Association of *Little cherry virus 1* (LChV1) with the Shirofugen Stunt Disease and Characterization of the Genome of a Divergent LChV1 Isolate

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ABSTRACT

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Double-stranded RNAs purified from the V2356 ('Successa') sour cherry source of the Shirofugen stunt disease (SSD) were sequenced using a 454 pyrosequencing multiplex approach. The 15,646 reads obtained were assembled into 279 contigs, 5 of which, totaling almost 16.9 kbp and 5,332 reads (34% of sample reads), showed high Blast scores and homology to *Little cherry virus 1* (LChV1). The five contigs were further assembled manually into three supercontigs spanning the full LChV1 genome with only two small gaps (17 and 55 bases). Completion of the sequencing of the viral genome was performed using targeted polymerase chain reaction and primers designed from the contigs. No

evidence for the presence of other viral agents in the V2356 source could be obtained in the remaining contigs or singletons. The V2356 LChV1 isolate is only ≈76% identical with the reference complete LChV1 sequences and, in particular, with the ITMAR isolate associated with the Kwanzan stunting syndrome. However, it is highly homologous (97 to 100% identity) in two short genome regions with divergent LChV1 from North America, providing the first complete sequence for such divergent isolates. Although not providing a definite proof, the failure to detect any other viral agent in the V2356 SSD source and the identification of LChV1 in a second, independent, source of the disease suggests that LChV1 isolates could be responsible for the SSD syndrome.

Additional keywords: *Closteroviridae*, deep sequencing, double-stranded RNA.

Despite extensive efforts by plant virologists worldwide, there are still many plant diseases or syndromes thought to be caused by viral agents for which the virus or viruses responsible have not yet been identified or described. This is particularly the case for vegetatively propagated crops such as stone or pome fruits (10,21). The difficulties encountered when trying to identify a viral causal agent stem, in large part, from the extreme diversity of the virus world (24,32). For woody crops, this complexity is compounded by the technical problems and constraints imposed by such hosts (e.g., the low and uneven viral concentrations or the presence of interfering substances such as tanins and polyphenols which greatly complicate or altogether prohibit viral purification or inoculation). Although the development of polyvalent polymerase chain reaction (PCR) assays (8,11) has provided a partial solution to these difficulties, testing for the presence of any virus in a plant sample still requires the availability and use of a broad range of polyvalent PCR assays.

Recently, the development of novel sequencing technologies (next-generation sequencing [NGS]) has drastically changed our

ability to generate thousands to millions of sequences from a given plant sample and allowed the development of new virus indexing strategies based on the nontargeted generation of sequences followed by bioinformatic analyses of the vast amount of data and the identification of viral sequences. These approaches have proven their utility for the rapid characterization of known or novel viral agents (1–3,5,16,27,33,34) or for more general, metagenomic or ecogenomic approaches (25).

In these approaches, a variety of nucleic acids extracted from the plant to be tested have been used, including single-stranded messenger RNAs (1–3,33,34), small interfering RNAs (14), double-stranded (ds)RNAs (1,2,5,25), and nucleic acids obtained from semipurified viral particle preparations (20). Among those, the analysis of dsRNAs presents the advantage of analyzing nucleic acids preparations enriched in viral sequences and, therefore, potentially requiring a sequencing effort of a lower scale to identify a viral agent with an RNA genome (1,2).

Shirofugen stunt is a syndrome of flowering cherry (*Prunus serrulata*) 'Shirofugen' indicators grafted with some sweet (*P. avium*) or sour (*P. cerasus*) cherry sources (6,15). It is characterized by strong rosetting with dwarfed and deformed leaves, reduced vigor, gradual necrosis at the union, and sometimes die-off of the indicator after a few vegetative cycles. The graft transmissibility of the disease and the type of symptoms strongly suggest a viral etiology but the causal agent has never been characterized. Symptoms on typical cherry indicator hosts are inconsistent. They are reported either as completely lacking (15) or consisting of atypical thin spotting or netting on older leaves that develops long after inoculation in 'F12-1', 'Bing', or 'Sam' sweet cherry indicators (6). The symptoms in the Shirofugen flowering

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The sequences reported in the present manuscript have been deposited in the GenBank database under the JX669615 (V2356) and JX961599 (Roma7) accession numbers.

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains one supplemental table.

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cherry indicator are reminiscent of the recently described “Kwanzan stunting” syndrome observed by Matic et al. (19) in the Kwanzan flowering cherry indicator. However, an early report suggests that the Shirofugen stunt agent causes only symptomless infection in *P. serrulata* ‘Kwanzan’ (15).

The Kwanzan stunting syndrome has been tentatively associated with isolates of *Little cherry virus 1* (LChV1), a tentative member of the *Closteroviridae* family (18), because two sources containing the disease agent were positive for LChV1 but tested negative in serological or molecular PCR assays for a range of other known agents infecting cherry (12). LChV1 is one of the two *Closteroviridae* members associated with the little cherry disease, the other being named *Little cherry virus 2* (LChV2) (7,12,26). The disease is mostly associated with sweet (*P. avium*) and sour (*P. cerasus*) cherry but a range of ornamental cherry species, including the oriental flowering cherry (*P. serrulata*) have been identified as symptomless hosts (12,22,23,31), although it is unclear whether the earlier reports refer to LChV1 or LChV2. Remarkably, in the case of the LChV1 associated with Kwanzan stunting, no symptoms typical of LChV1 infection were detected in either the original cherry hosts or inoculated indicators (19). Based on the similarity of symptoms with Kwanzan stunting, Matic et al. (19) suggested that the Shirofugen stunt syndrome could also be associated with LChV1 isolates.

In the present study, deep sequencing analysis of dsRNA molecules purified from a German sour cherry source of the Shirofugen stunt disease (SSD) was performed to try to identify the causal agent. Analysis of the sequences obtained allowed the identification of a single viral agent and the determination of its nearly complete genomic sequence. The virus identified is a LChV1 isolate which shows >23% nucleotide divergence from the ITMAR isolate associated with Kwanzan stunting described by Matic et al. (19).

MATERIALS AND METHODS

Virus isolates. The V2356 German sour cherry (*P. cerasus* L. ‘Successa’) source of the SSD (6) was maintained under greenhouse conditions by grafting on the cherry indicator *P. avium* ‘F12.1’. Another source of the SSD, the Roma7 sour cherry source from Italy, was also maintained on *P. avium* F12.1. LChV1 isolates 41/93 and 11/05 used as positive controls in some reverse-transcription (RT)-PCR experiments were kindly supplied by Dr. W. Jelkmann (Julius Kühn Institute, Dossenheim, Germany).

Total nucleic acid extraction and LChV1 detection by RT-PCR amplification. Total nucleic acid (TNA) extraction was performed according to the procedure 2 described by Foissac et al. (8). LChV1 was detected by a two-step RT-PCR. TNA samples (5 µl each) were submitted to RT using RNA-dependent RNA polymerase (RdRp)-R2i (Table 1) as reverse primer and the SuperscriptII Reverse Transcriptase according to the manufacturer’s recommendations (Invitrogen/Fisher Scientific, Illkirch, France). In a second step, 4 µl of cDNA was amplified in a final volume of 40 µl using 1 µM each primer (RdRp-F1i and RdRp-R2i) (Table 1) and 1 U of DyNAzymeII DNA polymerase (Finnzymes/Fisher Scientific). Fifty amplification cycles of 95°C for 30 s, 42°C for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C during 10 min, were then applied. Amplification products were analyzed by nondenaturing agarose gel electrophoresis.

dsRNA extraction, amplification, and pyrosequencing. dsRNAs were extracted from infected leaves and purified by CF11 cellulose batch chromatography following the method of Valverde et al. (30) modified by Gentit et al. (9). The purified dsRNAs were then submitted to a second round of CF11 purification and 3 µl of purified dsRNA was denatured during 5 min at 99°C and submitted to an RT initiated by a mixture of primers consisting of 1 mM dT₁₈ and 2 mM PcDNA₁₂ (5’ TGTGTTGGGTGTGTTTG

TABLE 1. Primers used in the present study

Primer name	Primer sequence (5’–3’) ^a	Temperature (°C)	Target ^b
LCh-F1	CTTCTGACTGTGACAGCCGTT	50	ORF1a (298–666)
LCh-R1	TGATGATATCAGCATCAGATGTC	...	...
LCh-F2	GCGTGCTCTTCAACGTCCAA	55	ORF1a (1,082–1,384)
LCh-R2	GAACCTCTGAGCATTGGGCTT	...	...
LCh-F3	GTTGGTTACATTCTTCAAGCT	49	ORF1a (1,797–2,129)
LCh-R3	CAAGTAAACGAAAATCATCA	...	...
LCh-F4	ACGATTGGACTACAAAGTTATTC	55	ORF1b (7,499–8,026)
LCh-R4	AGACACTCGCTAACATAGCCA	...	...
LCh-F5	AAGAATCTGCTAAGGGAGCGA	55	ORF4 (11,395–11,795)
LCh-R5	TACAGTGGGTGAAATCTGAA	...	...
LCh-F6	AGAGTGTTGACGAATATCA	49	ORF1b (6,970–7,318)
LCh-R6	CATTGAGTCTAGGAGCTGCA	...	...
LCh-F7	GGTATTAGTCCTGAAGTAGA	49	ORF3–ORF4 (10,128–10,577)
LCh-R7	AACATCACCACCACTGACTC	...	...
LCh-F8	ACTATTAAGTCTACTTTGAACC	49	ORF5 (12,465–12,817)
LCh-R8	GCAGATGTCCACTCTGCAG	...	...
LCh-F9	CGTATCTAGGTAGTTGGTAT	52	ORF6 (13,797–14,119)
LCh-R9	CAGGATGTCACTTCCAATTC	...	...
LCh-F10	GGTAGAGCTATTATGTTAGG	52	ORF6–ORF7 (15,107–15,612)
LCh-R10	AGTCATCGCAATATTGAATTAG	...	...
LCh-F11	GACTAACCATCACATCAATAATA	49	ORF6 (14,497–14,889)
LCh-R11	TGATACACTACTAAGTTTTC	...	...
LCh-F12	GACGAAAGCGGAAACCGAAAG	49	ORF4–ORF5 (11,867–12,169)
LCh-R12	CTCTTCTGTTGCATATTCCT	...	...
LCh-F13	GACAACGACACTAGCATCCA	50	ORF1b–ORF3 (8,470–8,763)
LCh-R13	CTTTGCTTGTTCGCCTAAC	...	...
LCh-F14	TTGTAGATGAATTGTTTGTG	49	ORF1a (6,301–6,537)
LCh-R14	ATAGTAAGTAACAGACATCAG	...	...
LCh-F15	GATGGTGTGGATATGTTGGCT	55	ORF8–3’ NCR (16,602–16,914)
LCh-R15	TATGACTAGAGAAGGTAAGCG	...	...
RdRp-F2i	ACTCTYCGDTTBGADTATTCIGAT	42	ORF1b (7,120–7,400)
RdRp-R2i	CGGCCAAACGRCCTTGGTTCCA	...	...

^a Y = C or T; R = A or G; D = A, G, or T; B = C, G or T; I = inosine.

^b Target gene and the position on the V2356 LChV1 genome are indicated; ORF = open reading frame; NCR = noncoding region.

GN₁₂ 3') using the SuperscriptII Reverse Transcriptase according to the manufacturer's instructions (Invitrogen). The dsRNAs were then submitted to a whole-genome amplification procedure, allowing at the same time their conversion to double-stranded cDNA and their tagging with multiplex identifier (MID) adaptors in order to allow their pooled sequencing with other samples. The 11-bp MID tags used can tolerate up to two sequencing errors and still allow reliable demultiplexing of samples. Complementary DNA were amplified using 2.5 µl of RT products in a 25-µl reaction containing 1× reaction buffer, 0.25 mM dNTPs, 1 µM primer (5' **ACGTACACACTTGTGTTGGGTGTGTTTGG** 3', MID Tag in bold italics), and 1 U of DyNAzym II DNA polymerase (Finnzymes). Amplification was performed as follows: 94°C for 1 min; 65°C for 0 s; 72°C for 45 s, with a slope of 5°C/s, followed by 40 cycles of 94°C for 0 s; 45°C for 0 s; 72°C for 5 min, with the same slope; and final steps of 5 min at 72°C and 5 min at 37°C. After removal of primers using the MinElute PCR Purification Kit (Qiagen, Courtaboeuf, France), the concentration of the amplification products was determined spectrophotometrically and 300 ng of PCR products were pooled with the amplified material from 11 other unrelated samples (see below) to be analyzed using one-eighth of a Roche 454 GS FLX Titanium sequencer run.

Pyrosequencing data manipulation, assembly, and annotation. All analyses and manipulation of 454 pyrosequencing data were performed using CLC Genomics Workbench 4.8 (<http://www.clcbio.com>). Sequencing reads were first demultiplexed in order to assign individual reads to the relevant sample. The adaptors containing the multiplexing tags were removed from the reads, which were then screened and trimmed on quality and length bases. Reads were finally assembled in contigs and annotated by BlastN and BlastX comparison with GenBank, using a conservative 10⁻³ *e*-value cut-off.

Targeted polishing and confirmation of the V2356 LChV1 sequence. The gaps between the contigs generated from the pyrosequencing reads and targeted regions for which sequence confirmation was sought were amplified from V2356 TNA with primers designed from the contigs (Table 1) using the two-step RT-PCR protocols described by Marais et al. (17). All amplification products were confirmed by nondenaturing agarose gel electrophoresis before being sequenced on both strands (GATC Biotech, Mulhouse, France). The sequences obtained were finally assembled with the pyrosequencing contigs to generate the complete genomic sequence of the V2356 LChV1 isolate.

Sequence comparisons and phylogenetic analyses. Multiple alignments of nucleotide or amino acid sequences were performed using the ClustalX program (29) as implemented in Mega 5.0 (28). Pairwise strict nucleotide and amino acid distances were computed using Mega 5.0 and phylogenetic trees were reconstructed using the neighbor joining in Mega 5.0.

RESULTS

Pyrosequencing of dsRNAs purified from the V2356 Shirofugen stunt source. dsRNAs were purified from fresh leaves of

the V2356 cherry source of the SSD (6). Following RT and amplification as described in the Materials and Methods section, the resulting cDNAs were pooled with those deriving from 11 other unrelated samples and sequenced using one-eighth of a run on a Roche 454 GS FLX Titanium sequencer. In total, 15,646 high-quality sequences with an average length of 175 nucleotides (nt) were thus obtained and assembled de novo, generating a total of 279 contigs (average length 402 nt) and leaving 5,116 singleton sequences that could not be assembled. All contigs and singletons were then annotated by automated BlastN and BlastX searches against GenBank and using a conservative *e*-value cut-off of 10⁻³. The sole clear viral signal concerned five contigs which showed highly significant BlastN or BlastX scores against LChV1 (Supplemental Table 1). No evidence for other viral agents could be identified in the remaining contigs or singletons.

The contigs annotated as belonging to a LChV1 isolate were 0.3–5 kb and represented 34% of the reads obtained from the V2356 sample. Taken together, they represented a total length of 16,894 nt or 99.7% of the LChV1 genome length. Although variable, the depth of sequencing was generally high, the contig with the lowest coverage still having an average 13× depth. These contigs could be further manually assembled into three super-contigs spanning the full LChV1 genome, with only two small internal gaps of 17 and 55 nt, respectively, as judged by comparison with the reference UW2 LChV1 isolate (13). The assembled sequence started at position 3 of the 5' LChV1 genome, missing only the 2 nt of the 5' noncoding region (NCR), and extended to the last 3' nucleotide compared with the genome of the reference.

Completion and polishing of the sequence of the LChV1 isolate identified in the V2356 source was performed by direct sequencing of RT-PCR fragments amplified from total nucleic acids extracts of the V2356 source, using primers designed to span the gaps identified or to target regions for which sequence confirmation was sought (Table 1). The overall final coverage of the polished genome was 85× and, essentially, no differences were observed between the initial genome assembly and the regions resequenced using confirmatory PCR products.

Genome organization of the identified LChV1. The assembled and polished genome of the LChV1 isolate identified in the Shirofungen stunt disease V2356 source (GenBank JX669615) was 16,929 nt long; it missed the last two 5' nucleotides when compared with the UW2 and ITMAR isolates (13,19). The gene order and the general genome organization were fully conserved with those isolates (Fig. 1) and insertion-deletion (indel) polymorphisms were observed at a limited number of positions in the genome, mostly in the 5' and 3' NCR as well as in the internal NCR between open reading frame (ORF)4 and ORF5 (coat protein [CP] gene) and between ORF6 (CP gene duplicate [CPd]) and ORF7. However, a few indel polymorphisms were observed within the genome ORFs. A 1-nt insertion in the HSP70 gene resulted in a frameshift followed immediately by a termination codon, resulting in a predicted HSP70 protein 67 amino acids shorter (≈14% of the whole protein) than for the UW2 or ITMAR

Fig. 1. Genome organization of the V2356 *Little cherry virus 1* isolate and nucleotide and amino acid divergence levels with the UW2 reference isolate (Y10237). Percentages of nucleotide divergence are given above the genome representation, the amino acid ones below. RdRp = RNA-dependent RNA polymerase; ORF = open reading frame; CP = coat protein; CPd = CP gene duplicate.

isolates. The corresponding region has 10× coverage in the pyrosequencing data and was confirmed by resequencing of an independently obtained PCR product, so that there was strong evidence that this mutation was not a sequencing artifact. In addition, a 2-nt deletion was observed 124 nt downstream of this insertion, a position that is still within the HSP70 ORF for the ITMAR and UW2 isolates, which further confirms that the end of the HSP70 gene is not functional in the V2356 isolate.

A 1-nt deletion was also observed at the end of ORF6 (CPd), resulting in a frameshift changing 4 amino acids and a premature termination, resulting in a predicted CPd protein that was 2 amino acids shorter than for the two reference isolates.

Although not affected by an indel polymorphism, the P4 protein encoded by ORF2 was also shorter than in the reference isolates, as a consequence of a T to A point mutation creating a stop codon. As a consequence, the V2356 P4 protein was 5 amino acids shorter than its counterpart in the ITMAR and UW2 isolates. Both this mutation and the indel polymorphism truncating ORF6 had >10× coverage in pyrosequencing and were independently confirmed by sequencing of PCR products, again confirming the reality of these mutations directly affecting the size of the viral ORFs.

Comparison of the sequence of the V2356 isolate with those of other LChV1 isolates. Compared with the two fully sequenced LChV1 isolates, UW2 and ITMAR (13,19), the genome of the V2356 isolate showed an overall nucleotide divergence of 23.5 and 23.8%, respectively. By contrast, these two isolates were much more closely related and showed only 5.2% nucleotide divergence over their complete genomes.

A comparison of each genomic region with the UW2 isolate (Fig. 1) showed that the most conserved region was the 5' NCR, followed by the 3' NCR. The various ORFs were clearly less constrained and showed significantly higher divergence values. The most divergent ORF was ORF6 (CPd, ≈28% nucleotide divergence between V2356 and the other two isolates) while the most conserved ones were ORF1b, coding for the polymerase, and ORF7, with 17.2 to 19% and 18.2% divergence, respectively. The same situation applied when considering the sequence of the encoded proteins but P4 encoded by ORF2 was also highly divergent (29.0% amino acid divergence). Essentially, similar divergence values were observed between the V2356 isolate and isolate ITMAR.

Blast analysis of the V2356 isolate sequence revealed, on the other hand, that closely related LChV1 isolates had previously been partially characterized from North America (4). A phylogenetic tree reconstructed using the short, 240-nt-long sequence in the ORF1b gene analyzed by these authors is shown in Figure 2. The V2356 clustered with 100% bootstrap support in the cluster comprising the North American isolates and away from the isolates from Eurasia (UW2, ITMAR, and 98Fi10R2 from Turkey) (4). Within the North American cluster, it associated with 99% bootstrap support to a small subcluster composed of the WACH1a and WACH1b (sweet cherry, state of Washington), CAJS1 (sweet cherry 'Bing', California), and BCOS1 (sweet cherry 'Lambert', British Columbia, Canada). In that small region, the nucleotide sequence of V2356 was 0.5 to 1.9% divergent from these isolates, compared with 17.6 to 18.1% divergence with the isolates from Eurasia.

In comparisons performed in the other region analyzed by Bajet et al. (4), a 232-bp fragment spanning the end of ORF8 and the beginning of the 3' NCR provided the same picture, with V2356 being identical to the CAJS1 isolate in this small region (result not shown).

Analysis of another source of the SSD. In order to evaluate the association of LChV1 with the SSD, another, independent source of the disease was analyzed for the presence of LChV1. A degenerated primer pair allowing the amplification of the short RdRp region targeted by Bajet et al. (4) was designed (Table 1). A fragment with the expected size was amplified from the V2356 isolate and for the two LChV1 isolates used as positive controls. Interestingly, the primers allowed the amplification of a similar fragment from the Roma7 source of SSD. The sequence of the amplicon (GenBank JX961599) was determined and is included in the unrooted phylogenetic tree shown in Figure 2. The Roma7 isolate clusters with the Eurasian isolates (UW2, ITMAR, and 98Fi10R2 from Turkey) and not with the V2356 isolate, suggesting that the association between SSD and LChV1 is not limited to V2356 type isolates.

DISCUSSION

The sequencing strategy used, which involved 454 pyrosequencing of multiplexed samples, allowed rapid and efficient discovery and characterization of a divergent LChV1 isolate in the German V2356 (Successa) sour cherry source of SSD. The quasi-totality of the virus genome sequence (99.7%) could be determined with high confidence, owing to the average 85× depth of sequencing coverage. Regions of low coverage or gaps could readily be determined using targeted PCR-based resequencing. The quality of the initial NGS genome assembly was demonstrated by the fact that essentially no differences were observed between the resequenced regions (a total of ≈6 kb) and the initial assembly. Given the multiplexing approach used, the sequencing cost per sample was ≈US\$250, making this approach a more comprehensive and viable alternative to biological indexing or to the use of multiple serological or molecular assays.

The LChV1 isolate identified showed high divergence (23.5 to 23.8%) from the two previously fully sequenced LChV1 isolates (13,19). The currently accepted sequence-based species discrimination criterion in the *Closteroviridae* family is a divergence of >25% in the amino acid sequences of relevant viral proteins (polymerase, CP, and HSP70) (18). Although a much lower value was observed in the ORF1b-encoded polymerase (6.4 and 9.9% for isolates UW2 and ITMAR, respectively) (Fig. 1) divergence levels close to 17 to 18% were observed for the HSP70 and 23% for the viral CP, with values >30% (30.7 to 30.8%) for the CP duplicate. These values indicated that the V2356 isolate should be considered as belonging to the LChV1 species but that it represents a very distant isolate from the isolates that had been previously completely sequenced.

Fig. 2. Unrooted phylogenetic tree calculated from the sequence of a partial fragment (240 nucleotides) of the polymerase gene of *Little cherry virus 1* (LChV1) isolates. The accession number of the sequences retrieved from GenBank is indicated, together with the isolate name. The tree was constructed by the neighbor-joining method from strict nucleotide identity distances and the statistical significance of branches was evaluated by bootstrap analysis (1,000 replicates). Only bootstrap values >70% are indicated. The scale bar represents 5% nucleotide divergence. The V2356 isolate is indicated by a black diamond, and the Roma7 isolate by a black triangle.

On the other hand, based on the very limited sequence information available, the V2356 isolate appeared to be very close to some of the North American isolates sequenced by Bajet et al. (4). Therefore, the V2356 sequence is likely to represent the first complete genomic sequence available for a second group of divergent LChV1 isolates. It also indicates that the distinction made by Bajet et al. (4) between Eurasian and North American LChV1 isolates is probably not a valid one because V2356 was identified in European sour cherry material. Alternatively, movement of plant material between continents could have resulted in redistribution of virus isolates from different clades. The WACH1a and WACH1b isolates, which are very close to V2356, are reported to have been associated with typical, severe little cherry disease symptoms but there is no indication about the symptoms induced by these isolates in cherry or in flowering cherry indicators.

Short LChV1 sequence fragments located at the end of ORF8 and the very beginning of the 3' NCR (FBN557588-91 and FM211646 from India and HE580104 from Poland) are available in GenBank. Unfortunately the sequenced region overlapped by only 131 nt with the region sequenced by Bajet et al. (4), making it difficult to draw any firm conclusions from comparisons on such a small sequence fragment (4). However, these comparisons point to the fact that the LChV1 isolates from India could represent a third distinct cluster of isolates, characterized by very different indel polymorphisms at the beginning of the 3' NCR (not shown).

Although not providing definitive proof, the failure to detect any other viral agent in the V2356 SSD source by the deep-sequencing approach used here suggests that the divergent LChV1 isolate identified is likely responsible for the Shirofugen stunt syndrome. This analysis was confirmed by the independent discovery of another LChV1 isolate in the second SSD source analyzed (Roma7). However, we cannot rule out the possibility that the syndrome could be caused either by a virus that could have escaped detection (a DNA virus or an RNA virus producing very little dsRNAs) or a viroid. However, the parallel with the findings of Matic et al. (19) tentatively associating another LChV1 isolate with a syndrome similar to the one studied here but developing in a different flowering indicator is enticing. These results run counter to old reports indicating that *P. serrulata*, the oriental flowering cherry, is a symptomless host of the LChV agent (12,22,23,31). However, the existence of two viruses, LChV1 and LChV2, associated with the little cherry disease was not known at the time so that it is not possible to know whether these old reports refer to LChV1 or LChV2. The analysis of further Shirofugen stunt or Kwanzan stunting isolates to evaluate their association with LChV1 seems the logical way to progress in our understanding of the agents responsible. However, formal association of LChV1 with these syndromes will ultimately require more direct experimental approaches, possibly involving infectious cDNA clones, in order to allow unambiguous inoculation of individual agents (35).

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