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BRIEF REPORT

Genetic variability and evolutionary analysis of parietaria mottle virus: role of selection and genetic exchange

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Abstract The genetic variability and evolution of parietaria mottle virus (PMoV) of the genus *Ilarvirus* was studied by analyzing nucleotide sequences of 2b and CP genes from isolates collected in different countries. Phylogenetic analysis showed that PMoV isolates clustered in different clades: one (clade I) composed of only Italian isolates and three clades (clades II-IV) including the Spanish isolates. The Greek isolate GrT-1 used in this study was in clade IV for the CP phylogenetic tree whereas it formed a separate branch in the 2b phylogenetic tree. The nucleotide sequence diversity of both the 2b and CP genes was low (0.062 ± 0.006 and 0.063 ± 0.006 for 2b and CP, respectively) but higher than those of other ilarviruses. Distribution of synonymous and nonsynonymous substitutions revealed that 2b and CP proteins are under purifying selection, with some positions under diversifying selection. Genetic exchange among Spanish isolates was also detected.

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Parietaria mottle virus (PMoV) is a member of the genus *Ilarvirus* (subgroup 1) of the family *Bromoviridae*. PMoV was detected first in parietaria plants (*Parietaria officinalis*) showing mottle symptoms in leaves [7] and later in tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) plants showing decoloration and necrosis symptoms in leaves and fruits [16, 21]. PMoV seems to be restricted to a few countries within the Mediterranean basin, and it has only been reported in Spain, Italy, France and Greece [4, 7, 22, 31]. In spite of PMoV infecting important crops such tomato and pepper, limited information regarding its geographic distribution, incidence or biological and genetic diversity is available. PMoV is transmitted by pollen, seeds and many insect vectors such as thrips or mirids (used for biological control) in a non-persistent manner [3]. The genome is composed of three single-stranded positive-sense RNAs [11]: RNA 1 encodes a putative replicase (p1); RNA 2 has two overlapping genes encoding a putative replicase (p2) and a protein of unknown function (2b) that is probably involved in viral movement and suppression of the posttranscriptional gene silencing pathway (PTGS) as in other members of the genus *Ilarvirus* and in the genus *Cucumovirus* (family *Bromoviridae*) in which this protein was detected [6, 8, 34]; RNA 3 has two non-overlapping genes encoding the movement protein (MP) and the coat protein (CP) [11, 24].

Knowledge about the genetic diversity of PMoV and the evolutionary mechanisms involved is essential for understanding the epidemiology of this virus and developing efficient strategies of disease control [13]. Viruses with RNA genomes have a high potential for rapid evolution

and adaptation, which is associated with their high mutation rate due to the lack of proofreading activity of the RNA polymerases involved in their replication. Mutation and recombination or reassortment of viruses with multipartite genomes are another cause of genetic diversity [9], which is subjected to natural selection, genetic drift and gene flow [25]. This work assesses the genetic diversity and structure of PMoV as well as the evolutionary forces shaping PMoV populations by analyzing the complete nucleotide sequences of genes encoding the CP and 2b proteins of PMoV isolates from different countries and hosts.

Twenty-four PMoV field isolates were collected from different countries in the main virus distribution area (Spain, Italy and Greece) and hosts (tomato, pepper and parietaria) (Table 1). Twenty-one Spanish isolates were collected in different provinces (Almería, Balearic Islands, Barcelona, Gerona, Guipúzcoa, Tarragona, Valencia, Vizcaya), hosts and years, including one Greek isolate (GR-1) recovered from an infected tomato plant collected in 2010; and two Italian isolates collected in infected tomato fields in Sicily in 2006 (ST-1) and Torino in 1979 (T32). Total RNA was extracted from PMoV-infected plants by using an RNeasy Plant Mini Kit (QIAGEN), following the manufacturer's instructions, and used for cDNA synthesis. In the reverse transcription step (RT), the cDNA was obtained using SuperScript II Reverse Transcriptase (Invitrogen) and 10 pmol of the reverse primers PMoV-CP Rv (5′ GAACCGTCGGTAACAACCAT 3′, position 1,990-1,969) and PMoV-2b Rv (5′ CCTCTCAAAGAGGCATCACAAC 3′, position 2,799-2,820) following the manufacturer's instructions. The complete sequences of the CP and 2b genes were amplified by PCR using Taq DNA polymerase (Invitrogen) and 50 pmol of primer pairs PMoV-CP For (5′ AGTCGCTTTTTGGAAATTAC 3′, position 1,281-1,300) and PMoV-CP Rv for CP and PMoV-2b For (5′ GTTCTAAAGCTTGTATTCGAA 3′, position 2,157-2,177) and PMoV-2bRev for 2b. Samples were subjected to an initial denaturing step of 4 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at

Table 1 Parietaria mottle virus isolates used in this study

Isolate	Original host	Geographic origin	Collection year	2b (GenBank accession no.)	CP (GenBank accession no.)
TT-1	Tomato	Tarragona, Spain	2007	KP234324	KP234332
TT-2	Tomato	Tarragona, Spain	2007	KP234309	KP234333
TT-3	Tomato	Tarragona, Spain	2007	KP234310	KP234334
TT-4	Tomato	Tarragona, Spain	2007	KP234318	KP234342
TT-5	Tomato	Tarragona, Spain	2012	KP234322	KP234346
BT-1	Tomato	Barcelona, Spain	2007	KP234306	KP234330
CR8*	Tomato	Barcelona, Spain	2002	FJ858203	FJ858204
BP-1	Pepper	Barcelona, Spain	2007	KP234305	KP234329
BT-2	Tomato	Barcelona, Spain	2012	KP234323	KP234347
BPar-1	Parietaria	Barcelona, Spain	2008	KP234313	KP234337
BPar-2	Parietaria	Barcelona, Spain	2008	KP234314	KP234338
BPar-3	Parietaria	Barcelona, Spain	2008	KP234315	KP234339
AP-1	Pepper	Almeria, Spain	2007	KP234302	KP234326
AP-2	Pepper	Almeria, Spain	2008	KP234303	KP234327
AP-3	Pepper	Almeria, Spain	2004	KP234304	KP234328
GT-1	Tomato	Gerona, Spain	2007	KP234307	KP234331
GT-2	Tomato	Gerona, Spain	2012	KP234308	KP234348
VT-1	Tomato	Valencia, Spain	2005	KP234311	KP234335
VT-2	Tomato	Valencia, Spain	2010	KP234312	KP234336
GuiT-1	Tomato	Guipúzcoa. Spain	2011	KP234316	KP234340
ViT-1	Tomato	Vizcaya, Spain	2011	KP234317	KP234341
BaT-1	Tomato	Baleares, Spain	2012	KP234319	KP234343
GrT-1	Tomato	Greece	2010	KP234320	KP234344
ST-1	Tomato	Sicily, Italy	2006	KP234325	KP234349
T32	Tomato	Torino, Italy	1979	KP234321	KP234345
Pe1*	Parietaria	Italy	1989	AY496069	U35145

* PMoV isolates whose nucleotides sequences were retrieved from GenBank

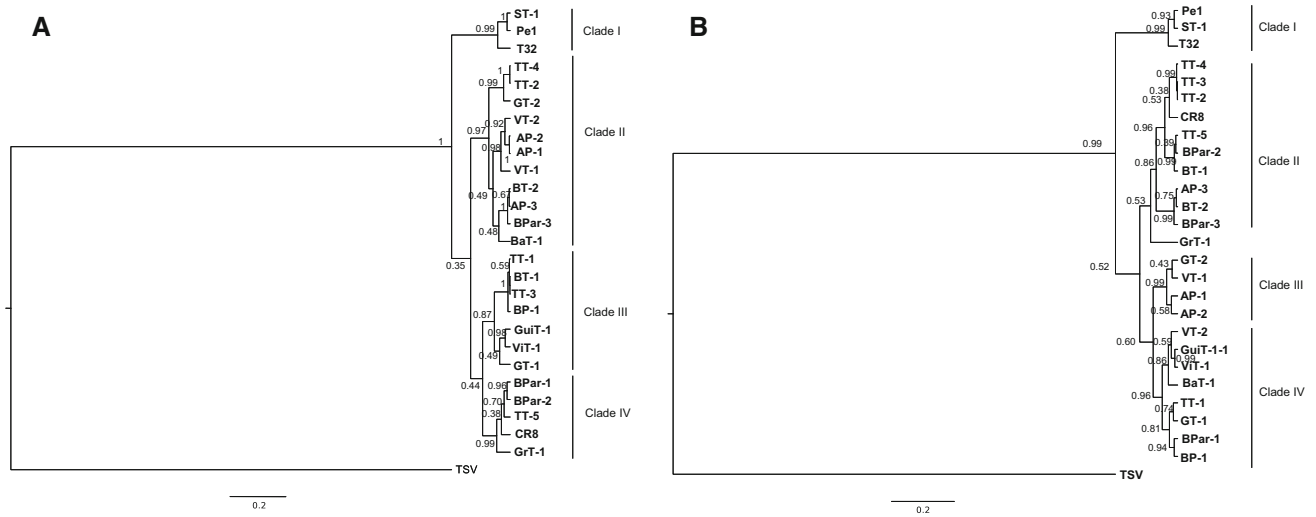

Fig. 1 Bayesian phylogenetic trees based on nucleotide sequences of genes encoding the CP (A) and 2b (B) proteins of parietaria mottle virus (PMoV). Node significance is indicated with posterior probability values. Trees were rooted by using tobacco streak virus (STV) as an outgroup

55 °C and 40 s at 72 °C, and a final extension of 7 min at 72 °C. RT-PCR products were purified using an UltraClean® PCR Clean-Up Kit (Mo-Bio), and the consensus nucleotide sequences of the amplification products were determined in both senses using an ABI 377 DNA sequencer (PerkinElmer) and deposited in GenBank under accession numbers KP23302 to 234325 for 2b gene and KP2343026 to KP234349 for the CP gene (Table 1). In addition, the nucleotide sequences of the 2b and CP genes from Spanish CR8 (FJ858203 and FJ858204) and Italian Pe1 (AY496069 and U35145) isolates were retrieved from GenBank and used in this study [11, 14, 33]. The nucleotide sequences were aligned at the amino acid level using the algorithms CLUSTAL W [19] and MUSCLE in MEGA v.6.0 [35]. The alignment was further corrected manually. The nucleotide substitution model that best fitted the sequence data was the Kimura two-parameter model (K2P) [18], and this was used to assess nucleotide distances among PMoV isolates and to infer their phylogenetic relationships by the maximum-likelihood (ML) and neighbour-joining (NJ) methods [32] with 1,000 bootstrap replicates. All of these analyses were performed with MEGA v.6.0. Bayesian phylogenetic analysis was performed using BEAST v.1.8.2 [10] with the GTR + Γ4 + I substitution model. Estimation was done using the relaxed uncorrelated exponential clock model with an exponential distribution set as the mean prior. This combination of the clock model and the mean prior distribution was found to be the most plausible model for calculating Bayes factor K = P(D|M1)/P(D|M2), where P(D|Mx) is the posterior probability of a model x given the data D. Two independent Markov chain Monte Carlo (MCMC) algorithms were completed with a chain length of 10^8 sampling every 1,000 trees to establish convergence of all parameters. The trees were combined and summarized onto a single target tree using LOGCOMBINER and TREEANNOTATOR v.1.8.2 implemented in the BEAST package, discarding the first 10 % of sampled trees as burn-in. In all cases, the trees were rooted with tobacco streak virus (TSV) 2b and CP sequences (JX463338 and JX463339, respectively), the ilarvirus subgroup I type member.

Phylogenetic trees generated by the different methods showed similar topology, but the highest resolution was obtained with the Bayesian analysis (Fig. 1). For both the CP and 2b genes, PMoV isolates clustered in several well-supported clades or lineages (posterior probability ≥0.80), with one (clade I) including the Italian isolates and three (clades II-IV) containing the Spanish isolates. The Greek isolate GrT-1 was in clade IV for CP, whereas it formed a separate branch in the 2b phylogenetic tree. GrT-1 was genetically closer to clades containing Spanish isolates (mean nucleotide distances of 0.057 ± 0.009, 0.051 ± 0.008 and 0.049 ± 0.008 for clades II, III and IV, respectively) than to clade I, containing Italian isolates (0.123 ± 0.017). The nucleotide sequence diversity of the overall PMoV population was low for both the CP and 2b genes, showing values of 0.063 ± 0.006 and 0.062 ± 0.006, respectively, which is in the range of most plant viruses [13, 36]. PMoV nucleotide sequence diversity is higher than those of other widespread ilarviruses such as American plum line pattern virus (APLPV), prunus necrotic ringspot virus (PNRSV) and citrus variegation virus (CVV) but lower than prune dwarf virus (PDV) [1, 5, 15, 17]. Since isolates infecting tomato and parietaria are present in different clades, it could be inferred that the phylogenetic relationships are not associated with the

infected host species. Incongruence among PMoV isolates was observed in CP and 2b phylogenetic trees (e.g., CR8 and BP-1) that could be evidence of genetic exchange among them or of the two genes being under different selection pressures. To investigate this, further studies of genetic exchange (recombination or reassortment) among PMoV isolates and signs of selection pressure exerted on the CP and 2b genes were done.

Genetic exchange (recombination or reassortment) among organisms increases their genome diversity and adaptability while removing deleterious mutations [27]. To determine the role of genetic exchange on PMoV evolution, the sequences of the 2b and CP genes of each isolate were concatenated and aligned. Analysis with the programs GARD [29] and RDP4, containing the recombination-detecting algorithms GENECONV, BOOTSCAN, MAXCHI, CHIMERA, SISCAN, 3SEQ, LARD, and RDP [23], showed three putative genetic exchange events (probably reassortment of genomic segments) among Spanish PMoV isolates detected by at least four algorithms (Table 2). Thus, the ancestor of isolates BP-1, TT-1, GT-1, GuiT-1 and ViT-1 may have resulted from one genetic exchange event with the CP from an unknown isolate and the 2b gene from an isolate similar to BaT-1. The other putative genetic exchange event might have occurred in an ancestor of isolates BT-1 and TT-3 with a CP like that of TT-1 and a 2b from an isolate similar to TT-2. Finally, the third genetic exchange event might have occurred in an ancestor of isolates TT-2 and TT-4, with the CP gene from an isolate similar to GeT-2 and the 2b gene from an isolate similar to CR8. Genetic exchange (mainly done by reassortment of different genomic segments on multipartite genomes) is frequent in plant viruses, including those of the family *Bromoviridae* [20], but this is the first report of this phenomenon in a member of genus *Ilarvirus.*

The role of natural selection was investigated using two approaches: i) computing separately the mean number of nonsynonymous substitutions per nonsynonymous site (N) and the mean number of synonymous substitutions per synonymous site (S) using the Pamilo-Bianchi-Li method [28] and ii) estimating the rates of nonsynonymous (N) and synonymous (S) changes at each codon by the FUBAR method [26] available on the Datamonkey server (http://www.datamonkey.org) [30]. N/S values were 0.200 for CP and 0.258 for 2b genes, indicating purifying selection in both cases. These values are in the same range as those obtained for other plant viruses [13]. Selection pressure analysis across the CP revealed that three positions are under diversifying selection and 44 positions are under purifying selection, whereas one position was under diversifying selection

Table 2 The most likely genetic exchange events supported by at least four algorithms in the RDP program

Virus isolate	Inference algorithm (*P*-value)								Recombination breakpoint*
	GENECONV	BOOTSCAN	MAXCHI	CHIMERA	SISCAN	3SEQ	LARD	RDP	
BP-1	-	-	2.989×10^{-03}	2.229×10^{-01}	6.658×10^{-06}	4.045×10^{-03}	-	-	640
GT-1	-	-	2.989×10^{-03}	2.229×10^{-01}	6.658×10^{-06}	4.045×10^{-03}	-	-	692
ViT-1	-	-	2.989×10^{-03}	2.229×10^{-01}	6.658×10^{-06}	4.045×10^{-03}	-	-	693
TT-1	-	-	2.989×10^{-03}	2.229×10^{-01}	6.658×10^{-06}	4.045×10^{-03}	-	-	659
GuiT-1	-	-	2.989×10^{-03}	2.229×10^{-01}	6.658×10^{-06}	4.045×10^{-03}	-	-	614
BT-1	5.756×10^{-04}	3.392×10^{-05}	1.704×10^{-06}	5.491×10^{-7}	6.154×10^{-9}	2.103×10^{-07}	-	-	614
TT-3	5.756×10^{-04}	3.392×10^{-05}	1.704×10^{-06}	5.491×10^{-7}	6.154×10^{-9}	2.103×10^{-07}	-	-	614
TT-4	-	-	1.400×10^{-02}	4.321×10^{-03}	5.194×10^{-04}	4.757×10^{-02}	-	-	610
TT-2	-	-	1.400×10^{-02}	4.321×10^{-03}	5.194×10^{-04}	4.757×10^{-02}	-	-	610

* Nucleotide position in concatenated sequences

and 34 were under purifying selection in the 2b gene (posterior probability $\geq$0.9). These results suggest that both genes are under evolutionary constrains, probably as a consequence of the essential roles they play in the virus life cycle. In the case of PNRSV, the CP is known to be a multifunctional protein involved in virus encapsidation and replication, whereas the 2b protein acts as an RNA silencing suppressor in the ilarvirus asparagus virus 2 (AV 2) [2, 34].

Some PMoV isolates showed differences in the size of their CP. Most PMoV isolates had a CP of 204 amino acids, except for isolates Pe1 and ST-1 from Italy, which had an addition of 16 amino acids in the N-terminal half part of the protein (220 amino acids). The difference of CP size among PMoV isolates was previously reported to be a consequence of a cytosine (C) deletion at position 1,366 of RNA 3 that resulted in a different start codon [12]. Initially, it was suggested that there had been a sequencing mistake for the Pe1 isolate, since it was the only nucleotide sequence available in GenBank lacking the C deletion at position 1,366. However, the identification in this work of a PMoV isolate without the C deletion in the CP gene rendering a protein of the same size than Pe1 isolate supports the existence of virus isolates with CPs of different lengths [12, 14]. The isolates with the longer CP had two amino acid changes in a putative RNA-binding domain containing arginine residues that are essential for the replication of ilarviruses (genic activation) [2], one of them resulting from the replacement of an arginine residue (basic) by a serine (polar).

In conclusion, this work provides information on the genetic variability and evolutionary history of PMoV, a virus infecting two economically important crops – tomato and pepper. Comparison between synonymous and non-synonymous substitutions showed that the 2b and CP genes are both under strong purifying selection. Incongruence in phylogenetic trees and different recombination-detecting algorithms indicated genetic exchanges (either recombination or reassortment) among Spanish isolates. The results presented here contribute to our understanding of virus epidemiology and can be useful for disease control strategies.

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