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Virology

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. Additionally, 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 peach-associated luteovirus (PaLV), nectarine stem pitting-associated virus (NSPaV), and a novel luteovirus, peach-associated luteovirus 2 (PaLV2), are present in all countries; the most prevalent virus was NSPaV, followed by PaLV. The genetic diversity of these viruses was also analyzed. 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 in either graft-inoculated GF305 indicators or 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 that these viruses are likely asymptomatic in peach under most circumstances.

Keywords: biological indexing, geographical distribution, high-throughput sequencing, *Luteovirus*, stone fruit

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 reassigned 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).

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Open reading frame 1 (ORF1) encodes a replication-association protein (P1), whereas ORF2 encodes the viral RNA-dependent RNA polymerase (RdRp). Following a −1 frameshift, RdRp is expressed as a P1-P2 fusion protein. ORFs 3a, 3, 4, and 5 are translated from subgenomic RNA1 (sgRNA1) (Domier and D'Arcy 2008; Smirnova et al. 2015). ORF3 codes for the coat protein (CP), whereas 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. 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 cherry-associated luteovirus (ChALV) (Lenz et al. 2017).

Prior to the present study, three *Prunus*-infecting luteoviruses had been described: nectarine stem pitting-associated virus (NSPaV) was the first luteovirus identified in peach (*Prunus persica*) by Bag et al. (2015) in the United States. Since then, NSPaV was reported to naturally infect peach in China, Hungary, South Korea, and Australia (Igori et al. 2017a; Jo et al. 2017; Krizbai et al. 2017; Lu et al. 2017) and *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 United States 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, which was initially isolated from nectarine trees showing extensive pitting on their woody cylinder (Bag et al. 2015), the authors pointed out the difficulty of correlating 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 nonexistent 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 coinfecting viruses (Lenz et al. 2017).

The discovery of stone fruit tree viruses using high-throughput sequencing (HTS) approaches has increased over the last two decades (Hou et al. 2020; Maliogka et al. 2018; Rubio et al. 2017). However, 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 *Prunus*-infecting luteovirus species were characterized at the molecular level. Furthermore, 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.

Materials 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 analyzed by HTS. For these trees, leaf samples were collected over the 2013 to 2021 period in various countries, regardless of the presence of symptoms (Table 1). Until used, fresh leaf tissues were desiccated over anhydrous $CaCl_2$ (Sigma Aldrich Chimie, Saint-Quentin-Fallavier, France) and stored at either room temperature or −80°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 from either 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), and Slovakia, Spain, and Turkey (50 each). These 350 peach trees were analyzed individually for the presence of some of the *Prunus*-infecting luteoviruses, including NSPaV, PaLV (known luteoviruses), and PaLV2 (a new luteovirus), whereas MaLV and PhaLV (the novel luteoviruses characterized in this work) were analyzed as pooled samples.

Double-stranded RNA extraction, library preparation, and sequencing

Double-stranded 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 II Reverse Transcriptase according to the 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 was amplified according to Marais et al. (2018) in a 50-µl reaction containing 10× buffer, 4 mM dNTPs, 1 µM primer MID tag, and 1.25 U Dream Taq DNA polymerase (Thermo Fisher Scientific). Random PCR amplification was performed for one cycle of 94°C for 1 min, 65°C for 0 s, 72°C for 45 s, and 40 cycles of 94°C for 0 s, 45°C for 0 s, 72°C for 5 min, and 1 final cycle of 5 min at 72°C and 5 min at 37°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 × 150 bp) (outsourced at the GetPlage INRAE platform, Toulouse, France, or Azenta, Leipzig, Germany).

Alternatively, dsRNAs were extracted from 1 g of leaf tissue (S5 and S6 samples; Table 1) using the CF11 cellulose protocol of De Paulo and Powell (1995) and converted into double-stranded cDNA using the Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, U.S.A.). 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 1 × 100 bp mode (SEQme.eu, Dobříš, Czech Republic).

HTS 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 × 150 bp). Alternatively, total RNAs were isolated from four leaves (100 mg) of the *P. armeniaca* sample (S2; Table 1) using the Plant/Fungi Total RNA purification kit (Norgen Biotek, Thorold, Ontario, Canada). 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 × 161 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 nonredundant (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 (minimum length fraction = 0.9; minimum 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

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 (10 min at 99°C) 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, U.S.A.) 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, U.S.A.). Obtained RACE products were 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 nucleotide or amino acid distances and randomized bootstrapping to evaluate branching validity. Mean diversities and genetic distances (p-distances calculated on nucleotide or amino acid 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 (TNAs) were extracted from *Prunus* leaves according to 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, TNAs were first submitted to a reverse transcription initiated by pdN_6 primers and using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Complementary DNAs were then amplified using specific primers and either the Dream Taq DNA polymerase (Thermo Fisher 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°C prior to chip-budding on GF305 peach indicator seedlings. The grafting assays were carried out using two twigs as budwood for every peach accession and 3 to 10 grafted seedlings per accession depending on twig size. Each grafted seedling was grafted with two bark pieces.

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

Index name	Species	Variety/cultivar	Nature/type	Symptoms	Collecting location	Country of origin	Collection year
S1	*Prunus mume*	Not known	Ornamental	Oak leaf mosaic	Kyoto, Japan	Japan	2015
S2	*Prunus armeniaca*	Jia Na Li	Cultivated	Mosaics, leaf and twig deformation	Germplasm[a], Czech Republic	China	2021
S3	*Prunus incisa*	na	Wild	No	Germplasm[b], France	Japan	2019
S4	*Prunus persica*	Henri Moulin	Cultivated	No	Germplasm[b], France	France	2019
S5	*Prunus cerasus*	Rannaja	Cultivated	No	Czech Republic	Moldova	2015
S6	*Prunus cerasus*	Cigany	Cultivated	Mosaic	Czech Republic	Hungary	2015
S7	*Prunus cerasus*	Amarelka Chvalkovicka	Cultivated	No	Germplasm[c], Czech Republic	Czech Republic	2013
S8	*Prunus mahaleb*	na	Wild	Bushy growth and shortened internodes	Aussois, France	France	2021
S9	*Prunus brigantina*	na	Wild	Bushy growth and shortened internodes	Névache mountain, France	France	2017

[a] Mendel University (Mendelu Lednice, Czech Republic).
[b] *Prunus* INRAE Biological Resource Center (BRC Toulenne, France).
[c] Research and Breeding Institute of Pomology (VŠÚO, Holovousy, Czech Republic); na: not applicable.

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 6 months to monitor the appearance of symptoms. After the first cycle of observation, the plants were stored at 2°C to induce artificial dormancy. After 3.5 months of dormancy, the graft-inoculated plants were cut back to 30 cm high and placed again in a 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 to 10 grafted seedlings per accession. For the other 14 accessions, grafted GF305 seedlings (3 to 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 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′ genome ends by RACE. The 5,822 nt contig for PmaLV, lacking only 10 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 S2. In addition to these complete genomic sequences, near-complete genomes were obtained from other *Prunus* samples, allowing for 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 cultivar 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 nt (nearly full length, in comparison with other *Prunus*-infecting luteoviral genomes) was identified. This contig shows only 48 to 73% nt identity with any known *Luteovirus* species, suggesting that 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 5,780, 5,748, and 5,726 nt, respectively. A near-complete genome of 5,822 nt is also available for PmaLV (S8), together with near-complete genomes of the MaLV isolates from

TABLE 2. Molecular features of representative *Prunus*-infecting luteoviruses

Virus	Genome size (nt)	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
AlLV1-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

[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; AlLV1: almond luteovirus 1; RdRp: RNA-dependent RNA polymerase; CP: coat protein; CP-RTD: CP-readthrough domain; MP: movement protein.

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* cultivar Cigany (5,689 nt, S6). The NSPaV scaffold detected in *P. cerasus* very likely represents the complete genome of this isolate (4,993 nt, S7). 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 completely covers the virus ORFs.

The genomes of ChLVA, MaLV, PaLV2, PhaLV, and PmaLV encode six to eight ORFs and have an organization similar to that of other members of the genus *Luteovirus* (Table 2; 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

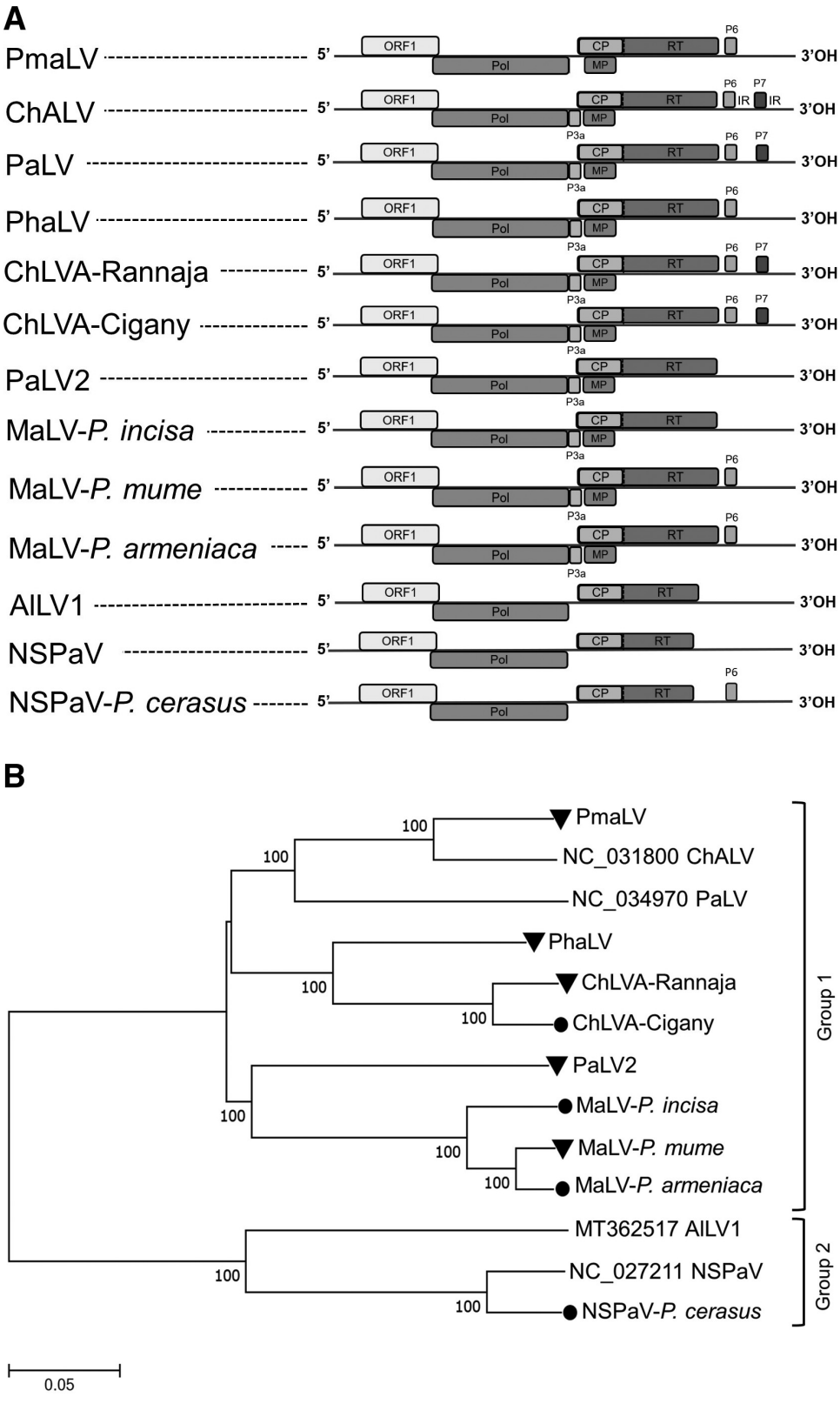

Fig. 1. A, Genomic organization of *Prunus*-infecting luteoviruses and **B,** phylogenetic tree based on their whole genome sequence alignment. The newly discovered viruses in this study are shown by triangles and the divergent variants by circles. The phylogenetic tree was constructed using the neighbor-joining 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; AlLV1: 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.

reported reference NSPaV isolate from *P. persica*, the NSPaV isolate reported here from *P. cerasus* has ORF6. There is thus both between-species and within-species 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 ORF4 and ORF3a and having a shorter ORF5, as previously reported for other NSPaV isolates and for almond luteovirus 1 (AlLV1) (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 *Prunus*-infecting luteoviruses divides them into two clades (Fig. 1B). Whereas NSPaV and AlLV1 form a distinct clade, the rest of the *Prunus*-infecting luteoviruses group 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 AlLV1. 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 amino acid identity was less than 90%, with the exception of PmaLV and ChALV, which showed 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 (NSPaV-*P. cerasus*, MaLV-*P. incisa*, MaLV-*P. armeniaca*, and ChLVA-Cigany) displayed more than 90% 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 *Prunus*-infecting 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 of 50 *P. persica* accessions were individually analyzed by dsRNA-based HTS indexing. On 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 were infected by NSPaV, compared with 38% for PaLV and 54% for PaLV2.

Resampling of the luteovirus-infected trees was performed in 2021, 2 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. 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, and 10/10 of 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 (Belgium, Czech Republic, Greece, Italy, Slovakia, Spain, and Turkey) were collected, seeking to maximize varietal diversity and without considering the presence of potential viral symptoms. All samples were tested by RT-PCR using virus-specific primers individually as above (Supplementary Table S1). Amplicons from positive samples were subjected to direct Sanger sequencing 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 NSPaV, 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 nucleotide 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

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, was 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 coinfecting 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%, whereas that of PaLV was 30%, and that for PaLV2 was 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 6 months after grafting. As expected, all GF305 plants grafted with the accession coinfected with ACLSV displayed the expected dark-green sunken

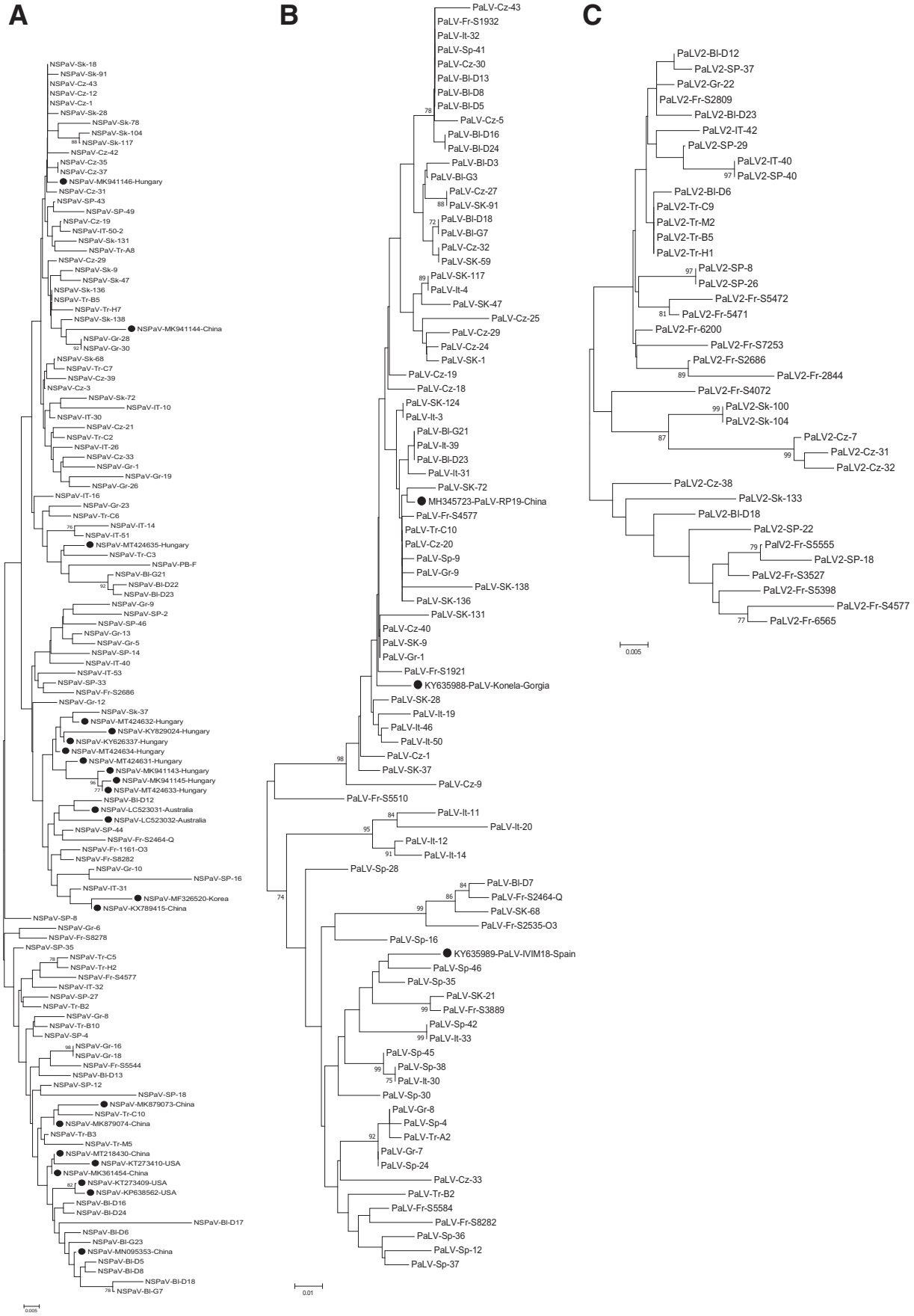

Fig. 2. Phylogenetic trees based on the alignment of the nucleotide sequences of the luteoviral PCR products generated from the positive samples from different countries. **A,** Nectarine stem pitting-associated virus. **B,** Peach-associated luteovirus. **C,** Peach-associated luteovirus 2. GenBank reference sequences are indicated by black dots. The geographical origin of the isolates is summarized as follows: SP: Spain; Tr: Turkey; Bl: Belgium; Gr: Greece; Fr: France; Cz: Czech Republic; IT: Italy; Sk: Slovakia. The phylogenetic trees were constructed using the 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% (B) nucleotide divergence.

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 × 6Y75 O3 and S3527 × 2Y16 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 × 6Y75 O3, no symptoms were expressed during this second growth cycle. For S3527 × 2Y16 O1, leaf reddening or chlorosis was observed again in 2/9 plants, but these symptoms were not correlated with NSPaV infection because positive trees were either symptomatic or asymptomatic. In addition, one case of stem necrosis (S1161 × 7Y8 O3) and one case of leaf chlorosis/reddening (S5555 × 4Y67 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 with the three previously reported *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 because 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% 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 the 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 AlLV1 (Bag et al. 2015; Khalili et al. 2020). Interestingly, we found no evidence for 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 mechanisms underlying its local and systemic movement therefore remain unclear. The absence of ORF6 was already known in NSPaV but

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

Geographical origin	Number of samples	Number of collection sites	Peach-infecting viruses		
			NSPaV[a]	PaLV[b]	PaLV2[c]
Belgium	26	1 germplasm	53%	57%	15%
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%

[a] NSPaV, nectarine stem pitting-associated virus.
[b] PaLV, peach-associated luteovirus.
[c] PaLV2, peach-associated luteovirus 2.

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

Peach accession	Infection status	Biological indexing Symptoms[a]		Luteovirus transmission Positive/grafted			Other viruses/viroids transmission Positive/grafted		
		Cycle 1	Cycle 2	PaLV2	NSPaV	PaLV	PNRSV	ACLSV	PLMVd
S2686 × 5Y70 O3	PaLV2-NSPaV-PNRSV	AS, 10/10	AS, 10/10	1/10	8/10	na	10/10	na	na
S4072 × 12Y24 Q	PaLV2-NSPaV	AS, 7/7	AS, 7/7	2/7	6/7	na	na	na	na
S3527 × 2Y16 O1	PaLV2-NSPaV	LC, LR, 2/9	LC, LR, 2/9	0/9	3/9	na	na	na	na
S5555 × 4Y67 O2	PaLV2-NSPaV-PaLV	AS, 10/10	LC, LR, 1/10	0/10	2/10	1/10	na	na	na
S2464 × 1Y16 Q	NSPaV-PaLV	AS, 10/10	AS, 10/10	na	7/10	3/10	na	na	na
S4617 × 2Y45 O2	NSPaV-PaLV	AS, 4/4	AS, 4/4	na	3/4	0/4	na	na	na
S2464 × 5Y76 O3	NSPaV-PaLV	AS, 10/10	AS, 10/10	na	5/10	4/10	na	na	na
S1932 × 1Y7 Q	PaLV-PNRSV-ACLSV-PLMVd	DGSM, 6/6	DGSM, 6/6	na	na	4/6	6/6	6/6	6/6
S1161 × 7Y8 O3	NSPaV	AS, 5/5	SN, 1/5	na	4/5	na	na	na	na
S8278 × 6Y75 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%

[a] AS, asymptomatic; LC, leaf chlorosis; LR, leaf reddening; /, 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.

was not confirmed in one isolate (sample S7; Table 2). We found no evidence for ORF6 in PaLV2, whereas it was present in two isolates of MaLV but absent from another one (sample S3; Table 2). The existence of 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 though 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 still be considered speculative because 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 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 suggest that the geographic distribution and prevalence of these viruses could 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 2 years indicate that these viruses have the ability to persist over extended periods 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 coinfecting viruses or viroids. 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, lacks 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 nonpropagative 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), whereas 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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