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1 **The expanding menagerie of *Prunus*-infecting luteoviruses**

2
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38

39 **Abstract**

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

60

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

62

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75 Introduction

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

85 Members of the genus *Luteovirus* are responsible for some of the most economically
86 important viral diseases in cereals (Miller and Rasochová 1997; Walls et al. 2019), and
87 have also been detected in many other crops or ornamental plants including fruit trees
88 (Bag et al. 2015; Igori et al. 2017b; Khalili et al. 2020; Lenz et al. 2017; Liu et al. 2018;
89 Shen et al. 2018; Wu et al. 2017). The genus *Luteovirus*, formerly belonging to the family
90 *Luteoviridae*, has recently been re-assigned to the family *Tombusviridae* (Miller and Lozier
91 2022). Its members have a single-stranded, messenger-sense RNA genome predicted to
92 encode four to six (potentially eight) proteins, depending on the viral species considered
93 (Bag et al. 2015; Hillman and Esteban, 2011; Lenz et al. 2017; Smirnova et al. 2015).
94 Open reading frame 1 (ORF1) encodes a replication-association protein (P1), while ORF2
95 encodes the viral RNA-dependent RNA polymerase (RdRp). Following a -1 frameshift,
96 RdRp is expressed as a P1-P2 fusion protein. ORF 3a, 3, 4, and 5 are translated from
97 sub-genomic RNA1 (sgRNA1) (Domier and D'Arcy 2008; Smirnova et al. 2015). ORF3

98 codes for the coat protein (CP), while ORF5 is expressed as a fusion to the CP following
99 the suppression of the leaky stop codon terminating ORF3. The small ORF3a, which is
100 located upstream of ORF3, is translated from a non-AUG start codon (Smirnova et al.
101 2015) and its P3a product has been shown to be implicated in viral movement. The ORF4,
102 which completely overlaps with the CP gene, encodes the movement protein (MP), and is
103 translated via leaky scanning of the ORF3 start codon due to its poor context for initiation
104 (Dinesh-Kumar and Miller 1993; Domier and D’Arcy 2008). A second subgenomic RNA,
105 sgRNA2, likely expresses the P6 protein (Kelly et al. 1994). ORF7 encodes the putative
106 P7 protein of unknown function and has been recently described in the genome of cherry-
107 associated luteovirus (ChALV) (Lenz et al. 2017).

108 Prior to the present study, three *Prunus*-infecting luteoviruses had been described:
109 nectarine stem pitting-associated virus (NSPaV) is the first luteovirus identified in peach
110 (*Prunus persica*) by Bag et al in the USA in 2015 (Bag et al. 2015). Since then, NSPaV
111 was reported naturally to infect peach in China, Hungary, South Korea, Australia (Igori et
112 al. 2017a; Jo et al. 2017; Krizbai et al. 2017; Lu et al. 2017), and in *P. mume* (Japanese
113 apricot) in Japan (Candresse et al. 2017). Furthermore, it has been experimentally shown
114 that NSPaV can infect *P. avium* (sweet cherry) and *P. tomentosa* (Nanking cherry)
115 (Villamor et al. 2016). Later, ChALV was characterized in *P. avium* and *P. cerasus* from
116 the Czech Republic (Lenz et al. 2017). Peach-associated luteovirus (PaLV) was initially
117 described in the USA from peach material imported from Georgia and Spain (Wu et al.
118 2017) and has since been reported, again from peach, in China, South Korea, Italy, and
119 Hungary (Barath et al. 2022; Igori et al. 2017b; Sorrentino et al. 2018; Zhou et al. 2018).

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

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

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

138 Using the HTS approach, we identified four new *Prunus*-infecting luteoviruses in the
139 present study. A fifth one was discovered following a screening approach of publicly
140 available *Prunus* RNA-Seq Sequence Read Archive (SRA) data. All five novel *Prunus*-
141 infecting luteovirus species were characterized at the molecular level. Besides, we
142 evaluated the peach-infecting luteoviruses for their graft transmissibility and, as a part of

143 a European field survey of peach trees, their prevalence, distribution, and genetic
144 variability.

145

146 **Material and methods**

147 **Plant material origin.** Fifty peach tree (*P. persica*) accessions introduced between 1937
148 and 2010 from different countries in the *Prunus* INRAE Biological Resource Center (BRC
149 Toulonne, France) were indexed by HTS. For each accession, five leaves from different
150 parts of the tree were collected in June 2019 and pooled in equal ratios, constituting the
151 sample analyzed by HTS. In addition, a few trees belonging to various *Prunus* species
152 were also analyzed by HTS. For these trees, leaf samples were collected over the 2013-
153 2021 period in various countries, regardless of the presence of symptoms (Table 1). Until
154 used, fresh leaf tissues were either desiccated over anhydrous CaCl₂ (Sigma Aldrich
155 Chimie, Saint-Quentin-Fallavier, France) and stored at room temperature or at -80°C.

156 To evaluate the prevalence of the luteoviruses identified in *P. persica*, samples from peach
157 trees originating from seven European countries (in addition to the 50 French samples
158 cited above) were obtained either from germplasm collections or production orchards.
159 Between 26 and 51 trees were thus sampled depending on the country: Belgium (26),
160 Greece (30), Czech Republic (43), Italy (51), Slovakia, Spain, and Turkey (50 each).
161 These 350 peach trees were analyzed individually for the presence of some of the *Prunus*-
162 infecting luteoviruses, including NSPaV, PaLV (known luteoviruses) and PaLV2 (a new
163 luteovirus), while MaLV and PhaLV (the novel luteoviruses characterized in this work)
164 where analyzed as pooled samples.

165 **Double-stranded RNA extraction, library preparation, and sequencing.** Double-
166 stranded RNAs (dsRNA) were purified from pooled leaves (S1, S3, S4, and S7 samples,
167 Table 1) by batch chromatography on cellulose CC41 (Whatman) as described (Marais et
168 al. 2018), and converted to cDNA using LDF primers (François et al. 2018, Supplementary
169 Table S1) and SuperScript™II Reverse Transcriptase according to manufacturer's
170 instructions (Invitrogen/Fisher Scientific, Illkirch, France). Each cDNA preparation was
171 subjected to a random PCR amplification using multiplex identifier (MID) adaptors
172 (François et al. 2018, Supplementary Table S1), allowing to sequence all the samples in
173 a multiplexed format. Five microliters of cDNA were amplified according to Marais et al.
174 (2018) in a 50 µl reaction containing 10× buffer, 4 mM dNTPs, 1 µM primer MID tag, 1.25
175 U Dream Taq DNA polymerase (Thermo Fisher Scientific). Random PCR amplification
176 was performed for one cycle of 94°C for 1 min; 65°C for 0 s; 72°C for 45 s, and 40 cycles
177 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
178 at 37°C. Following the purification of the PCR products using a MinElute PCR Purification
179 Kit (Qiagen SAS France, Courtaboeuf, France), PCR products were pooled equimolarly
180 before being sent for Illumina sequencing on a Hiseq3000 platform (2x150 bp)
181 [outsourced at the GetPlage INRAE platform (Toulouse, France) or Azenta (Leipzig,
182 Germany)].

183 Alternatively, dsRNAs were extracted from 1 g of leaf tissue (S5 and S6 samples, Table
184 1) using the CF11 cellulose protocol of De Paulo and Powell (DePaulo and Powell, 1995)
185 and converted into double-stranded cDNA using the Maxima H Minus Double-Stranded
186 cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The sequencing library was
187 prepared using the Illumina compatible MuSeek Library Preparation Kit (Thermo

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

190 **High throughput sequencing of total RNAs.** Total RNAs were extracted from
191 desiccated leaves of the *P. mahaleb* sample (S8, Table 1) using a modified CTAB
192 procedure (Chang et al. 1993), reverse-transcribed, ribodepleted, and sequenced
193 (HiSeq3000 2x150 bp). Alternatively, total RNAs were isolated from four leaves (100 mg)
194 of the *P. armeniaca* sample (S2, Table 1) using the Plant/Fungi Total RNA purification kit
195 (Norgen Biotek). Purified RNAs were ribodepleted using the QIAseq FastSelect-rRNA
196 Plant Kit (Qiagen) and a library prepared using the NEBNext Ultra II Directional RNA
197 Library Prep Kit before being sequenced in a multiplex run (NovaSeq6000, 2x 161 bp,
198 Institute of Experimental Botany, CAS, Olomouc, Czech Republic).

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

211 **Completion of the genome sequence of the identified new viruses.** In order to obtain
212 the complete genome sequence of the newly discovered viruses peach-associated
213 luteovirus 2 (PaLV2) and mume-associated luteovirus (MaLV), Rapid Amplification of
214 cDNA Ends (RACE) experiments were carried out for both 5' and 3' ends using the
215 SMARTer® RACE 5'/3'Kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) and
216 heat-denaturated (10 min at 99°C) dsRNAs as a template, following the manufacturer's
217 instructions. Alternatively, the cherry luteovirus A (ChLVA) genome termini amplification
218 was done using total RNAs and 5'- and 3'-RACE kits following the manufacturer's
219 recommendations (Invitrogen, Waltham, MA, USA) with the virus-specific primers
220 (Supplementary Table S1). Prior to the 3'-RACE, total RNAs were polyadenylated using
221 ATP and poly(U) polymerase following the manufacturer's recommendations (NEB,
222 Ipswich, MA, USA). Obtained RACE products sequenced (Eurofins Genomics, Ebersberg,
223 Germany) using the virus-specific primers. All specific RACE primers used were designed
224 from the sequence of the identified viral contigs and are listed in Supplementary Table S1.

225 **Data mining.** To uncover potential new luteoviruses in publicly available RNA-Seq data,
226 we performed an analysis on SRA using Serratus, an open-source cloud computing
227 infrastructure (Edgar et al. 2022) that seeks the closest matched SRA sequences to an
228 input virus using a 102 amino acid (aa) viral RNA-dependent RNA polymerase sequence
229 (RdRp palmpint). The sequence of the contig thus identified from a *Prunus humilis* SRA
230 from China (SRR12442710) has been deposited in GenBank under the BK061315
231 accession number.

232 **Phylogenetic, recombination and genetic population analyses.** Multiple alignments
233 of nucleotide (nt) or amino acid (aa) sequences were performed using the ClustalW

234 program (Thompson et al. 1994) implemented in Mega 7 (Kumar et al. 2016).
235 Phylogenetic trees were constructed using the neighbor-joining technique with strict nt or
236 aa distances and randomized bootstrapping to evaluate branching validity. Mean
237 diversities, and genetic distances (p-distances calculated on nt or aa identity) were
238 calculated using Mega 7. The RDP4 program (Martin et al. 2015) was used to search for
239 potential recombination events in the luteovirus genomic sequences obtained in this
240 study.

241 **Molecular detection of luteoviruses by RT-PCR for HTS validation, prevalence**
242 **determination, and genetic diversity analysis.** Total nucleic acids (TNA) were
243 extracted from *Prunus* leaves according to the procedure 1 described in Foissac et al.
244 (2005). The virus-specific primers were designed using the identified viral contigs
245 sequences (Supplementary Table S1) and used to detect the targeted viruses by two-step
246 RT-PCR assays. Briefly, TNA were first submitted to a reverse transcription initiated by
247 pdN₆ primers and using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo
248 Scientific). Complementary DNAs were then amplified using specific primers and either
249 the Dream Taq DNA polymerase (Thermo scientific) or the Advantage 2 polymerase mix
250 (Takara Bio Europe). Amplified products were analyzed by agarose gel electrophoresis
251 and Sanger sequenced on both strands (Eurofins). The PCR product sequences have
252 been deposited in the GenBank database under the accession numbers ON637949 to
253 ON638176.

254 **Graft transmission to GF305 peach indicator seedlings.** Based on their virome
255 composition, 24 peach trees of the INRAE *Prunus* BRC were selected for biological
256 indexing. New flush twigs were collected in June 2021 and kept at 4°C prior to chip-
257 budding on GF305 peach indicator seedlings. The grafting assays were carried out using

258 two twigs as budwood for every peach accession and 3-10 grafted seedlings per
259 accession depending on twig size. Each grafted seedling was grafted with two bark
260 pieces. In total, 199 GF305 plants were graft-inoculated in addition to five negative
261 controls self-grafted using healthy GF305 plants free of *Prunus* viruses and viroids. The
262 grafted plants were maintained under controlled greenhouse conditions for six months to
263 monitor the appearance of symptoms. After the first cycle of observation, the plants were
264 stored at 2°C to induce artificial dormancy. After 3.5 months of dormancy, the graft-
265 inoculated plants were cut back to 30 cm high and placed again in greenhouse for a
266 second cycle of observation.

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

275

276 **Results**

277 **Identification of four novel *Luteovirus* species and of new *Prunus* hosts for NSPaV.**

278 As part of a systematic effort to explore the virome of *Prunus* species, dsRNAs or total
279 RNAs extracted from a wide range of *Prunus* samples were analyzed by HTS. Following
280 reads quality trimming, *de novo* assembly and contigs annotation based on BlastX

281 analysis, several contigs with similarities to *Luteovirus* genus members were identified in
282 a range of samples. Contigs of interest were then assembled into scaffolds and extended
283 by successive rounds of residual reads mapping to yield finalized contigs spanning in
284 many cases near-complete genomes. A detailed analysis of the assembled genomes (see
285 below) revealed that four of them shared less than 90% aa identity in at least one of their
286 encoded proteins with known luteoviruses, which is below the molecular demarcation
287 threshold (10% aa divergence in any gene product) for new species in the genus
288 *Luteovirus* (Hillman and Esteban, 2011). Overall, four sequences representing potentially
289 four new species were thus identified in samples from *P. mume* (S1), *P. persica* (S4), *P.*
290 *cerasus* (S6), and *P. mahaleb* (S8) (Tables 1 and 2, Supplementary Table S2), with the
291 proposed names of mume-associated luteovirus (MaLV), peach-associated luteovirus 2
292 (PaLV2), cherry luteovirus A (ChLVA), and Prunus mahaleb-associated luteovirus
293 (PmaLV), respectively. The genomic sequences of the PaLV2, MaLV, and ChLVA isolates
294 were completed by filling internal gaps by PCR if needed, and by determining 5' and 3'
295 genome ends by RACE. The 5,822 nt contig for PmaLV, lacking only 10 nt and 40 nt at
296 the 5' and 3' ends respectively, as judged from a comparison with the most closely related
297 luteovirus, ChALV (NC_031800) was not completed. The corresponding genome
298 sequences have been deposited in the GenBank database under the accession numbers
299 ON408234 (PaLV2), ON408236 (MaLV), ON408238 (PmaLV) and ON146357 (ChLVA)
300 (Supplementary Table S2). The number of HTS reads mapped to each genome and the
301 average genome coverage are presented in Supplementary Table 2. In addition to these
302 complete genomic sequences, near-complete genomes were also obtained from other
303 *Prunus* samples, allowing the identification of divergent variants of MaLV in *P. armeniaca*
304 (sample S2) and *P. incisa* (sample S3), of ChLVA in a second *P. cerasus* from cv Cigany

305 (S6) and of a variant of NSPaV from *P. cerasus* (S7) (Table 1, Supplementary Table S2).
306 NSPaV infection was also identified in a *P. brigantina* sample (S9), but the low viral
307 concentration precluded the assembly of large contigs. The infection status of all samples
308 was in all cases validated using virus-specific RT-PCR assays and sequencing of the
309 amplicons. The near-complete genomic sequences of MaLV and NSPaV isolates have
310 been deposited in GenBank under the following accession numbers: ON408233 (NSPaV,
311 *P. cerasus*), ON408235 (MaLV, *P. incisa*) and ON408237 (MaLV, *P. armeniaca*),
312 (Supplementary Table S2).

313 **Identification of a novel *Luteovirus* species from publicly available *Prunus* RNASeq**
314 **data.** To uncover other luteoviruses infecting *Prunus*, the Serratus tool (Edgar et al. 2022)
315 was used with RdRp sequences of PaLV2 and MaLV, two of the four newly identified
316 viruses in this study, as queries. At the species level, only one RNAseq SRA (*P. humilis*
317 from China, SRR12442710) was identified, with a contig showing 83% aa identity in the
318 highly conserved RdRp motif with both queries, indicating that this sequence likely
319 represents a new species in the genus *Luteovirus* of this tentative agent. The SRA dataset
320 was downloaded and, following *de novo* assembly using CLC Genomics Workbench, a
321 large contig of 5,202 nt (nearly full-length, in comparison to other *Prunus*-infecting
322 luteoviral genomes) was identified. This contig shows only 48-73% nt identity with any
323 known luteovirus species, suggesting this isolate belongs to a novel species in the genus
324 *Luteovirus*. The sequence of this contig has been deposited in GenBank (BK061315) and
325 the name *Prunus humilis*-associated luteovirus (PhaLV) is proposed for the corresponding
326 novel species (Supplementary Table S2).

327 **Molecular characterization and phylogenetic affinities of the five novel**
328 **luteoviruses.** As indicated above, the full-length genomic sequences of PaLV2 (S4),
329 MaLV (S1) and ChLVA (S6) isolates were determined and shown to be respectively 5,780
330 nt, 5,748 nt, and 5,726 nt. A near-complete genome of 5,822 nt is also available for PmaLV
331 (S8), together with near-complete genomes of the MaLV isolates from *P. armeniaca*
332 (5,733 nt, S2) and *P. incisa* (5,705 nt, S3), as well as a near-complete genome for a
333 second ChLVA isolate from *P. cerasus* cv Cigany (5,689 nt, S6). The NSPaV scaffold
334 detected in *P. cerasus* represents very likely the complete genome of this isolate (4,993
335 nt, S7). The near-complete genome assembled from SRA data for PhaLV (5,202 nt) could
336 obviously not be completed by the RACE experiment but the available sequence covers
337 completely the virus open reading frames (ORFs).

338 The genomes of ChLVA, MaLV, PaLV2, PhaLV, and PmaLV encode six to eight ORFs
339 and have an organization similar to those of other members of the genus *Luteovirus* (Table
340 2 and Fig. 1A). The main variability observed concerns the short P6 and P7 ORFs, which
341 are missing in some viruses or isolates: ORF6 is absent in one isolate of MaLV (S3 from
342 *P. incisa*) and PaLV2 (Fig. 1A) and ORF7 is absent in most *Prunus*-infecting luteoviruses
343 with the exception of PaLV, ChALV and ChLVA (Table 2). Surprisingly, unlike the
344 previously reported reference NSPaV isolate from *P. persica*, the NSPaV isolate reported
345 here from *P. cerasus* has an ORF6. There is thus both between-species and within-
346 species presence-absence variability for these two small putative ORFs. The second main
347 divergence from the typical genomic organization for luteoviruses concerns NSPaV, with
348 the *P. cerasus* isolate lacking an ORF4 and an ORF3a and having a shorter ORF5, as
349 previously reported for other NSPaV isolates and for almond luteovirus 1 (AILV1) (Bag et
350 al. 2015; Khalili et al. 2020). ORF3a is also missing in the genome of PmaLV (Table 2).

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

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

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

372 **HTS virome characterization of peach accessions in INRAE *Prunus* BRC.** As part of
373 the *Prunus* virome characterization effort, a total number of 50 *P. persica* accessions were

374 individually analyzed by dsRNA-based HTS indexing. Upon demultiplexing and quality
375 trimming steps, an average of 1.5 million reads (range 0.24 to 5 million reads) were
376 obtained per individual sample. Apart from infrequent infections involving well-known
377 peach-infecting viruses such as apple chlorotic leafspot virus (*Betaflexiviridae*), prunus
378 necrotic ringspot virus (*Bromoviridae*), little cherry virus 1 (*Closteroviridae*), plum bark
379 necrosis stem pitting-associated virus (*Closteroviridae*) and peach latent mosaic viroid
380 (*Avsunviroidae*), BlastX analysis of the assembled contigs revealed that NSPaV, PaLV,
381 and the newly discovered PaLV2 showed high prevalence in the peach accessions
382 analyzed. The HTS reads datasets were also analyzed by mapping trimmed reads on
383 reference luteovirus genomes and the results were validated by RT-PCR using
384 corresponding virus-specific detection primers. Altogether, the results showed that 96%
385 of the 50 peach accessions are infected by NSPaV, compared to 38% for PaLV and 54%
386 for PaLV2.

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

398 **Geographical distribution, prevalence and genetic diversity of peach infecting**
399 **luteoviruses.** As shown above, three luteoviruses (NSPaV, PaLV, and the new PaLV2)
400 had high prevalence in the French peach BRC samples. To study the geographical
401 distribution of luteoviruses in peach in Europe, 350 peach samples originating from seven
402 countries including Belgium, Czech Republic, Greece, Italy, Slovakia, Spain, and Turkey
403 were collected trying to maximize varietal diversity and without taking into consideration
404 the presence of potential viral symptoms. All samples were tested by RT-PCR using virus-
405 specific primers individually as above (Supplementary Table S1). Amplicons from positive
406 samples were subjected to direct Sanger sequencing in order to confirm the specificity of
407 the amplification and assess the genetic diversity of the various viruses (see below).
408 Remarkably, all three viruses (NSPaV, PaLV and PaLV2) were identified in peach
409 samples from all seven countries, their incidences are shown in Table 3. On the contrary,
410 all tested peach samples were found negative for the *Prunus*-infecting luteoviruses not
411 reported so far in peach, including MaLV, and PhaLV. The most prevalent virus is NSPaV
412 with an average prevalence of 66% [range 27% (Italy) to 100% (Czech Republic)],
413 followed by PaLV with an average prevalence of 40% [range 6% (Turkey) to 88%
414 (Slovakia)] and finally PaLV2 with an average prevalence of 14% [range 3% (Greece) to
415 54% (France)]. In total, 216 different varieties out of 256 varieties (71 samples had no
416 information available on their variety) were found to be infected by either NSPaV or PaLV
417 or PaLV2. A subset of amplicons (up to 15 per virus and per country) were submitted to
418 Sanger sequencing and the nucleotide sequences, together with all available reference
419 sequences, were used to construct a phylogenetic tree for each virus (Fig. 2). A total of
420 103 amplicon sequences were thus generated for NSPaV, 87 for PaLV, and 38 for PaLV2.
421 The overall mean nt diversities in the short PCR fragments used for detection (3.7% +/-

422 0.006% for NSPaV and PaLV2, 7.1% +/- 0.009% for PaLV), as well as the topology of the
423 trees (Fig. 2) show a generally limited genetic variability between isolates originating from
424 different countries.

425 **Graft-transmissibility of peach-infecting luteoviruses.** In order to provide some
426 insights into the biology of the peach-infecting luteoviruses, their graft transmissibility to
427 GF305 peach seedling indicators as well as the symptoms induced were evaluated using
428 samples from the INRAE *Prunus* BRC for which a full HTS viral indexing had been
429 confirmed by specific RT-PCR assays. This included accessions with single or multiple
430 luteoviral infections, with or without mixed infections with other well-known *Prunus* viruses
431 or viroids (see above). A 100% transmission rate was observed for other co-infecting
432 viruses and viroids, including PNRSV, ACLSV and PLMVd, confirming the efficiency of
433 the transmission assay (Table 4). On the other hand, for 10 accessions based on the
434 individual testing of inoculated GF305, the overall rate of transmission of NSPaV was
435 estimated at 55.4%, while that of PaLV was 30% and that for PaLV2 at 8.3% (Table 4).
436 The rates of transmission from individual accessions were also quite variable but could
437 not be easily correlated with the infection status (single or multiple infections) of the
438 original peach accession. In GF305 grafted with the remaining 14 accessions tested as
439 composite pools of leaves, NSPaV was detected in 5 out of 14 pools, whereas PaLV was
440 only detected in 1 out of 4 pools and PaLV2 was not detected in the 2 relevant pools. A
441 visual inspection of the graft-inoculated GF305 plants was performed six months after
442 grafting. As expected, all GF305 plants grafted with the accession co-infected with ACLSV
443 displayed the expected dark green sunken mottle symptoms typical of ACLSV in this
444 widely used indicator (data not shown). On the contrary, most (7/9) of the GF305 plants

445 grafted with the other accessions showing various luteoviral combinations revealed no
446 visible symptoms on any of the grafted plants (Table 4). For two accessions (S8278
447 X6Y75 O3 and S3527 X2Y16 O1, Table 4), symptoms of leaf chlorosis, reddening, or
448 deformation could be observed in respectively 3/9 and 2/9 grafted plants. After 3.5 months
449 of cold-induced dormancy, a second round of observation was conducted (Table 4). In the
450 case of S8278 X6Y75 O3, no symptoms were expressed during this second growth cycle.
451 For S3527 X2Y16 O1, leaf reddening or chlorosis were observed again in 2/9 plants but
452 these symptoms were not correlated with NSPaV infection since positive trees were either
453 symptomatic or asymptomatic. In addition, one case of stem necrosis (S1161 X7Y8 O3)
454 and one case of leaf chlorosis/reddening (S5555 X4Y67 O2) were observed (Table 4), but
455 these were not associated with NSPaV infection.

456

457 Discussion

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

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

480 Unlike most other luteoviruses, the genome organization of *Prunus*-infecting luteoviruses
481 shows significant ORF presence/absence variability depending on virus or isolate (ORFs
482 3a, 4, 6 and 7, Fig. 1A and Table 2). P3a and P4 (MP) have been shown to be involved
483 in luteovirus movement (Ali et al. 2014; Ju et al. 2017; Smirnova et al. 2015). However,
484 these two proteins appear to be dispensable in at least some of the *Prunus*-infecting
485 luteoviruses as already described for NSPaV and AILV1 (Bag et al. 2015; Khalili et al.
486 2020). Interestingly, we found no evidence for an ORF3a in the PmaLV genome, although
487 it encodes an MP ORF. Despite being the most prevalent luteovirus in peach in our survey,
488 NSPaV lacks both ORF3a and ORF4, both of which are involved in movement. In cases

489 where it was found in single infection, it could not have been complemented for movement
490 by other viruses, and the mechanism(s) underlying its local and systemic movement
491 therefore remain unclear. The absence of an ORF6 was already known in NSPaV, but
492 was not confirmed in one isolate (sample S7, Table 2). We found no evidence for an ORF6
493 in PaLV2, while it was present in two isolates of MaLV but absent from another one
494 (sample S3, Table 2). The existence of an ORF7, downstream of ORF6, has been
495 proposed in the case of ChALV (Lenz et al. 2017) and the sequences reported here show
496 that ORF7 is also present in ChLVA. Even if P6 of BYDV-GAV has been shown to have
497 RNA silencing suppression activity in *N. benthamiana* (Liu et al. 2012), the existence of
498 both ORFs 6 and 7 should, however, still be considered speculative since the expression
499 of P6 and P7 *in planta* has yet to be demonstrated (Shen et al. 2006). Altogether, the
500 genomes of *Prunus*-infecting luteoviruses show significant gene composition variation in
501 when it comes to genes involved in RNA silencing suppression and movement. This
502 observation raises questions about possible biological peculiarities of woody *Prunus* hosts
503 and about the strategies used by *Prunus*-infecting luteoviruses to mount systemic
504 invasions of these hosts despite lacking the proteins used to that effect by other
505 luteoviruses.

506 Perhaps due to their relatively recent discovery, the geographical distribution and
507 prevalence of the *Prunus*-infecting luteoviruses are still poorly known. Obtaining the
508 complete genomes of novel viruses and of additional isolates for known ones has enabled
509 the development of specific diagnostic assays for each of them, allowing us to undertake
510 a systematic survey in European peaches involving 350 samples from eight countries.
511 NSPaV, PaLV, and the novel PaLV2 were identified in each country, a major change in

512 our vision of the geographic distribution of these viruses. Together with the absence of
513 obvious symptoms and the high prevalence rates observed, these results also suggest
514 that the geographic distribution and prevalence of these viruses may have been largely
515 underestimated and that they are likely present in many other *Prunus*-growing countries.
516 Sequencing of the amplicons generated during the survey indicated that similar to other
517 luteoviruses (Khine et al. 2020; Tian et al. 2019), the genetic diversity of NSPaV, PaLV,
518 and PaLV2 is relatively low. No clustering of isolates based on their geographical origin
519 was identified, a likely consequence of the trade of *Prunus* planting materials and of our
520 inability to detect these agents by widely used biological indexing (Bag et al. 2015).

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

527 The graft transmissibility of NSPaV had already been demonstrated (Villamor et al. 2016).
528 While confirming these results, the biological indexing experiments performed here on
529 GF305 peach indicator seedlings extend them to PaLV and to the newly identified PaLV2.
530 Surprisingly, graft transmissibility was not 100% for any of these luteoviruses, in contrast
531 to the other co-infecting viruses or viroid. This could be explained by an uneven
532 distribution in the original trees or, alternatively, by another unexpected effect such as the
533 imperfect junction of phloem tissues between the grafted bark pieces and the indicator
534 plants, which might limit transmission of the phloem-limited luteoviruses. It is noteworthy

535 that the virus with the highest graft transmission efficiency, NSPaV, misses ORFs 3a, 4,
536 and 6, which are implicated in viral movement in other luteoviruses, further questioning
537 how *Prunus*-infecting luteoviruses are able to spread in their hosts.

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

548 Another question unanswered to date and with relevance for risk assessment is whether
549 these viruses are transmitted by aphids. Aphids generally transmit luteoviruses in a
550 circulative non-propagative manner (Miller and Lozier, 2022). The mean genetic
551 diversities observed in the BRC orchard for the various viruses are of the same order as
552 their world diversities. This suggests that the observed high infection rates do not result
553 from a local epidemic spread driven by aphids. Similar to AILV1, NSPaV ORF5 is much
554 shorter than in other luteoviruses (Bag et al. 2015; Khalili et al. 2020), while the P3-P5
555 fusion protein is well known to be involved in aphid transmission of luteoviruses (Miller
556 and Lozier, 2022), directly raising the question of NSPaV aphid transmissibility. The

557 indexing experiments reported here have generated GF305 indicators with single
558 infections, which are excellent starting materials for further aphid transmission studies.

559 In conclusion, we identified five new luteoviruses from cultivated, wild and ornamental
560 *Prunus* species. We also identified new natural hosts of NSPaV and provided an inclusive
561 and expanded insight into the genetic diversity, geographical distribution, and prevalence
562 of peach-infecting luteoviruses. Taken together, the results obtained point to a lack of
563 pathogenicity of those viruses or to an ability to cause symptoms limited to some specific
564 and possibly infrequent situations. For future research, they also raise interesting
565 questions about the ability of these viruses to mount systemic infections in their *Prunus*
566 hosts despite lacking proteins contributing to the needed functions in other luteoviruses.

567

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576

577

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TABLES

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	<i>Prunus mume</i>	not known	Ornamental	Oak leaf mosaic	Kyoto, Japan	Japan	2015
S2	<i>Prunus armeniaca</i>	Jia Na Li	Cultivated	Mosaics, leaf and twig deformation	Germplasm ^a , Czech Republic	China	2021
S3	<i>Prunus incisa</i>	na	Wild	No	Germplasm ^b , France	Japan	2019
S4	<i>Prunus persica</i>	Henri Moulin	Cultivated	No	Germplasm ^b , France	France	2019
S5	<i>Prunus cerasus</i>	Rannaja	Cultivated	No	Czech Republic	Moldova	2015
S6	<i>Prunus cerasus</i>	Cigany	Cultivated	Mosaic	Czech Republic	Hungary	2015
S7	<i>Prunus cerasus</i>	Amarelka Chvalkovicka	Cultivated	No	Germplasm ^c , Czech Republic	Czech Republic	2013
S8	<i>Prunus mahaleb</i>	na	Wild	Bushy growth and shortened internodes	Aussois, France	France	2021
S9	<i>Prunus brigantina</i>	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 Toulence, France), ^c: Research and Breeding Institute of Pomology (VŠÚO, Holovousy, Czech Republic), na: not applicable

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- <i>P. mume</i>	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- <i>P. cerasus</i>	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

^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: CP-readthrough domain; MP: Movement protein.

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	PaLV	PaLV2
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%

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

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		Luteovirus transmission			Other viruses/viroids transmission		
		Symptoms ^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 □	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-PLMVd	DGSM, 6/6	DGSM, 6/6	Na	na	4/6	6/6	6/6	6/6
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%

^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

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 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.

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 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

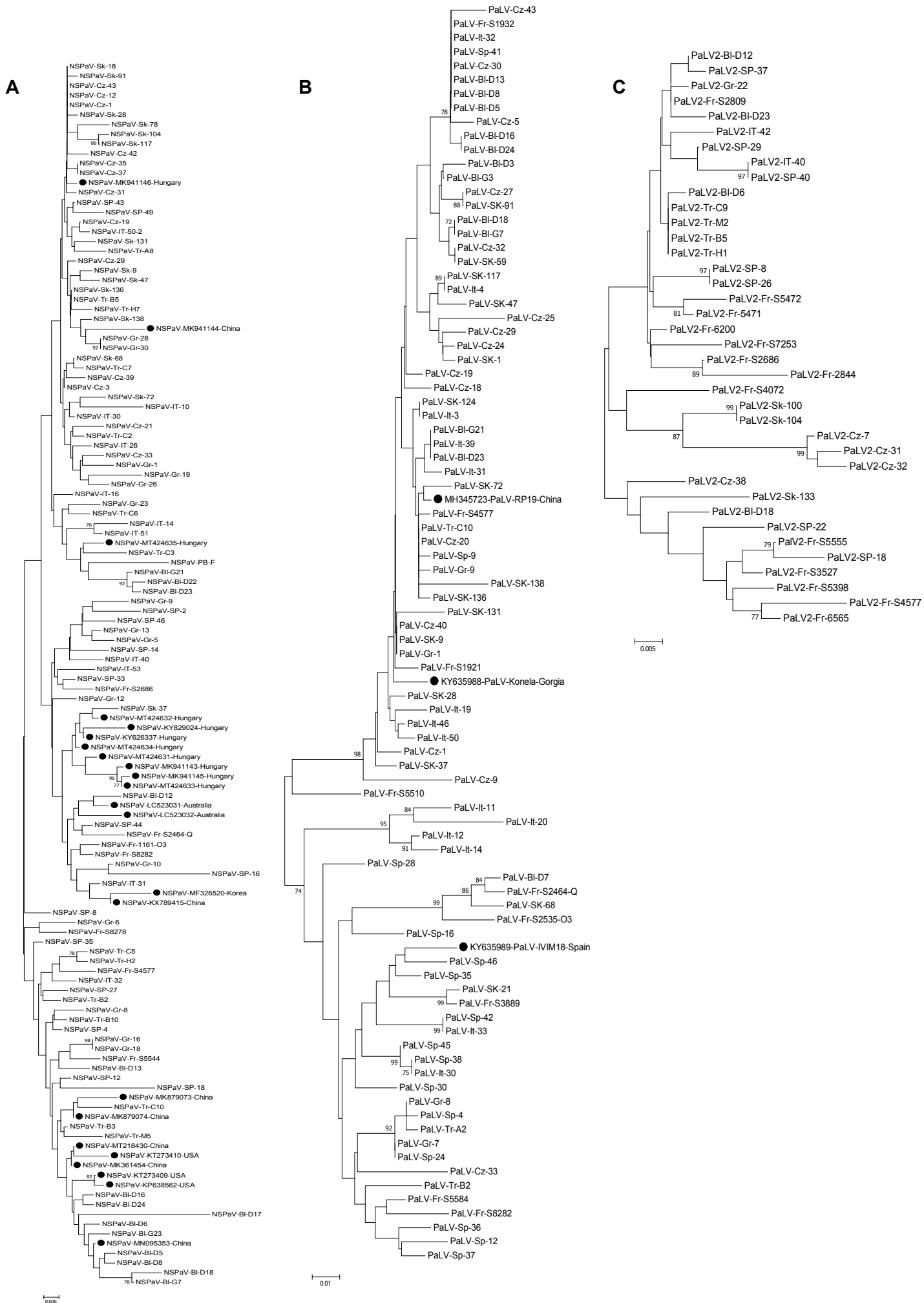
Supplementary Fig. S1. Phylogenetic trees based on the alignment of the P1-P2 (A) and P3-P5 (B) aa deduced sequences of *Prunus*-infecting luteoviruses. Phylogenetic trees were constructed using the neighbor joining method in MEGA7 and a strict aa identity distance. Bootstrap values (1,000 replicates)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

Supplementary Fig. S2. Pairwise aa identity of *Prunus*-infecting luteoviruses in P1-P2 (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.



Supplementary Table S1. List of primers used in this study

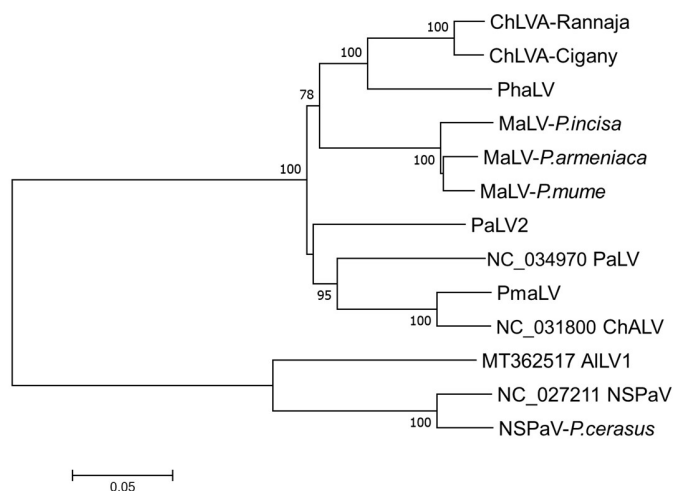
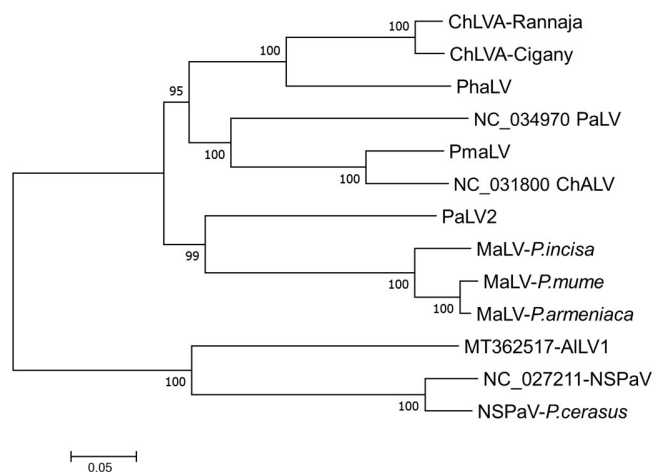
Primer name	Use	Sequence 5'-3'	Annealing temperature	PCR product size (nt)
MaLV-F	Detection	TCTACGAAGGATGATCAGTTCAA	55°C	549
MaLV-R	Detection	GAACAATTTGAATAGTTCCCTA		
MaLV-5RACE ^a	5' RACE	ACTCGAAGCGTAGATGAGCGAATC	70°C	150
MaLV-3RACE ^a	3' RACE	CTACCTAGTCAGGGGGATGGCTCACCATGTT	70°C	389
PaLV-F2	Detection	CTTTGGCGGCTAGGGCTTGCA	60°C	282
PaLV-R2	Detection	GAGAAGAGCCTCCGCTACCATTTA		
PaLV2-F	Detection	AGTCAGGTAGACGTCGTTGTAAA	61°C	365
PaLV2-R	Detection	TCTTCGGTGGTGCCTCATTCTC		
PaLV2-5RACE ^a	5' RACE	GTGCCCTCATTCTCCCCTCCCTTGACCT	70°C	881
PaLV2-3RACE ^a	3' RACE	GTGGTGGACTATCGTTGTGAGGTGTG	70°C	475
PhaLV-F	Detection	GTCCTCCATATCGTGAAGAGA	56°C	308
PhaLV-R	Detection	AAGCGGGTTGGACTTTGCTGT		
ChLVA-1492	5' RACE	ACGTTGGTATATAGGTATGACAC	60°C	191
ChLVA-1493	5' RACE	GCATTCCCATTCCCATTCTT	60°C	318
ChLVA-1494	3' RACE	AATTGGTAGTTCTGTTGTCA	60°C	530
ChLVA-1495	3' RACE	TTACGTGTTAGTTGAAGGT	60°C	415
ChLVA-1684	3' RACE	TGGTCACCTCGTTAAACAAC	60°C	487
NSPaV-F2	Detection	ACGACAAGGCGCACCCGCACCTC	62°C	335
NSPaV-R2	Detection	TCTGGGTGCAACTAGTGTCAATC		
LDF-087	S3 cDNA synthesis	TATGCTCGACCGCCNNNNNNNNNT	42°C	na
Tag718	S3 PCR for HTS	GGTCTTACATTATGCTCGACCGCC	45°C	na
LDF-042	S4 cDNA synthesis	CACTGAGCACCCCGGTCGCTATCA	42°C	na
Tag782	S4 PCR for HTS	CCCGGTCGCTATCANNNNNNNNNT	45°C	na
PcDNA12	S1, S7 cDNA synthesis	TTGGGTGTGTTTGGNNNNNNNNNT	42°C	na
MID-GENCO14	S1 PCR for HTS	CAAGAGTTTGTGTTGGGTGTGTTTGG	65°C-45°C	na
MID-GENCO6	S7 PCR for HTS	AGAGTCTTGTGTTGGGTGTGTTTGG	65°C-45°C	na

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 numbers
S1	MaLV- <i>P. mume</i>	dsRNA	33,113	18.6x	1.54	ON408236
S2	MaLV- <i>P. armeniaca</i>	RNA	122,714,448	40.8x	0.0015	ON408237
S3	MaLV- <i>P. incisa</i>	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- <i>P. cerasus</i>	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**B**

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

