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Short communication

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

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A B S T R A C T

Emaravirus is a recently established viral genus that includes two approved virus species: *European mountain ash ringspot-associated virus* (EMARaV) and *Fig mosaic virus* (FMV). Other described but unclassified viruses appear to share biological characteristics similar to emaraviruses, including segmented, negative-single stranded RNA genomes with enveloped virions approximately 80–200 nm in diameter. Sequence analysis of emaravirus genomes revealed the presence of conserved amino acid sequences in the RNA-dependent RNA polymerase gene (RdRp) denoted as pre-motif A, motifs A and C. Degenerate oligonucleotide primers were developed to these conserved sequences and were shown to amplify in reverse transcription-polymerase chain reaction assay (RT-PCR) DNA fragments of 276 bp and 360 bp in size. These primers efficiently detected emaraviruses with known sequences available in the database (FMV and EMARaV); they also detected viruses with limited sequence information such as Pigeonpea sterility mosaic virus (PPSMV) and Maize red stripe virus (MRSV). The degenerate primers designed on pre-motif A and motif A sequences successfully amplified the four species used as positive controls (276 bp), whereas those of motifs A and C failed to detect only MRSV. The amino acid sequences obtained from PPSMV and MRSV shared the highest identity with those of two other tentative species of the *Emaravirus* genus, Rose rosette virus (RRV) (69%) and Redbud yellow ringspot virus (RYRV) (60%), respectively. The phylogenetic tree constructed with 92 amino acid-long portions of polypeptide putatively encoded by RNA1 of definitive and tentative emaravirus species clustered PPSMV and MRSV in two separate clades close to RRV and Raspberry leaf blotch virus (RLBV), respectively. The newly developed degenerate primers have proved their efficacy in amplifying new emaravirus-specific sequences; accordingly, they could be useful in identifying new emaravirus-like species in nature.

Emaravirus is a recently established viral genus (Muehlbach and Mielke-Ehret, 2011) that is unassigned to any family of plant viruses and includes segmented, negative-single stranded RNA viruses with enveloped (double membrane-bodies, DMBs), approximately spherical particles of 80–200 nm in diameter. *European mountain ash ringspot-associated virus* (EMARaV) 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 another approved species (Adams and Carstens, 2012) of the genus *Emaravirus*, with a genome composed of six single-stranded negative-sense RNA segments, that has been recognized recently as the causal agent of fig mosaic disease (FMD) (Elbeaino et al., 2009a,b, 2012). Intracellular DMBs were identified as particles of the putative causal agents of fig mosaic and of the ringspot disease of European mountain ash (Bradfute et al., 1970; Martelli et al., 1993; Mielke and Muehlbach, 2007). In addition, the same structures were observed in infected cells of mosaic-affected plants of pigeonpea (*Cajanus cajan* L.) thought to be induced by Pigeonpea sterility mosaic virus (PPSMV), and of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.), induced by Maize red stripe virus (MRSV) (Kumar et al., 2002; Ahn et al., 1998; Jensen et al., 1996).

In addition to EMARaV, FMV, PPSMV and MRSV, three novel viruses with DMBs structures have recently been described: Rose rosette virus [RRV, Laney et al. (2011a)], Redbud yellow ringspot virus [RYRV, Laney et al. (2011b)], 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., 2011a,b); five for RLBV (McGavin et al., 2012); from

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four to six for MRSV (Jensen et al., 1996) and six for FMV and PPSMV (Ishikawa et al., 2012; Elbeaino et al., 2012; Kumar et al., 2002). In general, each segment of the genome contains a single ORF that encodes, in order, the polymerase, putative glycoprotein, putative nucleocapsid genes, and other proteins with unknown functions.

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

Many putative members of the genus *Emaravirus* share common structural, epidemiological, and molecular features with EMARaV and are now sequenced and characterized, with the exception of PPSMV and MRSV, for which the only sequence information available to date is limited to short fragments of RNA3. The possibility of identifying universal primers able to detect a large number of virus species of the same taxonomic group has the advantage of simplifying the diagnosis, detecting viruses with unknown sequences, and easing the task of classifying them. In this study, conserved motifs have been identified in the genome of definitive and tentative emaraviruses and two sets of genus-specific primers have been designed and RT-PCR detection methods optimized.

Infected plant material of European mountain ash (*Sorbus aucuparia*), pigeonpea (*Cajanus cajan*), maize (*Zea mais*), and fig (*Ficus carica*) used as controls in RT-PCR assays were provided by the University of Hamburg, Germany (EMARaV); the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, Andhra Pradesh, India (PPSMV); Kansas State University, Manhattan, KS, USA (MRSV); and the Mediterranean Agronomic Institute of Bari (MAIB), Italy (FMV). The extraction of viral templates was conducted in MAIB laboratories.

The design of primers was based on nucleotide and amino acid sequences of all RNA segments composing the genome of definitive and tentative emaraviruses available in GenBank; i.e., EMARaV, FMV, RRV, RLBV, and RYRV, and retrieved for sequence homology using the CLUSTALX program (Thompson et al., 1997). Viral species and accession numbers of sequences considered in this study are reported in Table 1.

Total nucleic acids (TNA) were extracted from 200 mg of infected leaf tissue using silica particles according to Foissac et al. (2001). Ten grams of leaf tissue also were used to recover dsRNAs templates by phenol/chloroform extraction and chromatography through cellulose CF-11 according to a protocol described by Elbeaino et al. (2009a). cDNAs were synthesized using 500 ng of TNA and 50 ng of dsRNA extracts mixed with 0.5 μl random hexamers primer according to manufacturer instruction (Roche Diagnostics, Mannheim, Germany).

cDNA mixtures (2.5 μl) were submitted to PCR amplification in 2.5 μl of 10× Taq polymerase buffer (Promega Corp., Madison, WI, USA), 2.5 μl of 25 μM $MgCl_2$, 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 primer pairs generated 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 s, annealing at 48 °C for 50 s, and extension at 72 °C for 40 s. 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 an ultraviolet illuminator. All PCR products were directly cloned into the pGEM-T Easy vector (Promega) according to manufacturer instructions, and selected clones were subjected to automated sequencing (Primm, Milan, Italy).

Homology with known proteins from Protein Information Resources (PIR, release 47.0) was determined using the FASTA (Pearson and 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.

Table 1
Sequence comparison of nucleotides and amino acids (bold) identity matrix of all RNA-encoded polyproteins of emaraviruses with their homologue. The accession numbers of all RNA segments 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)]. Sequence identity with MRSV and PPSMV RNA1 is based only on 276 nt and 636 nt, respectively, whereas that of RNA3 is based on 1329 nt and 764 nt, respectively. RNA5 of FMV and RLBV shared 35% (aa) and 11% (nt) identities.

RNA2 RNA1	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**	

RNA4 RNA3	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**	

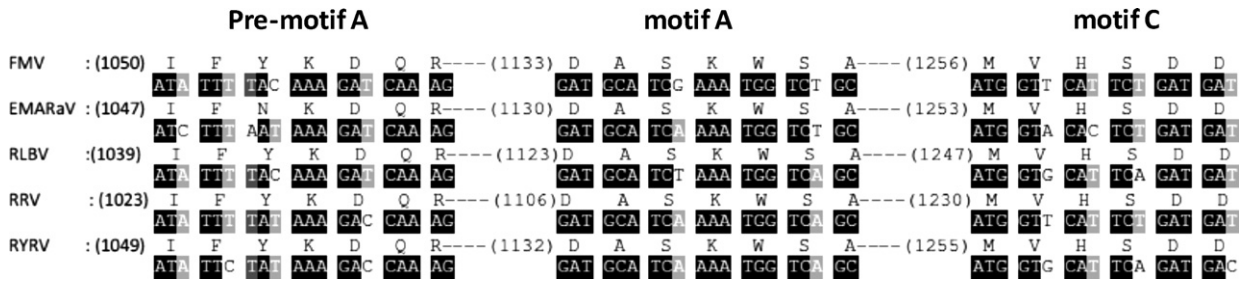

Fig. 1. Amino acid alignment between conserved RdRp pre-motifs A, motifs A and C of FMV (AM941711), EMARaV (AY653040), RLBV (CBZ42024), RRV (YP_004327589), and RYRV (AEO95760). Regions with high homology are highlighted in gray. Amino acid residues that are conserved in the majority of the viruses are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

Comparison of the nucleotide and predicted amino acid sequences of all RNA segments of definitive and tentative emaravirus species showed that RNA1 is the most conserved. Further analysis of RNA1 of FMV, EMARaV, RLBV, RRV, and RYRV revealed the presence of five highly conserved regions denoted as motifs (A–E) in the RdRp gene, similar to what was previously reported for FMV and EMARaV by Elbeaino et al. (2009a,b). Computer-assisted analysis showed that among the five analyzed regions, the pre-motif A, motifs A and C were the most conserved (Fig. 1). Accordingly, of the number of sense and anti-sense primers designed on nucleotide sequences of other motifs (D and E) with less conservation of definitive and putative members of *Emaravirus* genus, only the most reliable sets of primers (A–C) are reported.

RT-PCR 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) was shown to be effective for detecting FMV, EMARaV, PPSMV, and MRSV and yielded DNA fragments of the expected size (276–279 bp) (Fig. 2a). In addition, the second set of primers (motif A-sense and motif C-antisense) generated the expected amplicon (360–363 bp) in all viruses except MRSV (Fig. 2b). The viral nature of the two PCR-generated DNA fragments resulting from the amplifications of partial RdRp of FMV and EMARaV was ascertained by sequencing, and their sequences were found completely analogous to those present in GenBank.

The failure to detect 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, the level of degeneracy tends to increase from pre-motif A to motif E (data not shown). Moreover, it was not possible to validate these genus-specific primers on RRV, RLBV, and RYRV due to the lack of infected plant material in our laboratory; however, because these primers were designed based on sequences also conserved in their RdRp, it is highly probable that they could be effective in the detection of these viruses. The degenerate primers effectively amplified this region from PPSMV and MRSV, viruses without RNA1 sequence data prior to this report.

The newly identified sequences from RNA1 segments of PPSMV (636 bp) and MRSV (260 bp) were compared with the homologous regions of definitive and tentative emaraviruses present in GenBank. In the case of PPSMV, the highest nucleotide identity (67%) was with FMV and RRV (69% at the amino acid level with RRV), but for MRSV it was 70% with FMV (56% at amino acid level) (Table 1). The extensive sequence analysis conducted on all RNA segments composing the genome of definitive and tentative emaravirus species showed that the most conserved RNA1 segment has a level of nucleotide identity from 48% to 70%. The nucleotide identity in the other RNA segments was lower, with values from 38% to 59% for RNA2 and 30% to 57% for RNA3. RNA4, 5, and 6 showed no homology to any viral protein present in GenBank (Table 1). Accordingly, no conserved stretches of amino acids were identified during our sequence analysis of RNA2 and 3; therefore, no universal primers could be designed on these segments for the detection of emaraviruses.

The phylogenetic tree constructed with the sequences of partial RNA1 segments grouped the definitive and tentative emaraviruses in three different clusters and allocated MRSV close to RLBV and PPSMV close to RRV and FMV (Fig. 3), thus providing further evidence (together with their biological features) of their likely membership in the genus *Emaravirus*.

This is the first report of an RT-PCR assay that detects different species of genus *Emaravirus* using degenerate sets of primers. These same sets of primers could be effective for detecting other still-unknown emaraviruses because they were designed in very highly conserved genomic regions and could represent important sequence starters for further characterization of new emaraviruses.

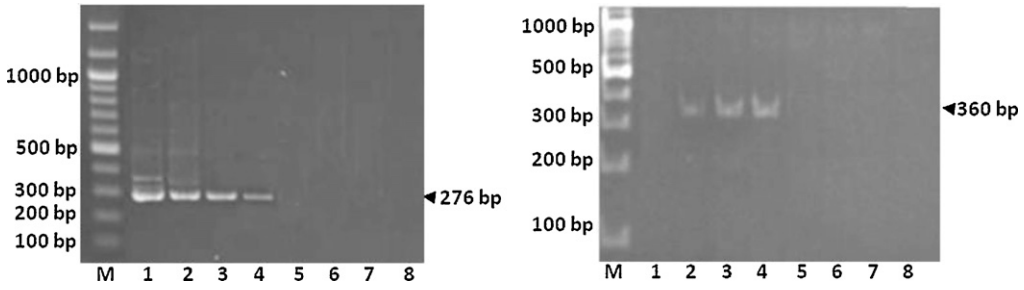

Fig. 2. Electropherogram showing RT-PCR amplifications was obtained using degenerate emaravirus-specific primers on leaf tissue extracts of plant material infected with MRSV (lane 1), PPSMV (lane 2), FMV (lane 3), and EMARaV (lane 4). All emaravirus-infected samples yielded expected DNA fragments of 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 sample infected with MRSV (right gel, lane 1) reacted negatively with primers of motifs A and C. With both primers, the healthy plant material (fig, pigeonpea, maize, and European mountain ash from lane 5 to 8, respectively) showed negative reactions. Lane M: DNA marker XIV (Fermentas Corp., Milan, Italy).

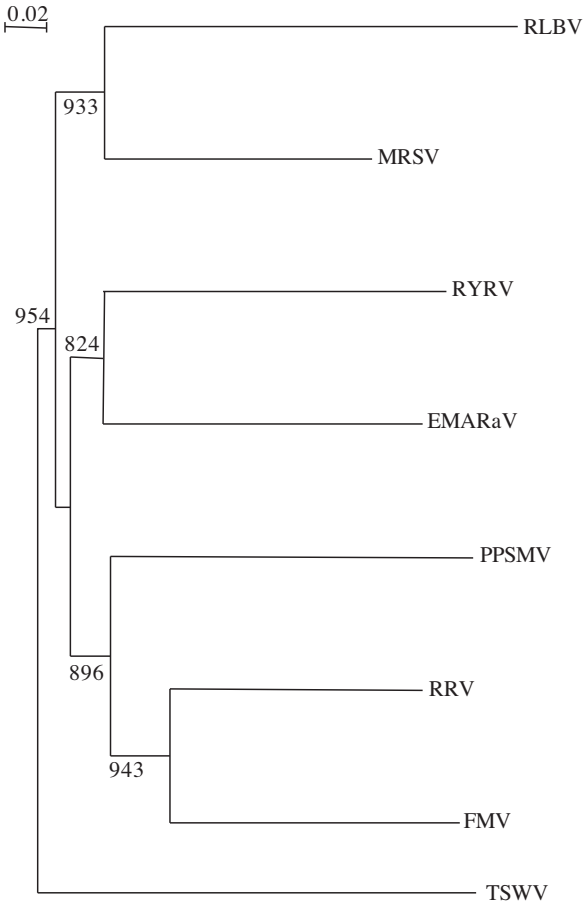

Fig. 3. Phylogenetic tree constructed with predicted aa sequences spanning the pre-motif A, motifs A and C region of RNA1 of assigned (FMV, EMARaV), and unassigned (RRV, RLBV, RYRV, PPSMV and MSV) emaraviruses. Bootstrap values are shown on the branches. Accession numbers of RNA1 segment of emaraviruses are those reported in Table 1. *Tomato spotted wilt virus* (TSWV) was used as an outgroup species (accession no. JQ284451).

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