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1   **ABSTRACT**

2   High-throughput sequencing of two lettuces showing virus-like symptoms in France provided

3   evidence of infection by members of the *Secoviridae* family. One plant (JG1) had a complex

4   mixed infection that involved, among others, a novel *Waikavirus* (lettuce waikavirus 1) and

5   two isolates of a sequivirus related to lettuce mottle virus (LeMoV). The second lettuce plant

6   (JG2) was singly infected by LeMoV. Complete genomic sequences were obtained for all four

7   isolates and, in addition, near complete genome sequences were obtained for other LeMoV or

8   LeMoV-related isolates (from French cultivated and wild lettuces, and from a Brazilian

9   cultivated lettuce) and for two isolates of another *Asteraceae*-infecting sequivirus, dandelion

10 yellow mosaic virus (DaYMV). Analysis of these genomic sequences allows to propose

11 tentative genome organization for the various viruses and to clarify their phylogenetic

12 relationships. Sequence and host range comparisons point to significant differences between

13 the two sequivirus isolates identified in the JG1 plant and LeMoV isolates from France and

14 Brazil, suggesting they belong to a novel species for which the name lettuce star mosaic virus

15 is proposed.

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is the most important annual leafy vegetable crop. It is primarily consumed fresh and is considered an important dietary vegetable worldwide. It belongs to the Asteraceae, the largest family of flowering plants. Cultivated lettuce was first documented on the walls of Egyptian tombs 4500 years ago and was soon cultivated in most parts of Europe and, later, in other parts of the world (De Vries 1997). The main producer of lettuce is Asia, especially India and China which, according to FAO in 2019, ranks first in annual production, followed by North and Central America, and Europe. In the European Union, Spain, Italy, Germany and France are the major lettuce-producing countries, with 74% of the total European lettuce production at almost 1.75 million tons in 2017 (Le Fustec 2020). In France, lettuce is grown year around in open fields, under plastic houses or glasshouses, with a total acreage of 8,300 hectares corresponding to about 500 million plants annually (Le Fustec 2020).

A number of pests and pathogens affect lettuce crops, including viruses belonging to the genera *Alfamovirus*, *Crinivirus*, *Cucumovirus*, *Cytorhabdovirus*, *Fabavirus, Ophiovirus*, *Polerovirus*, *Potyvirus*, *Sequivirus*, *Tombuvirus*, *Orthotospovirus* and *Varicosavirus* (Ciuffo et al. 2016; Ephytia 2019; Jadao et al. 2007; Lebeda et al. 2014; Moreno et al. 2012; Pavan et al. 2008). Many of these viruses cause epidemic diseases and considerable yield losses. Thanks to developments in high throughput sequencing (HTS) combined with specific bioinformatic tools (Lefebvre et al. 2019; Villamor et al. 2019), numerous plant viruses have been recently characterized, allowing major advances in the study of the etiology of viral diseases in perennial or annual crops (Wu et al. 2015; Maliogka et al. 2018; Maree et al. 2018). However, in many cases, past the identification and genome characterization of novel viral agents, the challenge is to demonstrate their potential implication in diseases and to

42   provide information about their biology, allowing to evaluate the risks these novel agents may

43   pose (Massart et al. 2017).

44   In recent years, several novel viruses have been characterized in lettuce crops (Ciuffo et al.

45   2016; Svanella-Dumas et al. 2018, Verbeek et al. 2014), while the main viruses infecting

46   lettuce in Europe or in Central America have been respectively reviewed in Moreno et al.

47   (2012) and Pavan et al. (2008). Lettuce mosaic virus (LMV, *Potyvirus*) is probably the most

48   important virus of lettuce worldwide (Pavan et al. 2008). LMV causes typical mosaic

49   symptoms that may occasionally turn necrotic. However, some other lettuce viruses can cause

50   almost indistinguishable mosaic symptoms, such as lettuce mottle virus (LeMoV) and

51   dandelion yellow mosaic virus (DaYMV), both of them from the genus *Sequivirus* in the

52   family *Secoviridae* (Jadao et al. 2007). DaYMV was the first sequivirus reported in lettuce

53   and in plants of dandelion (*Taraxacum officinale*) in different European countries (Kassanis

54   1944, 1947; Bos et al. 1983; Jadao et al. 2007). LeMoV, differing from DaYMV in host

55   range, is a distinct sequivirus infecting lettuce in Brazil and in Chile (Krause-Sakate et al.

56   2005; Jadao et al. 2007). In Brazil, and in particular in Sao Paulo State, LeMoV was in the

57   early 2000s the most frequent virus in lettuce crops (De Marchi et al. 2012, Krause-Sakate et

58   al. 2007) but it has so far never been reported outside of South America. For both DaYMV

59   and LeMoV, only very short, partial genome sequences are available (Jadao et al. 2007) and

60   there is very little information available on their genetic variability.

61   We report here the biological and molecular characterization by HTS of several secoviruses

62   from wild and cultivated lettuce in France and Brazil and from dandelion in Slovakia. Besides

63   providing the first complete genomic sequence for LeMoV and near-complete genome

64   sequences for DaYMV, the results presented here describe a novel *Sequivirus* and a novel

65   *Waikavirus* detected in French cultivated lettuces.

66

67   **MATERIALS AND METHODS**

68   **Plant samples and virus isolates**

69   To identify the etiological agent(s) involved, three cultivated lettuces (*Lactuca sativa* L.)

70   presenting systemic mosaic symptoms with some necrotic points were analyzed by HTS. The

71   first one (cv. Shangore, JG1) was collected in 2014 near Sainte-Livrade (southwestern

72   France) and the second one (cv. Guétary, JG2) came from an organic plot under a plastic

73   greenhouse and was collected in 2015 near Angers (western France). The last (21-015C,

74   variety unknown) was collected in southern France in 2022. The mosaic symptoms on the

75   JG2 plant, shown in Fig 1A, had more pronounced necrosis than those on the JG1 plant, while

76   besides mosaic, the 21-015C plant showed stunting and leaf deformation.

77   Several isolates of lettuce mottle virus (LeMoV) were analyzed. The P22 isolate was obtained

78   from an asymptomatic wild lettuce plant (*L. serriola*) collected in spring 2010 as part of a

79   plant virus metagenomic survey in Villenave d'Ornon (southwestern France) (Svanella-

80   Dumas et al. 2018). The second isolate (SP) was from a cultivated lettuce collected in 2013 in

81   Piedade County, Sao Paulo State, Brazil. This isolate was propagated in *Chenopodium quinoa*

82   or lettuce (cv. Trocadéro) plants by sap inoculation before HTS analysis. The third LeMoV

83   isolate, LIB3, was from a wild lettuce (*Lactuca saligna*) showing leaf yellowing and

84   discolorations collected in 2020 in Villenave d'Ornon.

85   Two isolates of dandelion yellow mosaic virus (DaYMV) in mixed infection, RNA24P-1 and

86   RNA24P-2, were identified by HTS from a dandelion plant collected in 2018 at the border of

87   a cultivated field near Bratislava, Slovakia.

88   **Host range study and sap inoculation of viruses**

89   In order to characterize the novel viruses identified, leaf pieces from the original JG1 plant

90   were ground 1:3 (wt/vol) in 0.05M potassium phosphate buffer pH 8.0. Activated charcoal

91 and carborundum (400 mesh) were added (100mg/ml homogenate) and the mixture was used

92 for sap-inoculation of plants of *L. sativa* (cv. Trocadero) or of ten plant species belonging to

93 the families *Asteraceae*, *Solanaceae*, *Chenopodiaceae* and *Amaranthaceae*. The LeMoV

94 isolate identified in the JG2 plant was sap-transmitted to lettuce (cv. Trocadéro), *Nicotiana*

95 *benthamiana* and *Gomphrena globosa* plants using the buffer described in Jadao et al. (2007).

96 The host range study was repeated at least twice for each viral isolate. All plants were kept

97 under greenhouse conditions and monitored for symptoms development up to one-month

98 post-inoculation.

99 The presence of the viruses in upper non-inoculated leaves of the test plants was evaluated

100 using specific RT-PCR assays for each virus using total nucleic acids (TNA) extracted from

101 fresh leaves according to protocol 1 of Foissac et al. (2005). The sequences of the various

102 amplification primers used [New waika-F / New waika-R (for the new waikavirus) and

103 NSeco-varA-F / NSeco-varA-R or NSeco-varB-F / NSeco-varB-R (that discriminate between

104 the two variants of the new sequivirus] are provided in Supplementary Table S1. These

105 primer pairs target fragments of respectively 385, 445 or 546 nucleotides (nt) in the viral

106 RNA-dependent RNA polymerase (RdRp) gene of each virus. The presence of LeMoV was

107 similarly checked using primer pair LeMoV3 (Jadao et al. 2007) and LeMoV4-multi

108 (Supplementary Table S1) which is adapted from Jadao et al. (2007) so as to improve

109 inclusiveness and to allow amplification of isolates JG2, P22, SP described here and of

110 previously analyzed Brazilian LeMoV isolates (DQ675190-91). This primer pair amplifies a

111 667 nt fragment of the RdRp gene.

112 For each RT-PCR assay, cDNA was first synthesized from 5µl of TNA using dT18 and pdN6

113 primers and the Revertaid Reverse Transcriptase (200U/µl) according to the manufacturer

114 recommendations (Thermo Fisher Scientific, Illkirch, France). In a second step, the PCR was

115 carried out using 3 µl of cDNA in a 25-µl reaction volume using 1 U of DyNAzymeII™ DNA

116 polymerase in accordance with the manufacturer recommendations (Thermo Fisher

117 Scientific). The annealing temperature and duration of the 72°C extension step are provided in

118 Supplementary Table S1. PCR products were analyzed by non-denaturing 1% agarose gel

119 electrophoresis with ethidium bromide staining and, if needed, directly sequenced (Eurofins

120 Genomics, Ebersberg bei München, Germany) to verify the specificity of the amplification

121 reaction.

122 **HTS analysis, genome assembly and completion of the genome of the various viral**

123 **isolates**

124 Double-stranded RNAs (dsRNAs) were purified from fresh symptomatic leaves of the

125 original JG1, JG2, P22 and LIB3 plants as described (Marais et al. 2018). Purified dsRNAs

126 were submitted to a random amplification (Marais et al. 2018) and sequenced on Illumina

127 platforms (2×250 or 2x150 nt). Total RNAs were purified from *N. benthamiana* leaves

128 infected by the LeMoV-JG2 isolate using the protocol of Chang et al. (1983) and sequenced

129 without ribodepletion an Illumina HiSeq2000 (2×150 nt). Additionally, total RNAs were

130 extracted from fully developed dandelion leaves as mentioned in Tomasechova et al. (2020)

131 and sequenced on an Illumina MiSeq (2×250 nt). Total RNAs extracted from the 21-015C

132 lettuce were similarly sequenced on the Illumina iSeq100 and MiSeq platforms (2x150nt).

133 Following demultiplexing and quality trimming, HTS reads from the various samples were

134 analyzed using CLC Genomics Workbench 8 and later versions

135 (https://www.qiagenbioinformatics.com) and the VirAnnot pipeline (Lefebvre et al. 2019). *De*

136 *novo* assembled contigs were annotated by BlastN and BlastX analyses against the GenBank

137 database using a 10^{-3} *e*-value cut-off. Viral contigs were further extended by several rounds of

138 mapping of residual reads and by integration of smaller contigs to yield scaffold covering

139 most of the viral genomes.

140   The 5′end of viral genomes was determined using a 5' RACE kit (Takara Bio

141   Europe/Clontech, Saint-Germain-en-Laye, France) and specific reverse primers designed on

142   the reconstructed scaffold of each virus (Supplementary Table S1). To complete the 3′ end of

143   the genomes of the sequiviruses, that do not contain a poly-A tail, TNA were extracted and

144   polyadenylated (Ambion/ThermoFisher Scientific, Illkirch, France). The polyadenylated

145   RNAs were then submitted to a reverse transcription using LD-polyT as a reverse primer and

146   the genome 3′end was then amplified by PCR using a forward-specific internal primer

147   designed from the genome scaffold sequenced contig (Supplementary Table S1) and the LD-

148   prime primer, following the protocol described by Youssef et al. (2011). The same procedure

149   was used to determine the *Waikavirus* 3' genome end, only omitting the polyadenylation step

150   since waikavirus genomes are polyadenylated.

151   Internal gaps and regions of low coverage were determined or confirmed by direct sequencing

152   of RT-PCR fragments obtained using internal primers designed from the sequences of

153   contigs. For LeMoV-JG2, the only gap identified was in the 3' part of the genome, in a C-rich

154   region with low coverage. Two combinations of primers (LeMoV-3Race1 / LeMoV-gap-R1

155   and LeMoV-3Race1 / LeMoV-gap-R2) (Supplementary Table S1), amplifying PCR products

156   of respectively 199 and 573 nt were used to confirm the genome sequence in this short region.

157   The nearly genomic sequence of the LeMoV isolate P22 was completed after designing six

158   pairs of primers (Supplementary Table S1) allowing to close six small internal gaps in the

159   HTS scaffold.

160   All amplified fragments were visualized on non-denaturing agarose gels and directly

161   submitted to Sanger sequencing on both strands (Eurofins Genomics) or, when needed,

162   sequenced after cloning in the pGEM-T Easy vector (Promega, Charbonnières-Les Bains,

163   France).

164 The nearly complete genome of the LeMoV isolate SP was obtained by RT-PCR-based

165 genome walking, using total RNAs extracted from infected *C. quinoa* leaves with the Total

166 RNA Purification Kit (Norgen Biotek, Thorold, Canada). The primers used (Supplementary

167 Table S1) were designed based on the genome sequence of the LeMoV P22 isolate. The one-

168 step RT-PCR reactions were performed using 2.5 µl of RNA, 1 µM of each primer and 0.03

169 units of Avian myeloblastosis virus reverse transcriptase (AMV RT, Promega, Charbonnières-

170 les-Bains, France) in a final volume of 25 µl of 1x PCR master mix (Fermentas, Vilnius,

171 Lithuania). The annealing temperature for each primer pair is provided in Supplementary

172 Table S1. PCR products were analyzed as described above, purified from agarose gels using

173 the QIAquick Gel Extraction Kit (Qiagen, Courtaboeuf, France) and sequenced using an ABI

174 3730 DNA Analyser (Applied Biosystems, Illkirch, France) using a BigDye Terminator v3.1

175 Cycle Sequencing kit (Applied Biosystems).

176 **Sequence comparisons and phylogenetic analyses**

177 Multiple nucleotide (nt) or deduced amino acid (aa) sequence alignments were performed

178 using ClustalW (Thompson et al. 1994) implemented in MEGA 7 (Kumar et al. 2016). Strict

179 identity nt or aa distances were calculated from these alignments and neighbor-joining

180 phylogenetic trees were reconstructed using Mega 7 with bootstrap analysis (500 replicates)

181 to evaluate branch validity.

182 **RESULTS**

183 **Viruses detected by HTS in the French JG1 and JG2 lettuces**

184 During the spring of 2014 and 2015, two symptomatic lettuces (JG1, cv. Shangore and JG2

185 cv. Guétary) showing systemic mosaic symptoms were observed during surveys. Both

186 varieties are resistant to lettuce downy mildew (*Bremia lactucae* Regel) but only JG2 is

187   resistant to lettuce mosaic virus (LMV) and to the lettuce aphid, *Nasonovia ribisnigri*. The

188   symptoms on the JG2 lettuce included some necrotic points (Fig. 1A) that were much less

189   present on the JG1 plant. Symptomatic leaves of both plants were used to mechanically

190   inoculate lettuce plants (cv. Trocadéro), which developed systemic mosaic symptoms,

191   confirming suspicions of viral infection. In order to identify the virus(es) involved, dsRNAs

192   were purified from 0.75g of fresh leaves from the original JG1 and JG2 plants and analyzed

193   by HTS. Following demultiplexing and quality trimming, the reads were assembled into

194   contigs that were annotated by BlastN and BlastX analyses, providing evidence for a mixed

195   infection by several agents for the JG1 lettuce and for a single infection in JG2 (Table 1).

196   For JG1, the contig integrating the most reads (26,980) and showing the highest average

197   coverage (449x), showed the highest BlastN identity (49% nt identity) with bellflower vein

198   chlorosis virus (BVCV, Seo et al. 2015), a recently described member of the genus

199   *Waikavirus*. A second contig, integrating 12,327 reads for a 283x average coverage, showed

200   the closest homology (54% nt identity) with carrot chordovirus 1 (Adams et al. 2014). The

201   complete genome of this viral isolate has since been completed, allowing the description of a

202   new viral species, lettuce chordovirus 1 (LeCV1, Svanella-Dumas et al. 2018) in the family

203   *Betaflexiviridae*. The last two contigs integrating respectively 4,198 and 5,227 reads (average

204   coverage of respectively 92x and 102x) showed highest BlastN nt identities of 54% with

205   parsnip yellow fleck virus (PYFV, Turnbull-Ross et al. 1993) and 47% with carrot necrotic

206   dieback virus (CNDV, Menzel et al. 2008), two members of the *Sequivirus* genus,

207   respectively. Following the extension of these two contigs by several rounds of reads mapping

208   and aggregation of additional smaller contigs, two scaffolds of respectively 8,961 nt and

209   9,158 nt, were reconstructed, showing 79.8% nt identity. Taken together these results showed

210   that the original JG1 lettuce had a mixed infection involving LeCV1, a tentatively novel

211   *Waikavirus* and two related and tentatively novel sequiviruses.

212   For JG2, the largest viral contig identified from dsRNA-derived reads, was 1,996-nt long (96x

213   average coverage, 891 reads) and showed 50% nt identity with CNDV (data not shown).

214   Given the very incomplete assembly of the viral genome, a second HTS run was performed

215   on this viral isolate using total RNAs purified from infected N. *benthamiana* leaves. Using

216   this second dataset, evidence for a single viral infection was obtained. A single long viral

217   contig was obtained and further extended by rounds of reads mapping into an 8,927-nt long

218   scaffold integrating 119,029 viral reads (1,790x average coverage) and sharing 50% nt

219   identity with CNDV (Table 1). Interestingly, this scaffold shared 85.4 and 86.3% nt identity,

220   respectively (98.4 and 97.9% aa identify, respectively) in a 562 nt region shared with the two

221   partial LeMoV sequences available (DQ675190 and DQ675191), suggesting that the viral

222   isolate identified in the JG2 plant might represent the first identification of LeMoV in

223   cultivated lettuce outside of south America.

224   **Genome organization of the *Waikavirus* identified in the JG1 lettuce**

225   Following further rounds of reads assembly and aggregation of shorter contigs, a nearly

226   complete genome scaffold of 11,852 nt was assembled for the tentative *Waikavirus* identified

227   in the JG1 plant. The missing terminal genome ends were determined by 5'-RACE and 3'

228   LD-polyT / LD-prime PCR (Youssef et al. 2011) using the purified dsRNAs from the original

229   plant as a template and specific primers designed from the scaffold sequence (Supplementary

230   Table S1). The completed genome is 11,929 nt in length, excluding the 3'poly (A) tail (Fig.

231   2A) and has been deposited in GenBank under the MT348710 accession number. The genome

232   organization is similar to that of other waikaviruses, with a single large open reading frame

233   (ORF) of 10,389 nt encoding a polyprotein of 3,463 aa (388.5 kDa). The 5' and 3'

234   untranslated regions (UTRs) are respectively 463 nt and 1,078 nt long. The presence of

235   additional potential ORFs has been reported for some waikaviruses (Shen et al. 1993; Firth et

236   al. 2008). The predicted short overlapping ORFX was identified starting at genome position

237 464, encoding an 88 aa protein sharing 73.3% identity with the corresponding protein of

238 BVCV and variable conservation levels with other genus members (Supplementary Figure 1).

239 On the other hand, the long 3' UTR of the JG1 waikavirus does not contain the small ORF2

240 and ORF3 described in rice tungro spherical virus (RTSV) by Thole et al. (1996). In

241 waikaviruses and sequiviruses, the single large polyprotein encodes functional proteins

242 released by post-translational cleavages mediated by the viral protease (Pro). From the N-

243 terminal polyprotein end, these are a P1 protein of unknown function, the three coat protein

244 subunits (CP1, CP2, and CP3), an NTP-binding helicase (NTB), the 3C-like protease and an

245 RdRp. The polyprotein cleavage sites have been experimentally identified for maize chlorotic

246 dwarf virus (MCDV) and RTSV (Reddick et al. 1997; Shen et al. 1993). They are relatively

247 conserved between genus members and generally involve a glutamine (Q) residue at the -1

248 position (Sekiguchi et al. 2005; Sanfaçon et al. 2011). Considering the homologies between

249 the lettuce virus polyprotein and those of other waikaviruses, it was possible to propose

250 tentative cleavage sites for its polyprotein using a ClustalW-generated multiple alignment of

251 the various polyproteins (Fig. 2 and Table 2). A comparison of the amino acid sequence at

252 each of the proteolytic cleavage sites of the lettuce virus (hereafter referred to as LWV1-JG1)

253 showed that glutamine was conserved at the -1 position, with different amino acids in the +1

254 position (Q/S, Q/T and Q/D) depending on the cleavage site. Comparisons between the

255 LWV1-JG1 polyprotein sequence and those of other waikaviruses confirmed the presence of

256 the expected conserved domains in the Hel-Pro-Pol module that is commonly used to

257 characterize picorna-like viruses (Le Gall et al. 2008). This includes the A "GAPGVGKS" (aa

258 1806 to 1813), B "DD" (aa 1857-1858) and C "KGKHCTSKYVFSCTN" (aa 1893 to 1907)

259 helicase motifs (Le Gall et al. 2008), the 3C-like protease catalytic triad of histidine, aspartate

260 and cysteine (Reddick et al. 1997) present at amino acids 2670, 2707 and 2801 and the A

261 "DYAKFDGIGSP" (aa 3149 to 3159), B "SGFSMTVIFNS" (aa 3210 to 3220) and C

262 "YGDD" (aa 3258 to 3261) motifs (Reddick et al. 1997; Le Gall et al. 2008) characterizing

263 the RdRp domain.

Genome organization of the sequivirus isolates identified in the JG1 lettuce

265 As indicated above, two long scaffolds representing sequivirus isolates could be reconstructed

266 for the JG1 plants, named here JG1-A (8,961 nt) and JG1-B (9,158 nt). The whole genome of

267 the JG1-B was completed by 5'-RACE and 3' LD-polyT / LD-prime PCR as described above

268 for the *Waikavirus* sequence, with the only exception that the genome 3' end was determined

269 after polyadenylation since sequiviruses do not possess a 3' polyA. No specific efforts were

270 made to complete the JG1-A genome sequence. The JG1-B complete genome is 10,183 nt

271 long and has been deposited in GenBank, together with the JG1-A contig sequence

272 (MT348706 and MT348705, respectively). As expected, the JG1-B genome harbors a single

273 large ORF of 9,054 nt encoding a predicted polyprotein of 3,018 aa (337.5 kDa), bordered by

274 5' and 3' UTRs of 150 nt and 980 nt, respectively (Fig. 2B). Conserved protein motifs were

275 identified at the expected locations in the JG1-B polyprotein. This includes the A

276 "GSPGVGKS" (aa 1430 to 1437), B "DD" (aa 1481 to 1482) and C "KGRTFSSKYIFSTTN"

277 (aa 1517 to 1531) helicase motifs, or the B "SGFPMTVIFNS" (aa 2555 to 2565) and C

278 "YGDD" (aa 2603 to 2606) RdRp motifs. However, polyprotein cleavage sites are much less

279 conserved between members of the *Sequivirus* genus (Sanfaçon et al. 2011), so that it was not

280 possible to propose candidates for all tentative cleavage sites of the JG1-B polyprotein (Fig.

281 2B and Table 2) from a comparison with those reported for PYFV (Turnbull-Ross et al.

282 1993). Nevertheless, four candidate sites liberating the P1 protein and the three CP subunits

283 could be proposed (N/P, Q/G, Q/G and Q/A) at aa position 383/384, 575/576, 799/800 and

284 1035/1036 of the polyprotein, respectively (Fig. 2B and Table 2). With its 79.8% overall nt

285 identity with the JG1-B sequence, the partial JG1-A isolate sequence revealed complete

286 conservation of the genome features outlined above.

Genome organization of the LeMoV isolate identified in the JG2 lettuce

287

288 The genome ends of the 8,927 nt contig for the LeMoV isolate assembled from the sequence

289 data generated for the JG2 lettuce were determined by 5'-RACE and 3' LD-polyT / LD-prime

290 PCR. As for the JG1-B sequivirus, it was necessary to add a poly-A tail at the 3' genome end.

291 The completed JG2 LeMoV genome has been deposited in GenBank (MT348707) and is

292 9,927 nt long and has 5' and 3' UTRs of respectively 158 and 704 nt (Fig. 2C). It harbors a

293 single large ORF of 9,066 nt encoding a predicted polyprotein of 3,022 aa (336.1 kDa), which

294 has all the expected conserved protein motifs at the expected positions. Indeed, all aa

295 sequences of motifs are similar to those identified for both isolates JG1-A and JG1-B except

296 for the motif C of the helicase region for which a serine replaces an arginine at the 6th

297 position. As for the JG1-B sequence, it was only possible to predict the four tentative

298 cleavage sites liberating the P1 protein and the three CP subunits (Fig. 2C and Table 2). Two

299 out of four predicted cleavage sites Q/G (577/578, between CP1 and CP2) and Q/A

300 (1037/103, between CP3 and Helicase) are identical to those predicted for JG1-B.

Genetic diversity of JG1-A/JG1-B-like isolates and of lettuce mottle virus (LeMoV)

301

302 Two nearly complete *Sequivirus* genome sequences related to the JG1-A/JG1-B isolates were

303 assembled from the reads obtained from a symptomatic cultivated lettuce (21-015C, cv.

304 unknown) collected in southern France in 2022. These two contigs are respectively 10107 nt

305 (21-015C-A, OQ446446) and 10066 nt (21-015C-B, OQ446447) and had an average

306 coverage of respectively 460 and 343x (Table 1). No specific effort was made to complete the

307 5' and 3' genome UTRs. The two sequences show 23.7% nt divergence with each other and

308 respectively 20.8% and 25% divergence with JG1-A. On the other hand, 21-015C-A shows

309 only 8.6% divergence with JG1-B, while the corresponding value for 21-015C-B is 23.9%.

310 Genomic organization and key protein motifs are conserved between these two new isolates

311   and the JG1 isolates, with only one polymorphism observed at the P1/CP1 tentative cleavage

312   site (Table 2)

313   Nearly complete genome sequences were obtained for five other LeMoV isolates, P22

314   assembled from the reads obtained from an asymptomatic wild lettuce plant (*L. serriola*)

315   collected in spring 2010 as part of a plant virus metagenomic survey in Villenave d'Ornon

316   (southwestern France) (Svanella-Dumas et al. 2018), SP assembled from the reads from a

317   cultivated lettuce collected in 2013 in Sao Paulo State, Brazil, 21-015C assembled from the

318   reads of the cultivated lettuce mentioned above and LIB3-A and LIB3-B assembled from the

319   reads from a wild *Lactuca saligna* collected in southwest France in 2020. The P22 contig of

320   9,012 nt (GenBank MT348708) integrates 2,517 reads (78x average coverage), while a large

321   part of the SP isolate genome (8,366 nt) was determined by primer walking along the genome

322   (GenBank MT348709). The LIB3-A (OQ446443) and LIB3-B (OQ446444) sequences are

323   9150 nt long while the 21-015C sequence (OQ446445) is 9807 nt long but contains a short 24

324   nt internal gap at the position of the C rich region in which a similar gap was closed by PCR

325   for the JG2 sequence. No specific efforts were made to determine the missing terminal

326   sequences for any of these five LeMoV sequences. The JG2 genomic sequence shows

327   between 11.1% and 14.4% nt divergence (2.0-3.5% aa divergence) with these 5 novel near

328   complete LeMoV sequences, which in turn show between 11.4% and 15% nucleotide

329   divergence (2.2-3.9% aa divergence) with each other. All conserved protein motifs and

330   predicted cleavage sites are identical for the various LeMoV isolates (Table 2).

331   **Genomic organization of a DaYMV isolate identified in a Slovak dandelion**

332   As described above, two viral contigs with homologies to sequiviruses could be assembled

333   from the reads obtained from the dandelion RNA24P plant from Slovakia. These contigs

334   respectively integrate 3,824 and 1,003 reads, corresponding to average coverages of 60-fold

335   and 15-fold, respectively and showed the highest homology (49% nt identity) with CNDV

336   (Table 2). Further assembly efforts yielded two large scaffolds named RNA24P-1 and

337   RNA24P-2 of 9,786 and 9,795 nt long, respectively, and sharing 77% and 81.7 % nt sequence

338   identity (95% and 96.2 % aa), respectively, with the only available DaYMV partial sequence

339   (DQ675189, 788 nt long). No specific efforts were made to determine the 5' and 3' terminal

340   regions of the nearly complete RNA24P-1 and RNA24P-2 genomes and both large contigs

341   have been deposited in GenBank (MT559283 and MT559284, respectively). These results

342   constitute the first identification of DaYMV isolates in dandelion in Slovakia. The RNA24P-1

343   genome organization is similar to that of other sequiviruses, with a single large ORF of 9,102

344   nt encoding a polyprotein of 3,035 aa (338.7 kDa). The incomplete 5' and 3' UTRs are

345   respectively 149 nt and 533 nt long (Fig. 2D), indicating that the amount of missing sequence

346   information is likely limited. The RNA24P-2 genome has an ORF of 9,105 nt encoding a

347   polyprotein which is one aa longer than that of RNA24P-1 in the 3C-like protease domain. All

348   polyprotein conserved amino acid motifs are identical to those identified for LeMoV isolates,

349   except for the helicase A motif in which a lysine replaces a serine at position 2. As for LSMV

350   and LeMoV, it was only possible to propose candidates for the first four polyprotein cleavage

351   sites of DaYMV (K/P, Q/G, Q/M/S and Q/G at aa positions 388/389, 580/581, 804/805 and

352   1041/1042, respectively; Fig. 2D and Table 2), with an M/S polymorphism between the two

353   isolates at the +1 position of the CP2-CP3 cleavage site.

354   **Sequence comparisons and phylogenetic relationships of the various viruses identified in**

355   **the analyzed wild and cultivated lettuces**

356   To clarify the taxonomic position of these various viruses identified above, sequence

357   comparisons and phylogenetic analyses were performed either with the taxonomically

358   relevant Pro-Pol region (defined as spanning the part of the polyprotein between the

359   conserved CG protease and GDD polymerase motifs) or with the coat protein subunits for all

360   viruses in the genera *Waikavirus* and *Sequivirus*, as well as for representative members of the

361 genera *Cheravirus*, *Comovirus*, *Cholivirus, Fabavirus*, *Nepovirus*, *Satsumavirus*,

362 *Stralaravirus, Torradovirus* and for unassigned viruses in the *Secoviridae* family (Sanfaçon et

363 al. 2020) (Fig. 3A and 3B). The trees unambiguously placed the JG1 *Waikavirus* with other

364 genus members with strong bootstrap support and the JG1-A/JG1-B-like and LeMoV

365 sequences within the *Sequivirus* genus, again with strong bootstrap support (Fig. 3A and 3B),

366 confirming the assignation of these various viruses to this genus. In the case of the

367 sequiviruses, only very short partial genome sequences were previously available for LeMoV

368 and DaYMV (Jadao et al. 2007). Integration of these partial sequences in the phylogenetic

369 trees confirm the identification of the JG2 isolate as LeMoV, together with the P22, SP, LIB3-

370 A, LIB3-B and 21-015C isolates, with a tight, bootstrap-supported clustering (Fig. 3A) and

371 shows that DaYMV unambiguously clusters away from the bootstrap-supported cluster

372 formed by the the JG1-A, JG1-B, 21-015C-A and 21-015C-B isolates, ruling out their

373 interpretation as DaYMV isolates (Fig. 3A).

374 The aa sequence identity levels between the JG1 *Waikavirus* and other waikaviruses are

375 below 64% and 56% for the Pro-Pol region and for the CPs, respectively (Supplementary

376 Table S2). Considering the species molecular discrimination criteria in the *Secoviridae* family

377 (less than 80% aa sequence identity in the Pro-Pol region and less than 75% identity in the

378 CPs) (Sanfaçon et al. 2009; Thompson et al. 2017), the isolate identified in the JG1 lettuce

379 should be considered as belonging to a novel species, for which the name lettuce waikavirus 1

380 (LWV1) is proposed.

381 Concerning the JG1-A/B and 21-015C-A/B isolates, they share with each other 86.2-97.5%

382 and 92.1-99.7% aa sequence identity in their Pro-Pol and CPs, respectively. They should thus

383 be considered as isolates belonging to the same virus species. As compared with other known

384 sequiviruses, they share at most 76.9% identity in the Pro-Pol region (with LeMoV isolates),

385 which is below the 80% species demarcation criterion (Sanfaçon et al. 2009; Thompson et al.

386 2017) (Supplementary Table S3). However, when comparing the CPs, an aa identity value of

387 82.2-84.2% is observed with LeMoV isolates, higher than the 76% species cut-off value. This

388 suggests that other species discrimination criteria need to be taken into consideration

389 (Thompson et al. 2017), in order to be able to decide on their taxonomic situation. We

390 therefore decided to compare the experimental host range of LeMoV with that of the JG1-A

391 and JG1-B isolates.

392 **Comparative host range analysis for various sequiviral isolates**

393 Despite repeated attempts using various buffers and inoculation conditions, all efforts to

394 transmit LWV1 from the JG1 plant to lettuce (cv. Trocadéro) failed (data not shown). The

395 same was observed for LeCV1 (Svanella-Dumas et al. 2018). These observations are

396 consistent with the notion that waikaviruses are not mechanically sap-transmissible because

397 they are confined to phloem tissue (Reavy et al. 1993). On the other hand, variants JG1-A and

398 JG1-B were readily transmitted from the JG1 plant and induced systemic mosaic and star

399 chlorosis symptoms on inoculated lettuce (cv. Trocadéro) (Figure 1F). These initial

400 transmission efforts also allowed to separate the JG1-A and JG1-B isolates as demonstrated

401 using isolate-specific RT-PCR assays (Supplementary Table 1). The JG1-A and JG1-B

402 isolates, as well as the JG2 LeMoV isolate, were sap-inoculated to plants belonging to ten

403 species in four families, some of which are reported as differential hosts between LeMoV and

404 DaYMV (Jadao et al. 1997, Bos et al. 1983; Table 3). The expression of symptoms was

405 monitored over a one-month period and the presence of the various viruses was verified in

406 non-inoculated upper parts of test plants by specific RT-PCR assays. The results of these

407 experiments are presented in Table 3.

408 In summary, the JG1-A and JG1-B variants were found to accumulate in six and seven of the

409 ten tested species, respectively. For both isolates, systemic mosaic symptoms were observed

410 on lettuce (cv. Trocadero), endive (*Chicorium endivia* cv. Anjou) (Fig. 1E) and common

411   sowthistle (*Sonchus olearaceus*), while asymptomatic accumulation was observed in *N.*

412   *benthamiana, N. clevelandii and Gomphrena globosa (*very occasionally only for JG1-A).

413   JG1-B, but not JG1-A, also accumulated in *C. quinoa* in which it induced yellow systemic

414   spots (Figure 1D).

415   In parallel experiments, inoculation of the JG2 LeMoV isolate yielded results only partially

416   comparable to those reported by Jadao et al. (1997) for the Brazilian AF197 isolate since

417   contrary to AF197, JG2 caused systemic mosaic symptoms in *C. endivia* and only caused

418   symptomless infection in *G. globosa* (Table 1). Another noteworthy difference is that contrary

419   to AF197, which systemically infected and caused local lesions and systemic symptoms in *C.*

420   *quinoa*, JG2 almost never infected this host. However, a *C. quinoa* plant was identified with

421   JG2 infection in independent experiments than those reported in Table 3. The virus from this

422   plant could then readily be transmitted to *C. quinoa* but with only a drastically reduced

423   efficiency to lettuce (not shown).

424   Taken together these results show that similar to the situation with LeMoV or with DaYMV,

425   there is significant inter-isolate variability in the host range of the JG1-A and JG1-B isolates.

426   The only host that consistently separated the JG1-A and JG1-B isolates from LeMoV isolates

427   was *N. clevelandii*, which was symptomlessly infected by the former, but not by the latter.

428   Differences also exist with DaYMV, for which multiple isolates are reported to cause local

429   lesions in *C. amaranticolor* and to be unable to infect *S. oleraceus* (Table 3, Bos et al. 1983).

430   **DISCUSSION**

431   In the current study, the use of HTS data in combination with 3' and 5' RACE and

432   confirmation by RT-PCR assays for regions of low coverage allowed us to assemble complete

433   genomic sequences for three *Secoviridae* family members from symptomatic lettuces

434   cultivated in France. Similar approaches were also used to obtain near-complete genome

435   sequences for additional isolates of the same viruses from wild or cultivated lettuces in France

436   or in Brazil as well as the first near-complete genomes for yet another *Secoviridae* member,

437   DaYMV from a dandelion from Slovakia.

438   For the *Waikavirus* found in the cultivated JG1 lettuce, its genome characterization and

439   phylogenetic analysis show that it belongs to a new species, for which the name lettuce

440   waikavirus 1 is proposed. The unsuccessful results at mechanical transmission suggest that

441   like RTSV, this new virus could be confined to phloem tissues and not mechanically

442   transmissible (Reavy et al. 1993). The biology and epidemiology of waikaviruses remain

443   poorly understood, likely in part due to their phloem restriction and reliance on specific

444   arthropod vectors for efficient transmission (Reddick et al. 1997). Some waikaviruses also

445   serve as helper viruses for sequiviruses or caulimoviruses (Elnagar et al. 1976, Thompson et

446   al. 2017). However, some other sequiviruses such as DaYMV and LeMoV are reported to be

447   transmitted by aphids in the absence of a helper virus (Jadao et al. 2007). In the case of the

448   JG1 plant, given the mixed infection involving both LWV1 and the JG1-A and JG1-B

449   sequivirus isolates, it is not possible to know whether transmission complementation effects

450   exist or not between these various *Secoviridae* members. However, the identification of the

451   21-015C-A and -B isolates in a wild lettuce and in the absence of a *Waikavirus* suggests that

452   similar to LeMoV these isolates do not require the assistance of a *Waikavirus* for

453   transmission.

454   Considering the 80% cut-off molecular criterion in the Pro-Pol region defining species within

455   the family *Secoviridae* (Sanfaçon et al. 2009; Thompson et al. 2017), the JG1-A, JG1-B, 21-

456   015C-A and 21-015C-B isolates should all be considered as belonging to a single new species

457   in the genus *Sequivirus*. However, when considering the 76% cut-off value that applies to the

458   CPs, they should be considered as isolates of LeMoV. They are however unambiguously

459   different from DaYMV, the only sequivirus known to date from lettuce in Europe. At the

460   same time, the JG2, P22, LIB3-A, LIB3-B and 21-015C isolates from cultivated and wild

461   lettuces are unambiguously European LeMoV isolates that tightly cluster with LeMoV

462   sequences from South America (Figure 3A & B). The host range analysis identified a single

463   consistent difference between LeMoV isolates and the JG1-A and JG1-B isolates, with the

464   former being unable to infect *N. clevelandii* (Table 3). Considering together the phylogenetic

465   analyses (Fig. 3), the species molecular criterion met for the Pol-Pro region, the differences in

466   the predicted cleavage sites liberating the 3 CPs (Fig. 2 and Table 2) and the host range

467   difference, we suggest that the JG1-A/B and 21-015C-A/B isolates could all be considered as

468   belonging to a distinct species, for which the name lettuce star mosaic virus (LSMV) is

469   proposed. LSMV is clearly pathogenic in lettuce, inducing star mosaic symptoms that may

470   turn to pin-point or star necrosis and are quite similar to those caused by LeMoV, while more

471   punctuated that the mosaics typically induced by lettuce mosaic virus (LMV). It should be

472   noted that in the absence of fully conclusive diagnostics at the time, older reports of the

473   presence of DaYMV in French lettuce crops (Blancard et al. 2003) might, in fact, have been

474   related to LeMoV or LSMV.

475   Previous surveys of lettuce crops in France suggest that LSMV or LeMoV are rare, even

476   though wild lettuce may possibly act as a reservoir in the absence of lettuce crops (hence the

477   HTS analysis of the JG1 and JG2 plants following the observation of unusual symptoms).

478   This situation is, at least for LeMoV, in contrast to that reported in the early 2000s in Brazil

479   where LeMoV was considered the first or second most important lettuce virus, with a reported

480   prevalence of 10.1%-26.1% under open field conditions in Sao Paulo state (Krause-Sakate et

481   al. 2008; De Marchi et al. 2012). However, this situation changed during the last years, with

482   LeMoV being now more rarely found and the main viral constraint now being thrips

483   transmitted orthotospoviruses (RKS, unpublished data). Identifying the causes underlying

484   these differences epidemiological trends, which might be related to differences in agricultural

485 practices, varietal choice, insect vector populations or other yet unidentified factors will

486 require further investigations. The availability of complete genomic sequences for LeMoV,

487 LSMV and DaYMV, the characterization and genome sequencing of LWV1 and the RT-PCR

488 assays developed here should provide useful tools for such studies and for a broader and more

489 thorough estimation of the distribution, prevalence and impact of these viruses on lettuce

490 crops worldwide.

502

LITERATURE CITED

503

504 Adams, I.P., Skelton, A., Macarthur, R., Hodges, T., Hinds, H., Flint, L., Nath, P.D.,
505   Boonham, N. and Fox, A. 2014. Carrot yellow leaf virus is associated with carrot internal
506   necrosis. PLoS ONE 9:e109125.

507 Blancard, D., Lot, H. and Maisonneuve, B. 2003. Maladies des salades : identifier, connaître
508   et maîtriser. INRA Editions, Paris, France, pp 311-312.

509 Bos, L., Huijberts, N., Huttinga, H. and Maat, D.Z. 1983. Further characterization of
510   dandelion yellow mosaic virus from lettuce and dandelion. Nether. J. Plant Pathol. 89:207-
511   222.

512 Candresse, T., Marais, A., Faure, C. and Gentit, P. 2013. Association of little cherry virus 1
513   (LChV1) with the Shirofugen stunt disease and characterization of the genome of a
514   divergent LChV1 isolate. Phytopathology 103:293-298.

515 Chang, S., Puryear, J. and Cairney J. 1993. A simple and efficient method for isolating RNA
516   from pine trees. Plant Mol. Biol. Rep. 11:113-116.

517 Ciuffo, M., Mammella, M., Vallino, M., Caciagli, P. and Turina, M. 2016. Molecular
518   identification and biogical characterization of a new potyvirus in lettuce. Arch. Virol.
519   161:2549-2554.

520 De Marchi, B.R., de Almeida Spadotti D.M., Leite de Oliveira, M., Martinello Sanches, M.,
521   Krause-Sakate, R. and Pavan, M.A. 2012. Levantamento revela a predominância do
522   Lettuce mottle virus em três regiões produtoras de alface no Estado de São Paulo. Summa
523   Phytopathol. 38:245-247.

524   de Vries, I.M. 1997. Origin and domestication of *Lactuca sativa* L. Genet. Resour. Crop Evol.

525   44:165-174.

526   Dullemans, A.M., Botermans, M., de Kock, M.J.D., de Krom, C.E, van der Lee, T.A.J.,

527   Roenhorst, J.W., Stulemeijer, I.J.E., Verbeek, M., Westenberg, M. and van der Vlugt,

528   R.A.A. 2020. Creation of a new genus in the family *Secoviridae* substantiated by sequence

529   variation of newly identified strawberry latent ringspot virus isolates. Arch. Virol. 165:21-

530   31.

531   Elnagar, S. and Murant, A.F. 1976. The role of the helper virus, anthriscus yellows, in the

532   transmission of parsnip yellow fleck virus by the aphid *Cavariella aegopodii*. Ann. Appl.

533   Biol. 84:169-181.

534   FAO 2019. The world lettuce economy. http://www.fao.org/faostat/en/#data

535   Firth, A.E. and Atkins, J.F. 2008. Bioinformatic analysis suggests that a conserved ORF in the

536   waikaviruses encodes an overlapping gene. Arch. Virol. 153:1379-1383.

537   Foissac, X., Svanella-Dumas, L., Gentit, P., Dulucq, M.J., Marais, A. and Candresse, T. 2005.

538   Polyvalent degenerate oligonucleotides reverse transcription-polymerase chain reaction: a

539   polyvalent detection and characterization tool for trichoviruses, capilloviruses, and

540   foveaviruses. Phytopathology 95:617-625.

541   Jadao, A.S., Krause-Sakate, R., Liberti, D., Pavan, M.A., Echer, M.M., Svanella-Dumas, L.,

542   Zerbini, F.M., Candresse, T. and Le Gall, O. 2007. Further characterization of two

543   sequiviruses infecting lettuce and development of specific RT-PCR primers. Arch. Virol.

544   152:999-1007.

545   Kassanis, B. 1944. A virus attacking lettuce and dandelion. Nature 154:6.

546   Kassanis, B. 1947. Studies on dandelion yellow mosaic and other virus diseases of lettuce.

547   Ann. Appl. Biol. 34:412-421.

548 Koloniuk, I. and Fránová, J. 2018. Complete nucleotide sequence and genome organization of
549   red clover associated virus 1 (RCaV1), a putative member of the genus *Waikavirus* (family
550   *Secoviridae*, order *Picornavirales*). Arch. Virol. 163:3447-3449.

551 Krause-Sakate, R., Firmino, A.C., Jadao, A.S., Pavan, M.A., da Silva, N., Hanai, S.M., Anbo,
552   R.H. and Nietzsche, T. 2008. Ocorrência generalizada do Lettuce mottle virus em três
553   regiões produtoras de alface comercial do Estado de São Paulo. Summa Phytopathol.
554   34:88-89.

555 Krause-Sakate, R., Jadao, A.S., Firmino, A.C., Pavan, M.A., Zerbini, F.M., Rosales, I.M.,
556   Bustamante, P. and Le Gall, O. 2005. First report of a lettuce-infecting sequivirus in Chile.
557   Plant Disease 89:1129.

558 Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: molecular evolutionary genetics
559   analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33:1870-1874.

560 Le Fustec, M. 2020. Laitues d'hiver 2019-2020: une fin de campagne marquée par l'impact de
561   la crise sanitaire sur la demande et les dernières récoltes. Agreste Infos Rapides 2020-048.
562   http://www.agreste.agriculture.gouv.fr

563 Le Gall, O., Sanfaçon, H., Ikegami, M., Iwanami, T., Jones, T., Karasev, A., Lehto, K.,
564   Wellink, J., Wetzel, T. and Yoshikawa, N. 2007. *Cheravirus* and *Sadwavirus*: two
565   unassigned genera of plant positive-sense single-stranded RNA viruses formerly
566   considered atypical members of the genus *Nepovirus* (family *Comoviridae*). Arch. Virol.
567   152:1767-1774.

568 Lebeda, A., Kristkova, E., Kitner, M., Mieslerova, B., Jemelkova, M. and Pink, D.A.C. 2014.
569   Wild *Lactuca* species, their genetic diversity, resistance to diseases and pests, and
570   exploitation in lettuce breeding. Eur. J. Plant Pathol. 138:597-640.

571 Lefebvre, M., Theil, S., Ma, Y. and Candresse, T. 2019. The VirAnnot pipeline: a resource for

572 automated viral diversity estimation and operational taxonomy units assignation for virome

573 sequencing data. Phytobiomes J. 3:256-259.

574 Maliogka, V.I., Minafra, A., Saldarelli, P., Ruiz-García, A.B., Glasa, M., Katis, N. and

575 Olmos, A. 2018. Recent advances on detection and characterization of fruit tree viruses

576 using high-throughput sequencing technologies. Viruses 10:436.

577 Marais, A., Faure, C., Bergey, B. and Candresse, T. 2018. Viral double-stranded RNAs

578 (dsRNAs) from plants: alternative nucleic acid substrates for high-throughput sequencing.

579 Meth. Mol. Biol. 1746:45-53.

580 Maree, H.J., Fox, A., al Rwahnih, M., Boonham, N. and Candresse, T. 2018. Application of

581 HTS for routine plant virus diagnostics: state of the art and challenges. Front. Plant Sci.

582 27:1082.

583 Menzel, W. and Vetten, H.J. 2008. Complete nucleotide sequence of an isolate of the

584 Anthriscus strain of Parsnip yellow fleck virus. Arch. Virol. 153:2173-2175.

585 Moreno, A. and Fereres, A. 2012. Virus diseases in lettuce in the Mediterranean basin. Adv.

586 Virus Res 84:247-248.

587 Pavan, M.A., Krause-Sakate, R., Silva, N., Zerbini, F.M. and Le Gall, O. 2008. Virus diseases

588 of lettuce in Brazil. Plant Viruses. Global Science Books, Viçosa, pp. 35-40.

589 Petrzik, K., Sanfaçon, H., Tzanetakis, I., van der Vlugt, R., Wetzel, T., Yoshikawa, N.,

590 Lefkowitz, E.J., Davison, A.J., Siddell, S.G., Simmonds, P., Adams, M.J., Smith, D.B.,

591 Orton, R.J. and Knowles, N.J. 2017. ICTV virus taxonomy profile: *Secoviridae*. J. Gen.

592 Virol. 98:529-531.

593 Reavy, B., Mayo, M.A., Turnbull-Ross, A.D. and Murant, A.F. 1993. Parsnip yellow fleck

594 and rice tungro sperical viruses resemble picornaviruses and represent two genera in a

595 proposed new plant picornavirus family (*Sequiviridae*). Arch. Virol. 131:441-446.

596   Reddick, B.B., Habera, L.F. and Law, M.D. 1997. Nucleotide sequence and taxonomy of
597   maize chlorotic dwarf virus within the family *Sequiviridae*. J. Gen. Virol. 78:1165-1174.

598   Sanfaçon, H., Dasgupta, I., Fuchs, M., Karasev, A., Petrzik, K., Thompson, J., Tzanetakis, I.,
599   van der Vlugt, R., Wetzel, T. and Yoshikawa, N. 2020. Proposed revision of the family
600   *Secoviridae* taxonomy to create three subgenera, "*Satsumavirus*", "*Stramovirus*" and
601   "*Cholivirus*", in the genus *Sadwavirus*. Arch. Virol. 165:527-533.

602   Sanfaçon, H., Iwanami, T., Karasev, A.V., van der Vlugt, R., Wellink, J., Wetzel, T. and
603   Yoshikawa, N. 2011. Family *Secoviridae*. In "Virus Taxonomy: Ninth Report of the
604   International Committee on Taxonomy of Viruses. King, A.M.Q., Adams, M.J., Carstens,
605   E.B. and Lefkowitz, E.J. Eds, Elsevier, Academic Press, pp 881-899.

606   Sanfaçon, H., Wellink, J., Le Gall, O., Karasev, A., van der Vlugt, R. and Wetzel, T. 2009.
607   *Secoviridae*: a proposed family of plant viruses within the order *Picornavirales* that
608   combines the families *Sequiviridae* and *Comoviridae*, the unassigned genera *Cheravirus*
609   and *Sadwavirus*, and the proposed genus *Torradovirus*. Arch. Virol. 154:899-907.

610   Seo, J.K., Kwak, H.R. and Lee, Y.J. 2015. Complete genome sequence of bellflower vein
611   chlorosis virus, a novel putative member of the genus *Waikavirus*. Arch. Virol. 160:3139-
612   3142.

613   Shen, P., Kaniewska, M., Smith, C. and Beachy, R.N. 1993. Nucleotide sequence and
614   genomic organization of rice tungro spherical virus. Virology 193:621-630.

615   Svanella-Dumas, L., Tsarmpopoulos, I., Marais, A., Theil, S., Faure, C., Gaudin, J. and
616   Candresse, T. (2018) Complete genome sequence of lettuce chordovirus 1 isolated from
617   cultivated lettuce in France. Arch. Virol. 163:2543-2545.

618 Thole, V. and Hull, R. 1996. Rice tungro spherical virus: nucleotide sequence of the 3'

619   genomic half and studies on the two small 3' open reading frames. Virus Genes 13:239-

620   246.

621 Thole, V. and Hull, R. 2002. Characterization of a protein from rice tungro spherical virus

622   with serine proteinase-like activity. J. Gen. Virol. 83:3179-3186.

623 Thompson, J., Higgins, D.G. and Gibson, T.J. 1994. CLUSTALW: improving the sensitivity

624   of progressive multiple sequence alignment through sequence weighting, position-specific

625   gap, penalties and weight matrix choice. Nucl. Acids Res. 22:4673-4680.

626 Thompson, J.R., Dasgupta, I., Fuchs, M., Iwanami, T., Karasev, A.V., Petrzik, K., Sanfaçon,

627   H., Tzanetakis, I., van der Vlugt, RA.A., Wetzel, T., Yoshikawa, N. and ICTV Report

628   Consortium 2017. ICTV Virus Taxonomy Profile: *Secoviridae*. J. Gen. Virol. 74:555-561.

629 Tomašechová, J., Hančinský, R., Predajňa, L., Kraic, J., Mihálik, D., Šoltys, K., Vávrová, S.,

630   Böhmer, M., Sabanadzovic, S. and Glasa, M. 2020. High-throughput sequencing reveals

631   bell pepper endornavirus infection in pepper (*Capsicum annum*) in Slovakia and enables its

632   further molecular characterization. Plants, 9:41.

633 Turnbull-Ross, A.D., Mayo, M.A., Reavy, B. and Murant, A.F. 1993. Sequence analysis of

634   the parsnip yellow fleck virus polyprotein: evidence of affinities with picornaviruses. J.

635   Gen. Virol. 74:555-561.

636 Turnbull-Ross, A.D., Reavy, B., Mayo, M.A. and Murant, A.F. 1992. The nucleotide

637   sequence of parsnip yellow fleck virus: a plant picorna-like virus. J. Gen. Virol. 73:3203-

638   3211.

639 Verbeek, M., Dullemans, A.M., van Raaij, H.M., Verhoeven, J.T.J., van der Vlugt, R.A.A.

640   2014. Lettuce necrotic leaf curl virus, a new plant virus infecting lettuce and a proposed

641   member of the genus *Torradovirus*. Arch. Virol. 159:801-805.

642   Villamor, D.E.V., Ho, T., al Rwahnih, M., Martin, R.R. and Tzanetakis, I.E. 2019. High

643   throughput sequencing for plant virus detection and discovery. Phytopathology 109:716-

644   725.

645   Wu, Q., Ding, S.W., Zhang, Y. and Zhu, S. 2015. Identification of viruses and viroids by

646   next-generation sequencing and homology-dependent and homology-independent

647   algorithms. Annu. Rev. Phytopathol. 53:425-444.

648

TABLE 1. List of viral isolates detected in various cultivated and wild lettuces and in the RNA24P dandelion plant.

Plant	Virus	Mapped reads	% Mapped reads	Average Coverage	BlastX best hit / (% of identity)
JG1 (a)	LWV1	26,980	33.7%	449x	Bellflower vein chlorosis virus (49%)
	LeCV1	12,327	15.4%	283x	Carrot chordovirus 1 (54%)
	LSMV-JG1-A	4,198	5.2%	92x	Parsnip yellow fleck virus (54%)
	LSMV-JG1-B	5,227	6.5%	102x	Carrot necrotic dieback virus (47%)
JG2 (b)	LeMoV	119,027	0.38%	1,790x	Carrot necrotic dieback virus (50%)
P22 (a)	LeMoV	2,517	29.3%	78x	Parsnip yellow fleck virus (47%)
LIB3 (a)	LeMoV LIB3-A	800.864	29.3%	9,107x	na
	LeMoV LIB3-B	365.279	13.2%	4,157x	na
21-015C (b)	LeMoV	143,557	2.6%	2,024x	na
	LSMV 21-015C-A	33,503	0.6%	460x	na
	LSMV 21-015C-B	24,895	0.4%	343x	na
RNA24P (b)	DaYMV-RNA24P-1	3,824	60.3%	60x	Carrot necrotic dieback virus (49%)
	DaYMV-RNA24P-2	1,003	15.6%	15x	Carrot necrotic dieback virus (49%)

For each viral contig, number of reads assigned to each isolate, the proportion of mapped reads and the average coverage are provided, together with information about the best BlastX hit. The results were obtained after HTS analysis of (a) double-stranded RNAs or (b) total RNAs from infected plants. LWV1: lettuce waikavirus 1, LeCV1: lettuce chordovirus 1, LSMV: lettuce star mosaic virus, LeMoV: lettuce mottle virus, DaYMV: dandelion yellow mosaic virus. na: does not apply

TABLE 2. Polyprotein dipeptides cleavage sites for viruses in the genera *Waikavirus* and *Sequivirus* and either (a) published or (b) predicted in the present work using a ClustalW-generated multiple alignment of the viral polyproteins.

Virus sequences per species in this study	Hypothetical P1 / CP1	CP1 / CP2	CP2 / CP3	CP3 / NTP-Protein Binding	NTP-Protein Binding / Pro[#]	Pro / RdRp[#]
RTSV NC_001632 (a)	Q/A	Q/S	Q/D	Q/M	Q/D	Q/A
MCDV NC_003626 (a)	Q/S	Q/M	Q/V	Q/M	Q/V	S/P
BVCV NC_027915 (a)	Q/S	Q/S	Q/G	K/D	Q/D	E/A
RcaV1 MH325329 (a)	Q/N	Q/N	Q/L	Q/M	Q/E	A/A
BraV1 NC_040586 (b)	Q/S	Q/A	Q/A	Q/M	Q/N	T/A
PWV LC_488189 (b)	Q/T	Q/A	Q/S	Q/K	Q/D	S/V
LWV1 MT348710 (b)	Q/S (700/701)	Q/S (922/923)	Q/S (1124/1125)	Q/T (1423/1424)	Q/D (2536/2537)	Q/S (2873/2874)
PYFV NC_003628 (a)	S/P	N/A	Q/A	Q/A	S/P	Q/S
CNDV NC_038320 (b)	G/S	Q/A	Q/S	Q/G	?	?
LeMoV-JG2 MT348707 (b)	E/P (385/386)	Q/G (577/578)	Q/N (801/802)	Q/A(1037/1038)	?	?
LeMoV-P22 MT348708 (b)	E/P	Q/G	Q/N	Q/A	?	?
LeMoV-SP MT348709 (b)	E/P	Q/G	Q/N	Q/A	?	?
LeMoV LIB3-A OQ446443 (b)	E/P	Q/G	Q/N	Q/A	?	?
LeMoV LIB3-B OQ446444 (b)	E/P	Q/G	Q/N	Q/A	?	?
LeMoV 21-015C OQ446445 (b)	E/P	Q/G	Q/N	Q/A	?	?
LSMV-JG1-A MT348705 (b)	N/P	Q/G	Q/G	Q/A	?	?
LSMV-JG1-B MT348706 (b)	N/P (383/384)	Q/G (575/576)	Q/G (799/800)	Q/A (1035/1036)	?	?
LSMV-21-015C-A OQ446446 (b)	N/P	Q/G	Q/G	Q/A	?	?
LSMV-21-015C-B OQ446447 (b)	D/P	Q/G	Q/A	Q/A	?	?
DaYMV-RNA24P-1 MT559283 (b)	K/P (388/389)	Q/G (580/581)	Q/M (804/805)	Q/G (1041/1042)	?	?
DaYMV-RNA24P-2 MT559284 (b)	K/P	Q/G	Q/S	Q/G	?	-

Amino acid positions of the cleavage sites on the polyprotein are provided in parentheses () for one representative isolate sequenced here of each species. #: a question mark indicates viruses for which it was not possible to identify a tentative dipeptide cleavage site.

TABLE 3. Infection and symptomatology of sequiviral isolates in various hosts following mechanical transmission.

	JG1-AT348705	JG1-BT348706	LeMoV-JG2	LeMoV-AF197(a)	DaYMV(b)
Chicorium endivia cv. Anjou	S **(9/11)**	S **(14/43)**	S **(33/60)**	-	S
Lactuca sativa cv. Trocadéro	S **(22/24)**	S **(13/18)**	S **(29/32)**	S	S
Sonchus olearaceus	S **(5/12)**	S **(13/29)**	S **(30/36)**	S	-
Nicotiana rustica	- (0/12)	- (0/33)	- (0/36)	-	(ll)(s)
Nicotiana glutinosa	- (0/12)	- (0/33)	- (0/48)	-	(ll)
Nicotiana benthamiana	s **(6/12)**	s **(27/36)**	s **(29/36)**	ll, s	s
Nicotiona clevelandii	s **(18/56)**	s **(11/67)**	- (0/48)	-	(ll)(s)
Chenopodium amaranticolor	- (0/11)	- (0/32)	- (0/48)	-	LL, (S)
Chenopodium quinoa	- (0/12)	S **(7/32)**	- (0/36)	LL, S	LL, (S)
Gomphrena globosa	s **(1/11)**	s **(11/55)**	s **(14/56)**	LL, S	(LL), (s)

The number of infected over inoculated plants is indicated in parentheses. Also shown are infection and symptoms reported by Jadao et al. (2007) for lettuce mottle virus (LeMoV) isolate AF197 (a) and reported in Bos et al. (1983) for several isolates of dandelion yellow mosaic virus (DaYMV) (b). -, no infection; S, systemic symptoms; LL, local lesions; s, latent systemic infection; ll, latent local infection. Symptoms noted in parentheses reflect inter-isolate variability.

LEGENDS TO THE FIGURES

Fig. 1. Symptoms caused by lettuce mottle virus (LeMoV) and lettuce star mosaic virus (LSMV) in various host plants. **A**, Necrotic mosaic symptoms observed on the original field-grown JG2 lettuce (cv. Guétary). **B**, Systemic necrotic lesions of LeMoV JG2 isolate on *Chicorum endivia* cv. Anjou 13 days post-inoculation (dpi). **C**, Systemic mosaic symptoms of LeMoV JG2 isolate on lettuce cv. Trocadéro at 13 dpi. The leaf on the left is from an uninoculated control plant. **D**, Ssymptoms of lettuce star mosaic virus (LSMV) isolate JG1-B on *Chenopodium quinoa* at 36 dpi. **E**, Symptoms of lettuce star mosaic virus (LSMV) isolate JG1-A on *C. endivia* cv. Anjou at 19 dpi. **F**, Symptoms of LSMV JG1-A (left) and JG1-B (right) in lettuce cv. Trocadéro.

Fig. 2. Schematic representation of the genomic organization drawn to scale of **A**, lettuce waikavirus 1 (LWV1) isolate JG1. **B**, lettuce star mosaic virus (LSMV) isolate JG1-B. **C**, lettuce mottle virus (LeMoV) isolate JG2. D, dandelion yellow mosaic virus (DaYMV) isolate RNA24P-1, partial. The short horizontal lines show the 5' and 3' untranslated regions, respectively. The large box is the long open reading frame with the coordinates of the beginning and the end mentioned. Predicted cleavage sites and their positions are shown as triangles under predicted mature proteins and uncertain cleavage sites by a question mark. P1, hypothetical protein 1; CP1, CP2, CP3, coat proteins 1, 2, and 3; NTB, NTP-binding helicase; PRO, 3C-like protease; RdRp, RNA-dependent RNA polymerase are indicated within the polyprotein. Predicted ORFX is indicated by a box for LWV1-JG1.

Fig. 3. Unrooted phylogenetic trees reconstructed using **A**, the Pro-Pol region, and **B**, the coat protein amino acid sequences from the members of the genera *Waikavirus* and *Sequivirus*, one representative species of other genera in family *Secoviridae*, and unassigned plant viruses showing sequence homology with the members of *Secoviridae*. The sequences of the viruses determined in the present work are indicated in bold font with black dots. The trees were constructed using the neighbor-joining method and the bootstrap values (500 replicates) greater than 70% are indicated next to the branches. The scale bar represents 10% aa divergence. The abbreviations and the accession numbers of the viruses used in this work are mentioned in Supplementary TABLE S4.
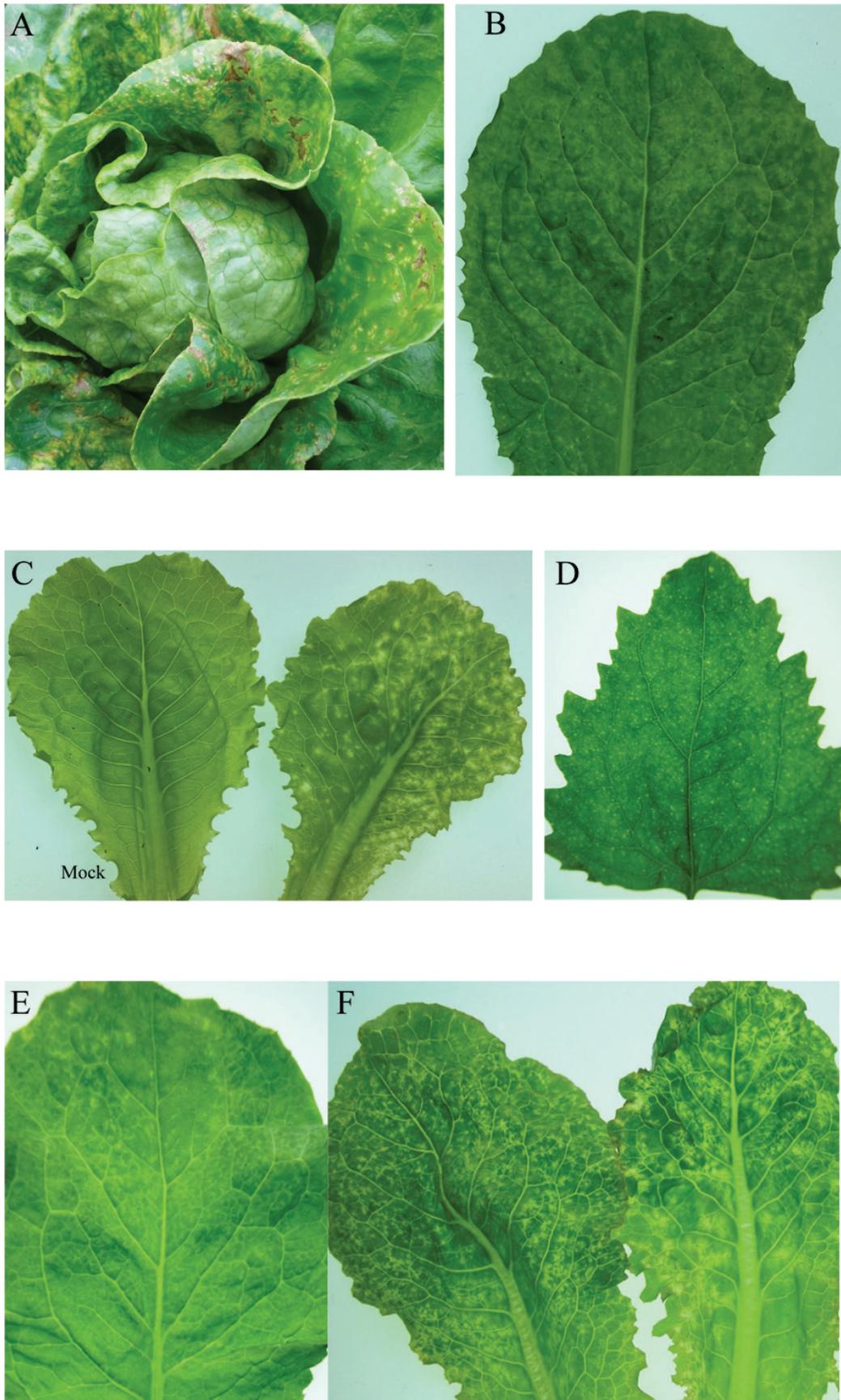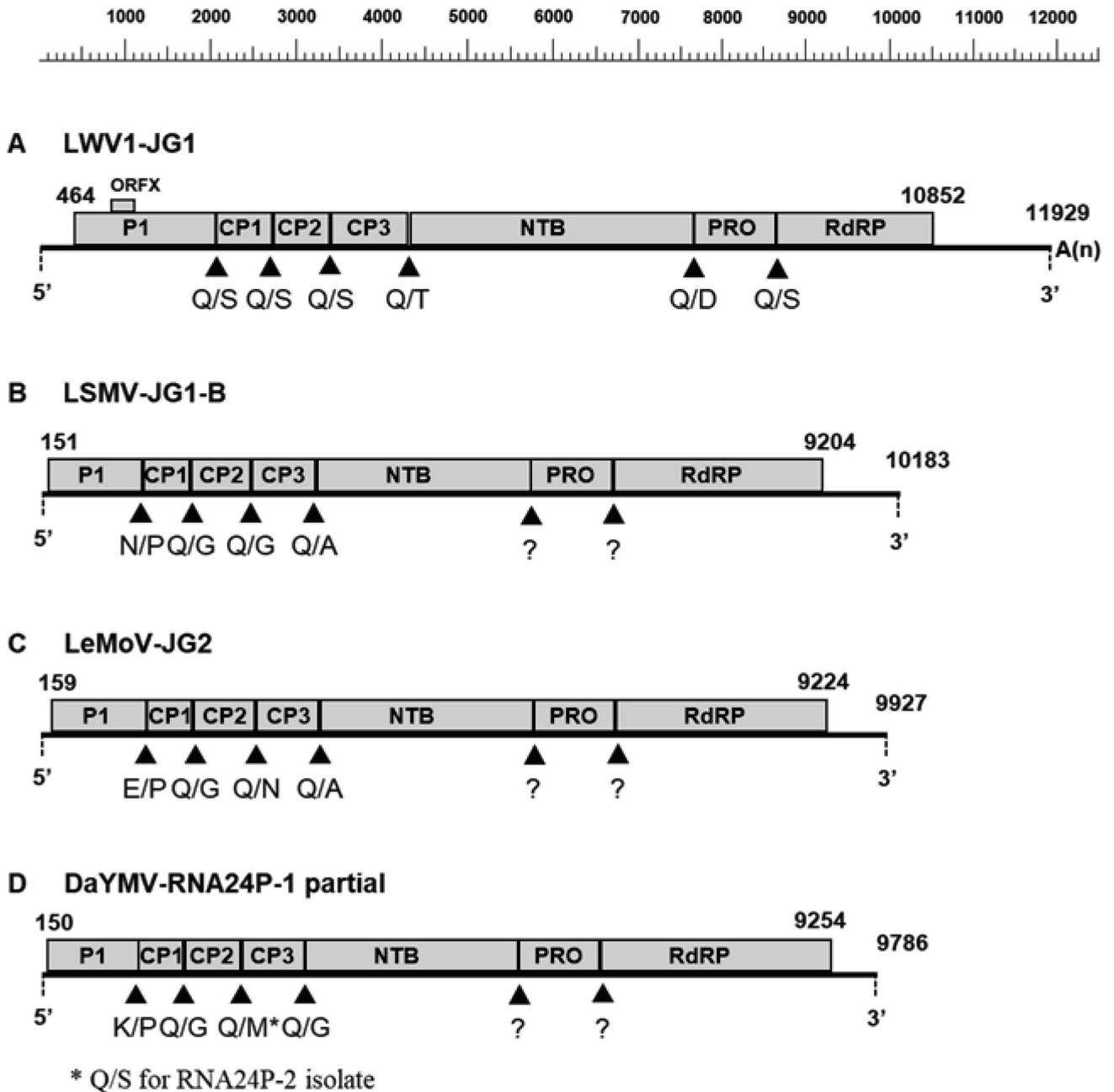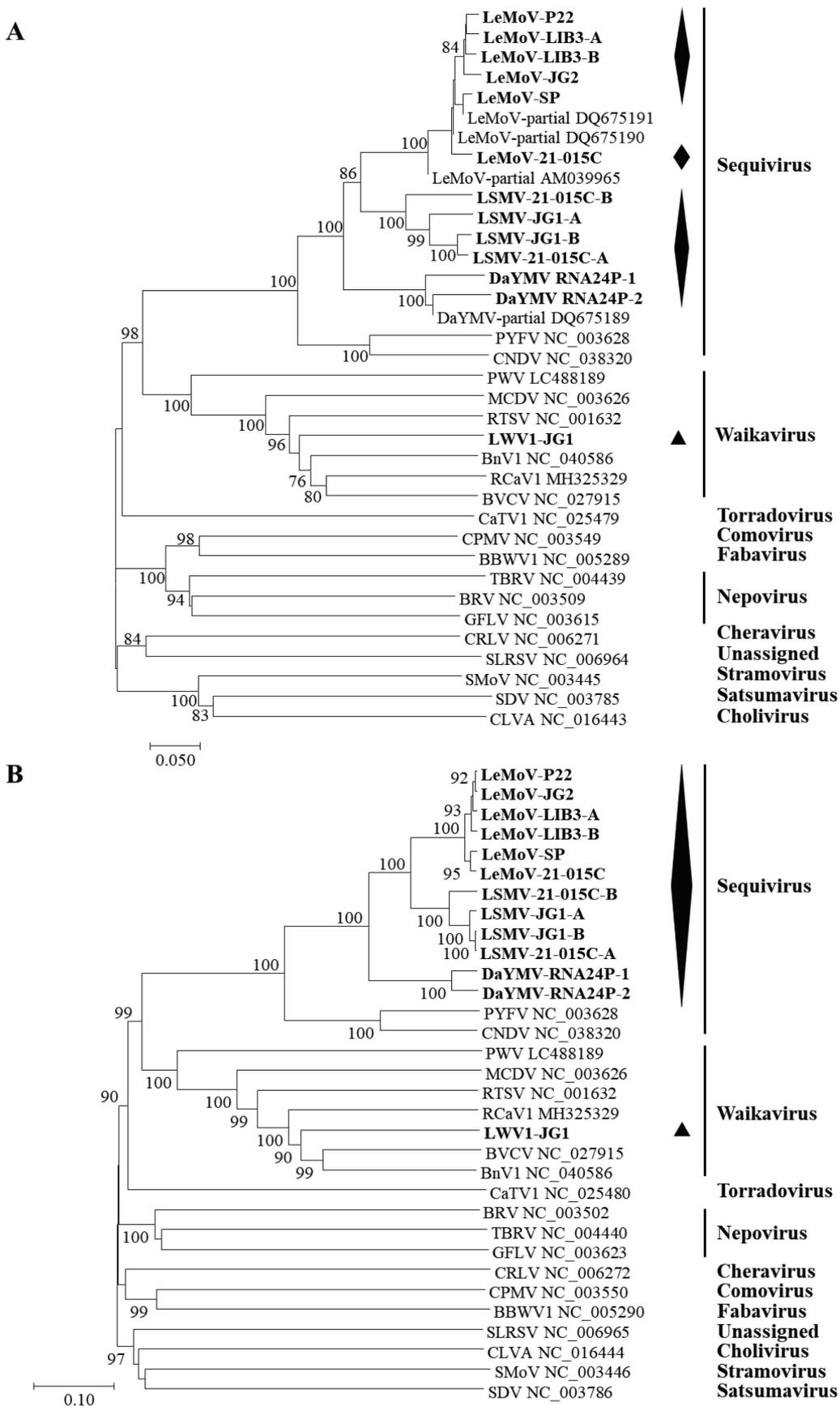
Figure 1

Figure 2

Figure 3

Supplementary table titles and Supplementary figure captions

Supplementary TABLE S1. Primers used for the detection and the molecular characterization of lettuce mottle virus (LeMoV) isolates JG2, P22 and SP, of lettuce star mosaic virus (LSMV) isolates JG1-A and JG1-B and of lettuce waikavirus 1 (LWV1) isolate JG1.

Supplementary TABLE S2. Pairwise percentages of amino acid identity for the CP (lower diagonal) and Pro-Pol (upper diagonal) regions of the polyproteins of Waikavirus genus members.

Supplementary TABLE S3. Pairwise percentages of identity for the CP (lower diagonal) and Prot-Pol (upper diagonal) regions of the polyproteins of Sequivirus genus members.

Supplementary TABLE S4. Description of the viral isolates used for the phylogenetic studies

Supplementary Figure 1. Multiple alignment of the predicted short overlapping ORFX from all members of the genus Waikavirus.