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Characterisation of the little cherry virus 1 isolate infecting almonds in the Czech Republic

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Abstract: *Little cherry virus 1* (LChV1) of the genus *Velarivirus* is an important pathogen associated with the little cherry disease. It infects various species of the genus *Prunus*, mainly the sweet cherry and the sour cherry. Recently, plums and apricots have also been reported as natural hosts. In the present work, the LChV1 isolate causing chlorotic mosaics in almonds is characterised. The nearly complete (+)ssRNA genome sequence of the Alm138 isolate, obtained by Sanger sequencing, is 16 878 nt long showing a typical velarivirus structure with 8 ORFs. Among them, the taxonomically important ORFs, ORF1a/1b encoding the polyprotein is 8 421 nt long, with a 0/+1 frameshift position at 6 923; ORF3 encoding the heat shock protein HSP70h is 1 656 nt long, and ORF6 encoding the coat protein duplicate is 1 989 nt long. The genome sequence showed its highest identity with LChV1 isolates Apr184R (96.2%), 19SP003B (92.2%) and Kyoto-2 (92.1%). A similar situation was also found for the ORF1, HSP70h, and CP nucleotide and amino acid sequences. A phylogenetic analysis identified Alm138 as a member of the G5 phylogenetic group, supporting its close relationships with the Czech Apr184R apricot isolate and the other members of this group. This report describes the first little cherry virus 1 isolate infecting almonds and confirms the presence of isolates of the G5 phylogroup within Europe.

Keywords: LChV-1; *Prunus dulcis* L.; Sanger sequencing; phylogeny

Little cherry virus 1 (LChV1) is a member of the *Velarivirus* genus in the *Closteroviridae* family of plant viruses. The virus, together with *Little cherry virus 2* and X-disease phytoplasma, is associated with the little cherry disease. This serious disease manifests itself by a deterioration in the fruit quality, size and taste, as well as leaf discolorations in susceptible sweet cherry and sour cherry cultivars. LChV1 is spread by the vegetative propagation of infected material; no vector was known up to now. Recently, other prunus hosts, such as plums, peaches, almonds, and apricots, have been described, but it had been believed that their infection by LChV1 was latent (Matic et al. 2007; Jelkmann & Eastwell 2011; Šafářová et al. 2016). The intensive study of LChV1 during the last decade showed a wide diversity of isolates and has led to their differentiation into five phylogenetic groups (G1–G5). The prevalent cherry isolates are distributed among all the phylogroups, and host specificity was not found (Katsiani et al. 2018).

MATERIAL AND METHODS

Sample collection. In May 2020, leaves showing chlorotic mosaics and chlorotic netting were collected from an almond tree (*Prunus dulcis* L.) at the locality Horní Věstonice, the Czech Republic.

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The leaves were kept in the cold, and the plant RNA was immediately isolated in the lab after transport.

RNA isolation and reverse transcription. The total RNA was isolated from two symptomatic leaves (100 mg of tissue) using a Plant/Fungi RNA Purification Kit (Norgen-Biotek, Canada) following the manufacturer's instructions to characterise the LChV1 isolate from this symptomatic plant. Three hundred ng of the isolated RNA was used for the synthesis of the first-strand cDNA. Reverse transcription using random hexamers and Bioscript reverse polymerase (Bioline, UK) was performed according to the manufacturer's instructions.

Polymerase chain reaction. Eight overlapping fragments covering the complete LChV1 genome were amplified using specific primer pairs [Figure 1, Table S1 in electronic supplementary material (see the electronic version)] in a subsequent polymerase chain reaction (PCR) analysis using MyFi polymerase (Bioline, UK). The reaction mix, in a total volume of 25 µL, consisted of a MyFi buffer (5×), specific primers (0.2 µM of each primer), MyFi polymerase (1 IU), and 2 µL of cDNA. The PCR programme consisted of 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 50–56 °C (depending on the used primer pair) for 1 min, 72 °C for 1–3 min (1 min per ca 1 kb length of the amplicon), and a final extension 72 °C for 7 minutes. The amplicons were analysed by 1% agarose gel electrophoresis in a 1× Tris-acetate-EDTA (TAE) buffer.

The detection of the potential co-infection by the Prunus necrotic ringspot virus (PNRSV), prune dwarf virus (PDV) and plum pox virus (PPV) was performed on the cDNA using specific primer pairs and original protocols as follows: PPV as described by Wetzel et al. (1991), PDV and PNRSV as described by Mekuria et al. (2003).

Sanger sequencing of the PCR amplicons. The PCR products were isolated using a QIAquick Gel Extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The isolated amplicons (one per each genomic fragment) were sequenced directly, bidirectionally, by Sanger sequencing using a BigDye™ Terminator (version 3.1) Cycle Sequencing Kit and an ABI PRISM3730 Genetic analyser in the Sequencing centre, Institute of Experimental Botany, AS CR, Olomouc.

Bioinformatic analysis. The obtained sequences were assembled into the final contig and the 5'- and 3'-end primers were cut off using Geneious Prime assembler (version 2020.01, https://www.geneious.com). The identity of the contig was verified by a BLASTN analysis (Altschul et al. 1997) against the GenBank database. The viral ORFs and the specific protein domains were identified using the Glimmer algorithm (Delcher et al. 2007) (implemented in Geneious Prime) and the National Center for Biotechnology Information (NCBI) Conserved Domains Database (CDD) (Marchler-Bauer et al. 2017). The comparisons of the almond LChV1 isolate and the other LChV1 isolates available in the GenBank database were carried out by the ClustalW program, and the distance analysis was carried out on the ClustalW derived matrices. The phylogenetic analysis was conducted using the neighbour-joining method, Tamura 3-parametric model, 1 000 bootstrap replicates, and the final tree was visualised using TreeExplorer, all in Mega11 (Tamura et al. 2021). The RDP4 program (Martin et al. 2015) was used to search for potential recombination events in the studied almond LChV1 genomic sequence.

RESULTS

An almond tree showing virus-like symptoms, chlorotic mosaics and chlorotic netting was repeat-

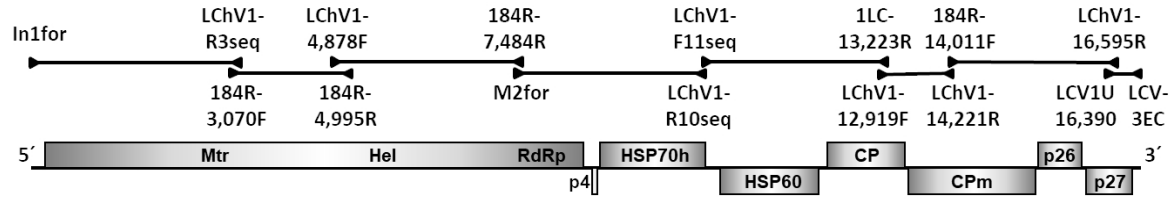

Figure 1. Genome map of the little cherry virus 1 (LChV1) Alm138 isolate with the position of the primers used for its amplification

Mtr – methyltransferase domain; Hel – helicase domain; RdRd – RNA dependent RNA polymerase domain; p4 – p4 gene; HSP70h – heat shock protein HSP70 homologue; HSP60 – heat shock protein HSP60; CP – coat protein; CPm – coat protein minor; p26 – hypothetical p26 protein; p27 – hypothetical p27 protein

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edly visually controlled over at least one decade (2010–2020). As the tree was previously detected as LChV1 positive by conventional RT-PCR, based on the partial ORF8 sequence (Šafářová et al. 2020), it was subjected to a detailed sequencing and genetic analysis with the aim to characterise the isolate infecting this very rare LChV1 host.

By Sanger sequencing, a 16 878-nt-long sequence covering the virus near-complete genome was obtained, and its identity was confirmed in the BLASTN analysis, showing a 96.24–73.97% identity with the complete sequences of the various LChV1 isolates available. The sequence of the isolate was deposited in the GenBank under the Acc. No. MZ570904.

The analysed Alm138 isolate has a genome structure typical of LChV1, harbouring eight ORFs and untranslated regions at the 5'- and 3'-ends (Figure 1). The biggest, ORF1, is 8 421 nt long (nt position 50–8 471) and consists of ORF1a and ORF1b that are expressed thanks to the characteristic ribosomal slippage identified at position 6 923. It encodes the polyprotein having the viral methyltransferase (aa position 684–998, pfam00978), helicase (aa position 2 011–2 268; cl26263), and RNA-dependent RNA polymerase (aa position 2 353–2 797; pfam00978) domains. ORF2 is 96 nt long (nt position 8 476–8 571) and encodes the hypothetical protein p4. ORF3 is 1 656 nt long (nt position 8 576–10 231) and encodes the heat shock protein HSP70h, displaying a nucleotide-binding domain of the HSP70 family (aa position 5–357; cd10170). ORF4 is 1 554 nt long (nt position 10 403–11 956) and encodes the heat shock protein HSP90 homologue, involved in the cell-to-cell movement, showing its typical viral Hsp90 superfamily domain (aa position 5–514; cl20248). ORF5 is 1 215 nt long (nt position 12 036–13 250) and encodes the coat protein. ORF6 is 1 989 nt long (nt position 13 256–15 244) and encodes a coat protein minor showing a typical closterovirus coat protein (CP) domain (aa position 510–642; cl03354); both CPs participate in the encapsidation of the viral genome. ORF7 (nt position 15 245–15 940) and ORF8 (nt position 15 977–16 696) are 696 nt and 720 nt long, encoding the p26 and p27 hypothetical proteins, respectively. The genome also contains the partial UTR regions 49 nt in length for the 5'-end, and 182 nt for the 3'-end, respectively.

The sequence comparisons with the LChV1 isolates available in the GenBank, using a p-distance analysis performed in MEGA11, showed the highest nt and aa identity with the Czech apricot Apr184R (Acc. No. MN242219), Japan flowering cherry Kyoto-2 (MG934545), Canadian 19SP003B (MZ291928), China sweet cherry BJ (MK775591), and Australian sweet cherry LV27S2 (LC523023) isolates (for detailed results on the complete genomes and selected ORFs, see Table 1). The topology of the phylogenetic tree constructed using the neighbour-joining method allowed for the discrimination of the isolates into five known phylogenetic groups, placing the Alm138 isolate into the G5 phylogenetic group in the closest relationship with the Apr184R isolate in the cluster containing isolates infecting the flowering and sweet cherry collected in Asia, Australia and Canada (Figure 2).

Table 1. Nucleotide and amino acid identity of the Alm138 isolate and the selected little cherry virus 1 (LChV1) representative isolates of each phylogroup within the complete genomes (CGs) and taxonomically important ORFs

LChV1 phylogroup	Isolate	CG, % nt	ORF1a/b, % nt (aa)	HSP70h, % nt (aa)	CP, % nt (aa)
G1	V2356	77.5	78.3 (85.8)	78.7 (84.9)	72.9 (74.3)
G2	YD	77.6	77.4 (84.9)	80.0 (86.8)	75.9 (76.5)
	Jerte	77.7	77.5 (85.1)	80.3 (87.5)	76.4 (76.9)
G3	UW2	76.1	76.5 (83.6)	77.7 (84.0)	73.0 (77.0)
	ITMAR	75.6	76.2 (81.9)	76.9 (83.7)	72.8 (76.0)
G4	GT15_3	73.5	73.5 (79.4)	75.7 (82.8)	70.1 (70.8)
G5	Apr184R	96.2	96.0 (96.2)	96.9 (97.5)	95.1 (92.6)
	Kyoto-2	92.1	92.0 (94.1)	93.4 (94.6)	91.0 (90.6)
	19SP003B	92.2	92.1 (93.9)	93.0 (94.9)	90.8 (89.9)
	BJ	92.0	92.0 (93.8)	92.8 (94.4)	90.5 (89.6)
	LV27S2	91.1	89.5 (90.9)	93.7 (95.3)	91.9 (90.8)

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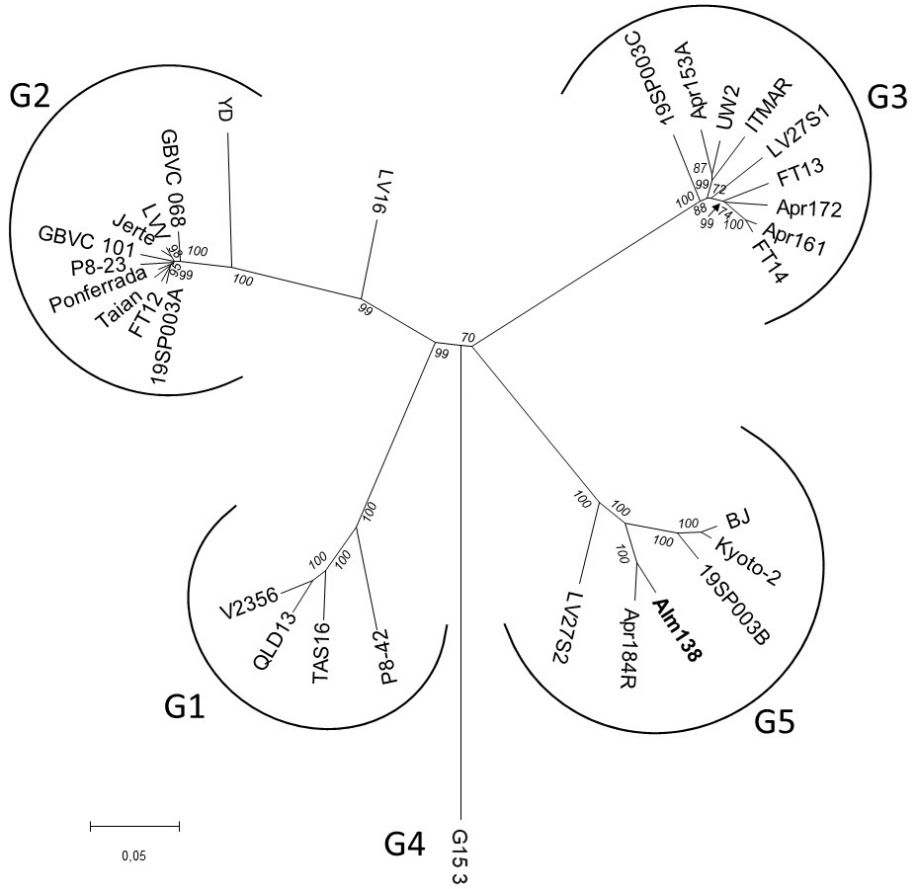

Figure 2. Unrooted neighbour-joining phylogenetic tree reconstructed using the complete nucleotide sequences of various little cherry virus 1 (LChV1) isolates

Bootstrap values ≥ 70% obtained from 1 000 replicates are shown; bar represents 5% nucleotide divergence. The virus sequences described by the name of isolate, details about the GenBank accession number and country of origin (two-letter international abbreviation) follow as: Alm138 – MZ570904 (CZ); 19SP003A – MZ291927 (CA); 19SP003B – MZ291928 (CA); 19SP003C – MZ291929 (CA); Apr153A – MN242215 (CZ); Apr161 – MN242217 (CZ); Apr172 – MN242218 (CZ); Apr184R – MN242219 (CZ); BJ – MK775591 (CN); FT12 – MN131067 (US); FT13 – MN131069 (HE); FT14 – MN131068 (US); G15_3 – LN794218 (GR); GBVC 101 – MK895512 (BE); GBVC 068 – MK895511 (BE); ITMAR – EU715989 (IT); Jerte – KX192366 (SP); Kyoto–2 – MG934545 (JP); LV16 – LC523021 (AU); LV27S1 – LC523022 (AU); LV27S2 – LC523023 (AU); LVV – LC523024 (AU); P8–23 – MH300060 (SP); P8–42 – MH300061 (SP); Ponferrada – KX192367 (SP); QLD13 – LC523019 (AU); Taian – KR736335 (CN); UW2 – NC 001836 (GE); TAS16 – LC523020 (AU); V2356 – JX669615(GE); YD – KR080325 (KR)

The analysis of the individual ORF1a/b, HSP70h, and CP nt and aa sequences brought the same phylogenetic position as the studied almond isolate. The recombinant analysis performed by the algorithms of the RDP4 software did not show any sign of a recombination event in the genome of the Alm138 isolate (results not shown). No unique changes in correlation with adaptation to the host or the symptomatic stage of the almond infection were identified throughout all the genetic analyses.

DISCUSSION

Little cherry virus 1 is generally considered a serious threat to the sour and sweet cherry production, being one of the causal agents of little cherry disease (Jelkman & Eastwell 2011). The negative impact of the disease on the fruit production and the health status of trees is the reason for its quarantine status and its spread needs to be controlled in cherry tree growing countries (EPPO 2022). Advanced molecular techniques and especially high-

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throughput sequencing over the last fifteen years have allowed the identification of other prunus hosts, such as the peach, almond or plum, even with very limited numbers or only one sample (Matic et al. 2007; Tahzima et al. 2019a, b). Generally, all these infections were not evaluated as dangerous as all the reported cases were without any visible symptoms observed on the infected trees. The situation reported for another new host, the apricot, shows that the virus can spread unnoticed as it was not expected to be able to infect anything other than the cherry host. The apricot had been, for a long time, considered as a non-host, and after the first report of the LChV1 presence in apricots in the Czech Republic, it was also reported in Hungary and Morocco, where the apricot trees were subsequently surveyed (Baráth et al. 2019; Tahzima et al. 2019b). In addition, the potential negative impact of LChV1 on the apricot fruit yield has been reported too (Šafářová et al. 2020).

The almond is still a rare, atypical, LChV1 host, but it does not mean that it should escape our attention. This study confirms the earlier findings, based only on the partial ORF8 sequences (Šafářová et al. 2020), about the presence of LChV1 isolates in G5, the so called non-European phylogroup in Europe. However, from an epidemiological viewpoint, based on the presented molecular analysis, the almond isolate does not show any specific evolutionary adaptation to this prunus species. Until now, the almond was considered as a non-symptomatic host, too. However, our 10-year long observation of the symptoms and repeated detection of LChV1 presence together with the PPV, PDV, and PNRSV absence show that the infection of almonds does not always have to be latent. Some cultivars could be sensitive to LChV1, which indicates a big risk with regards to the negative impacts of an infection on the fruit production in the future and the necessity of adhering to good practices and sanitation protocols during *Prunus* plant propagation.

To our knowledge, the described Alm138 isolate represents the first nearly-complete genome sequence of the LChV1 isolate infecting an almond tree.

REFERENCES

Altschul S.F., Madden T.L., Schaffer A.A., Zhang J.H., Zhang Z., Miller W., Lipman D.J. (1997): Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research, 25: 3389–3402.

Bajet N.B., Unruh T.R., Druffel K.L., Eastwell K.C. (2008): Occurrence of two little cherry viruses in sweet cherry in Washington State. Plant Disease, 92: 234–238.

Baráth D., Jaksa-Czotter N., Molnár J., Varga T., Balássy J., Szabó L.K., Kirilla Z., Tusnády G.E., Preininger É., Várallyay É. (2018): Small RNA NGS revealed the presence of Cherry virus A and Little cherry virus 1 on apricots in Hungary. Viruses, 10: 318. doi: 10.3390/v10060318

Delcher A.L., Bratke K.A., Powers E.C., Salzberg S.L. (2007): Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics, 23: 673–679.

EPPO (2022): *Little cherry virus 1* (LChV10). Available at https://gd.eppo.int/taxon/LCHV10 (accessed Jul 10, 2022).

Glasa M., Benedikova D., Predajna L. (2015): First report of little cherry virus-1 in Slovakia. Journal of Plant Pathology, 97: 542–542.

Jelkmann W., Eastwell K.C. (2011): *Little cherry virus-1* and *-2*. In: Hadidi A., Barba M., Candresse T., Jelkmann W. (eds). Virus and Virus-like Diseases of Pome and Stone Fruits. St. Paul, MN: American Phytopathological Society: 153–157.

Katsiani A.T., Maliogka V.I., Katis N., Svanella-Dumas L., Olmos A., Ruiz-Garcia A.B., Marais A., Faure C., Theil S., Lotos L., Candresse T. (2018): High-throughput sequencing reveals further diversity of *Little cherry virus 1* with implications for diagnostics. Viruses-Basel, 10: 385. doi: 10.3390/V10070385

Marchler-Bauer A., Bo Y., Han L., He J., Lanczycki C.J., Lu S., Chitsaz F., Derbyshire M.K., Geer R.C., Gonzales N.R., Gwadz M. (2017): CDD/SPARCLE: Functional classification of proteins via subfamily domain architectures. Nucleic Acids Research, 45: D200–D203.

Martin D.P., Murrell B., Golden M., Khoosal A., Muhire B. (2015): RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evolution, 1: vev003. doi: 10.1093/ve/vev003

Matic S., Myrta A., Minafra A. (2007): First report of little cherry virus 1 in cherry, plum, almond and peach in Italy. Journal of Plant Pathology, 89: S75–S75.

Mekuria G., Ramesh S.A., Alberts E., Bertozzi T., Wirthensohn M., Collins G., Sedgley M. (2003): Comparison of ELISA and RT-PCR for the detection of Prunus necrotic ring spot virus and prune dwarf virus in almond (*Prunus dulcis*). Journal of Virological Methods, 114: 65–69.

Rott M.E., Jelkmann W. (2001): Detection and partial characterization of a second closterovirus associated with little cherry disease, Little cherry virus-2. Phytopathology, 91: 261–267.

Šafářová D., Faure C., Candresse T., Navrátil M., Nečas T., Marais A. (2016): First report of *Little cherry virus 1*

https://doi.org/10.17221/63/2022-PPS

infecting apricot in the Czech Republic. Plant Disease, 101: 845–846.

Šafářová D., Ševčíková V., Neumanová K., Suchá J., Nečas T., Navrátil M. (2020): Molecular characterisation of little cherry virus 1 infecting apricots in the Czech Republic. European Journal of Plant Pathology, 158: 83–97.

Tahzima R., Foucart Y., Peusens G., Beliën T., Massart S., De Jonghe K. (2019a): High-throughput sequencing assists studies in genomic variability and epidemiology of little cherry virus 1 and 2 infecting *Prunus* spp. in Belgium. Viruses, 11: 592. doi: 10.3390/v11070592

Tahzima R., Qessaoui R., Foucart Y., Massart S., De Jonghe K. (2019b): First report of little cherry virus 1 infecting apricot (*Prunus armeniaca*) in Morocco (Africa). Plant Disease, 103: 2975. doi: 10.1094/PDIS-05-19-1082-PDN

Tamura K., Stecher G., Kumar S. (2021): MEGA11: Molecular evolutionary genetics analysis version 11. Molecular Biology and Evolution, 38: 3022–3027.

Wetzel T., Candresse T., Ravelonandro M., Dunez J. (1991): A polymerase chain reaction assay adapted to plum pox potyvirus detection. Journal of Virological Methods, 33: 355–366.

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