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Research Article

Positive selection, molecular recombination structure and phylogenetic reconstruction of members of the family Tombusviridae: Implication in virus taxonomy

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Abstract

A detailed study of putative recombination events and their evolution frequency in the whole genome of the currently known members of the family Tombusviridae, comprising 79 accessions retrieved from the international databases, was carried out by using the RECCO and RDP version 3.31β algorithms. The first program allowed the detection of potential recombination sites in seven out of eight virus genera (*Aureusvirus*, *Avenavirus*, *Carmovirus*, *Dianthovirus*, *Necrovirus*, *Panicovirus*, and *Tombusvirus*), the second program provided the same results except for genus *Dianthovirus*. On the other hand, both methods failed to detect recombination breakpoints in the genome of members of genus *Machlomovirus*. Furthermore, based on Fisher's Exact Test of Neutrality, positive selection exerted on protein-coding genes was detected in 17 accession pairs involving 15 different lineages. Except genera *Machlomovirus*, and *Panicovirus* along with unclassified Tombusviridae, all the other taxonomical genera and the unassigned *Tombusviridae* encompassed representatives under positive selection. The evolutionary history of all members of the Tombusviridae family showed that they segregated into eight distinct groups corresponding to the eight genera which constitute this family. The inferred phylogeny reshuffled the classification currently adopted by the International Committee on Taxonomy of Viruses. A reclassification was proposed.

Key words: bioinformatics, phylogeny, recombination, positive selection, Tombusviridae, sequence, taxonomy.

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Introduction

RNA recombination is one of the major factors responsible for the generation of new RNA viruses and retroviruses. The biological mechanisms of recombination differ across organisms, but in broad terms recombination results in the creation of mosaic sequences where the evolutionary history at each site may be different. Recombination, defined as the exchange of genetic information between two nucleotide sequences, is an important process that influences biological evolution at many different levels. Recombination explains a considerable amount of genetic diversity in natural populations and, in general, genes located in regions of the genome with low levels of recombination have low levels of polymorphism (Posada and Crandall, 2001). Recombination reshuffles existing variation and even creates new variants. It has been shown that RNA recombination enables the exchange of genetic material, not only between the same or similar viruses but also between distinctly different viruses (Worobey and Holmes, 1999). Sometimes, it also permits crossovers between viral and host RNA (Greene and Allison, 1994; Aaziz and Tepfer, 1999; Baroth *et al.*, 2000; Nagai *et al.*, 2003). Taking into account the structure of viral genomic molecules and the location of crossover sites, three basic types of RNA recombination were distinguished: homologous, aberrant homologous and non-homologous (Lai, 1992; Alejska *et al.*, 2001). The former two occur between two identical or similar RNAs (or between molecules displaying local homology), while the latter involves two different molecules. Most of the collected data suggest that RNA recombinants are formed according to a copy choice model (Alejska *et al.*, 2001). A viral replication complex starts nascent RNA strand synthesis on one template, called RNA donor, and then switches to another template, called RNA acceptor. Accordingly, two main factors are thought to affect RNA recombination: the structure of recombining molecules and the ability of the viral replicase to switch templates. Through generations, viral populations evolve under various selective forces at different regions and sites that display different functional constraints. A stringent and robust criterion for detecting adaptive evolution in a protein-coding gene is an accelerated nonsynonymous (d_N, amino acid replacing) rate relative to the synonymous (d_S, silent) rate of substitutions, with the rate ratio $\omega = d_N/d_S > 1$. As silent mutations do not change the amino acid whereas re-

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placement mutations do, the difference in their fixation rates provides a measure of selective pressure on the protein.

Amongst positive-strand plant RNA viruses, the family Tombusviridae encompasses several viruses with an important economical impact. According to the 8th ICTV (International Committee on Taxonomy of Viruses) report (Fauquet *et al.*, 2005), the family Tombusviridae includes the following genera: *Tombusvirus*, *Carmovirus*, *Necrovirus*, *Dianthovirus*, *Machlomovirus*, *Avenavirus*, *Aureusvirus* and *Panicovirus*. According to the Baltimore classification, the viruses in this family are classified as Type IV viruses, and are part of the luteovirus supergroup (Habili and Symons, 1989). The RNA is contained in an icosahedral (T = 3) capsid, composed of 180 units of a single coat protein 27-42 kDa in size; the virion measures 28-35 nm in diameter, and is not enveloped. All Tombusviridae have a positive- sense, single-stranded linear genome, with the exception of dianthoviruses, whose genome is bipartite. The genome is approximately 4-5.4 kb in length, depending on the genus. The 3' terminus is not polyadenylated. The 5' terminus is capped only in *Carnation mottle carmovirus*, *Red clover necrotic mosaic dianthovirus* and *Maize chlorotic mottle machlomovirus*. The genome encodes 4-6 ORFs. The polymerase ORF encodes an amber stop codon that is the site of a readthrough event within ORF 1 (except in dianthoviruses, where readthrough occurs *via* a frameshift), producing two products necessary for replication. There is no helicase encoded by the virus. The replication process of members of family Tombusviridae comprises the following steps: (i) the virus penetrates into the host cell, (ii) the viral genomic RNA is uncoated and released into the cytoplasm, (iii) the viral RNA is translated to produce the two proteins necessary for RNA synthesis (replication and transcription), (iv) a negative-sense complementary ssRNA is synthesized using the genome RNA as a template, (v) a new genomic RNA is synthesized using the negative-sense RNA as a template, (vi) the RNA-dependant RNA polymerase (RdRp) recognizes internal subgenomic promoters on the negative-sense RNA, to transcribe the 3' co-terminal subgenomic RNAs that will generate the capsid and movement protein, (vii) new virus particles are formed (White and Nagy, 2004).

The main objective of this work was to determine and characterize virus evolution mechanisms of the Tombusviridae based on the occurrence of putative recombination events and positive selection in their full-length genome. This was achieved by the analysis of 79 accessions obtained from GenBank. As a result, we propose a reclassification according to their predicted evolutionary history.

Material and Methods

The sequences of the entire genome of 79 accessions cataloged in GenBank were used in this study (Table 1).

The nucleotide sequences were aligned using programs CLUSTALW 2.0.9 and CLUSTALX 2.0.9 (Larkin *et al.*, 2007) with default configuration. Their phylogenetic relationships were determined with the Maximum-likelihood (ML) algorithm incorporated in the MEGA version 5 program (Tamura *et al.*, 2011) under assumption of the substitution models proposed by Jukes and Cantor (1969) (JC), Hasegawa *et al.* (1985) (HKY85), and Tamura and Nei (1993) (TN93). Bootstrap analyses with 500 replicates were performed to assess the robustness of the branches.

Using the MEGA4.1β program (Kumar *et al.*, 2008), positive selection was inferred by the counting method described by Nei and Gojobori (1986) and, later on, by Suzuki and Gojobori (1999). According to this method, the phylogenetic tree of sequences analyzed was used. For the parsimony method, the total numbers of synonymous (c_S) and nonsynonymous (c_N) substitutions as well as the average numbers of synonymous (s_S) and nonsynonymous (s_N) sites per codon over the phylogenetic tree for each codon site were computed according to the maximum parsimony principle (Fitch, 1971; Hartigan, 1973). The null hypothesis of selective neutrality ($r_S = r_N$ or $\omega = 1$) was tested for each site by computing the probability (p) of obtaining the observed or more biased values for c_S and c_N, which were assumed to follow a binomial distribution with the probabilities of occurrence of synonymous and nonsynonymous substitutions given by $s_S/(s_S + s_N)$ and $s_N/(s_S + s_N)$, respectively. Positive selection is inferred when $p < 0.05$ and $c_N/s_N > c_S/s_S$ (Suzuki, 2006).

Potential recombination events between diverged nucleotide sequences were explored using two programs: RDP v3.31β (Martin *et al.*, 2005b) and RECCO (Maydt and Lengauer, 2006). RDP incorporates several published recombination detection methods into a single suite of tools: RDP (Martin and Rybicki, 2000), GENECONV (Padidam *et al.*, 1999), BOOTSCAN (Martin *et al.*, 2005a), MAXCHI (Smith, 1992), CHIMAERA (Posada and Crandall, 2001), SISCAN (Gibbs *et al.*, 2000), and 3SEQ (Boni *et al.*, 2007). In all cases, default parameters were used. Only events predicted by more than half of the methods are considered as significant. The algorithm developed and described by Maydt and Lengauer (2006) as being a fast, simple and sensitive method for detecting recombination in a set of sequences and locating putative recombination breakpoints is based on cost minimization. This method has only two tunable parameters, recombination and mutation cost. In practice the only parameter considered is α, representing the cost of mutation relative to recombination. When α changes from 0 to 1, the cost of mutation weighted by α increases, and the cost for recombination weighted by 1 - α decreases. In other words, parameter α controls the ambiguity between mutation and recombination.

Table 1 - Current taxonomic status of the members of the family Tombusviridae included in the study and their accession numbers.

Genus (in bold), unclassified and unassigned viruses	Virus/Isolate	GenBank accession number
Aureusvirus	Cucumber leaf spot virus (CLSV)	NC_007816
	Cucumber leaf spot virus/Canada (CLSV/Canada)	EU127904
	Pothos latent virus/Pigeonpea (PoLV/Pigeonpea)	NC_000939
	Johnsongrass chlorotic stripe mosaic virus/Iran (JCSMV/Iran)	NC_005287
	Maize white line mosaic virus/USA (MaWLMV/USA)	NC_009533
Avenavirus	Oat chlorotic stunt virus (OCSV)	NC_003633
Carmovirus	Cardamine chlorotic fleck virus (CCFV)	NC_001600
	Carnation mottle virus/China (CarMoV/China)	NC_001265
	Carnation mottle virus (CarMoV)	X02986
	Carnation mottle virus/Indian (CarMoV/Indian)	AJ811998
	Cowpea mottle virus (CPMoV)	NC_003535
	Hibiscus chlorotic ringspot virus (HCRSV)	NC_003608
	Hibiscus chlorotic ringspot virus.Tw (HCRSV.Tw)	DQ392986
	Japanese iris necrotic ring virus (JINRV)	NC_002187
	Melon necrotic spot virus/Yamaguchi (MeNSV/Yamaguchi)	AB250687
	Melon necrotic spot virus/Nagasaki (MeNSV/Nagasaki)	AB250686
	Melon necrotic spot virus/Kochi (MeNSV/Kochi)	AB250685
	Melon necrotic spot virus/Chiba (MeNSV/Chiba)	AB250684
	Melon necrotic spot virus/Tottori (MeNSV/Tottori)	AB232925
	Melon necrotic spot virus/Kochi2 (MeNSV/Kochi2)	AB232926
	Melon necrotic spot virus (MeNSV)	NC_001504
	Melon necrotic spot virus/MNSV-ISR (MeNSV/MNSV-ISR)	DQ922807
	Melon necrotic spot virus/MNSV-Al (MeNSV/MNSV-Al)	DQ339157
	Melon necrotic spot virus/MNSV264 (MeNSV/MNSV264)	AY330700
	Melon necrotic spot virus/nK (MeNSV/nK)	AB044292
	Melon necrotic spot virus/NH (MeNSV/NH)	AB044291
	Melon necrotic spot virus/Malfa5 (MeNSV/Malfa5)	AY122286
	Pea stem necrosis virus/Japan (PSNV/Japan)	NC_004995
	Pelargonium flower break virus/MZ10 (PFBV/MZ10)	NC_005286
	Pelargonium flower break virus/SP18 (PFBV/SP18)	DQ256073
	Saguaro cactus virus (SCV)	NC_001780
	Turnip crinkle virus (TCV)	NC_003821
	Turnip crinkle virus/UK (TCV/UK)	AY312063
Unclassified	Angelonia flower break virus/Florida (AFBV/Florida)	NC_007733
Carmoviruses	Soybean yellow mottle mosaic virus/MS1-USA (SYMoMV/MS1-USA)	FJ707484
	Soybean yellow mottle mosaic virus/South Korea (SYMoMV/s.Korea)	NC_011643
Dianthovirus	Carnation ringspot virus RNA 1 (CarRSV-RNA 1)	NC_003530
	Carnation ringspot virus RNA 2 (CarRSV-RNA 2)	NC_003531
	Red clover necrotic mosaic virus RNA 1 (RCNMV-RNA 1)	NC_003756
	Red clover necrotic mosaic virus RNA 2 (RCNMV-RNA 2)	NC_003775
	Red clover necrotic mosaic virus RNA 1/Can (RCNMV-RNA 1/Can)	AB034916
	Red clover necrotic mosaic virus RNA 2/Can (RCNMV-RNA 2/Can)	AB034917
	Sweet clover necrotic mosaic virus RNA 1/59 (SCNMV-RNA 1/59)	NC_003806
	Sweet clover necrotic mosaic virus RNA 2/59 (SCNMV-RNA 2/59)	NC_003807
	Sweet clover necrotic mosaic virus RNA 2/38 (SCNMV-RNA 2/38)	S46027

Table 1 (cont.)

Genus (in bold), unclassified and unassigned viruses	Virus/Isolate	GenBank accession number
Unclassified dianthovirus	Rice virus X RNA 1 (RVX-RNA 1)	AB033715
Machlomovirus	Maize chlorotic mottle virus (MCMoV)	NC_003627
	Maize chlorotic mottle virus/Nebraska (MCMoV/Nebraska)	EU358605
Necrovirus	Beet black scorch virus (BBSV)	NC_004452
	Beet black scorch virus/Val25-Iran (BBSV/Val25-Iran)	EU545828
	Beet black scorch virus/CO-USA (BBSV/CO-USA)	EF153268
	Beet black scorch virus/Xinjiang (BBSV/Xinjiang)	AY626780
	Leek white stripe virus (LWSV)	NC_001822
	Olive latent virus 1/Citrus (OLV-1/Citrus)	NC_001721
	Olive latent virus 1/GM6-Portugal (OLV-1/GM6-Portugal)	DQ083996
	Tobacco necrosis virus A/FM1B (TNV-A/FM1B)	NC_001777
	Tobacco necrosis virus A/C (TNV-A/C)	AY546104
	Tobacco necrosis virus D/Hungarian (TNV-D/Hungarian)	NC_003487
	Tobacco necrosis virus D/Rhotamsted (TNV-D/Rhotamsted)	D00942
Unclassified necrovirus	Olive mild mosaic virus/GP-POrtugal (OMMV/GP-Portugal)	NC_006939
Panicovirus	Panicum mosaic virus (PMV)	NC_002598
Tombusvirus	Artichoke mottled crinkle virus/Bari (AMoCV/Bari)	NC_001339
	Carnation Italian ringspot virus (CarIRSV)	NC_003500
	Cucumber bulgarian latent virus (CBLV)	NC_004725
	Cucumber necrosis virus (CNV)	NC_001469
	Cymbidium ringspot virus (CymRSV)	NC_003532
	Grapevine algerian latent virus/nipplefruit (GALV/nipplefruit)	NC_011535
	Pear latent virus (PeLV)	NC_004723
	Tomato bushy stunt virus/Statice (TBSV/Statice)	AJ249740
	Tomato bushy stunt virus/Nipplefruit (TBSV/Nipplefruit)	AY579432
	Tomato bushy stunt virus/Pepper (TBSV/Pepper)	U80935
	Tomato bushy stunt virus/Cherry (TBSV/Cherry)	M21958
Unclassified Tombusviruses	Lisianthus necrosis virus/L (LNV/L)	NC_007983
	Lisianthus necrosis virus/Zantedeschia (LNV/Zantedeschia)	AM711119
	Pelargonium necrotic spot virus (PNSV)	NC_005285
Unassigned *Tombusviridae*	Maize necrotic streak virus (MaNSV)	NC_007729
	Pelargonium line pattern virus/PV-0193 (PLPV/PV-0193)	NC_007017
Unclassified *Tombusviridae*	Nootka lupine vein clearing virus/Alaska (NLVCV/Alaska)	NC_009017
	Pelargonium chlorotic ring pattern virus/ GR 57 (PCRPV/GR 57)	NC_005985

Results

Recombination events during Tombusviridae evolution

Examination of the RECCO program output regarding the occurrence of recombination events in the complete genome of the Tombusviridae family, revealed that three out of five aureusviruses were putative recombinants (PoLV.Pigeonpea, JCSMV.Iran, MaWLMV.USA). In contrast, CLSV (unknown isolate) and CLSV.Canada did not show any recombinant signal (Table 2). Within the genus *Aureusvirus*, the most frequently recombining virus was PoLV.Pigeonpea (33 putative recombination sites), whereas only 28 possible recombination signals were detected in the genome of viruses JCSMV.Iran and MaWLMV.USA. Similarly, the only representative of the genus *Avenavirus* (OCSV) was a potential recombinant with 175 putative sites. The RDP package confirmed these results for both genera. Among the carmoviruses, 14 out of 30 members were possible recombinants. According to RECCO, the most frequently recombining virus was JINRSV with 134 putative events, while MeNSV.Nagasaki

Table 2 - Determination of inferred putative recombination events and their frequency along the sequences of the entire genome of aureusviruses, one avenavirus and carmoviruses. Algorithm RDP v3.31β showed that only events supported by more than half of the different methods are reported. Nucleotide numbering corresponds to the aligned sequences. Abbreviations: NRS: -number of recombination sites, GIRE: -genomic interval of recombination events (the span of sequences in the viral genome where recombination events were predicted).

	Recombination determined by RECCO							Recombination determined by RDP v3.31β
Virus.isolate			Length of breakpoint				Genomic position of the longest breakpoint (size in nucleotide)	Putative parental (Major x Minor)
	NRS	GIRE (nt)	1 residue	2 residues	3 residues	> 3 residues		
PoLV.Pigeonpea	33	607-5320	10	8	3	12	2148-2184 (37)	OCSV x MeNSV/MNSV-264 CarRSV RNA 1 x MeNSV-Tottori CarRSV RNA 1 x MeNSV-Kochi 2
JCSMV.Iran	28	2457-5288	11	6	3	8	3790-3802 (13)	CarRSV RNA 1 x MeNSV-Tottori CarRSV RNA 1 x MeNSV-Kochi 2
MaWLMV.USA	28	3212-4043	18	3	1	6	3836-3853 (18)	OCSV x MeNSV/MNSV-264 CarRSV RNA 1 x MeNSV-Tottori
OCSV	175	766-5052	65	33	23	54	1295-1306 (12) 5036-5047 (12)	TCV.UK x MaWLMV.USA
CCFV	20	894-4681	7	2	3	8	1896-1908 (13)	/
CarMoV	5	1349-2124	0	0	1	4	1986-2045 (60)	/
CPMoV	14	1316-3143	4	6	2	2	2850-2854 (5)	/
JINRSV	134	689-5098	58	30	20	26	1736-1760 (25)	/
MeNSV.Nagasaki	2	623-4892	0	0	0	2	4847-4892 (46)	/
MeNSV.Kochi	4	1843-4694	1	0	0	3	3866-3907 (42)	/
MeNSV.MNSV-A1	3	619-4908	0	0	0	3	4864-4908 (45)	/
MeNSV.MNSV-264	5	4940-5131	3	0	2	0	4952-4954 (3) 5129-5131 (3)	/
MeNSV.NK	2	615-4908	0	1	0	1	615-874 (60)	SYMoMV/MS1-USA x TBSV.Statice
MeNSV.NH	5	615-4983	1	1	0	3	2070-2151 (82)	YMoMV/MS1-USA x TBSV.Statice
MeNSV.Malfa5	13	677-4831	0	0	2	11	1736-1796 (61)	/
PSNV.Japan	80	645-5174	34	15	9	22	4225-4266 (42)	/
SCV	61	665-4506	28	6	13	14	1887-1898 (12) 2761-2772 (12)	/
AFBV.Florida	87	665-5046	34	15	15	23	2151-2166 (16)	/

and MeNSV.NK had the lowest number of recombination sites (two putative sites). The RDP v3.31β algorithm confirmed the occurrence of possible recombination events only for accessions MeNSV.NK and MeNSV.NH. Recombination investigations of genus *Dianthovirus* based on RECCO analysis showed that only 80% of them were possible recombinants (CarRSV-RNA 1 and 2, RCNMV-RNA 1 and 2, RCNMV.Can. RNA 1 and 2, SCNMV.59.RNA 1, and RVX.RNA 1). in contrast, SCNMV.59.RNA 2 and SCNMV.38.RNA did not show any putative recombination signals. However, the RDP package did not predict recombination in the dianthoviruses (Table 3). Although the most frequently recombining necrovirus was RVX (166 putative sites), RCNMV.RNA 2 had only two putative sites. Based on RECCO analysis, 50% of the necroviruses (BBSV.Val25.Iran, LWSV, TNV-A.FMB, TNV-A.C, TNV-D.Hungarian, and OMMV-GP.Portugal,) were possible recombinants. Conversely, BBSV, BBSV.CO.USA, BBSV.Xinjiang, OLV-1.Citrus, OLV-1.GM6.Portugal, and TNV-D.Rhotamsted were not possible recombinants. These results were congruent with those obtained with the RDP package. While the most frequently recombining virus was LWSV (39 sites), TNV.A.C recombined into two sites. Regarding the sole representative of genus *Panicovirus* (PMV), the results obtained by the two methods (RECCO and RDP v3.31β) were incongruent. Indeed, with RECCO, 108 possible sites were detected, whereas no recombination signals were found with the RDP package. A similar situation was observed with regard to the newly proposed carmoviruses (NLVCV.Alaska, PCRPV.GR 57, PLPV.PV-0193). According to RECCO analysis, although NLVCV.Alaska was the most frequently recombining virus (65 sites), PLPV.PV-0193 recombined only into 38 sites (Table 4). Regarding the members of genus *Tombusvirus*, there was an agreement between the two methods indicating that 80% of the analyzed accessions were putative recombinants. While CBLV had the highest number of putative recombination signals (67 sites), TBSV.Cherry had only two recombination sites. Furthermore, it is noteworthy that the two representatives of genus *Machlomovirus* (MCMoV, and MCMoV.Nebraska) were not recombinants as assessed by the two methods of analysis used in this study. Seeking for the recombination frequency in the genome of the Tombusviridae, two-thirds of the aureusviruses (JCSMV.Iran, and MaWLMV.USA) showed that in most cases, their breakpoint length was a single residue. In contrast, the breakpoint length of most putative recombination sites of PoLV.Pigeonpea was between three and 37 nucleotides (Table 2). Also, the breakpoint length of the major recombination sites of the single representative of genus *Avenavirus* (OCSV) consisted of a single residue. In about 50% of the members of the genus *Carmovirus*, the length of their most detected recombination sites was a single residue. As opposed to that, the breakpoint interval of the remaining members exceeded three residues reaching a size as long as 82 residues (MeNSV.NH). In 62% of the investigated dianthoviruses, the breakpoint length exceeded three nucleotides reaching 100 residues (CarRSV.RNA 1) (Table 3). In the necroviruses, the breakpoint interval distribution was similar *i.e.*, 50% of the breakpoints consisted of a single residue, while the remaining breakpoints were between three and 77 nucleotides. For the sole member of the genus *Panicovirus* (PMV), most of the recombination sites had a breakpoint length of a single residue (45) (Table 3). As for the tombusviruses, 75% showed a breakpoint length exceeding three residues up to 161 nucleotides (AMoCV.Bari) (Table 4).

Nucleotide sequence analysis

Maximum composite likelihood estimate of the nucleotide substitution pattern were made using the MEGA4.1β program. The results for Tombusviridae showed that the rates of different transitional substitutions varied from 3.18 to 14.61, and those of transversional substitutions varied from 6.6 to 8.57. The nucleotide frequencies were: 0.269 (A), 0.258 (T/U), 0.207 (C), and 0.266 (G). The transition/transversion rate ratios were $k_1 = 1.705$ (purines) and $k_2 = 0.482$ (pyrimidines). The overall transition/transversion bias was $R = 0.547$, where $R = [AGk_1 + TCk_2]/[(A+G)(T+C)]$. There were a total of 1218 positions in the final dataset. In all these analyses, the codon positions included were first + second + third + noncoding. All positions containing gaps and missing data were excluded from the dataset (complete deletion option).

The MEGA4.1β program also incorporates the Tajima's Neutrality Test. The purpose of this test is to indentify sequences which do not fit the neutral theory model at equilibrium between mutation and genetic drift. Tajima's test compares a standardized measure of the total number of segregating sites (the polymorphic DNA sites) in the sampled DNA and the average number of mutations between pairs in the sample. Tajima's D was determined ($D = 5.280926$).

Positive selection

The high genetic stability of viruses can be attributed to negative or purifying selection to maintain the functional integrity of the viral genome. The degree of negative selection in genes, or the degree of functional constraint for the maintenance of the encoded protein sequence, can be estimated, as mentioned above, by the ratio between the nucleotide diversities in nonsynonymous and synonymous positions (d_N/d_S). For most coding genes the d_N/d_S ratio is < 1 which is consistent with negative selection against protein change. In contrast, a d_N/d_S ratio > 1 may be an indication that adaptive or positive selection is driving gene divergence. In this study, pairwise comparisons of all screened accessions showed that, none of the members of the genera *Machlomovirus* and *Panicovirus,* and unclassified

Table 3 - Determination of inferred putative recombination events and their frequency along the sequences of necroviruses, one panicovirus and dianthoviruses. Algorithm RDP v3.31β showed that only events supported by more than half of the different methods are reported. Nucleotide numbering corresponds to the aligned sequences. Abbreviations: NRS: -number of recombination sites, GIRE: -genomic interval of recombination events (the span of sequences in the viral genome where recombination events were predicted).

	Recombination determined by RECCO							Recombination determined by RDP v3.31β
Virus.isolate			Length of breakpoint				Genomic position of the longest breakpoint (size in nucleotide)	Putative parental (Major x Minor)
	NRS	GIRE (nt)	1 residue	2 residues	3 residues	> 3 residues		
CarRSV-RNA 1	19	709-4561	7	3	2	7	2581-2680 (100)	/
CarRSV-RNA 2	3	1826-2811	0	0	0	3	1826-1835 (10) 2802-2811 (10)	/
RCNMV-RNA 1	8	1297-4754	0	1	2	5	1479-1533 (55)	/
RCNMV-RNA 2	2	2696-2812	1	0	0	1	2803-2812 (10)	/
RCNMV-RNA1.Can	7	720-4218	2	0	0	5	1556-1644 (89)	/
RCNMV-RNA 2.Can	10	1730-2827	3	1	2	4	1730-1765 (36)	/
SCNMV-RNA 1.59	12	858-4443	2	2	1	7	3126-3193 (68)	/
RVX-RNA 1	166	439-5107	65	39	19	43	840-855 (16)	/
BBSV-Val25.Iran	4	800-4881	2	0	0	2	1435-1468 (34)	MeNSV.NH x LNV.Zantedeschia MeNSV.Malfa5 x MaNSV SCV x CNV MeNSV.Yamaguchi x CNV
LWSV	39	1027-4853	17	9	5	8	4846-4853 (8)	MeNSV.NH x LNV.Zantedeschia SCV x CNV
TNV-A.FM1B	3	2419-2789	3	0	2	1	2419-2496 (77)	OLV1.GM6-Portugal x BBSV.Xinjiang
TNV-A.C	2	2275-2619	0	0	0	2	2275-2619 (10)	OLV1.GM6-Portugal x BBSV.Xinjiang LWSV x TNV.D.Rhotamsted
TNV-D.Hungarian	4	2323-4287	2	0	0	2	3959-3964 (6)	MeNSV.NH x LNV.Zantedeschia MeNSV.Malfa5 x MaNSV BBSV x OMMV.GP.Portugal SCV x CNV
OMMV-GP.Portugal	20	935-4857	6	3	4	7	2116-2145 (30)	TNV.A.C x TNV.D.Hungarian TNV.A.C x TNV.D.Rhotamsted TNV.D.Rhotamsted x TNV.A.FM1B OLV1.GM6-Portugal x TNV.D.Hungarian
PMV	108	166-4751	45	28	12	23	1491-1505 (15)	/

Table 4 - Determination of inferred putative recombination events and their frequency along the sequences of the tombusviruses and tentative new members of genus *Carmovirus*. Algorithm RDP v3.31β showed that only events supported by more than half of the different methods are reported. Nucleotide numbering corresponds to the aligned sequences. Abbreviations: NRS: -number of recombination sites, GIRE: -genomic interval of recombination events (the span of sequences in the viral genome where recombination events were predicted).

Virus.isolate	Recombination determined by RECCO							Recombination determined by RDP v3.31β
			Length of breakpoint				Genomic position of the longest breakpoint (size in nucleotide)	Putative parental (Major x Minor)
	NRS	GIRE (nt)	1 residue	2 residues	3 residues	> 3 residues		
AMoCV.Bari	27	1074-4774	2	4	3	18	4614-4774 (161)	CymRSV x CBLV GALV.nipplefruit x MeNSV.Yamaguchi
CarIRSV	28	1998-5345	9	5	8	6	4775-4842 (68)	TBSV.pepper x PNSV TBSV.Statice x MeNSV.Tottori AMoCV.Bari x PNSV LNV.Zantedeschia x MaNSV SCV x CBLV PNSV x TBSV.Pepper CBLV x MeNSV.MNSV-ISR LNV.Zantedeschia x TBSV.Statice
CBLV	67	343-5390	16	17	10	24	1804-1836 (33)	TBSV.Pepper x JCSMV.Iran CarRSV.RNA 1 x MeNSV.Tottori GALV.nipplefruit x MaWLMV.USA PeLV x MaWLMV.USA LNV.Zantedeschia x MaNSV CymRSV x MaNSV
CNV	34	417-5280	8	2	6	18	2681-2714 (34)	CymRSV x MeNSV TBSV.Pepper x PNSV CymRSV x SCV LNV.L x TBSV.Pepper CymRSV x CBLV TBSV.Statice x LNV.L
CymRSV	44	675-5066	9	9	4	22	1719-1767 (49)	PNSV x TBSV.Pepper CarIRSV x CLSV.Israel TBSV.Statice x TCV.UK CNV x CLSV.Canada CNV x TCV.UK MeNSV.MNSV.ISR x PeLV
GALV.nipplefruit	45	369-5299	7	4	11	23	4481-4532 (52)	TBSV.Statice x CNV TBSV.nipplefruit x MaNSV TBSV.nipplefruit x TBSV.Pepper TBSV.Statice x PeLV CymRSV x CBLV CBLV x PNSV CymRSV x PeLV AMoCV.Bari x TBSN.Pepper TBSV.nipplefruit x PeLV PeLV x TBSV.Pepper TBSV.Pepper x CymRSV

Table 4 (cont.)			Recombination determined by RECCO					Recombination determined by RDP v3.31β
Virus.isolate			Length of breakpoint				Genomic position of the longest breakpoint (size in nucleotide)	Putative parental (Major x Minor)
	NRS	GIRE (nt)	1 residue	2 residues	3 residues	> 3 residues		
TBSV.statice	11	379-2743	1	0	1	9	1902-1931 (30)	GALV.nipplefruit x LNV.Zantedeschia CymRSV x CBLV CymRSV x PeLV TBSV.nipplefruit x TBSV.Pepper
TBSV.nipplefruit	7	618-1931	0	0	1	6	1550-1651 (102)	GALV.nipplefruit x LNV.Zantedeschia AMoCV.Bari x GALV.nipplefruit
TBSV.pepper	7	2167-4890	0	0	1	5	4852-4890 (39)	CymRSV x CBLV PeLV x AMoCV.Bari GALV.nipplefruit x LNV.Zantedeschia
TBSV.cherry	2	5324-5345	0	0	2	0	5324-5326 (3) 5343-5345 (3)	CymRSV x CBLV CarIRSV x AMoCV.Bari CymRSV x PeLV GALV.nipplefruit x LNV.Zantedeschia
MaNSV	54	547-5334	29	4	7	14	1932-1955 (24)	GALV.nipplefruit x MaWLMV.USA PeLV x MaWLMV.USA
PNSV	24	2025-5032	7	2	3	12	5009-5032 (23)	TBSV.Statice x MeNSV.Tottori CNV x CLSV.Canada AMoCV.Bari x TBSV.Cherry TBSV.nipplefruit x PeLV LNV.Zantedeschia x MaNSV TBSV.Statice x CNV CBLV x MeNSV.MNSV.ISR LNV.Zantedeschia x TBSV.Statice
NLVCV.alaska	65	771-5301	28	13	7	17	771-782 (12)	/
PCRPV.GR 57	45	1109-5104	11	17	4	13	2307-2316 (20)	/
PLPV.PV-0193	38	991-4908	20	7	2	9	2813-2821 (9)	/

Tombusviridae was under positive selection. On the contrary, the genera *Aureusvirus* (JCMSV.Iran), *Avenavirus* (OCSV), *Carmovirus* (CarMoV.China, CarMoV.Indian), *Dianthovirus* (CarRSV-RNA 2, RCNMV-RNA 2, SCNMV-RNA 2.59, SCNMV-RNA 2.38), *Necrovirus* (BBSV, BBSV.Val25.Iran), *Tombuvirus* (GALV.nipplefruit, PeLV, TBSV.Statice, PNSV) along with the unassigned Tombusviridae (PLPV.PV.0193) were under positive selection (Table 5). It is worth pointing out that, in the viruses with a segmented genome, positive selection was detected only in RNA 2, suggesting that probably reassortment events occurred. All these results were obtained by testing neutrality in sequence pairs with Fisher's Exact Test. The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) in favor of positive selection for each sequence pair was determined. Values of p less than 0.05 were considered significant at the 5% level. The variance of the difference ($d_N - d_S$) was computed using the bootstrap method (500 replicates). All analyses were made using the Nei-Gojobori method incorporated in the MEGA program. All positions containing gaps and missing data were excluded from the dataset (complete deletion option). The final dataset comprised a total of 234 positions.

Phylogenetic relationships

The phylogenetic relationships among members of the family Tombusviridae, based on the sequences of their complete genome, were inferred using a Maximum Likelihood algorithm under the assumption of three models of substitution (JC, HKY85, TN93). The topologies of the constructed trees were identical. The inferred phylogeny showed that each taxonomical genus in the family Tombusviridae constituted a homogenous group clearly distinct from the others. However, the results obtained in this study evidenced a few differences in terms of virus species composition within each taxonomical genus compared to the current classification adopted by the ICTV. In fact, three viruses considered by the ICTV as unassigned (PLPV.PV-0193) and unclassified Tombusviridae (NLVCV.Alaska, PCRPV.GR 57) showed a close phylogenetic relationship to known members of the genus *Carmovirus*. Moreover, the viruses belonging to this genus were divided into two distinct subgroups. The first subgroup comprised viruses: TCV, CCFV, JINRV, HCRSV, PLPV, PCRPV, NLVCV, SCV, AFBV, PFBV, CPMoV, SYMoMV and CarMoV, and the second subgroup encompassed viruses: MeNSV, and PSNV. Furthermore, it was proposed that genus *Necrovirus* should be constituted by two distinct subgroups named tentative Subgroup I (BBSV, TNV.D, LWSV) and tentative subgroup II (OMMV, TNV.A, OLV-1) (Figure 1). It should be noted that here OMMV is an integral part of subgroup I rather than an unclassified *Necrovirus*. In contrast, genus *Aureusvirus* encompassed members that evolved in a homogenous manner: CLSV, PoLV, MaWLMV, and JCSMV. Similarly, the following members of genus *Tombusvirus* also formed a coherent ensemble: MaNSV, CBLV, LNV.L, LNV.Zantedeschia, PeLV, CNV, CymRSV, AMoCV, TBSV.Statice, TBSV.Nipplefruit, TBSV.Pepper, TBSV.Cherry, GALV, PNSV, and CarIRSV. Their evolutionary history

Table 5 - Accession pairs in family Tombusviridae under positive selection with probability determined at the 5% level, based on Fisher's Exact Test of Neutrality and calculated $d_N - d_S$.

Accession pairs	Virus.isolate pairs	p value at 5% level	$d_N - d_S$
NC_007017/NC_003775	PLPV/PV-0193/ RCNMV-RNA 2	0.028	0.923
AB034917/NC_003633	RCNMV-RNA 2.Can/OCSV	0.016	2.325
NC_003531/NC_003633	CarRSV-RNA 2/ OCSV	0.039	1.820
S46027/NC_003633	SCNMV-RNA 2.38/ OCSV	0.041	1.847
NC_003775/NC_003633	PLPV.PV-0193/ OCSV	0.048	1.975
NC_003807/NC_003633	SCNMV-RNA 2.59/ OCSV	0.033	1.999
AB034917/NC_001265	RCNMV-RNA 2.Can/ CarMoV.China	0.034	1.620
AB034917/AJ249740	RCNMV-RNA 2.Can/ TBSV.Statice	0.029	1.921
NC_003531/NC_005287	CarRSV-RNA 2/ JCSMV.Iran	0.037	1.790
S46027/EU545828	SCNMV-RNA 2.38/ BBSV.Val25-Iran	0.045	1.682
AB034917/AJ811998	RCNMV-RNA 2/ CarMoV.Indian	0.031	1.679
AB034917/NC_011535	RCNMV-RNA 2/ GALV.nipplefruit	0.041	1.933
NC_003775/NC_011535	PLPV/PV-0193/ GALV.nipplefruit	0.033	1.921
NC_003807/NC_005285	SCNMV-RNA 2.59/ PNSV	0.049	1.773
S46027/NC_004723	SCNMV-RNA 2.38/ PeLV	0.047	1.735
S46027/NC_004452	SCNMV-RNA 2.38/ BBSV	0.043	1.749
NC_003807/NC_004452	SCNMV-RNA 2.59/ BBSV	0.050	1.654

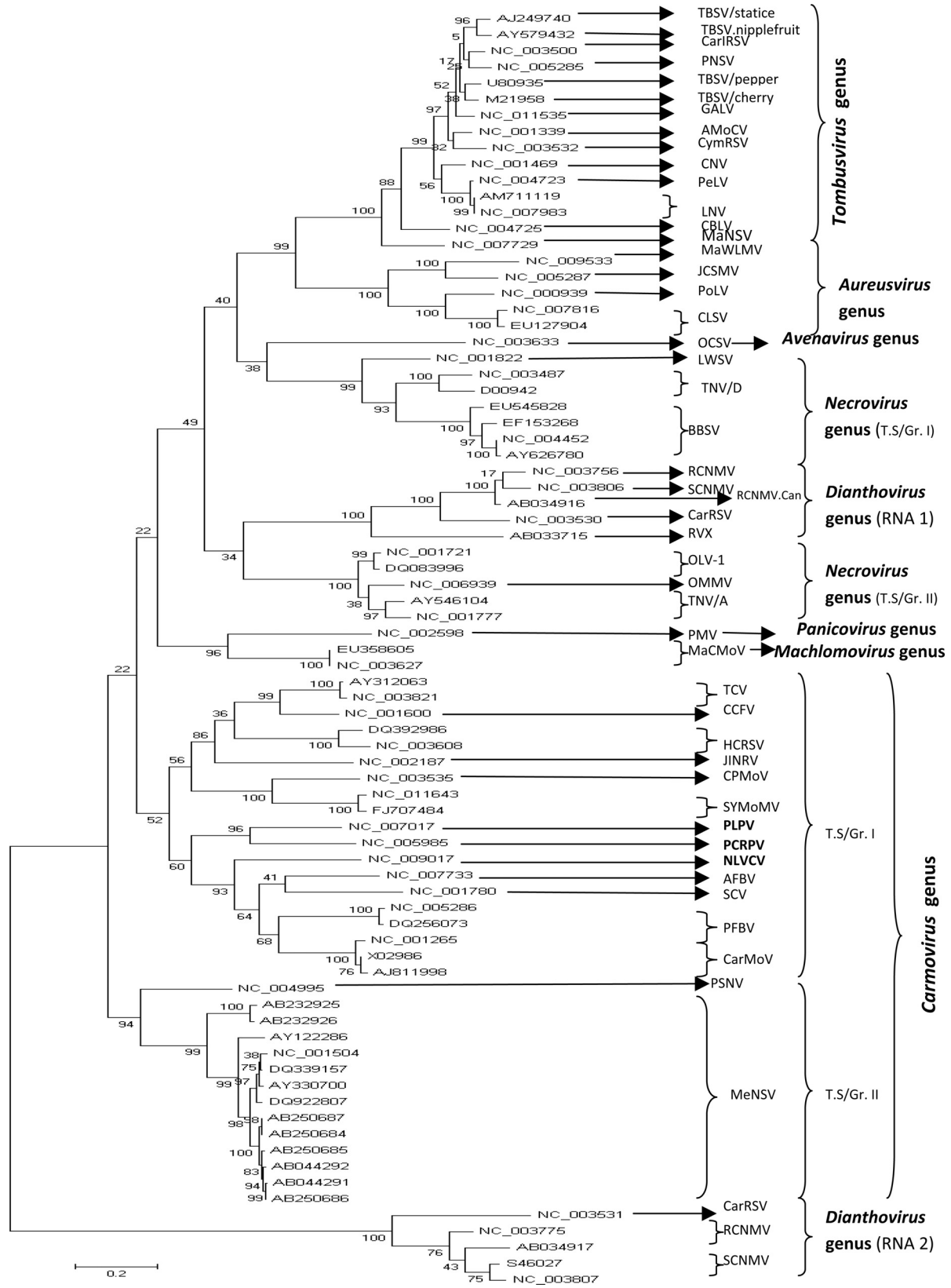

Figure 1 - Dendrogram depicting phylogenetic relationships among the studied members of the family Tombusviridae, based on their complete genome sequences. Eight clusters representing the eight genera were clearly delineated. While members of genera *Carmovirus*, *Necrovirus*, and *Dianthovirus* fell into two tentative distinct subgroups (T.S/Gr I, T.SGr. II), those of genera *Aureusvirus* and *Tombusvirus* constituted a homogenous ensemble. The tree was produced using the ML algorithm option of MEGA5.03. The numbers above the branches indicate the bootstrap confidence value. The scale bar shows the number of substitutions per nucleotide.

reshuffled the existing classification adopted by the ICTV since 2009. In fact, according to this classification, MaNSV was considered as an unassigned Tombusviridae, whereas LNV and PNSV were included in the unclassified *Tombusvirus* group. Concerning genus *Dianthovirus* which clearly was not monophyletic, the clustering pattern showed two distinct clades representing their RNAs 1 and 2, as illustrated in Figure 1. Originally, RVX was considered as an unclassified virus within genus *Dianthovirus*.

Discussion

This study evidenced the prediction of putative recombination events in the genome of several members of the family Tombusviridae and demonstrated that tombusviruses and carmoviruses are highly recombinant compared to viruses of the other genera. For this purpose, two methods were chosen (RECCO and RDP v.3.31β), based on the fact that they are appropriate for the mosaic structure of viruses as reported in previous works (Boulila, 2009; 2010). In this study, using the RECCO algorithm, it was demonstrated that the viruses belonging to the following genera contained putative recombination signals in their genome: *Aureusvirus*, *Avenavirus*, *Carmovirus*, *Dianthovirus*, *Necrovirus*, *Panicovirus*, and *Tombusvirus*. These results were in good agreement with those obtained by the RDP package except for members of genus *Diantovirus*. By both methods, the two representatives of genus *Machlomovirus* (MCMoV, MCMoV.Nebraska) were found to be non-recombinant. As revealed by RECCO, the most frequently recombining viruses were: OCSV, RVX.RNA 1, JINRSV, and PMV with 175, 166, 134, and 108 putative recombination sites, respectively. All of these recombination signals were constituted by a single residue. MeNSV.Nagasaki, MeNSV.NK, RCNMV.RNA 2, TNV-A.C, and TBSV.Cherry (2 sites), MeNSV.MNSV-Al, CarRSV-RNA 2, and TNV-A.FM1B (3 sites), MeNSV.Kochi, BBSV-Val25.Iran, and TNV-D.Hungarian (4 sites), CarMoV, MeNSV.MNSV-264, and MeNSV.NH (5 sites), RCNMV.RNA 1.Can, TBSV.nipplefruit, and TBSV.pepper (7 sites), and RCNMV.RNA 1 (8 sites) showed the lowest frequency of recombination breakpoints. In contrast, most of these breakpoints had an interval exceeding three nucleotides. Furthermore, this study showed that recombination may occur between viruses belonging to different genera. For example: *Oat chlorotic stunt avenavirus* (OCSV) and *Melon necrotic spot carmovirus* (MeNSV) may give rise to *Pothos latent aureusvirus* (PoLV). Similarly, OCSV itself may result from a recombination between *Turnip crinkle carmovirus* (TCV) and *Maize white line mosaic aureusvirus* (MaWLMV) (Table 2). Seemingly, these viruses could contain part of their sequences particularly in the coat protein-encoding gene of each other. Such an event was largely studied for *Cucumber necrosis tombusvirus* (CNV) and *Melon necrotic spot carmovirus* (MeNSV) (Riviere and Rochon, 1990).

On the other hand, investigations of selective pressure acting on protein expression of virus genes led to the identification of positive selection in 17 accession pairs involving 15 different lineages. It is worth mentioning that numerous viruses: JCSMV.Iran, OCSV, CarRSV-RNA 2, RCNMV-RNA 2.Can, BBSV.Val25.Iran, GALV.nipplefruit, TBSV.Statice, PNSV, and PLPV.PV.0193 evolved under both mechanisms: recombination and positive selection between which synergism might be occurring. Such a synergism between recombination and natural selection may have played a major role in Darwinian molecular evolution.

The evolutionary history of the Tombusviridae has shown that the 79 accessions split into eight clearly separated clusters representing the eight genera of the Tombusviridae family. From the present phylogenetic study, at least two taxonomic implications can be drawn: (i) three viruses (NLVCV.Alaska, PCRPV.GR 57, PLPV.PV-0193) currently considered by the ICTV as: one unassigned Tombusviridae (PLPV.PV-0193), and two unclassified Tombusviridae (NLVCV.Alaska and PCRPV.GR 57). All of them should be included in genus *Carmovirus*; (ii) In addition to the viruses belonging to genus *Carmovirus* which have formed two separated subgroups, the members of genera *Necrovirus*, and *Dianthovirus* evolved separately and divided into two distinct subgroups as shown in Figure 1. In contrast, members of genera *Aureusvirus*, and *Tombusvirus* formed separately a single ensemble. The evolutionary relationships among viruses are a reliable approach for classification. As stated by Stuart *et al.* (2004) (who reported similar results regarding the genetic divergence of components of genus *Necrovirus*), the comparison of complete genomes is a more balanced approach that should provide a more precise scheme of relatedness. On the other hand, it should be pointed out that, in genus *Dianthovirus*, the genetic divergence between RNAs 1 and 2 is correlated to the final products synthesized and their use by the virus to survive. For example: RNA silencing is a small RNA-guided sequence- specific gene activation mechanism in eukaryotes that is involved in different biological phenomena (*e.g.* development, heterochromatin formation and defense against molecular parasites such as viruses). Many viruses express suppressors to counteract RNA-silencing-mediated antiviral defenses. These RNA silencing suppressors have been identified in the following genera: *Aureusvirus*, *Carmovirus*, *Tombusvirus,* and *Dianthovirus* (Voinnet *e al*., 1999; Qu *et al.*, 2003; Mérai *et al.*, 2005; Takeda *et al.*, 2005). *Dianthovirus* uses a unique strategy to suppress RNA silencing. The dianthoviral suppressor consists of multiple components including P27, P88 (encoded by two ORFs in RNA 1) and viral RNA (Takeda *et al.*, 2005). Moreover, sequence variability of the coat protein-coding gene (RNA 1) may be linked to the interaction

between this structural protein and the host and vector which themselves show a major diversity among diathoviruses. In contrast, the ORF in RNA 2 encodes the movement protein. All these factors can influence the divergence between the two RNAs.

Finally, to the author's best knowledge, this is the largest study in the literature so far on recombination potentially occurring in the entire genome of all currently known members of the family Tombusviridae as well as positive selection operating on protein expression and their phylogenetic reconstruction. In addition, a reclassification based on their predicted evolutionary history, is proposed.

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