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ANNOTATED SEQUENCE RECORD

Partial nucleotide sequences of the RNA 1 and RNA 2 of lilac ring mottle virus confirm that this virus should be considered a member of subgroup 2 of the genus *Ilarvirus*

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Abstract Partial sequences of the RNA 1 and RNA 2 of LRMV are described. These confirm that LRMV is most closely related to members of the subgroup 2 ilarviruses. However, these sequences also show that LRMV is sufficiently different from other members of subgroup 2 so as to explain why oligonucleotide primers that had been used to amplify related subgroup 2 viruses did not amplify corresponding products from LRMV.

The genus *Ilarvirus* is one of six genera included in the family *Bromoviridae* [9]. Ilarviruses possess a tripartite genome of positive-sense ssRNAs [5] and are included in the alpha-like superfamily of viruses [12]. The RNA 1 is monocistronic, coding for a viral replicase. The RNA 2 is bicistronic in some members of the genus, but monocistronic in others. The open reading frame (ORF) proximal to the 5′ UTR codes for the viral polymerase. The second, smaller ORF found in some members of the genus is located toward the 3′ terminus of the molecule, and expressed through a subgenomic RNA that codes for a 2b protein [5]. The function of this protein has yet to be demonstrated in ilarviruses, but, based on similarities with the cucumoviruses, it is inferred to be involved in viral movement [27] and gene silencing [13]. The initial ORF of RNA 3 codes for the movement protein (MP), and the second ORF of RNA 3 codes for the coat protein (CP). The CP gene is expressed via a subgenomic RNA 4 [5]. Preparations of ilarviruses require the presence of either a few molecules of CP or the subgenomic RNA 4 for infectivity. This phenomenon of genome activation crosses the boundaries of both species and genera. The CP of any ilarvirus will activate the genome of any other ilarvirus, and the CP of the sole member of the genus *Alfamovirus* [alfalfa mosaic virus (AMV)] is capable of activating the genomes of ilarviruses and vice versa [2]. CP of ilarviruses and AMV possess a common arginine motif [1] located near the amino terminal end of the molecule which is involved in the binding of the CP to the genomic molecules during infection [2]. The RNAs of ilarviruses and AMV share similar 3′ terminal structures [2, 22] that are involved in this binding.

Species of ilarvirus were originally subdivided on the basis of serological relationships [6, 7, 15]. Serologically related viruses were assigned to the same subgroup, whereas ilarviruses for which no serological relationship could be demonstrated were assigned to additional, newly created, subgroups. Subgroup 2 contains the largest number of members and traditionally included the viruses asparagus virus-2 (AV-2), citrus leaf rugose virus (CiLRV), citrus variegation virus (CVV), elm mottle virus (EMoV), and tulare apple mosaic virus (TAMV) [6, 7, 15]. A serological relationship between members of subgroup 2 and spinach latent virus (SpLV) has now been demonstrated [8], although SpLV had originally been assigned to a unique subgroup. A serological relationship between EMoV and hydrangea mosaic virus (HdMV) had been reported [11], in contrast to the original description of HdMV [23], which reported no serological relationship with other then known species of ilarvirus. Sequencing of the original isolates of

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EMoV and HdMV showed them to be isolates of the same virus (EMoV) [19]. The difficulties in demonstrating serological relationships are perhaps not surprising as ilarviruses are "unpromising subjects for purification and raising of good antisera" [6]. Furthermore, many tests are completed in only one direction; i.e., antiserum to a novel virus tested against antigens of known viruses.

Lilac ring mottle virus (LRMV) was first described and characterized by van der Meer et al. [25]. It exhibits the properties of members of the genus *Ilarvirus*, but in serological tests it did not exhibit any relationship with EMoV, prunus necrotic ringspot (PNRSV), tobacco streak (TSV), or apple mosaic (ApMV) ilarviruses [25, 26]. In later work, a serological relationship between LRMV and a recently discovered ilarvirus, fragaria chiloensis latent virus (FCiLV), was detected [21]. However, sequence data [24] show that FCiLV is most closely related to prune dwarf virus (PDV) in subgroup 4. The complete nucleotide sequence of the RNA 3 of LRMV was published in 1995 [18], but the putative amino acid (aa) sequence of the CP did not show any obvious relationship with the sequences of the CPs of other ilarviruses that had been published at that time. As additional sequence data for other ilarviruses became available, subsequent phylogenies for the genus *Ilarvirus* involving the aa sequence of the CP placed LRMV as being related to, but outside, the cluster of ilarviruses that made up subgroup 2 of this genus [17, 20]. However, an examination of the molecular evolution of the family *Bromoviridae* based on the proteins encoded by the RNA 3 [4] clearly showed LRMV to be closely related to CVV and CiLRV. In this work, we describe partial sequences of the RNA 1 and RNA 2 of LRMV and confirm that LRMV is most closely related to members of the subgroup 2 ilarviruses.

Lyophilized tissue containing LRMV and antisera to the virus were the kind gift of Dr. D. Z. Maat, Wageningen. The virus was grown in *Chenopodium quinoa* Wild and purified according to van der Meer et al. [25]. RNA was extracted from both purified virus and lyophilized plant tissue using an RNeasy kit (QIAGEN, Valencia, CA, USA). Procedures used in cloning and sequencing have been described previously [19]. The initial clones produced for the RNA 1 and RNA 2 were amplified from viral RNA by reverse transcription PCR. Pairs of degenerate primers were designed using multiple alignments of the helicase region and the polymerase signatures [3, 14] of other ilarviruses, created using CLUSTAL X [10] and the CODEHOP program [16]. The downstream primer in each pair was used to synthesize cDNA, which was used in a PCR reaction with the corresponding upstream primers. Primer pair 1 (5′ CGAGAAGGCCAAGTAGTGGAARGA RRTNGA 3′ us; 5′ GAGCAGATGAAGGGCTGGTTRTR NGGRAA 3′ ds) was designed to amplify products from the RNA 1 (helicase region) of ilarviruses. Primer pair 2 (5′ GGAAGGAGATCGACTTCTCCAMNTTYGAYAA 3′ us; 5′ TGAACAGGAACTTGGAGCAGAYRAANGGYTG 3′ ds) was designed to amplify products from the RNA 2 (polymerase region) of ilarviruses. Products from these PCR reactions were cloned and sequenced. The sequence from these clones was used to design primers that allowed the sequence between the clone and the 3′ terminus of the virus to be determined by a 3′ RACE procedure. Attempts to determine the sequence between the clones and the 5′ terminus of the virus using 5′ RACE procedures were partially successful. The nucleotide (nt) sequences of the 5′ and 3′ RACE clones and the PCR fragments were assembled into a contiguous sequence using the program GeneJockey 2 (Biosoft, Ferguson, Missouri). Multiple alignments of sequences were completed using CLUSTAL X, and dendrograms were plotted using NJPLOT [10]. Paired comparisons between the nt sequences and the aa sequences of the putative products of the ORFs detected in these sequences were made using the FASTA program at the EMBL-EBI website (http://www.ebi.ac.uk/fasta33/).

The partial sequence for the RNA 1 was 2,031 nt in length. It contained a substantial length of the single ORF found on the RNA 1 of ilarviruses, including a helicase signature [3, 14] and a 3′ UTR of 163 nt. The sequence of the RNA 2 (2,821 nt) was almost full-length. It contained an ORF proximal to the 5′ UTR that coded for a protein of 791 aa (M_r 90,855) comparable in size to the P2 proteins of other ilarviruses and AMV and which contained a polymerase signature [3, 14]. The ORF proximal to the 3′ UTR codes for a putative 2b protein. A 3′ UTR of 190 nt, was present. As with other ilarviruses, the last 100 nt of the 3′ UTR are almost completely conserved between the RNA 1 and RNA 2, and also the RNA 3. In cloning and sequencing members of subgroup 2, we [19] had been able to use an octet of nucleotides (5′ GGAGATGC 3′) located at the 3′ terminus of the viral sequences to synthesize cDNA for most members of this subgroup except for TAMV. Attempts to use this octet to initiate cDNA synthesis with LRMV also failed, an observation that clearly can be explained by the mismatches between the terminal octet of LRMV (5′ GGAGTCGC 3′) and almost all other subgroup 2 viruses (5′ GGAGATGC 3′). The terminal octet of TAMV is (5′ GGAGAAGC 3′).

A putative translation of the sequence for the RNA 1 showed a helicase signature (EDGxxx…xxxSLRH) of 251 aa [3, 14] which, when aligned with the helicase signatures of subgroup 2 ilarviruses, showed many conserved regions, but there was one major difference. The signature for LRMV contained a 7-aa insertion (TTDECAL) at position 203 which was not present in the subgroup 2 viruses. The signature of all subgroup 2 viruses and LRMV differed from the helicase signature of TSV (subgroup 1) in that

TSV has a 6-aa insertion (SAAGSP) at position 22 and also has a single aa insertion (D) at position 152.

Putative translations of the ORF proximal to the 5′ terminus of the sequence of the RNA 2 showed aa that corresponded to the P2 protein [14] that occurs in ilarviruses and which included the polymerase signature of 104 aa [3, 14]. In previous work, six aa (K_{19}, K_{29}, P_{35}, N_{71}, R_{86}, and C_{91}) distributed along the polymerase signature of subgroup 2 viruses were shown to be completely conserved and to be distinct from those of other ilarviruses [19]. LRMV shared only two (P_{35} and R_{86}) out of these six amino acids. An alignment of the putative 2b protein of LRMV and subgroup 2 ilarviruses showed many differences (Fig. 1). However, there were regions of the 2b protein that are conserved amongst subgroup 2 viruses and LRMV. These conserved regions were absent in the 2b proteins of subgroup 1 ilarviruses (TSV, parietaria mottle virus, strawberry necrotic shock virus, and blackberry chlorotic ringsport virus) which, when aligned, showed conserved aa sequences completely different from those seen in the subgroup 2 viruses and LRMV. Only 7 aa, distributed along the length of the 2b protein, were conserved in both subgroup 1 and subgroup 2 viruses.

Scott et al. [19] used four pairs of primers to amplify regions of the RNA 1 and RNA 2 of subgroup 2 viruses. Attempts to amplify corresponding fragments from RNA of LRMV failed. Inspection of the nt sequence for LRMV RNA 1 and RNA 2 showed extensive mismatching between the primers used in the earlier work and the corresponding sites in these partial sequences.

Ilarviruses can be divided into two categories based on the presence or absence of a 2b ORF. Viruses in subgroup 1 and subgroup 2 both possess such an ORF. All other ilarviruses for which sequences for the genomic RNA 2 have been described, and which are assigned to other subgroups, do not. The work by Codoñer et al. [4] on the proteins coded for by the RNA 3 has already indicated that LRMV should be included in subgroup 2 rather than its current placing in subgroup 6 [5]. Comparison of the putative translation products coded for by the RNA 1 and RNA 2 of LRMV with corresponding data of other ilarviruses also places the virus in subgroup 2. Furthermore, the conserved

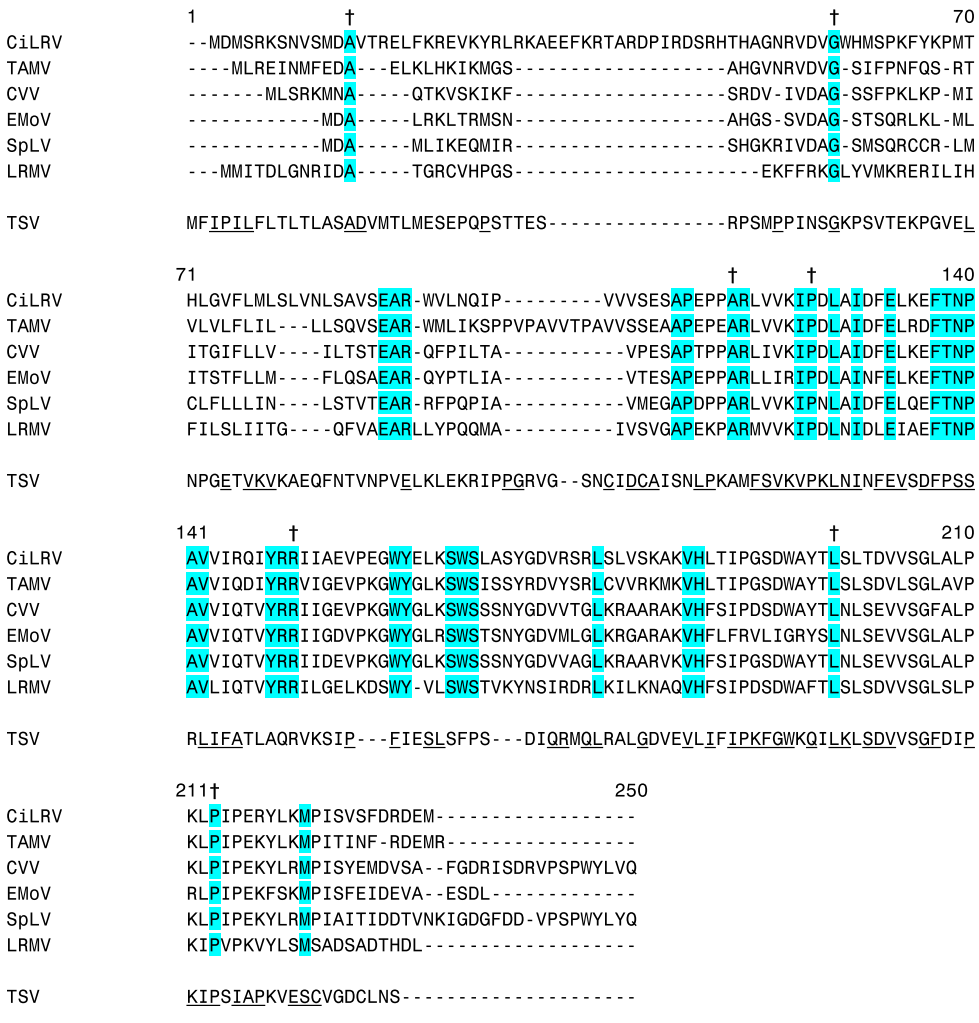

Fig. 1 A multiple alignment of the putative amino acid sequences of the 2b protein of lilac ring mottle virus (*LRMV*), members of subgroup 2 of the ilarviruses, and TSV (the type member of the genus *Ilarvirus* and a member of subgroup 1). Amino acids conserved among all subgroup 2 viruses and LRMV are surrounded by a gray box. Amino acids conserved among all subgroup 1 viruses are indicated by underlining aa in the sequence for tobacco streak virus (*TSV*). The seven aa conserved among the 2b proteins of both subgroup 1 and subgroup 2 ilarviruses are indicated by the symbol † above the alignment. The alignment was completed using Clustal X [10]. Sequences used in the comparison and acronyms are citrus leaf rugose virus (CiLRV) NC_003547, tulare apple mosaic virus (*TAMV*) NC_003834, citrus variegation virus (*CVV*) NC_009538, elm mottle virus (*EMoV*) NC_003568, spinach latent virus (*SpLV*) NC_003809, and TSV NC_003842

motifs in the aa sequences of the 2b proteins of LRMV and other subgroup 2 viruses confirms that LRMV should be grouped with viruses in subgroup 2 and not with the viruses in subgroup 1. However, molecular data for the RNA 1 and RNA 2 that we report here also show that LRMV is sufficiently different from other members of subgroup 2 so as to explain why oligonucleotide primers that had been used to amplify products from related subgroup 2 viruses did not amplify corresponding products from LRMV [19] and to suggest that LRMV has diverged further from some ancestral virus than have other members of the subgroup 2.

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