The sequencing of the complete genome of a Tomato black ring virus *(TBRV) and of the RNA2 of three* Grapevine chrome mosaic virus *(GCMV) isolates from grapevine reveals the possible recombinant origin of GCMV*

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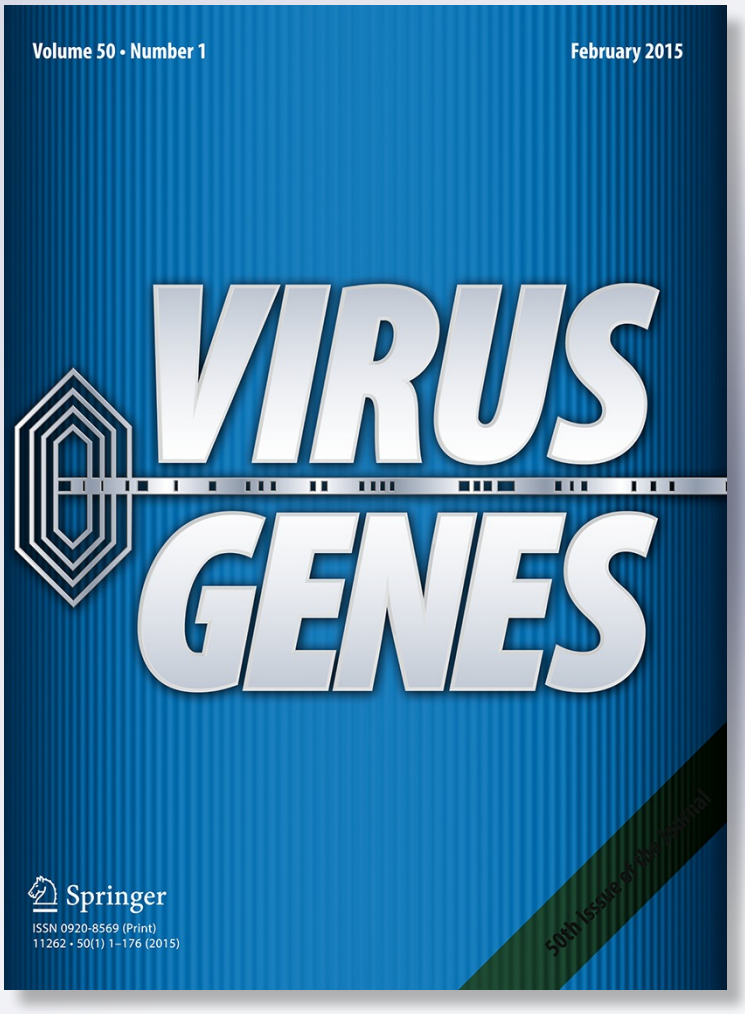

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The sequencing of the complete genome of a *Tomato black ring virus* (TBRV) and of the RNA2 of three *Grapevine chrome mosaic virus* (GCMV) isolates from grapevine reveals the possible recombinant origin of GCMV

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Abstract The complete genome of a *Tomato black ring virus* isolate (TBRV-Mirs) (RNA1, 7,366 nt and RNA2, 4,640 nt) and the RNA2 sequences (4,437; 4,445; and 4,442 nts) of three *Grapevine chrome mosaic virus* isolates (GCMV-H6, -H15, and -H27) were determined. All RNAs contained a single open reading frame encoding polyproteins of 254 kDa (p1) and 149 kDa (p2) for TBRV-Mirs RNA1 and RNA2, respectively, and 146 kDa for GCMV RNA2. p1 of TBRV-Mirs showed the highest identity with TBRV-MJ (94 %), *Beet ringspot virus* (BRSV, 82 %), and *Grapevine Anatolian ringspot virus* (GARSV, 66 %), while p2 showed the highest identity with TBRV isolates MJ (89 %) and ED (85 %), followed by BRSV (65 %), GCMV (58 %), and GARSV (57 %). The amino acid identity of RNA2 sequences of four GCMV isolates (three from this study and one from GenBank) ranged from 91 to 98 %, the homing protein being the most variable. The RDP3 program predicted putative intra-species recombination events for GCMV-H6 and recognized GCMV as a putative inter-species recombinant between GARSV and TBRV. In both cases, the recombination events were at the movement protein level.

Keywords Grapevine · *Tomato black ring virus* · *Grapevine chrome mosaic virus* · Sequence analysis · Recombination analysis

Nepovirus is a genus of positive single-stranded RNA viruses in the family *Secoviridae*, sub-family *Comovirinae* [1]. Virus particles are isometric, about 30 nm in size, have a capsid composed of 60 copies of a single protein type, and sediment as three components in sucrose gradients. All nepoviruses have a bipartite genome consisting of two linear single-stranded positive-sense RNAs (RNA1 and RNA2) with molecular mass of 2.4–2.8 $\times$ 10^6 and 1.3–2.4 $\times$ 10^6 Da, respectively [2]. The RNA molecules have a poly(A) tail at the 3′ end, and a viral protein genome linked (VPg, 3–6 $\times$ 10^3 Da) to the 5′ end [3]. Based on the size of RNA2, sequence similarity, and serological relationship, the genus *Nepovirus* is subdivided in three subgroups: (i) subgroup A has a RNA2 of 3,700–4,000 nucleotides (nts) in length, that in some species can be present in both M and B components; (ii) subgroup B has a RNA2 of 4,400–4,700 nts in length, present in the M component; and (iii) subgroup C has a RNA2 of 6,400–7,300 nts in length, present in M component particles that are sometimes barely separable from particles of B component [1].

To date, this genus includes at least 46 viruses infecting many plant families and causing diseases of economic importance in a wide range of cultivated annual, perennial, and woody plants. Grapevine (*Vitis vinifera*) hosts 15 different nepoviruses, which include some agents of "infectious degeneration" and "decline" [4]. With the exception of *Grapevine fanleaf virus* (GFLV), which is ubiquitous, all the other grapevine-infecting nepoviruses have a restricted geographical distribution. Nepoviruses in the subgroup A

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include GFLV, *Arabis mosaic virus* (ArMV), *Grapevine deformation virus* (GDeV), *Raspberry ringspot virus* (RpRSV), and *Tobacco ringspot virus* (TRSV); nepoviruses in the subgroup B include *Artichoke Italian latent virus* (AILV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine Anatolian ringspot virus* (GARSV), and *Tomato black ring virus* (TBRV); nepoviruses in the subgroup C include *Blueberry leaf mottle virus* (BLMoV), *Cherry latent ringspot virus* (CLRV), *Grapevine Bulgarian latent virus* (GBLV), *Grapevine Tunisian ringspot virus* (GTRSV), *Peach rosette mosaic virus* (PRMV), and *Tomato ringspot virus* (ToRSV) [5].

Of the four nepoviruses of subgroup B detected in grapevine, TBRV and AILV have a relatively wide host range, while GARSV and GCMV have only been found in grapevines [6]. To date, the complete sequence has been determined for one grapevine isolate each of GARSV [7, 8] and GCMV [9, 10], and for one isolate of TBRV from *Robinia pseudoacacia* (TBRV-MJ) [11]. For this last virus, the sequences of the complete RNA2 of a carrot isolate (TBRV-ED) [12] and of RNA fragments of some Polish (RNA-dependent RNA polymerase) [13] and Lithuanian (capsid protein) isolates (acc. number KF678365–73) from different plant species have been also determined. For AILV, the nt sequence is available only for a short fragment of RNA2 from an isolate from artichoke (acc. number X87254).

The amino acid (aa) sequence identity between TBRV-MJ and the other subgroup B nepoviruses is 83.7 % with BRSV and 61.8 % with GCMV in RNA1, whereas it is 93.6 % with TBRV-ED, 67.6 % with BRSV, and 58.8 % with GCMV in RNA2 [11]. In consideration of the high identity between GCMV and TBRV-ED in the MP domain (92 %), TBRV-ED was supposed to be a natural recombinant between TBRV-S (now BRSV) and GCMV [12].

The main purpose of this work was to contribute a study on the evolution of grapevine-infecting nepoviruses of subgroup B. For this reason, both genomic RNAs of a TBRV isolate from grapevine (denoted TBRV-Mirs) were completely sequenced for the first time, and compared with the sequences of other nepoviruses retrieved from GenBank. A preliminary analysis of TBRV sequences (RNA1 and RNA2) with those of GCMV from GenBank indicated that there were possible recombination events between the two viruses at the level of RNA2, this being in line with previous reports for other nepoviruses. Thus, the RNA2 of three additional GCMV isolates (denoted H6, H15, and H27) from our grape collection was sequenced and compared with that of TBRV for investigating the occurrence of recombination events that may have originated the different species.

The TBRV- and GCMV-infected vines under study were from a collection plot of the Mediterranean Agronomic Institute of Valenzano-Bari, Italy. All infected sources were Hungarian accessions of the French hybrid Mirs (TBRV-Mirs) and the European grape cultivars Dimont Pomaric (GCMV-H6), Italian Riesling (GCMV-H15), and Pinot noir (GCMV-H27). All virus isolates were transmitted by sap inoculation to *Nicotiana occidentalis* plants and maintained in a greenhouse at 24–25 °C.

Purified virus preparations were extracted from systemically infected *N. occidentalis* leaves according to Dunn and Hitchborn [14], and fractionated in 10–40 % linear sucrose density gradient columns.

Nucleic acids were extracted from purified virus preparations according to Diener and Schneider [15] and separated by electrophoresis in 1.2 % agarose gel under semi-denaturing conditions [16]. Gels were stained with red gel nucleic acid stain (Biotium, Hayward, CA, USA).

Viral RNAs were reverse-transcribed (RT) using random hexamers and/or an oligo(dT) primer according to Gubler and Hoffman [17]. Different sets of primers to be used in RT-PCR were designed for amplifying selected regions from RNA1 and RNA2 genomic templates. For designing primers, TBRV and GCMV sequences were retrieved from GenBank and aligned using CLUSTAL X 1.8 [18] for the identification of conserved nucleotide domains.

The 3′ termini of both RNAs were amplified using TBRV- or GCMV-specific primers in conjunction with an oligo(dT) primer, whereas the 5′ termini were amplified using the 5′ random amplification of cDNA ends (RACE)-PCR System (Roche Diagnostics, CA, USA) with TBRV- or GCMV-specific antisense primers. All PCR runs consisted of 35 cycles, with an initial denaturing temperature of 94 °C for 1 min, primer annealing at 50–55 °C (depending on the primer used) for 50 s, and an elongation time of 1 to 2 min at 72 °C, depending on amplicon size, with a final elongation at 72 °C for 7 min. 10 µl of the PCR reactions was electrophoresed in 1.2 % agarose gel in 1 × TAE buffer and stained with Red gel nucleic acid stain.

All amplicons were cloned into the Strata-Clone PCR cloning vector pUC18 (Agilent Technologies, CA, USA), introduced into *Escherichia coli* DH5α or SoloPACK cells and custom sequenced (Primm, Milan, Italy). Nucleotide and protein sequences were analyzed with the assistance of the DNA Strider 1.1 program [19]. Multiple alignments of nt and aa sequences were obtained using the default options in CLUSTAL X 1.8 [18]. A search for homologies with proteins from the protein information resources database (PIR, release 47.0) was done with the FASTA [18], BlastX, and BlastP programs [20]. The secondary structure of viral RNAs was analyzed with the Mfold program [21]. Phylogenetic trees were constructed using the neighbor-joining (NJ) method in the MEGA4 analysis package [22]. A

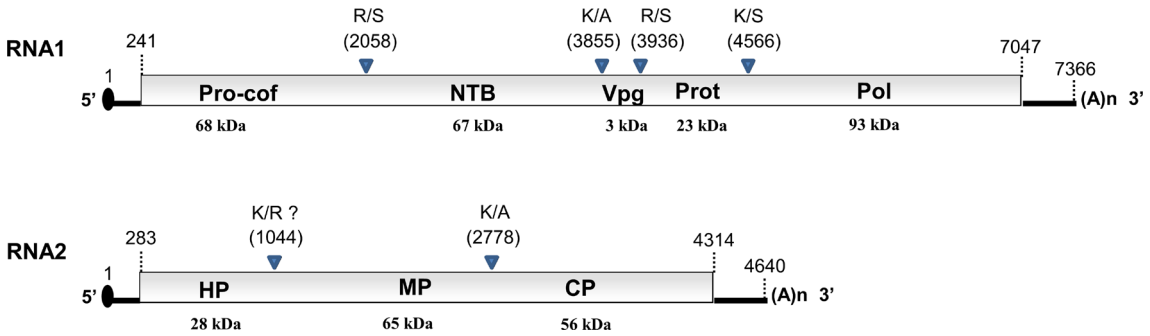

Fig. 1 Schematic representation of TBRV-Mirs RNA1 and RNA2 organization. *Boxes* represent RNA1- and RNA2-encoded polyproteins with their putative function and their estimated molecular weights shown inside and below the *boxes*, respectively. *Pro-cof* protease cofactor, *NTB* nucleotide triphosphate-binding protein, *VPg* viral genome-linked protein, *Prot* cysteine-like protease, *Pol* polymerase, *HP* homing protein, *MP* movement protein, *CP* capsid protein. Initiation and termination of protein translation are represented by *dotted lines* at the 5′ and 3′ termini of each RNA. The RNA1-encoded protein is cleaved by the viral protease at 4 sites to release 5 mature proteins. RNA2-encoded protein is cleaved at 2 sites to release 3 protein domains. Putative predicted cleavage sites are indicated above the *boxes*

bootstrap value for each node of NJ trees was calculated using 1,000 bootstrap replicates and a consensus tree was displayed by TREEVIEW [23].

RNA1 and RNA2 sequences were analyzed for the occurrence of putative recombination events using recombination detection program version 3 (RDP3) [24] with default parameters (the highest acceptable probability value = 0.05). The RDP3 package uses a suite of programs to detect the occurrence of robust recombination events [25], namely RDP [26], GENECONV [27], BOOTSCAN [26], MAXCHI [28], CHIMAERA [25], 3SEQ [29], SISCAN [30], and LARD [31]. BOOTSCAN, RDP, and SISCAN are phylogenetic methods; GENECONV, MAXCHI, CHIMAERA, and LARD are substitution methods; and PHYLPRO is a distance comparison method. Recombination sites identified by four or more programs and two or more types of methods were considered to be "significant recombination events" [32].

All GCMV and TBRV isolates in this study were purified from systemically infected *N. occidentalis* plants with average yields of 5–8 mg/kg of infected tissue. In sucrose gradient centrifugation virus preparations sedimented as three components, which consisted of empty shells (component T) and apparently intact particles (components M and B). In 1.2 % agarose gel electrophoresis, RNAs migrated as two distinct bands (RNA1 and RNA2).

The cloning operations conducted on TBRV-Mirs RNA1 and RNA2 spanned the full-length sequences of 7,366 and 4,640 nts, respectively, excluding the poly(A) tail. Both RNAs contained a single open reading frame (ORF), the same as with other nepoviruses.

RNA1: The 5′ untranslated regions (UTR) of TBRV-Mirs RNA1 and TBRV-MJ RNA1 had a slightly different size (240 and 239 nts, respectively), and 81 % sequence identity. Similarly, the 3′UTRs of TBRV-Mirs and TBRV-MJ showed a slightly different length (319 and 318 nts, respectively) and 93 % sequence identity.

TBRV-Mirs RNA1 coding region consisted of a single ORF (6,807 nt) extending from 240 to 7,047 nts. It encoded a polyprotein (p1) with a predicted molecular mass of ca. 254 kDa, comprising in the 5′ → 3′ direction the putative proteinase cofactor ($1A^{Pro\text{-}cof}$), the NTP-binding protein ($1B^{Hel}$), the viral genome-linked protein ($1C^{VPg}$), the proteinase ($1D^{Prot}$), and the RNA-dependent RNA polymerase ($1E^{Pol}$) (Fig. 1).

RNA2: The 5′UTR of RNA2 was 282-nt long, slightly different from those of TBRV-ED (299 nt) and TBRV-MJ (275 nt). Sequence identities with TBRV-MJ and TBRV-ED were 73 and 66 %, respectively. It is worth noting that TBRV-ED 5′UTR has an additional sequence of 26 nt from position 210 to 236 that is absent in the other two TBRV isolates, while TBRV-Mirs 5′UTR has seven additional nts compared with the other two isolates (Fig. 2a). Two repeats were present in both TBRV-Mirs and TBRV-MJ at positions 196–205 and 241–250, respectively, which were able to form a stable hairpin whose apical sequences (a decanucleotide: GGATAGCGCC) were remarkably conserved. TBRV-ED presented an additional repeat between positions 222 and 231 [12]; in each of these repeats, the bases around the conserved decanucleotide were identical in TBRV-Mirs and TBRV-MJ, but differed in TBRV-ED. This variability, however, did not preclude the possibility of forming the hairpin in the three RNAs (Fig. 2a).

The 3′UTR of TBRV-Mirs RNA2 was 326-nt long, excluding the poly(A) tail. Its size was identical to that of TBRV-MJ (326 nt), but longer than that of TBRV-ED (284 nt). It showed 92 % identity with the 3′UTR of TBRV-MJ and only 80 % with TBRV-ED. The latter missed a sequence of 39 nt, from position 60 to 99, compared to TBRV-Mirs and TBRV-MJ (Fig. 2b). The pairwise

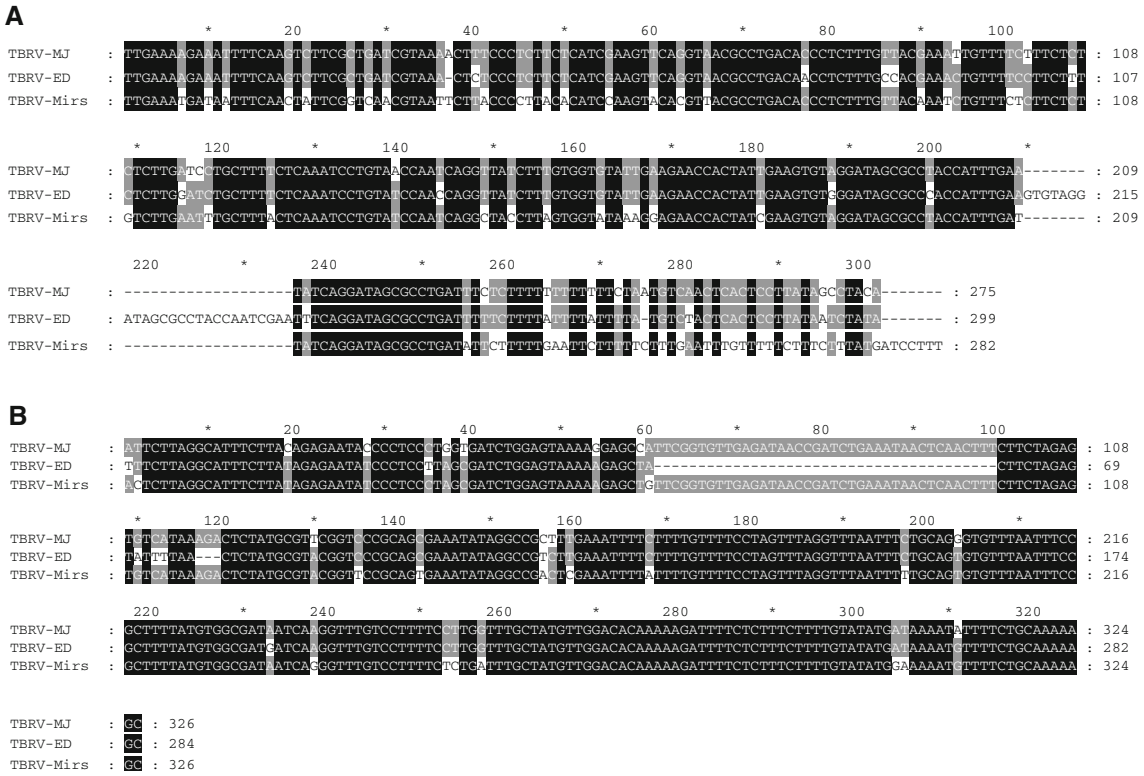

Fig. 2 Multiple nucleotide sequence alignment of the 5′UTR (**a**) and 3′UTR (**b**) of TBRV RNA2 isolates using the CLUSTAL X 1.8 program

alignment of both 5′ and 3′UTR regions of TBRV-Mirs RNA1 and RNA2 were found to be nearly identical (95 and 98 % nts identity, respectively), thus confirming what was already observed for other nepoviruses of subgroup B [11].

The coding region of TBRV-Mirs RNA2 was 4,032 nt in size, similar to its homologs of TBRV-MJ (4,032 nt) and TBRV-ED (4,037 nt). The single RNA2 ORF extended from nts 283 to 4,314 and coded for a polyprotein (p2) with a predicted molecular mass of ca. 149 kDa, comprising in the 5′ → 3′ direction the homing protein ($2A^{HP}$), the movement protein ($2B^{MP}$), and the coat protein ($2C^{CP}$) (Fig. 1).

Blast analysis of the aa sequence of the TBRV-Mirs polyproteins disclosed a high identity level with the comparable products of subgroup B nepoviruses, i.e., TBRV-MJ (94 %), BRSV (82 %), and GARSV (66 %) for p1; and TBRV-MJ (89 %), TBRV-ED (85 %), and BRSV (65 %) for p2 (Supplementary Table 1).

To study the relationships between TBRV-Mirs and the other subgroup B nepoviruses, bootstrapped dendrograms for the individual proteins (HP, MP, and CP) encoded by RNA2 were produced. To this aim, the full-length RNA2 sequences of the six subgroup B nepoviruses from GenBank [*Beet ringspot virus* (BRSV, X04062), *Cycas necrotic stunt virus* (CNSV, AB073148), GARSV (AY291207), GCMV (X15163), TBRV-MJ (AY157994), and TBRV-ED (X80831)] and those of the nepovirus objects of this study, i.e., TBRV-Mirs (HG939487), GCMV-H6 (HG939484), GCMV-H15 (HG939485), and GCMV-H27 (HG939486), were analyzed.

For subgroup B nepoviruses, the exact position of the cleavage site in the polyprotein p2 has been determined for MP and CP [33], whereas that between HP and MP is still uncertain. Based on the similarities with subgroup A nepoviruses, the preference for cleavage sites between a basic amino acid (lysine or arginine) and a hydrophobic amino acid (alanine, glycine, and serine) and the result of a predicting program for cleavage sites identification [34], the locus K/R at nts position 1,041–1,047 was assumed as the hypothetical HP/MP cleavage site for TBRV-Mirs p2 (Fig. 2).

Phylogenetic trees were constructed for each domain encoding individual proteins (HP, MP, and CP). In all dendrograms, CNSV was always clearly separated from the other species of subgroup B (Fig. 3). TBRV-Mirs clustered together with TBRV-MJ and TBRV-ED, and close to other grapevine-infecting nepoviruses of subgroup B. The position of TBRV isolates with regard to GCMV isolates in the different phylogenetic trees was apparently incongruent. In fact, whereas these two viruses were well separated in the HP and CP trees (an intermediate position was shown by GARSV and BRSV), they clustered close in the MP tree, suggesting that GCMV could be a putative recombinant.

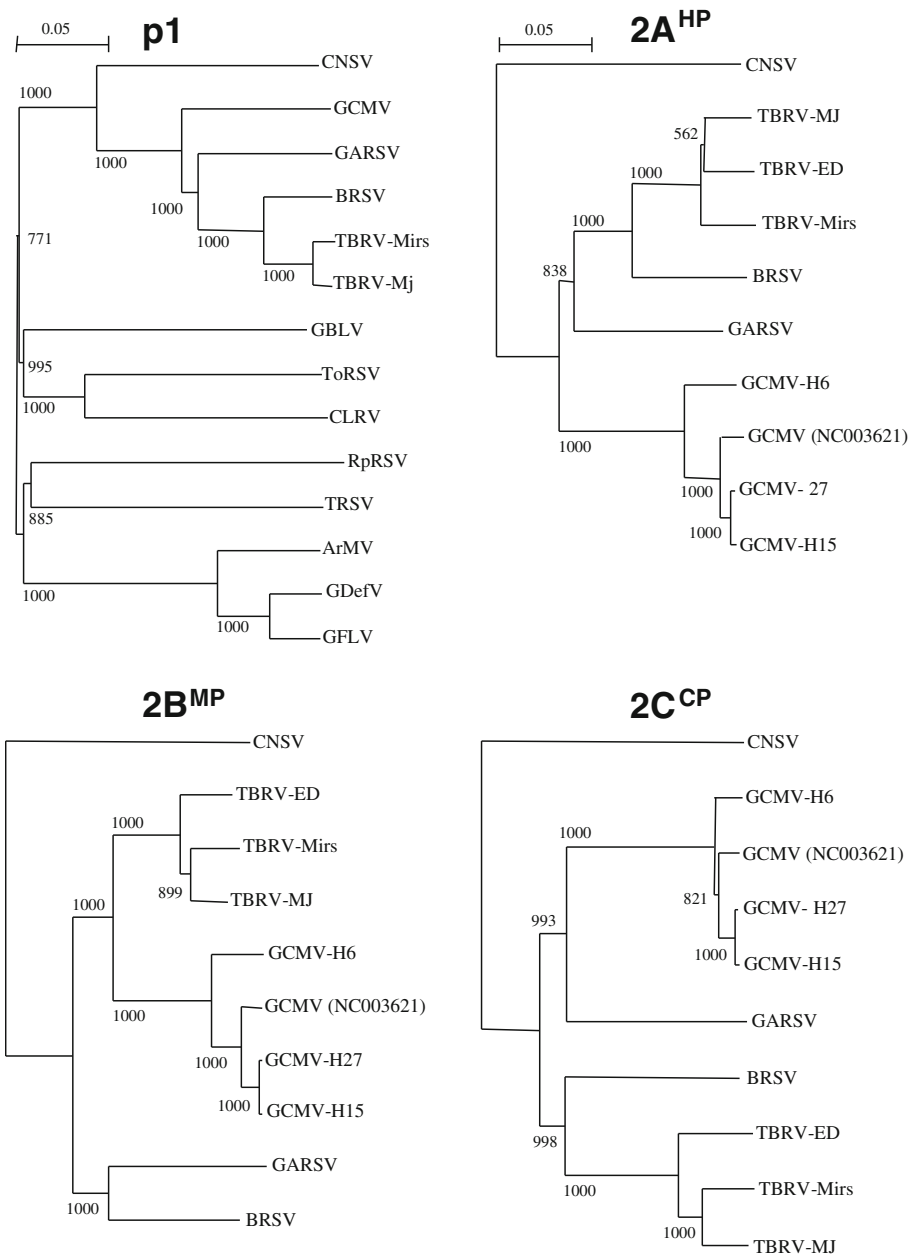

Fig. 3 Phylogenetic tree analysis based on amino acid sequences of p1 and different domains (HP, MP, and CP) of p2 polyproteins of subgroup B nepoviruses. Bootstrap values in percentages are based on 1,000 replicates for branches. (GenBank accession numbers of the sequences used in phylogenetic analysis are reported in the text)

A phylogenetic tree was also constructed based on the polyprotein (p1) sequences of different nepoviruses, including the new sequenced TBRV-Mirs isolate, five nepoviruses of the subgroup B [(BRSV (NC003693), CNSV (JN127336), GARSV (HE774604), GCMV (NC_003622), and TBRV-MJ (NC_004439)] and other eight nepoviruses infecting grapevine, all retrieved from GenBank [ToRSV (L19655), GBLV (NC_015492), GFLV (NC_003615), ArMV (AY303786), GDefV (HE613269), TRSV (NC_005097), RpRSV (NC_005 266), and CLRV (YP_004382746)]. As it was expected, TBRV-Mirs clustered together with TBRV-MJ and close to other nepoviruses of the subgroup B, in particular to BRSV and GARSV (Fig. 3).

GCMV RNA2 isolates (H6, H15, and H27) had full-length sequences of 4,437; 4,445; and 4,442 nts, respectively—excluding the poly(A) tail—and contained a single ORF, extending from nts 212–218 to 4,187–4,195 and encoding a polyprotein (p2) with a predicted molecular mass of ca. 146 kDa.

The 5′UTR of GCMV-H6 was 212-nt long, slightly shorter than that of GCMV-NC_003621 from GenBank (218 nt) and GCMV-H15 and H27 (217 nt). The nucleotide sequence analysis of the 5′UTR of the four GCMV isolates revealed an identity ranging from 83 to 94 %. Three repeated motifs were found in the 5′UTRs of the three new GCMV isolates between positions 143 and 207 similar to

Table 1 Intra-species (A) and inter-species (B) recombination crossover events in $2B^{MP}$ of GCMV-H6 isolate detected by the recombination detecting program RDP3

Isolates	Nt. position in $2B^{MP}$	Parental isolates (major × minor)	RDP3 (P value)[a]
(A) GCMV-H6	2,118–3,111	GCMV(NC_003621) × GCMV-H27	**R**GBMC3sS (1.062×10^{-23})
(B) GCMV-H6	1,390–2,650	GARSV × TBRV-Mirs	RG**B**MC3sS (8.487×10^{-28})

[a] RDP3-implemented methods supporting the corresponding recombination sites: R (RDP), G (GENECONV), B (BOOTSCAN), M (MAXCHI), C (CHIMAERA), 3s (3SEQ), and S (SISCAN). The reported P value within brackets is the lowest P value among those calculated using RDP3-implemented methods and the corresponding method is shown in bold

those previously observed for GCMV-NC_003621 [10]. The three repeats were able to form a stable hairpin whose apical sequence (a decanucleotide) was conserved. These repeats were at positions between nts 143–152, 169–178, and 198–207, respectively, and were conserved for all isolates, except for H6 and GCMV-NC_003621 in which an A was replaced by a T at position 173 in the second repeat. The 3′UTR of GCMV-H6 was 250-nt long, excluding the poly(A) tail, a size identical to those of GCMV-H15 and -H27 (250 nt), and shorter than that of GCMV-NC_003621 (251 nt). The 3′UTRs of isolates H6, H15, and H27 were 100 % identical to each other, and 95 % identical with that of GCMV-NC_003621.

GCMV-H6 p2 was 3,975 nt in length, like that of H27, but shorter than that of H15 (3,978 nt) and longer than that of GCMV-NC_003621 (3,972 nt). All RNA2 molecules comprised a single ORF encoding in the 5′ → 3′ direction the homing protein ($2A^{HP}$), the movement protein ($2B^{MP}$), and the coat protein ($2C^{CP}$).

Blast analysis conducted on the aa sequences of the different domains of the polyprotein p2 encoded by GCMV isolates showed the highest identities with nepoviruses of subgroup B. CP was the most conserved domain (94–98 %), followed by MP (89–98 %) and HP (86–98 %) (Supplementary Table 2). Sequence identity with the other species of subgroup B never exceeded 70 % in any of the domains. Intriguing was the high identity value (69–70 %) between GCMV and TBRV isolates at the MP level, if compared with the low values registered in HP (50–53 %) and CP (57–58 %) (Supplementary Table 2). This situation reflects what was already observed in the analysis of the phylogenetic trees of each single domain (Fig. 3) and further supports the hypothesis of a possible inter-specific recombination among viruses of subgroup B.

The RDP3 program [26] predicted a putative intra-species recombination event in the GCMV-H6 RNA2 sequence between nts 2,118 and 3,111 ($2B^{MP}$), involving GCMV-NC_003621 and GCMV-H27 as the major and minor parents, respectively (Table 1; Supplementary Fig. 1).

Furthermore, one significant inter-species recombination event was located in the $2B^{MP}$ domain of GCMV isolates, involving GARSV (as major parent) and TBRV (as minor parent). Recombination breakpoints in these isolates occurred at positions 1,390 (beginning breakpoint) to 2,650 nts (ending breakpoint) (Table 1). Each of the aforementioned recombination sites was predicted by at least seven different methods included in the RDP3 software package (Table 1).

Conversely, no significant inter-species recombination was observed by analyzing the RNA1 of the *nepovirus* species sequenced in this study (TBRV) and those of subgroup B retrieved from GenBank, including GCMV.

The high number (15) of nepoviruses infecting grapevines—six of which have no known hosts other than grapes (i.e., GFLV, GARSV, GDeV, GCMV, GTRSV, and GBLV), the mode of propagation of the vines (grafting), and the transmission by nematodes that favor the accumulation of different viral species in the same host make the *nepovirus*/grapevine associations particularly suitable for evolutionary studies. An important role in virus evolution is played by recombination, a particularly common phenomenon among nepoviruses [35–37], as confirmed by the recent identification of the recombinant origin of GDeV, having GFLV and ArMV as ancestors [38].

Comparable results were obtained in the course of the present investigation, for RDP3 predicted a highly significant inter-species recombination event in $2B^{MP}$ of GCMV RNA2, having GARSV and TBRV as putative major and minor parents, respectively. When and where this recombination may have occurred is difficult to hypothesize based on current knowledge. Nevertheless, it seems plausible that it took place in grapevines, since GARSV and GCMV have never been found in hosts other than *Vitis*, where mixed infections may have occurred following grafting or natural transmission by a yet unidentified vector.

The $2B^{MP}$ domain is an infrequent recombination site for nepoviruses. In fact, in subgroup A, where several inter-specific recombination cases have been reported [35–37], these events are much more frequent in $2A^{HP}$ than in $2B^{MP}$ and $2C^{CP}$. In the same subgroup, both $2B^{MP}$ and $2C^{CP}$ are highly conserved (95–99 % sequence identity at the aa level in MP and 93–99 % in CP) [38] and subjected to a strong negative selection. This situation seems different for

subgroup B nepoviruses, whose $2B^{MP}$ is less conserved than $2C^{CP}$, and thus is subjected to a higher positive (or adaptive) selection pressure. The intra-specific recombination predicted in the $2B^{MP}$ of GCMV-H6 that involved GCMV-NC_003621 and GCMV-H27 as parents seems to support this likelihood.

References

1. H. Sanfaçon, T. Iwanami, A.V. Karasev, R. Van der Vlugt, J. Wellink, T. Werzel, N. Yoshikawa, Family Secoviridae, pp. 881–899, in *Ninth Report of the International Committee on Taxonomy of Viruses*, ed. by A.M.Q. King, M.J. Adams, E.B. Carstend, E.J. Lefkowitz (Elsevier/Academic Press, San Diego, 2011), p. 1327
2. M. Mayo, D. Robinson, Nepoviruses: molecular biology and replication, pp. 139–185, in *The plant viruses*, ed. by B.D. Harrison, A.F. Murant (Springer Science, New York, 1996)
3. M. Serghini, M. Fuchs, M. Pinck, J. Reinbolt, B. Walter, L. Pinck, J. Gen. Virol. **71**, 1433 (1990)
4. G.P. Martelli, *Graft transmissible diseases of grapevines: handbook for detection and diagnosis* (Food and Agriculture Organization of the United Nations, Rome, 1993)
5. M. Digiaro, T. Elbeaino, G.P. Martelli, J. Virol. Methods **141**, 34 (2007)
6. G.P. Martelli, E. Boudon-Padieu, Directory of infectious diseases of grapevines. Options Mediterr. Ser. B **55**, 11–201 (2006)
7. N.A. Ghanem-Sabanadzovic, S. Sabanadzovic, M. Digiaro, G.P. Martelli, Virus Genes **30**, 335 (2005)
8. M. Digiaro, S. Nahdi, T. Elbeaino, Arch. Virol. **157**, 2013 (2012)
9. O. Le Gall, T. Candresse, V. Brault, J. Dunez, Nucl. Acids Res. **17**, 7795 (1989)
10. V. Brault, L. Hibrand, T. Candresse, O. Le Gall, J. Dunez, Nucl. Acids Res. **17**, 7809 (1989)
11. M. Jończyk, O. Le Gall, A. Pałucha, N. Borodynko, H. Pospieszny, Arch. Virol. **149**, 799 (2004)
12. O. Le Gall, M. Lanneau, T. Candresse, J. Dunez, J. Gen. Virol. **76**, 1279 (1995)
13. N. Rymelska, N. Borodynko, H. Pospiezny, B. Hasiow-Jaroszewska, Virus Genes **47**, 338 (2013)
14. D.B. Dunn, J.H. Hitchborn, Virol. **25**, 171 (1965)
15. T. Diener, I. Schneider, Arch. Biochem. Biophys. **124**, 401 (1968)
16. J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular cloning: a laboratory manual,* 2nd edn. (Cold Spring Harbor, 1989)
17. U. Gubler, B.J. Hoffman, A simple and very efficient method for generating cDNA libraries. Gene **25**, 263 (1983)
18. W.R. Pearson, D.J. Lipman, Proc. Natl. Acad. Sci. USA **85**, 2444 (1988)
19. C. Marck, Nucl. Acids Res. **16**, 1829 (1988)
20. S.F. Altschul, F. Stephen, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool. J. Mol. Biol. **215**, 403 (1990)
21. M. Zuker, Nucl. Acids Res. **31**, 3406 (2003)
22. K. Tamura, J. Dudley, M. Nei, S. Kumar, Mol. Biol. Evol. **24**, 1596 (2007)
23. R.D. Page, *TreeView* (Glasgow University, Glasgow, 2001)
24. D.P. Martin, P. Lemey, M. Lott, V. Moulton, D. Posada, P. Lefeuvre, Bioinform. **26**, 2462 (2010)
25. D. Posada, K.A. Crandall, Proc. Natl. Acad. Sci. USA **98**, 13757 (2001)
26. D.P. Martin, C. Williamson, D. Posada, Bioinform. **21**, 260 (2005)
27. M. Padidam, S. Sawyer, C.M. Fauquet, Virol. **265**, 218 (1999)
28. J.M. Smith, J. Mol. Evol. **34**, 126 (1992)
29. M.F. Boni, D. Posada, M.W. Feldman, Genet. **176**, 1035 (2007)
30. M.J. Gibbs, J.S. Armstrong, A.J. Gibbs, Bioinform. **16**, 573 (2000)
31. E.C. Holmes, M. Worobey, A. Rambaut, Mol. Biol. Evol. **16**, 405 (1999)
32. K. Ohshima, Y. Tomitaka, J.T. Wood, Y. Minematsu, H. Kajiyama, K. Tomimura, A.J. Gibbs, J. Gen. Virol. **88**, 298 (2007)
33. G. Demangeat, O. Hemmer, C. Fritsch, O. Le Gall, T. Candresse, J. Gen. Virol. **72**, 247 (1991)
34. G. Von Heijne, Nucl. Acids Res. **14**, 4683 (1986)
35. E. Vigne, A. Marmonier, M. Fuchs, Arch. Virol. **153**, 1771 (2008)
36. J. Jawhar, A. Minafra, P. La Notte, C. Pirolo, P. Saldarelli, D. Boscia, V. Savino, G.P. Martelli, pp 73–74, in *Ext. abstr 16th Meeting of International Council for the Study of Virus and Virus-like Diseases of the Grapevine*, ed. by Boudon-Padieu E. (Dijon, France, 2009)
37. T.A. Mekuria, L.R. Gutha, R.R. Martin, R.A. Naidu, Phytopathol. **99**, 1394 (2009)
38. T. Elbeaino, M. Digiaro, S. Ghebremeskel, G.P. Martelli, Virus Res. **166**, 136 (2012)