

Genomic sequences of blackberry chlorotic ringspot virus and strawberry necrotic shock virus and the phylogeny of viruses in subgroup 1 of the genus *Iilarvirus*

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Abstract Three members of subgroup 1 of the genus *Iilarvirus*: blackberry chlorotic ringspot (BCRV), strawberry necrotic shock (SNSV), and tobacco streak viruses (TSV), may infect *Rubus* and *Fragaria* species. All cause symptoms similar to those previously attributed to infection by TSV alone. Although similarities exist among the genomic sequences of the three, phylogenetic analysis shows them to be distinct viruses. These viruses and Parietaria mottle virus, the other currently accepted member of subgroup 1, appear to have evolved from a common ancestral virus, share conserved motifs in the products of the genomic RNAs, and constitute a distinct subgroup within the genus.

The genus *Iilarvirus* contains the greatest number of viral species in the family *Bromoviridae*: a family comprised of positive sense, single-stranded, RNA viruses with tripartite genomes encapsidated in icosahedral or quasi-icosahedral

virions. RNA 1 encodes a viral replicase (1a protein). The open reading frame (ORF) proximal to the 5' end of RNA 2 encodes an RNA dependent RNA polymerase (2a protein) [5]. In cucumoviruses and some ilarviruses (members of subgroups 1 and 2), an ORF proximal to the 3' end of RNA 2 encodes a protein (2b) that is suspected to be involved in the suppression of RNA interference based on potential functional homology with the corresponding protein of cucumoviruses [12]. RNA 3 codes for the movement (3a protein) and coat protein (CP). The latter is expressed via a subgenomic RNA 4. Species within the genus have been placed in subgroups based on serological relationships [5]. Nine species within the genus are known to infect rosaceous hosts [14]. Black raspberry latent virus (BLRV) [4] and strawberry necrotic shock virus (SNSV), [6] were reported to infect *Rubus* spp. (blackberry and raspberry) and *Fragaria* spp. (strawberry), respectively. Later work indicated serological relationships between these two viruses and tobacco streak virus (TSV), the type species of the genus, and the names BLRV and SNSV were subsequently relegated to the status of synonyms of TSV [10, 17]. With the availability of sequence data it has become clear that the assignment of some ilarviruses to subgroups in the genus on the basis of serological relationships was inappropriate and that ilarviruses could be grouped more reliably using molecular traits [15].

Molecular characterization of an ilarvirus isolated from both *Rubus* and *Fragaria* revealed that it was unique and the name strawberry necrotic shock virus (SNSV) was resurrected [19]. Several dozen plants, considered to be infected with the small fruit strain of 'TSV', were tested for both SNSV and TSV using virus-specific PCR assays but only SNSV was detected. This suggested that SNSV was the ilarvirus most frequently found infecting *Rubus* and *Fragaria* and that either TSV did not infect these species

The sequences reported in this manuscript are deposited with GenBank as accessions NC_008706, NC_008707, NC_008708 (SNSV) and NC_011553, NC_011554, NC_011555 (BCRV).

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(previous identifications were erroneous) or occurred in them only rarely. Recently, two isolates of TSV were detected in strawberry by molecular tests (GenBank accessions EF531708 and EF525175) confirming previous reports that TSV does infect at least *Fragaria* within the rosaceous small fruit crops.

Another ilarvirus, blackberry chlorotic ringspot virus (BCRV), was described in *Rubus* and rose [11, 20]. This virus showed no serological relationship with either BRLV or SNSV using antisera that in previous work had shown relationships between these two viruses and TSV [10, 17]. The incomplete sequence information obtained for BCRV and SNSV showed that the two viruses are closely related and are also close relatives of TSV. To confirm the relationships among these three viruses we completed the genomic sequences for BCRV and SNSV and compared the phylogeny of these viruses, with TSV and Parietaria mottle virus (PMoV), the other currently accepted members of subgroup 1 of the ilarviruses [8]. The partial sequences of Bacopa chlorosis virus (BaCV) and tomato necrotic spot virus (TNSV), two previously undescribed ilarviruses that share properties with PMoV [2, 13], were included in the comparison of subgroup-1 ilarviruses, where possible.

The genomic sequence of BCRV was completed using the techniques described previously [11] while the genomic sequence of SNSV was determined as described for *Fragaria chiloensis* latent virus (FCILV) [18]. Phylogenetic analyses were completed using CLUSTAL X with bootstrapping consisting of 1,000 pseudoreplicates and trees were plotted using NJPLOT [9]. Nucleotide (nt) and amino acid (aa) sequence comparisons were made with GatMat using default settings.

The RNA 1 of BCRV and SNSV are 3,479 and 3,429 nt in length, respectively. The complete nt sequences share 83% identity and the putative aa sequences of 1a proteins of BCRV (1,093 aa, M_r 124 kDa) and (SNSV (1,090 aa, M_r 123 kDa) share 92% identity. The methyltransferase signatures [3] of the respective 1a proteins occur at aa 97–186 for both BCRV and SNSV. This signature displays 98% identity between BCRV and SNSV and greater than 80% identity among the four members of subgroup 1. The helicase signature of the 1a protein [3] is found at residues 805–1,051 and 802–1,048 for BCRV and SNSV, respectively, and displays 93% identity between BCRV and SNSV and >68% identity among the subgroup 1 viruses.

The RNA 2 of BCRV and SNSV are 2,879 and 2,876 nt in length, respectively. The complete nt sequences share 81% identity and the putative aa sequences of the 2a proteins of BCRV (804 aa, M_r 92 kDa) and SNSV (803 aa, M_r 92 kDa) share 84% identity and display identities ranging from 67 to 70% with the putative 2a proteins of PMoV and TSV. The polymerase signatures [3] of the 2a proteins

occur at aa 460–563 for BCRV and aa 459–562 for SNSV, respectively. This signature displays 96% identity between BCRV and SNSV and more than 89% identity among the four members of subgroup 1. The 2b proteins of BCRV (211 aa, M_r 23.7 kDa) and SNSV (211 aa, M_r 23.5 kDa) share 80% identity and are slightly larger than those of

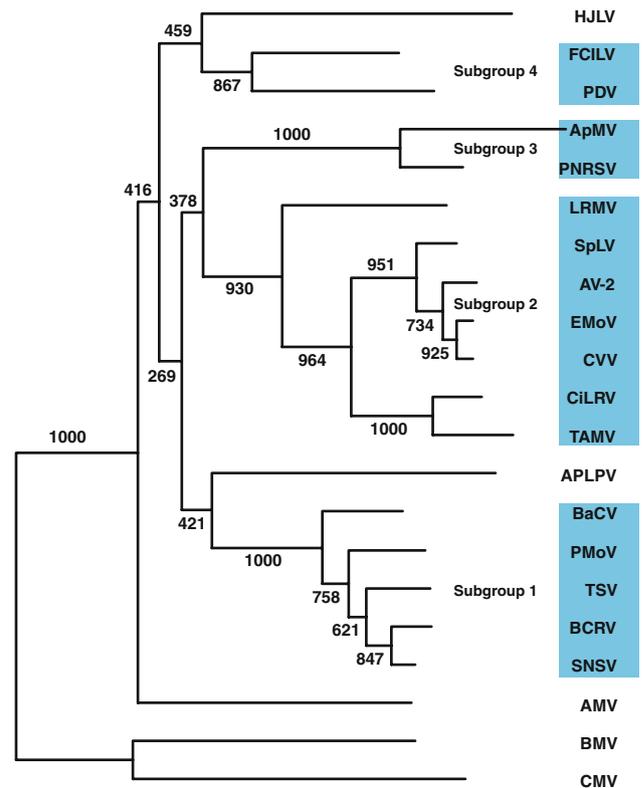


Fig. 1 A phylogenetic tree constructed from a multiple alignment of the putative aa sequences of the polymerase signature defined by Candresse et al. [3] of members of the genus *Iilarvirus* and alfalfa mosaic virus, and using cucumber mosaic and brome mosaic viruses as the outgroups. The acronyms of the viruses and the genomic sequences from which the polymerase signatures used in this comparison were extracted are: alfalfa mosaic virus (AMV), NC_002024; American plum line pattern virus (APLPV), NC_003452; apple mosaic virus (ApMV), NC_003465; asparagus virus 2 (AV-2), NC_011809; Bacopa chlorosis virus (BaCV), FJ607141; blackberry chlorotic ringspot virus (BCRV), NC_011554; brome mosaic virus (BMV), NC_002027; citrus leaf rugose virus (CiLRV), NC_003547; citrus variegation virus (CVV), NC_009538; cucumber mosaic virus (CMV), NC_002055; elm mottle virus (EMoV), NC003568; *Fragaria chiloensis* latent virus (FCILV), NC_006567; *Humulus japonicus* latent virus (HJLV), NC_006065; lilac ring mottle virus (LRMV), NC_003568; Parietaria mottle virus (PMoV), NC_005849; prune dwarf virus (PDV), NC_008037; Prunus necrotic ringspot virus (PNRSV), NC_004363; spinach latent virus (SpLV), NC_003809; strawberry necrotic shock virus (SNSV), NC_008707; tobacco streak virus (TSV), NC_003842; tulare apple mosaic virus (TAMV), NC_003834. Bootstrap values for individual branches are indicated using 1000 replicates. Bootstrap values of less than 70% are generally regarded as insignificant and the branches could be drawn as a polytomy but they are separated because of biological significance. The four currently accepted subgroups of ilarviruses are indicated

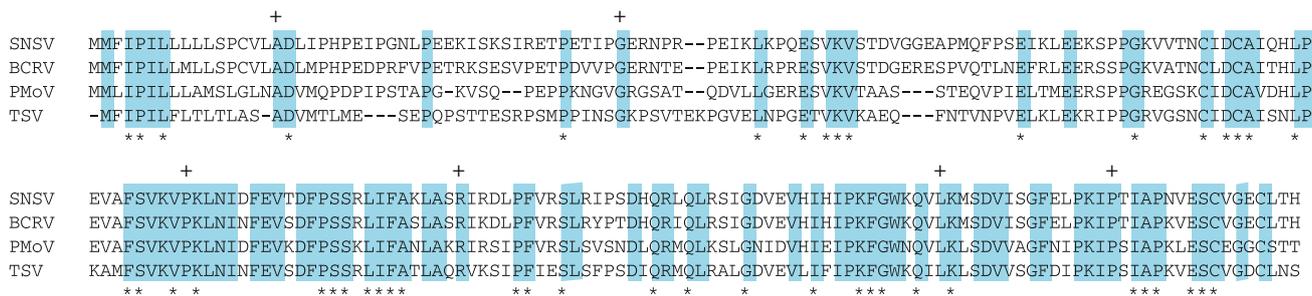


Fig. 2 Multiple alignment of the putative aa sequences of the 2b proteins of accepted subgroup 1 ilarviruses. Amino acids conserved among all viruses within the alignment are surrounded by a gray

background. Amino acids conserved in both subgroups 1 and 2 viruses are indicated by *plus*. An *asterisk* is placed beneath aa that are unique to members of subgroup 1. See also Ref. [16]

PMoV and TSV (both 205 aa, M_r 22 kDa) with which they share 54–62% identity.

The RNA 3 of BCRV and SNSV are 2,290 and 2,245 nt in length, respectively. They share 71% identity with each other and 68–71% with the sequences of the RNA 3 of PMoV and TSV. The putative 3a movement proteins of BCRV (299 aa, M_r 32.8 kDa) and SNSV (298 aa, M_r 32.3 kDa) are similar in size to that of PMoV (293 aa, M_r 32.1 kDa) but slightly larger than that of TSV (289 aa, M_r 31.6 kDa). The sequences of all four 3a proteins share identities of 66–74% with the greatest identity being between BCRV and SNSV. The putative CPs of BCRV, PMoV, SNSV, and TSV vary in length and size (BCRV–245 aa, M_r 27.1 kDa; PMoV–220 aa, M_r 26.2 kDa; SNSV–222 aa, M_r 24.4 kDa; TSV–220 aa, M_r 24.3 kDa) and share identities ranging from 45 (BCRV/PMoV) to 65% (SNSV/TSV).

In all phylogenetic trees constructed using the putative aa sequences of the products encoded by the individual ORFs, BCRV, PMoV, SNSV, and TSV cluster at the end of a single branch with bootstrap support of >93% as is shown with a tree constructed using the polymerase signature (Fig. 1). In this respect they resemble the manner in which viruses that are currently accepted members of subgroups 2 and 3 of the genus *Iilarvirus* cluster. The members of subgroup 4 cluster together at the end of a single branch but with bootstrap support of only 86%. Similar to members of subgroup 2 [15], conserved aa sequences are visible in the 1a and 2a proteins of the four members of subgroup 1 and BaCV. However, the relationships among viruses in subgroup 1 are different from those observed for members of subgroup 2. The polymerase signatures of viruses have been used to show relationships across a wide range of viral genera and species [3]. Three aa shared among all four subgroup 1 viruses plus BaCV, but not found in other ilarviruses or alfalfa mosaic virus (AMV), were identified at specific positions within the polymerase signature (D_{19} , H_{33} , A_{96}).

Conserved regions of aa sequence can be identified in the 2b, 3a and CPs of the four subgroup 1 ilarviruses plus

BaCV (Figs. 2, 3). Regions conserved in the 2b proteins of both subgroup 1 and subgroup 2 viruses can be identified, whereas other regions of the 2b protein of subgroup 1 viruses are conserved across all members of that subgroup but are distinct from regions at similar locations in the subgroup 2 viruses [16]. As reported for PMoV [7] most of the differences in the 3a proteins occur towards the carboxy (C) terminus of the protein (Fig. 3a). Deletions in the three aa at the C terminus of the 3a protein of AMV made the protein non-functional in cell-to-cell movement [21] and it has been suggested that differences in this region may influence and or reflect differences in host specificity [7]. The triplet of aa at the C terminus of the 3a protein in PMoV is completely different from the corresponding triplet in BCRV, SNSV, and TSV. The first aa in this triplet is conserved in BCRV, SNSV, and TSV but variation in the second and third aa could possibly be associated with differences in the host ranges of these three viruses.

The CPs of these five viruses share relatively low levels of identity (45–60%). An alignment of the CPs (Fig. 3b) centers on the arginine motif conserved in all ilarviruses and proposed as the site at which RNA binds during genome activation [1]. The amino (N) terminal regions of the different viruses vary. A pair of aa (MS) is at the start of the CPs of BCRV isolates from rose (DQ329378 and GQ325716), SNSV, PMoV_{TOM} and TNSV but not at the start of the CPs of the type isolates of BCRV, TSV, and PMoV. However, these aa are embedded downstream of the initiation codon in the CPs of both BCRV and TSV and align with CPs of BCRV isolates from rose and SNSV. Different computer programs used to identify the start of ORFs accept that these upstream initiation codons are in good context for translation in BCRV and TSV. Furthermore, multiple sequences in GenBank for the CPs of TSV show MNTL as the starting aa of the protein in agreement with the type sequence for TSV (NC_003842) used here. The difference between the aa at the beginning of PMoV_{TOM} and the type isolate of PMoV has already been noted [7] with the suggestion of either a cytosine deletion

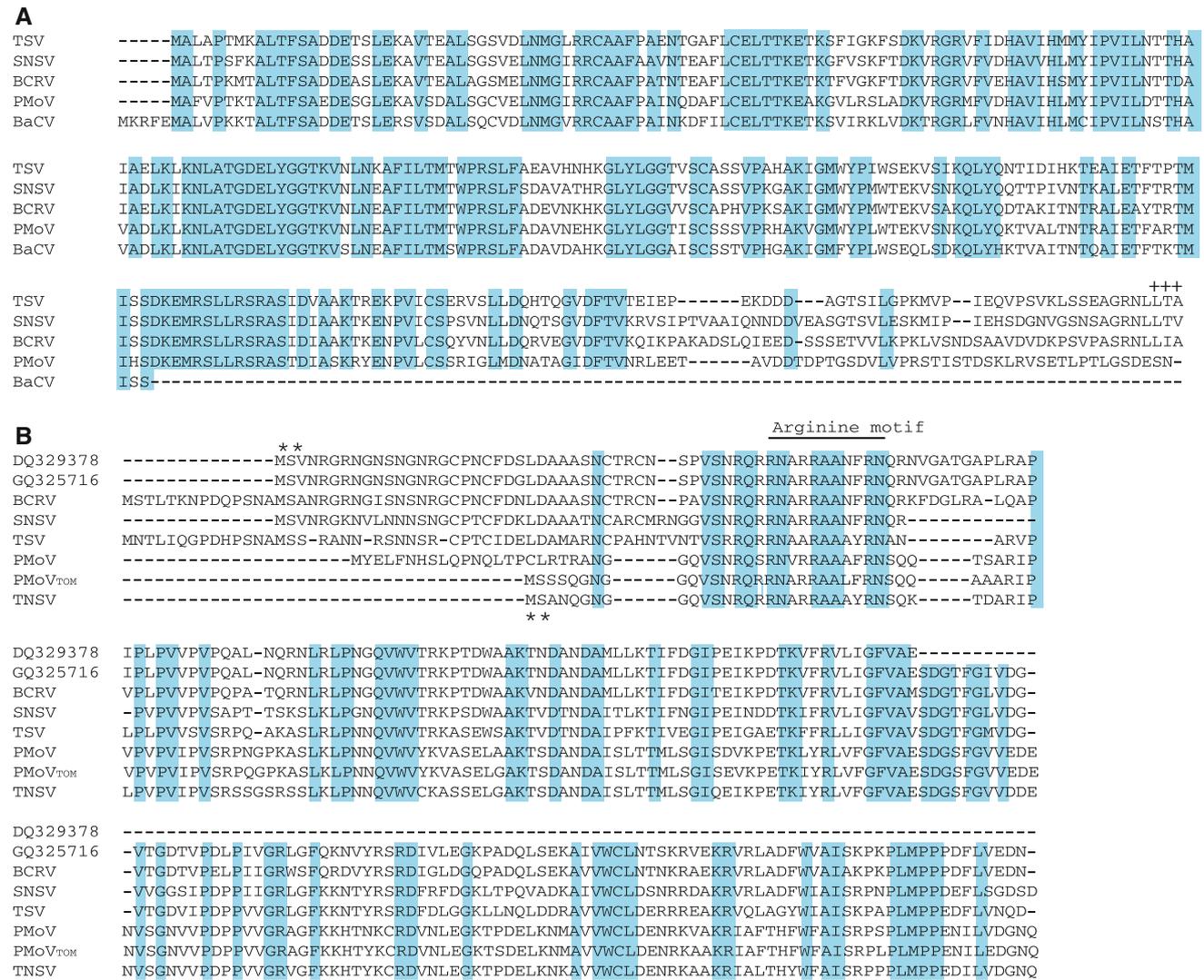


Fig. 3 Multiple alignments of the putative aa sequences of the 3a and 3b (CP) proteins of subgroup 1 ilarviruses. Amino acids conserved among all viruses within an alignment are surrounded by a gray background. **a** 3a proteins: There is little aa sequence conservation among the four viruses (BCRV, PMoV, SNSV, and TSV) towards the C terminus of the 3a proteins. The three amino acids at the C terminus that vary among BCRV, SNSV, and TSV are indicated by *plus*. The partial sequence for the 3a protein of BaCV is included for comparison. **b** Coat protein sequences of the subgroup 1 viruses

(type isolates of BCRV, PMoV, SNSV, and TSV) and TNSV (FJ236810). DQ329378 and GQ325716 are recently described sequences for BCRV isolates from rose. The arginine motif [1] is indicated by a line above that region of the sequence. Between this motif and the amino termini of the different viruses there are few conserved aa. The pair of aa (MS) common to BCRV, SNSV, and TSV is identified by *asterisks* above the alignment. The corresponding aa in PMoV_{TOM} isolates and TNSV are indicated by *asterisks* below the alignment

associated with tomato isolates or a mistake in the original sequence being responsible. The reasons for the differences in the putative CP sequences between the type isolate of BCRV and isolates from rose remain to be determined perhaps requiring sequencing of the CPs to resolve this issue.

In summary, the four viruses (BCRV, PMoV, SNSV, and TSV) that currently make up subgroup 1 of the genus *Iilarvirus*, together with BaCV, appear to have evolved from a common ancestral virus sharing conserved motifs in

both the products of the viral replicase, polymerase, and other proteins. Even though there are conserved motifs in the 2b, 3a and CPs of these viruses, there are sufficient differences in these virus proteins that interact intimately with the host for these viruses to be considered distinct species. Although members of a particular ilarvirus subgroup share many traits and also display properties similar to members of other subgroups, they can be distinguished at the molecular level. Furthermore, even though some members of the same subgroup can be found naturally

infecting the same host species with similar symptoms, molecular data allow for discrimination among the infecting agents.

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