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Evolutionary Dynamics of Cucumber Mosaic Virus Satellite RNA during Natural Epidemics in Italy

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The evolutionary dynamics of 22 variants of cucumber mosaic virus satellite RNA (CMV satRNA) isolated in Italy during virus epidemics from 1988 to 1993 were investigated on the basis of their primary structure and biological properties. Most of the variants were amplified from total nucleic acid preparations extracted from field-infected plants, thus representing wild isolates of CMV satRNA. Eleven variants were associated with subgroup II CMV strains, 10 with subgroup I and 1 with a mixed infection by both strains. When inoculated onto tomato seedlings, the variants induced the phenotype (necrogenic or ameliorative) predicted by their nucleotide sequence. Phylogenetic relationships between the satRNA variants were determined using the stationary Markov model, a stochastic model for evolution. For each satRNA, the Markov analysis gave a good correlation between position in the phylogenetic tree and biological properties. The variants with ameliorative and necrogenic phenotypes in tomato followed two different evolutionary dynamics in nature. Tfn-satRNA, a 390-nt-long molecule, followed a third type of evolutionary dynamic far apart from that of the shorter satRNA molecules (i.e., those in the 334- to 340-nt-length class). Average values of the mean constant rate of nucleotide substitutions/site ($K_{subs/site}$) indicated that in nature the variants tend to keep their heterogeneity unchanged from one epidemic episode to the other, even if the outbreaks occur in places very far from each other. This seems to be in agreement with the proposed maintenance of a functional molecular structure as a constraint to CMV satRNA evolution.

INTRODUCTION

Cucumber mosaic cucumovirus (CMV), the type species of the genus *Cucumovirus* (family Bromoviridae), is an isometric virus with a tripartite single-stranded RNA genome of positive polarity. Several strains of CMV support replication of a satellite RNA (satRNA) which depends on viral helper functions for its replication, encapsidation, and transmission (Collmer and Howell, 1992; Palukaitis *et al.,* 1992). The CMV satRNA is a small 332- to 405-nucleotide-long linear molecule that can sometimes affect CMV replication, pathogenesis, and symptom expression. Its biological activity (Collmer and Howell, 1992; Roossink *et al.,* 1992) is codetermined by the virus strain, host species and its cultivar, and by a number of other factors such as growing conditions, temperature, and photoperiod (Kaper, 1992, 1993; Wu *et al.,* 1993; White *et al.,* 1995; Kaper *et al.,* 1995).

A number of variants of CMV satRNA intensify symptoms induced by CMV in different plant species, whereas others attenuate them. Variants with attenuating effects have been extensively studied as potential or actual biocontrol agents for the prevention of CMV-induced diseases in tomato (see for review Tien and Wu, 1991; Kaper, 1993), due to difficulties in obtaining CMV-resistant lines by introgression of genes from wild species. This approach has become the basis for experimental and practical field strategies where the satellite-mediated protection can be achieved by either preinoculation of seedlings (cross-protection or "vaccination") or by transgenosis (Tien and Wu, 1991; Yie and Tien, 1993).

Encouraging results were obtained in several instances (Tien and Wu, 1991; Montasser *et al.,* 1991; Gallitelli *et al.,* 1991; Sayama *et al.,* 1993; Cillo *et al.,* 1995) and apparently no adverse effects were detected. Nevertheless, there is a justifiable concern about the safety of releasing CMV satRNA in large-scale agriculture for experimental evidence was obtained that deleterious satellite molecules can arise from benign ones by a single point mutation (Tepfer, 1993; Palukaitis and Roossinck, 1996). However, the possible ecological success of such mutants cannot be predicted and also whether the mutation is indeed likely to occur in nature and to affect the phenotype of individuals within a viral population is unknown.

Recurrent epidemics of CMV in Italy have offered the opportunity to analyze populations of CMV satRNA in a "natural laboratory." Here we show that evolutionary dynamics of a number of CMV satRNA variants can be studied using the stationary Markov model (Lanave *et al.,* 1984; Saccone *et al.,* 1990), a stochastic model for

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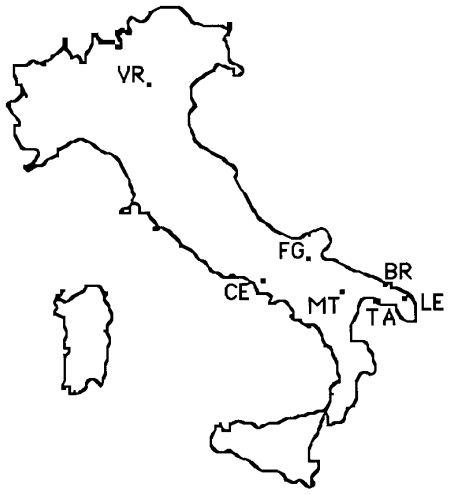

FIG. 1. Geographical map of Italy. Acronyms indicate the Italian provinces where the samples were collected. BR, Brindisi (Apulia); CE, Caserta (Campania); FG, Foggia (Apulia); LE, Lecce (Apulia); MT, Matera (Basilicata); TA, Taranto (Apulia); VR, Verona (Veneto).

evolution that gives a good correlation between phylogenetic relationships and biological activity.

MATERIALS AND METHODS

Collection and selection of field samples

Samples were collected from plant species showing disease symptoms in cropped areas of the Italian regions of Apulia, Basilicata, Calabria, Campania (southern Italy), and Veneto (northern Italy) (Fig. 1). Routinely, a crude tissue extract from these samples was viewed with the electron microscope and/or subjected to molecular hybridization (Crescenzi *et al.,* 1993b) with riboprobes to the following viruses: CMV, CMV-satRNA, potato Y potyvirus (PVY), alfalfa mosaic alfamovirus (AMV), and tomato spotted wilt tospovirus (TSWV). Samples naturally infected by CMV and CMV-satRNA were selected for this study and, unless otherwise stated, used for virus purification and to prepare total nucleic acid (TNA) extracts (see below). Fifteen of the 22 satRNA variants considered for this study were amplified by RT–PCR in TNA preparations extracted from naturally infected plants. In the four cases from *Nicotiana* hosts (samples 71, 80, 77, and 79 in Table 1), the poor condition of the sample required a single passage in tomato that was chosen as the experimental host. Since tobacco is regarded as a permissive host in supporting the replication of CMV satRNA to a high level (see for review Palukaitis *et al.,* 1992), these four isolates were purposely inoculated in tomato and not in tobacco to reduce the possibility for emergence of subliminal satRNA variants that could have been present in naturally infected *Nicotiana* plants. "Rutgers" tomato seedlings (grown under glasshouse at 22–24°) were mechanically inoculated at the cotiledon stage with plant sap extracted from naturally infected tobacco (isolates 77, 79, and 80) and *Nicotiana glauca* (isolate 71) tissues in 100 m*M* phosphate (Na_2-K) buffer, pH 7.2, and used as source for TNA extraction and virus purification.

The data of the previously characterized strains PG, TTS, and Tfn of CMV were also used. CMV-PG is a strain of CMV subgroup II isolated in Basilicata from tomato plants affected by lethal necrosis (Gallitelli *et al.,* 1988). The virus supports replication of a 334-nt satRNA (PG-satRNA) that codetermines a necrogenic phenotype in tomato (Kaper *et al.,* 1990). CMV-TTS was isolated in Apulia from severely stunted tomato plants (Grieco *et al.,* 1992); it belongs to CMV subgroup I and supports replication of a 339-nt satRNA (TTS-satRNA) that codetermines a stunting phenotype in tomato (Cillo *et al.,* 1994). CMV-Tfn was recovered in a commercial tomato field in Campania from plants with asymptomatic leaves but with fruits showing internal necrosis (Crescenzi *et al.,* 1993b). The virus belongs to CMV subgroup I and carries a 390-nt satRNA (Tfn-satRNA) that codetermines a transitory stunting in tomato seedlings inoculated at the cotiledon stage (Crescenzi *et al.,* 1992, 1993a). This biological behavior seems to map specifically on RNAs 1 and 2 of CMV-Tfn (Cillo *et al.,* 1994) since with heterologous strains of CMV, the Tfn-satRNA attenuates symptoms elicited in tomato (Crescenzi *et al.,* 1993a; F. Cillo and D. Gallitelli, unpublished results). The nucleotide sequence of PG, TTS, and Tfn satRNA variants was determined on preparations purified from naturally infected plants (Kaper *et al.,* 1990; Crescenzi *et al.,* 1992; Grieco *et al.,* 1992). Since their isolation, these viruses had been cultured only in Rutgers tomato seedlings grown under glasshouse.

Molecular and biological characterization of CMV isolates

TNA preparations were obtained from approximately 100 mg of naturally infected plant tissue according to White and Kaper (1989) and suspended in 50 μl nuclease-free water.

Naturally infected plant tissues used for TNA preparations were also used for CMV isolates purification according to Lot *et al.* (1972). The viruses were assigned to CMV subgroup I or II by molecular hybridization with subgroup-specific riboprobes (Crescenzi *et al.,* 1993b). The biological activity of each isolate was determined by inoculation onto 12 Rutgers tomato seedlings using 50 ng/ml purified virus suspended in 30 m*M* Na_2HPO_4. The plants were kept in a growth chamber (Heraeus HPS 500) at 25° and 70% RU under fluoresent lighting (ca. 20,000 lux), with a 16-hr photoperiod. Symptoms were recorded 12 days after inoculation.

Primer selection

Alignment of the CMV satRNA sequences available in the EMBL Database (release 84) with the PILEUP pro-

TABLE 1

Characteristics of the CMV satRNA Isolated in Italy during Virus Epidemics from 1988 to 1993

Isolate name (subgroup)	Natural infection				Experimental infection: Phenotype observed in tomato (Rutgers)	
	Year	Place	Host	Symptoms	+satRNA	−satRNA
TTS (I)	1989	BR	Tomato	ss	st	fi
Tfn (I)	1989	CE	Tomato	ifn	sy	fi
57 (II)	1993	CE	Tomato	ln	ln	fi
59 (I)	1993	CE	Pepper	ss, mo	ln	fi
8 (I + II)	1993	FG	Tomato	ln	ln	nt
12 (I)	1993	FG	Tomato	ln	ln	fi
18 (II)	1993	FG	Tomato	ln	ln	nt
27 (I)	1993	FG	Tomato	ln	ln	nt
71 (I)	1993	LE	*Nicotiana glauca*	mo	fi	fi
PG (II)	1988	MT	Tomato	ln	ln	fi
Ce (I)	1990	MT	Celery	bs, nlv, mo	ln	fi
8a (II)	1990	MT	Tomato	fi	fi	fi
16 (II)	1990	MT	Tomato	ln	ln	nt
22 (II)	1990	MT	Tomato	fi	fi	fi
1 (II)	1990	TA	Tomato	fi	fi	nt
2 (II)	1990	TA	Tomato	ln	ln	nt
4 (II)	1990	TA	Tomato	fi	fi	nt
6 (II)	1990	TA	Tomato	ln	ln	nt
7 (II)	1990	TA	Tomato	ln	ln	nt
77 (I)	1993	VR	Tobacco	syc, mo	ln	fi
79 (I)	1993	VR	Tobacco	syc, mo	ln	fi
80 (I)	1993	VR	Tobacco	syc, mo	ln	fi

Note. In bold, satRNA variants lacking the "necrogenic consensus" sequence (GA-GCUAAGGCUUA . . . UGCUAUGCUGAU; Devic *et al.,* 1990). bs, bushy stunt; fi, filimorphism; ifn, internal fruit necrosis; ln, lethal necrosis; mo, mosaic; nlv, necrosis of leaf veins; nt, not tested; st, stunting; ss, severe stunting; sy, symptomless; syc, severe yellow chlorosis.

gram (Anonymous, 1994) was used to design two degenerate deoxynucleotide primers, which should amplify all known CMV satRNA sequences. The antisense primer A was complementary to the last 18 nucleotides (5′-AAGGATCCGGGTCCTG(C,G,T)(A,G,T)(A,G,T)(A,G,T)GGAATG-3′) and the positive sense primer B was homologous to the first 28 nucleotides (5′-AAGGATCCGTTTTGTTTG(A,T)T(A,G)GAGAATTGCG(C,T)(A,G)GAG-3′) of CMV satRNA sequence (foreign sequences containing a *Bam*HI restriction site are underlined).

Reverse transcriptase–polymerase chain reaction (RT–PCR)

Three microliters of each TNA preparation (see above) was mixed with 50 pmol primer A and reverse-transcribed (Barbarossa *et al.,* 1993). Two microliters of this mixture was subjected to PCR amplification with 25 pmol primer A, 50 pmol primer B, and 5 units *Taq* polymerase (Promega Corp., U.S.A.) as recommended by the manufacturer. Complementary DNAs were amplified in a Perkin Elmer Cetus thermal cycler apparatus with 2 cycles at 94° for 1 min, 42° for 1 min, 72° for 1 min, followed by 35 cycles at 94° for 1 min, 55° for 1 min, 72° for 1 min. In the last cycle, time extension at 72° was 10 min.

cDNA cloning and sequencing

Amplified products were analyzed by electrophoresis through 1.2% agarose gel and visualized by staining with ethidium bromide. The bands corresponding to the expected products were excised and DNA was extracted using the QIAEX gel extraction kit (QIAGEN Inc., U.S.A.) following the manufacturer's protocol. The purified fragments were digested with the restriction enzyme *Bam*HI, ligated into *Bam*HI-cut, dephosphorylated pUC18 plasmid, and cloned in *Escherichia coli* strain DH5α as described by Grieco *et al.* (1992). The recombinant clones were identified by colony hybridization using a labelled cDNA probe (Sambrook *et al.,* 1989). Plasmid DNA was prepared and sequenced as previously described (Grieco *et al.,* 1989).

Phylogenetic analyses

The sequences obtained were first analyzed with the DNA Strider Program (Marck, 1988). The PILEUP and LINEUP programs (Anonymous, 1994) were used for alignment of the satellite nucleotide sequences. Multiple data sets generated by SEQBOOT were used to construct a phylogenetic tree by NEIGHBOR (UPGMA and Neigh-

bor-Joining methods) and CONSENSE programs in the PHYLIP package (Felsenstein, 1989) from distances computed by MARKOV (Saccone *et al.,* 1990). Parsimony trees were obtained with DNAPARS program using as input a multiple data set generated by SEQBOOT (Felsenstein, 1989).

RESULTS

CMV isolates and their associated satRNA variants

During CMV epidemics from 1988 to 1993, 126 CMV isolates were recovered in Italy from tomato and other plant species and characterized to various extents. A CMV-satRNA variant was associated with 30 of these isolates. Results from dot blot analysis and virus purification allowed the detection of a satRNA in 24 of the 30 isolates in naturally infected plants, whereas in the remaining six cases, a satRNA molecule emerged after a single passage onto "Xanthi" tobacco plants. These six satRNA variants were not considered for this analysis. Twenty-two of the other satRNA isolates were characterized in this study. Three of them (PG, TTS, and Tfn) were described previously as causing lethal necrosis, stunting, and fruit necrosis in tomato, respectively (Gallitelli *et al.,* 1988; Kaper *et al.,* 1990; Grieco *et al.,* 1992).

Dot-blot analysis with subgroup-specific riboprobes on RNA extracted from purified virions showed that 11 satRNA variants (Table 1) were associated with subgroup II CMV strains, 10 with subgroup I and 1 with mixed infection of strains belonging to both subgroups.

Primary nucleotide sequence determined on 22 satRNA molecules showed that 21 of 22 molecules were in the small length class (334–340 nt). The exception was the satRNA associated with CMV-Tfn that had a larger size (390 nt) (Crescenzi *et al.,* 1992; Cillo *et al.,* 1994). Fifteen of the 22 molecules showed the sequence block, defined as "necrogenic consensus" (*sensu* Devic *et al.,* 1990), usually associated with satRNA variants that are able to codetermine a necrogenic phenotype in tomato.

The plants from which the satRNA variants used in this study were obtained and sequenced showed the symptoms reported in Table 1. For 18 of these source plants, the CMV and CMV satRNA was purified from the naturally infected young leaf tissue and mechanically inoculated onto Rutgers tomato seedlings to determine the experimental phenotype, also shown in Table 1. Due to the poor condition of the four isolates from *Nicotiana* species, they were first amplified by a single passage in Rutgers tomato, for reasons described under Materials and Methods, and then inoculated onto tomato to determine the experimental phenotype. In these experiments 15 of the 22 isolates induced lethal necrosis in tomato, 5 induced filimorphism, 1 induced stunting, and 1 was symptomless. Many of these phenotypes were likely codetermined by the satRNA variant naturally present in the inoculum. This was experimentally proven for isolates PG, Tfn, and TTS (Kaper *et al.,* 1990; Crescenzi *et al.,* 1993a; Cillo *et al.,* 1994), and for isolates 12, 77, 79, 80, 57, 59, and Ce, that were deprived of the natural satRNA by fractionation through sucrose density gradient, and inoculated onto *Chenopodium quinoa* (Smith *et al.,* 1992). Local lesions induced on this host were excised, pooled together and inoculated onto tomato seedlings. In plants held at 25°, the isolates supporting a satRNA variant with necrogenic consensus induced lethal necrosis, i.e., a sequential necrosis of leaf veins, petioles, and stem that developed very early to reach the complete death of the plant within 12 days after inoculation. The presence/absence of a satRNA variant in the inoculum appeared unable to alter the phenotype elicited by isolates 71, 22, and 8a, a characteristic that remained unaltered also after seven sequential passages in tomato. If one considers only the variants isolated from tomato, a good correlation was observed between the natural and experimentally induced phenotype in this host.

Phylogenetic analysis of CMV satRNA variants with stochastic methods

The consensus tree (Fig. 2a) obtained with the UPGMA method (Sneath and Sokal, 1973) on the basis of distances calculated with the stationary Markov model showed that two primary branches depart from the bulk of all satRNA sequences, one leading to Tfn satRNA and the other to all 21 remaining satRNA sequences. On the whole, the 21 satRNA molecules clustered in three distinct groups (A, B, and B_1, in Fig. 2a), as indicated by the very significant values of bootstrap replicates placed at major nodes. Belonging in a group of any of the satRNAs was apparently not determined by the year, the place, or the host from which they were recovered. Rather, grouping correlated with the phenotype induced in tomato (i.e., necrogenic or not).

Group A comprised satRNA variants 22, TTS, 1, 4, and 8a isolated from tomato and variant 71 isolated from *N. glauca.* The values of bootstrap replicates at each node within group A ranged from 15 to 53, indicating poor differentiation within the group. From the Markov model the average value of the mean constant rate of substitutions/site ($K_{subs/site}$) was estimated for group A to be 0.060 $\pm$ 0.036 subs/site. None of these variants had the "necrogenic consensus" (*sensu* Devic *et al.,* 1990) and, except for TTS, they did not modulate the phenotype induced in tomato by the natural helper virus (Table 1). Devic *et al.* (1990) proposed the division of the nonnecrogenic CMV-satRNAs in two groups ("benign A" and "benign B") according to the differences from the necrogenic consensus of the nucleotide sequence between the *Asu*II site and the 3′ end. On the basis of this classification all these variants, which contain the *Asu*II site approximately at position 227, would be included in the benign B group.

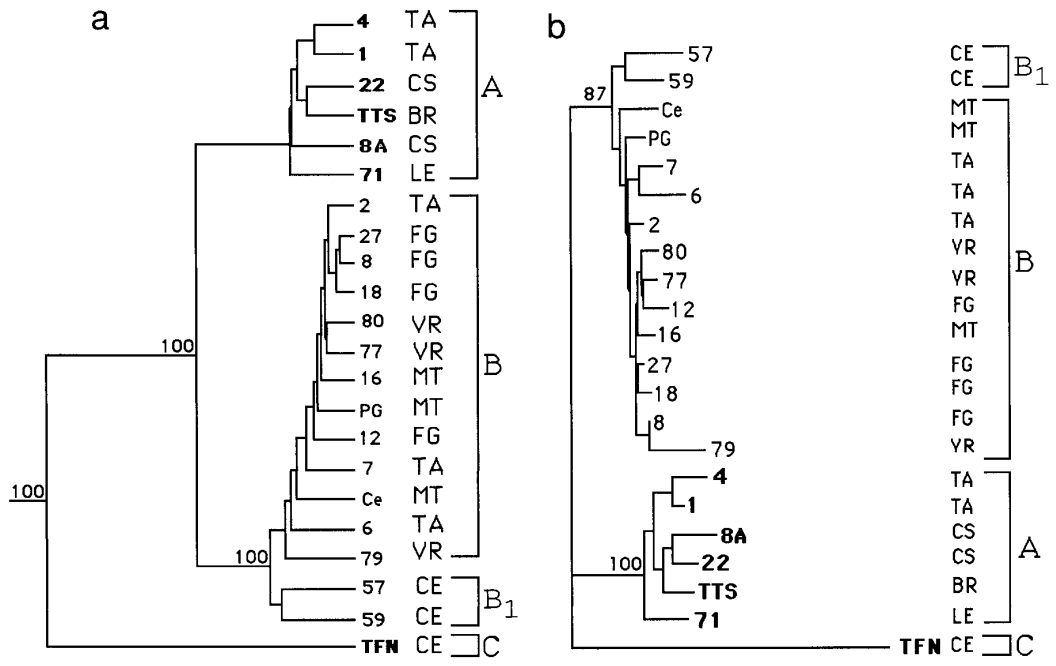

FIG. 2. Phylogenetic trees of the Italian satRNA variants. Distance matrix was calculated with MARKOV program and the trees were reconstructed with UPGMA (a) and Neighbor-Joining (b) methods. In bold, nonnecrogenic satRNA variants. The number above major nodes indicates the percentage of bootstrap replicates in which that node was recovered. Database accession numbers of sequence data: 4, X86415; 1, X86409; 22, X86412; TTS, X69136; 8A, X86408; 71, X86424; 2, X86410; 27, X86419; 8, X86420; 18, X86708; 80, X86421; 77, X86422; 16, X86413; PG, X86426; 12, X86416; 7, X86411; CE, X86425; 6, X86414; 79, X86417; 57, X86418; 59, X86423; TFN, X65455. Acronyms BR, CE, FG, LE, MT, TA, VR indicate Italian provinces as described in the legend to Fig. 1. A, B, B_1, and C show satRNA variant clusters (see text).

As already reported, TTS satRNA does not contain the necrogenic consensus (Grieco *et al.,* 1992) but it codetermines a stunting condition of tomato, a trait that is apparently regulated by a sequence domain located in the 5′ half of the molecule and by functions coded by RNAs 1 and 2 of subgroup I CMV strains (Cillo *et al.,* 1994).

Group B of Fig. 2a included satRNA variants 12, 77, 80, 27, 8, 18, 2, PG, 16, 7, Ce, 6, and 79 isolated from different places and from different hosts (Table 1). Values of bootstrap replicates ranged from 12 to 39 at nodes within group B, suggesting very similar, if not identical, evolutionary histories. All group B variants contained the necrogenic consensus and indeed, all tomato seedlings mechanically inoculated with CMV isolates supporting these molecules developed necrosis of leaves, petioles, and stem. The average value of $K_{subs/site}$ was 0.037 $\pm$ 0.020 subs/site.

Group B_1 comprised satRNA variants 57 and 59. Both of these variants were isolated from the same field but from different hosts and were supported by CMV isolates belonging to different subgroups. Both satRNAs codetermined a necrogenic phenotype in tomato. Because of this and the position within the tree, this group was considered a subbranching of group B. However, the average value of the $K_{subs/site}$ was higher (0.075 $\pm$ 0.045).

Finally, the evolutionary dynamic of the nonnecrogenic Tfn-satRNA variant was far apart from that of all other satRNA molecules. The average value of $K_{subs/site}$ calculated comparing Tfn-satRNA against each of other variants was 0.325 $\pm$ 0.126 and 0.292 $\pm$ 0.115 with group A and group B + B_1 variants, respectively. Similar phylogenetic results (Fig. 2b) were obtained also by the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). Values of bootstrap replicates ranged between 3 and 44 within group B and between 100 and 42 within group A.

Phylogenetic analysis of CMV satRNA variants with a deterministic method

Phylogenetic relationships among the CMV satRNA variants were inferred also by the Parsimony method. Using 0Y2 satRNA variant as outgroup (Fig. 3) two primary branches depart from the outgroup sequence, one leading to Tfn satRNA and the other to all 21 remaining satRNA sequences. Similarly to the trees obtained with UPGMA and NJ methods, these 21 satRNA molecules clustered in three distinct groups (A, B, and D in Fig. 3) and their position in the group correlated with the phenotype (necrogenic or not) induced in tomato. Comparing this tree with those of Figs. 2a and 2b, two main differences can be observed: (i) the lack of differentiation of satRNA variants 57 and 59 from the bulk of group B variants and (ii) differentiation of the Ce satRNA variant.

Evidence for the maintenance of genetic heterogeneity of CMV satRNA variants isolated in Italy over a period of 5 years

Due to recurrence of massive CMV outbreaks since 1988 in the bordering regions of Apulia and Basilicata

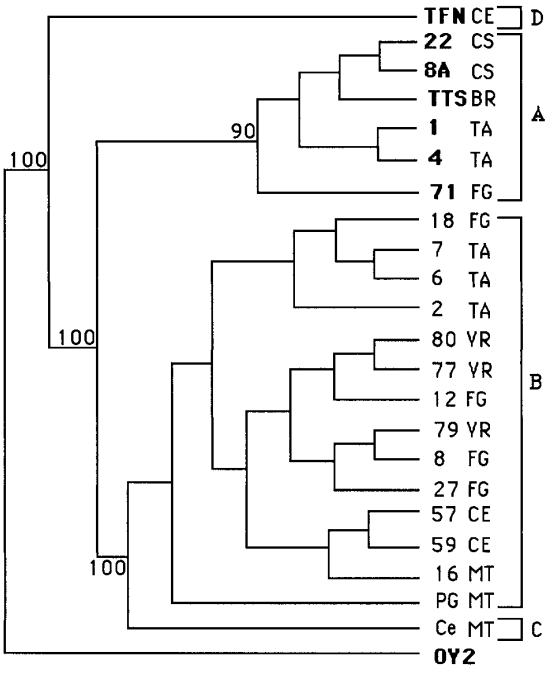

FIG. 3. Phylogenetic tree of the Italian satRNA variants. The tree was obtained with DNAPARS program using OY2 satRNA variant as outgroup. In bold, nonnecrogenic satRNA variants. The number above nodes indicates the percentage of bootstrap replicates in which that node was recovered. Database accession numbers of sequences are as described in the legend to Fig. 2. Database accession number of outgroup sequence data: OY2, M20845. Acronyms BR, CE, FG, LE, MT, TA, and VR indicate Italian provinces as described in the legend to Fig. 1. A, B, C, and D show satRNA variant clusters (see text).

(southern Italy) many tomato cropped areas were abandoned and new crops were established each year with a constant shift toward the northern part of Apulia. Therefore we were unable to estimate the evolution of satRNA variants from the same field. Nevertheless, Table 2 shows that some indications can be obtained considering only satRNA variants isolated in Apulia and Basilicata. For example, comparing PG (the first necrogenic variant characterized in 1988 in Italy) with the necrogenic variants isolated in 1990 and 1993, we obtained average values of $K_{subs/site}$ that are very similar. Molecules isolated in 1990 ($K_{subs/site} = 0.040$) and 1993 ($K_{subs/site} = 0.038$) had values that were also very similar to that ($K_{subs/site} = 0.046$) obtained with variants 77, 79, and 80 isolated from tobacco in the region Veneto (northern Italy) in 1993. This was also true comparing the value ($K_{subs/site} = 0.044$) obtained by intrapopulation analysis of necrogenic variants isolated in 1990 ($K_{subs/site} = 0.036$) with that computed by interpopulation analysis between variants of 1990 and 1993. The intrapopulation value of 1993 variants was lower ($K_{subs/site} = 0.020$) probably because it was obtained from variants isolated from a more homogeneous area. The tendency to maintain constant genetic heterogeneity within a population of satRNA variants from one year to the other can be also observed by comparing nonnecrogenic variants of 1990 with PG ($K_{subs/site} = 0.148$) and the other necrogenic variants isolated in 1990 ($K_{subs/site} = 0.149$) and 1993 ($K_{subs/site} = 0.143$). The intrapopulation value ($K_{subs/site} = 0.058$) of nonnecrogenic variants isolated in 1990 was also in the same range of those of necrogenic variants.

Computer analysis of CMV-satRNA variants gathered from the literature

To investigate whether the stationary Markov model could apply also to satRNA variants isolated and characterized in other laboratories, the published sequences of satRNAs $I_{17}N$, R, D, Y, K8, WL1, WL2, B1, B2, S, G, Ra, T43, 0Y2, J876, and D27 were aligned and subjected to the analysis. Figure 4a shows a consensus tree constructed with the UPGMA method on the basis of distance matrices calculated on 16 known satRNA variants having different biological characteristics. Although all the molecules analyzed obeyed the stationary condition, most of them had evolutionary histories that were more distant from each other than those of Italian isolates. Moreover, in this case the molecules showed only a weak tendency to group according to the biological activity. Two main branches can be observed in the tree, one leading to the ancestor of "large-size" molecules (0Y2, T43, D27, and J876) (average value of $K_{subs/site} = 0.090 \pm 0.044$) and the other to the ancestor of Y satRNA and of all remaining 11 variants. The average values of $K_{subs/site}$ for satRNA variants possessing or not the necrogenic consensus were 0.017 ± 0.011 and 0.075 ± 0.038, respectively. The evolutionary dynamic of Y satRNA separated it from all the other molecules possessing the necrogenic consensus. On the other hand, R satRNA that shows a biological activity of the ameliorative type — R satRNA is classified as benign A (*sensu:* Devic *et al.,* 1992) — appeared closer to D and $I_{17}N$ ($K_{subs/site} = 0.025$ and 0.012, respectively), which are necrogenic in tomato, than to WL1 ($K_{subs/site} = 0.158$) another benign A variant. Similar results were obtained contructing the tree with the NJ method (Fig. 4b). On the whole, the topology of

TABLE 2

Average Values of $K_{subs/site}$ between and within Some Selected Populations of CMV satRNA Variants

	Nonnecrogenic 1990[a]	Necrogenic 1990[b]	Necrogenic 1993[c]
Nonnecrogenic 1990[a]	0.058 ± 0.032		
Necrogenic 1990[b]	0.149 ± 0.070	0.044 ± 0.030	
Necrogenic 1993[c]	0.143 ± 0.069	0.036 ± 0.023	0.020 ± 0.012
PG	0.148 ± 0.071	0.040 ± 0.024	0.038 ± 0.021

[a] Variants 22, 1, 4, 8a.
[b] Variants 2, 16, 7, Ce, 6.
[c] Variants 12, 27, 8, 18.

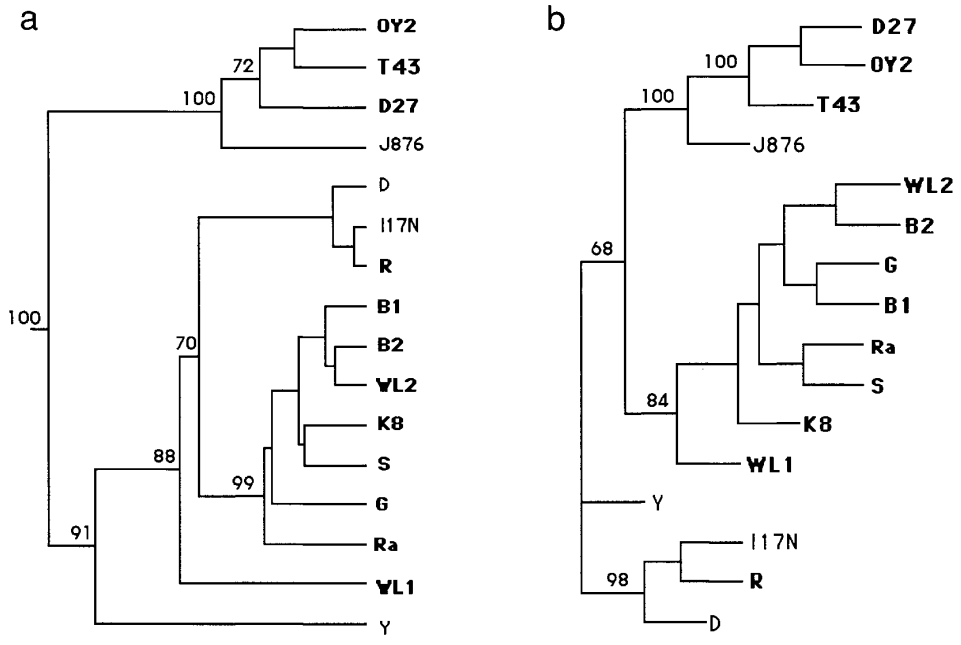

FIG. 4. Phylogenetic trees of satRNA variants gathered from literature. Distance matrix was calculated with the MARKOV program and the trees were recostructed with the UPGMA (a) and Neighbor-Joining (b) methods. In bold, nonnecrogenic satRNA variants. The number above nodes indicates the percentage of bootstrap replicates in which that node was recovered. Database accession numbers of sequence data: OY2, M20845; T43, D10039; D27, U31661; J876, U31660, D, M18772; I17N, M18867; R, M18871; B1, M16586; B2, M16587; WL2, M16590; K8, X53534; S, M14934; Ra, D00541; G, M16585; WL1, M16589; Y, M21745.

these trees is similar to that shown by Fraile and Garcia-Arenal (1991).

DISCUSSION

The "evolutionary dynamics" that governed CMV satRNA variants isolated in Italy during viral epidemics have been studied using the stationary Markov Model, a stochastic model of gene evolution.

According to this model, only the homologous set of sequences that fulfill the "stationary condition" can be used to determine evolutionary dynamics of a number of aligned sequences. Evolutionary dynamic is a process of evolution of a group of nucleotide sites that are probably subjected to similar physicobiological constraints and therefore to a similar evolutionary mechanism (Saccone *et al.,* 1990, 1993; Preparata and Saccone, 1991).

The stationary condition is fulfilled when the base frequencies in equivalent sites of both the extant molecules and their ancestors are constant. This condition can be achieved when the set of nucleotides considered is presumably subjected to the same environmental conditions and biological constraints. The important novelty introduced by the stationary Markov model is the absence of an oversimplified "special" assumption about the nucleotide substitution probabilities along sequences of homologous molecules (rate matrix). Indeed some of the stochastic methods routinely used to infer phylogenetic relationships from molecular data have their theoretical basis in the formula by Jukes and Cantor (1969) which uses the *a priori* assumption that each nucleotide has the same probability of being replaced by any of the other three, in all the molecules considered.

In the case of CMV satRNA we were in possession of the natural nucleotide sequences of several wild type variants isolated in Italy over a period of 6 years and therefore the method was fruitfully applied. Our data show that the Markov model establishes a clear relationship between position in the resulting phylogenetic tree and biological properties of the molecule. In order to make data comparable, we chose Rutgers tomato as the reference host to determine the biological properties of all the set of satRNA variants considered for this study. In some instances, this host was necessarily different from the natural one. However, at least in the cases in which the host shift was not necessary, symptoms from experimental data were in perfect agreement with those observed in natural infection.

Comparable but not unequivocal results were achieved with the Parsimony method because of its variation with the choice of a satRNA molecule to serve as the outgroup for the construction of the most parsimonious tree. This is a criterion that seems difficult to establish *a priori.* In considering this, the stochastic method used by the Markov model seems more free from such decisions that could condition the output of the results and probably applies particularly well to sets of molecules that, like those analyzed in this study, share a similar geographical origin.

In fact, the Markov model did not apply equally well to the analysis of more diverse satRNA variants of known sequence. This might be because these satRNA variants have very different geographical origins, different years of isolation, and their primary nucleotide sequences were probably determined after repeated subculturing in different hosts and under greenhouse conditions, two types of selection pressure that may have contributed to cause the loss of their original identity.

The phylogenetic trees reported in Figs. 2a and 2b show that satRNA variants isolated in Italy evolved along three different lineages. The bootstrap values of different branches among molecules within the same lineage and with similar biological activity are relatively low, indicating poor differentiation. This may reflect the fact that molecules of the same lineage have limited possibilities to accumulate substitutions, and this was also true for the larger Tfn-satRNA. Since the Markov model infers phylogenetic relationships by comparing only the sites that are in common for all the sequences and fulfill the stationary condition, it turns out that regions of the molecules with the possibility of accumulating substitutions are very likely the same in all the three lineages. In considering this, our data are in agreement with the proposed maintenance of a functional molecular structure as a constraint to CMV satRNA evolution (Fraile and Garcia-Arenal,1991; Tousignant and Kaper, 1993).

In conclusion, our data suggest that under epidemic conditions that have been in progression in Italy since 1987, the extant population of CMV satRNA variants had a propensity to maintain its original genetic heterogeneity from one year to the other. These results could find an explanation in the data of a previous field survey carried out in Basilicata and Campania (southern Italy) (Crescenzi *et al.,* 1993b). Very likely, most of the extant CMV populations and their satRNA complete the life cycle in cultivated crops and only in certain weed species.

Our data are also in partial agreement with those reported by Aranda *et al.* (1993) on the genetic structure and evolution of CMV satRNA during epidemics on tomatoes in eastern Spain. However, Aranda *et al.* (1993) concluded that due to quick evolution of CMV satRNA under field conditions extreme care should be taken in the use of molecules of a benign type as biological control agents. Although in principle we agree with this cautionary concept, it also should be taken into account that risks pointed out for a given satRNA variant may not be applicable to others and that such quick evolution of CMV satRNA under field conditions does not emerge from the results of this study. This consideration could contribute to alleviate the concern about the use of benign forms of CMV satRNA variants as biological control agents.

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