# Multiplex PCR and genome analysis of *Carnation mottle virus* Indian isolate†

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**The complete nucleotide sequence of an Indian isolate of *Carnation mottle virus* (CarMV) was determined to be 4005 bp in length, 2 bp longer than previous reports. The viral genome was amplified by RT–PCR using primers covering the entire genome length. Coat protein (CP) and movement protein (MP) genes were also amplified by multiplex RT–PCR from annual and perennial carnations. Identity of the amplified products was verified by sequencing. CP and MP genes of four Indian isolates were compared with the available sequences in the GenBank database. CP gene of annual and perennial carnations showed high similarity (95–100%) compared to isolates reported from different parts of the world. MP genes (p7 and p9) showed 95–98% nucleotide similarity while at the amino acid level similarity, of 86–98% for p7 and 92–98% for p9 respectively, was obtained. Recombination detection analysis indicated recombination in an isolate from annual carnation and division of CarMV isolates into six groups on the basis of recombination analysis.**

**Keywords:** Carnation, *Carnation mottle virus*, coat protein, complete genome, Indian isolate, movement protein, polyprotein.

*CARNATION mottle virus* (CarMV) is the type member of the genus *Carmovirus*[1], family *Tombusviridae*. It is one of the largest families having genera, including *Tombusvirus*, *Carmovirus*, *Dianthovirus*, *Necrovirus*, *Machlomovirus*, *Aureusvirus*, *Avenavirus* and *Panicovirus*, with virions exhibiting icosahedral symmetry and significant degree of sequence similarity with respect to replicase associated and coat protein (CP) genes[2,3]. Carmoviruses have one of the smallest genomes among positive-strand RNA viruses, the size being close to 4 kbp.

The complete nucleotide sequence of CarMV contains five open reading frames (ORFs). Although ORF1 (p27) and ORF2 (p86) share the same initiation codon, p86 is a translational readthrough product of the amber stop codon[4,5] at the end of p27. The readthrough part of p86 contains the typical GDD motif of plant virus RNA polymerases[6]. Except CarMV and *Cowpea mottle virus*, all other carmoviruses are not considered economically important pathogens[7].

In this article, the complete nucleotide sequence of an Indian isolate of CarMV was determined and compared with already available sequences of CarMV and other carmoviruses on the basis of nucleotide and amino acid sequences. All the three genes, i.e. CP and two movement protein (MP) genes were amplified using specific primers through multiplex RT–PCR. Sequencing of the RT–PCR-amplified capsid protein (CP) and MP (p7 and p9) genes confirmed the presence of CarMV in both annual and perennial carnations. ORFs p7, p9 and capsid protein of isolates reported from different parts of the world were also compared. Recombination Detection Programme (RDP) was used to determine the recombination events and phylogenetic analysis among different CarMV isolates.

## Materials and methods

### *Plant sample collection and virus isolation*

The annual and perennial carnation plants used in the present study were collected from carnation-growing areas of Himachal Pradesh, India. Pure cultures of the isolates used in present study were maintained separately on *Saponaria vaccaria*.

### *Primer designing*

The primers for CP and MP genes were designed on the basis of similarity of sequences in the database. For complete genome amplification four pairs of the oligonucleotide primers were synthesized according to the similarity of the two different complete genome sequences available in the database (Figure 1 and Table 1). The primers were designed such that restriction enzyme sites were present at the 5′ termini of these primers to generate appropriate linkers for combining the four cDNA products.

### *RNA isolation, cDNA synthesis and PCR amplification*

Viral RNA and total plant RNA were isolated using the RNAqueous kit (Ambion, USA) and eluted in 60 µl of nuclease-free Millipore water. Viral RNA was used for RT–PCR reactions.

†The sequences described in this work have been deposited in the EMBL database under accession numbers AJ811998, AJ844549, AJ844552, AJ844553 and from AJ844585 to AJ844587.

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**Table 1.** Primer sequence used for amplifying cDNAs of CarMV-Indian isolate

| Primer | Sequence | Product size |
|---|---|---|
| CarMV1UP | 5′-CGC AGA TCT GGG TAAGCT GGC GGG C-3′ | 1-1037 nt; cDNA1 |
| CarMV1DN | 5′-AAT CAG CTG ACT AGC TCC TTG TTG C-3′ | |
| CarMV2UP | 5′-AAT CAG CTG TCC GCA ACC TGA A-3′ | 1031-2304 nt; cDNA2 |
| CarMV2DN | 5′-AAT GGG CCC CAG CAC GTT GGT GTT GAT-3′ | |
| CarMV3UP | 5′-AGG GGG CCC CAG TGT GAA GCG GCA-3′ | 2304-2979 nt; cDNA3 |
| CarMV3DN | 5′-CCC AAG CTT GGG ATA ACA CCC GTG-3′ | |
| CarMV4UP | 5′-CCC AAG CTT TGA AGA CTG GGT CGT T-3′ | 2971-4005 nt; cDNA4 |
| CarMV4DN | 5′-CGC AGA TCT GGG CGG GGA AAG AGT A-3′ | |

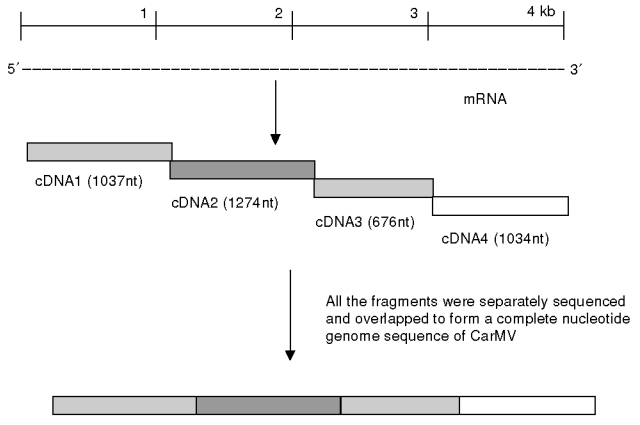


**Figure 1.** Strategy utilized for nucleotide sequencing of complete CarMV genome.

Multiplex RT–PCR was carried out for simultaneous amplification of three genes, i.e. CP and both MP (p7 and p9) genes. cDNA synthesis was carried out in a 50-µl volume µl of 10X First strand buffer (Amersham Pharmacia Biotech, USA), 2.5 mM dNTP mix, 200 ng down primer for CP and both MP genes, 40 units of ribonuclease inhibitor (Promega, USA), and 200 units Mu-MLV RT enzyme (Amersham Pharmacia Biotech). cDNA synthesis was then performed at 37°C for 1 h 15 min followed by enzyme inactivation at 70°C for 5 min.

PCR amplification of cDNA was carried out using 10 µl of cDNA prepared as described earlier, 5 µl of 10X PCR buffer (Genei, India), 3 µl of 10 mM dNTP mix, 200 ng of each primer, 1.5 units *Taq* DNA polymerase enzyme (Genei, India) in 50 µl reaction. The reaction consisted of 39 cycles with denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min 10 s and a final extension at 72°C for 10 min, carried out in Gene Amp PCR system 9700 (Applied Biosystems, USA).

For amplification of complete genome in four fragments, RT–PCR was carried out separately for each segment. The reaction mix for RT–PCR was similar as mentioned above. The annealing temperatures used for amplification of four different fragments were 48, 50, 50 and 45°C for 1 min, respectively. It was followed by extension at 72°C for 1 min 10 s, 1 min 30 s, 1 min and 1 min 30 s for amplification of four different fragments respectively. The PCR products (10 µl) were electrophoresed in 1% agarose gel prepared in 1% TAE buffer (0.04 mol/l Tris-acetate and 0.001 mol/l EDTA), stained with 0.5 µg/µl ethidium bromide and observed under UV light[8]. In all the RT–PCR reactions RNA extracted from healthy *Nicotiana clevelandii* leaves was used as a negative control.

### *cDNA cloning, nucleotide sequencing and analysis of the amplicon*

RT–PCR amplified fragments were cloned using the pGem-T easy vector system (Promega). Recombinant plasmid DNA was isolated for sequencing by the boiling method[9] and purified using the WIZARD DNA cleanup system (Promega). Both strands of the cloned DNA were sequenced by the dideoxy chain termination method[10] using an automated sequencer (ABI PRISM 310, Applied Biosystems, USA) and the ABI PRISM® Big Dye™ Terminator (version 3.0) Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Nucleotide sequences of the various genes of CarMV and other carmoviruses available at the EMBL nucleotide database were analysed using the BLAST (NCBI) program[11]. The comparison was also carried out for different genes, i.e. p86, p7, p9 and CP (p38). The nucleotide and amino acid sequence similarity was also compared among different tombusviruses. The BLASTP program was used for searching the amino acid sequence database. Parities comparison was performed using the ALIGN-2 program[12]. Multiple alignments were generated by the MULTALIN program[13].

Detection of potential recombinant sequences, identification of likely parental sequences, and localization of possible recombination breakpoints were carried out using the Bootscan[14], Chimaera[15], GENECONV[16], Maximum Chi Square[17] (MaxChi), RDP[18] and Sister Scan[19] (SiScan) methods as implemented in RDP2 (ref. 20). An alignment containing twenty-two CarMV CP and MP genes in continuation was scanned with the following default settings for Chimaera, GENECONV, MaxChi and SiScan detection methods no multiple comparison correction, window size = 10 and highest acceptable probability 0.001 and the list events detected by > 2 methods was kept with on set-

ting. For RDP analysis the window size was adjusted to 10, and 70–100% sequence sharing homology was adjusted with internal reference sequences. For Bootstrap, the settings were adjusted as follows: window size – 20, bootstrap replicate – 1000, random number seed – 3, cut-off percentage – 95 and Kimura (1980) model with *Ts*/*Tv* ratio of 2.0. Using the RDP software, we were able to test the detected statistical significance of the recombination events and identify crossing-over points in the CarMV annual carnation strain.

## Results

### *Pure culture maintenance and diagnostics of CarMV*

CarMV infection in annual and perennial carnations was confirmed by ELISA and RT–PCR. The virus was filtered through host range studies and purification was carried out as described earlier[21].

### *Molecular characterization*

The DNA fragments of expected sizes for CP (~ 1050 bp) and MP (~ 200 bp for p7 and ~ 300 bp for p9) genes were obtained as expected through multiplex RT–PCR for both annual and perennial carnations (Figure 2). Complete CarMV genome was amplified through RT–PCR and a fragment of expected size of 4.0 kb was observed. For complete CarMV genome sequence four fragments of sizes ~ 1.0, 1.2, 0.8 and 1.0 kb were obtained as expected. The first two fragments included the polyprotein, the third fragment had a part of the polyprotein, complete MP (p7 and p9) and a partial CP. The last fragment had part of CP and 3′ UTR.

All the four PCR-amplified fragments were cloned and sequenced. These sequenced fragments were overlapped and thus complete nucleotide sequence of CarMV was deduced, which was found to be 4005 bp in length, 2 bp longer than previous reports of 4003 bp available in database. The Indian isolate has an additional codon in the polyprotein region, which corresponds to the addition of one amino acid. It also has one nucleotide omission at the 5′ non-coding region compared to the previous sequences reported. The complete nucleotide sequence of CarMV was submitted to GenBank under the accession number AJ844549.

This is the fourth isolate to be completely sequenced (AJ811998) after the Spanish (AJ304989) and Chinese (NC_001265) isolates. The nucleotide sequence of the CarMV Indian isolate is 97% identical with the Spanish (AJ304989) and one Chinese (X029861) isolate, respectively, while with another Chinese isolate (AF192772) it showed 94% similarity.

Nucleotide sequence analysis of CP gene of perennial carnations showed 99% similarity with sequences reported from Spain and India, while only 95% similarity with the Yunnan (China) strain (AJ489479). CP gene of annual carnations showed 97% sequence similarity with strains from Spain (X029861), China (NC_001265), The Netherlands (AJ309496), USA (AJ309512) and Israel (AJ309498), while only 95% similarity with Yunnan (China) strains (AJ489479). When different CarMV strains were compared for CP gene, the nucleotide and amino acid sequence similarity was found to be almost similar (95–100 and 96–100% respectively). Sequence similarity values of different strains for CP genes of perennial and annual carnations are shown in Table 2.

MP genes, i.e. p7 and p9 of different Indian isolates were similar by 95–98% to the corresponding sequences in the database. When corresponding deduced amino acid sequences were compared, it was seen that the p9 gene showed almost similar per cent sequence similarity (92–98%) as for nucleotide (95–98), while the p7 gene showed slightly lower values (86–98) with the published sequences (Tables 3 and 4). In case of MP gene p7, different CarMV isolates when compared among themselves showed 94–100% nucleotide sequence similarity, while amino acid sequence similarity showed lower values ranging from 83 to 100%. MP gene p9 showed 94–100% nucleotide similarity, while amino acid similarity ranges between 91 and 100%.

### *Phylogenetic analysis*

Isolates indicating recombination and relative position of the inserts to the viral genome are reported in Table 5.

Eight CarMV isolates reported from Italy, Spain, France, Japan, The Netherlands, USA, Israel and an Indian isolate

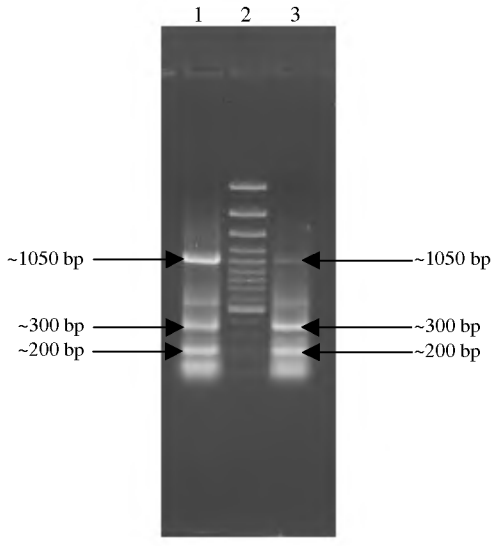


**Figure 2.** Gel electrophoresis of multiplex RT–PCR products for simultaneous amplification of CarMV CP and MP genes (p7 and p9). Lane 1, Amplicons for perennial carnations; lane 3, Amplicons for annual carntions; lane 2, 100-bp marker. Amplification of expected sizes for CP gene (~ 1050 bp), p7 (~ 200 bp) and p9 (~ 300 bp) was observed as expected. Total RNA from both perennial and annual carnations was used for RT–PCR. Arrows indicate RCR products.

**Table 2.** Per cent nucleotide (below diagonal) and amino acid (above diagonal) sequence similarities between CP of different CarMV isolates i nclusive of Indian isolates

| | In-a | In-1 | Ch-c | In-2 | Tw | In-3 | Yun | usa-2 | usa-1 | Sp-m | sp-s | Sp-l | Sp-I | Sp-4 | Sp-3 | Sp-2 | Sp-1 | It-2 | Isr-2 | It-1 | Isr-1 | Nl-2 | Nl-1 | Jap | Fr | Col | Aus | Dix | Ch-1 | Ch-2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| In-a | x | 97 | 96 | 97 | 97 | 96 | 96 | 97 | 97 | 96 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 96 | 97 |
| In-1 | 97 | x | 97 | 99 | 98 | 97 | 98 | 98 | 97 | 98 | 98 | 98 | 97 | 98 | 98 | 98 | 98 | 99 | 97 | 99 | 98 | 99 | 99 | 98 | 98 | 98 | 98 | 98 | 97 | 98 |
| Ch-c | 95 | 96 | x | 97 | 98 | 96 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 100 | 97 |
| In-2 | 96 | 99 | 96 | x | 98 | 96 | 97 | 97 | 97 | 97 | 98 | 98 | 97 | 97 | 98 | 98 | 97 | 98 | 97 | 98 | 97 | 98 | 98 | 97 | 97 | 97 | 97 | 97 | 97 | 97 |
| Tw | 95 | 97 | 95 | 97 | x | 98 | 98 | 97 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 98 | 99 | 98 | 99 | 98 | 99 | 99 | 98 | 98 | 98 | 98 | 98 | 98 | 98 |
| In-3 | 96 | 97 | 95 | 97 | 97 | x | 96 | 97 | 96 | 97 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 96 | 97 |
| Yun | 95 | 95 | 97 | 95 | 95 | 95 | x | 97 | 96 | 97 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 96 | 97 |
| USA2 | 97 | 97 | 95 | 97 | 96 | 97 | 95 | x | 97 | 97 | 97 | 98 | 97 | 97 | 97 | 98 | 97 | 98 | 97 | 98 | 97 | 98 | 98 | 97 | 97 | 97 | 97 | 97 | 97 | 97 |
| USA1 | 97 | 98 | 95 | 97 | 96 | 97 | 95 | 98 | x | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 98 | 97 | 98 | 97 | 98 | 98 | 97 | 97 | 97 | 97 | 97 | 96 | 97 |
| Sp-m | 95 | 96 | 96 | 96 | 96 | 95 | 95 | 95 | 95 | x | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 |
| Sp-s | 96 | 98 | 95 | 97 | 97 | 97 | 95 | 96 | 97 | 96 | x | 99 | 97 | 98 | 98 | 98 | 98 | 98 | 97 | 98 | 98 | 98 | 98 | 97 | 98 | 98 | 97 | 97 | 97 | 97 |
| Sp-l | 96 | 98 | 96 | 97 | 97 | 97 | 95 | 97 | 97 | 96 | 99 | x | 98 | 98 | 98 | 98 | 98 | 99 | 97 | 99 | 97 | 97 | 97 | 97 | 99 | 98 | 98 | 97 | 97 | 98 |
| Sp-i | 95 | 96 | 96 | 95 | 95 | 95 | 95 | 95 | 95 | 95 | 96 | 96 | x | 97 | 97 | 97 | 97 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 97 |
| Sp-4 | 96 | 97 | 95 | 97 | 96 | 97 | 95 | 96 | 96 | 95 | 97 | 97 | 95 | x | 100 | 98 | 100 | 98 | 98 | 98 | 100 | 98 | 98 | 99 | 100 | 100 | 99 | 97 | 97 | 99 |
| Sp-3 | 96 | 97 | 95 | 97 | 96 | 97 | 95 | 96 | 96 | 95 | 97 | 97 | 95 | 100 | x | 98 | 100 | 98 | 98 | 98 | 100 | 98 | 98 | 100 | 98 | 98 | 97 | 97 | 97 | 99 |
| Sp-2 | 96 | 97 | 95 | 97 | 97 | 97 | 95 | 96 | 97 | 95 | 98 | 98 | 95 | 96 | 96 | x | 98 | 99 | 97 | 99 | 98 | 99 | 99 | 98 | 98 | 98 | 98 | 98 | 97 | 98 |
| Sp-1 | 96 | 97 | 95 | 97 | 96 | 97 | 95 | 96 | 96 | 95 | 97 | 97 | 95 | 100 | 100 | 96 | x | 98 | 98 | 98 | 100 | 98 | 98 | 99 | 100 | 100 | 99 | 97 | 97 | 99 |
| It-2 | 96 | 98 | 95 | 97 | 97 | 97 | 95 | 97 | 97 | 96 | 98 | 98 | 95 | 97 | 97 | 97 | 97 | x | 98 | 99 | 98 | 99 | 99 | 98 | 98 | 98 | 98 | 98 | 97 | 98 |
| Isr-2 | 96 | 98 | 96 | 98 | 97 | 97 | 95 | 97 | 97 | 96 | 97 | 97 | 95 | 97 | 97 | 97 | 97 | 97 | x | 98 | 98 | 98 | 98 | 98 | 97 | 98 | 98 | 97 | 96 | 97 |
| It-1 | 97 | 98 | 95 | 98 | 97 | 97 | 95 | 98 | 98 | 96 | 97 | 97 | 95 | 97 | 97 | 97 | 97 | 97 | 98 | x | 98 | 99 | 99 | 98 | 98 | 98 | 98 | 98 | 97 | 98 |
| Isr-1 | 96 | 97 | 95 | 97 | 96 | 96 | 95 | 96 | 96 | 95 | 97 | 97 | 95 | 100 | 100 | 96 | 100 | 97 | 97 | x | 98 | 98 | 98 | 99 | 100 | 100 | 99 | 97 | 97 | 99 |
| Nl-2 | 97 | 98 | 96 | 98 | 97 | 97 | 97 | 97 | 97 | 96 | 99 | 99 | 96 | 97 | 97 | 98 | 97 | 98 | 98 | 98 | x | 99 | 98 | 98 | 98 | 98 | 98 | 98 | 97 | 98 |
| Nl-1 | 97 | 98 | 95 | 98 | 96 | 97 | 95 | 98 | 98 | 95 | 97 | 97 | 95 | 97 | 97 | 97 | 97 | 98 | 98 | 99 | 99 | x | 98 | 98 | 98 | 98 | 98 | 98 | 97 | 98 |
| Jap | 96 | 97 | 95 | 97 | 96 | 96 | 95 | 96 | 96 | 95 | 97 | 97 | 95 | 99 | 99 | 96 | 99 | 97 | 97 | 97 | 97 | 97 | x | 98 | 99 | 99 | 100 | 97 | 97 | 98 |
| Fr | 96 | 97 | 95 | 97 | 96 | 96 | 95 | 96 | 96 | 95 | 97 | 97 | 95 | 100 | 100 | 96 | 100 | 97 | 97 | 97 | 100 | 97 | 97 | x | 99 | 100 | 99 | 97 | 97 | 99 |
| Col | 96 | 97 | 95 | 97 | 96 | 96 | 95 | 96 | 96 | 95 | 97 | 97 | 95 | 100 | 100 | 96 | 100 | 97 | 97 | 97 | 100 | 97 | 97 | 99 | x | 100 | 99 | 97 | 97 | 99 |
| Col | 96 | 97 | 95 | 97 | 96 | 96 | 95 | 96 | 96 | 95 | 97 | 97 | 95 | 99 | 99 | 96 | 100 | 97 | 97 | 99 | 100 | 97 | 97 | 100 | 99 | x | 99 | 97 | 97 | 98 |
| Aus | 96 | 98 | 95 | 97 | 97 | 97 | 95 | 97 | 97 | 96 | 97 | 97 | 95 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 99 | 98 | 97 | 97 | 97 | 99 | x | 97 | 97 | 97 |
| Ch-1 | 95 | 96 | 100 | 96 | 95 | 95 | 97 | 95 | 95 | 96 | 95 | 96 | 96 | 95 | 95 | 95 | 95 | 95 | 96 | 95 | 95 | 96 | 95 | 95 | 95 | 95 | 95 | x | 97 | x |
| Ch-2 | 96 | 98 | 95 | 98 | 97 | 97 | 95 | 97 | 97 | 96 | 97 | 97 | 95 | 97 | 97 | 97 | 97 | 97 | 98 | 97 | 97 | 98 | 97 | 97 | 97 | 97 | 97 | 98 | 95 | x |

(perennial carnations, InP) were found to be free of recombination events. However, the Indian isolate characterized from annual carnation (InA) showed a recombination event at the both N- and C-terminal of CP and MP, which is supported by MaxChi and Chimaera. InA is major parent in one of the recombination event for the Isr1 daughter. The InP isolate was found to be the minor parent for daughter isolates from Columbia and Australia. One interesting feature was observed for isolates from Israel (Isr1 and Isr2). There are two isolates reported from Israel, one isolate (Isr1) was found to be derived as a result of extensive recombination events, while the other isolate (Isr2) was found to be completely conserved. Different CarMV strains can be classified into six different groups on the basis of RDP (Figure 3). Group I has two members, while groups III, V and VI has only one member, each. Groups II and IV have 9 and 8 members respectively. CarMV isolate infecting annual carnations (InA) forms a completely different group (group III), while Indian isolate infecting perennial carnations (InP) falls in group II.

## Discussion

CarMV is considered to be an economically important virus and because of its small genome, it is highly accessible to serve as an interesting model for understanding plant RNA virus genome structure, function and regulation. High percentage of CarMV incidence in carnations growing in northern India has been confirmed by ELISA[21].

We have already reported the MP and complete CP gene of CarMV infecting perennial carnations[21]. The virus was purified through host range and maintained as a single strain on *Saponaria vaccaria*. For diagnostic purpose and for the amplification of different genes simultaneously, multiplex RT–PCR was performed. Three genes, i.e. capsid protein and both MP (p7 and p9) genes of perennial and annual carnations were amplified using the technique. In addition to saving time and reagent costs[22], multiplex RT–PCR is being used increasingly because it improves the efficiency of diagnostic PCR[23]. Multiplex RT–PCR is generally used for the detection of different viruses, but here different genes from the genome of the same virus were amplified in one-tube RT–PCR reaction. In the near future possibility of multiplex PCR for simultaneous detection of several viruses for a particular crop and with simultaneous detection of other major plant pathogens such as viruses and viroids, bacteria, fungi is in the offing[24]. An increase in sensitivity would quite probably be achieved if multiplex PCR in a single tube were to be developed.

By multiplexing, amplifications of all the three genes were achieved, although the intensity of CP gene amplicon for annual carnations is less in comparison to perennial carnations under the reported amplification regime. This might be due to lack of primer specificity, annealing temperature difference or less cDNA synthesis. Amplification of CP gene was detected by Southern blotting (results not shown).

Although the complete CarMV genome cDNA was amplified by a single PCR in our trials, the results were not consistent (data not shown). A possible reason could be due to hampering of cDNA synthesis by strong secondary structures present on the viral template RNA. In order to obtain the complete genome a strategy was developed in which four fragments of cDNA were cloned and sequenced separately and assembled to obtain the complete sequence, which was found to be 4005 bp in length. In our experiments we found that all the four primers synthesized were specific for CarMV even when total plant RNA was used in reverse transcription reaction[25].

The complete genome of CarMV Indian isolate infecting perennial carnations was sequenced. It is the fifth isolate to be completely sequenced (AJ811998) after Spanish (AJ304989) and three Chinese isolates (AF192772, NC_001265 and X02986). The nucleotide sequence of InP isolate is 97% identical with the Spanish (AJ304989) and one Chinese isolate (X02986) respectively, while with another Chinese isolate (AF192772) it showed 94% sequence similarity. The Indian isolate has an additional codon in the polyprotein region, which corresponds to the addition of one amino acid. It also has one nucleotide omission at the 5′ non-coding region compared to the previous sequences reported. Therefore, the genomic sequences of different CarMV were found to be largely conserved in spite of their distant geographical sites of collection.

While studying sequence similarity translated nucleotide sequences were preferred in comparison to coding nucleotide sequences, since they contain more tightly con-

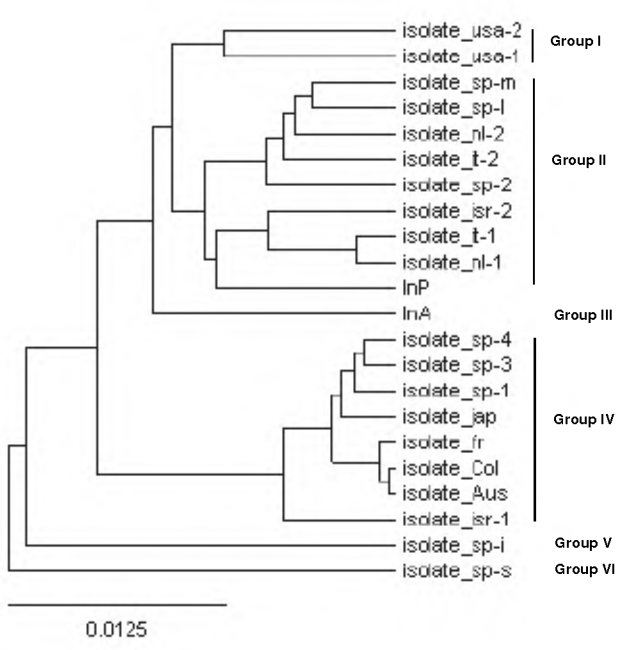


**Figure 3.** Dendrogram showing six different groups of CarMV isolates based on RDP.

**Table 3.** Per cent nucleotide (below diagonal) and amino acid (above diagonal) sequence similarities between p7 MP of different CarMV isolates incl usive of Indian isolates

| | In-a | In-1 | In-2 | In-3 | Ch-c | In-4 | usa-2 | usa-1 | Sp-s | Sp-m | Sp-1 | Sp-i | Sp-4 | Sp-3 | Sp-2 | Sp-1 | It-2 | Isr-2 | It-1 | Isr-1 | Nl-2 | Nl-1 | Jap | Fr | Col | Aus | Dix |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| In-a | x | 96 | 100 | 96 | 95 | 93 | 93 | 93 | 95 | 95 | 93 | 90 | 96 | 95 | 91 | 93 | 95 | 95 | 95 | 86 | 95 | 95 | 96 | 93 | 95 | 95 | 93 |
| In-1 | 98 | x | 96 | 96 | 95 | 96 | 93 | 96 | 95 | 95 | 93 | 90 | 96 | 91 | 98 | 93 | 95 | 95 | 95 | 86 | 95 | 95 | 96 | 93 | 95 | 95 | 96 |
| In-2 | 100 | 98 | x | 96 | 95 | 93 | 93 | 93 | 95 | 95 | 93 | 90 | 96 | 95 | 91 | 93 | 95 | 95 | 95 | 86 | 95 | 95 | 96 | 93 | 95 | 95 | 93 |
| In-3 | 98 | 97 | 98 | x | 95 | 93 | 93 | 93 | 95 | 95 | 93 | 93 | 96 | 95 | 91 | 93 | 95 | 95 | 95 | 86 | 95 | 95 | 96 | 93 | 95 | 95 | 93 |
| Ch-c | 96 | 96 | 96 | 96 | x | 96 | 98 | 98 | 96 | 96 | 95 | 91 | 98 | 96 | 96 | 98 | 96 | 96 | 96 | 88 | 100 | 96 | 98 | 95 | 96 | 96 | 98 |
| In-4 | 96 | 96 | 96 | 95 | 97 | x | 95 | 98 | 95 | 93 | 91 | 88 | 95 | 96 | 93 | 95 | 93 | 93 | 93 | 85 | 96 | 93 | 95 | 91 | 93 | 93 | 98 |
| usa-2 | 96 | 96 | 96 | 96 | 97 | 96 | x | 96 | 95 | 95 | 93 | 90 | 96 | 95 | 95 | 100 | 95 | 95 | 95 | 86 | 98 | 95 | 96 | 93 | 95 | 95 | 96 |
| usa-1 | 97 | 97 | 97 | 96 | 97 | 97 | 97 | x | 95 | 95 | 93 | 90 | 96 | 98 | 95 | 96 | 95 | 95 | 95 | 86 | 98 | 95 | 96 | 93 | 95 | 95 | 100 |
| Sp-s | 96 | 95 | 96 | 95 | 96 | 94 | 96 | 95 | x | 95 | 93 | 90 | 95 | 93 | 95 | 95 | 95 | 95 | 95 | 85 | 96 | 95 | 95 | 93 | 95 | 95 | 95 |
| Sp-m | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | x | 98 | 95 | 98 | 96 | 96 | 95 | 100 | 100 | 100 | 88 | 96 | 100 | 98 | 98 | 100 | 100 | 95 |
| Sp-1 | 96 | 96 | 96 | 96 | 96 | 96 | 95 | 96 | 95 | 98 | x | 96 | 96 | 95 | 95 | 93 | 98 | 98 | 98 | 86 | 95 | 98 | 86 | 100 | 98 | 98 | 93 |
| Sp-i | 95 | 95 | 95 | 96 | 95 | 95 | 94 | 95 | 94 | 97 | 98 | x | 93 | 91 | 91 | 90 | 95 | 95 | 95 | 83 | 91 | 95 | 93 | 96 | 95 | 95 | 90 |
| Sp-4 | 97 | 97 | 97 | 97 | 96 | 96 | 97 | 97 | 95 | 96 | 96 | 95 | x | 98 | 95 | 96 | 98 | 98 | 98 | 90 | 98 | 98 | 100 | 96 | 98 | 98 | 96 |
| Sp-3 | 98 | 98 | 98 | 97 | 97 | 97 | 97 | 98 | 95 | 97 | 97 | 96 | 98 | x | 93 | 95 | 96 | 96 | 96 | 88 | 96 | 96 | 98 | 95 | 96 | 96 | 98 |
| Sp-2 | 95 | 95 | 95 | 95 | 96 | 96 | 95 | 96 | 96 | 97 | 97 | 96 | 95 | 96 | x | 95 | 96 | 96 | 96 | 86 | 96 | 96 | 95 | 95 | 96 | 96 | 95 |
| Sp-1 | 96 | 96 | 96 | 96 | 97 | 96 | 100 | 97 | 96 | 96 | 95 | 94 | 97 | 97 | 95 | x | 95 | 95 | 95 | 86 | 98 | 95 | 96 | 93 | 95 | 95 | 96 |
| It-2 | 96 | 96 | 96 | 96 | 96 | 96 | 95 | 96 | 95 | 98 | 98 | 97 | 97 | 97 | 97 | 95 | x | 100 | 100 | 88 | 96 | 100 | 98 | 98 | 100 | 100 | 95 |
| Isr-2 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 100 | 98 | 97 | 96 | 97 | 97 | 96 | 98 | x | 100 | 88 | 96 | 100 | 98 | 98 | 100 | 100 | 95 |
| It-1 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 100 | 98 | 97 | 96 | 97 | 97 | 96 | 98 | 100 | x | 88 | 96 | 100 | 98 | 98 | 100 | 100 | 95 |
| Isr-1 | 95 | 95 | 95 | 96 | 94 | 93 | 94 | 95 | 93 | 94 | 94 | 93 | 95 | 96 | 93 | 94 | 94 | 94 | 94 | x | 88 | 88 | 90 | 86 | 88 | 88 | 86 |
| Nl-2 | 97 | 96 | 97 | 96 | 98 | 97 | 97 | 97 | 95 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 97 | 95 | x | 96 | 98 | 95 | 96 | 96 | 98 |
| Nl-1 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 100 | 98 | 97 | 96 | 97 | 97 | 96 | 98 | 100 | 100 | 94 | 97 | x | 98 | 98 | 100 | 100 | 95 |
| Jap | 98 | 97 | 98 | 97 | 98 | 96 | 97 | 97 | 95 | 97 | 97 | 96 | 98 | 98 | 96 | 97 | 97 | 97 | 97 | 96 | 97 | 97 | x | 96 | 98 | 98 | 96 |
| Fr | 96 | 96 | 96 | 96 | 96 | 96 | 95 | 96 | 95 | 98 | 100 | 98 | 96 | 97 | 97 | 95 | 98 | 98 | 98 | 94 | 97 | 98 | 97 | x | 98 | 98 | 93 |
| Col | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 100 | 98 | 97 | 96 | 97 | 97 | 96 | 98 | 100 | 100 | 94 | 97 | 100 | 97 | 98 | x | 100 | 95 |
| Aus | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 96 | 100 | 98 | 97 | 96 | 97 | 97 | 96 | 98 | 100 | 100 | 94 | 97 | 100 | 97 | 98 | 100 | x | 95 |
| Dix | 97 | 97 | 97 | 96 | 97 | 97 | 97 | 100 | 95 | 96 | 96 | 95 | 97 | 98 | 96 | 97 | 96 | 96 | 96 | 95 | 97 | 96 | 97 | 96 | 96 | 96 | x |

**Table 4.** Per cent nucleotide (below diagonal) and amino acid (above diagonal) sequence similarities between p9 MP of different CarMV isolates inclusive of I ndian isolates

| | In-a | In-1 | In-2 | Ch-c | In-3 | usa-2 | usa-1 | Sp-s | Sp-m | Sp-l | Sp-i | Sp-4 | Sp-3 | Sp-2 | Sp-1 | It-2 | Isr-2 | It-1 | Isr-1 | Nl-2 | Nl-1 | Jap | Fr | Col | Aus | Dix |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| In-a | X | 97 | 97 | 96 | 94 | 98 | 97 | 97 | 97 | 97 | 96 | 97 | 98 | 97 | 98 | 96 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 98 |
| In-1 | 98 | X | 100 | 96 | 92 | 98 | 97 | 97 | 97 | 97 | 98 | 97 | 98 | 97 | 98 | 96 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 98 |
| In-2 | 98 | 100 | X | 96 | 92 | 98 | 97 | 97 | 97 | 97 | 98 | 97 | 98 | 97 | 98 | 96 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 98 |
| Ch-c | 96 | 96 | 96 | X | 94 | 97 | 96 | 96 | 98 | 98 | 97 | 96 | 97 | 98 | 97 | 97 | 98 | 98 | 95 | 98 | 98 | 98 | 98 | 98 | 98 | 97 |
| In-3 | 95 | 96 | 96 | 94 | X | 94 | 92 | 92 | 95 | 95 | 94 | 92 | 94 | 95 | 94 | 94 | 95 | 95 | 91 | 95 | 95 | 92 | 95 | 95 | 95 | 94 |
| usa-2 | 98 | 98 | 98 | 98 | 96 | X | 98 | 98 | 98 | 98 | 97 | 98 | 100 | 98 | 100 | 97 | 98 | 98 | 97 | 98 | 98 | 98 | 98 | 98 | 98 | 100 |
| usa-1 | 96 | 97 | 97 | 96 | 94 | 98 | X | 97 | 97 | 97 | 96 | 97 | 98 | 97 | 98 | 96 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 98 |
| Sp-s | 97 | 98 | 98 | 96 | 94 | 98 | 96 | X | 97 | 97 | 96 | 97 | 98 | 97 | 98 | 96 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 98 |
| Sp-m | 97 | 97 | 97 | 97 | 96 | 98 | 97 | 97 | X | 100 | 98 | 97 | 98 | 100 | 98 | 98 | 100 | 100 | 96 | 100 | 100 | 97 | 100 | 100 | 100 | 98 |
| Sp-l | 98 | 98 | 98 | 98 | 96 | 99 | 98 | 98 | 99 | X | 98 | 97 | 98 | 100 | 98 | 98 | 100 | 100 | 96 | 100 | 100 | 97 | 100 | 100 | 100 | 98 |
| Sp-i | 97 | 98 | 98 | 97 | 96 | 98 | 97 | 97 | 99 | 99 | X | 96 | 97 | 98 | 97 | 97 | 98 | 98 | 95 | 98 | 98 | 96 | 98 | 98 | 98 | 97 |
| Sp-4 | 98 | 98 | 98 | 97 | 96 | 99 | 98 | 98 | 98 | 99 | 98 | X | 98 | 97 | 98 | 98 | 97 | 97 | 96 | 97 | 97 | 97 | 97 | 97 | 97 | 98 |
| Sp-3 | 98 | 98 | 98 | 96 | 95 | 98 | 97 | 97 | 98 | 98 | 98 | 98 | X | 98 | 100 | 97 | 98 | 98 | 97 | 98 | 98 | 98 | 98 | 98 | 98 | 100 |
| Sp-2 | 98 | 98 | 98 | 97 | 96 | 98 | 97 | 97 | 99 | 99 | 99 | 98 | 99 | X | 98 | 98 | 100 | 100 | 96 | 100 | 100 | 97 | 100 | 100 | 100 | 98 |
| Sp-1 | 98 | 98 | 98 | 98 | 96 | 100 | 98 | 98 | 98 | 99 | 98 | 99 | 98 | 98 | X | 97 | 98 | 98 | 97 | 98 | 98 | 98 | 98 | 98 | 98 | 100 |
| It-2 | 97 | 97 | 97 | 96 | 95 | 98 | 96 | 96 | 98 | 98 | 98 | 98 | 98 | 99 | 98 | X | 98 | 98 | 95 | 98 | 98 | 96 | 98 | 98 | 98 | 97 |
| Isr-2 | 97 | 97 | 97 | 97 | 96 | 98 | 97 | 97 | 98 | 99 | 99 | 98 | 98 | 99 | 98 | 98 | X | 100 | 96 | 100 | 100 | 97 | 100 | 100 | 100 | 98 |
| It-1 | 97 | 97 | 97 | 97 | 96 | 98 | 97 | 97 | 100 | 99 | 99 | 98 | 98 | 99 | 98 | 98 | 100 | X | 96 | 100 | 100 | 97 | 100 | 100 | 100 | 98 |
| Isr-1 | 97 | 97 | 97 | 95 | 94 | 97 | 96 | 96 | 100 | 97 | 97 | 97 | 97 | 97 | 97 | 96 | 97 | 97 | X | 96 | 96 | 96 | 96 | 96 | 96 | 97 |
| Nl-2 | 98 | 98 | 98 | 97 | 96 | 98 | 97 | 97 | 97 | 99 | 99 | 98 | 99 | 100 | 98 | 99 | 99 | 99 | 97 | X | 100 | 97 | 100 | 100 | 100 | 98 |
| Nl-1 | 97 | 97 | 97 | 97 | 96 | 98 | 97 | 97 | 99 | 99 | 99 | 98 | 98 | 99 | 98 | 98 | 100 | 100 | 97 | 99 | X | 97 | 100 | 100 | 100 | 98 |
| Jap | 97 | 98 | 98 | 96 | 95 | 98 | 96 | 97 | 100 | 98 | 97 | 98 | 97 | 97 | 98 | 96 | 97 | 97 | 96 | 97 | 97 | X | 97 | 97 | 97 | 98 |
| Fr | 97 | 97 | 97 | 97 | 96 | 98 | 97 | 97 | 97 | 99 | 99 | 98 | 98 | 99 | 98 | 98 | 100 | 100 | 97 | 99 | 100 | 97 | X | 100 | 100 | 98 |
| Col | 97 | 97 | 97 | 97 | 96 | 98 | 97 | 97 | 100 | 99 | 99 | 98 | 98 | 99 | 98 | 98 | 100 | 100 | 97 | 99 | 100 | 97 | 100 | x | 100 | 98 |
| Aus | 97 | 97 | 97 | 97 | 96 | 98 | 97 | 97 | 100 | 99 | 99 | 98 | 98 | 99 | 98 | 98 | 100 | 100 | 97 | 99 | 100 | 97 | 100 | 100 | x | 98 |
| Dix | 97 | 98 | 98 | 96 | 95 | 98 | 99 | 97 | 100 | 98 | 98 | 98 | 98 | 98 | 98 | 97 | 98 | 98 | 96 | 98 | 98 | 97 | 98 | 98 | 98 | x |

**Table 5.** Different recombination events observed using RDP in twenty-two CarMV isolates reported from different parts of the world

| Isolate | Major parent | Minor parent | Detected by program |
|---|---|---|---|
| Sp-4 | Nl-1 | Aus | MaxChi, Chimaera |
| Sp-4 | Isr-2 | Col | MaxChi, Chimaera |
| Sp-4 | Isr-2 | Aus | MaxChi, Chimaera |
| Sp-1 | Fr | USA-2 | RDP, GENECONV, MaxChi, Chimaera, SiScan |
| Sp-1 | Fr | USA-2 | RDP, GENECONV, MaxChi, Chimaera, SiScan |
| Isr-1 | Isr-2 | Sp-1 | GENECONV, MaxChi |
| Isr-1 | In-A | Sp-1 | GENECONV, MaxChi |
| Col | It-1 | InP | MaxChi, Chimaera, SiScan |
| Aus | It-1 | InP | MaxChi, Chimaera |
| InA | Sp-s | Sp-2 | MaxChi, Chimaera |

Aus, *Carnation mottle virus*, isolate Australia AJ309492; Col, *Carnation mottle virus*, isolate Columbia AJ309493; Fr, *Carnation mottle virus*, isolate France AJ309494; InA, *Carnation mottle virus*, Annual carnations isolate; InP, *Carnation mottle virus*, Perennial carnations isolate AJ811998; Isr-1, *Carnation mottle virus*, Isolate Israel AJ309498; Isr-2, *Carnation mottle virus*, Isolate Israel AJ309501; It-1, *Carnation mottle virus*, isolate Italy AJ309499; Nl-1, *Carnation mottle virus*, isolate The Netherlands AJ309496; Sp-1, *Carnation mottle virus*, isolate Spain AJ309503; Sp-2, *Carnation mottle virus*, isolate Spain AJ309504; Sp-4, *Carnation mottle virus*, isolate Spain AJ309506; USA-2, *Carnation mottle virus*, isolate USA AJ309512.

served sequences. But an exception with respect to the p7 gene was observed, where amino acid similarity decreases to the range of 85–95% compared to nucleotide similarity of 95–98%. The Indian isolates showed minimum amino acid similarity with an Israeli isolate (AJ309498), while maximum similarity was observed with Spanish and Japanese isolates. Comparison between the different strains available in the database in terms of nucleotide as well as amino acid similarity has been presented in Table 3.

MP gene p9 showed 92–98% amino acid similarity with the available sequences in the database. One of the Indian isolates (AJ584842) showed minimum amino acid similarity (92%) with isolates from Japan (AJ309495) and Spain (AJ309506), while all other Indian isolates showed maximum similarity (98%) with isolates from USA (AJ309512) and Spain (AJ309505) (Table 4). Thus it can be concluded that MP genes of different Indian isolates were found to be closely related to isolates reported from Spain and USA.

The most conserved region was found to be located in the capsid protein gene. The different isolates reported from other parts of the world and all the Indian isolates, including annual carnation isolate were found to show high level of similarity with respect to both nucleotide (95–98%) as well as amino acid (96–99%) sequences (Table 2).

The amino acid sequence from each CarMV complete genome ORF was compared with the homologous one from each carmovirus. In general, the CarMV replicase protein was found most conserved in other carmoviruses, except in the case of viruses that have higher identity in nucleotide sequence with CarMV.

For recombination studies, MP and CP genes at a stretch were taken because in the genome they are present in the same fashion. There are few events observed for CarMV isolates, suggesting the high level of genetic stability in CarMV isolates. This is supported by the fact that carnation cuttings are propagated vegetatively and once established in the cuttings, the virus will remain there for generations to come[20]. The recombination event observed in InA indicates the necessity for successful survival in carnations growing from seeds. Both major and minor parents are strains from Spain (Sp-s and Sp-2 respectively). An Israeli isolate showing recombinant events was supported by the phylogenetic tree where it forms a separate group. The isolate might be the natural variant of CarMV. The prerequisite for successful recombination is that both parental strains must co-infect the same host[26–28].

CarMV isolates can be divided into six different groups on the basis of RDP, which involved six different parameters for the determination of phylogenetic studies[13]. Group I has isolates from USA only, while group III has viral isolate infecting seed-grown carnations. One interesting feature observed is that while group II has members showing no recombination, group IV members show recombination in the genes taken under consideration. Groups V and VI have one member each and both are from Spain.

The data presented here indicate: (i) Multiplex RT–PCR is increasingly becoming the choice of reaction system because of economic aspects and time-saving properties. (ii) This is the first study on complete CarMV genome of an Indian isolate. Further it was concluded that the CarMV genome is highly conserved and its isolates separated in time and space, present high genetic stability as reported earlier[29]. High genetic stability of CarMV may be because of global trade of carnation and its vegetative propagation that has helped in dispersal of the virus around the world.

(iii) CarMV isolates can be divided into six groups based on RDP, which involved generation of data on the basis of six different parameters.


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