Emaravirus-specific degenerate PCR primers allowed the identification of partial RNA-dependent RNA polymerase sequences of Maize red stripe virus and Pigeonpea sterility mosaic virus

Toufic Elbeaino[a], Anna Whitfield[b], Mamta Sharma[c], Michele Digiaro[a]

[a] Istituto Agronomico Mediterraneo di Bari, Via Ceglie 9, 70010 Valenzano, Bari, Italy

[b] Kansas State University, Department of Plant Pathology, Manhattan, KS, USA

[c] International Crop Research Institute for the Semi-Arid Tropics, Patancheru 502324, Andhra Pradesh, India

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Emaravirus-specific degenerate PCR primers allowed the identification of RdRp sequences of Maize red stripe virus (MRSV) and Pigeonpea sterility mosaic virus (PPSMV)

Toufic Elbeaino[a], Anna Whitfield[b], Mamta Sharma[c] and Michele Digiaro[a],

[a]*Istituto Agronomico Mediterraneo di Bari, Via Ceglie 9, 70010 Valenzano, Bari, Italy.*
[b]*Kansas State University, Department of Plant Pathology, Manhattan, KS, USA.*
[c]*International Crop Research Institute for the Semi-Arid Tropics, Patancheru 502324, Andhra Pradesh, India.*

Abstract

Local areas of conserved amino acids sequences in the RNA dependent RNA polymerase gene (RdRp), denoted as pre-motif A, motifs A and C, of viruses putatively belonging to the newly established genus *Emaravirus*, were used for designating sets of downstream and upstream degenerate oligonucleotide primers able to amplify in reverse transcription-polymerase chain reaction assay (RT-PCR) DNA fragments of 276 bp and 360 bp in size. These primers were efficient to detect Emaraviruses with known sequences available in database, i.e., *Fig mosaic virus*

(FMV) and *European mountain ash ringspot-associated virus* (EMARaV), together with Pigeonpea sterility mosaic virus (PPSMV) and Maize red stripe virus (MRSV), of which only short sequences limitedly to their RNA3 segments constitute the unique molecular information available in Genbank. In particular, the degenerate primers designated on pre-motif A and motif A sequences were successful to amplify (276 bp) the four Emaraviruses used as positive controls while those of motifs A and C have failed to detect only MRSV. The amino acids sequences obtained from PPSMV and MRSV shared the highest identity with those of RRV (69%) and RYRV (60%), respectively. The phylogenetic tree constructed with RNA1 sequences of Emaravirus-like viruses clustered PPSMV and MRSV in two separate clades close to RRV and RLBV, respectively. The newly developed degenerate primers have proved their efficacy to amplify new Emaravirus-specific sequences and accordingly they could be useful to fish-out new Emaravirus-like viruses present in nature similarly to those reported in the literature and commonly used for virus identification.

Keywords: *Emaravirus; RdRp*; degenerate primers; RT-PCR, sequencing, phylogeny.

1. Introduction

Emaravirus is a recently established viral genus (Muehlbach and Mielke-Ehret, 2011), which includes segmented, negative-single stranded RNA viruses with enveloped (double membraned-bodies, DMBs), approximately spherical, particles of 80-200 nm in diameter. The genus deems its' acronym to the *European mountain ash ringspot-associated virus* (EMARaV), firstly identified and is the type species of this genus, which has a genome composed of four RNA segments (Mielke and Muehlbach, 2007). *Fig mosaic virus* (FMV) is an another approved species (Adams and Carstens, 2012) of the recently established genus *Emaravirus* with a genome composed of six single-stranded negative-sense RNA that has recently been recognised as the causal agent of fig mosaic disease (FMD) (Elbeaino *et al*., 2009a; 2009b, 2012). Intracellular DMBs were identified as particles of the putative causal agents of fig mosaic and of the ringspot disease of European mountain ash (Mielke and Muehlbach, 2007; Elbeaino *et al*., 2009a; 2009b). In addition, the same structures were also observed in infected cells of mosaic-diseased plants of pigeonpea (*Cajanus cajan* L.), maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.), thought to be induced by Pigeonpea sterility mosaic virus (PPSMV) and Maize red stripe virus (MRSV) (Kumar *et al*., 2002; Ahn *et al*., 1998; Jensen *et al*., 1996).

Besides EMARaV, FMV, PPSMV and MRSV, three novel viruses with DMBs structures have recently joined the list, i.e. Redbud yellow ringspot virus [RYRV, Laney *et al.* (2011)], Rose rosette virus [RRV, Laney *et al*. (2012)] and Raspberry leaf blotch virus [RLBV, McGavin *et al*. (2012)]. Most of these viruses are vectored in nature by eriophyid mites and have a multipartite RNA genome, with a number of segments differing among species, i.e. four in the case of EMARaV, RRV and RYRV (Mielke and Muehlbach, 2007; Laney *et al*., 2011), five for RLBV (McGavin *et al*., 2012), six for FMV and PPSMV (Elbeaino *et al*., 2012; Kumar *et al*., 2002) and up to seven for MRSV (Jensen *et al*., 1996). In general, each segment of the genome contains a single ORF that encodes, in order, the polymerase, putative glycoprotein, putative nucleocapsid genes together with other RNA-encoding proteins with unknown functions.

Members of genus *Emaravirus*, an unassigned genus to any family of plant viruses, share some distant similarities with viruses belonging to the family *Bunyaviridae* and the genus *Tenuivirus* (Elbeaino et al., 2009a, 2009b). However, the number of genome segments (at least four) distinguishes the genus *Emaravirus* from bunyaviruses and sequence analyses do not allow classification within either the family *Bunyaviridae* or the genus *Tenuivirus* (Muehlbach and Mielke-Ehret, 2011).

All yet unassigned viruses are considered as potential members of the genus *Emaravirus* because of their structural, epidemiological and molecular features similar to EMARaV; whereas most of them was fully or almost completely sequenced and characterized. Uniquely, PPSMV and MRSV had remained unexplored molecularly whose only short nucleotides sequence restricted to their RNA3 segment constitute the unique information available in Genbank. This lack of molecular

information on their genomes has always penalised their definite classification in a genus of plant viruses and in particular to *Emaravirus* genus.

Noteworthy mentioning that these viruses (DMBs, Emaravirus-like viruses) have been so long observed in parenchymatic cells of infected plants species (Bradfute et al., 1970; Kim and Martin, 1978; Appiano, 1982; Gergerich and Kim, 1983; Martelli et al., 1993; Ahn et al., 1996).

Nevertheless, the difficult identification of these viruses using most frequently serological and\or molecular techniques has long hindered the knowledge of their nature, classification and true incidence. Accordingly, PPSMV and MRSV make part of this case, and the possibility to identify universal primers able to detect a large number of virus species of the same taxonomic group (*Emaravirus*) has the advantage to simplify the diagnosis, to allow the detection of viruses whose the sequence is still unknown and as a result to ease the task of their classification.

Based on previous experiences reported in the literature in developing degenerate genus-specific primers for the detection of plant viruses belonging to the same taxonomic affiliation; analogously, in this study attempts were made to identify possible conserved motifs in newly identified Emaraviruses genomes and to design universal primers for their detection.

2. Materials and methods

2.1. Virus sources

The different virus used as positive controls in RT-PCR assays were procured from different origins: EMARaV (University of Hamburg, Germany), PPSMV (International Crop Research Institute for the Semi-Arid Tropics, ICRISAT, India), MRSV (Kansas State University, USA) and FMV (Mediterranean Agronomic Institute of Bari, MAIB, Italy).

2.2. Alignment of RNA segments and design of degenerate primers for Emaraviruses detection

Available nucleotide and amino acids sequences of all RNA segments composing the genome of all Emaravirus-like viruses, i.e. EMARaV, FMV, RRV, RLBV and RYRV (very short nucleotides sequence of PPSMV and MRSV were however used), were retrieved from GenBank and assembled using the Strider 1.1 Program (Marck, 1988). The alignment for homology using the nucleotide and the amino acid sequences was done with the Clustal X Program (Thompson et al., 1997). Viral species and accession numbers of sequences considered in this study are reported in table 1.

2.3. Extraction of total nucleic acid (TNA) and double-stranded RNA (dsRNA)

Total nucleic acids (TNA) were extracted from 200 mg of infected phloematic tissues, homogenised in 1 ml grinding buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc pH 5 and 2.5% w/v PVP-40) and purified using silica particles according to Foissac et al. (2001).

Leaf vein tissues (10 g) from infected plant materials were used to recover dsRNAs by phenol/chloroform extraction and chromatography through cellulose CF-11 column in the presence of 17% ethanol (Elbeaino et al., 2009a). Extracts were followed by selective digestion by DNase and RNase (Dodds, 1993).

2.4. Virus cDNA synthesis

Five hundred ng of TNA or 50 ng of dsRNA extracts were mixed with 0.5 µl random hexamers primer (Roche Diagnostics, Mannheim, Germany) (0.5 µg\µl) and 2 µl of sterile water,

denatured at 95°C for 5 min and kept in ice for 3 min. Reverse transcription was carried out for 1 h at 39°C in 1 µl (200 Units\µl) of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 4 µl buffer 5× (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 µM MgCl2), 2 µl of 100 mM DTT and 0.5 µl of 10 mM dNTPs. A final step for enzyme denaturation was conducted at 70°C for 10 min.

2.5. PCR

cDNA mixtures (2.5 µl) were submitted to PCR amplification in 2.5 µl of 10× Taq polymerase buffer (Promega Corp., Madison, USA), 2.5 µl of 25 µM MgCl2, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM primer "Pre-motif A-sense" (5'- AT(AC) TT(TC) (AT)A(TC) AAA GA(TC) CAA AG-3') in conjunction with primer "Motif A-antisense" (5'- GC(AT) GA CCA TTT (ATC)GA TGC ATC- 3') and\or primer "Motif A-sense" (5'- GAT GCA TC(GAT) AAA TGG TC(TA) GC- 3') in conjunction with primer "Motif C-antisense" (5'- ATC ATC (AT) GA (GA) TG (TAC) ACC AT- 3') and 0.2 µl of Taq polymerase (5 U\µl) in a final volume of 25 µl. In PCR, these two sets of primers pairs generate two DNA fragments of 270 bp and 360 bp in size (Fig. 2). The cDNA amplification was carried out in a Perkin Elmer Cetus Thermal Cycler apparatus with 35 cycles, after an initial denaturation at 94°C for 5 min. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 48°C for 50 sec, and extension at 72°C for 40 sec. Final elongation was carried out at 72°C for 7 min. PCR products were electrophoresed in 1.2% agarose-TBE gel, stained with ethidium bromide and visualized under ultraviolet illuminator. Amplicon size was determined by comparison with DNA XIV markers (Fermentas, Milan, Italy).

2.6. Computer-assisted analysis

All PCR products were directly cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions, and used to transform *Escherichia coli* DH5a competent cells. Selected clones were subjected to automated sequencing (Primm) and sequences were assembled with the Strider 1.1 program (Marck, 1988). Alignment for nucleotide and amino acid homology was done with the CLUSTALX program (Thompson et al., 1997). Homology with known proteins from the Protein Information Resources (PIR, release 47.0) was determined using the FASTA (Pearson & Lipman, 1988) and BLAST (Altschul et al., 1990) programs. Tentative phylogenetic trees were constructed using the NJPLOT package (Perrière and Gouy, 1996) with 1000 bootstrap replicates

3. Results

3.1. Design of oligonucleotide degenerate primers for Emaravirus-like viruses detection

Comparison of the nucleotides and predicted amino acid sequence of all RNA segments of Emaravirus-like viruses with their homologue showed that RNA1 is the most conserved segments among all others analyzed, for which that of FMV, EMARaV, RLBV, RRV and RYRV revealed the presence of five highly conserved regions denoted as motifs (A–E) in the RdRp gene, similarly to what has been previously reported with FMV and EMARaV by Elbeaino et al., (2009a, 2009b). Computer-assisted analysis showed that among all five analyzed regions, the pre-motif A, motifs A and C were the most conserved ones (data not shown) (Fig. 1). Accordingly, of a number of sense and anti-sense primers designed on these conserved sequences of members of *Emaravirus* genus, the most reliable sets of primers in several preliminary tests were here reported.

3.2. Amplification of partial RdRp gene

RT-PCR assay conducted on reverse-transcribed RNA templates extracted with Silica particles, using the first set of oligonucleotide degenerate primers (pre-motif A- sense and motif A-antisense) showed to be effective to detect FMV, EMARaV, PPSMV and MRSV and yielded DNA fragments of expected size (276-279 bp) (Fig. 2a). Furthermore, the second set of primers (motif A-sense and motif C-antisense) generated the expected amplicon (360-363) in all viruses, except for MRSV (Fig. 2b). Similar PCR results were obtained when dsRNA extracts were used as a template; nevertheless nonspecific DNA amplification, which were observed with RNA templates, here became unnoticeable in the agarose gel due to the removal of plant nucleic acids an other contaminants (data not shown).

3.3. *Sequence and phylogenetic analyses*

The newly identified sequences from RNA1 segments of PPSMV (636 bp) and MRSV (260 bp) were compared with homologous regions of Emaravirus-like viruses present in Genbank.
In the case of PPSMV, the highest nucleotides identity reached 67% and was found with FMV and RRV, while at the amino acids level, PPSM shared 67% and 69% identity with FMV and RRV, respectively (Tab. 1). Almost similar to PPSMV, 70% and 56%, were the values of nucleotides and amino acids sequence identity that MRSV shared with RRV, respectively.

The extensive sequence analysis conducted on all RNA segments, separately, composing the genome of Emaravirus-like viruses showed that the RdRp gene (RNA1 segment) is the most conserved segment with 68% and 67% identity (with FMV and RRV) at the nucleotides and amino acids level, respectively (Tab. 1).
Results also showed that the range of identity found in other RNA segments of Emaraviurses was lower than that found in RNA1 and reached insignificant homologies, though RNA4, 5 and 6, showed no homology to any viral protein present in Genbank. Accordingly, no conserved stretches of amino acids were identified during our sequence analyses and therefore no primers were found suitable to be designed on these segments for the detection of Emaraviruses.
The phylogenetic tree constructed with sequences of RNA1 segments, in particular with those identified of PPSMV and MRSV, showed that Emaraviruses are grouped in three different clusters and further confirm the relatedness of these viruses to a same taxonomical affiliation (Fig. 3).

4. Discussion

Although *Emaravirus* is a novel viral genus with characteristics rather consolidated, whose member species share similar morphological, symptomatological and epidemiological features, likely resulting in a largely homogenous genetic composition.
The search for genomic portions highly conserved, here in our case, is of great interest since it theoretically should enable to: a) draw sets of genus-specific primers capable of detecting the presence of different emaraviruses in infected plants, regardless of the viral species involved; b) detect and sequence genomic portions of viruses considered to be potential members of the genus *Emaravirus* that have been only partially (as in the case study of PPSMV and MRSV) or not at all characterized; c) provide new elements for the definition of such viral genus still in formation.
The analysis carried out in this study on the sequences of the genomic region of species already assigned to the genus *Emaravirus* (FMV, EMARaV) and other species of recent acquisition likely belonging to the same genus (RRV, RLBV and RYRSV), allowed us to highlight the presence of several conserved motifs in the RdRp (RNA1), which were used as basis for the design of degenerate primers. Their application in virus diagnosis enabled us to detect the presence of different Emaraviruses in infected plants, as well as to amplify unknown genomic portions from PPSMV and MRSV infected tissues. Sequencing of partial RdRp genes of these two viruses (ca. 636 nt of PPSMV and 276 nt of MRSV) allowed us to highlight their resemblance to RRV and

RLBV as shown in the phylogenetic tree drawn with sequences of the RdRp, thus providing further evidence of the likely membership of these two viruses to the genus *Emaravirus*.
The failure detection of MRSV with the second set of primers used (motif A sense and motif C antisense) could indicate that the sequence of MRSV differs slightly from other Emaraviruses at the motif C region. In fact, rolling down with sequence from pre-motif A to motif E, the level of degeneracy at motifs sites tends to increase (data not shown). However, it was not possible to validate these Emaravirus genus-specific primers on the other viruses (RRV, RLBV and RYR) due to the lack of infected plant material by these viruses in our laboratory. Though, we do believe that their efficacy to detect the untested remaining viruses will not come less than that approved on PPSMV and MRSV, especially that they were initially designed on conserved and already known sequences at primers sites in the RdRp gene.
This is the first report of a RT-PCR assay that detects different species of genus *Emaravirus* using degenerate sets of primers. In our experience this method detected four distinct Emaravirus-like viruses and would theoretically be suitable for the detection of all remained viruses. It cannot be excluded that these same set of primers could be effective also for detecting other still unknown Emaraviruses, since they were designed in very are highly conserved genomic regions, and could represent important sequence starters for their further characterization.

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Fig. 1. Amino acid alignment between conserved RdRp pre-motifs A, motifs A and C of FMV (AM941711), EMARaV (AY653040), RLBV (CBZ42024), RRV (YP_004327589) and RYRV (AEO95760). Regions with high homology are highlighted in gray. Amino acid residues that are conserved in the majority of the viruses are highlighted in black. Numbers correspond to the position of the amino acids in the RdRp region.

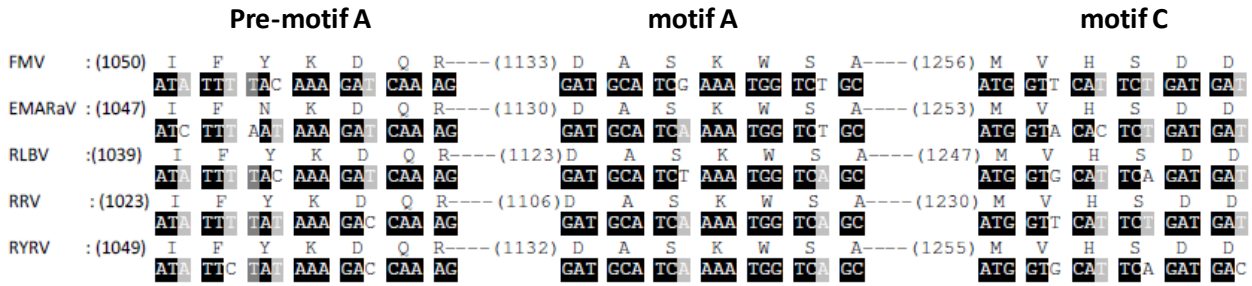

Fig. 2. Electropherogram showing RT-PCR amplifications obtained by using degenerate Emaravirus-specific primers on leaf tissue extracts of plant material infected with MRSV (lane 1), PPSMV (lane 2), FMV (lane 3) and EMARaV (lane 4). All Emaravirus-infected samples yielded expected DNA fragments of ca. 276 bp and 360 bp in size (lanes 1–4) with pre-motif A sense and motif A antisense (left gel) and motif A sense and motif C antisense (right gel) in RT-PCR. Solely, MRSV infected sample (right gel, lane 1) react negatively with primers of motifs A and C. With both primers, the healthy plant material showed negative reactions (lanes 5-7). Lane M: DNA marker XIV (Fermentas Corp., Italy).

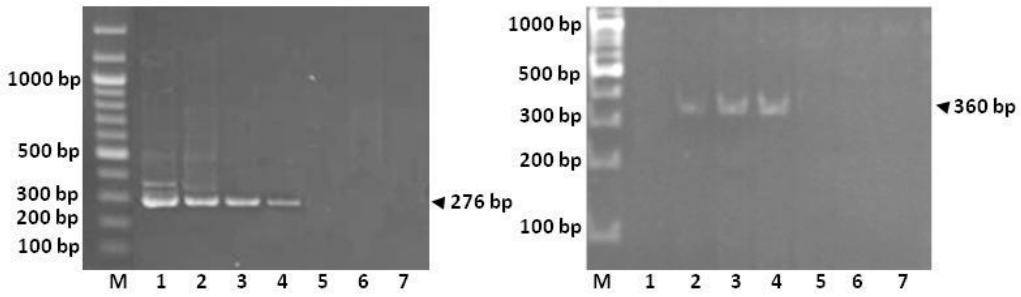

Fig. 3. Phylogenetic tree constructed with predicted aa sequences spanning the pre-motif A, motifs A and C region of RNA1 of assigned (FMV, EMARaV) and unassigned (RRV, RLBV, RYRV, PPSMV and MSV) Emaraviruses. Bootstrap values are shown on the branches. Accessions numbers of RNA1 segment of Emaraviruses are those reported in table 1. *Tomato spotted wilt virus* (TSWV) was used as an outgroup specie (Accession n°. JQ284451).

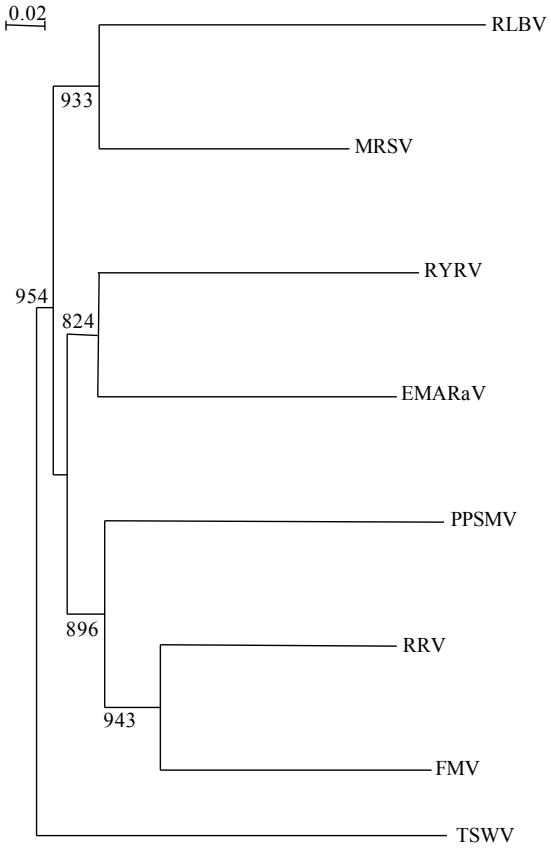

Table 1. Sequence comparison of nucleotides and amino acids (bold) identity matrix of all RNA-encoded polyproteins of Emaraviruses with their homologue. All RNA segments (RNA1 to RNA6) and their accession numbers are reported between brackets: FMV [RNA1 (AM941711), RNA2 (FM864225), RNA3 (FM991954), RNA4 (FM992851), RNA5 (HE803826), RNA6 (HE803827)]; EMARaV [RNA1 (NC_013105), RNA2 (NC_013106), RNA3 (DQ831831), RNA4 (NC_013107)]; MRSV [RNA1 (HE817771), RNA3 (U60141)]; PPSMV [RNA1 (HE817772), RNA3 (AJ439561)]; RLBV [RNA1 (FR823299), RNA2 (FR823300), RNA3 (FR823301), RNA4 (FR823302), RNA5 (FR823303)]; RRV [RNA1 (HQ871942), RNA2 (HQ871943), RNA3 (NC_015300), RNA4 (HQ871945)] and RYRV [RNA1 (JF795479), RNA2 (JF795480), RNA3 (JF795481), RNA4 (JF795482)].

RNA1/RNA2

	FMV	EMARaV	MRSV*	PPSMV**	RLBV	RRV	RYRV
FMV		46 **36**	-	-	39 **24**	59 **49**	50 **38**
EMARaV	55 **48**		-	-	38 **23**	47 **35**	48 **41**
MRSV*	63 **55**	66 54		-	-	-	-
PPSMV**	67 **67**	64 **65**	63 **59**		-	-	-
RLBV	49 **31**	48 **32**	65 **55**	58 **52**		39 **22**	38 **22**
RRV	68 **67**	55 **48**	70 **56**	67 **69**	48 **32**		51 **38**
RYRV	55 **46**	59 **53**	66 **60**	64 **63**	49 **33**	55 **46**	

* 276 nt and ** 636 nt

RNA3/RNA4

	FMV	EMARaV	MRSV	PPSMV	RLBV	RRV	RYRV
FMV		35 **5**	-	-	35 **16**	61 **59**	46 **27**
EMARaV	46 **37**		-	-	27 **7**	34 **5**	38 **5**
MRSV	41 **25**	40 **21**		-	-	-	-
PPSMV	31 **20**	30 **16**	34 **12**		-	-	-
RLBV	33 **20**	34 **20**	51 **32**	34 **11**		37 **20**	37 **20**
RRV	57 **59**	43 **31**	40 **26**	31 **21**	34 **19**		47 **29**
RYRV	44 **36**	49 **44**	42 **24**	34 **21**	37 **18**	43 **32**	

RNA5 of FLM and RLBV showed 35% (aa) and 11% (nt)