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# An Advanced One-Step RT-LAMP for Rapid Detection of *Little cherry virus 2* Combined with High-Throughput Sequence-Based Phylogenomics Reveal Divergent Flowering Cherry Isolates

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## Abstract

Little cherry virus 2 (LChV-2, genus *Ampelovirus*) is considered to be the main causal agent of the economically damaging little cherry disease, which can only be controlled by removal of infected trees. The widespread viral disease of sweet cherry (*Prunus avium* L.) is affecting the survival of long-standing orchards in North America and Europe, hence the dire need for an early and accurate diagnosis to establish a sound disease control strategy. The endemic presence of LChV-2 is mainly confirmed using laborious time-consuming reverse-transcription (RT-PCR). A rapid reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay targeting a conserved region of the coat protein was developed and compared with conventional RT-PCR for the specific detection of LChV-2. This affordable assay, combined with a simple RNA extraction, deploys desirable characteristics such as higher ability for faster (<15 min), more analytically sensitive (100-fold), and robust broad-range diagnosis of LChV-2 isolates from sweet cherry, ornamental flowering cherry displaying heterogenous viral etiology and, for the first time, newly identified potential insect vectors. Moreover, use of Sanger and total RNA high-throughput sequencing as complementary metaviromics approaches confirmed the LChV-2 RT-LAMP detection of divergent LChV-2 isolates in new hosts and the relationship of their whole-genome was exhaustively inferred using maximum-likelihood phylogenomics. This entails unprecedented critical understanding of a novel evolutionary clade further expanding LChV-2 viral diversity. In conclusion, this highly effective diagnostic platform facilitates strategical support for early in-field testing to reliably prevent dissemination of new LChV-2 outbreaks from propagative plant stocks or newly postulated insect vectors. Validated results and major advantages are herein thoroughly discussed, in light of the knowledge required to increase the potential accuracy of future diagnostics and the essential epidemiological considerations to proactively safeguard cherries and *Prunus* horticultural crop systems from little cherry disease.

*Keywords*: *Ampelovirus*, *Closteroviridae*, epidemiology, high-throughput sequencing, little cherry disease management, phylogenetics, point-of-care diagnostics

Little cherry virus 2 (LChV-2, genus *Ampelovirus*), the main viral agent of little cherry disease (LChD), is an important phloem-limited flexuous virus within the family *Closteroviridae* (Fuchs et al. 2020; Karasev 2000). LChD is a global economically important graft-transmissible disease associated with two distinct viruses that negatively impact cherry commercial production and industries (European Food Safety Authority 2017; Galinato et al. 2019). Since its first reported emergence in Canada in the Kootenay Valley of British Columbia in 1933, and later in other parts of North America, Little cherry disease has rapidly spread and has extensively impacted the fruit yield and quality of sweet (*Prunus avium* L.) and sour cherry (*Prunus cerasus* L.) production areas in the American Pacific Northwest and in Europe (Foster and Lott 1947; Foster and MacSewan 1949; Foster et al. 1951; Jelkmann et al. 2008; Keim-Konrad and Jelkmann 1996; Milbrath and Williams 1956; Theilmann et al. 2002, 2004; Welsh and Wilks 1951; Wilde 1960, 1962; Wilks and Milbrath 1956; Wilks and Reeves 1960; Wilks and Welsh 1964). Little cherry virus 1 (LChV-1), a member of the genus *Velarivirus* of the same family, is another widespread pathogen associated with LChD (Katsiani et al. 2015). Both Little cherry virus 1 and 2 have been identified and fully characterized as distinct culprits (Jelkmann et al. 1997; Rott and Jelkmann 2001, 2004). A third agent, namely Western X phytoplasma, has also been linked with the disease complex (Blodgett et al. 1950; Wilks and Milbrath 1956). LChV-2 is transmitted in a semipersistent mode, although inefficiently, by at least two mealybug species (Hemiptera, Pseudococcidae), namely the grape mealybug (*Pseudococcus maritimus* Ehrhorn; Mekuria et al. 2014) and the apple mealybug (*Phenacoccus aceris* Signoret; Raine et al. 1986). Nonetheless, dissemination of this virus occurs mainly through exchange and propagation of conducive plant material or by infected grafts.

Ascribed characteristic symptoms of LChV-2, produced by infected trees, are early reddening or bronzing of leaves; development of small drupes; imperfect ripening; and unsuitable taste. LChV-2 has been detected in several other members of temperate *Prunus* spp. as natural host species, including numerous cultivars of ornamental flowering cherry (*Prunus serrulata* L.), which can be infected – often latently and without acute symptoms – by both viruses and other viral agents, leading to further confounding of the diagnosis (Bajet et al. 2008; Eppler 1998; Komorowska and Cieslinska 2008; Mekuria et al. 2014; Posnette 1965; Rao et al. 2011; Tahzima et al. 2017, 2019a; Voncina et al. 2016; Yorston et al. 1981). Accurate diagnosis of LChD etiology is arduous also because visual symptoms may take years to appear; can vary depending on weather, season, and cultivar or variety; and can be confused with other disease or nutrient deficiencies (Galinato et al. 2019). So far, all cultivated cherries show susceptibility to LChV-2. Control measures such as prophylactic methods, monitoring of vectors, and massive insecticide applications against mealybugs are being used to counter the LChD pathogen (Peusens et al. 2017). In the circumstances

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where cultivars resistance remains unavailable and without treatment possibilities, early *in planta* virus-testing becomes essentially desirable. Hence, the effectiveness of a sound and sustainable integrated pest management strategy of the LChD pathogen is highly dependent on the immediate availability of a fast, sensitive, specific, and inexpensive diagnostic. Since the well-documented unprecedented epidemics, the incidence of the LChD pathogen in American and European sweet cherry orchards and in other *Prunus* species has increased in the last few years, revealing more diversity and indicating that the already wide distribution of LChV-2 is still expanding (Jelkmann and Eastwell 2011; Rubio et al. 2020). As of the writing of this article, only a few genetically heterogeneous LChV-2 genomes, representing different genetic lineages from narrow host ranges, have been publicly available. Henceforth, rapid discovery of new LChV-2 genomes using high-throughput sequencing (HTS) may therefore be a valuable avenue to uncover further viral diversity to mitigate the dissemination of the LChD pathogen.

Diagnostics for LChV-2 detection include antibody-based assays, reverse-transcription recombinase polymerase amplification, real-time- and, mainly, reverse-transcriptase PCR (RT-PCR), which remains the gold standard and the most popular of the molecular tests (Diaz-Lara et al. 2020; Eastwell et al. 1996; Eastwell and Bernardy 2001; Isogai et al. 2004; Mekuria et al. 2014; Theilmann et al. 2002; Vitushkina et al. 1997; Zong et al. 2014). Most of the prevalent methods suffer inherent shortcomings, such as skilled workforce requirements and immobility, cross reactivity with taxonomically unrelated pathogenic species due to inadequate specificity, expensive chemicals and materials, or the practice of normalization to ensure the accuracy of results (Li et al. 2013; Nassuth et al. 2000).

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is an extensively used low-cost and portable point-of-care diagnostic technology that enables isothermal amplification of targeted nucleic acids (Notomi et al. 2000). Its desirable features lie mainly in its short reaction time at thermostable incubation and user-friendliness. It can be adequately implemented *in situ* with minimal staffing. RT-LAMP robustness is improved by its modulated speed using four primers (and two additional optional loop primers), hence its enhanced thermostable specificity (Boonham et al. 2008; Harper et al. 2010; Lu et al. 2015; Nagamine et al. 2002; Tomlinson and Boonham 2008; Wong et al. 2018). The results of the LAMP test that allows simple endpoint formats can be visualized under different systems (Francois et al. 2011; Notomi et al. 2015; Okiro et al. 2019; Shen et al. 2014). Several (RT-)LAMP assays have been readily designated for a broad taxonomic scope of plant viruses, i.e., for the genera *Comovirus*, *Crinivirus, Geminivirus*, *Ilarvirus*, *Potyvirus*, *Tobamovirus*, *Tospovirus*, and *Velarivirus*, as well as for a few viroids (Boubourakas et al. 2009; Candresse et al. 1998; Fukuta et al. 2003a, b, 2013; Hadersdorfer et al. 2011; Lenarčič et al. 2013; Nie 2005; Okuda et al. 2005, 2015; Przewodowska et al. 2015; Sarkes et al. 2020; Shen et al. 2014; Silva et al. 2015; Tahzima et al. 2019b; Varga and James, 2006; Walsh and Pietersen 2013; Wang et al. 2014; Wei et al. 2012; Zhang et al. 2011; Zhao et al. 2010, 2016). Nevertheless, plant virus mobile point-of-care diagnostics that integrate the entire process from sample-to-results consultation, practiced in parallel with a characterization of genetic diversity, is only seldomly done (Rubio et al. 2020).

In this article, a rapid procedure and reliable one-step RT-LAMP for specific detection of LChV-2 was designed and validated in support of field disease management. This portable diagnostic test was compared with the prevalent RT-PCR approach to demonstrate an innovative tool for use in field diagnosis – and it was shown superior to existing technologies for accurate tracking of diverse LChV-2 isolates in potential new hosts species. The sensitivity and robustness of this assay was also assessed using various types of samples, including numerous species of insect vectors for the first time, which contributes to fill knowledge gaps toward better understanding of the little cherry disease. The unparalleled point-of-care assay used in this preliminary LChV-2 surveillance for insect vectors, addressed in this research, is an important tool for identifying viral circulation and potential entry points, therefore contributing to prevent outbreaks. This assay complies with phytosanitary regulations, and, finally, constitutes a suitable asset for sustainable epidemiological field investigations as well as a preventive management strategy against the infectious little cherry disease.

## Materials and Methods

**Sampling, plant, insect materials, and RNA extraction.** During growing seasons 2016 to 2018, an intensive survey was conducted across Belgium to monitor the incidence and spread of LChV-1 and LChV-2 in sweet cherry (*P. avium* L.), flowering cherry (*P. serrulata* L.), and plum (*Prunus domestica* L.) trees. Leaves from symptomatic and asymptomatic host plants were collected in commercial and private orchards where LChV-2 was prevalent, and in urban lane trees. Total RNA was isolated from cambial scrapings of midrib leaf samples using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, Overijse, Belgium). In total, 142 adult insects (*Hemiptera*, *Sternorrhyncha*, *Aphidoidea*, *Pseudococcidae*, and *Coccoidea*) were sampled throughout the growing season (April to October 2018) in LChV-2–infested cherry orchards by passing a sweep net through the vegetation using alternate back- and forehand strokes. Specimens were collected in nets or directly on plants with an aspirator, morphologically identified at species level, conserved in 1.5-ml vials containing 70% EtOH, and stored at –20°C. LChV-2 detection tests were conducted on a subset of five specimens for each species. The RNA quality and quantity were assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, The Netherlands) as described in Tahzima et al. (2019b). Additionally, total RNA from virus-tested healthy greenhouse plants was used as a negative (matrix) control. The final concentration of total RNA was adjusted to 50 ng/µl with RNase-free Milli-Q water (Millipore, Billerica, MA, U.S.A.) using the Nanodrop spectrophotometer. Crude leaf extracts were prepared by taking 1 cm of leaf midrib from LChV-2 infected trees exhibiting either obvious or no symptoms. These samples were immediately bead-disrupted in OptiGene lysis buffer (OptiGene Ltd., Horsham, U.K.) and subsequently 10-fold serially diluted in RNase-free Milli-Q water. The RNA and sample extracts were cryopreserved (–70°C). All LChV-2 isolates and other common *Prunus*-associated pathogens used in the validation experiments are detailed later in Table 2.

**RT-PCR–based LChV-2 detection.** To detect LChV-2 from *Prunus* spp. tree samples, conventional RT-PCR was performed according to the conditions described by Eastwell and Bernardy (2001). cDNA was prepared from 10-fold diluted total RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Temse, Belgium). Amplification was

**Table 1.** Little cherry virus 2 (LChV-2)-specific primer sets used for reverse transcription loop-mediated isothermal amplification assay (nucleotide [nt] position corresponds to the nt sequence of the LChV-2 USA6b isolate, GenBank accession number AF531505)

| Primers | Description | Length | Sequence (5′-3′) | 5′ | 3′ |
|---|---|---|---|---|---|
| LChV2CP_F3 | External forward primer | 19-nt | CGGTCAGGTTTTGGTGATG | 13,664 | 13,682 |
| LChV2CP_B3 | External backward primer | 20-nt | ACAATACTGGGAGAGATAGC | 13,858 | 13,842 |
| LChV2CP_FIP | Forward inner primer | 51-mer | CACCTTCTTTTCCATCTATGACGATTTT TTGACTTCAATGAACACAAAAGC | 13,731 | – |
| LChV2CP_BIP | Backward inner primer | 54-mer | CAGTTACGGACGTGTGAGAGATTTTTC TCATGAACCTACGCATG | – | 13,779 |
| LChV2CP_LF | Forward loop primer | 20-nt | TCTACTACACCTTGGCGACA | 13,708 | 13,728 |
| LChV2CP_LB | Backward loop primer | 22-nt | GAACCCATTTTCTGGAACACCG | 13,790 | 13,812 |

**Table 2.** List of Little cherry virus 2 (LChV-2) isolates and host plants used for LChV-2 loop-mediated isothermal amplification (LAMP) detection and optimization, and nontarget *Prunus*-associated viruses and cellular organisms using RT-LAMP and validated with diagnostic reverse transcription-PCR (RT-PCR)[a]

| Viruses (family) and organisms | Host | Geographical origin | Year of isolation | Reference ID | LChV-2 CP RT-LAMP[b] | LChV-2 RdRp RT-PCR[c] |
|---|---|---|---|---|---|---|
| Ampelovirus (*Closteroviridae*) | | | | | | |
| LChV-2 LC5 (AF416335) | *Prunus avium* | Canada (JKI) | 2015 | AF416335 | Positive | Positive |
| LChV-2 USA6b (AF531505) | *P. avium* | Canada | 2015 | AF531505 | Positive | Positive |
| LChV-2 TAKB | *P. avium* (wild) | Belgium | 2016 | GBVC_LChV2_071 | Positive | Positive |
| LChV-2 DCP448 | *P. avium* (low stem) | Belgium | 2016 | GBVC_LChV2_036 | Positive | Positive |
| LChV-2 DCP450 | *P. avium* (low stem) | Belgium | 2016 | GBVC_LChV2_038 | Positive | Positive |
| LChV-2 DCP451 | *P. avium* (low stem) | Belgium | 2016 | GBVC_LChV2_039 | Positive | Positive |
| LChV-2 DCP424 | *P. avium* (low stem) | Belgium | 2016 | GBVC_LChV2_040 | Positive | Positive |
| LChV-2 MV17GE | *P. avium* (high stem) | Belgium | 2017 | GBVC_LChV2_043 | Positive | Positive |
| LChV-2 MV17HE1 | *P. avium* (high stem) | Belgium | 2017 | GBVC_LChV2_050 | Positive | Positive |
| LChV-2 MV17ST1 | *P. avium* (high stem) | Belgium | 2017 | GBVC_LChV2_053 | Positive | Positive |
| LChV-2 MV17STK2 | *P. avium* (high stem) | Belgium | 2017 | GBVC_LChV2_059 | Positive | Positive |
| LChV-2 HIZ | *Prunus serrulata* (high stem) | Belgium | 2018 | GBVC_LChV2_070 | Positive | Positive |
| LChV-2 40856 | *P. avium* | Switzerland | 2017 | GBVC_LCHV2_065 | Positive | Positive |
| LChV-2 900247 | *P. avium* | Netherlands | 2018 | NL900247 | Positive | Positive |
| LChV-2 226 | *P. avium* | Turkey | 2018 | GBVC_LChV2_066 | Positive | Positive |
| LChV-2 JO2 | *Prunus domestica* | Jordan | 2018 | GBVC_LChV2_062 | Positive | Positive |
| LChV2 JO6 | *P. avium* | Jordan | 2018 | GBVC_LChV2_064 | Positive | Positive |
| LChV-2 INSK_S2A1 | *Myzus persicae*[d] | Belgium | 2018 | GBVC_LChV-2 S2A1 | Positive | Positive |
| LChV-2 INSK_B1S2 | *Coccus hesperidum*[e] | Belgium | 2018 | GBVC_LChV-2 S8B2 | Positive | Positive |
| LChV-2 INSK_S8B2 | *C. hesperidum*[e] | Belgium | 2018 | GBVC_LChV-2 S8B2 | Positive | Positive |
| LChV-2 INSK_3 | *Phenacoccus aceris*[e] | Belgium | 2018 | GBVC_LChV-2 INSK_3 | Positive | Positive |
| LChV-2 (13) D10 | *P. avium* | Germany | – | 13D10 | Positive | Positive |
| LChV-2 (45) 28393 | *P. avium* | Switzerland | – | CHE28393 | Positive | Positive |
| LChV-2 (12) Kyoto5 | *Prunus cerasifera* | Japan | – | Kyoto5 | Positive | Positive |
| LChV-2 (11) Kyoto2 | *P. cerasifera* | Japan | – | Kyoto2 | Positive | Positive |
| Closterovirus (*Closteroviridae*) | | | | | | |
| *Citrus tristeza virus* (CTV) | *Citrus* sp. | Spain | – | GBVC CTV_04 | Negative | Negative |
| *Beat yellows virus* (BYV) | *Beta vulgaris* | Germany | – | DSMZ PV0981 | Negative | Negative |
| *Barley yellow dwarf virus* (BYDV) | *Hordeum vulgare* | Belgium | – | GBVC_BYDV_01 | Negative | Negative |
| Capillovirus (*Betaflexiviridae*) | | | | | | |
| *Cherry virus A* (CVA) | *P. avium* | Belgium | 2016 | GBVC_CVA_001 | Negative | Negative |
| Ilarvirus (*Bromoviridae*) | | | | | | |
| *Prunus necrotic ringspot virus* (PNRSV) | *Prunus cerasus* | Germany | – | GBVC_PNRSV_001 | Negative | Negative |
| *Prune dwarf virus* (PDV) | *P. avium* | Belgium | 2017 | GNBC_PDV_001 | Negative | Negative |
| Fabavirus (*Secoviridae*) | | | | | | |
| *Prunus virus F* (PrVF) | *P. avium* | Belgium | 2018 | GBVC_PrVF_001 | Negative | Negative |
| Potyvirus (*Potiviridae*) | | | | | | |
| *Plum pox virus* (PPV) | *P. domestica* | Germany | 2014 | GBVC_PPV_07 | Negative | Negative |
| Nepovirus (*Secoviridae*) | | | | | | |
| *Tobacco ringspot virus* (TRSV) | *Phaseolus vulgaris* | U.S.A. | – | DSMZ PV0236 | Negative | Negative |
| *Tomato ringspot virus* (ToRSV) | *Pelargonium* sp. | Denmark | – | DSMZ PV0049 | Negative | Negative |
| *Cherry leafroll virus* (CLRV) | *Vitis vinifera* | Germany | – | DSMZ PV0797 | Negative | Negative |
| Robigovirus (*Betaflexiviridae*) | | | | | | |
| *Cherry necrotic rusty mottle virus* (CNRMV) | *P. avium* | Belgium | 2018 | GBVC_PrVF_001 | Negative | Negative |
| *Cherry green ring mottle virus* (CGRMV) | *P. avium* | Austria | 2018 | GBVC_ CGRMV _001 | Negative | Negative |
| Velarivirus (*Closteroviridae*) | | | | | | |
| LChV-1 B2 | *P. avium* | Belgium | 2015 | GBVC_LChV1_022 | Negative | Negative |
| *Grapevine leafroll-associated virus 7* (GRLaV-7) | *V. vinifera* | Switzerland | 2016 | CHE40855 | Negative | Negative |
| Bacteria | | | | | | |
| *Pseudomonas syringae* | *P. avium* | Belgium | 2014 | GBBC 1987 | Negative | Negative |
| *Pseudomonas morsprunorum* | *P. cerasus* | Belgium | 2015 | GBBC 3047 | Negative | Negative |
| *Agrobacterium tumefaciens* | *Prunus* sp. | Belgium | – | LMG 167 | Negative | Negative |
| Fungi | | | | | | |
| *Monilinia laxa* | *P. domestica* | Netherlands | – | CBS 489.50 | Negative | Negative |
| *Monilinia fructigena* | *Malus pumila* | Netherlands | 1996 | CBS 101502 | Negative | Negative |
| *Botrytis cinerea* | *Malus* sp. | Belgium | 2006 | PCF 260 | Negative | Negative |
| *Cladosporium herbarium* | *Solanum tuberosum* | Belgium | 2017 | – | Negative | Negative |

[a] Reference material and collections. GBVC and GBBC, ILVO Virus and Bacteria collections, respectively, Belgium. JKI, Julius Kühn Institute collection, Neustadt, Germany. CBS, Fungal collection 1013 Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. DSMZ, Leibniz Institute, German Collection of Microorganisms and Cell Cultures.
[b] CP, coat protein.
[c] RdRp, RNA-dependent RNA polymerase.
[d] Hemiptera (family Aphididae).
[e] Hemiptera (family Coccidae).

carried out using primer pair PLC26L (Fwd 5′-GCAGTACGTTC GATAAGAG-3′) and PLC26R (Rev 5′-AACCACTTGATAGT GTCCT-3′) (Eastwell and Bernardy 2001), targeting a 409-bp fragment of the RNA-dependent RNA polymerase (RdRp) genomic region. The PCR reactions were carried out in a total volume of 25 µl of PCR mixture containing 10 µM of primers, 2.5 µl of 10× FastStart Taq DNA Polymerase reaction mix (Sigma-Aldrich, Overijse, Belgium), and 0.2 µl of FastStart Taq DNA polymerase (5 U/µl; Roche, Vilvoorde, Belgium), and 2 µl of 10× diluted cDNA RNase-free Milli-Q water in a ABI9700 GeneAmp Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.) under the following RT-PCR thermal conditions: 4 min at 94°C for initial denaturation; 40 cycles of 1 min at 94°C, 1 min at 53°C, 1 min at 72°C, 5 min at 72°C for final extension; and 4 min at 94°C for initial denaturation. Target-specific amplification was confirmed by gel electrophoresis using 2% agarose gel stained with 0.06 µl/ml Midori Green Advanced Stain (Nippon Genetics Europe, Düren, Germany) or were visualized with a fluorescence camera under EPI blue light (470 nm) according to the manufacturer's instructions (Azure Biosystems Inc., Dublin, CA, U.S.A.).

**LChV-2 primers and RT-LAMP assay design.** The LChV-2 conserved genomic region open reading frame (ORF) 5, coding for the capsid protein (CP) gene, was selected for the primer design amplification target. The nucleotide (nt) sequences, which spanned a 500-bp fragment of the CP genomic region from all LChV-2–available sequences (NCBI accession nos. AF531505, AF416335, MG881767, MF069043, KP410831, HQ412772, and EU153101), were downloaded from NCBI's GenBank and aligned (Fig. 1) to identify conserved LChV-2 genomic subregions using the software MEGA v.7 (https://www.megasoftware.net/; Kumar et al. 2016). From this alignment, a consensus sequence was determined for LChV-2–specific primer design using the software Primer Explorer v.5 (Eiken Chemical Co., Ltd., Tokyo, Japan; https://primerexplorer.jp/lampv5/index.html). The designed primers were also submitted to the BLASTn online platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi) as additional *in silico* quality control. The primer sequences and positions of the RT-LAMP primers are shown in Table 1. Primers were synthesized at Sigma-Aldrich (Overijse, Belgium). All RT-LAMP assay reactions were performed in a single tube containing a total volume of 25 µl in a Genie II thermocycler (OptiGene Ltd.). The reaction mixture consisted of 1.6 µM of primers LChV2CP_FIP and LChV2CP_BIP, 0.2 µM of primers LChV2CP_F3 and LChV2CP_B3, and 0.6 µM of primers LChV2CP_LF and LChV2CP_LB, as well as 15.8 µl of Isothermal Mastermix ISO-004 containing fast GspSSD 2.0 DNA Polymerase, thermostable inorganic pyrophosphatase, optimized reaction buffer, $MgCl_2$, deoxynucleotide monomers, and a ds-DNA binding dye (FAM). In addition, 0.2 µl of AMV (100 U) RT-001 (OptiGene Ltd.) was added to accelerate the RT reaction and improve the analytical sensitivity. Three microliters of 10× diluted template and RNase-free Milli-Q water were added. To find the optimal isothermal conditions, the RT-LAMP reaction containing all primers was performed through a temperature gradient ranging from 60, 63, 65, 66, and 67 to 68°C with total RNA or crude extracts in a Genie II device (OptiGene Ltd.).

**Analytical specificity and relative sensitivity of the LChV-2 RT-LAMP assay.** The analytical specificity of the LAMP reaction was evaluated by including 50 ng/µl total RNA or DNA of a selection of the most relevant targets (inclusivity) and nontarget organisms (exclusivity) that also infect cherry; RNA or DNA of these organisms was isolated from infected plants or pure cultures, respectively (Table 2). Total RNA and DNA extracted from those viruses and *Prunus*-associated organisms was used as a template in the CP-based LAMP and RdRp-based RT-PCR assays. The analytical specificity of both detection protocols was evaluated using total RNA and crude extracts and was tested three times independently. The relative analytical sensitivity was also assessed on cherry samples collected on LChV-2–infected field-grown cherry trees and insects from different origins (Table 2) and was compared with the analytical sensitivity of the RT-PCR protocol described above using Genie II (OptiGene Ltd.) and an ABI9700 GeneAmp Thermal Cycler (Applied Biosystems, Foster, CA, U.S.A.), respectively. To compare the sensitivity of both protocols, serial 10-fold dilutions of extracted RNA were amplified three times independently. Total RNA from healthy plants was used for a negative (matrix) control and RNase-free Milli-Q water was used for a technical control.

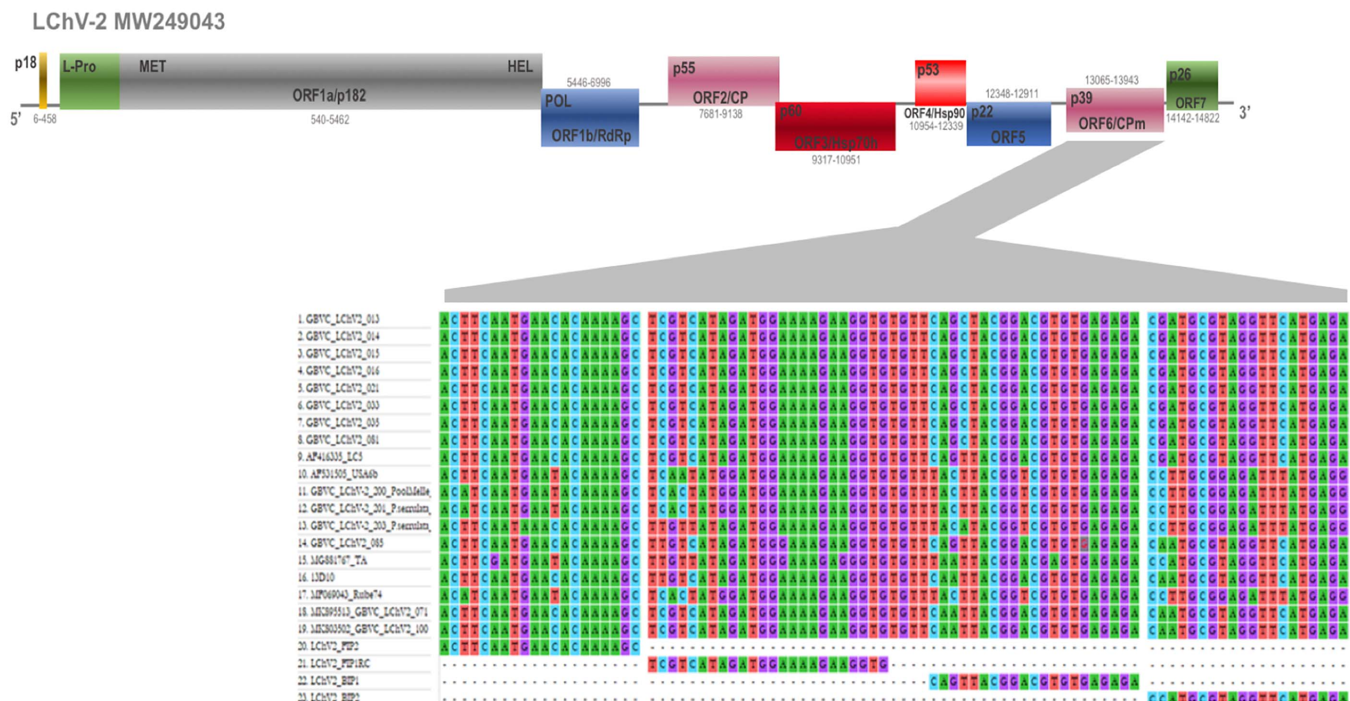


**Fig. 1.** Genomic map organization of Little cherry virus 2 (LChV-2) isolate (GenBank accession number MW249043) and position of LChV-2 reverse transcription loop-mediated isothermal amplification (RT-LAMP) primers (without loop primers) within the coat protein (CP) genomic region. Multiple sequence alignment using sequences from Belgian and GenBank isolates of LChV-2 (AF531505, AF416335, MG881767, and MF069043) used to design the RT-LAMP assay in this study. ORF, open reading frame.

**Total RNA high-throughput sequencing and bioinformatics analysis.** Total RNA was extracted from 100 mg of fresh leaf material infected by LChV-2 using the Spectrum Total Plant RNA Kit (Sigma Aldrich N.V). Quantification and quality controls were done with a Nanodrop ND-1000 spectrophotometer and a Quantus fluorometer (QuantiFluor RNA System kit, Promega Benelux B.V.) followed by RNA purification (NucleoSpin RNA Clean-up XS; Macherey-Nagel, Germany). Library preparation and rRNA depletion were done externally (Admera Health, South Plainfield, NJ, U.S.A.) using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, MA, U.S.A.) and Ribo-Zero Plant kit (New England BioLabs), respectively, followed by NextSeq sequencing (2 × 150-bp read length, 2 × 20 M reads per sample; Illumina; https://www.illumina.com/systems/sequencing-platforms/nextseq.html). The obtained sequence reads were subjected to quality filtering, adapter removal, and a standardized bioinformatics analysis strategy using the tools Cutadapt (https://cutadapt.readthedocs.io/en/stable/), Pear (https://gitlab.com/ilvo/phbn-wp2-training/tree/master/CL_ILVO_1), SortmeRNA (https://bioinfo.lifl.fr/RNA/sortmerna/), and the Virus-Detect pipeline (http://virusdetect.feilab.net/cgi-bin/virusdetect/index.cgi; Zheng et al. 2017). To determine the presence of viral species, the consensus sequences of the complete genomes were obtained through reference-based read mapping with the tool CLC Genomics Workbench 12 (Qiagen, Hilden, Germany).

**Sequences and phylogenetic analysis.** The obtained Sanger sequences from the partial RdRp and CP genomic regions were assembled, aligned, and analyzed using the platform BioNumerics Seven (v.7.6.1; Applied Maths, https://www.applied-maths.com/bionumerics). Sequence similarity was confirmed by similarity search using the tool BLASTn in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition to Belgian sequences, a selection of nt sequences of the RdRp of all representative LChV-2 from different countries and host plants was retrieved from GenBank, then aligned and used for phylogenetic analyses and molecular evolutionary genetics analysis with the software MEGA v.7.0 (Kumar et al. 2016). The deduced amino acid sequences of the RdRp and coat protein (CP) gene were obtained with the open reading frame online finder tool ORFfinder from https://www.ncbi.nlm.nih.gov/orffinder/. Phylogenetic and phylogenomic trees were generated from nt alignments of partial and full genome sequences, respectively, using maximum-likelihood (ML) algorithms with assessment of the confidence of branching patterns by bootstrap analysis with 1,000 pseudo-random iterations to test the robustness of the internal branches. The identification and sequence accession numbers of the LChV-2 isolates of this study, together with all other *Prunus*-related viruses included for this analysis, are available in the GenBank under Taxid No. 154339 (Table 2).

## Results

**LChV-2 RT-PCR–based detection.** The preliminary diagnostic test confirmed the presence of LChV-2 in RNA and crude extracts using conventional RT-PCR. LChV-2 was also detected, using RT-PCR, in the apple mealybug (*P. aceris*, Pseudococcidae), a known insect vector of LChV-2; a potential vector, brown soft scale, a common soft-scale species (*Coccus hesperidum* L., Coccidae); and in a less likely vector, the aphid (*Myzus persicae* Sulzer, Aphididae; Table 2).

**Specificity validation of the LChV-2 RT-LAMP assay.** The *in silico* BLASTn analysis of the synthesized LChV-2 LAMP CP-specific primers validated the absence of homology with sequences from other viral species of the family *Closteroviridae*. The LChV-2 RT-LAMP primer set, while being the best-fit trade-off for the targeted conserved CP genomic region, exhibited only little variability with a highly divergent isolate (GenBank accession number MF069043 Rube74; Fig. 1), yet did not hinder consistent specific and exact recognition of a broader range of strains. Considering the *in silico* extrapolations at the time of investigation and our adjusted polyvalent amplification settings to elude false negatives, this primers set is enabling persistent detection of LChV-2 isolates from various geographical origins and host plant species considered in this study. The performance of the LAMP-selected primers was assessed on a selection of well-characterized LChV-2 isolates using the described primers set (Tables 1 and 2), on total RNA as well as via direct detection from crude extracts. The ideal incubation temperature of the LAMP reaction was 67°C (Supplementary Fig. S1). Subsequently, all LAMP validations were performed at the 67°C optimum. A positive fluorescent signal was observed, indicating that the target CP gene of most LChV isolates can be successfully and rapidly amplified within 10 to 20 min. The promptness of our assay, being ensured by using six primers, makes it distinctively quicker than most plant virus LAMP assays designed up to now. Equivalent to RT-PCR, our results, based on amplification plots of serially diluted LChV-2 infected samples, revealed that the target CP gene of most LChV-2 isolates can be rapidly and efficiently amplified within the first 15 min (Supplementary Figs. S1, S2, S3, and S4). To assess and validate the specificity of our LChV-2 LAMP design, the optimized procedure was extended to a broad range of *Prunus*-associated organisms including non-European LChV-2 isolates, LChV-1, further *Closteroviridae* and stone fruit viruses, and on economically important bacteria and fungi. When using the GENIE II instrument, the LAMP instrument displays a unique melting peak with matching melting temperature ($T_m$ values) of 84°C ± 0.09 for LChV-2 isolates (insets, Supplementary Figs. S1, S2, S3, and S4). All LChV-2 isolates, whether from purified RNA or crude extracts, including from insects, were consistently detected with a mean detection time inferior to 15 min and at different sampling points in the year, whereas all non-LChV-2 RNA or DNA samples were not detected. Clearly, this indicates that the designed sequence-specific primers were suitable for robust LChV-2 detection under the stable settings and thereby secure the high specificity of this whole assay. In absence of false detection, the RT-PCR and the LAMP assay for all samples considered showed similar specificity. RNA or DNA isolated from nontarget organisms did not express amplification or cross reactivity.

**Assessment of sensitivity.** LChV-2 was detected in undiluted samples as well as in serial dilutions of $10^{-1}$ to $10^{-6}$ after RNA extraction from insects and plant samples. The detection limit was either equivalent or 100 times higher in comparison with the one-step RT-PCR method of Eastwell and Bernardy (2001) (Fig. 2). Direct detection on serial dilution of crude leaf extracts of the same samples gave reliable results for the $10^{-1}$ to $10^{-4}$ dilutions in the LAMP procedure, whereas the detection threshold of the compared conventional one-step RT-PCR was $10^{-2}$ (Fig. 2), or 100-times less. Undiluted crude extract could sporadically give a low nonspecific melting curve (Supplementary Fig. S2), but was avoided by systematically using direct dilution in the assay. All negative plant matrices and nontemplate references never showed any positive signal. An important feature of the LAMP method is the very short detection time. Conventional LChV-2 virus-testing requires long and laborious RNA extraction from tested plant samples, which makes it difficult to apply under field conditions. These shortcomings were also highlighted with respect to other plant virus LAMP methods (Budziszewska et al. 2016; He and Xu 2011; Iseki et al. 2007; Shen et al. 2014; Silva et al. 2015). When using diluted crude extract, our new LAMP assay remains more sensitive than RT-PCR with distinctive amplification patterns despite the occasional occurrence of an additional nonspecific small peak (Supplementary Fig. S2). A number of factors inherent to the biological material or to instrument stability might have accounted for this slight deviation. The improved sensitivity of the new LAMP method makes it ideal for use as a frontline screening assay, because LChV-2–infected plants can contain low concentrations of LChV-2, sometimes below the RT-PCR detection threshold. Moreover, a supplementary feature of our advanced test is that while the RT-PCR did not always detect LChV-2 attributable to factors such as seasonal variations (mainly during end of summer) and the woody plant matrix properties (*Prunus* sp.), these inadequacies were not observed in our LAMP assay.

**LChV-2 RT-LAMP field diagnostic performance for epidemiological applications.** The specific detection of LChV-2 in field samples demonstrated successfully that our on-site LAMP protocol is feasible as a potential portable assay. Its flexible robustness

constitutes a valuable advantage for straightforward field use, where it could be more difficult to control reaction conditions. The result of detection assay was unaffected when using either decimal dilutions of RNA or crude extracts from sweet cherry, or potential insect vectors (Supplementary Figs. S2 and S4). No false LChV-2 LAMP amplification was noticed in non-LChV-2–infected samples nor in samples containing nontarget organisms, allowing visual effortless interpretation without special equipment. The diagnostic robustness and repeatability of the implemented LChV-2 LAMP method were demonstrated using different isolates from different plant and insect extracts, and from various geographic regions. All isolates were shown positive based on amplification measures and no amplification was observed in healthy plants. Furthermore, from our screening for potential vectors, specimens from three common species gave a positive signal for the presence of LChV-2 using the RT-LAMP. Lastly, these results were validated by RT-PCR, showing the pertinence of this new test in forefront epidemiological characterization studies.

**High-throughput sequencing and phylogenomic inferences of divergent LChV-2 isolates.** In this section, a representative selection of diverse LChV-2 samples from flowering cherry (*P. serrulata*; accession no. MW249041, MW249042, and MW249043) were subjected to total RNA high-throughput sequencing and their whole genomes were recovered. The presence of LChV-2 and other known fruit tree viruses was also confirmed by conventional RT-PCR with specific primers and validated by Sanger sequencing (Table 3). Additionally, sequence analysis of genomic portions of the RT-PCR products and full genomes of several Belgian LChV-2 isolates was conducted to look at the degree of variability among all Belgian isolates and the extent of their genetic relationship to corresponding sequenced LChV-2 sequences in GenBank (Fig. 3; Table 4). Whole-genome sequence comparison of these three isolates from *P. serrulata* was determined and revealed a significantly high nt and amino acid heterogeneity (>20%) with all LChV-2 isolates except isolate Rube74. ML phylogenetics based on partial sequence (nt) comparisons and phylogenomics of the four LChV-2 reference genomes were analyzed (Fig. 3A and B) with estimates of the genetic diversity and evolutionary relationships among new and genetically divergent LChV-2 isolates from various hosts, which proved them most closely related (>99%) to LChV-2 Rube74 (GenBank accession number MF069043, Czech Republic). Remarkably, this endorses a distinct phylogenetic clade and solid evidence of further expanded LChV-2 diversity. The deduced phylogenomic relationships are in accordance with the overall level of divergence of these isolates with respect to all other LChV-2 complete reference genomes (Table 4). This corresponds with sequence (nt) comparisons carried out in previous studies (Theilmann et al. 2002,

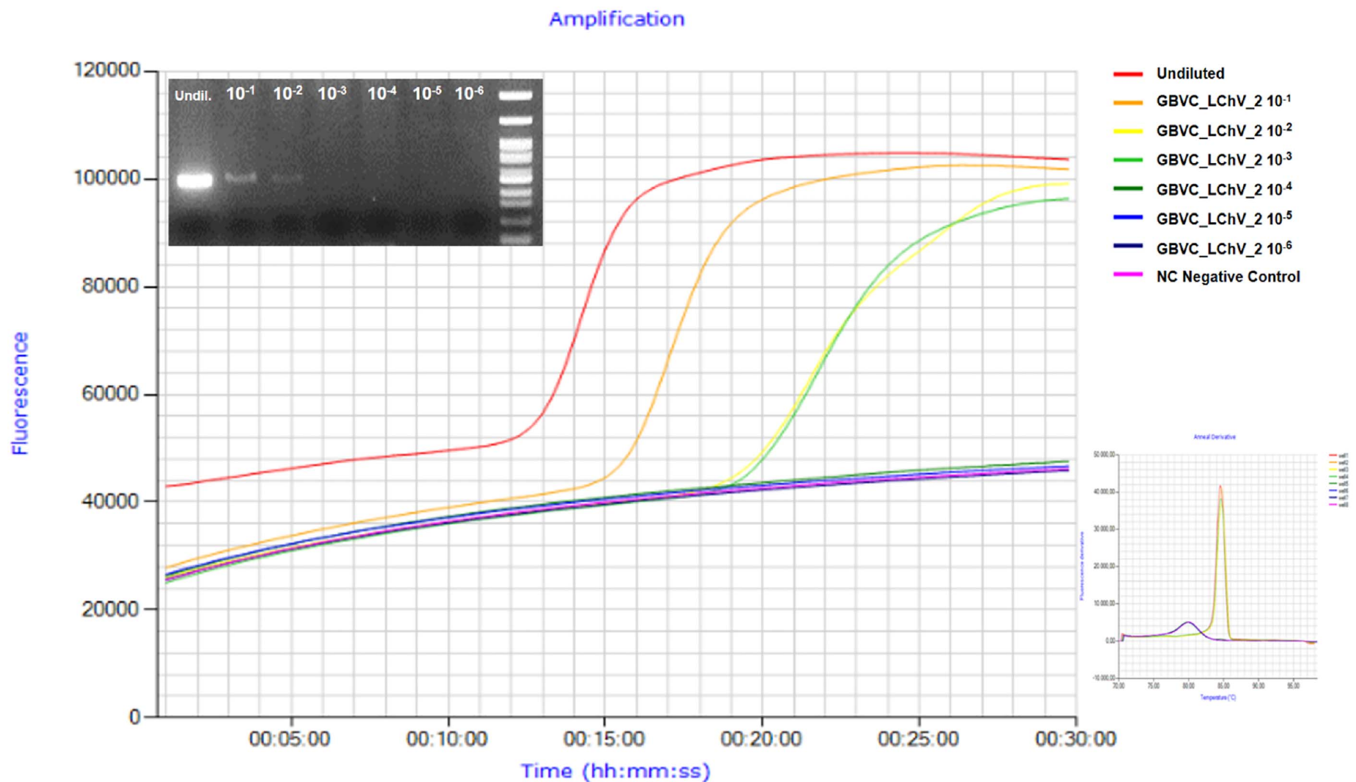


**Fig. 2.** Sensitivity–amplification plot of LChV-2 loop-mediated isothermal amplification of undiluted and serially diluted Little cherry virus 2 (LChV-2) RNA ($10^{-1}$ to $10^{-6}$). *NC* = dd$H_2O$ negative control. (Inset plot) Annealing derivative with specific melting curves. Agarose gel shows specific amplification bands (409 bp, undiluted to $10^{-2}$). A 100-bp molecular weight marker (ladder) is used in the right lane.

**Table 3.** List of isolates used for Little cherry virus 2 (LChV-2) loop-mediated isothermal amplification (LAMP) detection from diverse infected host plants, and identified with reverse transcription-PCR (RT-PCR) and high-throughput sequencing results

| Sample | No. of trees | Origin | Host | Symptoms | Total (raw) reads number | LChV-2 RdRp RT-PCR | LChV-2 CP RT-LAMP | Presence of other viruses[a] |
|---|---|---|---|---|---|---|---|---|
| KnokkePlum (P2) | 50 | Belgium | *Prunus domestica* | Yellowing | 18,521,058 | Negative | Negative | LChV1/ CVA/ CNRMV |
| MW249041 | 50 | Belgium | *Prunus serrulata* | No | 3,064,973 | Positive | Positive | LChV1 |
| MW249043 | 2 | Belgium | *P. serrulata* | Reddening | 1,189,144 | Positive | Positive | N/A |
| MW249042 | 1 | Belgium | *P. serrulata* | Reddening | 822,360 | Positive | Positive | LChV1 |
| 14 A1 | 6 | Morocco | *Prunus armeniaca* | Yellowing | 7,071,696 | Negative | Negative | LChV1/CVA |

[a] Detection as described in Tahzima et al. (2019a, b). N/A, not applicable.

2004). Intercluster comparative analysis of the nt sequences obtained from all encoded ORFs showed that the genetic distances among the four phylogenetic clusters were important and gradually increased toward the 3′-end of the genome, with the highest values encountered in the HSP90h (23%), CP regions (24%), and P26 (28%) ORFs.

## Discussion

Strategic and prophylactic management of little cherry disease, which can only be controlled by removing affected *Prunus* trees, relies mainly on prevention – particularly monitoring, correct virus identification, presence of weed reservoirs and insect vectors, and most importantly controlling for phytosanitary statuses of planting material – to restrict its dissemination. Nevertheless, LChV-2 and its insect vectors have, for many years, exerted great pressure on cherry production. Given the importance of *Prunus* species worldwide, LChD affecting these species has become a significant economic burden because of its wide geographic distribution. Owing to fruit yield losses and/or quality deterioration caused in various host species, cherry growers and stone fruit industry stakeholders are being urged to develop and implement rapid and robust on-site pathogen detection tools to reduce the time needed for plant testing as well as possible costly consequences of delay during certification or removal and destruction of horticultural materials. Severe LChV-2 outbreaks, documented as of 2019, underline the importance for prompt diagnosis of this viral pathogen (EPPO 2007; Galinato et al. 2019).

The heralded LAMP assay has been used for advanced molecular detection and triggered on-site diagnosis of plant pathogens including

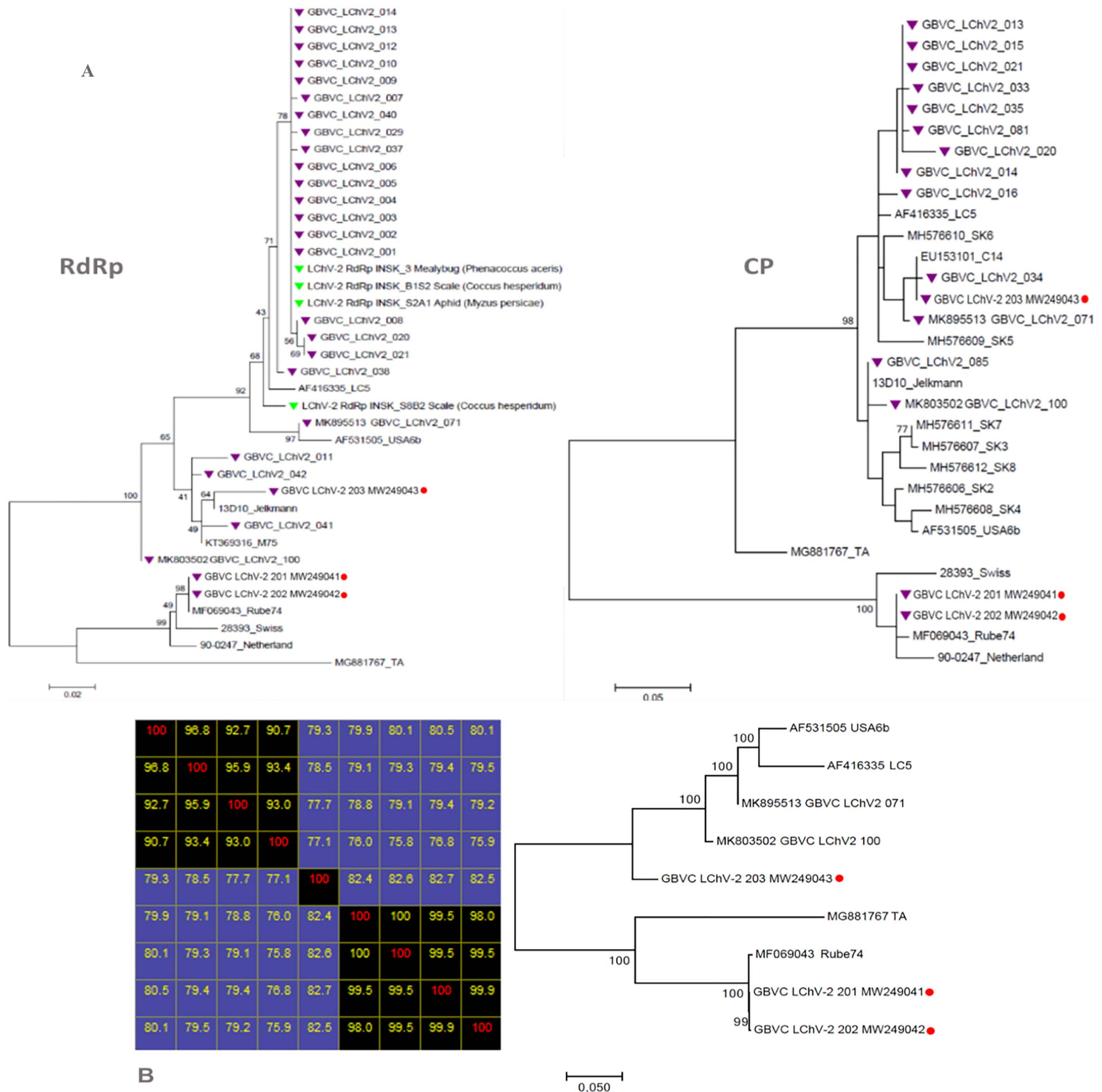


**Fig. 3.** Maximum-likelihood phylogenetic tree inferred from **A,** partial RNA-dependent RNA polymerase (RdRp) and coat protein (CP; purple triangles) and **B,** full genome nucleotide (nt) sequences of the Little cherry virus 2 (LChV-2) Belgian (red points) and LChV-2 isolates from GenBank with a similarity matrix (nt). The GenBank accessions are indicated together with the isolate name, host plant, and cultivar. Phylogenomic analysis (using the software MEGA v.7.0) included most of the available LChV-2 sequences. Branch lengths on the phylogenetic tree represent the genetic distance, and the numbers at the branches represent the percentage of replicates in which the branch topology was observed after 1,000 bootstrap replicates (only values >70% are shown).

viral diseases (Bonants et al. 2019; Fu et al. 2011; Khan et al. 2018; Tomlinson et al. 2010; Wong et al. 2018). The LAMP technology holds the following advantages: high specificity and high amplification efficiency; accurate results yielded from limited starting material and mostly without misdiagnosis or expensive infrastructure; and only a small portable instrument is needed (Fukuta et al. 2003a, b; Mori et al. 2001; Nagamine et al. 2002; Park et al. 2013; Tomita et al. 2008). Fast in situ diagnostics are an important focus for the deployment of control measures, especially for environmental monitoring of quarantine pathogens and warning systems (Boonham et al. 2008; Congdon et al. 2019; Okiro et al. 2019; Panno et al. 2020).

This study is, to our knowledge, the first attempt to develop and implement a rush-to-result portable LAMP assay for reliable detection of LChV-2 in naturally infected plant material as well as potentially invasive or emerging LChV-2 vector species that can be extended as a key prospective measure. We have described the development and optimization of an easy-to-use, fast LAMP method for specific and sensitive on-field detection of LChV-2. This LAMP procedure involves a direct single reaction tube assay on RNA or diverse crude biological matrix without intolerance to inhibitory substances. The use of the robust AMV polymerase overcomes these obstacles, which allows our assay to be performed in one step while maintaining its activity using plant and insect tissues harboring notorious impeding inhibitors (Rubio et al. 2020). Importantly, calibration of such assay, using serial dilution of in vitro transcripts to further help better determine analytical sensitivity, is recommended. Nevertheless, our assay affords direct stable visual detection of LChV-2 within 10 to 15 min, as compared with the more time-consuming procedure (90 to 180 min) that requires LChV-2 RT-PCR assays run under stringent nonisothermal cycling conditions, often after a tedious RNA extraction procedure in the laboratory (Eastwell and Bernardy 2001; Rao et al. 2011; Rott and Jelkmann 2001). Undoubtedly, this constitutes a major benefit as it shortens the diagnostic procedure and significantly reduces the risk of false-positives from unintended carry-over contamination, one of the few reported LAMP deficiencies (Lenarčič et al. 2013; Lu et al. 2015; Wong et al. 2018).

In this regard, our advanced one-step LAMP assay enables reproducible diagnostics for a wide and representative range of LChV-2 isolates and from different types of material (RNA, crude leaf mixture, insects), achieving dramatically increased specificity and inclusiveness with analogous or improved relative analytical sensitivity compared with the available RT-PCR protocols. No amplification products or cross reactivities were observed for RNA or DNA templates isolated from a range of closely related plant viruses or nontarget *Prunus* organisms. These results corroborate observations from other plant viruses detected in 2014 and 2015 using RT-LAMP (Fan et al. 2015; Shen et al. 2014). Furthermore, in our study, this broadly functional assay did not show amplification from healthy *Prunus* matrix. Thus, our new one-step LChV-2 LAMP assay efficiently accomplishes immediate detection and represents a potentially simple but also inexpensive method to track LChV-2 infections in diverse host plants such as sweet cherry trees from commercial orchards, in public green ornamental trees, and directly in insects requiring entomological surveillance in remote areas. While LChV-2 detection in the apple mealybug *P. aceris*, a reported LChV-2 pest vector, was expected (Raine et al. 1986; Jelkmann et al. 1995; Rott and Jelkmann 2004), detection in a newly suggested candidate insect vector (*C. hesperidum* L.), a common *Prunus* soft scale species belonging to the same superfamily (García-Morales et al. 2016) and described to transmit phloem-limited viruses of the genus *Ampelovirus* (Martelli et al. 2002), can open interesting research avenues. Nevertheless, despite some life-cycle commonalities between

**Table 4.** Nucleotide and amino acid identity percentages (%) between the Little cherry virus 2 (LChV-2) isolates from *Prunus serrulata* (GenBank accession number MW249041-43) from Belgium and all representative available LChV-2 full genome sequences (GenBank)[a]

| Genomic region | USA6b (AF531505) | LC5 (AF416335) | LChV2_100 (MK803502) | LChV2_071 (MK895513) | LChV-2-TA (MG881767) | Rube74 (MF069043) |
|---|---|---|---|---|---|---|
| LChV-2 (MW249041) | 83.55 | 86.36 | 86.56 | 84.53 | 84.05 | 99.58 |
| ORF0 (p18) | 95.79[b] (97.33) | N/A | 90.71 (95.08) | 94.24 (94) | 81.26 (75.33) | 99.56 (100) |
| ORF1a (p182/ POLYPRO) | 84.04 (82.82) | 81.81 (77.60) | 84.56 (82.27) | 85.09 (82.38) | 82.90 (88.41) | 99.76 (99.76) |
| ORF1b (RdRP_2) | 86.26 (93.59) | 86.53 (94.37) | 89.28 (95.53) | 87.35 (94.95) | 89.72 (96.84) | 99.87 (100) |
| ORF2 (p55) | 75.27 (81.24) | 76.40 (81.24) | 77.10 (81.24) | 75.96 (82.27) | 83.21 (87.63) | 99.52 (99.38) |
| ORF3 (p60/HSP70h) | 78.26 (87.68) | 78.27 (88.05) | 80.34 (88.97) | 79.72 (88.79) | 84.59 (93.01) | 99.63 (99.63) |
| ORF4 (p53/HSP90h) | 76.54 (80.83) | 77.93 (81.05) | 78.71 (82.79) | 77.35 (82.57) | 82.60 (88.91) | 99.57 (99.57) |
| ORF5 (p22) | 79.12 (85.56) | 80.00 (87.17) | 80.53 (87.17) | 80.71 (86.63) | 85.11 (94.12) | 99.29 (100) |
| ORF6 (p39/CP) | 88.23 (81.06) | 76.01 (80.50) | 76.43 (78.55) | 77.34 (78.83) | 75.58 (80.22) | 99.35 (99.72) |
| ORF7 (p26) | 95.59 (95.58) | 73.51 (71.21) | 93.39 (92.48) | 93.98 (92.92) | 72.33 (69.03) | 99.41 (99.56) |
| LChV-2 (MW249042) | 83.58 | 83.38 | 86.58 | 85.55 | 84.05 | 99.52 |
| ORF0 (p18) | 95.79 (97.33) | N/A | 90.71 (95.08) | 94.24 (94) | 81.26 (75.33) | 99.56 (100) |
| ORF1a (p182/ POLYPRO) | 84.04 (82.76) | 81.81 (77.51) | 84.56 (82.33) | 85.09 (82.44) | 82.88 (88.35) | 99.74 (99.70) |
| ORF1b (RdRP_2) | 86.26 (93.59) | 86.53 (94.37) | 89.28 (95.53) | 87.35 (94.95) | 89.72 (93.59) | 99.87 (100) |
| ORF2 (p55) | 75.27 (81.03) | 76.40 (81.03) | 76.97 (81.03) | 75.96 (82.06) | 83.21 (87.42) | 99.38 (99.18) |
| ORF3 (p60/HSP70h) | 78.38 (87.68) | 78.08 (88.05) | 80.47 (88.97) | 79.55 (88.79) | 84.71 (93.01) | 99.51 (99.63) |
| ORF4 (p53/HSP90h) | 76.61 (80.83) | 77.61 (81.05) | 78.37 (82.79) | 77.42 (82.57) | 82.49 (88.70) | 99.49 (99.35) |
| ORF5 (p22) | 79.12 (85.56) | 80.00 (87.17) | 80.53 (87.17) | 80.71 (86.63) | 85.11 (94.12) | 99.29 (100) |
| ORF6 (p39/CP) | 81.51 (81.06) | 76.01 (80.50) | 76.43 (78.55) | 77.34 (78.83) | 75.58 (80.22) | 99.35 (99.72) |
| ORF7 (p26) | 95.59 (95.58) | 73.51 (69.03) | 93.39 (92.48) | 93.98 (92.92) | 72.33 (69.03) | 99.41 (99.56) |
| LChV-2 (MW249043) | 90.75 | 89.16 | 94.31 | 92.59 | 79.96 | 85.36 |
| ORF0 (p18) | 95.57 (97.33) | N/A | 90.71 (95.08) | 94.01 (94.00) | 81.26 (75.33) | 99.78 (100) |
| ORF1a (p182/ POLYPRO) | 84.87 (86.70) | 84.47 (82.97) | 92.39 (87.56) | 88.92 (87.07) | 78.58 (85.37) | 84.22 (90.80) |
| ORF1b (RdRP_2) | 92.65 (95.93) | 93.56 (96.71) | 96.39(97.87) | 93.87 (97.09) | 84.25 (92.65) | 87.12 (94.19) |
| ORF2 (p55) | 92.25 (92.37) | 91.90 (91.13) | 97.94 (97.11) | 93.96 (93.81) | 74.95 (81.24) | 76.28 (80.41) |
| ORF3 (p60/HSP70h) | 92.84 (97.43) | 91.62 (96.69) | 97.19 (98.16) | 95.11 (98.53) | 79.99 (88.05) | 79.01 (87.13) |
| ORF4 (p53/HSP90h) | 92.14 (95.66) | 91.63 (95.66) | 96.18 (97.18) | 95.02 (97.18) | 76.90 (82.57) | 76.98 (81.05) |
| ORF5 (p22) | 92.20 (95.19) | 92.20 (94.65) | 95.39 (97.86) | 94.86 (97.33) | 80.85 (88.24) | 79.65 (86.10) |
| ORF6 (p21/CP) | 92.04 (88.05) | 87.94 (88.84) | 91.25 (88.45) | 91.01 (88.45) | 82.59 (86.06) | 87.44 (71.71) |
| ORF7 (p26) | 92.04 (77.88) | 72.96 (69.35) | 91.13 (75.22) | 91.01 (75.66) | 82.59 (60.18) | 79.95 (81.42) |

[a] ORF, open reading frame; N/A, not applicable.
[b] nt (amino acids).

*C. hesperidum* and *P. aceris*, little information is available on its biology and further transmission investigations need to be undertaken to confirm its proper role as meaningful LChV-2 vector.

Hitherto, only four genetically distinct isolates of LChV-2 have been fully sequenced and described, including isolates from different *Prunus* species. Sweet cherry and flowering cherry are known to be conducive host plants or reservoirs of LChV-2 (Reeves et al. 1955; Wilks and Reeves 1960) and our molecular as well as the transmission electron microscopy study confirmed the presence of the virus in two more new *P. serrulata* varieties, namely 'Kwanzan' and 'Hizakura', where 'Kwanzan' was already shown to asymptomatically harbor LChV-1 (Matić et al. 2009).

In this work, sequences corresponding to the partial RdRp and CP genes were determined from Belgian LChV-2 isolates originating from different host plants or insects. These were analyzed along with published homologous genomic regions from other LChV-2 isolates. ML phylogenetic analysis of both genes revealed the segregation of four evolutionary distinct groups showing no host or geography-based clustering. Mean genetic distances among the three clusters were high, with the CP region showing the highest divergence, although intragroup variability levels were lower. Several new LChV-2 variants fully sequenced genomes from different isolates were discovered in 2019 using different HTS approaches (Tahzima et al. 2019a). Remarkably, our results revealed the striking presence of further genomic diversity within the LChV-2 viral species, detected, for the first time, by using the RT-LAMP technique. Intercluster comparative whole-genome analysis, backed with in-depth characterization and well-supported phylogenomics, revealed new insights into the high intrahost and intraspecies diversities of LChV-2 that might help elucidate its pathogenicity and uncover epidemiological or quarantine implications worldwide.

Lastly, applications of HTS applications to fruit tree virology has allowed the discovery of new and sometimes divergent *Prunus*-infecting virus genomes, allowing further study of viral diversity (Villamor et al. 2016). Overall, within this extensively HTS supported study, whole genomic sequences of several LChV-2 isolates detected using our LAMP assay, including genetically divergent ones from different host plants, were also retrieved to ascertain their identification using the broader scope of HTS. For this purpose, nt and amino acid sequence comparative metaviromics of all available LChV-2 isolates have shown a high intraclade conservation in the 5′-terminal and the 3′-terminal ends of their genomic regions, while a significant variability was observed in the variable ORF2 to ORF5 spanning the replication and the morpho-modules of the newly sequenced LChV-2 isolates from *P. serrulata*. Variability in the same positions is also observed in the LChV-2 genomic sequences of isolate Rube74 and TA from Czech Republic and China, respectively, for which two unique whole-genome sequences are publicly available. However, the biological significance of this genomic diversity remains undetermined, although it has been hypothesized that genetic differences in these genomic modules might affect the efficiency of viral transmission and interaction (Ng and Falk 2006). The global LChV-2 high genetic diversity and the highly divergent isolates grouped in a new phylogenomic clade reported here for the first time could affect the reliable detection of viral isolates. Therefore, our analysis also showed that the LChV-2 specific primers used in this RT-LAMP scheme targeting the CP gene likely exhibit the highest detection range. Although growing evidence suggests that LChV-2 isolates could be largely latent in many of their hosts (Jelkmann et al. 2008; Rott and Jelkmann 2004), it is still included in many certification and quarantine schemes and several LChV-2 isolates have been tentatively associated with specific syndromes in sweet cherry and in other *Prunus* species (European Food Safety Authority 2017; USDA NASS 2017). Furthermore, evidence of mixed infection was confirmed by HTS and RT-PCR with different LChV-2 and LChV-1 genotypes being identified in two samples from *P. serrulata*, respectively. Taken together, these results would seem to exclude the unique contribution of LChV-2 isolates analyzed in this study to the LChD pathogen. No clear conclusive link can be drawn concerning their potential pathogenicity because, as frequently observed in other pathosystems, *Prunus* species hosting these complex viral entities were coinfected with several other graft- and vector-transmissible viruses addressing the persisting question of their potential prevalence and contribution to the virology of LChD. Further HTS-based investigations on LChV-2 genetic diversity on various *Prunus* hosts are clearly necessary to experimentally validate this hypothesis.

This LChV-2 LAMP assay, therefore, also represents an unrivalled complementary tool for predicting possible LChD outbreaks by generating real-time molecular epidemiological information emphasizing the benefits of including metaviromics HTS analyses as a crucial broad-spectrum tool for assessing the sanitary status of *Prunus* plant materials (Massart et al. 2017). This practical approach will help growers adopt strategical and sustainable decisions for managing LChV2-infected orchards, resulting in better financial outcomes. Indeed, while the advent of HTS diagnostic space has undoubtedly become prominent in plant health and virology, allowing identification of multiple viral pathogens in a single analysis without any previous knowledge of their nature (Al Rwahnih et al. 2015; Massart et al. 2014), several inherent limitations still need to be overcome before HTS can be implemented for integral point-of-care purposes. Unfortunately, owing to the observed and reported frequent mixed infections in ornamental and stone fruit *Prunus* trees (Marais et al. 2016; Rott and Jelkmann 2001), the inability of HTS to unequivocally dissociate any other virus species or variants from the specific symptoms associated with LChV-2 or even with *Prunus*-specific expressed phenotype ultimately imposes the need for multifaceted strategies to properly address the protracted burdens of the pathogenicity and symptomatology of LChD. Furthermore, HTS is limited by its requirements for sophisticated laboratory features, proprietary informatics, and a reliable power supply, and workflows remain complex, including sample preparation, multiple analysis steps, and several quality checks using bio-analysis equipment (Massart et al. 2017). The cumbersome and costly nature of these analytics make them, at present, inappropriate for most routine point-of-care diagnostic uses; however, future developments might make them affordable for comparative LChV-2 diagnostics in conjunction with metaviromics applications to help determine adequate or unique primers (Adams et al. 2018; Bonants et al. 2019; Maree et al. 2018).

Because advances of diagnostics in woody perennials such as *Prunus* species are generally evolving at a slower pace than in other horticultural crops species (Martinelli et al. 2015), this LChV-2 RT-LAMP test is an expedient, inexpensive, and functional tool that has the potential to break through diagnostic limitations in the *Prunus* horticultural sector and improve the phytosanitary status of grafting material from commercial nursery stock. Overall, it can be implemented both during field visits in vulnerable horticultural sites and in rigorous, high-tech research laboratories. This versatile and adaptable assay is therefore recommended as a viable frontline platform to enhance epidemiological forecasting targeting little cherry disease.


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