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A proposed new *Tombusviridae* genus featuring extremely long 5' untranslated regions and a luteo/polerovirus-like gene block

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Tombusviridae is a large family of single-stranded, positive-sense RNA plant viruses with uncapped, non-polyadenylated genomes encoding 4−7 open reading frames (ORFs). Previously, we discovered, by high-throughput sequencing of maize and teosinte RNA, a novel genome of a virus we call Maize-associated tombusvirus (MaTV). Here we determined the precise termini of the MaTV genome by using 5' and 3' rapid amplification of cDNA ends (RACE). In GenBank, we discovered eleven other nearly complete viral genomes with MaTV-like genome organizations and related RNA-dependent RNA polymerase (RdRp) sequences. These genomes came from diverse plant, fungal, invertebrate and vertebrate organisms, and some have been found in multiple organisms across the globe. The available 5' untranslated regions (UTRs) of these genomes are remarkably long: at least 438 to 727 nucleotides (nt), in contrast to those of other tombusvirids, which are <150 nt. Moreover these UTRs contain 6 to 12 AUG triplets that are unlikely to be start codons, because - with the possible exception of MaTV - there are no large or conserved ORFs in the 5' UTRs. Such features suggest an internal ribosome entry site (IRES), but the only conserved features we found were that the 50 nt upstream of and adjacent to the ORF1 start codon are cytosine-rich and guanosine-poor. ORF2 (RdRp gene) appears to be translated by in-frame ribosomal readthrough of the ORF1 stop codon. In all twelve genomes we identified RNA structures known in other tombusvirids to facilitate this readthrough. ORF4 overlaps with ORF3 (coat protein gene) and may initiate with a non-AUG start codon. ORF5 is predicted to be translated by readthrough of the ORF3 stop codon. The proteins encoded by ORFs 4 and 5 diverge highly from each other and from those of the similarly organized luteo- and poleroviruses. We also found no obvious 3' cap-independent translation elements, which are present in other tombusvirids. The twelve genomes diverge sufficiently from other tombusvirids to warrant classification in a new genus. Because they contain two leaky stop codons and a potential leaky start codon, we propose to name this genus *Rimosavirus* (*rimosa* = leaky in Latin).

KEYWORDS

metagenomics, stop codon readthrough, leaky scanning, internal ribosome entry site, untranslated regions, luteovirus

1 Introduction

Metagenomics has revolutionized virus discovery. Searching for viruses by sequencing total RNA from environmental samples (metagenomics), such as soil (1), seawater (2), or organisms has resulted in an exponential increase in known viruses, or viral genomes in the past decade (3, 4). The viruses associated with these newly discovered genomes are mostly uncharacterized, but the obvious viral nature of the genomes indicate that those viruses exist (5). Because virus particles can be highly abundant and stable, viruses isolated from an organism may not actually infect that organism, but may be just "hitching a ride". For example, plant viruses have been identified in human intestinal microbiome (6), bat guano (7), and in aphid vectors that transmit them (8) but which are not infected by them. Hence, viruses known only by sequence and the organism from which they are isolated are labeled as "associated" with that organism.

In large scale metagenomics projects, thousands of viral genome sequences have been automatically assembled, annotated, and deposited in GenBank, in some cases with very little direct scrutiny by humans (9, 10). Because of the numerous noncanonical translation mechanisms used by many RNA viruses, these autoannotated genomes are often mis-annotated (11). Also, if the viral genomes are not of interest to the sequencers, or if the sequencers simply lack the time to study these viral genomes, they may remain essentially undiscovered in GenBank. Here we describe several genomes of viruses in the *Tombusviridae* family (tombusvirids) that appear to fall in this category.

The *Tombusviridae* family contains over 100 virus species (tombusvirids) in eighteen genera officially named by the International Committee on Virus Taxonomy (ICTV). This large and diverse family includes many economically costly pathogens, such as maize chlorotic mottle virus, which has devastated maize production in mixed infection with a potyvirus in East Africa (12), and the barley yellow dwarf viruses which comprise the most ubiquitous viruses of wheat, barley and oat, worldwide (13, 14). In addition, tombusvirids have proved to be excellent models. For example, the first X-ray crystal structure of an icosahedral virus was that of tomato bushy stunt virus (for which the family is named) (15). Also the roles of host proteins and subcellular structures in every stage of the virus life cycle are better understood for TBSV than almost any other RNA virus (16–18). Tombusvirids contain a positive strand RNA genome of 4–6 kb which lacks a 5' cap and a poly(A) tail, as the genome terminates in CCC (19, 20). *Dianthovirus* is the only tombusvirid genus that has a bipartite genome (21).

The tombusvirid genome encodes 4–7 open reading frames (ORFs). ORF1 encodes a replication protein that lines membrane-bound replication vesicles (18). ORF2 encodes the RNA-dependent RNA polymerase (RdRp) and is translated as a long C-terminal extension of ORF1 via ribosomal readthrough of – or frameshift around – the ORF1 stop codon (22–25). Downstream ORFs encode movement proteins, suppressors of RNA silencing, coat protein and vector transmission components (19, 26–28). They are translated – often via various leaky start and stop codons (23, 29–31) – from subgenomic mRNAs that are 5'-truncated versions of genomic RNA (32–34). To allow translation of the uncapped genomic and subgenomic mRNAs, a 3' cap-independent translation element (3' CITE) is present in the 5' end of the 3' UTR (35, 36). The 3' CITE is followed at the 3' end by RNA structures and sequences required for RNA replication (20), and in some cases structures that regulate readthrough or frameshifting at the ORF1 stop codon via long-distance base pairing (37, 38).

After discovery by deep sequencing of the genome of the novel tombusvirid maize-associated tombusvirus (MaTV) in maize and teosinte leaves (39), we searched GenBank for related sequences. Here we describe eleven other viral genomes, all found by metagenomic sequencing, that are related to the MaTV. Based on (i) genome organization, (ii) sequences of RNA-dependent RNA polymerase (RdRp) and coat protein (CP), and (iii) conserved RNA secondary structures, all of these viruses clearly belong in the *Tombusviridae* family. However their sequences and genome features, such as extremely long 5' UTR, place them in a clade sufficiently distinct to merit classification in a new genus.

2 Materials and methods

2.1 Rapid amplification of cDNA ends

Sequences of oligonucleotides used for RACE are listed in Supplementary Table S1.

2.1.1 Source of RNA

The RNA used for RACE was from the same total RNA extract from maize leaf that was used for sequencing the MaTV genome (39). The leaf was collected from an unhealthy, possibly diseased maize plant near Irapuato, Mexico in October 2017. Total RNA was extracted using a Zymo Direct-zol RNA Miniprep Plus kit (Zymo Research, Irvine, CA, USA), depleted for ribosomal RNA using an Illumina Ribo-Zero rRNA Removal Kit (Plant Leaf) (Illumina, San Diego, CA, USA), concentrated with a Zymo RNA Clean & Concentrator-5 kit, and stored at −80°C. Details are described in Lappe et al. (39).

2.1.2 First-strand cDNA synthesis for 5' RACE

Using Millipore Sigma's 5'/3' RACE Kit, 2nd Generation, MaTV cDNA was synthesized using random primers on 1 μg of the above total maize leaf RNA. Following the kit's instructions, the reaction mixture was incubated at 55°C for 60 minutes and for another 5 minutes at 85°C. Immediately after first-strand cDNA synthesis, the cDNA products were purified using Sigma's High Pure PCR Product Purification Kit, following the specific instructions for cDNA purification outlined in the 5' RACE protocol as opposed to the protocol that comes with the purification kit.

2.1.3 PCR on the cDNA sample for 5' RACE

PCR was carried out following the purification step after first-strand cDNA synthesis, using Sigma-Aldrich's Expand High

Fidelity PCR System. Using the Expand High Fidelity Buffer with 15 mM MgCl$_2$, thermal cycler PCR conditions were set according to Sigma's RACE protocol with an altered annealing temperature to accommodate both the MaTV-specific primer and the oligo dT anchor primer in the RACE kit. Three rounds of PCR were conducted on the samples using a different nested primer each round (Nested primers 1, 2, and 3), without purifying the PCR product or diluting the PCR product before the next round was started. The final PCR product was subjected to gel electrophoresis and gel extraction followed by ethanol precipitation and resuspension in 50 μl of nuclease-free water. The resulting DNA was sequenced at the Iowa State University DNA Facility using MaTV-specific 5' RACE sequencing primer, 5R1 (Supplementary Table S1).

2.1.4 Ligation of an artificial poly(A) tail to MaTV RNA for 3' RACE

The Millipore Sigma 3' RACE kit utilizes a poly(A) tail on the RNA sample. Because viruses in the family *Tombusviridae*, which includes MaTV, do not have a poly(A) tail, an oligonucleotide [3'poly(A)] containing a poly(A) tract was ligated onto the 3' end of the total RNA sample using NEB's T4 RNA Ligase 1 kit. This sequence is complementary to the oligo d(T) anchor primer from the RACE kit and was designed with both a 5' and 3' phosphate to prevent the oligo from ligating to itself and ligating to RNA in the total RNA sample that already possesses a poly(A) tail. Using 1 μl of the total RNA sample including Thermo Fisher's RNaseOut RNA inhibitor, the reaction mixture was incubated at 25°C for 2 hours and the reaction was stopped by column cleanup using the NEB Monarch RNA Cleanup Kit.

2.1.5 First-strand cDNA synthesis for 3' RACE

Following Millipore Sigma's 5'/3' RACE Kit, 2nd Generation, 5ul of the newly polyadenylated total RNA sample containing the ligated oligo(dT) anchor primer was used at a concentration of 100 ng/μl. After incubation at 55°C for 60 min immediately followed by incubation at 85°C for 5 min, the cDNA was ready for PCR. No purification step was necessary after cDNA synthesis.

2.1.6 PCR on the cDNA sample for 3' RACE

PCR was carried out immediately following first-strand cDNA synthesis using Sigma-Aldrich's Expand High Fidelity PCR System. Using the Expand High Fidelity Buffer with 15 mM MgCl$_2$, thermal cycler conditions were set according to Sigma's RACE protocol altered to accommodate both the MaTV-specific primer, 3F1 (Supplementary Table S1), and the oligo dT anchor primer in the RACE kit. The PCR product was ethanol precipitated and resuspended in 50 μl of nuclease-free water.

2.1.7 Gel electrophoresis and gel extraction

16 μl of purified PCR product at a concentration of 100 ng/μl was run on a 2% agarose gel for 30 minutes at 150V to isolate the MaTV cDNA produced from the total RNA sample. A visible band at ~400 bp (estimated product size at the 3' end of MaTV genome) was gel extracted using Qiagen's QIAquick PCR & Gel Cleanup Kit.

After measuring the concentration of cDNA in the eluted sample after gel extraction (~5 ng/μl), an additional round of PCR was conducted to increase the cDNA concentration (~300 ng/μl).

2.1.8 3' end sequencing of MaTV

Following PCR and purification of the MaTV cDNA, the samples were sent to Iowa State University's DNA Sequencing Facility for dideoxy sequencing using MaTV-specific primer 3F2 (Supplementary Table S1) that was nested 3' of the MaTV-specific primer used for PCR.

2.2 Multiple sequence alignment and phylogeny prediction

The RNA-dependent RNA polymerase (RdRp), coat protein (CP), and read-through domain (RTD) amino acid sequences of select viruses from families *Tombusviridae* and Solemoviridae were aligned with Muscle v3.8.31 using default parameters in SnapGene. RNA alignments were imaged using Jalview (40, 41). For phylogenetic tree construction, multiple sequence alignments were passed to FastTree 2.1.11 with the flags "-lg -gamma" to predict phylogenies where branch split reliability values are estimated with the Shimodaira-Hasegawa test (42). The resultant trees were drawn with FigTree v1.4.4 and re-rooted using the nearest relative outside of the taxonomic group of the compared sequences (43): Providence virus (NC_014126.1) for the RdRp tree and Ourmia melon virus (NC_011070.1) for the coat protein tree. Accession numbers are the GenBank accession numbers from which the respective amino acid sequences were taken.

2.3 RNA structures

RNA secondary structures were predicted using MFOLD (44), Vienna package (45), Scanfold2 (46) under default parameters, in iterations with multiple sequence alignments and inspection with the human eye. Secondary structures were drawn using RNAcanvas (47).

3 Results

3.1 Identification of viruses similar to MaTV: a new genus

Previously, we discovered and assembled genomic sequences of MaTV from maize (GenBank no. OK0180181) and teosinte (OK0180182). These genomes are 99.5% identical and none of the base differences affect lengths of open reading frames (ORFs) (39). Thus they are isolates of the same virus; for all subsequent comparisons in this paper, we use the maize isolate. To identify other viruses related to MaTV, we performed a BLAST search of GenBank seeking matches with the RNA-dependent RNA polymerase (RdRp) gene (ORF2) of MaTV. We use the RdRp

because a major component of RNA virus classification is based on sequence similarities of the RdRps, as it is the key replication protein encoded by all RNA viruses (48, 49). We also searched for genes with similarity to MaTV ORF4 which seemed to be unique to this genus, when compared to known tombusvirids.

The BLAST searches revealed nearly complete genomes of eleven other viruses with substantial sequence similarity in the RdRp ORF and similar genome organizations to that of MaTV (Figure 1). This includes apple virus E (AVE), which we reported previously as being similar to MaTV (39). Like the MaTV genome,
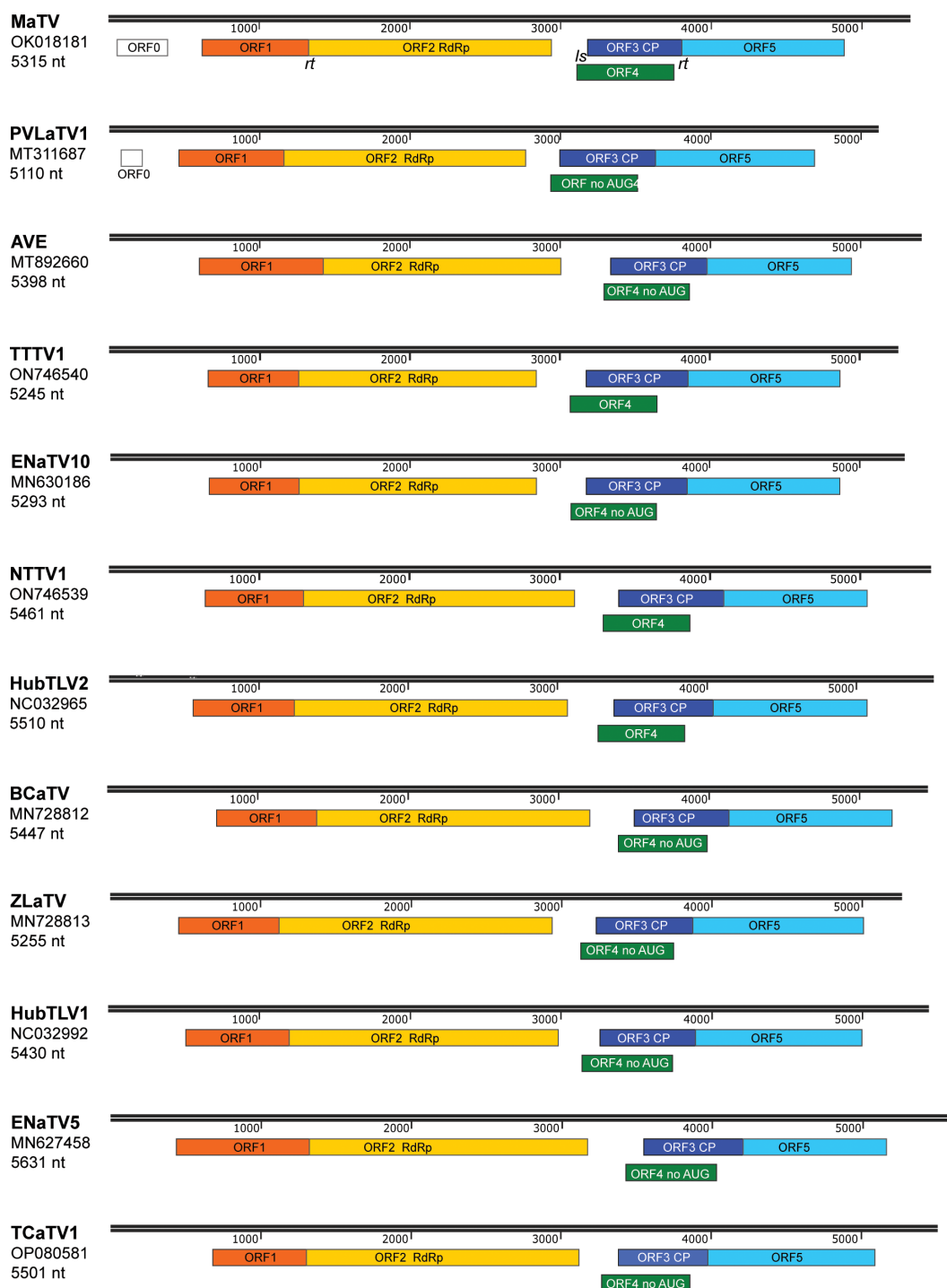
FIGURE 1

Maps of genomes in proposed new genus, *Rimosavirus*. Virus acronym, GenBank accession numbers used throughout this manuscript, and length of genome sequence for that accession number are shown at left. Colored boxes indicate ORFs, with functions of the protein products of ORF2 (RdRp) and ORF3 (CP) indicated. Scale bar (in nt) is indicated in black above each genome map. ORFs 2 and 5 are translated by in-frame readthrough (rt) of the ORF1 and ORF3 stop codons, respectively. ORFs 4 of the indicated viruses (no AUG) lack an in-frame AUG start codon upstream of ORF3 AUG start codon (see text), which is predicted to be translated from a subgenomic mRNA via leaky scanning (ls).

all eleven of these genomes were discovered in metagenomics sequencing projects using Illumina sequencing (9, 10, 50–52). As discussed below, all of the genomes were misannotated because ORFs were assumed to begin with an AUG codon. Yet, by comparison with other members of the *Tombusviridae*, there appear to be two ORFs (ORFs 2 and 5) that are translated via in-frame readthrough of the stop codon of the preceding ORF. Thus, these ORFs begin immediately after the stop codon rather than at the first AUG codon of the ORF. Because these genomes were found in large, exploratory metagenomics sequencing projects, we do not know what hosts they infect or if any symptoms were associated with these viruses, but they have been found associated with a remarkable variety of organisms including plants, fungi, invertebrates and vertebrates (Table 1). Some virus species have been found more than once, in organisms of diverse kingdoms across the globe (Table 1).

In addition to high sequence similarity in the RdRp ORF, these genomes share the following features. (i) They contain an extremely long putative 5' untranslated region (UTR) ranging from at least 438 nt to 727 nt, although most are almost certainly longer than listed because it is unlikely that most have been sequenced to the 5' end (below); (ii) The presumed first translated ORF (ORF1, MW 23–34 kDa, Supplementary Table S2) is followed immediately by ORF2, which encodes the RdRp (59–69 kDa) in an arrangement

that suggests translation of ORF2 via ribosomal readthrough of the ORF1 stop codon. This arrangement is present in 15 of the 18 *Tombusviridae* genera, the exceptions being *Luteovirus*, *Dianthovirus* and *Umbravirus* genera, members of which employ a -1 ribosomal frameshift for translation of the RdRp. (iii) ORF2 is followed by a noncoding region followed by ORF3, which encodes the coat protein (CP, 22–26 kDa), and an overlapping ORF4 (~17–20 kDa) (Figure 1; Supplementary Table S2). The stop codon of ORF3 is followed immediately in the same reading frame by ORF5, suggesting translation of ORF5 via readthrough of the ORF3 stop codon to generate a 34–41 kDa C-terminal extension to the CP (Figure 1; Supplementary Table S2). This arrangement of ORFs 3, 4, and 5 resembles that of viruses in the genera *Luteovirus* (*Tombusviridae*) and *Polerovirus* (*Solemoviridae*) (53), with the exception that ORF4 likely initiates upstream of ORF3 in these new viruses. In all luteo- polero- and enamoviruses (L/P/E viruses), ORF5 has been shown to be translated via readthrough of the ORF3 stop codon (31, 54, 55), and the protein product of ORF5 (the readthrough domain – RTD) is required for aphid transmission (26, 56–58), and participates in virus cell to cell movement in the luteo- and poleroviruses (59–61). In the new tombusvirids reported here, ORF5 is followed by a 3' UTR with minimum lengths of 229 to 458 nt, but it is unlikely that the 3' UTR sequences are complete all the way to the 3' end, except for those of MaTV and possibly

TABLE 1 Genomes and sources of viruses in this study.

Viral genome	Acronym	Organism[a]	Location[a]	GenBank no.	Reference[a]
Maize-associated tombusvirus	MaTV	maize, teosinte tuatara[b]	Irapuato, Mexico; Takpourewa, New Zealand	OK018181.2 OK018182.1	(39, 51)
Plasmopara viticola lesion-associated tombus-like virus 1	PVLaTV1	grapevine downy mildew	Italy	MT311687.1	(50)
Apple virus E	AVE	apple	USA	MT892660.1	None found
Taian Tombu tick virus 1	TTTV1	*Haemaphysalis longicornis* (Asian long-horned tick), *H. concinna*	Shandong & Jilin, China	ON746540.1	(52)
Erysiphe necator-associated tombus-like virus 10	ENaTV10	grapevine powdery mildew	Spain	MN630186.1	None found
Nanning Tombu tick virus 1	NTTV1	*Rhipicephalus sanguineus* (brown dog tick)	Guangxi, China	ON746539.1	(52)
Hubei tombus-like virus 2	HubTLV2	pill worm	China	NC032965.1	(9)
Brassica caulorapa tombusvirus	BCaTV	kohlrabi, tuatara[b], *H. longicornis*	Zhenjiang City, China; Takpourewa, NZ; Jiangsu, China	MN728812.1	(10, 51, 52)
Zizania latifolia tombusvirus	ZLaTV	Manchurian wild rice, tuatara[b], *H. longicornis*	Zhenjiang City, China; Takpourewa, NZ; Beijing & Henan, China	MN728813.1	(10, 51, 52)
Hubei tombus-like virus 1	HubTLV1	Chinese land snail	China	NC032992.1	(9)
Erysiphe necator-associated tombus-like virus 5	ENaTV5	grapevine powdery mildew	Spain	MN627458.1	None found
Tuatara cloaca-associated tombusvirus 1	TCaTV1	tuatara (*Sphenodon punctatus*)	Takpourewa, NZ	OP080581.1	(51)

[a]For viral genomes found in more than one location, the named virus was first found in the first organism listed at the first location listed by the authors of the first citation listed.
[b]Viral genomes found in tuatara (all from the island of Takpourewa, New Zealand) have genome sequence with >93% sequence identity to the listed viral genome.

Erysiphe necator-associated tombus-like virus 10 (ENaTV10) (discussed below).
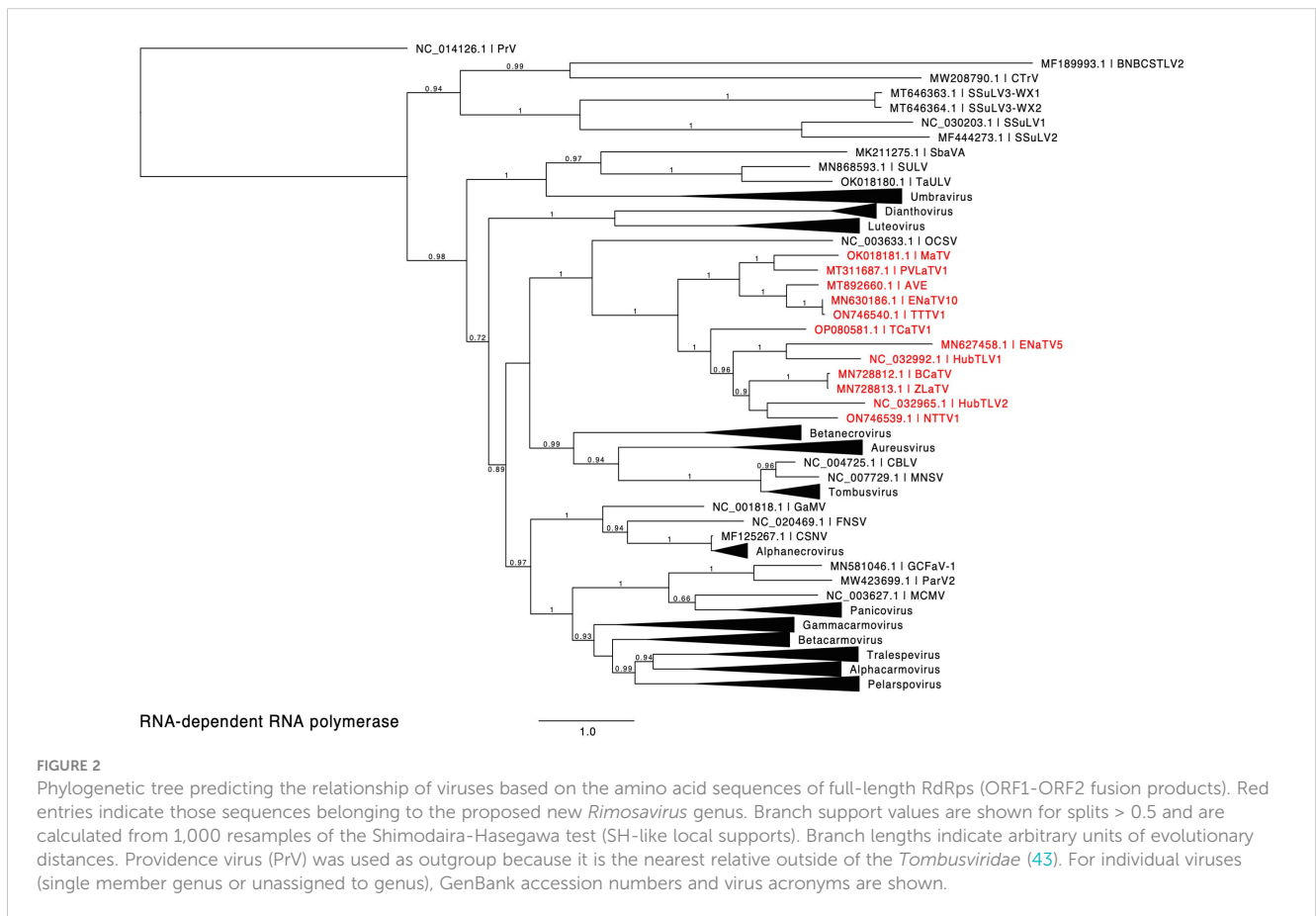
Whole genome comparisons revealed that Taian tombu tick virus 1 (TTTV1) and ENaTV10 are 95% identical, thus they are strains of the same virus even though one was obtained from an invertebrate parasite of mammals and the other from a plant pathogenic fungus. MaTV and *Plasmopara viticola* lesion-associated tombus-like virus 1 (PVLaTV1) genomes also show close similarity at 68.5% nucleotide sequence identity (73.4% identity in the RdRp ORF), but are clearly different species based on ICTV demarcation criteria for tombusvirids, which is <85% amino acid (aa) sequence identity in the CP, as the CPs show 71.2% aa identity (76.6% nt identity).

We propose that these twelve viruses comprise a new genus in the *Tombusviridae* family. This is based on the distance of the clade in the phylogenetic tree of RdRp sequences from the nearest relative, oat necrotic dwarf virus (genus *Avenavirus*) (Figure 2), and their distinctive genome organizations, which differ in the same ways from those of other tombusvirids. Because all twelve of these viruses have two probable leaky stop codons (at the ends of ORFs 1 and 3), and may initiate translation of the ORF3 via ribosomal leaky scanning, we propose to call this new genus *Rimosavirus* (*rimosa*, Latin for leaky). While this name is only provisional, for convenience throughout this manuscript, we refer to the twelve viruses on which this paper focuses (Table 1) as rimosaviruses.

3.2 5′ and 3′ untranslated regions

As mentioned above, it is not clear if any of the rimosaviral genomes were sequenced completely to the 5′ and 3′ ends. To determine the terminal sequences of MaTV, we performed 5′ and 3′ RACE (rapid amplification of cDNA ends) (62) on the same preparation of total maize RNA from which the nearly complete MaTV genome sequence was obtained. RACE revealed 19 additional nt at the 5′ end (GAAAAUAUUUAGGGUACUA) and 62 nt at the 3′ end (UUCCAAACUGCUCAGUAAUGAGAAC UUCAAUUACAGUACAGCUAGACAGAUCUGUAAUGCCC) that were not found in the Illumina sequence assembly (accession no. OK018181.1) (39). That sequence has been updated to include the above RACE results (accession no. OK018181.2). The complete genome length is 5315 nt with 622 nt upstream of ORF1, which we call the 5′ UTR (below), and a 434 nt 3′ UTR. Assuming the same length of sequence is present at the ends of the 99% identical teosinte isolate, the GenBank sequence (OK018182.1) lacks 20 nt and 50 nt at the 5′ and 3′ ends, respectively, but we have not performed 5′ or 3′ RACE on that sample.

FIGURE 2
Phylogenetic tree predicting the relationship of viruses based on the amino acid sequences of full-length RdRps (ORF1-ORF2 fusion products). Red entries indicate those sequences belonging to the proposed new *Rimosavirus* genus. Branch support values are shown for splits > 0.5 and are calculated from 1,000 resamples of the Shimodaira-Hasegawa test (SH-like local supports). Branch lengths indicate arbitrary units of evolutionary distances. Providence virus (PrV) was used as outgroup because it is the nearest relative outside of the *Tombusviridae* (43). For individual viruses (single member genus or unassigned to genus), GenBank accession numbers and virus acronyms are shown.

3.2.1 5' untranslated region

The 5' end of the MaTV genome starts with GAAAAUAU, which is highly similar to that of apple virus E (AVE), GAAAAUCU. These sequences resemble the 5' ends of other tombusvirids which start with a purine (usually a G) followed by a purine-rich, C-poor tract. Thus, the GenBank sequence of AVE (accession no. MT892660) appears to have a complete 5' end. The GenBank sequence of PLVaTV1 (MT311687) appears to be missing 18 nt from its 5' terminus, based on alignment with its close relative MaTV (Supplementary Figure S1). The 5' ends of the other nine rimosavirus sequences found in GenBank do not begin with a similar sequence, and alignments (Supplementary Figure S1) suggest numerous bases are missing from the 5' ends.

The 5' termini of characterized tombusvirid genomes form a stem-loop of modest stability (63, 64). We predicted the secondary structures of the 5'-terminal 40 nt of MaTV and AVE genomes and indeed found such stem-loops (Figure 3). For comparison, the known terminal structures of tombusvirids barley yellow dwarf virus (BYDV, *Luteovirus*) and saguaro cactus virus (SCV, *Carmovirus*) are shown. This further supports that the MaTV and AVE 5' ends are complete.

We predict that the sequence upstream of ORF1 is untranslated in rimosaviruses based on the following observations. (i) There are no conserved ORFs upstream of ORF1 with the following possible exception. MaTV contains a predicted AUG-initiating ORF of 336 nt (112 codons), that we call ORF0, starting at nt 85, but it is absent in the other rimosaviruses, save for a truncated version of ORF0 (177 nt, 58 codons) in PVLaTV1 (Figure 1; Supplementary Figure S2B). (ii) There are 6 to 12 AUG triplets scattered in different positions among the rimosavirus genomes upstream of the ORF1 start codon

(Table 2; Supplementary Figure S1), including upstream and downstream of ORF0 in MaTV and PVLaTV1, but they result in short, non-conserved ORFs. (iii) Finally, ORF1 is the 5'-proximal ORF in all other tombusvirids.

Such a 5' UTR consisting of many hundreds of bases (Table 2) and containing numerous nontranslated AUG triplets suggests presence of an internal ribosome entry site (IRES) or possibly a long ribosomal shunt system. IRESes in the genomes of picornaviruses, hepaciviruses and many other animal RNA viruses are highly structured RNAs that co-opt various host proteins to recruit the ribosome shortly upstream of the first actual start codon for highly efficient cap-independent translation (65–67). In shunting, scanning ribosomes jump across a long structured stem-loop, then rejoin the mRNA to scan to the start codon (68). However, we detect little sequence conservation among the 5' UTRs of the twelve rimosaviruses, nor were we able to predict any conserved secondary structure, as would be expected for an IRES or a shunting mechanism. One conserved feature is that the sequence upstream of and near the ORF1 AUG is G-poor and C-rich (Figure 4). Specifically, in all twelve viruses, the 24 nt immediately upstream of ORF1 start codon have at most one guanosine base (Table 2). The C-richness usually extends 50 or more bases upstream of the ORF1 AUG (Figure 4). Certain IRESes have cytosine- or pyrimidine-tracts at about this position relative to the start codon (69), and the region just upstream of well-translated start codons in plants is enriched in C-rich tracts (70). In summary, the 5' UTR is enigmatic, as it contains numerous AUG codons and small nonconserved ORFs, that seem to rule out a conventional scanning mechanism for initiation of translation of ORF1, but it appears to lack conserved secondary structures or sequences (except potential C-rich motif), as are known in IRESes or shunting structures.

3.2.2 3' untranslated region

The 3' terminus of MaTV is CCC, the trinucleotide present at the 3' end of virtually all tombusvirid genomes for which the 3' sequence has been confirmed. The eleven posted rimosavirus sequences other than that of MaTV do not end in CCC, so we predict they are incomplete. To estimate roughly how many bases are missing from the 3' ends, we took advantage of known structures near the 3' end. Tombusvirids have a distinct conserved secondary structure at the 3' end in which the final four bases, usually GCCC, form a pseudoknot by base pairing to a GGGC bulge in a nearby upstream stem-loop (20, 71–74). Indeed, we predict these structures in MaTV RNA, with the bulged stem-loop terminating 61 nt upstream of the 3' end of the genome (Figure 5A).

To estimate how many bases are missing from the 3' ends of the other rimosaviruses, we searched for the 3'-proximal GGGC tract in a bulged stem-loop in each genome. All but two viral genomes gave a discrete stem-loop with the GGGC bulge (Figure 5B). (Although weak intra-bulge base pairs were predicted in HubTLV2). The two exceptions, BCaTV and ZLaTV, have much shorter 3' UTRs in the GenBank sequences (229 nt and 247 nt, respectively) than the others, which range from 374 to 474 nt (Table 2); thus the posted BCaTV and ZLaTV 3' UTR sequences are both probably incomplete to the extent that they lack the entire GGGC bulged stem-loop. Based on the number of bases downstream of this bulged stem-loop in the other
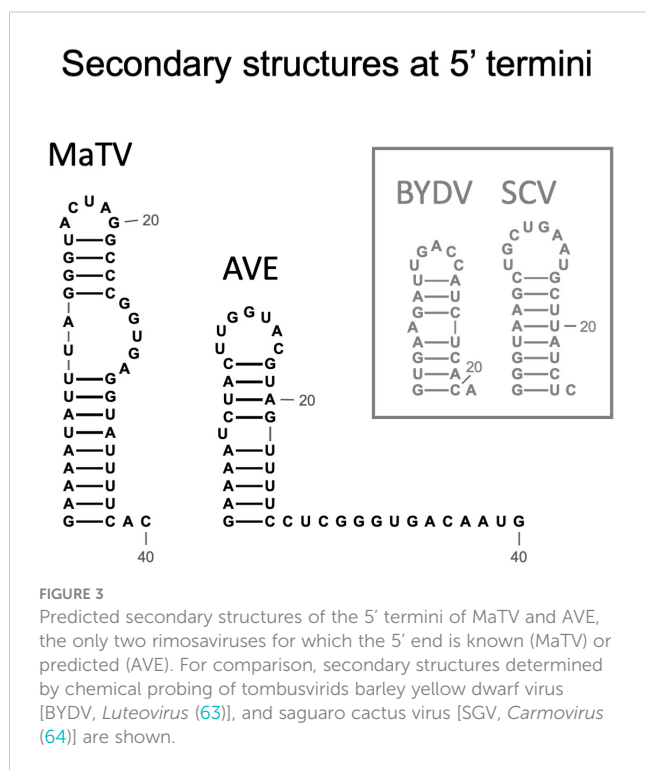

FIGURE 3
Predicted secondary structures of the 5' termini of MaTV and AVE, the only two rimosaviruses for which the 5' end is known (MaTV) or predicted (AVE). For comparison, secondary structures determined by chemical probing of tombusvirids barley yellow dwarf virus [BYDV, *Luteovirus* (63)], and saguaro cactus virus [SGV, *Carmovirus* (64)] are shown.

FIGURE 4
Base compositions flanking the ORF1 start codons (AUG). Plots depicting relative nucleotide frequencies calculated using a sliding window approach plus or minus one-hundred fifty or one-hundred nucleotides relative to the ORF1 start codon, respectively. The X-axis shows genomic position, the Y-axis represents the frequency of each base in a 50 nucleotide window as depicted by the colors in the legend.

rimosavirus sequences, we roughly estimated the number of bases that would be missing from each 3' end if the number of bases from the bulged stem-loop to the 3' end is the same as for MaTV (Table 2). However, the HubTLV2 and ENaTV10 3'UTRs extend beyond the predicted position for the terminal GCCC sequence and do not terminate in GCCC (Figure 5C). An additional stem-loop adjacent to the terminal GCCC is much larger than that of MaTV, giving HubTLV2 and ENaTV10 longer 3'UTRs (Supplementary Figure S3). ENaTV10 has an additional 21 nt 3' of a GCCC tract. We speculate that adapter bases used for sequencing were not trimmed from what is the true 3' end of the viral genome (GCCC). Interestingly, the predicted terminal six bases, AAGCCC can form a six-base pseudoknot with the upstream GGGCUU bulge, instead of the usual four base pairs (Supplementary Figure S3).

Tombusvirid RNAs are uncapped (76), so they carry a cap-independent translation element (CITE) usually in the 5' end of the 3' untranslated region (36). In this region of the rimosavirus genomes we did not identify any secondary structures conserved across all twelve viruses. We predict structures conserved between closely related viruses MaTV and PVLaTV1, and between ENaTV10 and TTTV1 (Supplementary Figure S4). However, these two pairs of structures differ from each other and do not resemble known CITEs. That said, CITEs can be difficult to identify, given the variety of structures possible, varying from a bulged stem-loop with some conserved motifs to more complex branched structures (77–80). Thus, we cannot rule out the presence of a 3' CITE in the 3' UTRs of rimosaviruses, given that the 3' UTRs are certainly long enough to contain a 3' CITE.

TABLE 2 Properties of rimosavirus genomes.

virus	length of sequencedgenome[a]	uAUGs[b]	Gs/Cs in 24 nt 5' of ORF1	5' UTR length[c]	3' UTR length	Approx. 3' nt absent[d]	In-frame ORF4 AUG[g]	(PX)n repeats in RTD[h]
MaTV	5315 nt	12	0G/10C	622 nt	434 nt	0	yes	4 + 6
PVLaTV1	5110 nt	9	1G/7C	464 nt	418 nt	15	no	2 + 5
AVE	5398 nt	11	0G/12C	597 nt	458nt	13	no	0
TTTV1	5245 nt	8	0G/9C	662 nt	374 nt	32	yes	3
ENaTV10	5293 nt	8	1G/6C	658 nt	425 nt	-21[e]	no	3
NTTV1	5461 nt	6	0G/11C	641 nt	412 nt	?	yes	2
HubTLV2	5510 nt	7	1G/14C	562 nt	437 nt	2?	yes	3
BCaTV	5447 nt	8	0G/9C	727 nt	229 nt	?[f]	no	7
ZLaTV	5255 nt	6	0G/9C	457 nt	247 nt	?[f]	no	0
HubTLV1	5430 nt	9	0G/11C	515 nt	435 nt	~6–8	no	2 + 3 + 2
ENaTV5	5631 nt	12	0G/14C	438 nt	474 nt	~6–8	no	0
TCaTV1	5501 nt	10	0G/13C	680 nt	410 nt	~27–29	no	2

[a]Sequence of Genbank accession numbers in Table 1. [b]AUGs upstream of ORF1 start codon. [c]5' UTR is defined as sequence upstream of ORF1 start codon. All but MaTV and AVE are likely incomplete. [d]Nts missing estimated by alignments of sequences and secondary structures from the conserved GGGC-bulged stem-loop to the 3' end of Genbank sequence (Figure 5, Supplementary Figure S3). [e]Appears to have nt beyond the 3' terminal GCCC. [f]3' UTR sequence is too incomplete to have GGGC bulge. [g]AUG start codon. hn = number of consecutive alternating proline repeats [(PX)n] in the readthrough domain (RTD) encoded by the first 200 nt of ORF5 (3'-adjacent to ORF3 stop codon) (P, proline; X, any amino acid). Plus signs indicate separate blocks of (PX)n repeats.

3.3 Rimosavirus ORFs and encoded proteins

3.3.1 ORF0

As mentioned above, MaTV and PLaTLV1 encode what we call ORF0, although that of PLaTLV1 is only 60% as long as that of MaTV (Supplementary Figure S2A). This includes a 37 codon block of high sequence similarity (28/37 amino acids are identical; Supplementary Figure S2B). Close inspection reveals three codons that vary in the wobble position, conserving would-be amino acid sequence (Supplementary Figure S2C). This may indicate selection for amino acid and thus that ORF0 encodes functional protein. However, numerous other differences alter amino acid sequences, and there are indels outside of the conserved coding region that completely alter amino acid sequence (including introduction of the stop codon that truncates the PLaTLV1 ORF0), while the nucleotide sequence remains highly conserved. These observations, combined with the absence of ORF0 in the other rimosaviruses leads us to think that ORF0 is unlikely to encode functional protein.

3.3.2 ORFs 1–2

ORF1 is predicted to be translated as the protein P1 and also fused with ORF2, via occasional stop codon readthrough, to produce P1-P2 protein. As expected, P2 contains the RdRp active site with the highly conserved D(X)3φD, SG(X)$_3$T(X)$_3$N(X)$_{25}$GDD motifs (81). In addition to forming a clade distinct from those of other tombusvirids (Figure 2), the RdRps also fall into two subclades with MaTV, PVLaTV1, ENaTV10, TTTV and AVE in one group. The other seven rimosaviruses contain a 67 to 81 amino acid insertion between

the positions of amino acids 85 and 86 in the RdRps of MaTV, PVLaTV1, ENaTV10, TTTV and AVE (Supplementary Figure S5).

3.3.3 ORF3

ORF3 encodes the coat protein, as evidenced by homology with those of other tombusvirids. The CPs of the twelve rimosaviruses fall into a distinct clade like the RdRps, but some relationships within the proposed genus differ from those of the RdRps (Figure 6). For example, the CPs of TTTV1/ENaTV10 are in a distant branch from MaTV and PLVaTV1, but the RdRps of these four viruses fall in the same subclade (Figure 2). While the RdRps of BCaTV and ZLaTV are closely related, their CPs are not. Interestingly the CPs of other tombusvirids – with the exception of luteoviruses – are more closely related to those of viruses in genus *Sobemovirus* of the *Solemoviridae* family (e.g. rice yellow mottle virus, RYMV), than they are to those of the rimosaviruses (Figure 6).

3.3.4 ORF4

Poleroviruses and most luteoviruses (but not enamoviruses) encode a movement protein (MP) in ORF4, that overlaps with ORF3, initiating a few nucleotides downstream of the ORF3 start codon but in a different frame and terminating shortly before the ORF3 stop codon (82). In these genera, ORF4 is translated from the same subgenomic mRNA (sgRNA1) as ORF3 (83, 84), via leaky scanning, in which some scanning ribosomes skip the ORF3 AUG and instead initiate on the ORF4 AUG codon (29, 85). In contrast, in four of the rimosaviruses, what we call ORF4 appears to initiate with an AUG codon upstream of the ORF3 start codon. In the other eight, there is a similar ORF overlapping with most of ORF3, but it
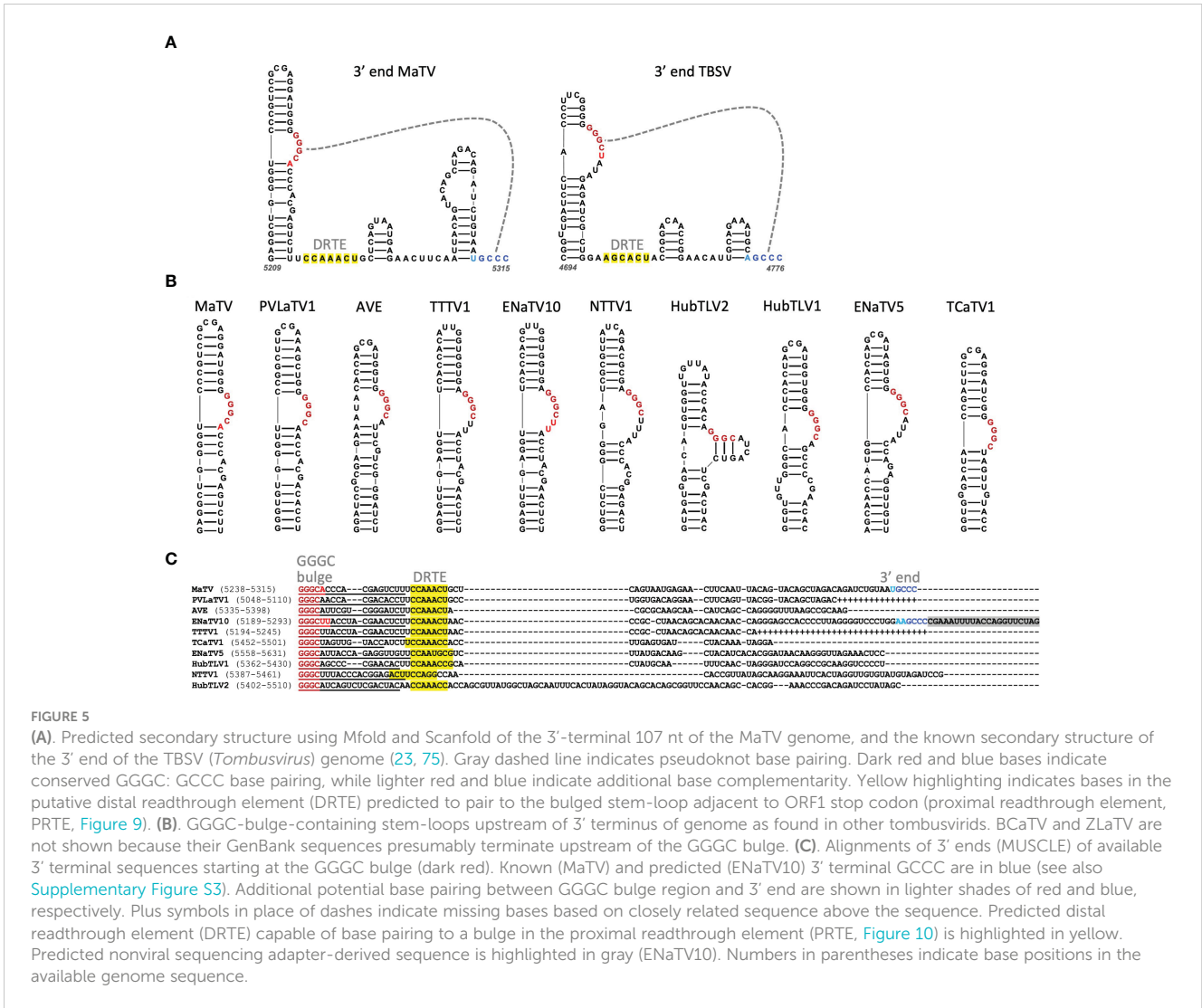
FIGURE 5
(A). Predicted secondary structure using Mfold and Scanfold of the 3'-terminal 107 nt of the MaTV genome, and the known secondary structure of the 3' end of the TBSV (*Tombusvirus*) genome (23, 75). Gray dashed line indicates pseudoknot base pairing. Dark red and blue bases indicate conserved GGGC: GCCC base pairing, while lighter red and blue indicate additional base complementarity. Yellow highlighting indicates bases in the putative distal readthrough element (DRTE) predicted to pair to the bulged stem-loop adjacent to ORF1 stop codon (proximal readthrough element, PRTE, Figure 9). **(B)**. GGGC-bulge-containing stem-loops upstream of 3' terminus of genome as found in other tombusvirids. BCaTV and ZLaTV are not shown because their GenBank sequences presumably terminate upstream of the GGGC bulge. **(C)**. Alignments of 3' ends (MUSCLE) of available 3' terminal sequences starting at the GGGC bulge (dark red). Known (MaTV) and predicted (ENaTV10) 3' terminal GCCC are in blue (see also Supplementary Figure S3). Additional potential base pairing between GGGC bulge region and 3' end are shown in lighter shades of red and blue, respectively. Plus symbols in place of dashes indicate missing bases based on closely related sequence above the sequence. Predicted distal readthrough element (DRTE) capable of base pairing to a bulge in the proximal readthrough element (PRTE, Figure 10) is highlighted in yellow. Predicted nonviral sequencing adapter-derived sequence is highlighted in gray (ENaTV10). Numbers in parentheses indicate base positions in the available genome sequence.

lacks an in-frame methionine start codon and appears to be disrupted by frameshift mutations upstream of the ORF3 overlap (Figure 7). Upon aligning the RNA sequence at the beginning of MaTV ORF4 with the closely related PVLaTV1 sequence, we see an AUG in PVLaTV1 sequence that aligns with the second AUG of MaTV ORF4 as does the remaining sequence until position 48 at which a U insertion occurs in PVLaTV1 RNA (Figure 7B). This places the upstream portion of what would encode PVLaTV1 ORF4 out of frame with the rest of the ORF. A U insertion at a similar position also disrupts ORF4 in ENaTV10 ORF4 relative to the 95% identical TTTV1 genome, which has an intact AUG-initiated ORF4 (Figure 7B). Similar frameshift mutations near the 5' end of ORF4 may explain why the ORFs 4 of AVE, BCaTV, ZLaTV, HubTLV1, ENaTV5 and TCaTV1 also appear to lack an in-frame AUG start codon (Supplementary Figure S6).

Other possibilities were considered. If the 5' end of the sgRNA1 is downstream of the predicted ORF4 AUG start codon but upstream of the ORF3 start codon, then we would expect that ORF4 would initiate at the next AUG downstream of the ORF3 start codon, as in the luteo- and poleroviruses (29). However, AVE,

BCaTV1, TCaTV1 and PVLaTV1 have no AUG codons anywhere in ORF4, and ORFs 4 of MaTV and HubTLV2 have no AUG codons downstream of the ORF3 start codon (methionine residues, Figure 7A). The AUG codons in ORFs 4 of the other rimosaviruses are in the middle or C-terminus of the ORF and not in conserved locations (Figure 7A). Thus, initiation by leaky scanning downstream of the ORF3 start codon is highly unlikely. Instead, the most plausible explanation is that ORF4 initiates with a non-AUG codon. The non-AUG codons ACG, AUC, AUU, AUA, UUG, CUG, GUG have been observed to serve as start codons, albeit much less efficiently than AUG (86–88). To allow translation of ORF4 in all twelve rimosaviruses, a non-AUG start codon would have to be located downstream of the homologous positions to the frame-disrupting insertions in ENaTV10 and PVLaTV1 and an in-frame stop codon in AVE (Figure 7C), and upstream of the ORF3 AUG start codon, as initiation of translation at a non-AUG is not likely to take place downstream of the highly efficient AUG. Alignment of this portion of the rimosavirus genome revealed numerous non-AUG codons, known to be capable of initiation (Figure 7C). Thus, we propose that ORF4 is translated by initiation
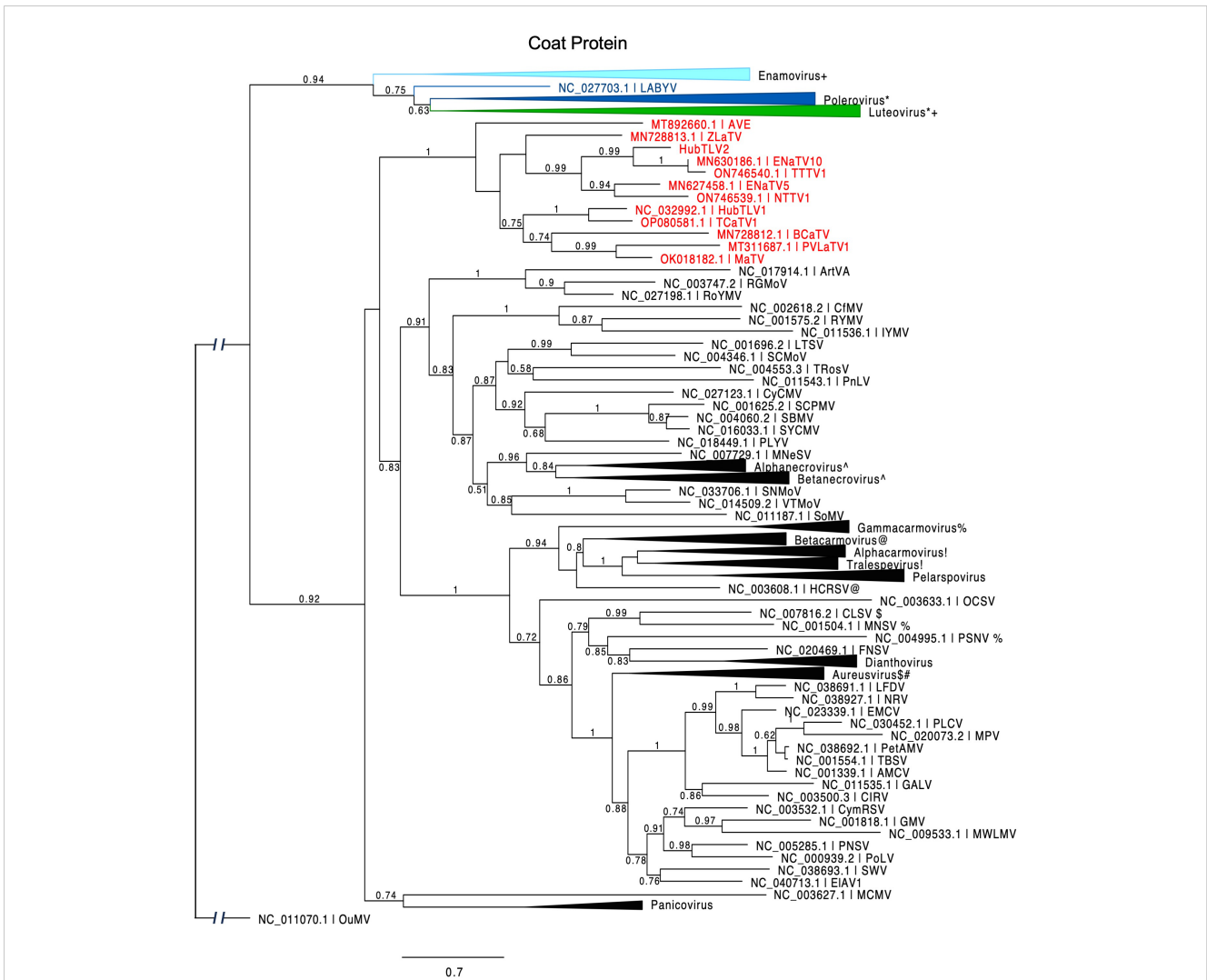
FIGURE 6

Phylogenetic tree predicting the relationship of viral coat proteins (ORF3) in *Tombusviridae* and *Solemoviridae*. Red entries indicate those members of the proposed new *Rimosavirus* genus. Each collapsed tree consists primarily of sequences belonging to the indicated genus. Modifier symbols (+, *, ^, %, etc.) indicate genera where one or more CP sequences from one genus are grouped with the other genus containing same modifier symbol. Branch support values are shown for splits > 0.5 and are calculated from 1,000 resamples of the Shimodaira-Hasegawa test (SH-like local supports). Branch lengths indicate arbitrary units of evolutionary distances. Spell-outs of viral acronym can be found in GenBank via the indicated accession number. Because solemovirids (*Pisuviricota*) and tombusvirids (*Kitrinoviricota*) belong to separate phyla, the outgroup CP sequence (Ourmia melon virus, OuMV) was chosen from a third phylum, *Lenarviricota*.

at one of these non-AUG start codons. This arrangement allows leaky scanning initiation at the AUG of ORF3, resembling the arrangement of ORF3a in luteo- and poleroviruses, which also initiates with a non-AUG codon and overlaps with ORF3 (89).

Regardless of the precise start site, it is clear that ORF4 is present in all 12 rimosaviruses (Figure 1). Starting from the potential non-AUG start codon closest to the CP AUG (Figure 7C), we translated ORFs 4 *in silico* and aligned the protein sequences with those of polero- and luteoviruses. The phylogenetic tree generated from this alignment revealed the rimosavirus P4 proteins diverge highly from those of the polero/luteoviruses and from each other (Figure 8). One rimosavirus, AVE, clusters with a luteovirus clade, but it is so divergent and bootstrap value so low (<0.5) that this branch assignment is not certain.

3.3.5 ORF5

The arrangement of rimosavirus ORFs 3 and 5 resembles that in the L/P/E genera because ORF5 appears to be translated by readthrough of the ORF3 stop codon, creating a large C-terminal readthrough domain (RTD) extension to the CP. The L/P/E RTDs all are more closely related to each other than to any of those from proposed genus *Rimosavirus*, despite the fact that luteoviruses (*Tombusviridae*), and polero- and enamoviruses (both *Solemoviridae*) belong to different families. Interestingly, polerovirus RTDs (dark blue in Figure 9) fall into two major subclades. One clade also includes a luteovirus (green) RTD (bean leafroll virus, BLRV), while the other polerovirus clade includes enamovirus RTDs (light blue). The RTDs of the rimosaviruses (red, Figure 9) all diverge highly from those of L/P/E viruses and from
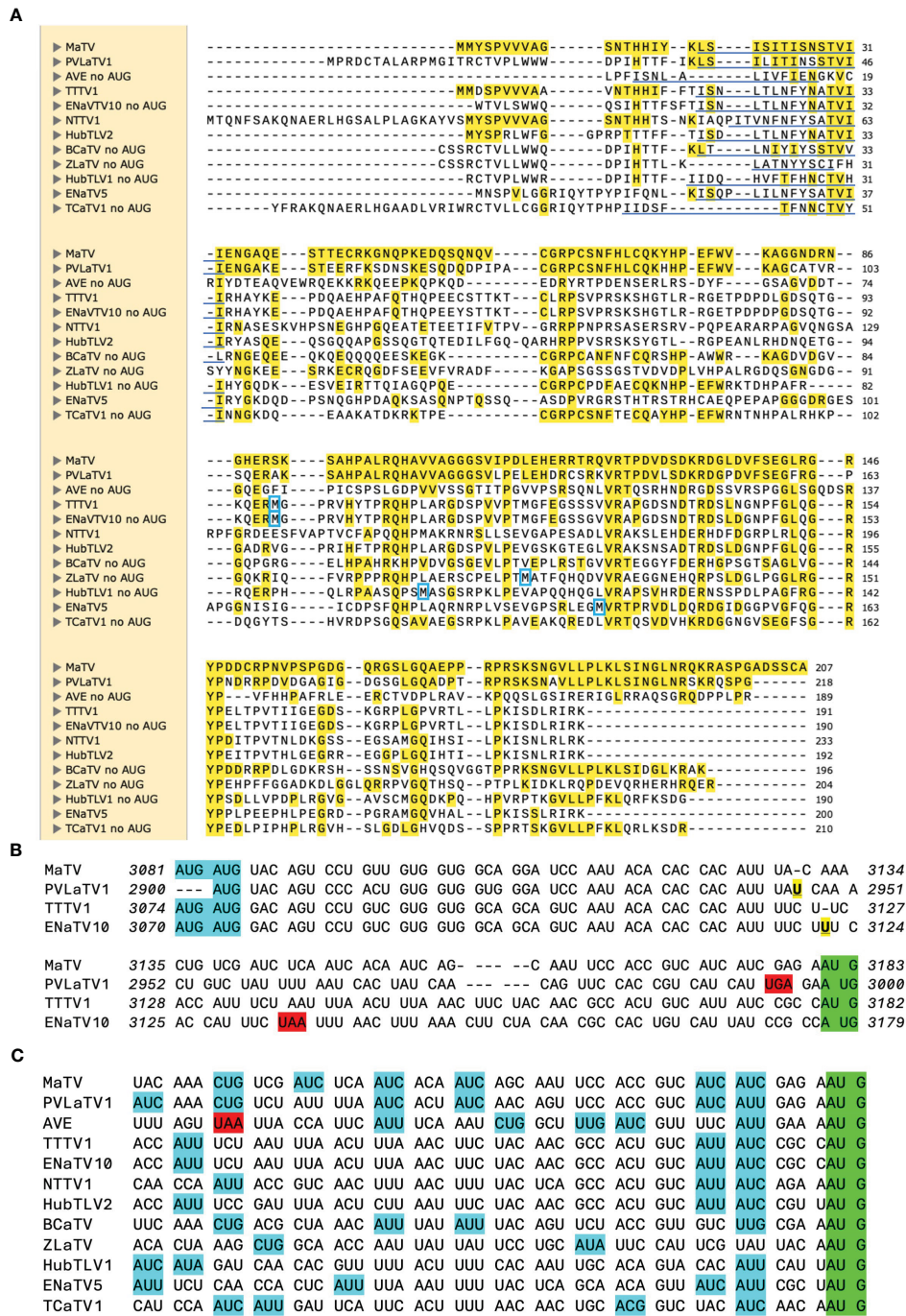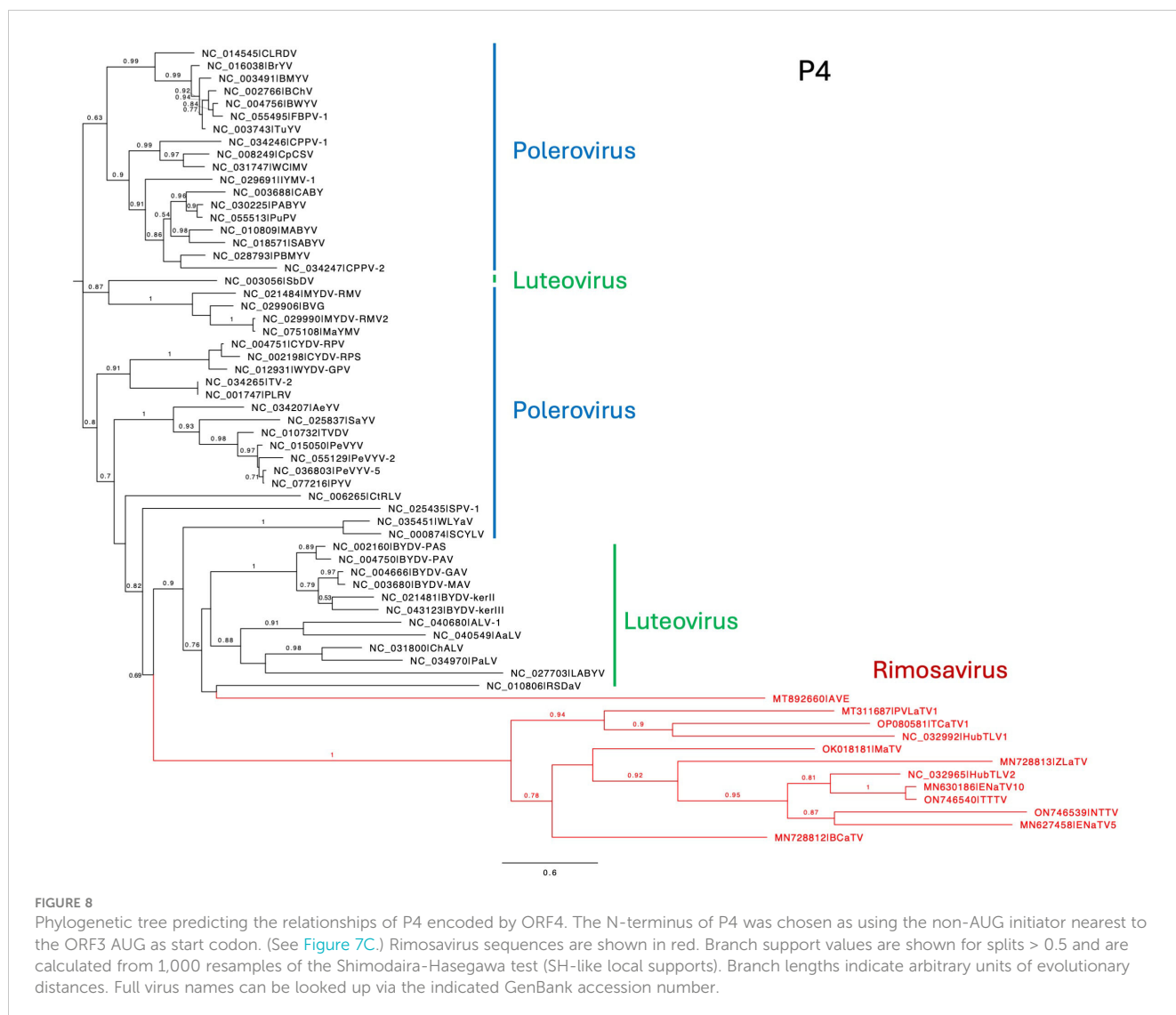
FIGURE 7

ORF4 comparisons. (A). Alignment (MUSCLE) of predicted P4 proteins, starting from an in-frame methionine or immediately downstream of an in-frame stop codon. However, actual predicted N-terminus of P4 is predicted to be from translation initiation at a non-AUG codon in the underlined region [per ungapped nt alignment, (C)]. First met translated from an AUG located downstream (in the ORF4 frame) of the ORF3 AUG is indicate by blue box. MaTV, PVLaTV1, AVE, HubTLV2, BCaTV and TCaTV1 have no such AUGs (i.e. no internal mets). Yellow highlighting: aa identical to that of MaTV P4. (B). Alignment of the regions of ORF4 from their potential AUG start codons (blue) to the ORF3 start codons in a different frame (green), with spacing to show codons starting from the AUGs. The U insertions (bold, underlined, yellow) in PVLaTV1 relative to MaTV, and in ENaTV10 relative to TTTV1, disrupt the reading frames, leading to stop codons (red). For PVLaTV1 and ENaTV10, the potential initiator AUGs are in a different frame than the amino termi in panel (A). Italic numbering indicates genomic positions of first and last nucleotide on each line. See Supplementary Figure S6 for alignments of this portion of all 12 rimosavirus genomes without codon spacing. (C) Possible non-AUG start codons (blue) (86−88) for ORF4 are shown in the 52 nt upstream of the ORF3 start codon (green). Stop codon is highlighted in red.

FIGURE 8
Phylogenetic tree predicting the relationships of P4 encoded by ORF4. The N-terminus of P4 was chosen as using the non-AUG initiator nearest to the ORF3 AUG as start codon. (See Figure 7C.) Rimosavirus sequences are shown in red. Branch support values are shown for splits > 0.5 and are calculated from 1,000 resamples of the Shimodaira-Hasegawa test (SH-like local supports). Branch lengths indicate arbitrary units of evolutionary distances. Full virus names can be looked up via the indicated GenBank accession number.

each other. Although some bootstrap values are low, RTDs of the branches representing HubTLV2/ENaTV10/TTTV1, MaTV/PLVaTV1, AVE, and ZLaTV all are more divergent from each other than all of the divergence among all the L/P/E viruses.

Features of ORF5 and the RTD it encodes reflect these extreme differences from the L/P/E RTDs. For example, ORF5 of L/P/E virus RNAs contains about eight to sixteen direct repeats of the sequence CCXXXX (X = any base) shortly downstream of the ORF3 stop codon. This encodes an alternating proline repeat (PX)n, which is thought to serve as a spacer between the CP and functional domains of the RTD protein (58, 59, 90). However, most rimosaviruses have few, if any CCXXXX or PX repeats in the RNA and encoded protein, respectively (Table 2).

3.4 Readthrough elements

Readthrough of stop codons in viral RNAs is usually facilitated by RNA structures located immediately 3' of the stop codon (23, 31, 54, 55, 91, 92). The ~100 nt tract adjacent to the ORF1

stop codons of all twelve rimosaviruses are well-conserved in sequence and secondary structure (Figure 10). This UAG-proximal structure consists of a stem-loop with four to five helices separated by bulged regions, including a distal bulge with a run of 3 C's, and a more proximal bulge with the consensus RGUUUGG (red, Figure 10). We predict this conserved sequence base pairs with downstream sequences to form a pseudoknotted structure that facilitates readthrough, as shown for other tombusvirids (17, 23). Indeed, in the 3' UTR, just downstream of the GGGC bulged stem-loop is a conserved CCAAAYY sequence in a region predicted to be single stranded (Figure 4C, Supplementary Figure S3). This is the exact position of the distal readthrough element (DRTE) which base pairs to a bulge in the stop codon-proximal readthrough element (PRTE) to facilitate readthrough in carnation Italian ringspot virus (CIRV, genus *Tombusvirus*) (23). A DRTE is also at or near this position in the genomes of at least six other tombusvirid genera, all base pairing to the PRTE with different sequences (23).

For readthrough of the predicted leaky ORF3 stop codon, we look to research on RNA sequences and structures that control readthrough of the homologous stop codon in L/P/E viruses.
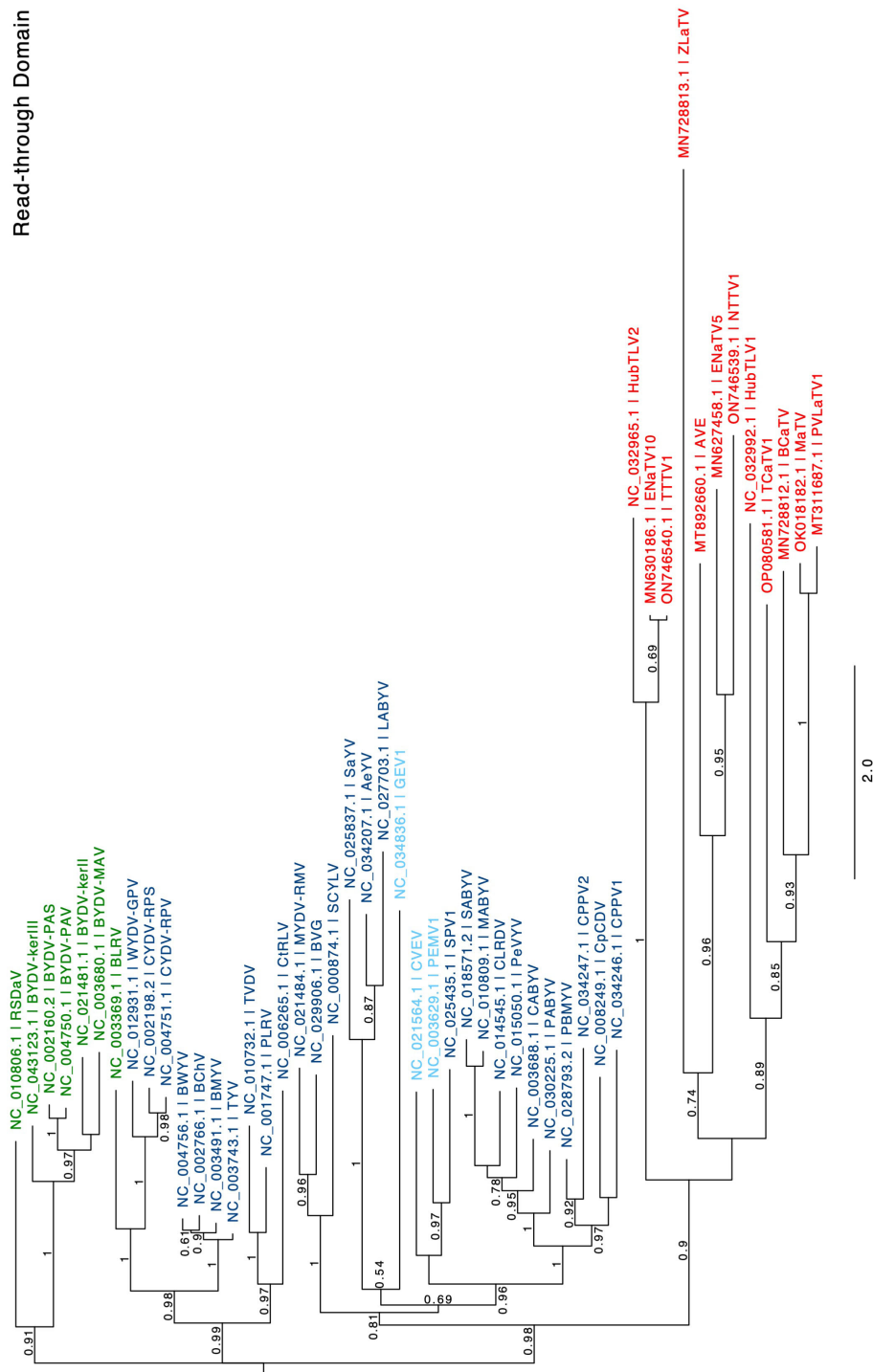
FIGURE 9
Phylogenetic tree predicting the relationships of RTDs (ORF5) based on the amino acid sequences. Genus is color coded in red for *Rimosavirus*, green for *Luteovirus*, dark blue for *Polerovirus* and light blue for *Enamovirus*. Branch support values are shown for splits > 0.5 and are calculated from 1,000 resamples of the Shimodaira-Hasegawa test (SH-like local supports). Branch lengths indicate arbitrary units of evolutionary distances. Full virus names can be looked up via the indicated GenBank accession number.

Mutagenesis of the $(CCXXXX)_{8-16}$ repeat sequence prevented efficient readthrough for BYDV (54) and PLRV (55). Andy White's lab then did a more comprehensive analysis of the Pea enation mosaic virus 1 (PEMV1) (*Enamovirus*) readthrough structure which revealed four separate long-distance base pairings that coaxially stack in the readthrough-facilitating structure (31). That publication also revealed a different arrangement of long-distance base pairings needed for PLRV readthrough. Finally, they showed that local base pairing can compete with the long-distance base pairing to perhaps comprise a switch to regulate readthrough efficiency (31). We predict diverse
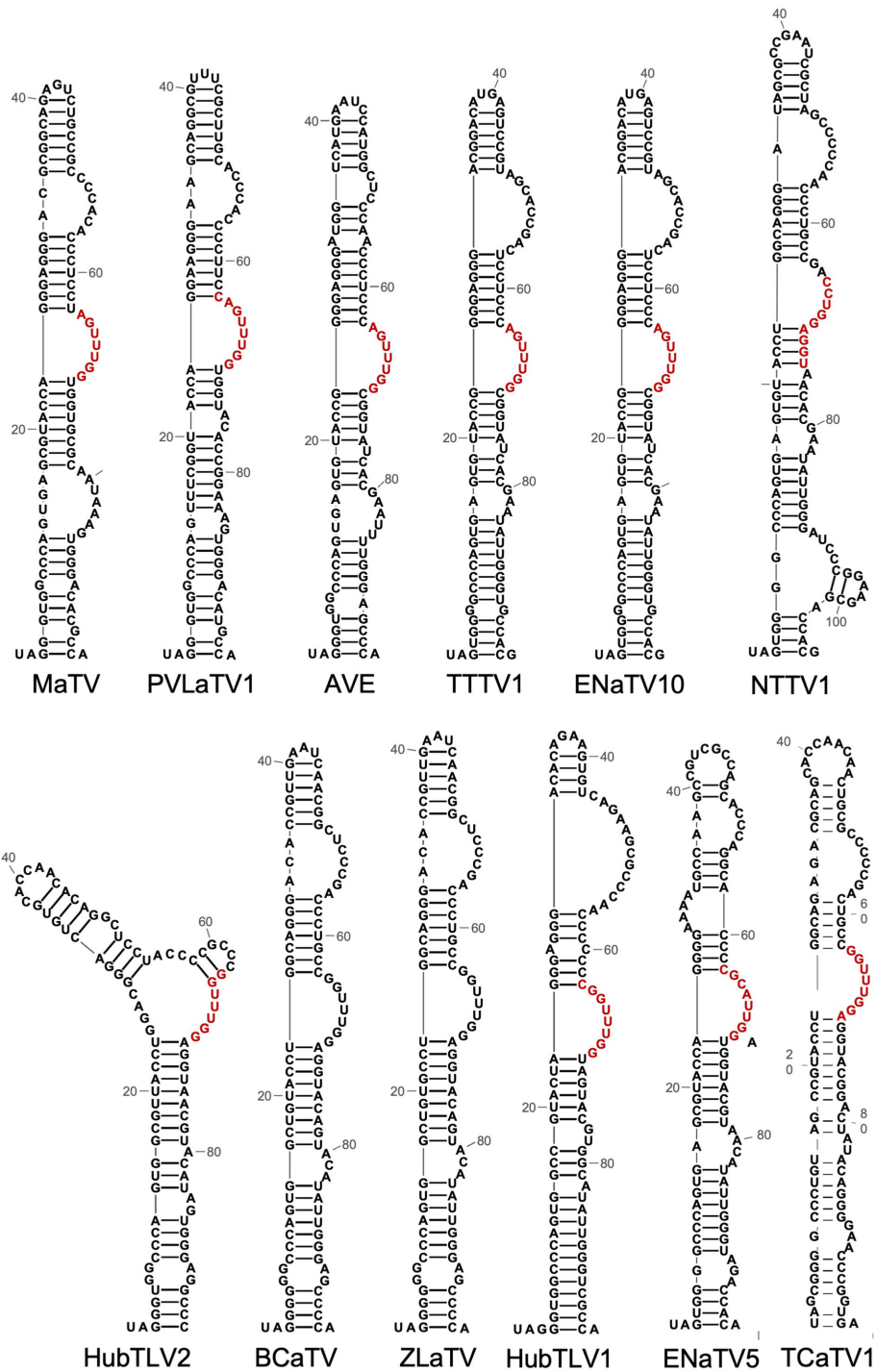
FIGURE 10
Predicted secondary structures (RNA Alifold, Mfold) of sequences beginning with the putatively leaky ORF1 stop codon (UAG in all rimosaviruses). Based on studies of other tombusvirids, these structures comprise the proximal readthrough element (PRTE). Bases in red are predicted to base pair to the distal readthrough element (DRTE, highlighted in yellow in Figure 5). Note the co-variations in which the sequences vary but maintain at least seven consecutive base pairs in all 10 viruses where the base pairing can be predicted. This long-distance base pairing cannot be predicted for BCaTV and ZLaTV PRTEs because the available genome sequences lack the region containing the PRTE.

stem-loop structures adjacent to the ORF5 stop codon, with all except MaTV, PVLaTV1 and AVE having a G-C-rich helix that contains a G-C base pair 7 nt downstream of the stop codon (boxed helices, Supplementary Figure S7). Thus, the RTD for rimosaviruses is novel,

not only in its high amino acid sequence variation in its encoded RTD, but also for lack of obvious conserved sequence (e.g. CCXXXX repeats) or obvious L/P/E-like secondary structure to facilitate the CP ORF5 stop codon readthrough.

4 Discussion

4.1 Remarkable distribution and diversity of sources of rimosaviruses

Rimosavirus sequences were collected from diverse organisms around the globe in large environmental metagenomics sequencing projects with no reports of actual hosts in which they replicate or disease symptoms they cause. Because of their worldwide distribution, it is perhaps surprising that the viruses associated with these genomes have not been discovered previously. It is remarkable that the genome of the virus we call MaTV because its genome was found first in maize and its ancestor teosinte in Mexico, was also found in the cloaca of a tuatara on the tiny uninhabited island of Takpourewa (also known as Stephens Island) in New Zealand (Table 1), a wildlife refuge on which no crops are cultivated (93). Moreover, BCaTV and ZLaTV sequences, first described in kohlrabi and Manchurian wild rice, respectively, in China (10) were also found in the Asian long-horned tick in China (52) and in the cloaca of a tuatara in New Zealand (51).

These wide distributions suggest these viruses may have wide host ranges, and perhaps rather cryptic symptomatology. However, the host range is likely limited to plants, based on their clear membership in the *Tombusviridae* family, despite the fact that several were isolated from various invertebrates, a reptile, and from plant pathogenic fungi (Table 1). The rimosaviruses associated with *Erisyphe necator* (powdery mildew of grapevine) and *Plasmopara viticola* (downy mildew of grapevine) may have infected plant material contaminating the mildew preparation for sequencing, or the mildew may be a vector of the virus. The tombusvirid cucumber necrosis virus is transmitted by zoospores of the soil fungus *Olpidium bornovanus* (94). Recently, cucumber mosaic virus (not a tombusvirid), which has a wide plant host range, was shown to infect and replicate in the plant pathogenic fungus *Rhizoctonia solani* (95). Thus, we cannot rule out that these apparent plant viruses may infect the mildews with which they associate.

The animal-associated rimosavirus genomes may have been acquired from plant material in the diet of Chinese land snails (HubTLV1) or pill worms (HubTLV2). For the carnivorous tuatara, the rimosavirus found in its cloaca could be derived from a herbivorous insect in its diet. Plant viruses, including tombusvirids, have been found in other carnivores such as dragonflies (7) and bats (7), and were assumed to have been obtained this way. Ticks are blood feeders, but even in that case, plant viral sequences, including those of tombusvirids have been identified in the human blood virome, albeit at very low abundance (96). This wide diversity of associations by rimosaviruses is a testament to the high abundance and particle stability of tombusvirids. Clearly, additional experiments are necessary to determine the actual hosts in which these rimosaviruses replicate.

4.2 Gene organization and protein function

As mentioned above, the rimosavirus genome encodes P1 and P2 almost certainly via a readthrough mechanism, which is standard for most tombusvirids. In TBSV, and probably all tombusvirids, P1 binds viral RNA and lines the membrane-bound replication vesicles, while P1-P2 fusion has the RNA-dependent RNA polymerase activity to replicate the genome and transcribe subgenomic mRNA (16). ORF3 clearly encodes the CP to form the T=3 icosahedral virion, based on sequence similarity to other tombusvirids.

We expect P4 plays a role in virus movement and other functions, based on the fact that ORF4, which also overlaps with ORF3 in polero- and luteoviruses, encodes a movement protein (MP) in these viruses (26, 97, 98). It can also boost virus infection by suppressing (i) antiviral RNA silencing (99), (ii) host catalase activity (100), and (iii) thiamine synthesis (101). ORF4 differs (i) markedly in sequence compared to that of the luteo/ poleroviruses, (ii) in that we predict its translation initiates upstream instead of downstream of the ORF3 start codon, and (iii) at a non-AUG start codon. Thirdly, we detected no ORF3a, which encodes another MP in the ORF4-encoding viruses (89, 102).

While unlikely, it cannot be ruled out that ORF4 may not be translated in the rimosaviruses that lack an in-frame AUG start codon for ORF4. Recently some luteoviruses were discovered that lack ORFs 4 and 3a (103, 104). None of the polero-like enamoviruses encode ORFs 3a or 4 (89). For some enamoviruses, the movement functions are provided by a co-infecting umbravirus (105, 106), but for other enamoviruses and the luteoviruses lacking ORFs 3a and 4, no co-infecting partner is known. Thus these and many rimosaviruses may have found other ways to move within the host plant, perhaps by commandeering a host phloem protein as has been observed recently for certain umbravirus-like viruses in the *Tombusviridae* (107). However, given the presence of ORF4 overlapping with most of ORF3 in all twelve rimosavirus genomes, we favor our hypothesis that ORF4 is translated via initiation at a non-AUG shortly upstream of the ORF3 AUG start codon.

Based on its position and homology to ORF5 in L/P/E viruses, ORF5 is highly likely to be translated by readthrough of the ORF3 stop codon, and thus encodes the RTD. In the L/P/E/s, the RTD is essential for the persistent, circulative, nonreplicative transmission by aphids (26, 58, 60, 108, 109). Thus, we speculate that the rimosaviruses may be also transmitted by aphids, but given the extreme sequence differences of many of the rimosavirus RTDs from those of L/P/Es, we wonder if some rimosaviruses may be transmitted by other insect species or possibly non-insect vectors. For example, the unrelated beet necrotic yellow vein virus (*Benyviridae*) encodes an RTD extension on the CP that facilitates fungal transmission of the virus (110). In the poleroviruses, the C-terminal half of the RTD also has been shown to play a role in virus movement in the phloem (111–113). Thus, it appears that ORFs 3a, 4, and C-terminus of ORF5 may act together to ensure efficient, phloem-limited virus movement in the infected plant (102). Other functions are possible. Recently, a CP-RTD protein of an ilarvirus was shown to have silencing suppressor activity (114). The role(s), if any, the rimosavirus RTD plays in vector transmission, virus movement, or silencing suppression is one of the many interesting questions about this cryptic genus that remains to be answered.

Finally, based on the phylogenetic tree showing that all L/P/E RTDs fall into one clade that is less diverse than the branches that

include only rimosaviruses, we propose that rimosavirus RTDs have a very ancient origin, and/or have been undergoing more rapid selection and evolution than the L/P/E RTDs. The polero- and enamoviruses have a replication apparatus so different from the rimosaviruses and luteoviruses that they fall into a different phylum (*Pisuviricota*) from the *Tombusviridae* (*Kitrinoviricota*) (53). Thus, we speculate that a sobemo-like ancestor of the polero- and enamoviruses may have acquired its RTD by recombination in mixed infection with an ancestral rimosa-like or luteo-like virus.

4.3 Noncanonical translation

4.3.1 Potential IRES in the 5′ UTR

Perhaps the most unusual feature of rimosaviruses is the long tract at the 5′ end upstream of the ORF1 initiation codon. MaTV encodes a significant size ORF (ORF0) in this region, and PVLaTV1 encodes a truncated version of this ORF, however (i) there are AUGs upstream of ORF0, (ii) no ORF of substantial size is present or conserved upstream of ORF1 in the other rimosaviruses, and (iii) there are numerous AUGs scattered at different positions upstream of ORF1 in all rimosavirus genomes (Supplementary Figure S1). Thus, we speculate that the 5′UTR may have IRES or ribosome shunting activity, which would be novel, because genomes in the other tombusvirid genera contain a short 5′ UTR (maximum 142 nt in BYDV), relying on the 3′ CITE that facilitates translation by ribosome scanning from the 5′ end. The rimosavirus 5′ UTR has potential to contain an IRES or shunting structure, but we found no significant, conserved secondary structures in the 5′ UTRs. A conserved G-poor tract immediately upstream of the ORF1 start codon is a feature in some translation enhancers such as the tobacco mosaic virus 5′ leader (115), and we also found the tract upstream of and including the G-poor tract is C-rich, which is a feature in some IRESes (116). Although the 3′ UTR is long enough to encode a 3′ CITE (36), we found no secondary structures in the 3′ UTR that resembled known CITEs. Additional computational approaches and of course lab experiments are necessary to determine how translation initiates on rimosavirus genomes.

4.3.2 Leaky start and stop codons

One translation feature that is not mysterious is the secondary structure that controls readthrough of the ORF1 stop codon to allow translation of the RdRp encoded in ORF2. All twelve viruses have obvious bulged stem-loops adjacent to the ORF1 stop codon that can base pair to a 3′ distal readthrough element (DRTE) as has been shown to facilitate readthrough in many tombusviruses (23). Also, the stem-loop near the 3′ end, with the GGGC bulge capable of pseudoknot base pairing to the extreme 3′ terminal bases GCCC resembles the replication structure present in all other studied tombusvirid genomes (20, 71).

By analogy with luteo- and poleroviruses (29, 117), we speculate that ORFs 4, 3 and 5 are translated from sgRNA1, initiating upstream of the ORF3 start codon, as the CP of all tombusvirids is translated from a sgRNA. If our hypothesis of non-AUG translation initiation of ORF4 is correct, then the 5′ end of sgRNA1 must be downstream of any AUG triplets located upstream of the ORF3 AUG, because if the

5′ end of sgRNA1 included those AUGs, scanning ribosomes would initiate at those, rather than the predicted non-AUG start codon and subsequent AUG initiator of ORF3. Bearing this in mind, we sought conserved sequences and secondary structures that may be required for generating the 5′ end of sgRNA1, because such structures have been shown to be required for sgRNA1 synthesis in many tombusvirids (34, 118). Indeed, a conserved sequence and stem-loop is predicted in this region of rimosavirus genomes (underlined bases in Supplementary Figure S6). Co-variations in the few base differences among these sequences support the existence of the stem-loop, implying that base pairing is required, even if the sequences that comprise those base pairs vary (compare underlined regions in PVLaTV1 and AVE in Supplementary Figure S6).

In conclusion, phylogenetic comparisons reveal that the twelve genome sequences found in GenBank described here clearly belong to viruses in a new genus in the *Tombusviridae* family. The biological properties of the viruses associated with these genomes, such as host range, symptomatology, vector specificity, remain to be elucidated. It is clear that the diverse catalog of noncanonical translation mechanisms that are a hallmark of tombusvirid gene expression is enriched by this puzzling new collection of viral genomes.

Author's note

A paper published online after this manuscript was accepted identifies genomes of more probable rimosaviruses: Kim, J., Jeon, E. J., Jun, M., Lee, D.-S., Lee, S.-J., and Lim, S. (2014). Complete genome sequences of two tombusviruslike viruses identified in *Echinacea purpurea* seeds. *Virus Genes* doi: 10.1007/s11262-024-02092-5.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, OK018181.2.

Author contributions

ZL: Formal analysis, Visualization, Writing – original draft, Writing – review & editing. LH: Investigation, Methodology, Writing – review & editing. ES: Formal analysis, Visualization, Writing – review & editing. WM: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fviro.2024.1422934/full#supplementary-material

SUPPLEMENTARY FIGURE 1
5' UTR alignments using default parameters for MUSCLE version 3.8.1551 in SnapGene. Sequences end at the start codon for ORF1. Bases with ≥50% sequence identity at each position are shaded in yellow, with intensity of shading proportional to number of sequences in which the base is conserved at each position. All AUG triplets are highlighted in green.

SUPPLEMENTARY FIGURE 2
Alignment of ORF0 and P0 sequences of MaTV and PLaTLV1. (A) Alignments of complete ORFs. Note PLaTLV1 ORF0 is 60% as long as MaTV ORF0. Identical bases are highlighted in blue. (B) Alignment of P0 encoded by ORF0. (C) Codons encoding the region of highly conserved potential

amino acids. Base differences in wobble position that do not change amino acid are in green. Base differences that change the encoded amino acid are in red. In all three panels, gold box outlines the nts (A, C) that encode the conserved block of 37 codons, or the 37 amino acid sequence itself (B). Note that most of the identical bases in the first row of the alignment in (A) are in different reading frames, hence no aa sequence identity is encoded until bases 70/52 for MaTV and PLaTLV1 ORFs 0, respectively.

SUPPLEMENTARY FIGURE 3
Alignment of amino acid sequences of RdRp domains encoded by ORF2. Alignment was made using MUSCLE version 3.8.1551 in SnapGene using default parameters.

SUPPLEMENTARY FIGURE 4
Predicted (MFOLD) secondary structures of the 3' termini of GenBank sequences of the indicated viral genomes. HubTLV2 sequence is likely incomplete, as it does not terminate in GCCC. Predicted 3' terminus of ENaTV10 genome is indicated. Bases we speculate are adapter-derived are in gray. In this portion of the genome, TTTV1 differs from ENaTV10 at only one base position (indicated) and the TTTV1 GenBank sequence terminates before the actual predicted 3' end, which is the same as that predicted for ENaTV10. Dashed line indicates pseudoknot base pairing conserved in all tombusvirids (dark red and dark blue). Extended potential base pairing beyond conserved 4 base pairs is indicated in lighter shades of red and blue. Downstream translational readthrough element (DTRE), predicted to base pair to a bulge in the stem-loop that facilitates readthrough of the ORF1 stop codon are highlighted in yellow.

SUPPLEMENTARY FIGURE 5
Predicted secondary structures of regions in the 5' end of the 3' UTRs of the indicated viruses. Sequences begin with the ORF5 stop codon.

SUPPLEMENTARY FIGURE 6
Alignment of nucleotide sequences of the AUGs that might be predicted to start ORF4, except for frame changes caused by indels before ORF3 in most cases, through the start codon of ORF3, which overlaps ORF4 in a different frame. Shade of yellow highlighting increases with increased sequence identity at each position. The two underlined tracts are predicted (MFOLD) to base pair to each other to form a stem-loop that may contribute to generating the 5' end of sgRNA1.

SUPPLEMENTARY FIGURE 7
Predicted base pairing 3' proximal to the CP ORF stop codon (UAG in red). 203 nt were used in each prediction. Base numbering starts with the first base of the stop codon. Only the UAG-proximal helices are shown. Dashed line indicates long structured region not shown. Box indicates G-C-rich base-paired region starting with the G-C base pair 7 nt downstream of the stop codon. TTTV1 and ENAV10 sequences are identical with the exception of the blue U in ENaTV10 vs A in TTTV1 at position UAG +3.

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