In GF305 grafted with the remaining 14 accessions tested as composite pools of leaves, NSPaV was detected in 5 out of 14 pools, whereas PaLV was only detected in 1 out of 4 pools and PaLV2 was not detected in the 2 relevant pools. A visual inspection of the graft-inoculated GF305 plants was performed six months after grafting. As expected, all GF305 plants grafted with the accession co-infected with ACLSV displayed the expected dark green sunken mottle symptoms typical of ACLSV in this widely used indicator (data not shown). On the contrary, most (7/9) of the GF305 plants grafted with the other accessions showing various luteoviral combinations revealed no visible symptoms on any of the grafted plants (Table 4). For two accessions (S8278 X6Y75 O3 and S3527 X2Y16 O1, Table 4), symptoms of leaf chlorosis, reddening, or deformation could be observed in respectively $3 / 9$ and $2 / 9$ grafted plants. After 3.5 months of cold-induced dormancy, a second round of observation was conducted (Table 4). In the case of S8278 X6Y75 O3, no symptoms were expressed during this second growth cycle. For S3527 X2Y16 O1, leaf reddening or chlorosis were observed again in 2/9 plants but these symptoms were not correlated with NSPaV infection since positive trees were either symptomatic or asymptomatic. In addition, one case of stem necrosis (S1161 X7Y8 O3) and one case of leaf chlorosis/reddening (S5555 X4Y67 O2) were observed (Table 4), but these were not associated with NSPaV infection.

## Discussion

This study describes five novel luteoviruses identified from different Prunus species. Compared to the previously reported three Prunus-infecting luteoviruses (NSPaV, PaLV and ChALV), these results provide further evidence of the power of HTS-approaches for the discovery of unknown viruses, even in situations of latent or mixed infections. However, the in silico discovered PhaLV should be considered with caution since it has not been possible to experimentally validate its presence in this host. However, the fact that PhaLV could also be identified in RNASeq data independently generated (PRJNA683804) is in favor of the existence of PhaLV in P. humilis.

Compared with all other known luteoviruses, the five viruses characterized here share less than $90 \%$ of aa sequence identity in at least one of their proteins, which is the currently accepted molecular species demarcation criteria in the genus Luteovirus (Hillman and Esteban, 2011). Phylogenetic analyses demonstrate their close affinities with previously described ChALV and PaLV with which they form a monophyletic clade. We also identified divergent isolates for MaLV, ChLVA, and NSPaV. The discovery of isolates of MaLV in $P$. mume, $P$. incisa, and $P$. armeniaca indicates the ability of this virus to infect a range of ornamental, wild, and cultivated Prunus species. We also identified variants of NSPaV in $P$. cerasus and $P$. brigantina, representing new hosts and, in the case of $P$. brigantina, the first report of a wild NSPaV host. GenBank data available to date indicate rather narrow natural host ranges for Prunus-infecting luteoviruses. On the other hand, experimental graft inoculations have demonstrated that NSPaV is able to infect $P$. tomentosa and Bing cherry ( $P$. avium) indicators (Villamor et al. 2016), suggesting the possibility of a broader natural host range as reported here.

Unlike most other luteoviruses, the genome organization of Prunus-infecting luteoviruses shows significant ORF presence/absence variability depending on virus or isolate (ORFs 3a, 4, 6 and 7, Fig. 1A and Table 2). P3a and P4 (MP) have been shown to be involved in luteovirus movement (Ali et al. 2014; Ju et al. 2017; Smirnova et al. 2015). However, these two proteins appear to be dispensable in at least some of the Prunus-infecting luteoviruses as already described for NSPaV and AILV1 (Bag et al. 2015; Khalili et al. 2020). Interestingly, we found no evidence for an ORF3a in the PmaLV genome, although it encodes an MP ORF. Despite being the most prevalent luteovirus in peach in our survey, NSPaV lacks both ORF3a and ORF4, both of which are involved in movement. In cases where it was found in single infection, it could not have been complemented for movement by other viruses, and the mechanism(s) underlying its local and systemic movement therefore remain unclear. The absence of an ORF6 was already known in NSPaV, but was not confirmed in one isolate (sample S7, Table 2). We found no evidence for an ORF6 in PaLV2, while it was present in two isolates of MaLV but absent from another one (sample S3, Table 2). The existence of an ORF7, downstream of ORF6, has been proposed in the case of ChALV (Lenz et al. 2017) and the sequences reported here show that ORF7 is also present in ChLVA. Even if P6 of BYDV-GAV has been shown to have RNA silencing suppression activity in N. benthamiana (Liu et al. 2012), the existence of both ORFs 6 and 7 should, however, still be considered speculative since the expression of P6 and P7 in planta has yet to be demonstrated (Shen et al. 2006). Altogether, the genomes of Prunus-infecting luteoviruses show significant gene composition variation in when it comes to genes involved in RNA silencing suppression and movement. This observation raises questions about possible biological peculiarities of woody Prunus hosts and about the strategies used by Prunus-infecting luteoviruses to mount systemic invasions of these hosts despite lacking the proteins used to that effect by other luteoviruses.

Perhaps due to their relatively recent discovery, the geographical distribution and prevalence of the Prunus-infecting luteoviruses are still poorly known. Obtaining the complete genomes of novel viruses and of additional isolates for known ones has enabled the development of specific diagnostic assays for each of them, allowing us to undertake a systematic survey in European peaches involving 350 samples from eight countries. NSPaV, PaLV, and the novel PaLV2 were identified in each country, a major change in
our vision of the geographic distribution of these viruses. Together with the absence of obvious symptoms and the high prevalence rates observed, these results also suggest that the geographic distribution and prevalence of these viruses may have been largely underestimated and that they are likely present in many other Prunus-growing countries. Sequencing of the amplicons generated during the survey indicated that similar to other luteoviruses (Khine et al. 2020; Tian et al. 2019), the genetic diversity of NSPaV, PaLV, and PaLV2 is relatively low. No clustering of isolates based on their geographical origin was identified, a likely consequence of the trade of Prunus planting materials and of our inability to detect these agents by widely used biological indexing (Bag et al. 2015).

The results of the retesting of peach trees after two years indicate that these viruses have the ability to persist over extended periods of time in infected Prunus hosts. However, PaLV, NSPaV, and PaLV2 were in some cases not re-detected in previously positively tested trees, possibly due to an uneven distribution of infection within host trees. Such a situation is already known for many Prunus-infecting viruses (Barba et al. 2011; Büttner et al. 2011; Myrta et al. 2011; Quiot et al. 1995; Salem et al. 2003).

The graft transmissibility of NSPaV had already been demonstrated (Villamor et al. 2016). While confirming these results, the biological indexing experiments performed here on GF305 peach indicator seedlings extend them to PaLV and to the newly identified PaLV2. Surprisingly, graft transmissibility was not $100 \%$ for any of these luteoviruses, in contrast to the other co-infecting viruses or viroid. This could be explained by an uneven distribution in the original trees or, alternatively, by another unexpected effect such as the imperfect junction of phloem tissues between the grafted bark pieces and the indicator plants, which might limit transmission of the phloem-limited luteoviruses. It is noteworthy
that the virus with the highest graft transmission efficiency, NSPaV, misses ORFs 3a, 4, and 6, which are implicated in viral movement in other luteoviruses, further questioning how Prunus-infecting luteoviruses are able to spread in their hosts.

Whereas most species of the genus Luteovirus are responsible for symptoms and yield reduction (Miller and Lozier, 2022), there are significant uncertainties about the pathogenicity of NSPaV and PaLV. In the present work, none of the analyzed NSPaV, PaLV, or PaLV2 isolates induced clear or reproducible symptoms, alone or in combination, in the widely used GF305 peach indicator. Likewise, detailed symptoms observation of a wide range of orchard-grown peach varieties infected by various combinations of NSPaV, PaLV and PaLV2 failed to identify stem pitting or other unusual symptoms. Taken together, all results reported here suggest an absence of pathogenicity of these viruses in peach under a wide range of situations. Therefore, we suggest that these viruses should likely be considered harmless until proven otherwise in an unambiguous fashion.

Another question unanswered to date and with relevance for risk assessment is whether these viruses are transmitted by aphids. Aphids generally transmit luteoviruses in a circulative non-propagative manner (Miller and Lozier, 2022). The mean genetic diversities observed in the BRC orchard for the various viruses are of the same order as their world diversities. This suggests that the observed high infection rates do not result from a local epidemic spread driven by aphids. Similar to AILV1, NSPaV ORF5 is much shorter than in other luteoviruses (Bag et al. 2015; Khalili et al. 2020), while the P3-P5 fusion protein is well known to be involved in aphid transmission of luteoviruses (Miller and Lozier, 2022), directly raising the question of NSPaV aphid transmissibility. The
indexing experiments reported here have generated GF305 indicators with single infections, which are excellent starting materials for further aphid transmission studies. In conclusion, we identified five new luteoviruses from cultivated, wild and ornamental Prunus species. We also identified new natural hosts of NSPaV and provided an inclusive and expanded insight into the genetic diversity, geographical distribution, and prevalence of peach-infecting luteoviruses. Taken together, the results obtained point to a lack of pathogenicity of those viruses or to an ability to cause symptoms limited to some specific and possibly infrequent situations. For future research, they also raise interesting questions about the ability of these viruses to mount systemic infections in their Prunus hosts despite lacking proteins contributing to the needed functions in other luteoviruses.

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## TABLES

TABLE 1. List of Prunus samples from which luteovirus genomes were reconstructed in the present work

| Index <br> name | Species | Variety / <br> Cultivar | Nature/type | Symptoms | Collecting location |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- |

${ }^{\text {a: }}$ Mendel University (Mendelu Lednice, Czech Republic), ${ }^{\text {b: Prunus INRAE Biological Resource Center (BRC Toulenne, }}$
France), c: Research and Breeding Institute of Pomology (VŠÚO, Holovousy, Czech Republic), na: not applicable

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TABLE 2. Molecular features of representative Prunus-infecting luteoviruses

| Virus | Genome <br> size ( $n t$ ) | Protein size (aa) |  |  |  |  |  |  |  | Reference |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | P1 | RdRp-fusion | P3a | P3 (CP) | CP-RTD | MP | P6 | P7 |  |
| ChLVA-Rannaja | 5,726 | 364 | 890 | 47 | 196 | 630 | 170 | 37 | 71 | This study |
| MaLV-P. mume | 5,748 | 368 | 895 | 48 | 197 | 642 | 175 | 74 | na | This study |
| PaLV2 | 5,780 | 364 | 890 | 48 | 195 | 640 | 170 | na | na | This study |
| PmaLV | 5,822 a | 364 | 890 | Na | 198 | 647 | 147 | 38 | na | This study |
| PhaLV | 5,202 a | 364 | 890 | 48 | 196 | 632 | 172 | 50 | na | This study |
| NSPaV-P. cerasus | 4,993 a | 328 | 847 | Na | 206 | 526 | na | 62 | na | This study |
| PaLV-NC034970 | 5,819 | 364 | 890 | 49 | 199 | 670 | 177 | 56 | 49 | Wu et al. 2017 |
| ChALV-NC031800 | 5,857 | 364 | 890 | 45 | 198 | 647 | 175 | 79 | 79 | Lenz et al. 2017 |
| AILV1-MT362517 | 5,047 | 329 | 848 | Na | 204 | 550 | na | na | na | Khalili et al. 2020 |
| NSPaV-NC027211 | 4,991 | 328 | 847 | Na | 206 | 526 | na | na | na | Bag et al. 2015 |

$\overline{\mathrm{a}}=$ not completed by Race experiments; na: not applicable; NSPaV: nectarine stem pitting-associated virus; PmaLV: Prunus mahaleb-associated luteovirus; PhaLV: Prunus humilis-associated luteovirus; PaLV2: peach-associated luteovirus 2; MaLV: mume-associated luteovirus; ChLVA: cherry luteovirus A; PaLV: peach-associated luteovirus; ChALV: cherry-associated luteovirus; AILV1: almond luteovirus 1; RdRp: RNA-dependent RNA polymerase; CP: Coat protein; CP-RTD: CPreadthrough domain; MP: Movement protein.

TABLE 3. Peach-infecting luteovirus incidence in Prunus persica in eight European countries

| Geographical <br> origin | Number of <br> samples | Number of collection <br> sites | Peach infecting viruses |  |  |
| :--- | :--- | :--- | :--- | :--- | :--- |
| Belgium | 26 | 1 germplasm | NSPaV | PaLV | PaLV2 |
| Czech Republic | 43 | 1 germplasm + 6 orchards | $100 \%$ | $60 \%$ | $9 \%$ |
| France | 50 | 1 germplasm | $96 \%$ | $38 \%$ | $54 \%$ |
| Greece | 30 | 5 orchards | $53 \%$ | $13 \%$ | $3 \%$ |
| Italy | 51 | 1 germplasm | $27 \%$ | $26 \%$ | $4 \%$ |
| Slovakia | 50 | 1 germplasm +5 orchards | $88 \%$ | $88 \%$ | $6 \%$ |
| Spain | 50 | 44 orchards | $68 \%$ | $30 \%$ | $10 \%$ |
| Turkey | 50 | 4 orchards | $42 \%$ | $6 \%$ | $8 \%$ |

NSPaV: nectarine stem pitting-associated virus; PaLV: peach-associated luteovirus;
PaLV2: peach-associated luteovirus 2

## Maryam Khalili

Phytopathology
TABLE 4. Graft transmission experiments of NSPaV, PaLV and PaLV2 on GF305 peach seedling indicator plants

| Peach accession | Infection status | Biological indexing |  | Luteovirus transmission |  |  | Other viruses/viroids transmission |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | Symptoms ${ }^{\text {a }}$ |  | Positive / Grafted |  |  | Positive / Grafted |  |  |
|  |  | Cycle 1 | Cycle 2 | PaLV2 | NSPaV | PaLV | PNRSV | ACLSV | PLMVd |
| S2686 X5Y70 O3 | PaLV2-NSPaV-PNRSV | AS, 10/10 | AS, 10/10 | 1/10 | 8/10 | na | 10/10 | na | na |
| S4072 X12Y24 Q | PaLV2-NSPaV | AS, 7/7 | AS, 7/7 | 2/7 | 6/7 | na | na | na | na |
| S3527 X2Y16 O1 | PaLV2-NSPaV | LC, LR, 2/9 $\square$ | LC, LR, 2/9 | 0/9 | 3/9 | na | na | na | na |
| S5555 X4Y67 O2 | PaLV2-NSPaV-PaLV | AS, 10/10 | LC, LR, 1/10 | 0/10 | 2/10 | 1/10 | na | na | na |
| S2464 X1Y16 Q | NSPaV-PaLV | AS, 10/10 | AS, 10/10 | Na | 7/10 | 3/10 | na | na | na |
| S4617 X2Y45 O2 | NSPaV-PaLV | AS, 4/4 | AS, 4/4 | Na | 3/4 | 0/4 | na | na | na |
| S2464 X5Y76 O3 | NSPaV-PaLV | AS, 10/10 | AS, 10/10 | Na | 5/10 | 4/10 | na | Na | na |
| S1932 X1Y7 Q | PaLV-PNRSV-ACLSV- | DGSM, 6/6 | DGSM, 6/6 | Na | na | 4/6 | 6/6 | 6/6 | 6/6 |
|  | PLMVd |  |  |  |  |  |  |  |  |
| S1161 X7Y8 O3 | NSPaV | AS, 5/5 | SN, 1/5 | Na | 4/5 | na | na | Na | na |
| S8278 X6Y75 O3 | NSPaV | LD, 3/9 | AS, 9/9 | na | 3/9 | na | na | Na | na |
| Overall transmission rate |  |  |  | 8.3\% | 55.4\% | 30\% | 100\% | 100\% | 100\% |

${ }^{\text {a AS: asymptomatic, LC: leaf chlorosis, LR: leaf reddening, } \square \text { : decline and death, DGSM: dark green sunken mottle, LD: leaf deformation, SN: stem necrosis }}$
na: does not apply; NSPaV: nectarine stem pitting-associated virus; PaLV2: peach-associated luteovirus 2; PaLV: peach-associated luteovirus; ACLSV: apple chlorotic leaf spot virus; PNRSV: prunus necrotic ringspot virus; PLMVd: peach latent mosaic viroid

## FIGURE CAPTIONS

Fig. 1. Genomic organization Prunus-infecting luteoviruses (A) and phylogenetic tree based on their whole genome sequence alignment (B). The newly discovered viruses in this study are shown by triangles and the divergent variants by circles. The phylogenetic tree was constructed using the neighbor joining method in MEGA7 and a strict nucleotide identity distance. Bootstrap values (1,000 replicates) less than $70 \%$ were removed. PmaLV: Prunus mahaleb-associated luteovirus; ChALV: cherry-associated luteovirus; PaLV: peach-associated luteovirus; PhaLV: Prunus humilis-associated luteovirus; ChLVA: cherry luteovirus A; PaLV2: peach-associated luteovirus 2; MaLV: mume associated luteovirus; AILV1: almond luteovirus 1; NSPaV: nectarine stem pittingassociated virus. The scale bar represents 5\% nucleotide divergence. ORF1: open reading frame 1 Pol: RNA-dependent RNA polymerase; MP: movement protein; CP: coat protein; RT: readthrough domain.

Fig. 2. Phylogenetic trees based on the alignment of the nucleotide sequences of the luteoviral PCR products generated from the positive samples from different countries. A. Nectarine stem pitting-associated virus. B. Peach-associated luteovirus. C. Peach-associated luteovirus 2. GenBank reference sequences are indicated by black dots. The geographical origin of the isolates is summarized as follows: SP: Spain; Tr : Turkey; BI: Belgium; Gr: Greece; Fr: France; Cz: Czech Republic; IT: Italy; Sk: Slovakia. The phylogenetic trees were constructed using neighbor joining method in MEGA7 and strict nucleotide identity distances. Bootstrap values (1,000 replicates) less than 70\% are not shown. The scale bars represent $0.5 \%$ ( $A$ and $C$ ) or $1 \%$ nucleotide divergence (B).

## SUPPLEMENTARY FIGURE CAPTIONS

Supplementary Fig. S1. Phylogenetic trees based on the alignment of the P1-P2 (A) and P3-P5 (B) aa deduced sequences of Prunus-infecting luteoviruses. Phylogenetic trees were constructed using the neighbor joining method in MEGA7 and a strict aa identity distance. Bootstrap values (1,000 replicates) less than $70 \%$ were removed. The scale bars represent 5\% aa divergence

Supplementary Fig. S2. Pairwise aa identity of Prunus-infecting luteoviruses in P1P2 (CP-RTD) fusion protein (A) and in P3-P5 (B) (viral replicase)

## SUPPLEMENTARY TABLE TITLES

Supplementary Table S1. List of primers used in this study
Supplementary Table S2. Methods used for HTS, number of trimmed reads, average coverage and mapped reads percent to the reference genome of novel Prunus-infecting luteoviruses and new isolates.


B


Fig. 1. Genomic organization Prunus-infecting luteoviruses (A) and phylogenetic tree based on their whole genome sequence alignment (B). The newly discovered viruses in this study are shown by triangles and the divergent variants by circles. The phylogenetic tree was constructed using the neighbor joining method in MEGA7 and a strict nucleotide identity distance. Bootstrap values (1,000 replicates) less than $70 \%$ were removed. PmaLV: Prunus mahaleb-associated luteovirus; ChALV: cherry-associated luteovirus; PaLV: peachassociated luteovirus; PhaLV: Prunus humilis-associated luteovirus; ChLVA: cherry luteovirus A; PaLV2: peach-associated luteovirus 2; MaLV: mume associated luteovirus; AILV1: almond luteovirus 1; NSPaV: nectarine stem pitting-associated virus. The scale bar represents $5 \%$ nucleotide divergence. ORF1: open reading frame 1 Pol: RNA-dependent RNA polymerase; MP: movement protein; CP: coat protein; RT: readthrough domain.


Supplementary Table S1. List of primers used in this study

| Primer name | Use | Sequence 5'-3' | Annealing <br> temperature | PCR product <br> size (nt) |
| :--- | :--- | :--- | :--- | :--- |
| MaLV-F | Detection | TCTACGAAGGATGATCAGTTCAA | $55^{\circ} \mathrm{C}$ | 549 |
| MaLV-R | Detection | GAACAATTTGAATAGTTCCCTA |  |  |
| MaLV-5RACE |  |  |  |  |

\# This primer was used in conjunction with the universal primer provided by the 5' and 3' rapid amplification of cDNA ends kit (Takara Bio Europe)

Supplementary Table S2. Methods used for HTS, number of trimmed reads, average coverage and mapped reads percent to the reference genome of novel Prunus-infecting luteoviruses and new isolates.

| Sample | Virus-isolate | Method | Trimmed reads | Average coverage | mapped reads (\%) | Accession <br> numbers |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| S1 | MaLV-P. mume | dsRNA | 33,113 | 18.6x | 1.54 | ON408236 |
| S2 | MaLV-P. armeniaca | RNA | 122,714,448 | 40.8x | 0.0015 | ON408237 |
| S3 | MaLV-P. incisa | dsRNA | 415,410 | 855.7x | 1.18 | ON408235 |
| S4 | PaLV2 | dsRNA | 945,443 | 128.22x | 0.69 | ON408234 |
| S5 | ChLVA-Rannaja | dsRNA | 25,890,504 | 29.7x | 0.007 | ON146357 |
| S6 | ChLVA-Cigany | dsRNA | 25,944,878 | 20.2x | 0.005 | ON146356 |
| S7 | NSPaV-P. cerasus | dsRNA | 720,762 | 1x | 0.01 | ON408233 |
| S8 | PmaLV | RNA | 58,149,924 | 155.45x | 0.01 | ON408238 |
| na | PhaLV | Datamining | 124,143,971 | 51.6x | 0.002 | BK061315 |

na: not applicable; MaLV: mume-associated luteovirus; PaLV2: peach-associated luteovirus 2; ChLVA: cherry luteovirus A; NSPaV: nectarine stem pitting-associated virus; PmaLV: prunus mahaleb-associated luteovirus; PhaLV: prunus humilis-associated luteovirus

A


$$
\longmapsto
$$

B


$$
\vdash_{0.05}
$$

## Supplementary Fig. S1. Phylogenetic trees based on the alignment of the P1-P2 (A)

 and P3-P5 (B) aa deduced sequences of Prunus infecting luteoviruses. Phylogenetic trees were constructed using the neighbor joining method in MEGA7 and a strict aa identity distance. Bootstrap values (1,000 replicates) less than $70 \%$ were removed. The scale bars represent 5\% aa divergence
## A

| MaLV-P.mume | 100 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MaLV-P.armeniaca | 97 | 100 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| MaLV-P.incisa | 96 | 96 | 100 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| PaLV2-P.persica | 84 | 84 | 83 | 100 |  |  |  |  |  |  |  |  |  |  |  |  |  |
| ChLVA-Rannaja | 84 | 84 | 83 | 84 | 100 |  |  |  |  |  |  |  |  |  |  |  |  |
| ChLVA-Cigany | 85 | 84 | 84 | 84 | 97 | 100 |  |  |  |  |  |  |  |  |  |  |  |
| PhaLV | 83 | 84 | 83 | 83 | 88 | 88 | 100 |  |  |  |  |  |  |  |  |  |  |
| PmaLV | 82 | 81 | 81 | 83 | 82 | 82 | 82 | 100 |  |  |  |  |  |  |  |  |  |
| NSPaV-P.cerasus | 52 | 53 | 52 | 53 | 52 | 53 | 53 | 53 | 100 |  |  |  |  |  |  |  |  |
| NC_031800-ChALV | 82 | 82 | 81 | 84 | 82 | 82 | 81 | 95 | 52 | 100 |  |  |  |  |  |  |  |
| NC_034970-PaLV | 84 | 83 | 83 | 84 | 82 | 82 | 81 | 85 | 52 | 85 | 100 |  |  |  |  |  |  |
| NC_027211-NSPaV | 52 | 53 | 52 | 54 | 52 | 53 | 53 | 54 | 94 | 52 | 53 | 100 |  |  |  |  |  |
| MT362517-AILV1 | 53 | 53 | 53 | 54 | 53 | 53 | 53 | 54 | 79 | 53 | 53 | 79 | 100 |  |  |  |  |
| NC_040680-ALV1 | 63 | 63 | 62 | 62 | 61 | 62 | 62 | 64 | 53 | 63 | 63 | 53 | 51 | 100 |  |  |  |
| NC_040549-AaLV | 62 | 62 | 61 | 63 | 61 | 61 | 62 | 64 | 53 | 63 | 63 | 53 | 53 | 64 | 100 |  |  |
| NC_010806-RSDaV | 55 | 55 | 55 | 56 | 55 | 55 | 56 | 56 | 51 | 57 | 55 | 51 | 52 | 53 | 55 | 10 |  |
| NC_004750-BYDV-PAV | 52 | 52 | 52 | 52 | 52 | 52 | 52 | 53 | 49 | 53 | 51 | 49 | 50 | 51 | 51 |  |  |

B

| MaLV-P.mume | 100 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MaLV-P.armeniaca | 98 | 100 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| MaLV-P.incisa | 90 | 92 | 100 |  |  |  |  |  |  |  |  |  |  |  |  |  |
| PaLV2-P.persica | 62 | 62 | 62 | 100 |  |  |  |  |  |  |  |  |  |  |  |  |
| ChLVA-Rannaja | 54 | 54 | 55 | 60 | 100 |  |  |  |  |  |  |  |  |  |  |  |
| ChLVA-Cigany | 54 | 55 | 55 | 59 | 96 | 100 |  |  |  |  |  |  |  |  |  |  |
| PhaLV | 53 | 54 | 54 | 58 | 76 | 76 | 100 |  |  |  |  |  |  |  |  |  |
| PmaLV | 54 | 55 | 54 | 58 | 61 | 61 | 62 | 100 |  |  |  |  |  |  |  |  |
| NSPaV-P.cerasus | 30 | 30 | 30 | 31 | 30 | 29 | 31 | 31 | 100 |  |  |  |  |  |  |  |
| NC_031800-ChALV | 54 | 55 | 54 | 57 | 61 | 61 | 59 | 88 | 31 | 100 |  |  |  |  |  |  |
| NC_034970-PaLV | 52 | 52 | 52 | 55 | 58 | 58 | 59 | 65 | 31 | 65 | 100 |  |  |  |  |  |
| NC_027211-NSPaV | 30 | 30 | 29 | 30 | 29 | 29 | 30 | 30 | 92 | 30 | 30 | 100 |  |  |  |  |
| MT362517-AILV1 | 30 | 30 | 31 | 33 | 31 | 31 | 32 | 32 | 58 | 32 | 33 | 58 | 100 |  |  |  |
| NC_040680-ALV1 | 36 | 36 | 35 | 37 | 37 | 37 | 36 | 38 | 29 | 37 | 35 | 29 | 30 | 100 |  |  |
| NC_040549-AaLV | 38 | 38 | 37 | 37 | 39 | 39 | 39 | 38 | 28 | 38 | 37 | 28 | 31 | 47 | 100 |  |
| NC_010806-RSDaV | 35 | 36 | 35 | 36 | 36 | 36 | 36 | 39 | 35 | 37 | 37 | 34 | 31 | 34 | 36 | 100 |
| NC_004750-BYDV-PAV | 31 | 31 | 31 | 32 | 33 | 33 | 34 | 33 | 31 | 33 | 32 | 30 | 30 | 31 | 30 | 31100 |

Supplementary Fig. S2. Pairwise aa identity of Prunus-infecting luteoviruses in P1-
P2 (CP-RTD) fusion protein (A) and in P3-P5 (B) (viral replicase)

