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ORIGINAL PAPER

Discovery and molecular characterization of a novel trichovirus infecting sweet cherry

Eric Brewer[1] · Mengji Cao[1,2] · Benjamin Gutierrez[3] · Margarita Bateman[4] · Ruhui Li[1]

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Abstract

Contigs with the highest sequence similarity (73%) to *Apricot pseudo-chlorotic leaf spot virus* (genus *Trichovirus*, family *Betaflexiviridae*) were identified by high-throughput sequencing from a symptomless sweet cherry accession. The complete genome sequence of this new virus is 7460 nucleotides, excluding the 3′ poly(A) tail. Its genome organization is very similar to several trichoviruses infecting fruit trees, with three open reading frames encoding putative replicase, movement protein and coat protein (CP). The virus shares amino acid sequence identities of 60–73% at replicase and 53–76% at CP with other trichoviruses. Phylogenetic analyses group it and other *trichoviruses* in a cluster. These results support that this virus, which is tentatively named cherry latent virus 1, should be considered a new member in the genus *Trichovirus*.

Keywords High-throughput sequencing · Cherry latent virus 1 · Genome sequence · *Trichovirus* · *Betaflexiviridae*

Introduction

Sweet cherry (*Prunus avium* L.) is an economically important fruit crop worldwide. It is one of the most popular fresh fruit in many temperate countries since it has both desirable culinary attributes and high levels of bioactive compounds with antioxidant characteristics [1].

Many viruses infect sweet cherry [2]. Although most infections are latent, some, especially the mix infections, cause diseases that reduce yield and market value of the crop. Genus *Trichovirus* in the family *Betaflexiviridae* is a group of plant viruses consisting of a single stranded RNA genome of 5.9–9.5 kb encapsidated in flexuous filamentous particles of 640–890 × 10–12 nm [3]. *Apple chlorotic leaf spot virus* (ACLSV) and *Cherry mottle leaf virus* (CMLV) are two trichoviruses that have been reported to infect sweet cherry and cause diseases [4, 5]. Both viruses have filamentous morphology and are mechanically transmitted. However, the ACLSV genome contains only three open reading frames (ORF) and has no known insect vector [4], while CMLV encodes four putative proteins and is transmitted by eriophyid mites (*Eriophyes inaequalis*) [5].

In this study, a new member of the genus *Trichovirus* was identified from a sweet cherry accession. The complete genome of the virus was determined, and its relationship with other trichoviruses was analyzed.

Materials and methods

Virus source, bioassay and detection of known viruses

Two accessions of *Prunus* spp. that were imported to the U.S. from the Republic of Georgia were used in a high-throughput sequencing (HTS) evaluation study. One accession was cherry cultivar ‘Mskhvil Nakota’ and another was

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✉ Ruhui Li
ruhui.li@usda.gov

[1] USDA-ARS, National Germplasm Resources Laboratory, Beltsville, MD 20705, USA

[2] National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, Chongqing 400712, China

[3] USDA-ARS, Plant Genetic Resources Unit, Geneva, NY 14456, USA

[4] Plant Protection and Quarantine, USDA-APHIS, Miami, FL 33122, USA

apricot cultivar 'Farbel'. The two accessions were established by grafting scion buds onto peach rootstock. They were also grafted onto following three seedlings of each of eight indicator plants for symptom expression in the greenhouse: *P. armeniaca* 'Tilton', *P. dulcis* 'Peerless', *P. persica* GF305, *P. salicina* Shiro, *P. serrulata* 'Kwanzan' and *P. avium* 'Bing', 'Sam' and 'Canindex'. The original plants were also tested by ELISA and RT-PCR/PCR using pathogen-specific or genus-specific primers for the presence of known viruses, viroids and phytoplasma (Supplementary Table 1).

RNA isolation, high-throughput sequencing and sequence analyses

Total RNAs were extracted by RNAeasy Plant Mini Kit (Qiagen) from leaves of both accessions and pooled into one sample, P3. The sample was subjected to HTS using Illumina NexSeq 500 system (SeqMatic). Analyses of total paired-end reads were performed using CLC Genomics Workbench 9.5.2 (Qiagen). The raw reads were filtered to remove failed reads, and qualified reads were assembled de novo into contigs with a cut-off of 200 nucleotides (nt). Contigs were annotated by Blastx comparisons to local Viruses_NR and viroid databases downloaded from NCBI Genbank and a phytoplasma database provided by Yan Zhao of USDA-ARS.

Virus genome amplification, cloning and sequencing

To obtain the genomic sequence of new virus identified by HTS, RT-PCR was conducted by SuperScript ™ III One-Step RT-PCR System (Thermo Fisher Scientific) using specific primers designed based on the contig sequences (Table 1). The 5′-end sequence was obtained using a 5′ RACE System Kit (Thermo Fisher Scientific), and the

Table 1 Primers used to amplify genome of cherry latent virus 1

Primer name	Sequence (5′–3′)	Position[a] (nt)	Amplicon size (bp)
P3CLV1-5endR2	TCTAATGCTAGAGCCTCCTAACC	496–474	496 by 5′ RACE
P3CLV1-5endR1	CTAGAAGGAAAGCATCCATTTGC	656–634	656 by 5′ RACE
P3CLV1F1	TGAACAGACTCACACAGTCTC	177–197	1266
P3CLV1R1	GCTGACACACTTGAGTTCTCC	1443–1423	
P3CLV1F2	ACCCTATGGAAGCACCCTGGAG	1273–1295	1132
P3CLV1R2	TGTCTTTGTGCATTCCAAGTGTG	2405–2383	
P3CLV1F3	AGTCTCTTCCCCTTACACAACTC	2184–2207	1382
P3CLV1R3	TGGTAGACCTTGAGAGGACG	3566–3547	
P3CLV1F4	TCCTAGCTAATGACTGGAGTG	3378–3398	1332
P3CLV1R4	TTGGAGAGCTTGGTTTCCTC	4710–4691	
P3CLV1F5	TGAACCAAGCCATGAATCATGAG	4458–4480	1339
P3CLV1R5	TGAATCATGAGGTTGGAGG	5797–5779	
P3CLV1F6	ACTGAATTGGAGTGGCTGTCTG	5645–5666	1334
P3CLV1R6	TGATCATGCCCACCACATCC	6979–6960	
P3CLV1-3endF1	AGACAAGGAGGTTTGAGGAC	6664–6683	1000 with Oligo (dT)
P3CLV1DetF1[b]	GTCCTTCTTAAAGCGACAAGTACC	1643–1666	496
P3CLV1DetR1[b]	ACCAAACCATGCGTGCAGATACTC	2139–2116	
P3RobigoDetF1[b]	TGCTGTTCAAGAGTCAGGAAACC		696
P3RobigoDetR1[b]	AGACTTCCTGATCACCTTCTTTCC		
PDV1[b]	GGAAAACCTACTGCCCGTTC		540
PDV4[b]	ATCGAGTGTTGGAGGTACTGAGT		
HSVd-VP19[b]	TGGGGAATTCTCGAGTYGCCG		292
HSVd-VP20[b]	AGAGGAWTCCGCGGCAGAGG		

[a]The position of each primer on the genome of cherry latent virus 1 (CLV-1)

[b]The primers used for the detection of CLV-1 (P3CLV1Det), new robigovirus (P3RobigoDet), prune dwarf virus (PDV) and hop stunt viroid (HSVd), respectively

3′-terminus was amplified by a virus-specific forward and oligo-dT primers. Amplicons were isolated and cloned into pGEM-T Easy Vector (Promega), and plasmid DNAs isolated from overnight cultures of selected colonies were sequenced by MCLAB.

Sequence and phylogenetic analyses

Sequence analyses were performed using CLC Genomics Workbench. The conserved domains were identified by Motif Scan at https://myhits.isb-sib.ch/cgi-bin/motif_scan. Phylogenetic analyses were performed by MEGA7.1 [6]. Recombination analysis was performed using RDP4.95 [7].

Survey of the virus in cherry germplasm

To detect for the new virus in additional cherry, total nucleic acids were extracted from 175 samples, 56 flowering cherries from the U.S. National Arboretum, Washington, D.C., 108 sour cherries from the USDA Plant Genetic Resources Unit in Geneva, NY and 11 sweet cherries from Maryland, were tested for CLV-1 by a CTAB method [8] and tested by RT-PCR using virus-specific primers designed according to the sequence obtained in this study (Table 1).

Results and discussion

Reactions of indicator plants and results of laboratory detection

Neither the original plant nor the indicator plants showed any symptoms in the screen house and green house conditions. Prune dwarf virus (PDV, genus *Ilarvirus,* family *Bromoviridae*) and hop stunt viroid (HSVd, genus *Hostuviroid*, family *Pospiviroidae*) were only detected by quarantine indexing in the sweet cherry accession.

Virus identification by high-throughput sequencing

A total of 36,327,573 RNA reads were obtained from the P3 sample. De novo assembly of the RNA reads generated 67,988 contigs (> 200 nt). A Blastx search of the contigs against the Virus_NR database revealed the following: three contigs (3265, 2506 and 1935 nt) with the highest amino acid (aa) sequence identities (95–97%) to the gene products of PDV; two contigs (2644 and 4799 nt) with the highest aa sequence identities (76–80%) to the gene products of *Apricot pseudo-chlorotic leaf spot virus* (APCLSV, genus *Trichovirus,* family *Betaflexiviridae*); a large contig (8349 nt) with high aa sequence identities (53–54%) to replicase of the four cherry robigoviruses (geuns *Robigovirus*, family *Betaflexiviridae*) [9]; and a short contig (292 nt) with the highest nt similarity (84%) to HSVd. The identities of the two betaflexiviruses identified in the P3 sample were less than the species threshold of 80% aa identity at replicase and coat protein (CP) when compared to other betaflexiviruses [3], suggesting that they might be new members of the family. No phytoplasma was identified in the sample.

RT-PCR detection of viruses and viroid

The presence of the viruses and viroid in each accession was determined by RT-PCR using pathogen-specific primers (Table 1). PDV, two novel viruses and HSVd identified by HTS in the P3 sample were detected only in the cherry accession (Supplementary Fig. 1). None of the viruses and HSVd was detected in apricot accession. In this study, the new trichovirus named cherry latent virus 1 (CLV-1) was characterized.

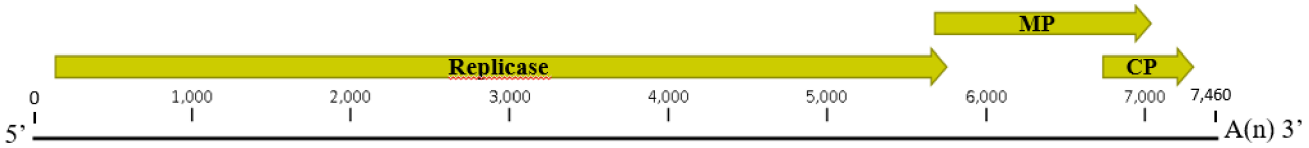
Analyses of genomic sequence

The complete genomic sequence of CLV-1 is 7460 nt (GenBank # MK770441), excluding the 3′ poly(A) tail. Its genome consists of three overlapping open reading frames (ORF) organized in the same arrangement as those of APCLSV, ACLSV, peach chlorotic leaf spot virus (PCLSV), grapevine berry inner necrosis virus and grapevine Pinot gris virus of the genus *Trichovirus* (Fig. 1a) [4, 5, 10–13]. ORF1 (nt 140-5783) encodes a putative replicase of 1872 aa (215 kDa) which contains viral methyltransferase (PF01660, aa 43–336), 2OG-Fe(II) oxygenase (PF13532, aa 723–826), peptidase_C34 (PF05413, aa 831–922), viral RNA helicase (PF01443, aa 1046–1294) and RNA-dependent RNA polymerase (PF009780, aa 1460–1774) domains. ORF2 (nt 5673–7043) codes for a putative movement protein of 456 aa (51 kDa), which might also act as a suppressor of RNA silencing [14]. ORF3 (nt 6730–7308) encodes a putative coat protein (CP) of 192 aa (21 kDa) that is crucial for infectivity [15].

Phylogenetic and recombination analyses

Comparisons of genomic and individual protein sequences among selected members of *Betaflexiviridae* confirm that CLV-1 is most closely related to APCLSV, ACLSV and PCLSV (Fig. 1b). The sequence identities between CLV-1

A



B

Species[1]	GenBank Accession No.	Genome (nt[2])	Replicase (aa[3])	Movement Protein (aa)	Coat Protein (aa)
APCLSV	NC_006946	68.6	73.5	58.4	75.5
ACLSV	NC_001409	64.4	67.6	57.4	72.4
PCLSV	MH084695	65.0	67.7	56.6	69.3
CMLV	NC_002500	60.9	62.6	47.7	51.6
PcMV	NC_011552	60.9	60.7	49.6	53.2
GBINV	NC_015220	46.6	39.1	33.5	32.8
GPGV	NC_015782	47.1	40.1	32.6	36.5

[1] APCLSV, apricot pseudo-chlorotic leaf spot virus; ACLSV, apple chlorotic leaf spot virus; PCLSV, peach chlorotic leaf spot virus; CMLV, cherry mottle leaf virus; PcMV, peach mosaic virus; GBINV, grapevine berry inner necrosis virus; GPGV, grapevine Pinot gris virus. [2] nt, nucleotide. [3] aa, amino acid.

Fig. 1 Schematic genomic organization of cherry latent virus 1 (CLV-1) (A) and percentage identities of the genomic and putative gene product sequences between CLV-1 and other members of the genus *Trichovirus* in the family *Betafexiviridae* (BMP—movement protein; CP—coat protein)

and other trichoviruses are 46.6–68.6% at the whole genome sequence level, 47.0–54.6% at the replicase aa sequence level and 32.8–75.5% at the CP aa sequence level. These values are below the species demarcation criteria (< 72% genome sequence identity or < 80% aa sequence identity of replicase or CP), indicating that CLV-1 is a novel virus [3]. Phylogenetic analyses showed that CLV-1 and five other trichoviruses infecting *Prunus* spp. form a clade distinct from two grapevine trichoviruses and members of the other genera in the *Betaflexiviridae* family (Fig. 2). Topologies of phylogenetic trees changed slightly when the aa sequences of the putative proteins were analyzed, but a close relationship of these trichoviruses was retained (data not shown).

Recombination analysis conducted using the complete genomic sequences of CLV-1 and 107 other betaflexiviruses revealed that CLV-1 was a recombinant of two carlaviruses, Sambucus virus E (major parent) and Helleborus net necrosis virus (minor virus), at the region of nt 1438–1883. The recombination event was detected by GENEGONV (5.783×10^{-6}), MaxChi (1.516×10^{-5}) and Chimaera (5.776×10^{-3}) of the RDP4 package. This event was also detected in the similar region of the APCLSV genome, supporting that the two viruses are most closely related.

Graft transmission and survey results

CLV-1 was detected by RT-PCR using primers P3CLV-1DetF1 and P3CLV1DetR1 (Table 1) in three 'Kwanzan' cherry trees inoculated with infected cherry material (Fig. 3), indicating the virus is graft transmissible. The virus was not detected in any of the 175 samples, indicating its infection is not common. The genomic sequence obtained in this study contributes to the taxonomy of the genus *Trichovirus*, and the characterization and detection of CLV-1 alerts Prunus quarantine and clean stock programs about a new virus.

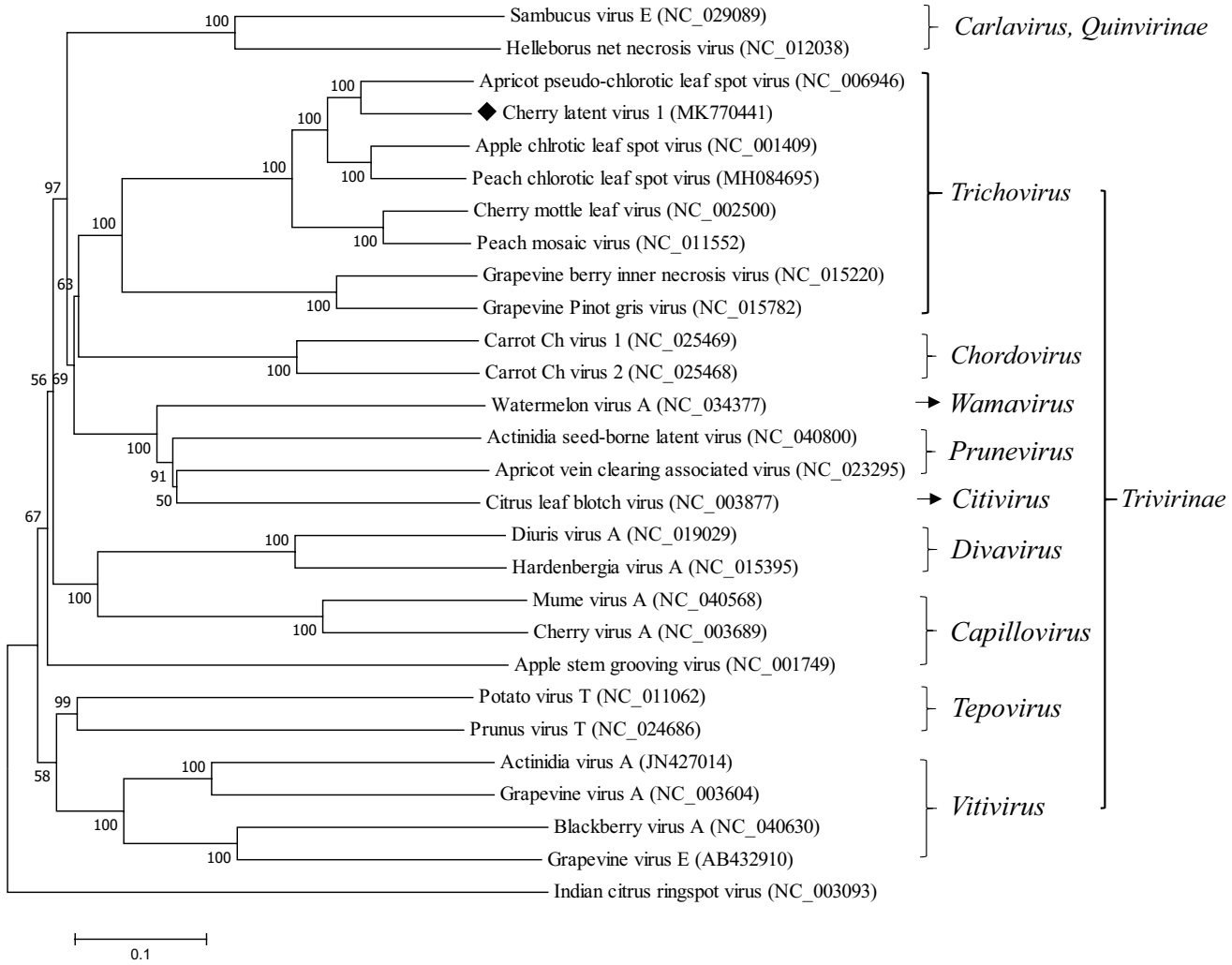


Fig. 2 Maximum likelihood tree based on replicase amino acid sequences of cherry latent virus 1, members of the genus *Trichovirus* and selected members of the family *Betafexiviridae*. Bootstrap analysis was applied using 1000 replicates. The diamond indicates the virus characterized in this study. Scale represents genetic distance. Indian citrus ringspot virus, a member of the family *Alphaflexiviridae,* was used as an outgroup

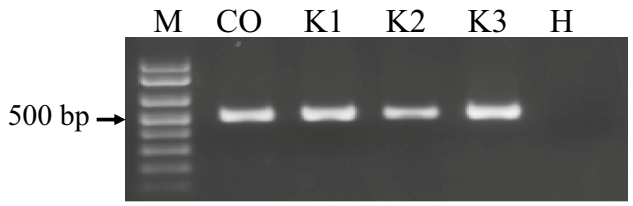


Fig. 3 Detection of cherry latent virus 1 by RT-PCR in cherry trees grafted with an infected cherry accession from Republic of Georgia. Lanes M 1 kb plus DNA ladder, CO original cherry accession; K1–K3) cv. 'Kwanzan' trees grafted with the infected cherry. H uninoculated 'Kwanzan'. Arrow indicates the size of the DNA marker

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Author contributions This work was designed by RL and MC; performed by EB, RL, MC, MB and BG; and written by RL and EB.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any research involving human or animal participants.

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