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ABSTRACT

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Fig mosaic virus (FMV) is a multipartite negative-sense RNA virus infecting fig trees worldwide. FMV is transmitted by vegetative propagation and grafting of plant materials, and by the eriophyid mite *Aceria ficus*. In this work, the genetic variation and evolutionary mechanisms shaping FMV populations were characterized. Nucleotide sequences from four genomic regions (each within the genomic RNAs 1, 2, 3, and 4) from FMV isolates from different countries were determined and analyzed. FMV genetic variation was low, as is seen for many other plant viruses. Phylogenetic analysis showed some geographically distant FMV isolates which clustered together, suggesting long-distance migration. The extent of migration was limited, although varied, between countries, such that FMV populations of different countries were genetically differentiated. Analysis using several recombination algorithms suggests that genomes of some FMV isolates originated by reassortment of genomic RNAs from different genetically similar isolates. Comparison between nonsynonymous and synonymous substitutions showed selection acting on some amino acids; however, most evolved neutrally. This and neutrality tests together with the limited gene flow suggest that genetic drift plays an important role in shaping FMV populations.

RNA virus populations, including those within an individual virus-infected host or those in different host individuals, are heterogeneous in nature. For RNA viruses, this is often attributed to their large population sizes, short generation times, and high mutation rates from error-prone replication by the viral RNA-dependent RNA polymerase (RdRp), which lacks a proofreading activity (9). Additional sources which can give rise to genetic variation include genome recombination and reassortment (39). The genetic diversity and structure of virus populations are limited and shaped by natural selection, genetic drift, and gene flow (37). The effects of these evolutionary mechanisms are also affected by the virus biology (e.g., host type and range, and means and extent of spread), the ecological environment, and population parameters (e.g., population size and history of population bottlenecks). Understanding the factors involved in the genetic diversity and structure of virus populations is fundamental for designing effective strategies for disease control or virus eradication (1).

Like many animal viruses and bacteriophages, some RNA plant viruses have measurably evolving populations (11,20) whereas others evolve more slowly and have genetically stable populations (19). Two key aspects affecting virus evolution are the means of spread and host type. Plant viruses mostly utilize specific vectors for their plant-to-plant transmission (40). Plant-to-plant spread of viruses among herbaceous annual plants may be rapid and the life of the plant host is relatively short. Thus, these viruses are constantly infecting and adapting to new plant hosts. By contrast, the chronic infections in woody perennial plants can last for decades, with or without observable symptoms (44). In addition, most perennial crop plants are vegetatively propagated. If source plants used for propagation are virus infected, this provides opportunity for efficient transfer and maintenance of the chronic infections in the offspring or cuttings. These chronic infections provide ideal settings for the long-term evolution of the viruses within a host plant and, presently, little information is available regarding their evolution.

In this work, we studied *Fig mosaic virus* (FMV), which affects fig (*Ficus carica* L.) trees worldwide (12). The fig tree is a perennial, one of the earliest plants domesticated by humans, and is grown throughout the world in temperate to tropical environments (27). FMV is efficiently transmitted by vegetative propagation and grafting but is not seed transmitted. FMV is also specifically transmitted plant to plant by the eriophyid mite *Aceria ficus* (16). FMV is a hexapartite, negative-strand single-stranded RNA genome virus (24). Each anti-genomic segment is monocistronic. RNA 1 encodes for an RdRp, RNA 2 encodes a putative glycoprotein precursor, RNA 3 encodes a nucleocapsid (NP) protein, RNA 4 encodes a putative movement protein, and RNA 5 and RNA 6 encode proteins of unknown functions (13,24,25,52). The genome organization and the deduced amino acid sequences of FMV-encoded proteins are similar to those of another eriophyid mite-transmitted virus, *European mountain ash ringspot associated virus* (EMARAV). Based on this, the genus *Emaravirus* (unassigned family) was proposed which contains EMARAV and tentatively three other species, one of which is FMV (12,24, 38,52).

In this study, we estimated the genetic variation and population structure of FMV isolates collected from diverse worldwide locations by analyzing the nucleotide sequences of four regions, one

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains one supplemental figure and one supplemental table.

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each of genomic RNAs 1, 2, 3, and 4. The roles of the evolutionary factors recombination, selection, genetic drift, and gene flow were examined.

MATERIALS AND METHODS

Virus isolates. Samples were collected from 37 FMV isolates from California, Turkey, and Israel (Fig. 1). In all, 21 FMV isolates were from California, including 10 collected from different commercial orchards and 11 obtained from the United States Department of Agriculture (USDA) National Clonal Germplasm Repository, University of California (UC), Davis. The repository currently has 190 fig accessions from different parts of the world (47), and all of these plants show visible symptoms of the fig mosaic disease (52). Ten isolates were collected from different regions of Turkey and six isolates were obtained from trees imported from Israel by the California Department of Food and Agriculture, Riverside. Nucleotide sequences from 16 additional

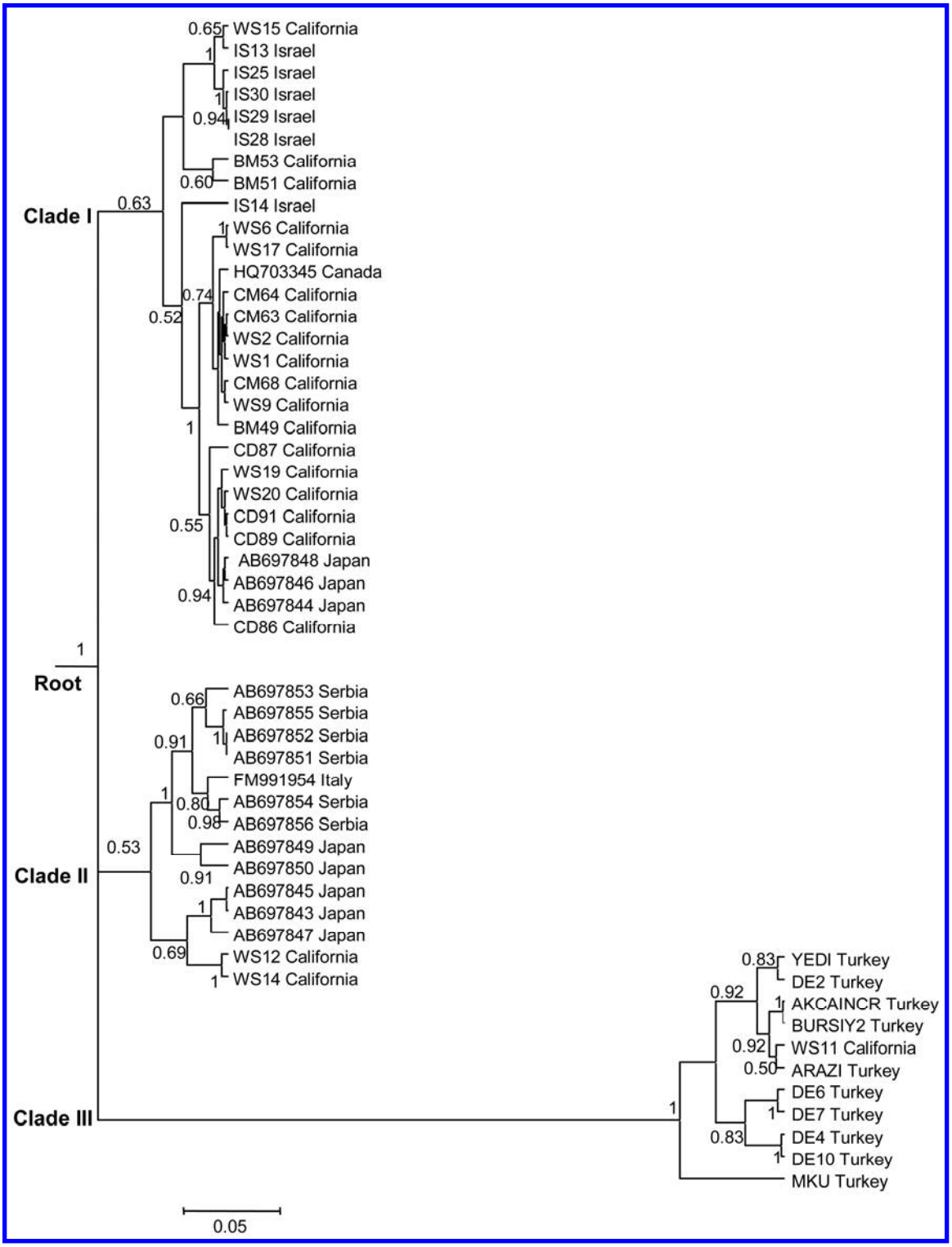

Fig. 1. Bayesian phylogenetic tree of the nucleocapsid gene of *Fig mosaic virus* isolates from California, Canada, Italy, Serbia, Turkey, Israel, and Japan. Node significance was indicated by posterior probability values (≥0.50). California isolates obtained from a germplasm collection are named as WS followed by a number, whereas the other Californian isolates are from commercial fields and given a B or C. Sequences from GenBank are indicated by their respective numbers.

isolates (1 from Canada, 1 from Italy, 8 from Japan, and 6 from Serbia) were retrieved from GenBank (accession numbers in Figure 1).

Genotyping. Total RNA was extracted from symptomatic fig leaves by using the Qiagen RNeasy Plant mini kit (Qiagen Sciences, MD). Oligonucleotide primers were designed for the genomic RNAs 1, 2, 3, and 4 (Table 1). A one-step reverse-transcription polymerase chain reaction (RT-PCR) was performed at 47°C for 30 min (reverse transcription); then, 35 cycles of 94°C for 30 s, 51°C for 45 s, and 72°C for 1 min; and a final extension for 7 min at 72°C. The amplified products were analyzed by agarose gel electrophoresis and stained with SYBR Gold (Invitrogen, Molecular Probes Inc., Eugene, OR) as per the manufacturer's instructions. Bands were cut from the gels and DNA was extracted by using the Minelute Gel extraction kit (Qiagen Sciences). The purified RT-PCR products were directly sequenced with the respective primers, using the ABI 3730 Capillary Electrophoresis Genetic Analyzer at the UC DNA Sequencing facility, UC Davis. No ambiguous sites were found, indicating a within-isolate homogeneous population and absence of mixed infections. Nucleotide sequences were deposited in GenBank under accession numbers KC182474 to KC182510 and KC295716 to KC295790.

Nucleotide sequence analysis. Multiple sequence alignments were performed with the algorithm CLUSTAL W (29) implemented in the program MEGA 5.05 (49). The nucleotide substitution model which best fits the sequences and the nucleotide divergence to correct superimposed substitutions (42) was estimated with MEGA 5.05. The best nucleotide substitution model (that with the lowest Akaike Information Criterion value) was the general time reversible (GTR) with nonuniformity of evolutionary rates among sites. It is modeled by using a discrete γ distribution (+G) with five rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I). The estimate of γ shape parameter was 0.29 and the estimated fraction of invariant sites was 0.56.

The aligned sequences of the *NP* gene of FMV RNA 3 were used to infer a Bayesian phylogenetic tree (node significance was estimated with posterior probabilities) by using the program BEAST version 1.7.4 (10) using the estimated GTR + Γ_4 + I substitution model. The MCMC was run for 10^8 samplings every 1,000 trees to ensure convergence of all parameters. The BEAST output was analyzed using TRACER, version 1.5 (tree.bio.ed.ac.uk/software/tracer) with satisfaction of the effective sample sizes (posterior = 324.747, likelihood = 11,795.484). The sample of the trees was summarized into the maximum clade credibility phylogeny using TREEANNOTATOR, version 1.7.4 (beast.bio.ed.ac.uk/TreeAnnotator), discarding the first 10% of sampled trees as burn-in. Nucleotide diversities (mean nucleotide distances between sequence pairs) of the FMV *NP* gene within and between geographic populations (standard errors were calculated with 1,000 bootstrap replicates) were estimated with MEGA 5.05. The program DNASP 5.0 (30) was used to estimate genetic differentiation between populations with three permutation-based statistical tests (Ks*, Z*, and S_{nn}) (22,23) as well as the level of gene flow with the statistic F_{st}, which has values between 0 and 1 for complete and absence of gene flow (53).

The program RDP3, which contains the recombination-detecting algorithms GENECONV, BOOTSCAN, MAXCHI, SISCAN, 3SEQ, LARD, and RDP (33), was used to search for possible recombination or reassortment events by analysis of the concatenated FMV genomic regions located in RNAs 1, 2, 3, and 4, respectively.

The degree of selective constraints at the amino acid level was estimated with MEGA 5.05 by analyzing separately the rate of nonsynonymous (dN) and synonymous (dS) substitutions with the Pamilo-Bianchi-Li method (41). The difference between dN and dS provides information on the sign and intensity of selection. Selection across the genomic coding regions was studied by estimation of the rates of dN and dS at each codon using the fixed effects likelihood method (28) implemented in the Datamonkey Server (http://www.datamonkey.org/). The program DNASP 5.10 (30) was used to evaluate the importance of natural selection to shape an FMV population by testing the mutation neutrality hypothesis with several statistics: Tajima's D, based on the differences between the number of segregating sites and the average number of nucleotide differences (48); Fu and Li's D test, based on the differences between the number of singletons (mutations appearing only once among the sequences) and the total number of mutations (18); and Fu and Li's F test, based on the differences between the number of singletons and the average number of nucleotide differences between every pair of sequences (18). Significant deviation from the neutral hypothesis would indicate selection but this can also be produced by a rapid growth of the viral population after a bottleneck event.

TABLE 1. Primers used to reverse-transcription polymerase chain reaction (RT-PCR) amplify regions of the four *Fig mosaic virus* (FMV) genomic RNAs

Primer sequence[a]	Genomic region[b]	Amplified region (nt)[c]	Size (nt)[d]
GTTATGGCTATATATTCTGATTATTC	RNA 1	2,170–2,537	367
TCAAACTTGTATGGTGTGTAATA	(RdRp)		
AGATGTGGGAAAATCATATGCT	RNA 2	1,535–2,107	572
AGACCAACTTGCAGGCTTTT	(GP)		
GTCATGTTGATACATGTGCTGC	RNA 3	347–1,220	873
CACACTTACACATCTTACATCATCT	(NP)		
GATCTTGTTGGAAACACAATA	RNA 4	490–1,073	583
GCTTTGGCAGATTCTATT	(MP)		

[a] Oligonucleotide primers used. The upper sequence corresponds to the forward primer complementary to the negative strand and the lower to the reverse primer complementary to the positive strand.

[b] Genomic regions where the primers are located: RNA-dependent RNA polymerase (RdRp) encoded in RNA 1, glycoprotein (GP) encoded in RNA 2, nucleocapsid (NP) protein encoded in RNA 3, and a protein of unknown function encoded in RNA 4.

[c] Nucleotide (nt) positions of the primers in the respective FMV genomic RNAs are indicated.

[d] Expected size (nt) of the RT-PCR products.

RESULTS AND DISCUSSION

Genetic structure and variation of FMV. The genetic structure and the degree of genetic variation must be considered for designing and evaluating strategies of disease control (1). For example, plant breeding to obtain resistant cultivars should be assessed with different viral isolates covering the virus genetic spectrum because resistance would depend on specific virus–host interactions (17). To estimate the genetic structure of FMV, the phylogenetic relationships of the *NP* gene of 53 FMV isolates from different countries were inferred. This analysis showed three well-resolved main clades (Fig. 1). Clade I was composed of all isolates collected in field in California, 10 of 13 isolates collected from the California germplasm collection, the Canadian isolate, all isolates from Israel, and 3 isolates from Japan. Clade II contained two isolates from the California germplasm and isolates from Italy, Serbia, and Japan. Finally, clade III was composed of all isolates from Turkey and one from the California germplasm collection.

The close genetic relationships between some geographically distant isolates suggest long-distance migration, probably due to the international traffic of propagative fig material. This has been also observed for other plant viruses (34,45). The phylogeny topology showed that at least three independent introductions of divergent FMV isolates have occurred in California, most likely via importation of germplasm material. However, only one of the three introductions (clade I) has spread in the field. Nucleotide

variation was low, with the highest nucleotide distance between two isolates being 0.057 and a mean nucleotide distance of 0.030 ± 0.005. This is in the range seen for an equivalent genomic region of EMARAV (26) and most plant viruses (19). The mean nucleotide distance for several viruses infecting woody perennial and annual crops (8,15,34,45,46,51) is shown in Supplemental Figure 1. There seems to be no correlation between the host type and the nucleotide variation. For example, the mean nucleotide distance was low for two members of the family *Closteroviridae*, *Citrus tristeza virus* (CTV) and *Cucurbit yellow stunting disorder virus*, infecting woody and herbaceous plants, respectively, whereas it was much higher for two members of the family *Secoviridae*, *Grapevine fan leaf virus* and *Broad bean wilt virus* 2, infecting woody and herbaceous plants, respectively.

Genetic diversity and gene flow of geographically distinct FMV populations. The great ability of RNA viruses to evolve rapidly with respect to cellular organisms implies that epidemiological and evolutionary processes occur on a similar temporal scale and can interact with each other (21). Comparison between virus populations from different geographic areas can provide relevant information to understand the emergence, epidemiology, short- and long-distance movement, and gene flow of viruses which can be implemented in disease control strategies based on limiting virus dispersion.

The genetic diversity of the FMV *NP* gene for populations from six geographic areas was estimated (Table 2). Our analysis showed that the nucleotide diversity of FMV in each area was very low (0.009 for Israel, ≈0.015 for California and Serbia, and ≈0.030 for Turkey and Japan) whereas those between most areas were a little higher. It is difficult to compare the nucleotide diversity with other plant viruses occupying the same geographic area because these studies are scarce and differ in sampling size, location, and date. In California, nucleotide diversity of *Cucumber mosaic virus*, CTV, and *Citrus psorosis virus* have been estimated as ≈0.030 (31,34,46), which is also low in comparison with bacteriophages, animal viruses, and some plant viruses (14,20).

Genetic differentiation was evaluated with the statistical tests Ks*, Z*, and S_{nn}, and the extent of genetic differentiation and, therefore, gene flow was estimated with the coefficient F_{st}. When samples collected from the USDA Germplasm collection (located at UC Davis) were compared with the isolates collected from commercial fields in California, the F_{st} value was equal to –0.021 and the three statistical tests were nonsignificant. This indicates that the isolates from the Germplasm collection and from commercial fields are closely related and, from a genetic view, can be considered as the same population. The results of these analyses when compared for FMV isolates from different geographic regions are shown in Table 3. In all cases, the tests Ks*, Z*, and S_{nn} gave significant values suggesting strong genetic differentiation. F_{st} values were >0.600 between California, Serbia, and Israel FMV isolates, suggesting very infrequent gene flow between these populations. They were ≈0.400 between Turkey and Serbia or Israel FMV isolates, and between Japan and Serbia or Israel FMV isolates, suggesting moderate gene flow. Finally, F_{st} values <0.120 were estimated between California and Japan FMV isolates and between them and Turkey FMV isolates, which suggests a certain gene flow. These results agree with those obtained in our phylogenetic analysis (Fig. 1).

Reassortment or recombination for FMV. Recombination and reassortment (pseudorecombination) events were analyzed because these can promote genome diversity and adaptability or offset fitness decrease by accumulation of deleterious mutations in bottleneck events (3,39). The analysis of the concatenated four genomic regions located in the genomic segments 1, 2, 3, and 4, respectively, suggested that some FMV isolates could have arisen via genome segment reassortment between co-infecting different FMV isolates (Fig. 2). The Turkish FMV isolates ARAZI and YEDI seem to have acquired RNA 2 from an unknown FMV isolate whereas the other three segments could have originated from an ancestor of isolate BM49. The Californian isolates WS19, WS20, CD86, CD87, CD89, and CD91 most likely originated from four different reassortment events between the ancestors of FMV isolates IS28 and CM68. Thus, WS19 and WS20 evolved from IS28-like RNAs 1 and 4 and CM68-like RNAs 2 and 3; CD86 from IS28-like RNAs 1 and 2 and CM68-like RNAs 3 and 4; CD87 from an IS28-like RNA 1 and CM68-like RNAs 2, 3, and 4; and CD89 and CD91 from IS28-like RNAs 1, 2, and 4 and a CM68-like RNA 3. Finally, FMV isolates CM63 and CM68 seem to have acquired RNA 1 from a WS11 ancestor and the other three RNAs from a CM64 ancestor, whereas isolate WS1 likely acquired RNA 1 from a CAN01 ancestor and the other three RNAs from an unknown FMV isolate. Reassortment has been found in other negative-sense plant RNA viruses (e.g., *Tomato spotted wilt virus*) (50) and other multipartite plant viruses (2,31,36). By contrast, no recombination events were found within any genomic segment analyzed here; however, the genome regions analyzed here are relatively small and, thus, we cannot rule out that recombination could have happened in the non-analyzed genomic regions. However, recombination in negative-sense RNA viruses is reported to be rare (4), in contrast to positive-sense RNA viruses (5,35) or DNA viruses (7).

Genetic variation and selective pressure for different genomic regions of FMV. Natural selection and genetic drift are two main evolutionary mechanisms limiting genetic variation of virus populations (37). The genetic variation for the four genomic regions of FMV was determined (Table 4). The coding regions of RNAs 3 and 4 had relatively low genetic variation (nucleotide diversity ≈0.020 and proportion of polymorphic sites ≈0.090) whereas the

TABLE 3. Genetic differentiation and gene flow of the nucleocapsid gene between *Fig mosaic virus* (FMV) populations from different geographic locations[a]

Location	Isolates	California	Serbia	Turkey	Israel
California	21	…	…	…	…
Serbia	6	0.637*	…	…	…
Turkey	10	0.118*	0.465*	…	…
Israel	6	0.625*	0.706*	0.403*	…
Japan	8	0.114*	0.400*	0.097*	0.451*

[a] Values correspond to F_{st} statistic which estimates gene flow between FMV populations. An asterisk (*) means that the genetic differentiation was statistically significant according to the three tests: K_{st}*, Z*, and S_{nn}.

TABLE 2. Nucleotide diversity of the nucleocapsid gene within and between *Fig mosaic virus* (FMV) populations from different geographic areas[a]

Areas	Isolates	California	Serbia	Turkey	Israel	Japan
California	21	**0.013 ± 0.003**	…	…	…	…
Serbia	6	0.040 ± 0.008	**0.014 ± 0.003**	…	…	…
Turkey	10	0.024 ± 0.004	0.043 ± 0.007	**0.029 ± 0.007**	…	…
Israel	6	0.029 ± 0.007	0.042 ± 0.009	0.032 ± 0.006	**0.009 ± 0.002**	…
Japan	8	0.025 ± 0.004	0.040 ± 0.006	0.043 ± 0.007	0.038 ± 0.008	**0.032 ± 0.006**

[a] Nucleotide diversity (mean nucleotide distance between pairs of sequences) is shown within populations (on the diagonal, in bold) or between populations (below the diagonal). Standard errors are indicated.

regions of RNAs 1 and 2 showed a nucleotide diversity approximately five times higher (≈0.100) and approximately three times more polymorphic sites (≈0.265).

It is assumed that the potential for mutation rates is the same along the genomic RNAs and that the differences seen in genetic variation then must result from different selective pressures exerted in the RNA coding regions. To evaluate the sign and intensity of selection at both nucleotide and amino acid levels, the dS and dN nucleotide substitutions were computed separately. The genomic regions analyzed from RNAs 3 and 4 had dN values much lower (0.003 and 0.002, respectively) than those for RNA 1 (0.013) and RNA 2 (0.026), suggesting a very strong negative selection probably due to functional or structural constrictions of the encoded proteins. RNAs 3 and 4 showed lower dS values (≈0.063) than those for RNA 2 (0.150) and RNA 3 (0.262), also suggesting negative selection at the nucleotide level. Selection at the nucleotide level could occur by thermodynamic stability of RNA (secondary structure), codon usage bias for translation efficiency, activation of gene silencing, and RNA–RNA or RNA–protein interactions (6).

Statistical analysis of the ratio of nonsynonymous and synonymous changes for individual codons showed that a total of 90 of 1,905 sites in the four genomic regions were under negative selection (Table 4; Supplemental Table 1). These sites could be involved in functional properties or perhaps be critical structural domains. In RNA 2, four sites were identified to be under positive selection, which could be the result of an adaptation of FMV to an environment change. This has been described for other plant viruses (e.g., some isolates of *Tomato spotted wilt virus* had a positively selected amino acid change which correlated with the ability for these isolates to break resistance conferred by the gene *Sw-5* in tomato) (32). The sites under selection identified in this work can serve as a guide for functional studies based on directed mutagenesis and reverse genetics.

To assess the role of natural selection at the population level, three different neutrality tests were performed. They gave negative values for the four FMV genomic regions, although they did not show significant deviation from neutrality, suggesting that genetic drift would have an important role in shaping the observed FMV populations. This is in accordance with the codon

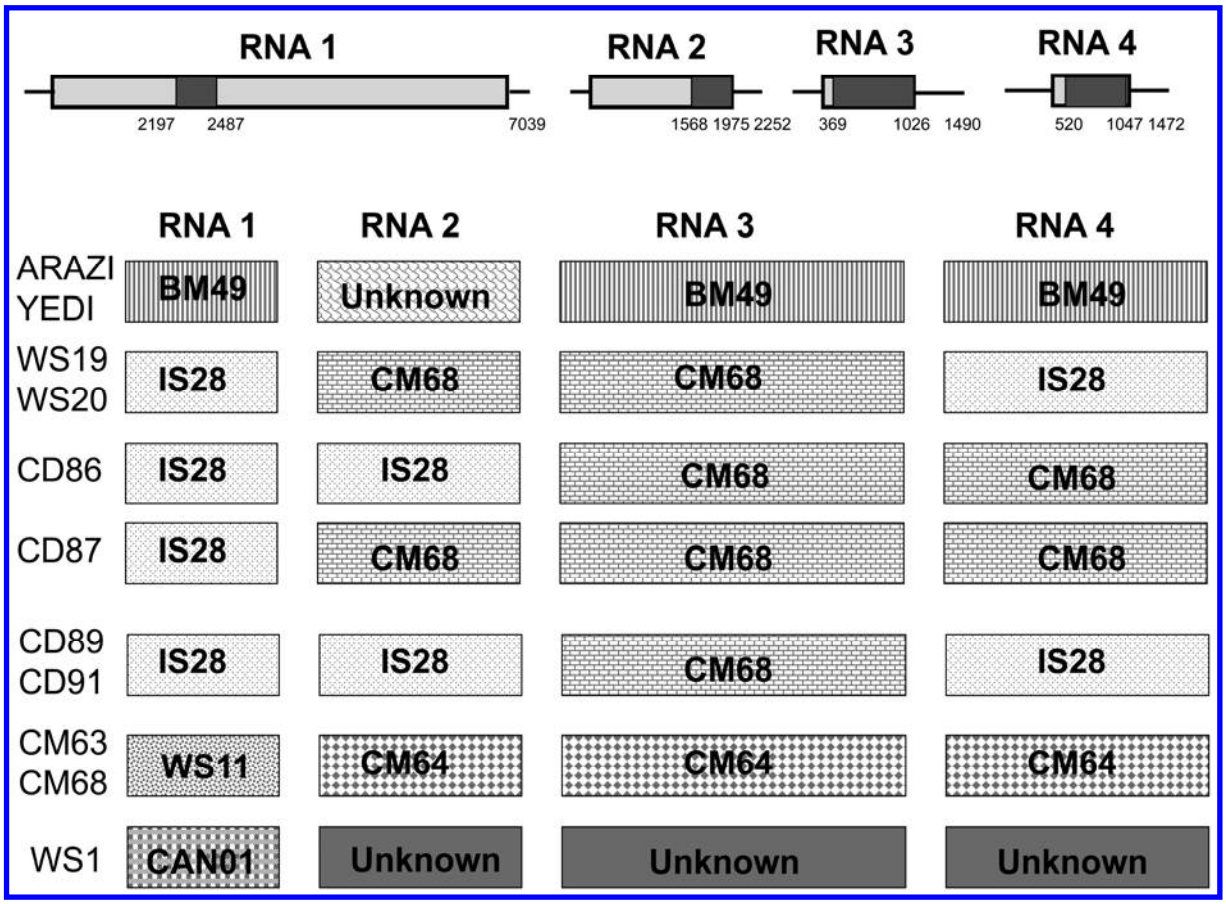

Fig. 2. Schematic representation of the recombination analysis of concatenated *Fig mosaic virus* (FMV) sequences RNA 1 + RNA 2 + RNA 3 + RNA 4. Possible parental sequences are indicated in the boxes with different patterns. FMV isolate names are given at left.

TABLE 4. Population genetic parameters and neutrality tests calculated for the four *Fig mosaic virus* (FMV) genomic regions[a]

Genomic region	*n*	S	π	dS	dN	dN/dS	Ne	Po	Tajima's D	Fu and Li's D	Fu and Li's F
RNA 1	291	0.261	0.111 ± 0.030	0.262 ± 0.054	0.013 ± 0.006	0.050	19	0	–0.135	–0.206	–0.216
RNA 2	408	0.270	0.099 ± 0.017	0.150 ± 0.021	0.026 ± 0.006	0.173	38	4	–0.836	–0759	–0.927
RNA 3	678	0.093	0.023 ± 0.005	0.065 ± 0.010	0.003 ± 0.001	0.046	13	0	–0.731	–0.916	–1.010
RNA 4	528	0.089	0.022 ± 0.005	0.062 ± 0.013	0.002 ± 0.001	0.032	20	0	–0.836	–1.353	–1.397

[a] Abbreviations: *n* = number of sites, S = number of segregating (polymorphic) sites, π = nucleotide diversity (mean nucleotide differences per site between sequence pairs), dS = frequency of synonymous substitution per site, dN = frequency of nonsynonymous substitution per site, Ne = number of negatively selected codons, and Po = number of positively selected codons.

selection analyses, which showed that 95.1% of the sites were under neutral evolution. This has also been observed in some plant virus populations (8) whereas, in others cases, negative selection seems have played a major role (43).

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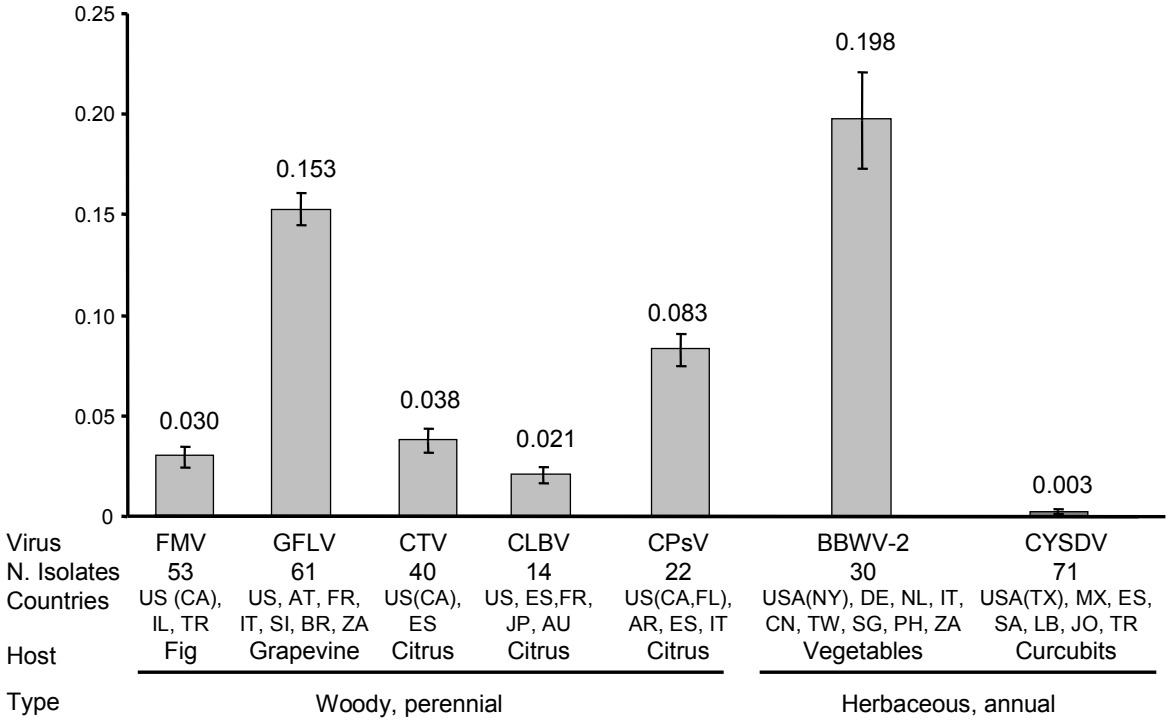

Supplemental Figure 1. Nucleotide diversity of the coat protein or NP genes of worldwide isolates of different plant viruses: *Fig mosaic virus* (FMV), *Grapevine fan leaf virus* (GFLV), *Citrus tristeza virus* (CTV), *Citrus leaf blotch virus* (CBLV), *Citrus psorosis virus* (CPsV), *Broad bean wilt virus 2* (BBWV-2) and *Cucurbit yellow stunting disorder virus* (CYSDV). Countries are indicated with two-letter country codes defined in ISO 3166-1 and USA states with the two-letter codes. Vertical bars indicate standard errors.

Supplemental Table 1. Amino acid sites under natural selection.

RNA[a]	ORF[b]	GenBank[c]	Sites under selection[d] Negative	Positive
1	RdRp	AM941711	775, 776, 778, 783, 785, 787, 791, 794, 795, 796, 797, 798, 799, 802, 803, 808, 811, 812, 814	
2	GP	FM864225	508, 509, 513, 514, 515, 516, 518, 520, 522, 526, 527, 528, 533, 540, 543, 544, 547, 554, 555, 558, 560, 565, 570, 573, 575, 577, 579,580, 582, 600, 602, 604, 606, 614, 617, 621, 627, 632	538, 620, 621, 535
3	NP	FM991954	98, 103, 129, 133, 134, 147, 163, 168, 170, 218, 226, 283, 309	
4	MP	FM992851	147, 150, 155, 162, 163, 172, 188, 206, 242, 243, 253, 256, 261, 267, 279, 291, 299, 303, 310, 316	

[a]genomic RNAs.

[b]Open reading frames: RdRP= RNA dependent RNA polymerase, GP= glycoprotein. precursor, NP= nucleocapsid protein, MP=putative movement protein.

[c]GenBank accessions of FMV isolate GR10 used as reference.

[d]Sites under negative and positive selection.