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Genetic Variability and Evolution of the Satellite RNA of Cucumber Mosaic Virus during Natural Epidemics

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The genetic structure of populations of cucumber mosaic virus (CMV) satellite RNA (satRNA) and its evolution were analyzed during the course of a CMV epidemic in tomatoes in eastern Spain. A total of 62 variants of CMV-satRNA from epidemic episodes in 1989, 1990, and 1991 were characterized by RNase protection assay (RPA); RPA patterns defined 60 haplotypes in the CMV-satRNA population. RPA of nine CMV-satRNAs of known sequences showed that numbers of nucleotide substitutions per site (d_{ij}) between different satRNAs can be estimated from RPA data. Thus, d_{ij} were estimated for any possible pair of field CMV-satRNA types, and nucleotide diversities within and between yearly subpopulations were calculated. Also, phylogenetic relationships among CMV-satRNAs were derived from RPA data (by parsimony) or from $\hat{d}_{ij}$ (by neighbor joining). From these analyses, a model for the evolution of CMV-satRNAs in field epidemics can be built. High genetic variability of CMV-satRNA results in very heterogeneous populations, even compared with those of other RNA genomes. The high diversity of the population is maintained through time by the continuous generation of variants by mutation, counterbalanced by negative selection; this results in a certain replacement of haplotypes from year to year. The sequential accumulation of mutations in CMV-satRNA leads to fast genetic divergence to reach what appears to be an upper permitted threshold.

Cucumber mosaic virus (*Cucumovirus*) (CMV) consists of small isometric particles that contain three genomic RNAs, plus the subgenomic messenger RNA for the coat protein (29). Some isolates of CMV also encapsidate a small (335 to 390 nucleotides [nt] long) linear RNA that depends on CMV (helper virus) for its replication, dispersion within the infected plant (25), encapsidation, and transmission. This satellite RNA (CMV-satRNA) is able to variously modify the replication and pathogenesis of CMV in a complex way that depends on the strain of CMV, strain of CMV-satRNA, and species of host plant (1a, 33). A number of characterized variants of CMV-satRNA are able to attenuate the symptoms induced by CMV and/or depress its accumulation in different host species, and it has been proposed that these attenuating CMV-satRNAs could be used in the control of CMV-induced diseases, either in classical cross-protection programs or in transgenic plants expressing the satRNA (10, 35, 36).

It has been shown that CMV-satRNA isolates consist of populations of molecules heterogeneous in nucleotide sequence (19, 26), and that new variants arise in serial passage experiments (9, 20). Nothing has been reported, though, on the genetic variability and evolution of CMV-satRNAs under natural field conditions. It should be pointed out that the frequency of CMV isolates carrying satRNAs in the field is usually low (17) and that CMV plus satRNA epidemics have been reported, to our knowledge, only three times. On all three occasions, the presence of satRNA resulted in a necrotic syndrome on tomatoes: its first occurrence in Alsace, France (1972) (23), led to the first description of a CMV-satRNA (16); the other two reported instances occurred in southern Italy (6) and eastern Spain (13) in the late 1980s. The epidemic in eastern Spain gave us the opportunity to analyze the evolution of CMV-satRNA in the field. We present here data on the population structure of CMV-satRNA, on its high variability, and on its fast evolution to reach what appears to be an upper threshold for genetic divergence.

MATERIALS AND METHODS

Virus and satRNA isolates. Tomato plants showing symptoms that had been associated with infection by CMV, with or without satRNA (i.e., stunting and filimorphism, stunting and leaf curl, or systemic necrosis [13]), were sampled in different tomato fields (two to four according to years) in a range of 30 km, at two localities (Benifaió and Cullera) less than 40 km south of Valencia, eastern Spain, in June 1989, June 1990, and June 1991. To avoid virus and/or satRNA variation or contamination during greenhouse multiplication, CMV virions were purified (21) directly from field-infected plants. A CMV preparation obtained from the terminal leaves of a single tomato branch was considered a CMV isolate (named 89/*i*, 90/*i*, and 91/*i*; $i = 1, 2, \ldots, n$). From some plants, different isolates were obtained from branches showing different symptoms. Virion RNAs (6 to 12 μg) from CMV isolates were separated by electrophoresis in semidenaturing polyacrylamide gels (9), which enabled the separation of satRNA variants present in a single CMV isolate (satRNA variants were named 89/*i.j*, 90/*i.j*, and 91/*i.j*; i and $j = 1, 2, \ldots, n$). satRNA variants were detected by staining the gel with 0.05% toluidine blue, each band was excised from the gel, and the CMV-satRNA variants were eluted (30) and analyzed separately. A CMV isolate with a satRNA (Gr-satRNA) isolated in 1990 from a necrotic tomato in Greece was kindly provided by C. Varveri. Nine CMV-satRNA variants from four CMV isolates were also obtained in 1990 from *Nicotiana glauca* plants collected within a 30-km radius of Valencia.

Reference CMV-satRNAs of known sequences were B1-,

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TABLE 1. Details of the CMV-satRNA population studied and of subpopulations for 1989, 1990, and 1991

Subpopulation	No. of:				
	Tomato samples	CMV isolates	Isolates with satRNA present	CMV-satRNA variants	CMV-satRNA haplotypes in RPA
1989	24	24	12[a]	18	18
1990	25	22	21	32	30
1991	25	11	9	12	12
Total	74	57	42	62	60

[a] Because of the amount of sample available, only 10 were analyzed.

B2-, B3-, G-, WL1-, and WL2-satRNA (8), K8-satRNA (5), Ix-satRNA (26), and R- and $I_{17}N$-satRNA (11).

Comparative analysis of CMV-satRNAs. Each satRNA variant was characterized by the pattern of fragments generated by RNase digestion of mismatches (RNase protection assay [RPA]) in hybrids made with a cRNA probe from a full-length clone of B2-satRNA (the gift of J. J. Bernal). RPA was performed by the method of Winter et al. (37), except that RNase digestions were done at 15°C. The fragments of the probe that were protected from RNase digestion were separated by electrophoresis in 8% polyacrylamide–8 M urea sequencing gels; for each RPA, two electrophoreses, in which the xylene cyanol marker dye migrated to 20 and 40 cm, respectively, from the origin, were run. Molecular size markers were included in each gel. For each RPA, the number and size (in nucleotides) of fragments were recorded, and a certain set of fragments defined an RPA pattern. Only the presence or absence of fragments was considered, and no attention was paid to the relative intensities of the fragment bands. RPA patterns were used to differentiate between satRNAs with different nucleotide sequences; thus, a given RPA pattern defined a haplotype (*sensu* Nei 1987; p. 259 in reference 28). The reproducibility of the method was assessed with 10 CMV-satRNAs of known sequences and with 40 of the field CMV-satRNAs; with each of these, RPA was performed at least three times, and no variation in the pattern was observed. Thus, a certain RNA sequence always gave a certain RPA pattern. Fragments in an RPA pattern were considered taxonomically informative characters and were used as input data for maximum parsimony inference of phylogenetic relationships among haplotypes. Also, the sets of presences or absences of fragments in the RPA patterns were used as data to estimate nucleotide substitution values per site between any two haplotypes. Estimated nucleotide substitution values per site were used to calculate intrapopulational and interpopulational nucleotide diversity values (28) and to infer phylogenetic relationships among haplotypes by neighbor joining. All phylogenetic analyses were done with the PHYLIP package (version 3.4) of J. Felsenstein.

RESULTS

Genetic structure of CMV-satRNA populations. During 1989, 1990, and 1991, a total of 57 isolates of CMV were obtained, of which 42 (74%) were supporting satRNAs (Table 1). From these 42 CMV isolates, 62 electrophoretic variants of CMV-satRNA were obtained, ranging from 1 to 5 per CMV isolate. The frequency of CMV isolates with satRNAs increased from 0.5 in 1989 to 0.8 to 0.9 in 1990 and 1991. If the CMV isolate associated with five satRNA variants (89/3) is excluded, the ratio of satRNA variants to CMV isolates was about 1.4 for all 3 years. All satRNAs found belonged to the 340-nt-length class, according to their electrophoretic mobilities under fully denaturing conditions (not shown). When the 62 satRNA variants were analyzed by RPA (see Fig. 1 for examples), the number of haplotypes obtained was 60 (Table 1) and only two haplotypes were present twice (in 1990). Thus, the ratio of satRNA haplotypes to satRNA variants was almost 1. It is worth pointing out that semidenaturing electrophoretic separation of CMV-satRNA variants occasionally had a higher discriminative power for variants than did RPA, as satRNA variants 90/17.1 and 90/17.2 gave the same RPA pattern. When a CMV isolate supported more than one satRNA variant, the comparison of the RPA patterns suggested that this was reflecting two phenomena: evolution of satRNA within the infected plant (e.g., 90/13.1 and 90/13.2, 90/14.1 and 90/14.2, or 90/15.1 and 90/15.2 [lanes 4 to 9 in Fig. 1]) and superinfection

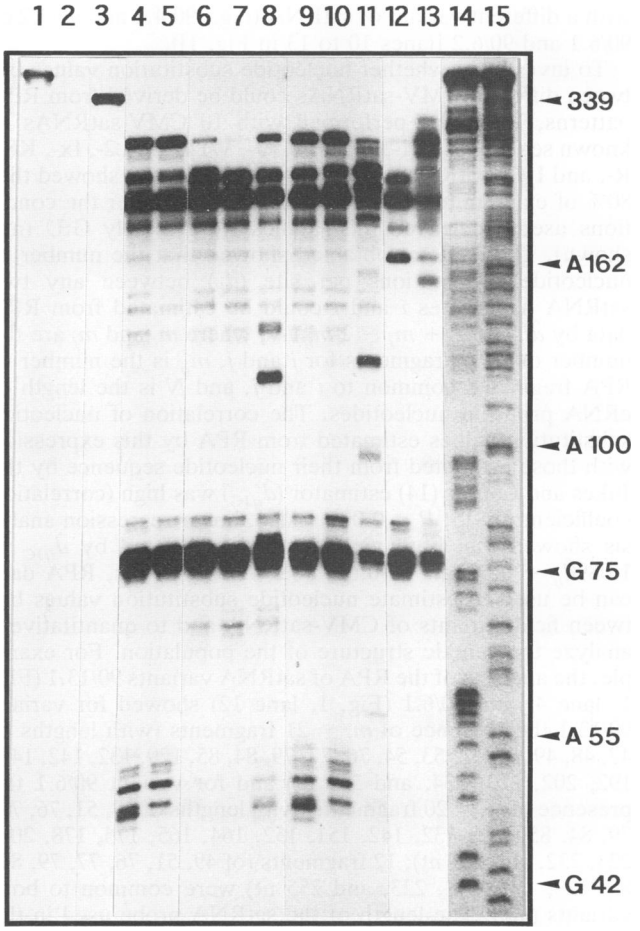

FIG. 1. Autoradiography of RPA patterns for CMV-satRNA variants from CMV isolates 90/13 (lanes 4 and 5), 90/14 (lanes 6 and 7), 90/15 (lanes 8 and 9), 90/4 (lanes 10 and 11), and 90/6 (lanes 12 and 13). Lane 1, undigested probe; lane 2, digested, unhybridized probe; lane 3, perfectly matched duplex: RPA pattern of B2-satRNA. Molecular size markers are from the sequencing reaction (8) of 5′-end-labelled B2-satRNA transcripts carried out with RNase T_1 (lane 14) or RNase φM (lane 15). Electrophoresis was carried out until xylene cyanol migrated to 20 cm from the origin. Undigested probe (lane 1) has 28 extra nt at the 5′ end, compared with B2-satRNA (lane 3).

with a different variant of satRNA (e.g., 90/4.1 and 90/4.2 or 90/6.1 and 90/6.2 [lanes 10 to 13 in Fig. 1]).

To investigate whether nucleotide substitution values between different CMV-satRNAs could be derived from RPA patterns, RPA was performed with 10 CMV-satRNAs of known sequences (B1-, B2-, B3-, G-, WL1-, WL2-, Ix-, K8-, R-, and I_{17}N-satRNA). Allocation of fragments showed that 80% of existing mismatches were cleaved under the conditions used; uncleaved mismatches were mostly G:U (not shown). The data set obtained showed that the number of nucleotide substitutions per site (d_{ij}) between any two satRNA haplotypes i and j could be estimated from RPA data by $\hat{d}_{ij} = (m_i + m_j - 2\,m_{ij})/N$, where m_i and m_j are the number of RPA fragments for i and j, m_{ij} is the number of RPA fragments common to i and j, and N is the length of cRNA probe in nucleotides. The correlation of nucleotide substitution values estimated from RPA by this expression with those estimated from their nucleotide sequence by the Jukes and Cantor (14) estimator ($\hat{d}_{ijJC}$) was high (correlation coefficient, 0.815; $P \leq 0.001$). Also, linear regression analysis showed that both estimators were related by $\hat{d}_{ijJC} = 1.089\hat{d}_{ij} + 0.004$ ($r^2 = 0.66$; $P \leq 0.0001$). Thus, RPA data can be used to estimate nucleotide substitution values between field variants of CMV-satRNA and to quantitatively analyze the genetic structure of the population. For example, the analysis of the RPA of satRNA variants 90/13.1 (Fig. 1, lane 4) and 90/6.1 (Fig. 1, lane 12) showed for variant 90/13.1 the presence of $m_i = 21$ fragments (with lengths of 47, 48, 49, 51, 52, 53, 54, 76, 77, 79, 84, 85, 129, 132, 142, 149, 192, 202, 223, 234, and 255 nt) and for variant 90/6.1 the presence of $m_j = 20$ fragments (with lengths of 49, 51, 76, 77, 79, 84, 85, 129, 132, 142, 151, 152, 164, 165, 176, 178, 200, 223, 232, and 255 nt); 12 fragments (of 49, 51, 76, 77, 79, 84, 85, 129, 132, 142, 223, and 255 nt) were common to both variants (m_{ij}). The length of the satRNA probe used in the RPA was 339 nt. Thus, for 90/13.1 and 90/6.1, $\hat{d}_{ij} = (21 + 20 - 24)/339 = 0.0501$. All fragment bands were recorded, even faint ones that do not show in Fig. 1. Note that Fig. 1 shows the gel with a xylene cyanol run of 20 cm, and that the longer fragments were recorded from a gel with a xylene cyanol run of 40 cm. Note also that fragments smaller than 40 nt were not considered, to minimize the probability of taking as the same fragment in i and j fragments of equal length originating from the cleavage of different mismatches in sequences i and j. The original data on the presence or absence of fragments on each RPA are not shown but are available from the authors upon request.

Nucleotide substitutions per site for field CMV-satRNAs ranged from 0.003 (for 91/2.1 and 91/2.2) to 0.142 (for 89/15.1 and 91/3.2). This maximum value is approached by maximum values found for the 1989 and 1990 subpopulations (0.130 and 0.133, respectively). The average number of nucleotide substitutions between any two satRNA haplotypes in the population (intrapopulational diversity) is 0.0644. This value is similar to that found for each of the 1989, 1990, and 1991 subpopulations (Table 2). For satRNA variants obtained from the same CMV isolate, $\hat{d}_{ij}$ values were either of the same order as the average ones cited above (e.g., for variants of 89/15, 89/16, 90/4, and 90/6) or much smaller (even an order of magnitude smaller for variants of 90/13, 90/17, 91/2, and 91/3). This may again reflect situations of superinfection of tomato plants with different satRNAs or generation of new variants within the infected plant, respectively. The net average number of nucleotide substitutions per site between any two haplotypes from subpopulations of two different years (interpopulational diversity) is much smaller than values for haplotypes of the same year; also, these interyear values show an increase with time (Table 2).

TABLE 2. Nucleotide diversity within and between subpopulations of CMV-satRNAs[a]

Subpopulation	Diversity value for subpopulation		
	1989	1990	1991
1989	0.05670		
1990	0.00442	0.06252	
1991	0.01561	0.00552	0.05367

[a] Within-population diversity, $\hat{D}_k = (n_k/n_{k-1})\Sigma\, x_i x_j \hat{d}_{ij}$, where n_k is the number of satRNAs in population k, x_i and x_j are the frequencies of haplotypes i and j in population k, and $\hat{d}_{ij}$ is the estimated number of nucleotide substitutions per site. Between-population diversity for populations k and l, $\hat{D}_{kl} = \Sigma x_i x_j \hat{d}_{ij} - (1/2)\,(\hat{D}_k + \hat{D}_l)$, where $\hat{d}_{ij}$ is the number of nucleotide substitutions between the ith haplotype from k and the jth haplotype from l and x_i and x_j are the frequencies of haplotypes i and j in populations k and l, respectively (after Nei [p. 276 in reference 28]).

Relationships among CMV-satRNA variants. Phylogenetic relationships between the CMV-satRNA haplotypes were inferred by Wagner parsimony (3, 18), with the presence or absence of fragments in the RPA patterns as input data, and by neighbor joining (34) with the estimated $\hat{d}_{ij}$ values. Similar results were obtained with both methods. For each of the 1989, 1990, and 1991 subpopulations, the same evolutionary pattern was found: CMV-satRNA variants diverge from putative ancestor types through a few evolutionary lines. For each line, haplotypes are found at, or very near, each node, indicating divergence by the sequential accumulation of mutations (Fig. 2A, for 1990). When 24 haplotypes randomly chosen from the 3 years were analyzed together, more evolutionary lines appeared, but intermediate types were still apparent for some of them. CMV-satRNAs from the same year are not more related to each other than they are to other years' variants. Haplotypes from *N. glauca* are grouped with the tomato variants.

DISCUSSION

An epidemic of CMV with associated satRNAs in tomato crops has been in progress in eastern Spain since 1986 (13). The data we present here show that CMV isolates from tomato plants, collected in a small area late in the springs of 1989, 1990, and 1991, frequently have an associated satRNA. The frequency of CMV isolates containing satRNA in the CMV population increased from 0.5 in 1989 to 0.8 to 0.9 in 1990 and 1991. Before 1986, CMV-satRNA had not been found in CMV isolates from crops or weeds, either in the eastern region or in other agricultural regions of Spain (1, 13, 22). The observed increase in the frequency of CMV isolates with a satRNA may reflect the spread of CMV-satRNA in the CMV population from one or a few foci at the beginning of the epidemic, to reach a frequency close to 1. With a single exception (isolate 89/3, having five satRNA variants), in CMV isolates with a satRNA one to three electrophoretic variants of satRNA are found. The ratio of variants of satRNA to isolates of CMV did not vary for the period considered here.

RPA of the 62 CMV-satRNA variants obtained resulted in 60 different patterns (haplotypes). The high ratio of haplotypes to variants shows a high ability of CMV-satRNA to vary under field conditions, in agreement with reports of variation under experimental conditions (9, 20, 26). The

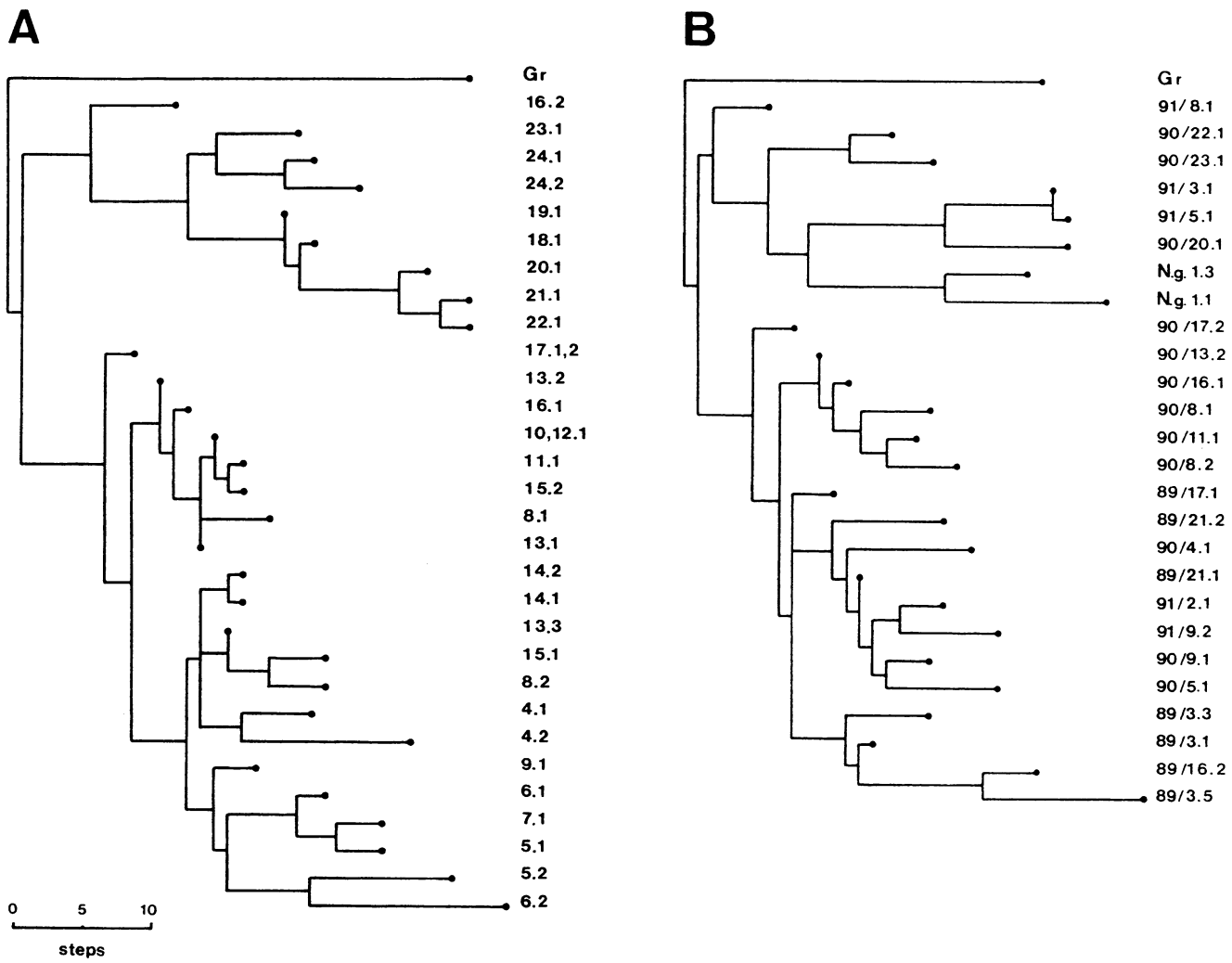

FIG. 2. Most-parsimonious phylogenetic trees for CMV-satRNA variants from 1990 from tomatoes (A) or from CMV-satRNA variants randomly sampled from the 1989, 1990, and 1991 tomato subpopulations and from *N. glauca* plants (N.g.) (B). A CMV-satRNA from Greece (Gr) was used as the outgroup. Horizontal branch lengths are to scale; vertical separation is for clarity only.

comparison of the RPA patterns of CMV-satRNA variants from the same CMV isolate, as well as analyses derived from them, suggests that when two satRNAs are found associated with one CMV isolate two different phenomena may have occurred: (i) one of the variants may have evolved in the infected plant to generate the second one (this could be the case for the very similar satRNA variants from CMV isolates 90/13, 90/14, and 90/15 and would again agree with the reported ability of CMV-satRNAs to generate new variants in planta during passage experiments), and (ii) in other cases, the satRNA variants associated with a CMV isolate were very different (e.g., for CMV isolate 90/4 or 90/6) and their presence in the same tomato branch could rather be interpreted as due to superinfection by aphid transmission. In many instances, superinfection of plants is supported by RPA analysis of the RNAs of the helper virus (unpublished data). This second phenomenon could indicate that cross-protection, reported for CMV-satRNAs (7, 24, 35), is not operating between satRNA variants naturally dispersed during an epidemic outbreak.

The analysis of the RPA patterns of 10 CMV-satRNAs of known sequences showed that, at least for CMV-satRNA, RPA data can be used to estimate values of nucleotide substitutions per site, d_{ij}, between different haplotypes i and j. This has also been shown in a recent simulation study (2). An estimator for d_{ij} was developed and applied to the field CMV-satRNA variants, and $\hat{d}_{ij}$ values were used to calculate nucleotide diversity values within and between subpopulations. Diversity values were similar for the subpopulations representing each of the three years; thus, the genetic heterogeneity of the CMV-satRNA population was maintained during the period studied. The CMV-satRNA populations were very heterogeneous: intrapopulation diversity values were high (5 to 10 times higher) compared with those reported for other plant or animal RNA viruses (27, 31, 32). Values for net nucleotide diversities between yearly subpopulations were much smaller than values for yearly subpopulations and increased with time, indicating a small but clear replacement of haplotypes in the population. Thus, a high proportion, but not all, of the variants present in one epidemic episode would be found, and/or be parentals for new variants, in the next episode. The persistence of haplo-

types is also supported by phylogenetic analyses that do not show a closer relationship among types from the same year than among types from different years. This persistence of haplotypes does not reflect the observed phenotypic evolution in the field, with necrogenic satRNAs being replaced by nonnecrogenic ones (13). Given the discontinuity in time of tomato crops, and the breakdown of aphid populations in the fall, bottlenecks in the CMV plus satRNA populations would be expected, and the question arises as to how persistence of a large number of satRNA haplotypes between epidemic episodes can be explained. It must be pointed out that CMV-satRNA generally does not multiply as efficiently as its helper virus in all host plants for CMV (12, 15). The only solanaceous weed in the area known to harbor CMV plus satRNA that was examined provided CMV-satRNA variants that were grouped with the tomato ones, indicating that this weed could be a reservoir for satRNA for tomato.

The maximum value of $\hat{d}_{ij}$ estimated from the variants analyzed over 3 years is 0.142; this maximum value could also be found between types from the same year subpopulation and is similar to that found among 23 CMV-satRNA variants isolated from different parts of the world (4). Thus, under field conditions, CMV-satRNA may evolve quickly to reach what appears to be an upper threshold for genetic divergence; our data show that the sequential accumulation of mutations plays an important role in this evolution. It has been shown that the maintenance of a functional molecular structure is a constraint to CMV-satRNA evolution (4), and negative selection could be acting on variant CMV-satRNA generated by mutation.

CMV-satRNAs of an attenuating phenotype have been proposed, and used, as control agents for CMV (7, 24, 35). The results presented here, in addition to providing an evolutionary model for CMV-satRNA, represent a cautionary tale for the use of untamed CMV-satRNAs for the control of CMV-induced diseases in the field.

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