Identification and characterization of two novel genomic RNA segments of fig mosaic virus, RNA5 and RNA6

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INTRODUCTION

Common fig (Ficus carica) is an important fruit tree species grown in many geographical areas, most notably in the Mediterranean littoral zone (Anonymous, 2008). Trees of this species throughout the world are commonly afflicted with fig mosaic disease (FMD), a disease first described approximately 80 years ago (Condit & Horne, 1933) and first seen in Japan approximately 50 years ago (Komuro, 1962). The identity of the causal agent of the disease has long been elusive, although the eriophyid mite Aceria ficus was implicated in its transmission a long time ago (Flock & Wallace, 1955).

Recently, a novel negative-strand RNA virus was detected in FMD-symptomatic fig trees in various countries, including Italy, USA, Turkey and Japan (Elbeaino et al., 2009a; Walia et al., 2009; Çaglayan et al., 2010; Ishikawa et al., 2012). The nucleotide sequence of this virus has significant identity to that of European mountain ash ringspot-associated virus (EMARaV) (Benthack et al., 2005). Based on its consistent association with symptoms of FMD (Elbeaino et al., 2009a; Walia et al., 2009) and on its mite transmissibility (Çaglayan et al., 2010), the virus has been recognized as a causal agent of FMD and named fig mosaic virus (FMV). Elbeaino et al. (2009b) reported that the FMV genome of an Italian isolate consists of four negative-strand RNA segments. Based on the sequence homologies of their nucleocapsid protein (NP) genes and on their eriophyid mite transmissibility, FMV and EMARaV are thought to be closely related to a group of unclassified negative-strand RNA viruses that includes rose rosette virus (RRV; Laney et al., 2011), raspberry leaf blotch virus (RLBV; McGavin et al., 2012), pigeonpea sterility mosaic virus (PPSMV; Kumar et al., 2003) and maize red stripe virus (MRSV; Skare et al., 2006). These viruses have been suggested to collectively comprise a new genus Emaravirus (Elbeaino et al., 2009b; Laney et al., 2011; McGavin et al., 2012).

Although the genome sequences PPSMV and MRSV have not yet been thoroughly analysed, the genomes of all putative members of the genus Emaravirus are thought to consist of four or five negative-strand RNA segments.
(Mielke & Muhlbach, 2007; Elbeaino et al., 2009b; Laney et al., 2011; McGavin et al., 2012). Based on their sequences, RNA1, RNA2, RNA3 and RNA4 are thought to be complementary sense genes for RNA-dependent RNA polymerase (RdRp), glycoprotein, NP and movement protein, respectively. Among the putative members of the genus *Emaravirus*, only RLBV possesses a fifth RNA segment; this segment (known as RNA5) is a complementary sense gene for a protein (known as p5) of unknown function (McGavin et al., 2012).

A common feature of segmented, negative-stranded RNA viruses is conservation of, and high inverted complementarity between, the nucleotide sequences at the 3'- and 5'-terminals (Nguyen & Haenni, 2003; Walpita & Flick, 2005). This feature appears to be common also to viruses of the proposed *Emaravirus* genus: for EMARaV, FMV, RRV and RLBV, each end of each genomic segment has a highly conserved 13 nt stretch that is almost perfectly complementary to that at the opposite end (**AGUAGGUUCUCC**-**GGAGUUCACUACU**-3') (Mielke & Muhlbach, 2007; Elbeaino et al., 2009b; Laney et al., 2011; McGavin et al., 2012). These complementary terminal sequences enable viral RNA genomes to form a panhandle structure that is involved in their replication and translation (Barr et al., 2003; Kohl et al., 2004; Barr & Wertz, 2005; Gultyaev et al., 2010).

In the present study, we identified two previously unrecognized FMV RNA segments in fig leaves with typical FMD symptoms using RT-PCR with primers based on the 3'- and 5'-terminal 13 nt stretches. These novel RNA segments were found in all of the 14 individual fig plants tested. We found that the structural organization of these RNA segments, designated RNA5 and RNA6, was similar to that of other FMV genome segments: each contains a single ORF on the viral complementary RNA strand (vcRNA) flanked by UTRs that include the conserved, complementary terminal sequences. Moreover, phylogenetic analyses revealed similar evolutionary patterns among RNA3, RNA4, RNA5 and RNA6. These findings suggest that RNA5 and RNA6 are genomic RNA segments of FMV.

### RESULTS

#### Detection of FMV by RT-PCR analysis

Fourteen individual fig trees suspected to be infected with FMV were collected from Japan (eight trees) and Serbia (six trees) (Table 1). Total RNA was extracted from symptomatic leaves of these fig plants and used as a template for RT-PCR amplification with the primer set E5-s/E5-a, which can be used to detect FMV (Elbeaino et al., 2009a). An approximately 300 bp fragment, derived from RNA1, was amplified from each of the 14 individual trees (Fig. 1a). These amplified fragments were 86.5–92.3 % identical in their nucleotide sequences to that of an Italian FMV isolate (Gr10) reported previously (Table S3a, available in JGV Online), confirming that all of the plants were infected with FMV. The 14 FMV isolates were designated as shown in Table 1.

#### Simultaneous amplification of FMV RNA segments with terminal sequence-specific primers

In an attempt to simultaneously amplify all four of the known viral RNA segments of FMV (RNA1, RNA2, RNA3 and RNA4), we performed RT-PCR using primers based on the conserved 13 nt stretches found at the 3'- and 5'-termini of FMV genomic segments. The sequences of these primers, designated 3TerE for the 5'-termination and 5TerE for the 3'-termination, were **AGUAGGUUCUCC**- and **GGAGUUCACUACU**-3', respectively. Unexpectedly, however, electrophoretic analysis of the PCR products suggested that five or six fragments rather than four were amplified from fig leaves infected with the JS1 and SB1 isolates (Fig. S1). While three of the fragments (approx. 7, 2.2 and 1.5 kbp) were similar in size to those corresponding to the four known...

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**Table 1. Collection of FMV isolates from Japan and Serbia**

<table>
<thead>
<tr>
<th>No.</th>
<th>Geographical origin (provinces)</th>
<th>Varieties of figs</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Japan (Shimane)</td>
<td>Houraishi</td>
<td>JS1</td>
</tr>
<tr>
<td>2</td>
<td>Japan (Fukuoka)</td>
<td>Houraishi</td>
<td>JF1</td>
</tr>
<tr>
<td>3</td>
<td>Japan (Fukuoka)*</td>
<td>Houraishi</td>
<td>JFT4</td>
</tr>
<tr>
<td>4*</td>
<td>Japan</td>
<td>Athens</td>
<td>JTT-At</td>
</tr>
<tr>
<td>5*</td>
<td>Japan</td>
<td>King</td>
<td>JTT-K</td>
</tr>
<tr>
<td>6*</td>
<td>Japan</td>
<td>Lisa</td>
<td>JTT-Li</td>
</tr>
<tr>
<td>7*</td>
<td>Japan</td>
<td>Panachee</td>
<td>JTT-Pa</td>
</tr>
<tr>
<td>8†</td>
<td>Japan</td>
<td>Violette de Sollies</td>
<td>JTT-Vi</td>
</tr>
<tr>
<td>9</td>
<td>Serbia</td>
<td></td>
<td>SB1</td>
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<tr>
<td>10</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>14</td>
<td>Serbia</td>
<td></td>
<td>SB2-6</td>
</tr>
</tbody>
</table>

*Represents a different commune from no. 2.
†Represents the isolate derived from fig trees that are maintained in our laboratories.

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**Fig. 1. Electrophoresis of RT-PCR products obtained from 14 FMV isolates using the primers E5-s and E5-a (a) or the terminus-specific primers 3TerE and 5TerE (b). H, Asymptomatic fig leaves from Japan. Lane numbers correspond to isolate numbers shown in Table 1.**
FMV genomic RNA segments (RNA1: 7039 nt, RNA2: 2252 nt, RNA3: 1490 nt and RNA4: 1472 nt) (Elbeaino et al., 2009b), the lengths of the other two fragments (approx. 1.7 and 1.2 kbp) did not correspond to any known FMV genomic RNA segments. Furthermore, when RT-PCR was carried out using healthy fig samples, none of the five or six fragments were amplified, indicating that the two novel fragments did not result from non-specific amplification.

We suspected that the two unexpected, FMV-specific fragments originated from previously undiscovered, additional genomic RNA segments of FMV. To confirm that these putative RNA segments were present in other FMV isolates, we performed RT-PCR with the 3TerE/5TerE primer set on the other 12 FMV isolates. Like JS1 and SB1, all of the FMV isolates yielded novel bands at approximately 1.7 and 1.2 kbp, as well as bands corresponding to the known FMV genomic segments (Fig. 1b).

Cloning and sequencing of all of the RT-PCR amplified fragments from JS1 and SB1 confirmed that the 7 and 2.2 kbp fragments were derived from FMV RNA1 and RNA2, respectively; the fragments from JS1 were 88.9 and 92.7% identical in sequence to RNA1 and RNA2 from Gr10, respectively, while those from SB1 were 91.7 and 94.0% identical in sequence to RNA1 and RNA2 from Gr10, respectively. Sequencing also confirmed that the 1.5 kbp fragment was derived from FMV RNA3 and RNA4 (Table S2). In contrast, the nucleotide sequences of the novel 1.7 and 1.2 kbp fragments had no significant homology with the four known FMV RNA segments.

We next used rapid amplification of cDNA ends (RACE) analysis to obtain the full-length sequences of the amplified fragments from JS1 and SB1. This analysis confirmed that the 1.7 and 1.2 kbp RNA segments had the same 13 nt stretches at their termini as those conserved in RNA1–4 of FMV, with no substitutions, deletions or additions. These data suggested that the RT-PCR amplified 1.7 and 1.2 kbp fragments were previously unrecognized genomic RNA segments, designated RNA5 and RNA6, respectively.

Sequence analysis of RNA5 and RNA6 RNA5 of JS1 is 1752 nt in length and the vcRNA contained one ORF encoding a predicted 502 aa, 59 kDa protein (hereafter called p5). The polarity of FMV genomic RNAs was determined in the previous study (Elbeaino et al., 2009a). The ORF was flanked by a 57 nt 3′-UTR and an 186 nt 5′-UTR in the viral RNA (vRNA) (Fig. 2; Table S2). RNA5 of SB1 was similar to that of JS1, except that its encoded p5 protein was shorter (486 aa; 57 kDa) because its ORF contained four multiple-nucleotide deletions (Fig. 2). These deletions were 6, 12, 9 and 21 nt in length and corresponded to nt 234–239, 250–261, 346–354 and 1045–1065 of JS1 RNA5, respectively.

Sequence analysis of the 1.7 kbp RNA5 fragments of the other 12 FMV isolates showed that they were nearly identical to RNA5 of JS1 and SB1 in their structural organization and UTR lengths. Nine of the segments had the same pattern of nucleotide deletions in their p5-encoding ORF as that
the RNA6 sequences of the 14 isolates (Fig. S2d). Terminal nucleotide stretches were also conserved among ORFs longer than 200 nt were present. Segment-specific identities in all 14 isolates (Table S2), and no other sense or antisense among the 14 FMV isolates and the Gr10 isolate. The overall amino acid sequences of RNA3, RNA4, RNA5 and RNA6 were compared to the corresponding nucleotide and deduced sequences of FMV isolates

Comparison of nucleotide and amino acid sequences of FMV isolates

To evaluate the genetic diversity of the novel RNA segments, we compared the corresponding nucleotide and deduced amino acid sequences of RNA3, RNA4, RNA5 and RNA6 among the 14 FMV isolates and the Gr10 isolate. The overall nucleotide sequence identities of RNA3 and RNA4 among the isolates were 93.0–99.9 and 93.8–99.9 %, respectively (Table S3b, c), and the NP (encoded by RNA3) and p4 (encoded by RNA4) amino acid sequences exhibited 97.8–100 % identity among the isolates. The nucleotide sequence identities of RNA5 and RNA6 among the isolates were slightly lower than those of RNA3 and RNA4, at 84.4–100 and 90.0–99.8 %, respectively (Table S3d, e), and the predicted amino acid sequence identities of p5 and p6 among the isolates were also lower, at 81.9–100 and 87.1–100 %, respectively. The overall mean genetic distances of RNA3, RNA4, RNA5 and RNA6 among the 14 isolates sequenced in this study were 4.7, 3.3, 8.9 and 5.5 % for nucleotide sequences and 1.1, 1.0, 10.8 and 7.3 % for amino acid sequences, respectively. Thus, among these four RNA segments, RNA5 exhibited the highest genetic diversity.

Northern blot analysis

To further characterize RNA5 and RNA6, we performed Northern blot analysis with sense riboprobes (for detection of vRNA) and antisense riboprobes (for detection of vcRNA). In a previously described Northern blot analysis (Elbeaino et al., 2009a), clear signals were detected only with sense riboprobes. In our experiments, however, probes of both senses yielded signals corresponding to full-length RNA3, RNA5 and RNA6 in infected figs (Fig. 3), confirming that RNA5 and RNA6 vcRNAs are produced during infection and, hence, that RNA5 and RNA6 replicate in plants. We assume that the apparent disagreement between our results and those presented in the previous report might arise from a difference in the sensitivities of the riboprobes used for detection or in the vcRNA/vRNA ratio, which might vary with the plant variety or growth conditions.

Evolutionary association analysis of FMV RNA3, RNA4, RNA5 and RNA6

For segmented viruses, phylogenetic trees constructed from different genomic segments of a particular virus exhibit somewhat similar topologies (Carroll et al., 2010; Tentchev et al., 2011). To determine whether RNA5 and RNA6 would exhibit a similar evolutionary association with other FMV genomic RNA segments, we constructed and compared phylogenetic trees based on the full-length RNA3, RNA4, RNA5 and RNA6 sequences of the 14 FMV isolates sequenced in this study and the Gr10 isolate. To root the RNA3 and RNA4 trees, the RRV nucleotide sequences were used as an outgroup. No appropriate outgroup was available for the RNA5 or RNA6 tree.

The four constructed trees shared similar topologies, most notably between RNA3 and RNA4 (Fig. S3), between RNA3 and RNA6 (Fig. 4a), and between RNA4 and RNA5 (Fig. 4b). In other words, the relative phylogenetic positions of RNA3, RNA4, RNA5 and RNA6 of each FMV isolate are similar. However, for several of the isolates, one of the four RNAs differed in its phylogenetic position relative to the other three (e.g. RNA5 of JF1 and RNA3 of
JTT-Ki), probably because of reassortment between the RNAs, which has been reported for segmented RNA viruses, including tomato spotted wilt virus (Tentchev et al., 2011).

In general, the FMV isolates were clustered geographically within each tree. Six Japanese isolates (JS1, JF1, JFT4, JTT-At, JTT-Li and JTT-Ki) formed a cluster supported by a bootstrap value of 100%, while all Serbian isolates and Gr10 fell into another cluster with high bootstrap values (>72%). However, the phylogenetic positions of JTT-Pa and JTT-Vi differed depending on the RNA segment, probably poorly supported by bootstrap values. Distinct clustering of JTT-Pa and JTT-Vi might be hampered by their distant relationships to the other FMV isolates. Despite these exceptions, the considerable evolutionary association between RNA5 and RNA6, and known FMV genomic segments allows us to conclude that RNA5 and RNA6 are additional genomic segments of FMV.

**DISCUSSION**

Our findings demonstrate that two novel RNAs, named RNA5 and RNA6, are associated with FMV, in addition to its four previously described genomic RNA segments (RNA1–RNA4). Several pieces of evidence strongly suggest that RNA5 and RNA6 are novel genomic RNA segments of FMV. First, these two RNAs are associated with all of our 14 FMV isolates originating from two geographically distant countries, Japan and Serbia (Fig. 1b). Second, these novel RNAs and the four known FMV genomic RNA segments are highly concordant in their structural organization; they each have a single ORF on the vcRNA, and they each
have complementary termini including conserved 3′- and 5′-terminal 13 nt stretches (Fig. 3). Third, the complementary strands of RNA5 and RNA6 were detectable by Northern blot analysis, indicating replication of these RNAs in infected figs (Fig. 3). Finally, RNA5 and RNA6 exhibit a molecular evolutionary pattern similar to that of known FMV genomic RNA segments (Fig. 4).

RNA5 and RNA6 have no detectable similarity to other RNA segments other than in their 3′- and 5′-terminal regions, which contain the conserved 13 nt stretches seen in other FMV genomic RNA segments. Thus, they are quite unlikely to be defective RNAs or truncated or rearranged versions of RNAs derived from helper virus genomes (Simon et al., 2004). The possibility that RNA5 and RNA6 are satellite RNAs cannot be totally dismissed, but the complete association of RNA5 and RNA6 with FMV isolates originating from geographically distant countries indicates that these RNA segments are necessary for the FMV infection cycle, supporting the conclusion that they are genomic RNAs.

In a previous Northern blot study of the Gr10 isolate of FMV using a segment of the 3′-terminal 13 nt sequence as a probe, Elbeaino et al. (2009b) detected only four genomic RNA segments. However, a close examination of the Northern blot published in that report reveals faint signals close in size to full-length RNA3 and RNA4, which are equivalent in length to RNA5 and RNA6. Pieces of evidence provided in this study led us to suspect that they may represent RNA5 and RNA6 of Gr10.

Although FMV is closely related to EMARaV, RRV, RLBV, PPSMV and MRSV, no RNA segments corresponding to RNA5 or RNA6 have been found in these viruses, with a single exception: RNA5 has been reported in RLBV (McGavin et al., 2012). However, the predicted p5 proteins of RLBV and FMV are only approximately 10% similar in amino acid sequence, precluding the determination of whether the two proteins are homologous. If additional genomic RNA segments are present in the other putative members of the genus *Emaravirus*, as suggested by our findings, they should be detectable by RT-PCR using the 3TerE/5TerE primer set used in our study, as the *Emaravirus* terminal 13 nt segments are conserved (Mielke & Müelhbach, 2007; Laney et al., 2011; McGavin et al., 2012).

The number of genomic segments is an important criterion for taxonomic classification of segmented viruses because each segment is likely to reflect a different biological property. In fact, division of the unassigned genus *Tenuiviruses*, which contains negative-strand viruses with different numbers of genomic segments, into multiple genera according to the number of genomic segments has been proposed (King et al., 2011). The precise taxonomic allocation of the viruses in the genus *Emaravirus*, which would contain members with different numbers of genomic segments, is debatable.

In conclusion, we have demonstrated that FMV contains two previously unidentified genomic RNA segments and, thus, contains six genomic RNA segments rather than four. The discovery of these novel FMV genomic segments will shed light on the nature not only of FMV, but also of the other poorly understood members of the genus *Emaravirus*. Further studies are needed to decipher the roles of the proteins encoded by the newly identified genomic RNA segments of FMV, RNA5 and RNA6.

**METHODS**

**Plant material and preparation of total RNA.** Symptomatic leaves of 14 individual fig trees were collected from two different countries, Japan and Serbia. Asymptomatic fig leaves from Japan were used as a negative control. These leaves were ground to a fine powder in liquid nitrogen and the total RNA was extracted using RNeasy mini kits (Qiagen) according to the manufacturer’s instructions.

**RT-PCR.** FMV was detected by RT-PCR analysis using E5-s and E5-a primers as described previously (Elbeaino et al., 2009a; Ishikawa et al., 2012). For simultaneous amplification of genomic RNA segments of FMV, the conserved 13 nt stretches found at the ends of FMV genomic segments were used as primers specific for the termini of genomic RNA segments. The 5TerE and 3TerE primer sequences were 5′-AGUAGUGUUCUCC-3′ and 5′-GGAGUUACUACU-3′, respectively (Table S1). RT reactions were carried out using Superscript III Reverse Transcriptase (Invitrogen), 3TerE primer and 1 μg total RNA in 20 μl reaction mixtures for 30 min at 45 °C. Then, 4 μl of each RT reaction was removed and used in a 50 μl PCR with Takara La Taq (Takara), 5TerE and 3TerE primers in a thermal cycler set for an initial denaturation step at 94 °C (3 min); 35 cycles of denaturation at 94 °C (15 s), annealing at 45 °C (30 s) and elongation at 68 °C (7 min); and a final elongation step at 68 °C (7 min).

**RACE.** To identify the 5′- and 3′-terminal sequences of each RNA segment, 5′-RACE analysis was performed using the 5′-RACE System, version 2.0 (Invitrogen). The RT reactions were carried out at 42 °C using a gene-specific primer 1 (gsp1) (Table S1) and 1 μg total RNA from FMV-infected fig leaf and were stopped after 30 min by incubation of the reaction mixture at 70 °C for 15 min. The anbridged anchor primer in the RACE kit and a gene-specific primer 2 (gsp2) (Table S1) were used for PCR in a thermal cycler set for an initial denaturation step at 94 °C (2 min); 35 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s) and elongation at 68 °C (1 min); and a final elongation step at 68 °C (1 min). The PCR products were cloned into the TOPO vector as described below.

**Cloning and sequencing of PCR products.** The simultaneously RT-PCR-amplified fragments of the JS1 and SB1 FMV isolates were purified using an UltraClean 15 DNA Purification kit (Mo Bio) and subcloned into the TOPO vector using a TOPO TA Cloning kit (Invitrogen) according to the manufacturer’s instructions. The selected clones were sequenced with M13 forward and reverse primers or with primers specific for internal sequences of RNA1 and RNA2 of JS1 and SB1 (Table S1). For each amplified fragment, at least four clones were sequenced using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems).

Amplified fragments derived from other FMV isolates were treated with ExoSAP-IT (GE Healthcare) to remove unincorporated dNTPs and PCR primers, and then directly sequenced using an ABI 3130xl genetic analyser (Applied Biosystems) with the primers described above. The GenBank database was searched using the BLAST program (National Centre for Technology Information), and domains in p5 and p6 were predicted using the Pfam program (http://pfam.sanger.ac.uk/).
Northern blot hybridization. For probe preparation, the regions of JSJ RNA3, RNA5 and RNA6 corresponding to nt 280–1280, 100–1100 and 120–1120, respectively, were amplified using primer pairs based on the RNA sequences of JSJ RNA3, RNA5 and RNA6, respectively (RNA3nor-s/RNA3nor-a, RNA5nor-s/RNA5nor-a and RNA6nor-s/RNA6nor-a, respectively; Table S1). The amplified fragments were cloned into the pGEM-T Easy vector (Promega) according to the manufacturer’s instructions. After digestion of the resulting vector constructs with Ncol or SalI, the RNA probes were transcribed using T7 polymerase or SP6 polymerase, respectively.

The above probes were used for Northern blot analysis of FMV RNA expression as described previously (Senshu et al., 2009). After total RNA was isolated from fig leaves using RNeasy mini kits (Qiagen) according to the manufacturer’s instructions, 1 μg total RNA was electrophoresed on a 1 % MOPS-formaldehyde agarose gels. The RNA was then hybridized to the probes via overnight incubation at 68 °C. The hybridized bands were detected using the digoxigenin detection system (Roche Diagnostics).

Comparison of nucleotide and amino acid sequences of FMV isolates. The nucleotide and amino acid sequences of RNA1 and RNA2 of JSJ (GenBank accession numbers AB697826 and AB697828), SB1 (AB697827 and AB697829) and Gr10 (AM941711 and FM864225) were compared to each other. The nucleotide and amino acid sequences of RNA3, RNA4, RNA5 and RNA6 of 14 FMV isolates from Japan and Serbia (GenBank accession numbers are provided in Table S2) and of RNA1, RNA3 and RNA4 of the Gr10 isolate (FM991954 and FM992851, respectively) were analysed. The CLUSTAL W program (Thompson et al., 1994) at the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-j.html) was used to create nucleotide sequence alignments and MEGA software version 5.0 (Tamura et al., 2011) was used for phylogenetic analysis and calculation of overall mean genetic distances. The phylogenetic trees were constructed using the neighbour-joining method with the p-distance model of evolution (Takahashi & Nei, 2000) and bootstrap analyses were performed with 1000 replicates. To root the RNA3 and RNA4 trees, RRV nucleotide sequences (GenBank accession numbers HQ871944 and HQ871945, respectively) were used.

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