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BRIEF REPORT

Sequence analysis of RNA 2 and RNA 3 of lilac leaf chlorosis virus: a putative new member of the genus *Ilarvirus*

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Abstract RNA 2 and RNA 3 of lilac leaf chlorosis virus (LLCV) were sequenced and shown to be 2,762 nucleotides (nt) and 2,117 nts in length, respectively. RNA 2 encodes a putative 807-amino-acid (aa) RNA-dependent RNA polymerase associated protein with an estimated M_r of 92.75 kDa. RNA 3 is bicistronic, with ORF1 encoding a putative movement protein (277 aa, M_r 31.45 kDa) and ORF2 encoding the putative coat protein (221 aa, M_r 24.37 kDa). The genome organization is similar to that typical for members of the genus *Ilarvirus*. Phylogenetic analyses indicate a close evolutionary relationship between LLCV, ApMV, and PNRSV.

The genus *Ilarvirus* is one of five genera within the family *Bromoviridae* [17], although there is evidence that alfalfa mosaic virus (AMV) may be a true ilarvirus and the separate genus *Alfamovirus* may not be required [4, 24]. *Bromoviridae* is an important taxon of plant RNA viruses, with members distributed worldwide and causing severe and economically important diseases in various crops [5]. Their genomes consist of three segments of positive-sense, single-stranded RNA, packaged in separate virions that may contain also sgRNAs, defective RNAs, or satellite RNAs [5, 17]. This is a complex family of viruses, with classification made even more difficult by common recombination events [2, 5]. If more and more viruses within the family are identified and their genomes characterized, more accurate classification and better understanding of the evolution of members of the family *Bromoviridae* can be achieved.

Common lilac (*Syringa vulgaris* L.) is an ornamental shrub that is cultivated and sold commercially [25]. The plant is affected by several viruses including lilac ring mottle virus (LRMV), an ilarvirus [22, 23, 26]. In this study, a putative new member of the genus *Ilarvirus* was detected in lilac. The relationship of this virus to members of the genus *Ilarvirus* is described.

Cuttings of common lilac (*S. vulgaris*) with symptomatic leaves were submitted for testing at the Sidney Laboratory, Canadian Food Inspection Agency. The cuttings were obtained from two nurseries and were collected during inspections of plants imported from the USA, one set from a nursery in the province of Quebec (3828-01), and the other from the province of Alberta (3831-01). The symptoms observed on the leaves were similar and common to both sources and included leaf abnormality, chlorosis, and edge necrosis. The associated virus has been named lilac leaf chlorosis virus (LLCV).

Symptomatic leaves of 3828-01 were ground in a buffer consisting of 0.025 M phosphate buffer (pH 7.0) with 0.5% nicotine, for use as inoculum. The leaves of herbaceous indicator plants at the 6-leaf stage were dusted lightly with the abrasive powder corundum (grit 600) and gently rub-inoculated with the inoculum described above. Herbaceous plants included *Chenopodium amaranticolor*, *C. quinoa* subsp. *quinoa*, *C. quinoa* subsp. *milleanum*, *Nicotiana occidentalis* '37B', *N. occidentalis* subsp. *obliqua*, *N. benthamiana*, and *Cucumis sativus*. Leaves from infected *C. quinoa* subsp. *quinoa* were used for dsRNA extractions as described by Morris and Dodds [14].

The nucleotide sequences have been deposited in EMBL Nucleotide Sequence Database under accession numbers FN669168 (RNA 2) and FN669169 (RNA 3).

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Formvar-carbon-coated copper grids were used in leaf dip preparations that allowed particles to bind to the grid. The grids were washed and stained with 2% uranyl acetate. Prunus necrotic ring spot virus (PNRSV) antiserum was used in immunoelectron microscopy (IEM) as described by Van Regenmortel [27].

Double-stranded (ds) RNA bands were gel-purified and extracted using standard phenol:chloroform gel extraction procedures [19], and the purified dsRNA was used as a template for cDNA production. Several strategies were used initially to generate cDNA; (1) RT-PCR using random hexamers, (2) RT with an oligo(dT) primer followed by PCR with a combination of random hexamers and the oligo(dT) primer, and (3) a modified version of the single-primer amplification technique (SPAT) with the ligation primer A (5′-PO_4-AGGTCTCGTAGACCGTG CACC-NH_2-3′) as described by Attoui et al. [1]. Subsequently, further nucleotide (nt) sequence determination was carried out by cDNA walking using reverse virus-specific primers combined with random hexamers in RT-PCR to generate overlapping cDNA fragments. The 5′ terminial of the virus segments were determined using the 5′ RACE System, version 2.0 (cat. #18374-058, Invitrogen, Burlington, ON, Canada), with SPAT used to generate the 3′ terminus and also confirm the sequence of the 5′ terminus. Amplified cDNA fragments were gel-purified and extracted using a MinElute Gel Extraction Kit (cat. #28604, Qiagen, Mississauga, ON, Canada). The purified cDNA fragments were ligated into the pCR-TOPO vector and cloned using the TOPO TA Cloning Kit as directed by the supplier (Invitrogen).

Sequencing and sequence analysis were carried out as described by James and Varga [13]. Overlapping clones of plasmids with virus-derived cDNA inserts were used to generate the nucleotide sequence of LLCV. Each region was covered by 2–5 independently generated clones. Bidirectional sequencing was carried out using the M13F and M13R primers. The molecular weights of the deduced proteins were determined by analysis with the program Compute pI/Mw tool, http://au.expasy.org/tools/pi_tool.html [7]. Phylogenetic analyses were carried out using the neighbor-joining method described by Saitou and Nei [18], within ClustalX (version 1.83), and with a bootstrap of 1,000 replicates. Trees were produced using NJPLOT [16]. Phylogenetic networks were created using the SplitsTree4 program developed by Huson and Bryant [12], located at http://www.splitstree.org/. Nucleotide alignments of the concatenated complete genome segments RNA 2 and RNA 3 were produced in ClustalX (1.83) using default settings. The NeighborNet distance transformation (OrdinaryLeastSquares Variance option with ConvexHull splits transformation) was used to generate potential networks.

LLCV-derived sequences were used to design several oligonucleotide primers that targeted the RNA-dependent RNA polymerase (RdRp)- and coat protein (CP)-encoding regions of RNA 2 and RNA 3, respectively. The best primers of those evaluated were LCP-F1 (5′-AGAGCCC GACAGAGAGCTCGA-3′) and LCP-R1 (5′-ACGCGTTC ACTCTGAGTAGGATC-3′), targeting the CP-encoding region (0.2 μM each) and amplifying a 271-bp product. RT was carried out at 42°C for 45 min, followed by 5 min at 99°C; and the cycling conditions for PCR were 95°C for 30 s, 53°C for 45 s, and 72°C for 1 min, for 30 cycles, followed by a final extension step at 72°C for 7 min. RT-PCR reliability was evaluated by screening non-symptomatic and presumed healthy lilac as well as lilac infected with LLCV isolates 3828-01 and 3831-01. Primer specificity was confirmed by screening plants infected with several members of the genus *Ilarvirus* [17], including American plum line pattern virus (APLPV, isolates 1819-01 and 3273-01), apple mosaic virus (ApMV, isolates 1148-10 and 1162-03), Prunus necrotic ringspot virus (PNRSV, isolate 3137-03), and tobacco streak virus, type species (TSV, isolate 3077-02).

Approximately 10 days post-inoculation (dpi), systemic symptoms including chlorosis and distortion were observed on leaves of *C. quinoa* (subsp. *quinoa* and *milleanum*). At 16 dpi, symptoms on *C. quinoa* had progressed to necrosis. Chlorosis, mottling, and epinasty were observed also on leaves of *C. amaranticolor*. *N. benthamiana*, *N. occidentalis* ('37B' and subsp. *obliqua*), and *C. sativus* were confirmed as symptomless hosts by RT-PCR. Spherical virions, approximately 23–28 nm in diameter, were observed in leaf dip preparations of symptomatic *C. quinoa* subsp. *quinoa* leaves. No decoration was observed in IEM analysis with polyclonal antibodies to PNRSV.

The entire sequence of RNA 2 of LLCV consists of 2,762 nucleotides (nts) (accession number FN669168). LLCV RNA 2 has a 5′ untranslated region (UTR) consisting of 138 nt, and a 3′ UTR 203 nt in size. BLAST analysis of the entire RNA 2 revealed 66% identity across a 2,257-nt segment with the corresponding region of the RNA 2 of PNRSV, and 67% identity across a 1,893-nt segment with the corresponding region of ApMV. No sequence matches were observed after BLAST analysis of either UTR. RNA 2 has a single open reading frame (ORF) starting with an AUG codon at nts 139-141 and is terminated by a stop codon (UGA) at nts 2,560–2,562. The ORF consists of 2,421 nts (62 and 58% identity to PNRSV and ApMV, respectively) and encodes a protein consisting of 807 deduced amino acid residues (aa), M_r 92.746 kDa (58 and 56% identity to PNRSV and ApMV, respectively). Identities of 44% (nt) and 31% (aa) were observed when compared with the corresponding region of LRMV. This ORF encodes the putative RdRp and contains the highly

conserved RdRp signature sequence of glycine-aspartic acid-aspartic acid (GDD) at aa position 633–635 of the deduced 807-aa protein. This motif is contained within an aa region that is highly conserved among ilarviruses (AS**GDD**SLI) and constitutes the N-terminus of the polymerase signature region described by Candresse et al. [3] and Ge et al. [8]. The peptides S(K/T)FDKSQ and FE(A/T)KFPHNQP are present in the palm subdomain [15] of the polymerase region of LLCV, except that glutamine (Q) in the latter peptide is replaced by methionine (M). This substitution was confirmed by bidirectional sequencing of four independently derived cDNA clones. No evidence for a valid 2b ORF could be found in the RNA 2 of LLCV.

The RNA 3 of LLCV consists of 2,117 nts (accession number FN669169) and is bicistronic, with an ORF at the 5′ end encoding the putative movement protein (MP) and an ORF proximal to the 3′ terminus encoding the putative CP. ORF1, which encodes the putative MP, begins with a start codon at nt position 327 and is terminated by a stop codon (UAA) at nts 1,158–1,160, encoding a 277-aa protein with M_r 31.45 kDa. This protein contains the putative RNA-binding domain described by Herranz and Pallás [11] starting at aa position 55 of the protein. Within this domain, there are basic residues that are conserved among LLCV, ApMV and PNRSV (KxxxxK/RxxxKxxK/RxxxxK/RxxxxxK/RxK/R). The triplet consensus sequence of L/M-V/R-L/M seen in most members of the family *Bromoviridae* [11] is replaced by L-K-L in LLCV, which is similar to ApMV. ORF2 encodes the putative CP, begins at nt 1,259, and is terminated by a stop codon (UAG) at nt position 1,922–1,924. The deduced CP consists of 221 aa with M_r 24.37 kDa. Three UTRs were identified: a 5′ UTR (326 nts), an intergenic UTR (101 nts) separating the MP gene from the CP gene, and a 3′ UTR (196 nts). The last 100 nts of the 3′ UTRs of LLCV RNA 2 and RNA 3 were 72% identical, similar to PNRSV (73%) but less than that observed with Parietaria mottle virus (PMoV) [6] and LRMV [23], representing subgroups 1 and 2, respectively. The essential AUGC motif, box 3 [9] or box 2 [21], is present at the 3′ terminus of the 3′UTR of the RNA 3 of LLCV. The nt and aa sequences of the MP region of LLCV are most closely related to those of PNRSV (identities of 54 and 58%, respectively) and ApMV (identities of 52 and 54%, respectively). Similarly, the CP nt and aa sequences

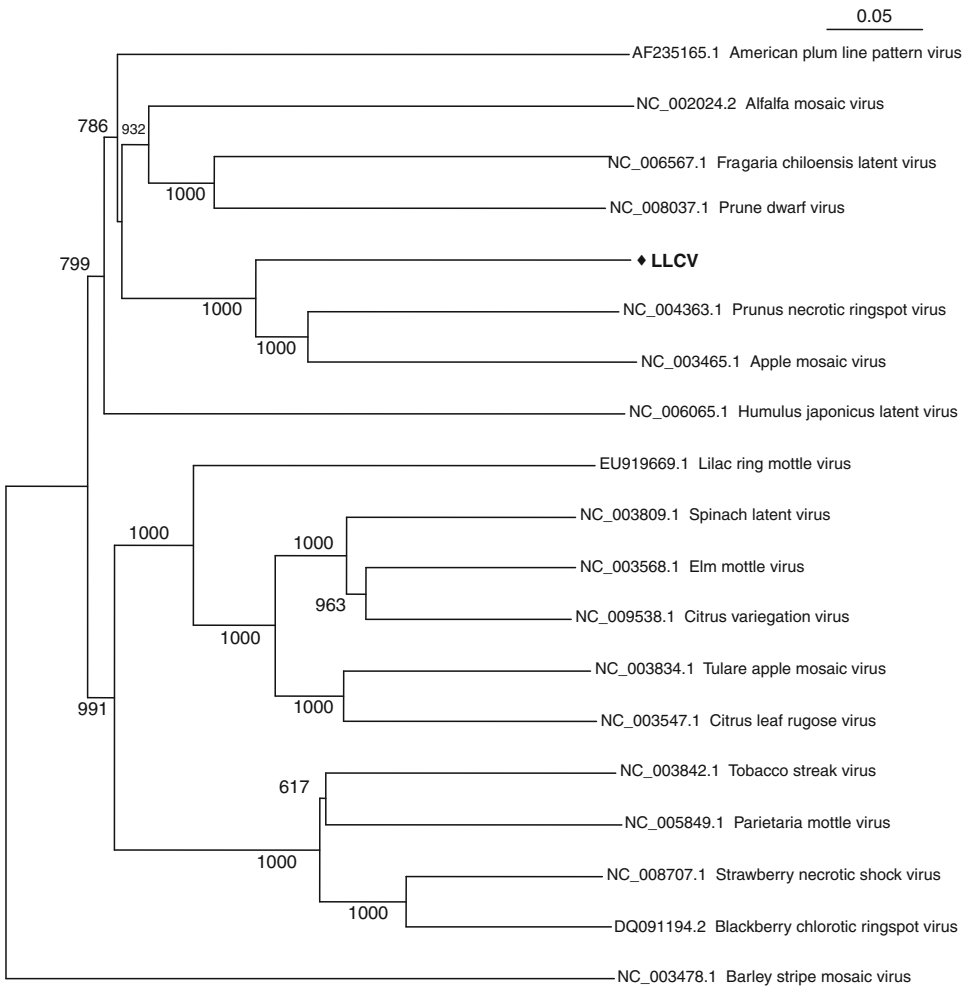

Fig. 1 Phylogenetic analysis based on alignments of the nucleotide sequence of the RNA-dependent RNA polymerase gene of lilac leaf chlorosis virus (*LLCV*) and members of the genus *Ilarvirus*, family *Bromoviridae*, with barley stripe mosaic virus as an outgroup. The tree was produced using NJPLOT. Bootstrap values out of 1,000 replicates are shown, and the *scale bar* indicates the number of substitutions per residue

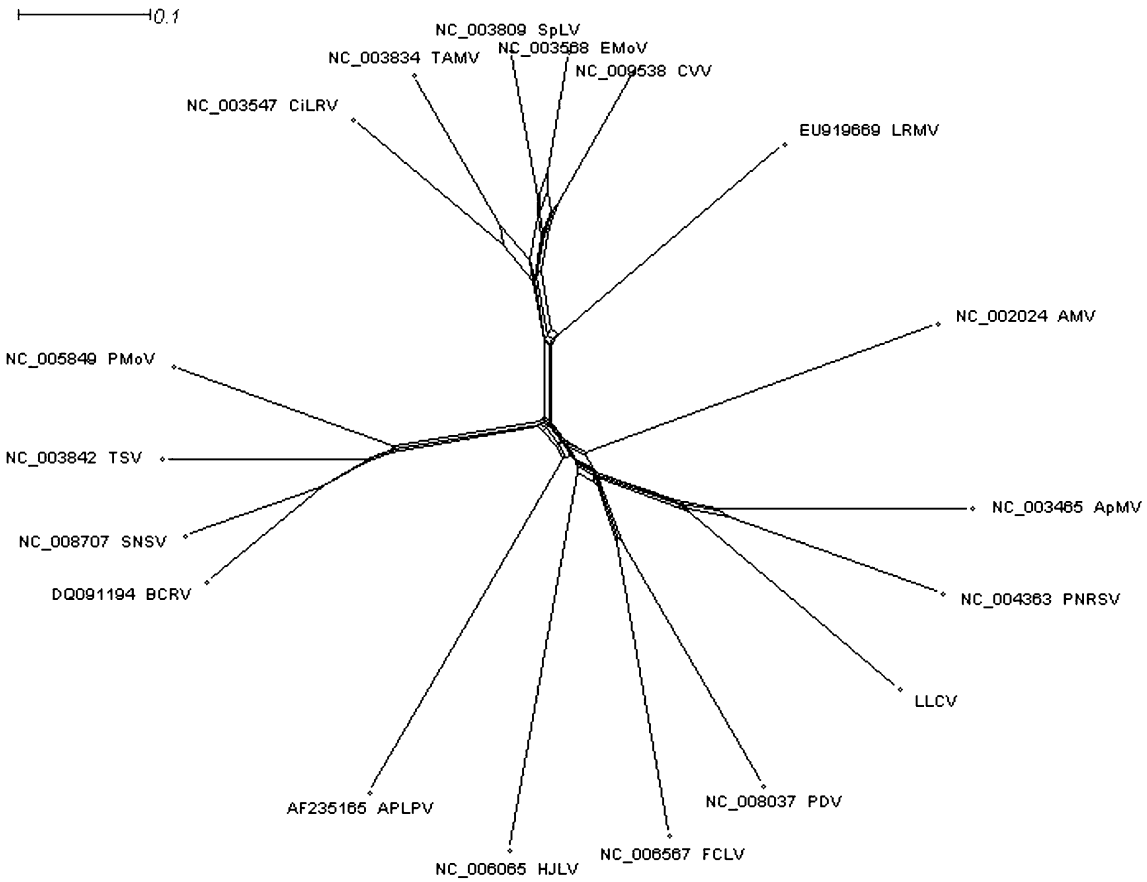

Fig. 2 An unrooted neighbor-net constructed from concatenation of the RNA 2 and RNA 3 sequences of lilac leaf chlorosis virus (*LLCV*) and members of the genus *Ilarvirus*, family *Bromoviridae*. The network indicates the existence of 3 nodes of evolution, with LLCV sharing ancestors common to apple mosaic virus (*ApMV*) and Prunus necrotic ring spot virus (*PNRSV*). Lilac ring mottle virus (*LRMV*), another ilarvirus infecting lilac, is linked to a different and distinct node. Other ilarviruses included in the comparison were: alfalfa mosaic virus (*AMV*), prune dwarf virus (*PDV*), Fragaria chiloensis latent virus (*FCLV*), Humulus japonicus latent virus (*HJLV*), American plum line pattern virus (*APLPV*), blackberry chlorotic ringspot virus (*BCRV*), strawberry necrotic shock virus (*SNSV*), tobacco streak virus (*TSV*), Parietaria mottle virus (*PMoV*), citrus leaf rugose virus (*CLRV*), Tulare apple mosaic virus (*TAMV*), spinach latent virus (*SLV*), and citrus variegation virus (*CVV*). The GenBank accession numbers are provided

were most similar to those of PNRSV (identities of 58 and 50%, respectively) and ApMV (identities of 56 and 45%, respectively). The low aa identity may explain the lack of an IEM reaction between LLCV virions and PNRSV polyclonal antibodies. In the case of LRMV, the other ilarvirus infecting lilac, identities ranged from 44 to 45% for the nt sequences of RNA 2 and RNA 3, and 17–22% for the deduced aa sequences. A putative zinc finger domain [9, 10, 20] was found in the cysteine-rich N-terminus of the CP (aa 3–17) of LLCV (**C**KL**C**G**HTH**A**GG**C**VK**C**). The bolded residues are conserved among isolates of LLCV, ApMV, and PNRSV. Downstream of this region is an Arg-rich region that may comprise a RNA-binding protein site [21].

Phylogenetic analyses based on both the nt sequence and the aa sequence of the RNA 2 ORF (encoding the RdRp) and the ORF1 and ORF2 regions of RNA 3 consistently indicated a close relationship of LLCV to PNRSV and ApMV. The phylogeny tree (Fig. 1) based on the nt sequence of the ORF associated with the RdRp-encoding region of RNA 2 shows the close relationship between LLCV, PNRSV, and ApMV, with high bootstrap values supporting this relationship.

Unrooted neighbor-net analysis carried out using the concatenated data set of the complete nt sequences of the RNA 2 and RNA 3 of LLCV and some known members of the genus *Ilarvirus* (Fig. 2) showed three major branches or subgroups. The network connections support the existence of recombination events in the evolution of ilarviruses, as indicated by Codoñer and Elena [5]. Again, a close evolutionary linkage is observed between LLCV, ApMV, and PNRSV. LLCV is distinct from LRMV and appears to have ancestors in common with ApMV and PNRSV.

The primers LCP-F1 and LCP-R1 successfully amplified an LLCV-specific 271-bp fragment in all cases (Fig. 3a, b). The primers were used effectively to detect the virus in

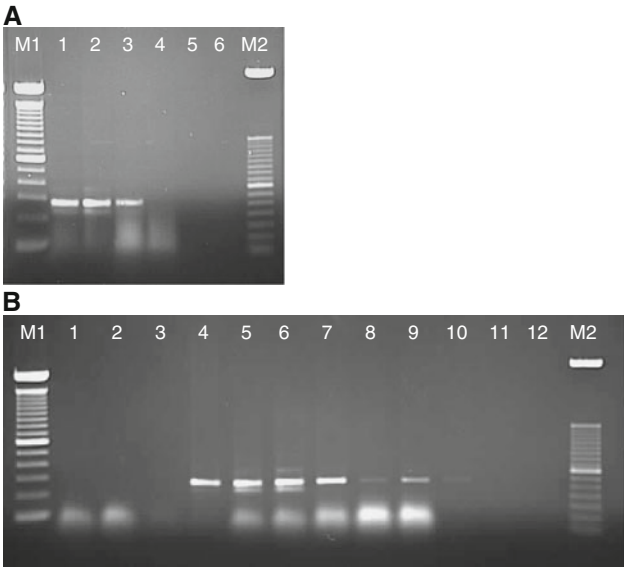

Fig. 3 **a** Agarose gel analysis of lilac leaf chlorosis virus (LLCV)-specific RT-PCR. *Lane M1* 100-bp DNA ladder, *lane 1* LLCV isolate 3831-01 in lilac, *lane 2* isolate 3828 in lilac, *lane 3* isolate 3828-01 in *C. quinoa*, *lane 4* healthy *C. quinoa*, *lane 5* water control in RT step, *lane 6* water control in PCR step, *lane M2* 50-bp DNA ladder. **b** Agarose gel analysis of a RT-PCR assay to determine the herbaceous host range of lilac leaf chlorosis virus (LLCV). *Lane M1* 100-bp DNA ladder, *lane 1* healthy *Nicotiana benthamiana*, *lane 2* healthy *N. occidentalis* '37B', *lane 3* healthy *Chenopodium quinoa* subsp. *quinoa*, *lane 4* infected *C. amaranticolor*, *lane 5* infected *C. quinoa* (subsp. *quinoa*), *lane 6* infected *C. quinoa* (subsp. *milleanum*), *lane 7* infected *N. benthamiana*, *lane 8* infected *N. occidentalis* subsp. *obliqua*, *lane 9* infected *N. occidentalis* '37B', *lane 10* infected *Cucumis sativus*, *lane 11* water control in RT step, *lane 12* water control in PCR step, *lane M2* 50-bp DNA ladder

C. quinoa and both isolates of the virus in lilac (Fig. 3a). Healthy lilac samples were always negative (data not shown). The amplified fragments associated with LLCV isolates 3831-01 and 3828-01 in lilac were gel-purified, cloned, and sequenced, and they were found to be 99% identical. The RT-PCR assay also allowed confirmation of infection in the non-symptomatic herbaceous hosts *N. benthamiana*, *N. occidentalis* (subspp. '37B' and *obliqua*), and *Cucumis sativus* (Fig. 3b). In evaluations of primer specificity, negative results were obtained in RT-PCR screening of plants infected with APLPV, ApMV, PNRSV, and TSV (data not shown).

The range of subgroups (3–10) that have been described for the genus *Ilarvirus* [2, 4, 17] attests to the complexity of this genus. Recent analyses [2, 5] seem to indicate that three subgroups may be sufficient to encompass the genetic diversity that characterizes members of the genus. This study provides support for possible classification of members of the genus into three subgroups. Phylogenetic network analysis [12] with simultaneous analysis of sequences of both RNA2 and RNA3 of several ilarviruses generated three main branches or subgroups. Two branches form very tight clusters and correspond to subgroups 1 and 2 according to Roossinck et al. [24]. The third main branch consists of members of subgroups 3, 4, and 5, plus Fragaria chiloensis latent virus (FCLV) of subgroup 6 [24], or essentially the subgroup-1 members proposed by Boulila et al. [2].

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