An Advanced One-Step RT-LAMP for Rapid Detection of *Little cherry virus 2* Combined with HTS-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 (LChD), 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 towards a sound disease control strategy. The endemic presence of LChV-2 is mainly confirmed using laborious time-consuming RT-PCR. A rapid RT-LAMP assay targeting a conserved region of the coat protein (CP) 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 (HTS) 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 current knowledge ensuing future diagnostic potentials and essential epidemiological considerations to proactively safeguard cherries and *Prunus* horticultural crop systems from little cherry disease.

INTRODUCTION

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* (Karasev, 2000; Fuchs et al., 2020). LChD is a global economically important graft transmissible disease associated with two distinct viruses that negatively impact cherry commercial production and industries (Galinato et al., 2019; EFSA, 2017). Since its first reported emergence in Canada (1933) in the Kootenay Valley of British Columbia 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 et al., 1947,1949, 1951; Milbrath et al., 1956; Welsh et al., 1951; Wilde, 1960, 1962; Wilks, 1960, Wilks et al., 1956, 1960; Keim-Konrad and Jelkmann 1996; Theilmann et al., 2002[a,b], 2004; Jelkmann et al., 2008). 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 viruses, little cherry virus 1 and 2, have been identified and fully characterized as distinct culprits (Jelkmann et al., 1997; Rott and Jelkmann, 2001). A third agent, namely Western X phytoplasma, has also been linked with the disease complex (Blodgett et al., 1950, Wilks et al., 1956). LChV-2 is transmitted in a semi-persistent mode, although inefficiently, by at least two mealybugs 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 of 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 (*P. serrulata* L.) which can be infected - often latently and without acute symptoms - by both viruses and other viral agents, leading to further compound diagnosis (Posnette 1965; Yorston et al., 1981; Eppler 1998; Bajet et al., 2008; Komorowska et al., 2008; Rao et al., 2011; Mekuria et al., 2014; Voncina et al., 2016 ; Tahzima et al., 2017, 2019[b]). Accurate diagnosis of LChD aetiology 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 currently used to counter LChD (Peusens et al., 2017). In the circumstances 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 LChD is highly dependent on the immediate availability of a fast, sensitive, specific and inexpensive diagnostic. Since the well-documented unprecedented epidemics, the incidence of LChD in American and European sweet cherry orchards and in other *Prunus* species has increased in recent years, revealing more diversity and indicating that the already wide distribution of LChV-2 is still expanding (Hadidi et al., 2011, Rubio et al., 2020). Concurrent with the writing of this paper, only few genetically heterogeneous LChV-2 genomes, representing different genetic lineages from narrow host ranges, are publicly available. Henceforth, rapid discovery of new LChV-2 genomes using HTS may therefore be a valuable avenue to uncover further viral diversity to mitigate the dissemination of LChD.

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

RT-LAMP (Reverse transcription loop-mediated isothermal amplification) 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 its 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 (Nagamine et al., 2002; Boonham et al., 2008; Harper et al., 2010; Tomlinson et al., 2008; Lu et al., 2015; Wong et al., 2017). The results of the LAMP test that allows simple endpoint formats can be visualized under different systems (Francois et al., 2011; Shen et al., 2014; Notomi et al., 2015; Okiro et al., 2019). 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 (Wei et al., 2012; Boubourakas et al., 2009; Candresse et al., 1998; Fukuta et al., 2003[a,b], 2013; Nie et al., 2005; Varga and James, 2006; Zhao et al., 2010; Zhang et al., 2011; Lenarcic et al., 2012; Walsh et al., 2013; Shen et al., 2014; Wang et al., 2014; Hadersdorfer et al., 2011; Przewodowska et al., 2015; Okuda et al., 2005, 2015; Silva et al., 2015; Zhao et al., 2016; Tahzima et al., 2019[a]; Sarkes et al., 2020). Nevertheless, plant virus mobile point-of-care diagnostic integrating the whole process from sample-to-results consultation in parallel with characterization of genetic diversity remains still scarcely adopted (Rubio et al., 2020).

In this paper, 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 ensuing this powerful innovative tool for field diagnosis and 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 towards 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 preventive management strategies 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 (*Prunus avium* L.), flowering cherry (*P. serrulata* L.) and plum (*P. 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 Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, 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 previously described (Tahzima et al., 2019a). Additionally, total RNA from virus-tested healthy greenhouse plants was used as negative (matrix) control. The final concentration of total RNA was adjusted to 50 ng/µl with RNase-free milliQ water using Nanodrop. 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, UK) and subsequently tenfold serially diluted in RNase-free milliQ 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 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 tenfold diluted total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Temse, Belgium). Amplification was carried out using primer pair PLC26L (Fwd 5′-GCAGTACGTTCGATAAGAG-3′) and PLC26R (Rev 5′-AACCACTTGATAGTGTCCT-3′) (Eastwell and Bernardy, 2001), targeting a 409-bp fragment of the RNA-dependent polymerase (RdRp) genomic region. The PCR reactions were carried out in a total volume of 25 µl of PCR mixture containing 10 µM primers, 2.5 µl 10x FastStart™ Taq DNA Polymerase reaction mix and 0.2 µl of FastStart Taq DNA polymerase (5U/µl) (Roche, Vilvoorde, Belgium), 2 µl of 10x diluted cDNA

RNase-free milliQ water in a ABI9700 GeneAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA) 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 (470nm) according to the manufacturer's instructions (Azure Biosystems Inc., Dublin, CA, USA).

LChV-2 Primers and RT-LAMP Assay Design. The LChV-2 conserved genomic region ORF5, coding for capsid protein (CP) gene, was selected as for primer design amplification target. The nucleotide sequences, which spanned a 500-bp fragment of the CP genomic region from all LChV-2 available sequences (NCBI accession numbers AF531505, AF416335, MG881767, MF069043, KP410831, HQ412772, EU153101) was downloaded from GenBank and was aligned (Fig. 1) to identify conserved LChV-2 genomic sub-regions using MEGA 7 software (Kumar et al., 2016). From this alignment, a consensus sequence was determined for LChV-2-specific primer design (Primer Explorer V5 software (https://primerexplorer.jp/lampv5/index.html, Eiken Chemical Co, Ltd, Tokyo, Japan). The designed primers were also submitted to the BLASTn online platform (http://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, Horsham, UK). 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, 15.8 µl of Isothermal Mastermix ISO-004 containing a fast GspSSD 2.0 DNA Polymerase, a thermostable inorganic pyrophosphatase, optimized reaction buffer, $MgCl_2$, deoxynucleotide monomers (dNTPs) and a ds-DNA binding dye (FAM). In addition, 0.2 µl of AMV (100U) RT-001 (OptiGene Ltd, Horsham, UK) was added to accelerate the RT reaction and improve the analytical sensitivity. Three microliters of 10x diluted template and RNase-free milliQ 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°C, 63°C, 65°C, 66°C, 67°C to 68°C with total RNA or crude extracts in a Genie® II device (OptiGene Ltd, Horsham, UK).

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 non-target 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 to the analytical sensitivity of the RT-PCR protocol described above using Genie® II (OptiGene Ltd, Horsham, UK) and ABI9700 GeneAmp Thermal Cycler (Applied Biosystems, Foster, CA, USA), respectively. To compare the sensitivity of both protocols, serial tenfold dilutions of extracted RNA were amplified three times independently. Total RNA from healthy plants and RNase-free milliQ water were used as negative (matrix) control and technical control, respectively.

Total RNA High-Througput 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 Nanodrop ND-1000 spectrophotometer and Quantus (QuantiFluor® RNA System kit, Promega Benelux B.V.) followed by RNA-purification (NucleoSpin® RNA Clean-up XS; Machery-Nagel, Germany). Library preparation and rRNA-depletion were done externally (Admera Health, NJ, USA) using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® and Ribozero Plant kit, respectively, followed by NextSeq sequencing (2x150bp read length, 2x20M reads per sample). The obtained sequence reads were subjected to quality filtering, adapter removal and a standardized bioinformatics analysis strategy using Cutadapt, Pear, SortmeRNA and the VirusDetect pipeline (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 in 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 BioNumerics 7 (Applied Maths version 7.6.1). Sequence similarity was confirmed by similarity search using 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, aligned and used for phylogenetic analyses and molecular evolutionary genetics analysis with the MEGA 7.0 (Kumar et al., 2016). The deduced amino acid sequences of the RdRp and coat protein gene were obtained with the open reading frame finder ORFfinder online tool from NCBI.nih.gov/ORFfinder. Phylogenetic and phylogenomic trees were generated from nucleotide 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 1000 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: 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 in the apple mealybug (*Phenacoccus aceris, Pseudococcidae*) using RT-PCR, a known insect vectors of LChV-2 as well as in a potential vector, namely a common soft scale species (*Coccus hesperidium* L.*, Coccidae*) and a less likely vectors such as aphids (*M. 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 (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 all currently described LChV-2 isolates from various geographical origin and host plants 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 (Table 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 (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 minutes (Fig. S1-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 as well as 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 (inserts, Fig. S1 to Fig. 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 minutes 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 non-target 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 to 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

313 10^{-4} dilutions in the LAMP procedure, whereas the detection threshold of the compared
314 conventional one-step RT-PCR was 10^{-2} (Fig.2), or 100 times less. Undiluted crude
315 extract could sporadically give a low non-specific melting curve (Fig.S2), but was
316 avoided by systematically using direct dilution in the assay. All negative plant matrices
317 and non-template references never showed any positive signal. An important feature
318 of the LAMP method is the very short detection time. Conventional LChV-2 virus-testing
319 requires long and laborious RNA extraction from tested plant samples, which makes it
320 difficult to apply under field conditions. These shortcomings were also highlighted with
321 respect to other plant virus LAMP methods (He and Xu, 2011; Iseki et al., 2007; Shen
322 et al., 2014; Silva et al., 2015; Budziszewska et al., 2016). When using diluted crude
323 extract, our new LAMP assay remains more sensitive than RT-PCR with distinctive
324 amplification patterns despite the occasional occurrence of an additional nonspecific
325 small peak (Fig.S2). A number of factors probably inherent to the biological material or
326 instrument stability might have accounted for this slight deviation. The improved
327 sensitivity of the new LAMP method makes it ideal for use as a frontline screening
328 assay, since LChV-2 infected plants can contain low concentrations of LChV-2,
329 sometimes below the RT-PCR detection threshold. Moreover, a supplementary feature
330 of our advanced test is that while the RT-PCR did not always detect LChV-2 due to
331 some factors such as seasonal variations (mainly during end of summer) and the
332 woody plant matrix properties (*Prunus* sp.), these inadequacies were not observed in
333 our LAMP assay.
334
335 **LChV-2 RT-LAMP Field Diagnostic Performance for Epidemiological Applications**. The
336 specific detection of LChV-2 in field samples demonstrated successfully that our on-
337 site LAMP protocol is feasible as a potential portable assay. Its flexible robustness
338 constitutes a valuable advantage for straightforward field use, where it could be more
339 difficult to control reaction conditions. The result of detection assay was unaffected
340 when using either decimal dilutions of RNA or crude extracts from sweet cherry, or
341 potential insect vectors (Fig. S2 and Fig. S4). No false LChV-2 LAMP amplification was
342 noticed in non LChV-2 infected samples nor in samples containing non-target
343 organisms, allowing visual effortless interpretation without special equipment. The
344 diagnostic robustness and repeatability of the implemented LChV-2 LAMP method
345 were demonstrated using different isolates from different plant and insect extracts, and
346 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, 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 all currently available corresponding sequenced LChV-2 sequences in GenBank (Fig. 3 and Table 4). Whole-genome sequence comparison of these 3 isolates from *P. serrulata* was determined and revealed a significantly high nucleotide and amino acid heterogeneity (>20%) with all LChV-2 isolates except isolate Rube74. Maximum likelihood 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 proved them most closely related (>99%) to LChV-2 Rube74 (GenBank accession 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[a,b], 2004). Inter-cluster comparative analysis of the nucleotide sequences obtained from all encoded ORFs showed that the genetic distances among the four phylogenetic clusters were important and gradually increased towards 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 – in order 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 due to 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 in order 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. Recent severe LChV-2 outbreaks underline the importance for prompt diagnosis of this viral pathogen (Galinato et al., 2019).

The herald advent of LAMP assay has been used for advanced molecular detection and triggered on-site diagnosis of plant pathogens including viral diseases (Tomlinson et al., 2010; Fu et al., 2010; Khan et al., 2018; Bonants et al., 2019; Wong et al., 2017). The LAMP technology holds the appealing advantages of high specificity and high amplification efficiency, yielding accurate results from limited starting material mostly without misdiagnosis or expensive infrastructure, using only a small portable instrument (Mori et al., 2001; Nagamine et al., 2002; Fukuta et al., 2003; Tomita et al., 2008; Park et al., 2013). Currently, 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; Okiro et al., 2019; Congdon et al., 2019; Panno et al., 2020).

This study is, to our knowledge, the first attempt to develop and implement a crush-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 *invitro* transcripts to further help better determine analytical sensitivity is recommended. Nevertheless, our assay affords direct stable visual detection of LChV-2 within 10-15 min, as compared to the more time-consuming assay (90-180 min) required using the LChV-2 RT-PCR assays run under stringent non-isothermal cycling conditions often after a tedious RNA extraction procedure in the laboratory (Eastwell and Bernardy, 2001; Rott and Jelkmann, 2001; Rao et al., 2011). Undoubtedly, this constitutes a major benefit as it shortens the diagnostic procedure and significantly reduces the risk of false positive due to unintended carry-over contamination, one of the few reported LAMP deficiencies (Lenarcic et al., 2013; Lu et al., 2015; Wong et al., 2017).

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 type of material (RNA, crude leaf mixture, insects), achieving dramatically increased specificity and inclusiveness with analogous or improved relative analytical sensitivity compared to currently available RT-PCR protocols. No amplification products or cross-reactivity were observed for RNA or DNA templates isolated from a range of closely related plant viruses or non-target *Prunus* organisms. These results corroborate observations from other recent plant viruses detected using RT-LAMP (Shen et al., 2014; Fan et al., 2015). 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 fitting 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, 2005), detection in a newly suggested candidate insect vector (*C. hesperidium* L.), a common *Prunus* soft scale species belonging to the same superfamily (Garcia-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 *C. hesperidum* and *P. aceris*, little information

449 is available on its biology and further transmission investigations need to be
450 undertaken to confirm its proper role as meaningful LChV-2 vector.
451 Hitherto, only four genetically distinct isolates of LChV-2 have been fully
452 sequenced and described, including isolates from different *Prunus* species. Sweet
453 cherry and flowering cherry are known to be conducive host plants or reservoirs of
454 LChV-2 (Reeves et al., 1955; Wilks and Reeves, 1960) and our molecular as well as
455 the TEM study confirmed the presence of the virus in two more new *Prunus serrulata*
456 varieties, namely Kwanzan and Hizakura, where Kwanzan was already shown to
457 asymptomatically harbor LChV-1 (Matic et al., 2009).
458 In the present work, sequences corresponding to the partial RNA-dependent RNA
459 polymerase (RdRp) and coat protein (CP) genes were determined from Belgian LChV-
460 2 isolates originating from different host plants or insects. These were analyzed along
461 with published homologous genomic regions from other LChV-2 isolates. Maximum
462 likelihood phylogenetic analysis of both genes revealed the segregation of four
463 evolutionary distinct groups showing no host or geography-based clustering. Mean
464 genetic distances among the three clusters were high, with the CP region showing the
465 highest divergence, although intragroup variability levels were lower. Several new
466 LChV-2 variants fully sequenced genomes from different isolates were recently
467 discovered using different HTS approaches (Tahzima et al., 2019[b]). Remarkably, our
468 results revealed the striking presence of further genomic diversity within the LChV-2
469 viral species, detected for the first time using RT-LAMP. Inter-cluster comparative
470 whole-genome analysis backed with in-depth characterization and well supported
471 phylogenomics revealed new insights into the high intra-host and intra-species
472 diversities of LChV-2 which might help elucidate its pathogenicity and uncover
473 epidemiological or quarantine implications worldwide.
474 Lastly, current applications of high throughput sequencing (HTS) applications to
475 fruit tree virology has allowed the discovery of new and sometimes divergent *Prunus*-
476 infecting virus genomes, allowing further study of viral diversity (Villamor et al., 2016).
477 Overall, within this extensively HTS supported study, whole genomic sequences of,
478 several LChV-2 isolates detected using our LAMP assay, including genetically
479 divergent ones from different host plants, were also retrieved to ascertain their
480 identification using the broader scope of HTS. For this purpose, nucleotide and amino
481 acid sequence comparative metaviromics of all available LChV-2 isolates have shown
482 a high intra-clade conservation in the 5′-terminal and the 3′-terminal ends of their

genomic regions, whereas, 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 are also observed in the LChV-2 genomic sequences of isolate Rube74 and TA from Czech Republic and China respectively, for which 2 unique whole genomic 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 (Rott and Jelkmann, 2005; Jelkmann et al., 2008), 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 (EFSA, 2017; USDA, 2017). Furthermore, evidence of mixed infection was confirmed by HTS and RT-PCR with different LChV-2 and LChV-1 genotypes were 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 the present study to the LChD. 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 co-infected with several other graft- and vector-transmissible viruses addressing the persisting question of their potential prevalence and contribution in the LChD virology. 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 (Massart et al., 2014; Al Rwahnih et al., 2015), 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 (Rott and Jelkmann, 2001; Marais et al., 2016), 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 current 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, whereas workflows remain still 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 find out adequate or unique primers (Maree et al., 2018; Adams et al., 2018; Bonants et al., 2019).

Presently, since 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 expedite, inexpensive and functional tool that has the potential to break through current 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 as well as 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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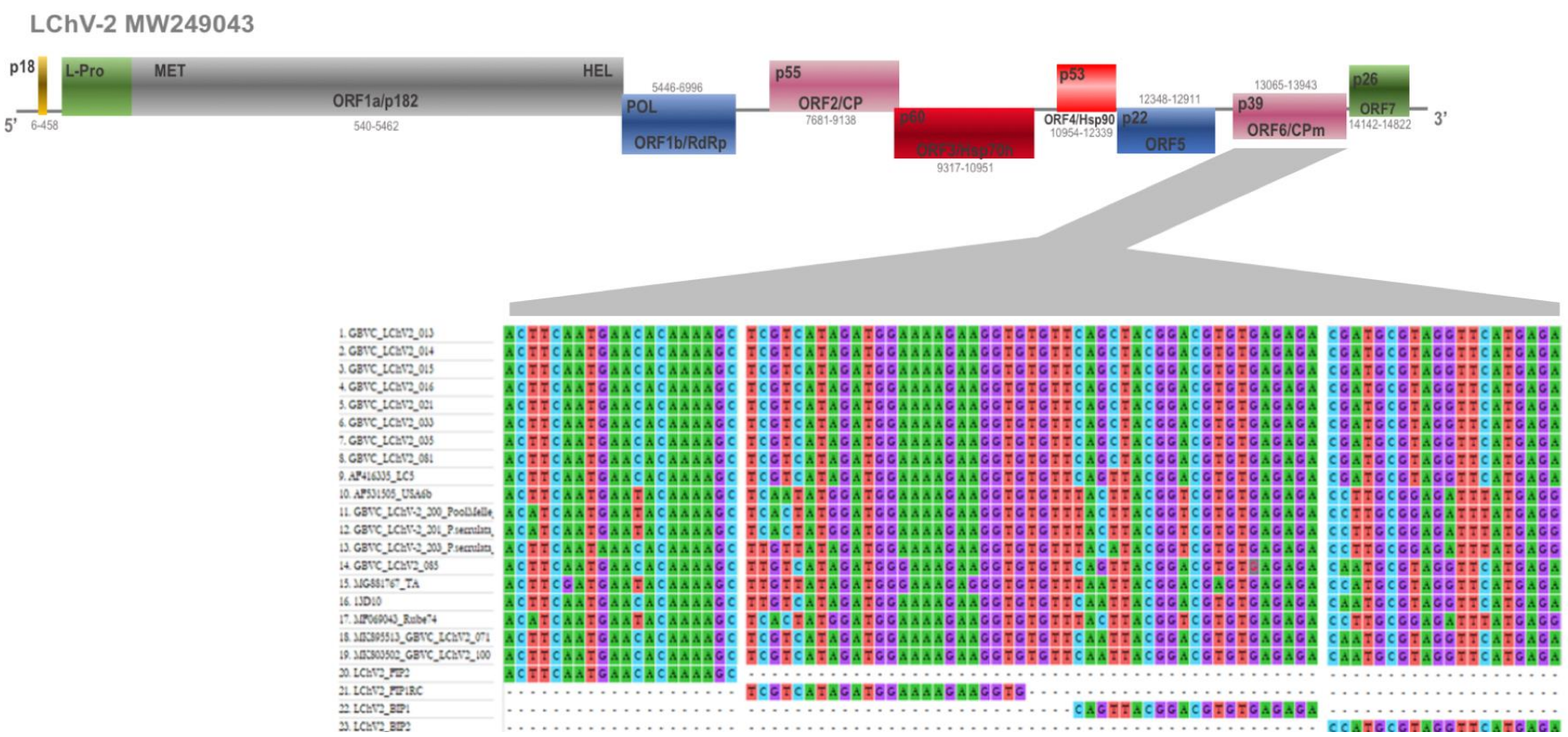


Fig. 1. Genomic map organization of LChV-2 isolate (GenBank accession MW249043) and position of LChV-2 RT-LAMP primers (without loop primers) within the coat protein genomic region. Multiple sequence alignment using sequences from Belgian and GenBank isolates of LChV-2 (AF531505, AF416335, MG881767, MF069043) used to design the RT-LAMP assay in this study.

Table 1. LChV-2 specific primer sets used for RT-LAMP assay (Nucleotide position correspond to the nt sequence of the LChV-2 USA6b isolate, Genbank® accession AF531505).

Primers	Description	Length	Sequence (5′-3′)	5′	3′
LChV2CP_F3	External forward primer	19nt	CGGTCAGGTTTTGGTGATG	13664	13682
LChV2CP_B3	External backward primer	20nt	ACAATACTGGGAGAGATAGC	13858	13842
LChV2CP_FIP	Forward inner primer	51mer	CACCTTCTTTTCCATCTATGACGATTTTTTGACTTCAATGAACACAAAAGC	13731	-
LChV2CP_BIP	Backward inner primer	54mer	CAGTTACGGACGTGTGAGAGATTTTTCTCATGAACCTACGCATG	-	13779
LChV2CP_LF	Forward loop primer	20nt	TCTACTACACCTTGGCGACA	13708	13728
LChV2CP_LB	Backward loop primer	22nt	GAACCCATTTTCTGGAACACCG	13790	13812

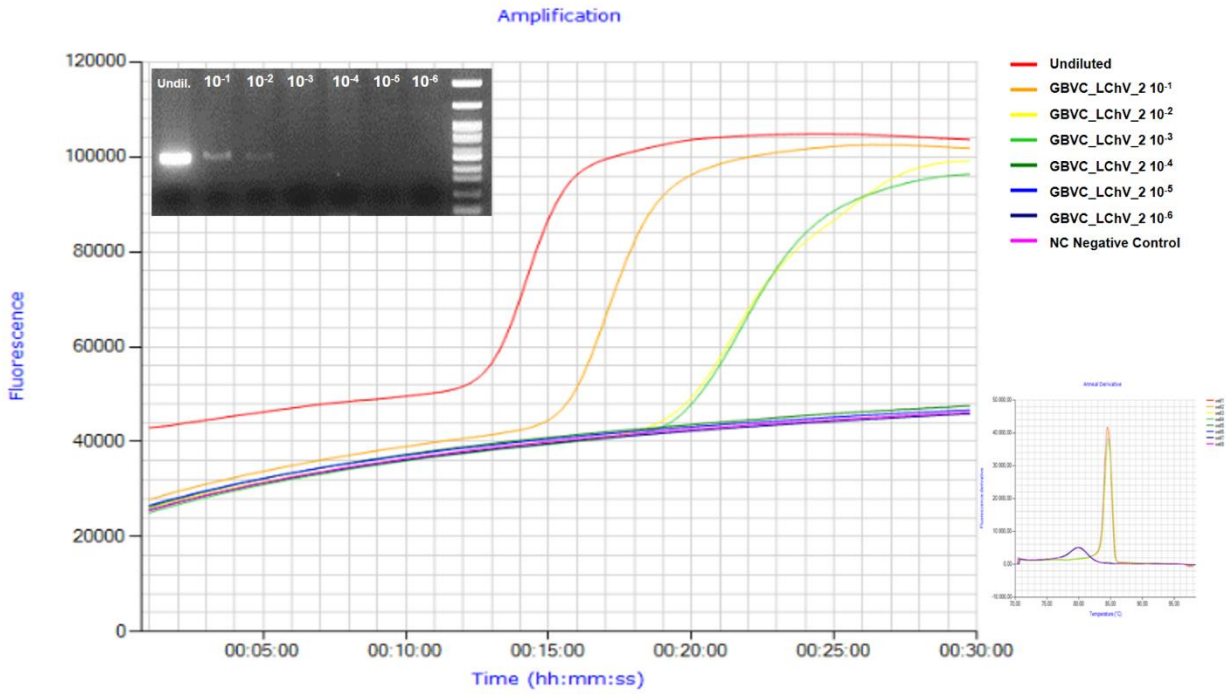


Fig. 2. Sensitivity – Amplification plot of LChV-2 loop-mediated isothermal amplification of undiluted and serially diluted LChV-2 RNA (10^{-1} to 10^{-6}). NC = ddH_2O negative control. Insert plot shows the annealing derivative with specific melting curves. Agarose gel showing specific amplification bands (409 bp, Undil. to 10^{-2}). Ladder 100b.

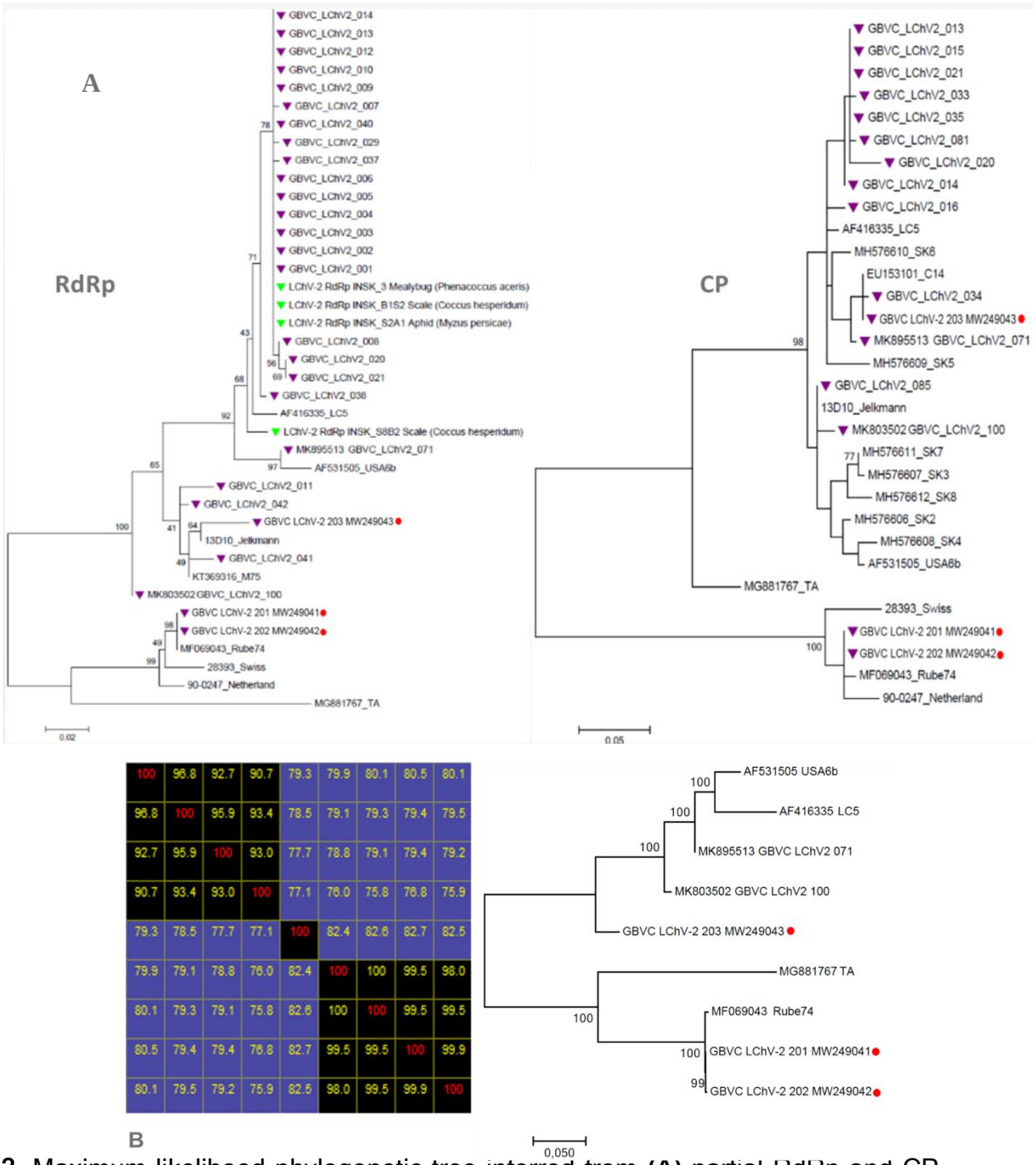


Fig. 3. Maximum likelihood phylogenetic tree inferred from **(A)** partial RdRp and CP (purple triangles) and **(B)** full genome nucleotide sequences of the LChV-2 Belgian (red points) and LChV-2 isolates from GenBank with similarity matrix (nt). The GenBank accessions are indicated together with the isolate name, host plant and cultivar. Phylogenomic analysis (MEGA 7.0) included most of the available LChV-2 sequences. Branch lengths on the phylogenetic tree represent the genetic distance, the numbers at the branches represent the percentage of replicates in which the topology of the branch was observed after 1000 bootstrap replicates (only values >70% are shown).

1010 **Table 2.** List of LChV-2 isolates and host plants used for LChV-2 LAMP detection and optimization, and non-target *Prunus* associated
1011 viruses and cellular organisms using the LAMP and validated with diagnostic RT-PCR.
1012

Viruses (Family) and Organisms	Host	Geographical Origin	Year of Isolation	Reference ID	LChV-2 CP RT-LAMP	LChV-2 RdRp RT-PCR
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	*P. 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	*P. 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**	Belgium	2018	GBVC_LChV-2 S2A1	Positive	Positive
LChV-2 INSK_B1S2	*Coccus hesperidum***	Belgium	2018	GBVC_LChV-2 S8B2	Positive	Positive
LChV-2 INSK_S8B2	*Coccus hesperidum***	Belgium	2018	GBVC_LChV-2 S8B2	Positive	Positive
LChV-2 INSK_3	*Phenacoccus aceris***	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*	Zwitserland		CHE28393	Positive	Positive
LChV-2 (12) Kyoto5	*P. 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)	*P. 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 leafrol 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)	*Vitis 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

Reference material and collections. GBVC and GBBC - ILVO Virus and Bacteria collections, respectively, Belgium. JKI - Julius Kühn Institute collection, Neustadt, Germany. CBS – Fungal collection. Centraalbureau voor Schimmelcultures, Utrecht, The Nederlands. DSMZ – Leibniz Institute - German Collection of Microorganisms and Cell Cultures. Hemiptera (Family **Aphididae, **Coccidae*).

Table 3. List of isolates used for LChV-2 LAMP detection from diverse infected host plants, and identified with RT-PCR and high-throughput sequencing (HTS) results.

Sample	Nbr of Trees	Origin	Host	Symptoms	Total (raw) Reads Number	LChV-2 RdRp RT-PCR	LChV-2 CP RT-LAMP	Presence* of other viruses
KnokkePlum (P2)	50	Belgium	*P. domestica*	Yellowing	18,521,058	Negative	Negative	LChV1 / CVA / CNRMV
MW249041	50	Belgium	*P. 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	*P. armeniaca*	Yellowing	7,071,696	Negative	Negative	LChV1/CVA

- Detection as described in Tahzima et al., 2019 [a,b]

Table 4. Nucleotide and amino acid identity percentages (%) between the LChV-2 isolates from *P. serrulata* (GenBank accession MW249041-43) from Belgium and all representative available LChV-2 full genome sequences (GenBank).

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*(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)

*nt (a.a).

Supplementary Material

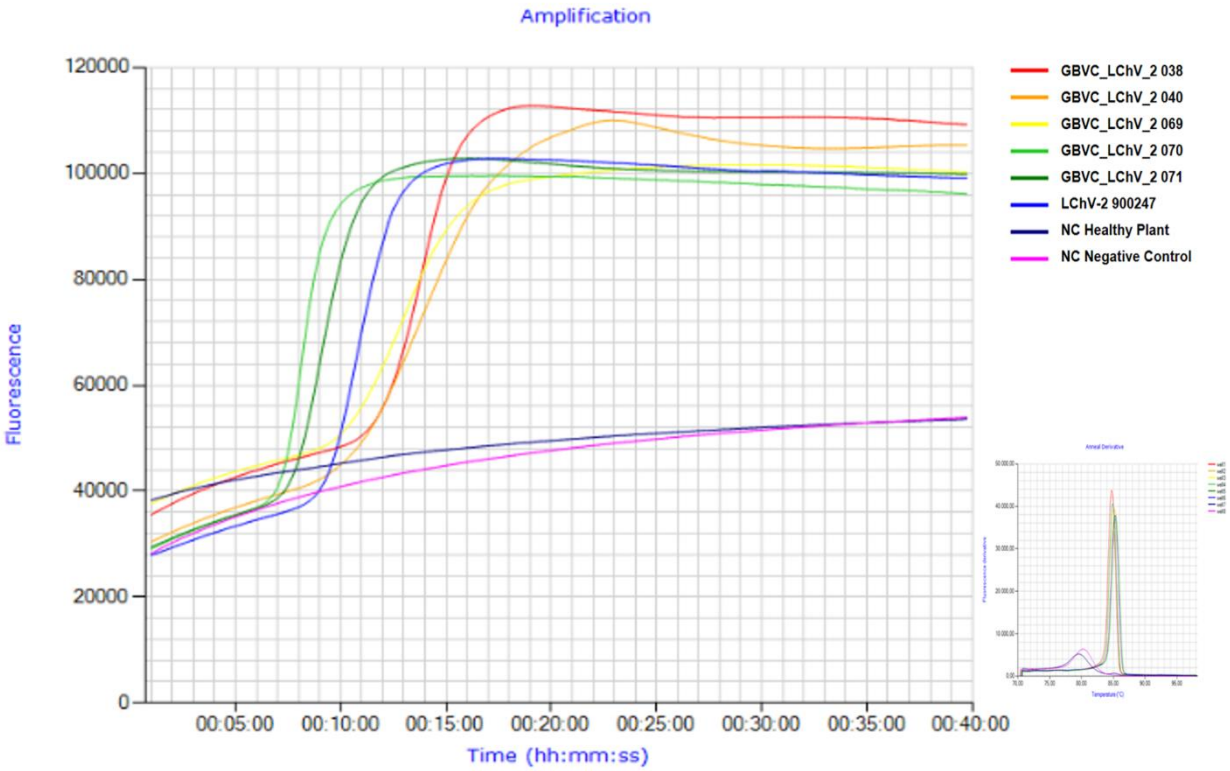


Fig. S1. LChV-2 Loop-mediated isothermal amplification of LChV-2 isolates RNA samples. NC = Healthy *P. avium* RNA extract and ddH_2O negative controls. Insert plot shows the annealing derivative with specific melting curves.

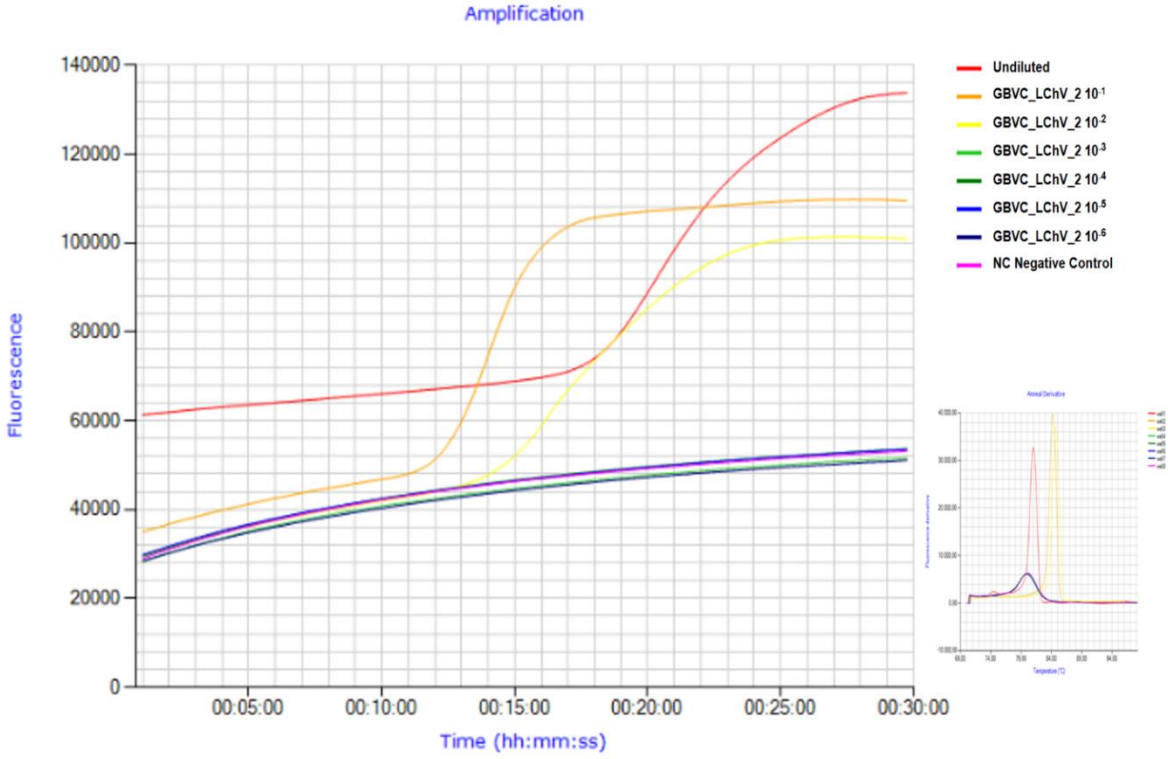


Fig. S2. Sensitivity – Amplification plot of LChV-2 loop-mediated isothermal amplification of undiluted and serially diluted LChV-2 (10^{-1} to 10^{-6}) from infected crude plant material. NC = ddH_2O negative control. Insert plot shows the annealing derivative with specific melting curves.

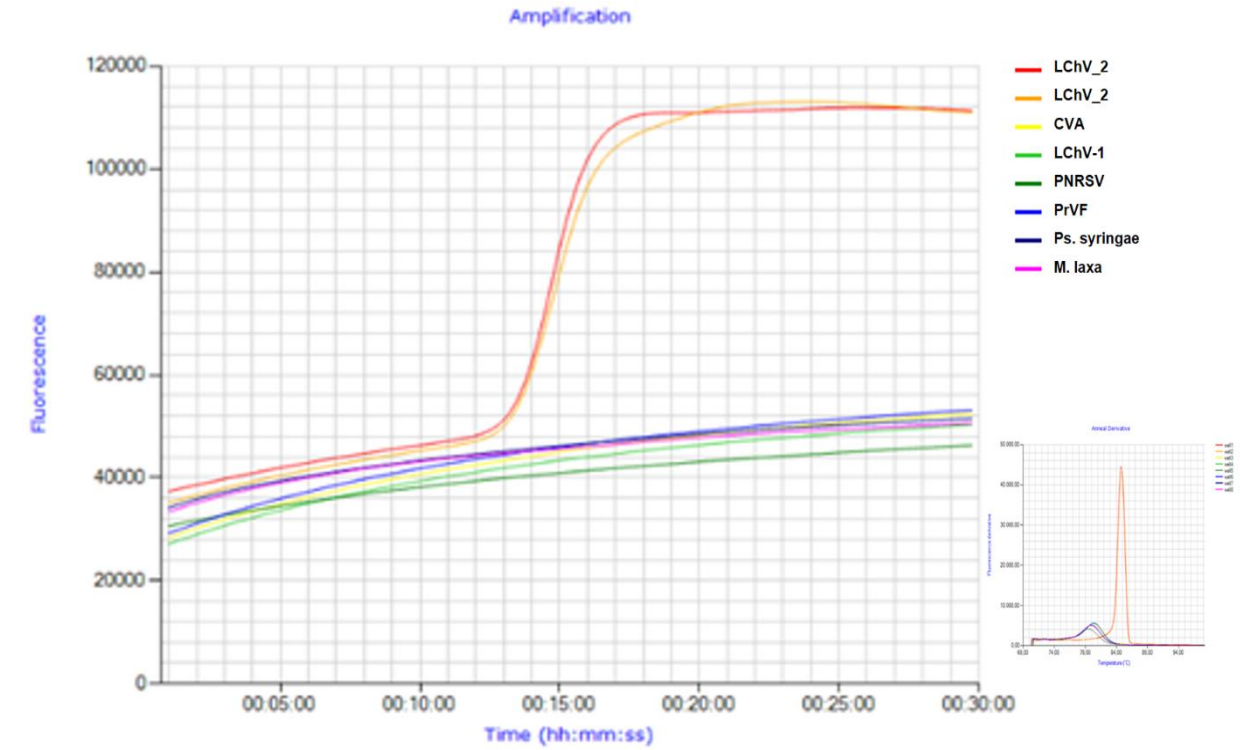


Fig. S3. Specificity – Results of LChV-2 loop-mediated isothermal amplification of LChV-2 and Non-LChV-2 *Prunus* associated organisms. Insert plot shows the annealing derivative with specific melting curves.

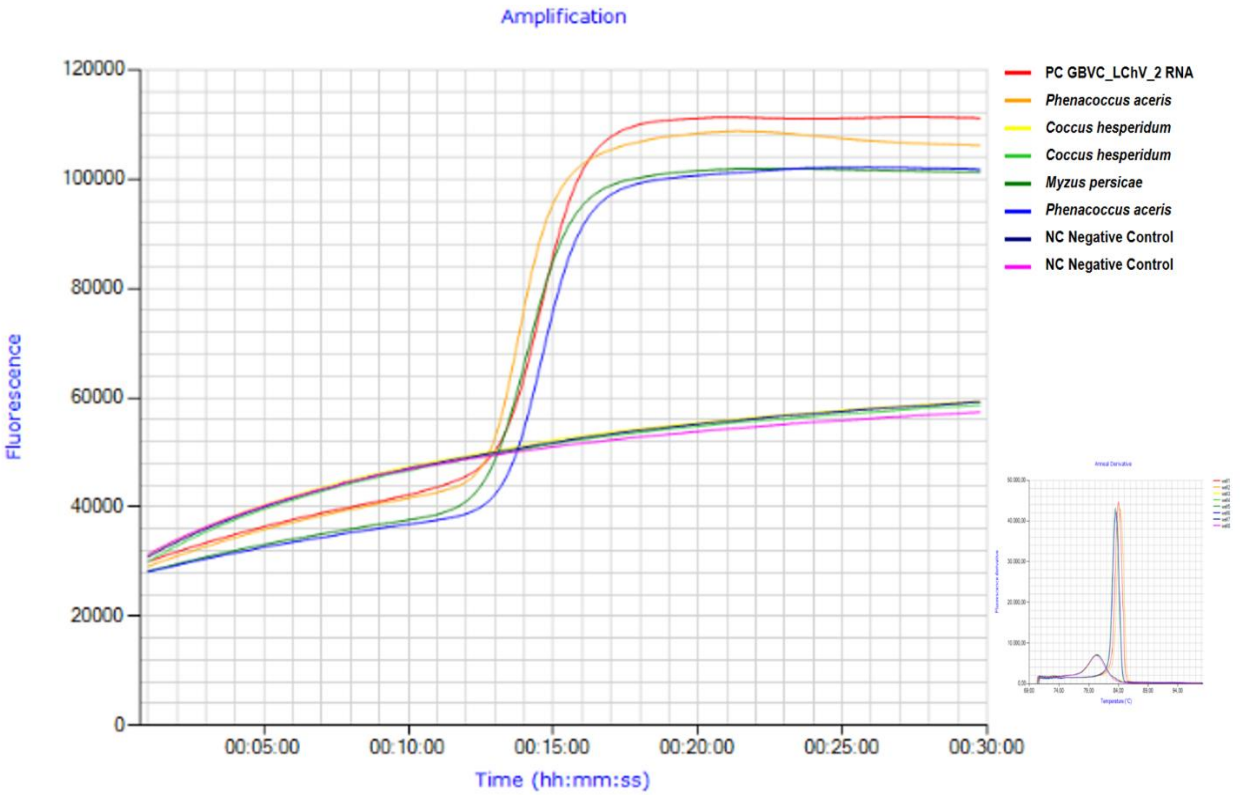
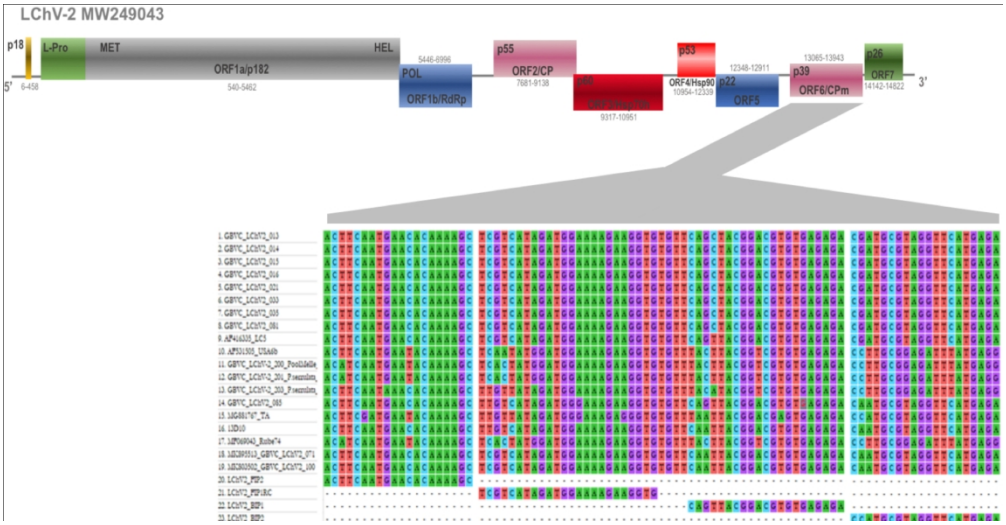


Fig. S4. LChV-2 loop-mediated isothermal amplification performed directly on known and potential insect vectors carrying. PC = LChV-2 Positive RNA control. NC = ddH_2O negative controls.



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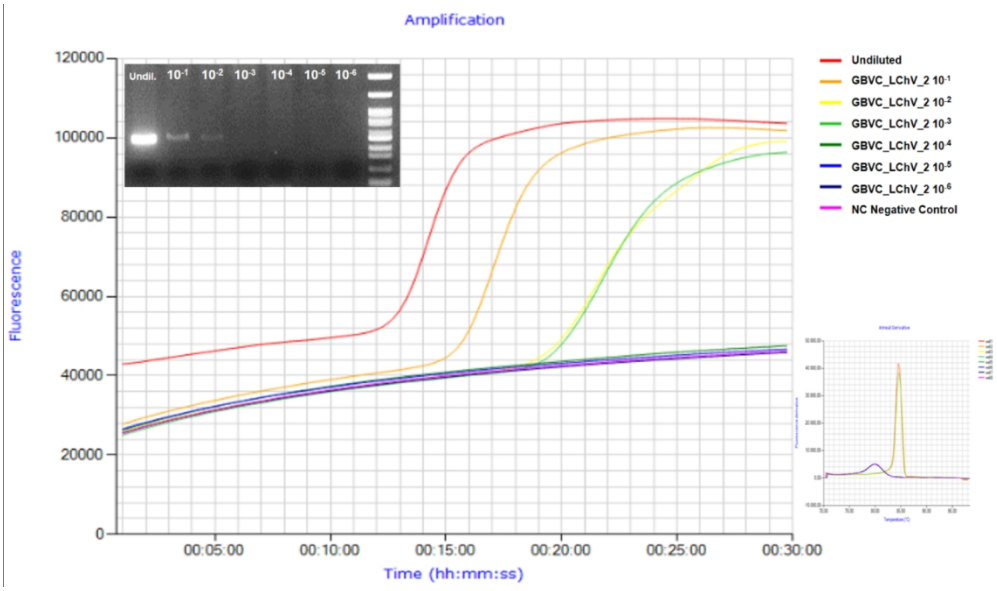

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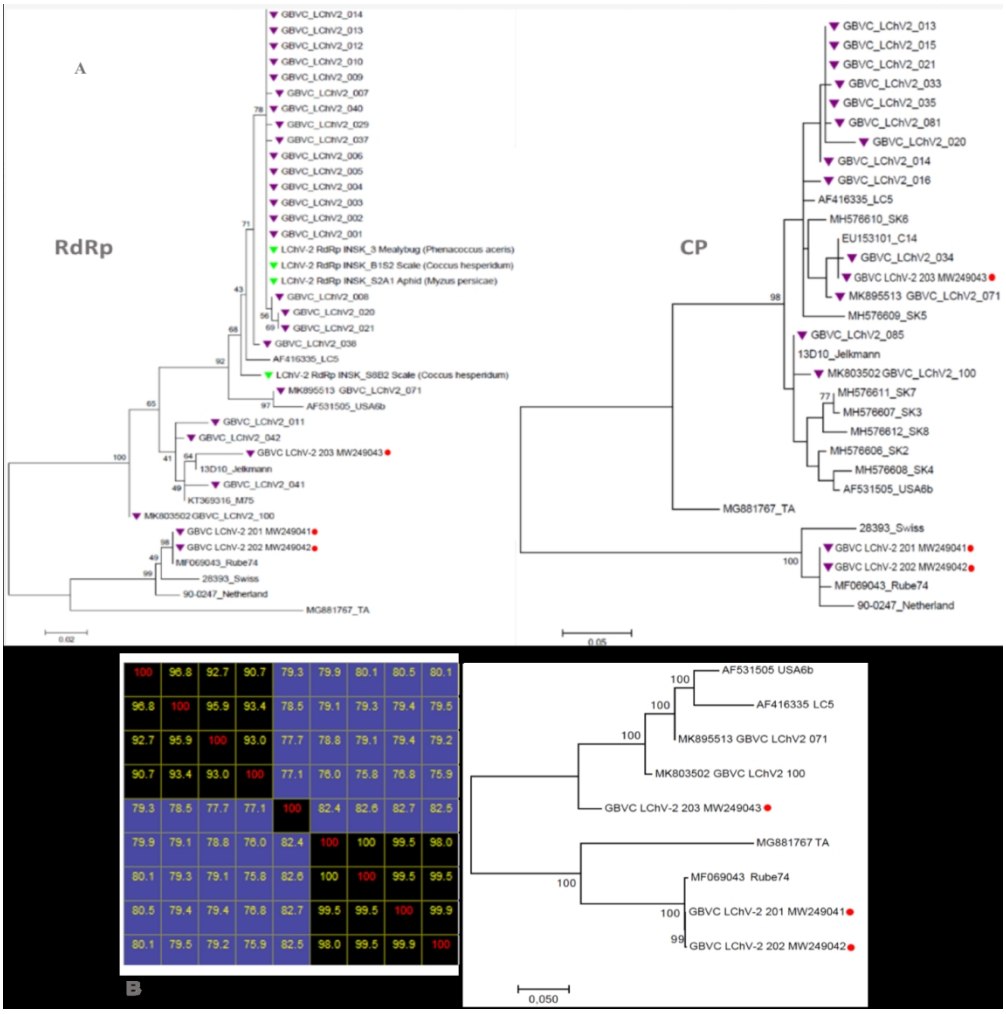

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An Advanced One-Step RT-LAMP for Rapid Detection of *Little cherry virus 2* Combined with HTS-based Phylogenomics Reveal Divergent Flowering Cherry Isolates

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Extra Supplementary Material

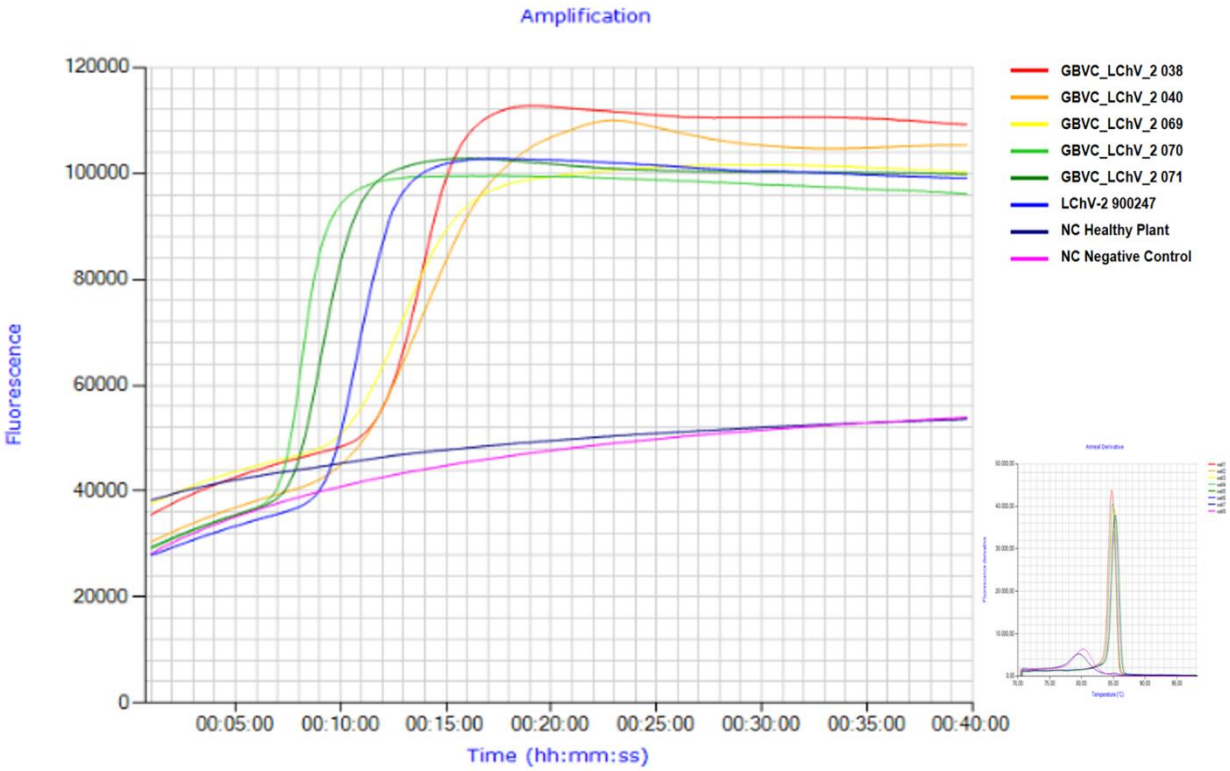

Fig. S1. LChV-2 Loop-mediated isothermal amplification of LChV-2 isolates RNA samples. NC = Healthy *P. avium* RNA extract and ddH_2O negative controls. Insert plot shows the annealing derivative with specific melting curves.

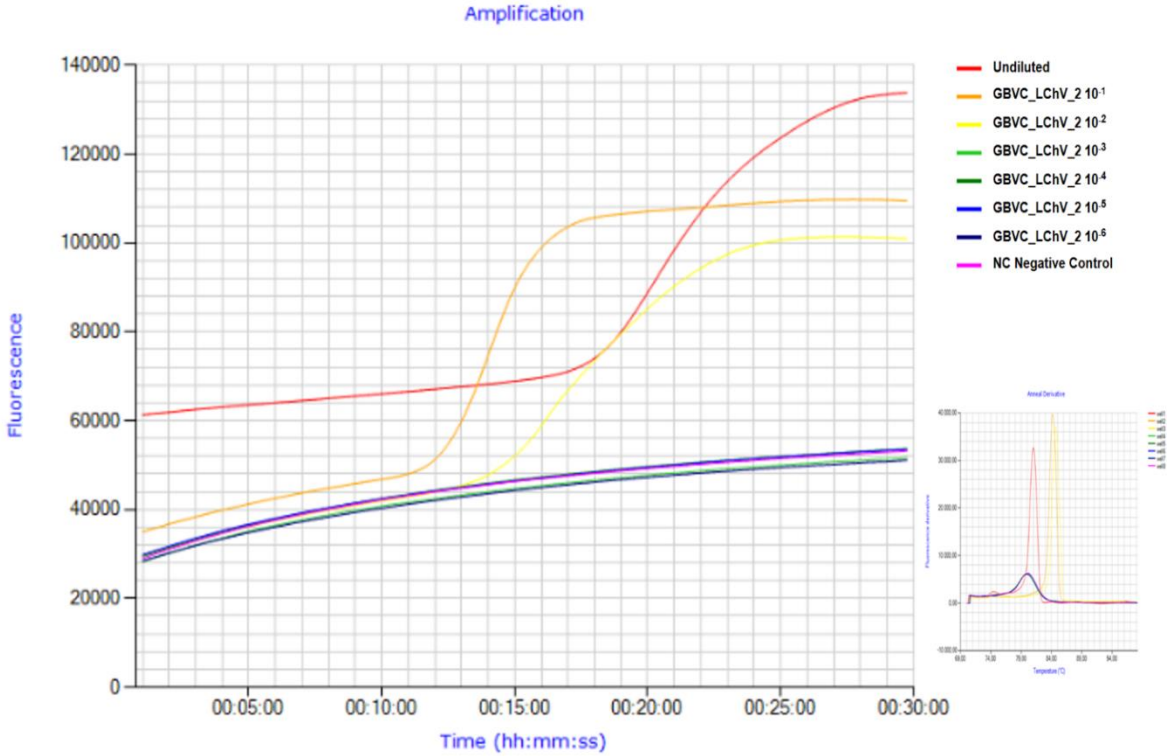

Fig. S2. Sensitivity – Amplification plot of LChV-2 loop-mediated isothermal amplification of undiluted and serially diluted LChV-2 (10^{-1} to 10^{-6}) from infected crude plant material. NC = ddH_2O negative control. Insert plot shows the annealing derivative with specific melting curves.

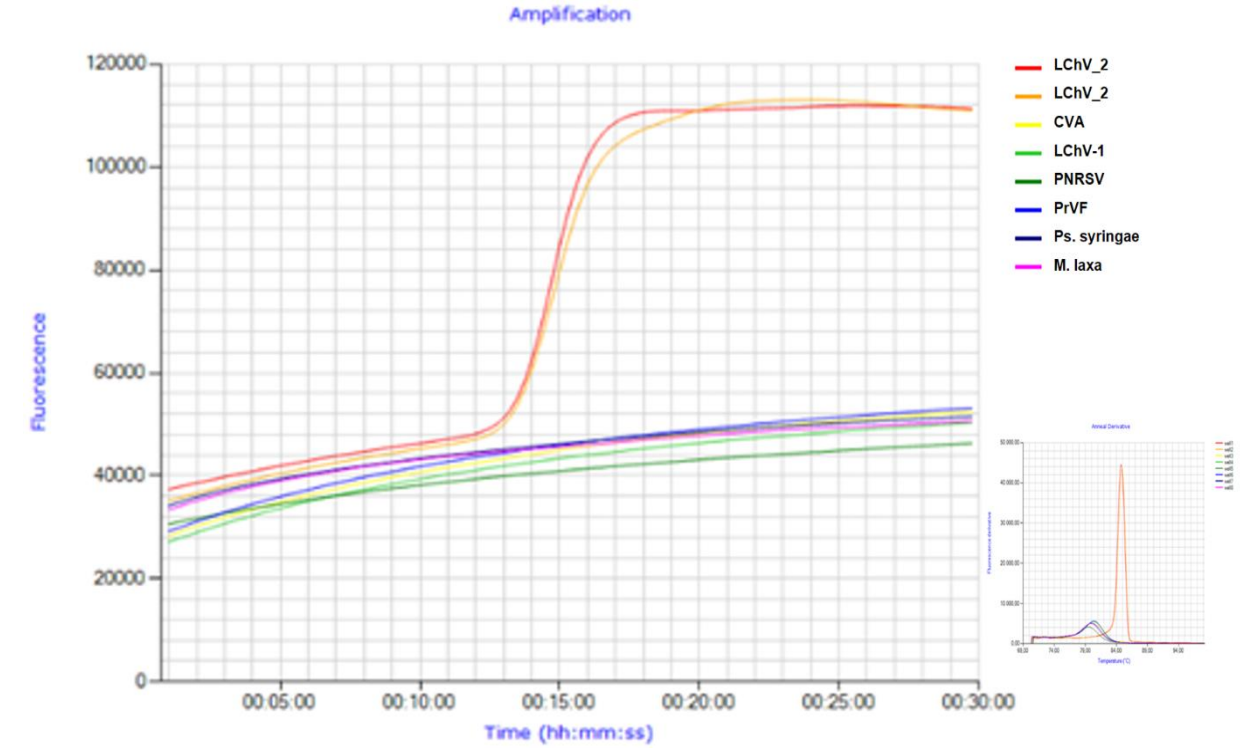

Fig. S3. Specificity – Results of LChV-2 loop-mediated isothermal amplification of LChV-2 and Non-LChV-2 *Prunus* associated organisms. Insert plot shows the annealing derivative with specific melting curves.

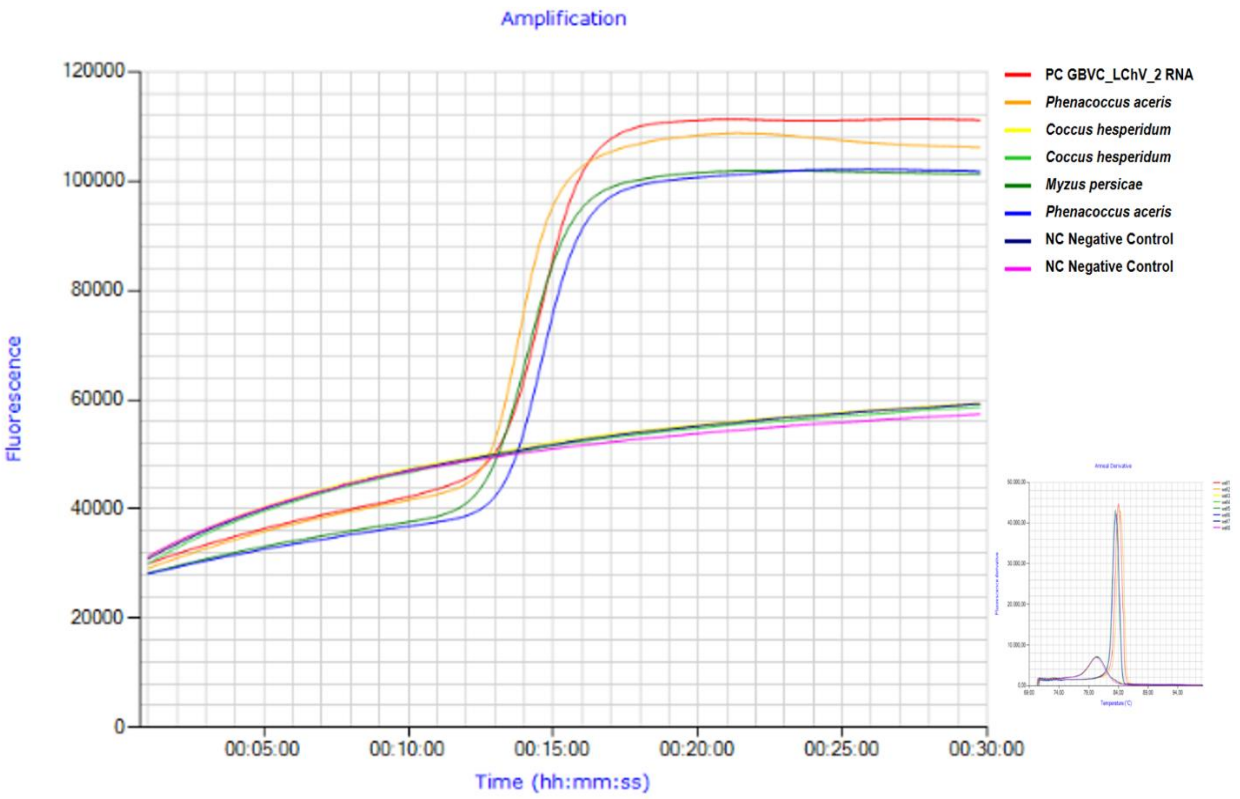

Fig. S4. LChV-2 loop-mediated isothermal amplification performed directly on known and potential insect vectors carrying. PC = LChV-2 Positive RNA control. NC = ddH_2O negative controls.