Genetic Structure of the Population of *Pepino mosaic virus* Infecting Tomato Crops in Spain

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ABSTRACT

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The population structure of *Pepino mosaic virus* (PepMV), which has caused severe epidemics in tomato in Spain since 2000, was analyzed. Isolates were characterized by the nucleotide sequence of the triple gene block and coat protein gene and, for a subset of isolates, a part of the RNA-dependent RNA polymerase gene. The full-length sequence of the genomic RNA of a *Solanum muricatum* isolate from Peru also was determined. In spite of high symptom diversity, the Spanish population of PepMV mostly comprised highly similar isolates belonging to the strain

reported in Europe (European tomato strain), which has been the most prevalent genotype in Spain. The Spanish PepMV population was not structured spatially or temporally. Also, isolates highly similar to those from nontomato hosts from Peru (Peruvian strain) or to isolate US2 from the United States (US2 strain) were detected at lower frequency relative to the European strain. These two strains were detected in peninsular Spain only in 2004, but the Peruvian strain has been detected in the Canary Islands since 2000. These results suggest that PepMV was introduced into Spain more than once. Isolates from the Peruvian and US2 strains always were found in mixed infections with the European tomato strain, and interstrain recombinants were detected. The presence of different strains of the virus, and of recombinant isolates, should be considered for the development of control strategies based on genetic resistance.

A new viral disease of tomato plants, characterized by a yellow mosaic of the leaves, was described in The Netherlands in 1999. Shortly after, it was shown that the disease was caused by *Pepino mosaic virus* (PepMV, genus *Potexvirus*) (36). Tomato infection by PepMV soon was reported from other countries in Europe (3, 18,26,30,39) and North America (8). In Spain, the virus was first identified in 2000 (13), when the initial outbreak occurred in the geographically isolated tomato-growing areas of the province of Murcia (southeastern [SE] Spain), where tomato is grown throughout the year in open fields, greenhouses, or screen houses. In SE Spain, PepMV-infected tomato plants showed symptoms only from autumn through spring. Symptoms disappeared with warm temperatures in late spring, suggesting that symptom expression is highly dependent on temperature or light conditions, which was confirmed experimentally (*unpublished data*). PepMV infection of tomato plants in SE Spain was associated with a wide variety of leaf symptoms of different severity, including mild and severe mosaics, yellow mosaic and spots on the leaves, and leaf lamina distortion. Fruit showed mosaics, particularly when ripe, and fruit symptoms occurred with or without leaf symptoms. Infected plants often senesced prematurely and stem pith tissues were disorganized. PepMV infection also was found in asymptomatic tomato plants.

The initial localization of PepMV suggested a single introduction in Murcia. In recent years, PepMV has caused severe epidemics in tomato crops in SE Spain and in all tomato-growing

areas in the eastern coastal plains of Spain along the Mediterranean Sea. By 2000, PepMV also was detected in the Canary Islands, with infected tomato plants showing symptoms similar to those in peninsular Spain. Geographic isolation of the Canary Islands suggested a separate introduction.

PepMV infection in tomato had not been reported prior to the recent epidemic in Europe. PepMV was described in 1974 infecting pepino in Peru (12). Pepino (*Solanum muricatum* Ait.) is a traditional Andean crop grown for its fruits which are consumed as fresh fruit or in salads. Only recently has pepino been introduced as a crop in Europe, Australia, and the United States. Pepino was the only known natural host of PepMV, which induced a characteristic yellow mosaic and distortion of the leaves. Experimentally, tomato was an asymptomatic host of the virus (12). Previously, only the amino acid sequence for the coat protein and a partial nucleotide sequence for the RNA-dependent RNA polymerase gene have been reported for the original pepino isolates of PepMV (26,37). More recently, PepMV was isolated in Peru from different wild species of the genus *Lycopersicon* (34). A Peruvian isolate from *Lycopersicon peruvianum* Mill., which infected tomato asymptotically, was characterized and the complete nucleotide sequence determined (19). Nucleotide sequences reported for PepMV isolates from tomato crops in various European countries were ~99% similar (1,3,26,37), and are clearly different (~96% identity) from the Peruvian *L. peruvianum* isolate. More recently, distinct nucleotide sequences were reported for PepMV isolated from diseased tomato plants in North America (21). Hence, different strains of PepMV exist, possibly associated with specific hosts or geographic origins.

RNA viruses have a high potential for genetic variation, due to high mutation and recombination rates, which has been suggested to increase the ability to adapt to new environments (4,5). How-

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ever, high genetic conservation is the rule for natural populations of most plant viruses (9), even when confronted with strong selection pressures, such as those imposed by widespread deployment of resistant cultivars (10). High genetic conservation of RNA plant virus populations could be explained by strong functional constraints on multifunctional proteins encoded by viral genomes and by a high frequency of deleterious or lethal mutations, among other reasons (20,32).

The ongoing epidemic of PepMV in Spain presented the opportunity to analyze genetic variability and population structure of a virus during demographic expansion following recent introduction to a new niche (i.e., domestic tomato crops). Our results show that European PepMV isolates pathogenic for tomato belong to a single clade sharing a common ancestor with the Peruvian isolates that are asymptomatic in this host plant. In spite of symptom diversity, the epidemic of PepMV in SE Spain was largely due to the demographic expansion of a single genetic type. The presence of other genotypes did not correlate with phenotype. Furthermore, it appears that recombination among different strains is contributing to the generation of increased genetic variability.

MATERIALS AND METHODS

Virus isolates, bioassays, and preparations. We define here a virus isolate as the virus preparation obtained from one field-infected plant. Field surveys were conducted in the tomato-growing areas affected by the PepMV epidemic since it was first declared in 2000 through February 2004. Tomato plants, symptomatic or asymptomatic, were sampled randomly in affected tomato fields in all provinces where PepMV outbreaks were reported. Sampling locations included open fields, greenhouses, and screen houses. PepMV infection was detected by enzyme-linked immunosorbent assay (ELISA) using a commercial antiserum (DSMZ As-0554; Biologische Bundesanstalt, Braunschweig, Germany). In addition, an isolate was obtained from freeze-dried material from an infected tomato plant sampled in Murcia during 1998. A Peruvian PepMV isolate from *Solanum muricatum* Ait., collected in 1974 and multiplied in *Nicotiana glutinosa* L., was generously provided by R. Mumford (Central Science Laboratory, York, UK).

To determine whether different symptoms shown by tomato plants in the field could be reproduced, isolates from tomato plants were sap inoculated onto three tomato plants each of cvs. Rutgers or Marmande Cuarenteno germinated from heat-treated seeds (24 h at 80°C, dry heat). The inoculated plants were maintained in a growth chamber (27 and 22°C, day and night, respectively; 14 h of light; and 64% relative humidity) in Valencia or in a greenhouse (25 and 15°C, 16 h of light) in Madrid. Symptoms were recorded over a 4-week period, and the presence of PepMV was verified by ELISA.

Virion or RNA preparations were obtained directly from field-infected material or from tomato plants used for symptom assays.

Virus particles were purified as described by Aguilar et al. (1). Virions were disrupted in 4% sodium dodecyl sulfate, 0.1 M Tris-HCl, pH 9.0, with bentonite at 1 mg/ml and virion RNA extracted with phenol-chloroform. Total RNA from infected plants was obtained using the RNeasy kit (AMBION; Austin, TX) according to the manufacturer's instructions.

Nucleotide sequence determination and analyses. Genetic characterization of PepMV isolates was done by determination of the nucleotide (nt) sequence for three genomic regions: a part of the RNA-dependent RNA polymerase (RdRp) gene, the triple-gene block (TGB), and the capsid protein (CP) gene. Virion RNA, or total RNA preparations from leaves, was reverse transcribed and polymerase chain reaction (PCR) amplified using the Superscript III One-Step reverse-transcription (RT)-PCR with Platinum Taq kit (Invitrogen, Carlsbad, CA) and appropriate primers. A 624-nt region in the RdRp gene (open reading frame [ORF]1) was amplified using primers Pep3 and Pep4; a 1,317-nt region encompassing the complete TGB was amplified using primers PepTGB-D and PepTGB-R; and an 845-nt region, including the complete CP gene, was amplified using primers PepCP-D and PepCP-R. PepTGB-D and PepTGB-R did not amplify the American-type isolates. Therefore, primers PepUSTGB-D1 and PepUSTGB-R were used to amplify the TGB region of these isolates. Primers PepRecB-D and PepRec-R were used to amplify a 1,028-nt region, including the 3' half of the TGB and the 5' half of the CP ORF. The sequences of these primers and their positions relative to PepMV Sp-13 isolate (accession no. AF484251) and US2 isolate (accession no. AY509927) are listed in Table 1.

Genomic RNA of the *S. muricatum* isolate was extracted from virions and used as template. TGB and CP genes were RT-PCR amplified using Superscript III One-Step RT-PCR with Platinum Taq kit (Invitrogen) with PepTGB-D and PepTGB-R (TGB), and PepCP-D and PepCP-R (CP). The RdRp gene was amplified using SMART PCR cDNA synthesis kit (Clontech, Mountain View, CA) with primer Pep4. RT-PCR-amplified fragments were gel purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA). Nucleotide sequences of the PCR products were determined using an ABI Prism 3100 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA). Sequences determined in this work can be found in the EMBL database under accession nos. AM042564 to AM042592, AM041929 to AM041992, AM040183 to AM040192, AM113787 to AM113850, AM042007 to AM042012, and AM109896.

Multiple sequence alignments were done using CLUSTAL X (35) with the default parameters. The alignments were used to calculate pairwise genetic distances by Kimura's two-parameter method (15). Phylogenetic analyses were done by the neighbor-joining (31) and parsimony (6) methods implemented with CLUSTAL X and DAMBE (40) and displayed by TreeView (29). Robustness of inferred evolutionary relationships was assessed by 1,000 bootstrap replicates. Pairwise synonymous and nonsynony-

TABLE 1. Primers used for reverse-transcription polymerase chain reaction amplification of different regions in the genome of *Pepino mosaic virus* (PepMV)

Primer name	Primer sequence 5' → 3'	Position
Pep3 ^a	ATG AGG TTG TCT GGT GAA	3,897–3,914
Pep4 ^a	AAT TCC GTG CAC AAC TAT	4,504–4,521
PepTGB-D ^a	GGA GAA GCA ATG AGA CTT	4,356–4,376
PepTGB-R ^a	GGT GCA CTT GAA GTG GCA G	5,655–5,673
PepCP-D ^{a,b}	CAC ACC AGA AGT GCT TAA AGC A	5,522–5,543
PepCP-R ^{a,b}	CTC TGA TTA AGT TTC GAG TG	6,348–6,367
PepUSTGB-D1 ^c	TCA CAA ACT CCA TCA AGG	4,335–4,356
PepUSTGB-R ^c	TTA GAA GCT GTA GGT TGG TTT T	5,632–5,653
PepRecB-D ^a	GAA CTA AAT GCC AGG TCT	5,110–5,127
PepRec-R ^a	GTG ACT CCA TCG AAG AAG T	6,120–6,138
RecRepl-R ^a	TGA GTT CTG AAG GAA GTG TA	4,774–4,793

^a Positions numbered as in the sequence of PepMV Sp-13 (accession no. AF484251).

^b Primers PepCP-D and PepCP-R are primers PepMV TGB F and PepMV UTR R, respectively, as described by Mumford and Metcalfe (28).

^c Positions numbered as in the sequence of PepMV US2 (accession no. AY509927).

mous substitutions were calculated according to the method of Nei and Gojobori (28). Within and between population diversities were estimated according to Nei. (27) using MEGA version 3.0 (17). Putative recombination events were identified by two complementary methods. The method described by Jacobsen and Eastel (11) is based on the analysis of phylogenetic incompatibility between pairs of informative positions within a set of aligned nucleotide sequences, scored against a randomized data set. The method described by Sawyer (33) analyzes the probability that sequence fragments between polymorphic sites come from the same parent sequence.

RESULTS

Diversity of the Spanish PepMV population. The nucleotide sequence for the RdRp (624 nt), TGB (1,317 nt), and CP (845 nt) genes was determined for a set of 28 isolates (sampled between 2000 and 2004) from all regions in Spain where PepMV has been reported, and for the 1998 Murcia isolate collected before the virus was first identified in Spain. These genomic regions were chosen to represent viral genes encoding proteins with distinct functions. The sequence of the TGB and CP genes also were determined for another 36 isolates from Murcia sampled between 2000 and 2004. In all, four epidemic episodes in Murcia (2000, 2001, 2003, and 2004) were represented by 5, 10, 15, and 19 isolates, respectively. Hence, two overlapping data sets were analyzed: one sampled the whole Spanish population of PepMV and another sampled successive epidemic outbreaks in the tomato-growing area where PepMV was first reported in Spain. It is

important to note that analyses of both sets were highly congruent (described below).

Analyses based on the RdRp, TGB, or CP genes yielded similar results. Most Spanish isolates of PepMV were highly similar and belonged to a single phylogenetic cluster (Fig. 1). Within this cluster, isolates did not group according to geographical origin in peninsular Spain or the Canary Islands, or to symptoms on field infected or experimentally infected plants (Fig. 1; data not shown). This cluster included the 1998 Murcia isolate and all Spanish isolates collected between 2000 and 2003 in peninsular Spain (Fig. 1; data not shown). This same cluster also included the PepMV isolates from tomato isolated in 2000–01 in other European countries (i.e., from the United Kingdom, accession no. AF340024, and France, accession no. AJ438767) (Fig. 1). We refer to this cluster as the European tomato strain of PepMV.

Genetic distance among isolates within the European tomato strain was unrelated to location or year of collection. For all three genomic regions some haplotypes occurred more than once, often in isolates collected in different years or from different geographic origins. For the CP gene, the two most frequent haplotypes accounted for 7 and 2 of the isolates collected between 2000 and 2004 out of 29 isolates analyzed (31%) (Fig. 1). Genetic diversity for the European tomato strain was very low, with a total within-population nucleotide diversity of 0.0076 ± 0.0018 , 0.0075 ± 0.0011 , and 0.0057 ± 0.0009 for the RdRp, TGB, and CP genes, respectively. Functional constraints on the evolution of the represented portion of the RdRp, the nonoverlapping portions of the TGB, and the entire CP, were analyzed by the ratio of genetic diversity at nonsynonymous and synonymous sites (Table 2).

Fig. 1. Neighbor-joining phylogenetic trees for three genomic regions of Spanish isolates of *Pepino mosaic virus* (PepMV): RNA-dependent RNA polymerase gene (RdRp), triple-gene block (TGB), and coat protein (CP) gene. Twenty-nine isolates representing all Spanish regions sampled were analyzed. Isolates are identified by a two-letter code indicative of geographic origin (Al: Alicante, Am: Almeria, Ba: Barcelona, Cl: Canary Islands, Mu: Murcia, Na: Navarra) followed by the year of collection and an ordinal. SM74; isolate from *Solanum muricatum*, 1974, sequenced in this work. Sequences for other PepMV isolates obtained from public data bases (shown in italics) were included. Tomato isolates were from France (Fr, AJ438767), United Kingdom (UK, AF340024), Spain (Sp-13, AF484251; LE.00, AJ606359; LE.02, AJ606360), and the United States (US1, AY509926; US2, AY509927), or from *Lycopersicon peruvianum* from Peru (LP.01, AJ606361). Similar results were obtained with maximum parsimony analyses.

RdRp was the most constrained protein, as shown by the d_{NS}/d_S ratios and by the significantly lower diversity at nonsynonymous sites compared with the two other genomic regions. The least constrained protein was the CP, due to a significantly lower diversity at synonymous sites than for the other two regions. In the absence of recombination, genetic drift should be the same for all genes and mutation rates may be assumed to be similar over stretches as long as those considered here; therefore, these results indicate a stronger selection at synonymous sites of the CP gene compared with synonymous sites elsewhere in the genome.

Within- and between-population diversity for the populations representing the epidemic episodes of 2000, 2001, and 2003 in Murcia had values that were similar to those found for the set of the European tomato strain isolates from all regions. For both genomic regions analyzed (TGB and the CP), diversity values were similar (Table 3). This indicates a single population, undifferentiated with respect to yearly epidemic outbreaks. However, the within-population diversity for the 2004 population was more than an order of magnitude (between 12 and 30 times) greater, which also was the case for diversity between the 2004 population and those of 2000, 2001, and 2003 (Table 3). These results indicate that new genotypes were present in the 2004 population. Phylogenetic analyses showed that, in the 2004 population from Murcia, two new genetic types of PepMV were present (Fig. 1). One type was represented by 8 of 19 isolates for the CP gene, which were broadly divergent from the European tomato strain (77% nucleotide sequence identity), and most similar (99% nucleotide sequence identity) to US2 (accession no. AY509927). We refer to this second genetic type as the US2 strain. The RdRp gene region determined for two isolates with a US2 strain-like CP differed broadly from RdRps of both the US2 strain (isolate Mu 04.15) (Fig. 1) and the European tomato strain (isolate Mu 04.17) (Fig. 1).

The third genotype of PepMV was represented by 2 of 19 isolates from Murcia collected in 2004. This novel genotype had TGB sequences that clustered with that reported for the *L. peruvianum* isolate (accession no. AJ606361). These sequences also clustered with the sequence of the *S. muricatum* isolate from Peru, which was nearly identical to that from *L. peruvianum* (Fig. 1). Although isolates of this type were found in Murcia only in 2004, they were in the Canary Islands since 2000 (e.g., isolate CI 00.1) (Fig. 1). Sequences from this type defined a third strain, here named the Peruvian strain. The European tomato and Peruvian strains formed a single clade with a high bootstrap support under all the analyses (Fig. 1; data not shown).

Because symptom induction by PepMV in tomato is highly dependent on environmental conditions, tomato plants showing PepMV-like symptoms, as well as asymptomatic plants, were screened for PepMV infection. PepMV was detected in a considerable fraction of asymptomatic plants (11 of 20 analyzed), but

detection in symptomatic plants was much higher (42 of 46 analyzed). There was a significant association ($P = 0.001$) between symptom expression and PepMV infection as detected by ELISA. Isolates from tomato plants exhibiting different symptoms in the field, including isolates from ELISA-positive asymptomatic plants, were assayed for symptom induction at two locations. In Valencia, assays were done in a growth chamber with temperature and light conditions similar to those in the production greenhouses of Murcia at the time of symptom expression (winter). In Madrid, with much colder winters, these conditions were met in an experimental greenhouse. In all, 45 isolates, 41 from peninsular Spain and 4 from the Canary Islands, were assayed. Field symptoms were reproduced for 24 of 34 isolates from plants showing the most frequent symptom of leaf lamina reduction, distortion, and mosaic; 2 of 4 isolates from plants showing yellow mosaic; and 1 of 4 isolates from plants showing leaf yellows. One of three isolates from asymptomatic plants were asymptomatic. Hence, the capacity to induce different symptoms seems to be a property of the isolate. This is indicative of biological diversity within the Spanish population of PepMV. Isolates of the European tomato strain were found to be asymptomatic or to induce leaf lamina reduction, distortion, and mosaic, as well as all the other different symptoms (data not shown). Hence, the observed biological diversity of PepMV isolates does not correlate with different genotypes.

Mixed infections and recombination among the strains occur in the PepMV population of Murcia. Sequences belonging to the Peruvian strain initially were identified in analyses of the TGB fragment of isolates CI 00.1, CI 01.3, Mu 04.12, and Mu 04.19. However, the sequences of the RdRp and CP genes for these isolates clustered within the European tomato strain (Fig. 1; data not shown). Because the sequences for these three genomic regions were derived from three independent RT-PCR events, this could be due to these isolates being recombinants between the European tomato and Peruvian strains or because the original field plants were infected with both strains. To discriminate between these two possibilities, a third fragment was RT-PCR amplified from these samples using primers Pep3 and RecRepl-R (Table 1), so that an 896-nt fragment overlapping the TGB and RdRp ORFs was obtained. The nucleotide sequences of these fragments showed no evidence of recombination (data not shown). On the contrary, the TGB and RdRp were of the European tomato strain, indicating mixed infection in these plants.

Similarly, sequences belonging to the US2 strain were identified for the CP RT-PCR-amplified fragment of isolates Mu 04.7, Mu 04.8, Mu 04.9, Mu 04.10, Mu 04.12, Mu 04.15, Mu 04.17, and Mu 04.18, whereas the TGB amplified from the same samples was either of the European tomato strain or of the Peruvian strain (isolate Mu 04.12). Because the European tomato and U.S. strains differ considerably at this region, new primers were designed

TABLE 2. Nucleotide diversity at nonsynonymous (d_{NS}) and synonymous (d_S) positions in three genomic regions of *Pepino mosaic virus* of the European tomato strain

	d_{NS}	d_S	d_{NS}/d_S
RNA-dependent RNA polymerase	0.0019 ± 0.0012	0.0281 ± 0.0080	0.0676
Triple gene block ^a	0.0035 ± 0.0008	0.0206 ± 0.0041	0.1699
Coat protein	0.0034 ± 0.0009	0.0125 ± 0.0027	0.2720

^a Only nonoverlapping regions were considered.

TABLE 3. Nucleotide diversity of *Pepino mosaic virus* populations within and between different epidemic outbreaks in southeastern Spain

	2000	2001	2003	2004
2000	0.0085 ± 0.0020	0.0090 ± 0.0015	0.0064 ± 0.0012	0.0692 ± 0.0050
2001	...	0.0070 ± 0.0014	0.0072 ± 0.0015	0.0691 ± 0.0050
2003	...	...	0.0040 ± 0.0011	0.0678 ± 0.0049
2004	...	...	...	0.1017 ± 0.0075

based on the US2 sequence to amplify the TGB of these isolates (primers PepUSTGB-D1 and PepUSTGB-R) (Table 1): The amplified fragments showed sequences of the US2 strain (data not shown; accession no. AM042007-AM042012). Again, this could indicate mixed infections or recombination. A third genomic fragment, overlapping the TGB and CP, was RT-PCR amplified using primers PepRecB-D and PepRec-R. Both Sawyer (33) and Jacobsen and Eastel (11) tests for recombination showed a significant departure ($P = 0.05$) from the respective null hypotheses of random distribution of the length of fragments defined by polymorphisms, or of compatible informative sites. The distribution of pairs of incompatible informative sites along the analyzed genomic region is shown by black dots in the matrix of Figure 2. The pairs of phylogenetically incompatible sites were not distributed randomly along the sequence, which provided evidence for gene conversion. The pairs of incompatible sites did not cluster according to the different genes represented in the analyzed genomic region. When isolates Mu 04.8 and Mu 04.9 were removed from the analyzed set of isolates, the analyses no longer provided evidence for recombination, indicating that these two isolates were the only recombinant isolates (data not shown).

DISCUSSION

Knowledge of the epidemiology and evolution of pathogen populations is essential for understanding the emergence of new diseases. With these two goals in mind, we characterized the population structure of PepMV infecting tomato crops in Spain. PepMV is a typical instance of an emergent plant pathogen. Prior to 1999, PepMV was described only as a pathogen of *S. muricatum* (pepino) in Peru, and Peruvian isolates from pepino and other solanaceous hosts were asymptomatic in tomato (12,16,34).

The population structure of PepMV in Spain showed that a single genetic type, the European tomato strain, was highly prevalent (more than 80% of the population). Isolates of this strain were found even before the first epidemic outbreak in Murcia and since the first outbreak in the Canary Islands. Hence, isolates of the European tomato strain are at the origin of the current epidemic. Molecular characterization based on the analysis of a

547-nt fragment from the RNA polymerase gene or an 844-nt fragment in the CP gene of tomato-infecting PepMV isolates from Europe and North America (26,37) showed a high nucleotide sequence identity (above 99%) among all isolates. Collectively, molecular analyses of PepMV from tomato indicates that demographic expansion of a single strain is the cause of the PepMV epidemic in Europe and North America, as had been suggested previously (37).

The highly conserved, undifferentiated population of the European tomato strain in Spain is compatible with demographic expansion during the epidemic. In an expanding population, selection for the fittest genotype would result in fixation as a single clone (7,25). Year-round availability of the tomato host, as well as seed transmission and high survival potential of PepMV in plant debris in the soil (C. Jordá, *unpublished data*), would contribute to the high population numbers needed for selection to operate. In agreement with this interpretation, most polymorphism occurred at silent positions in the three analyzed genes.

Isolates of the European tomato strain clustered together with the Peruvian isolates from other solanaceous hosts. The near identity of the genomic RNAs of an isolate obtained from pepino in 1974 (12; sequence from this work) and another isolate sampled from *L. peruvianum* in 2002 (19) could suggest that the Peruvian population of nontomato isolates of PepMV also could be highly conserved. The widespread occurrence of PepMV in wild *Lycopersicon* spp. in Peru, as well as in tomato (34), suggests that Peru is the origin of both the European tomato strain and the Peruvian strain. It has been shown that isolates asymptomatic on tomato from wild *Lycopersicon* spp. or from *S. muricatum* accumulated in tomato less efficiently than virulent isolates (34). Although these comparisons included a limited number of PepMV isolates from tomato, they suggest an increased fitness in this host linked to virulence. This could explain the demographic expansion of the European tomato strain. Interestingly, some isolates from *L. peruvianum* accumulated in tomato to the same levels as those from tomato and caused similarly severe symptoms (34), supporting the hypothesis of a Peruvian origin of the European tomato strain. Unfortunately, no sequence data for those isolates are available. An extended host range of Spanish isolates from tomato compared with the original isolate from pepino (12,14,22) also could facilitate demographic expansion. We have not attempted to identify the mutation or mutations leading to virulence in tomato, but changes in host range or in virulence to a particular host may be due to relatively few mutations (23,38). All of the various symptoms associated with PepMV infection in the field were reproduced in the greenhouse by infection with isolates of the European tomato strain. Thus, no association between genotype and phenotype was found.

Two other genotypes, the Peruvian and US2 strains, were detected in Murcia only in 2004. Detection of the Peruvian strain in the Canary Islands in 2000 suggests an independent introduction of PepMV in the Canary Islands and in peninsular Spain. Isolate 3253 of Martínez-Culebras et al. (22), collected in the Canary Islands in 1999, had an RdRp region 98% identical to those of isolates US1 and US2 (21). This suggests an early presence of the US2 strain in Spain. Restriction-endonuclease analyses of 107 PepMV isolates in the same RdRp region analyzed here yielded three different restriction fragment length polymorphism (RFLP) profiles, (P1, P2, and P3) which seemed to correspond to the European tomato, Peruvian, and US2 strains, respectively (22). Isolates with P2 or P3 RFLP profiles were collected both in Murcia and in the Canary Islands since 2000. Hence, if the correspondence between the RFLP profiles of Martínez-Culebras et al. (22) and our strains were concordant, it would indicate the presence of all three PepMV strains in peninsular Spain and in the Canary Islands since the first important epidemic outbreaks in tomato in 2000. If true, our data would indicate an increase of the US2 strain in the population in 2004.

Fig. 2. Distribution of phylogenetically incompatible positions along the concatenated partial sequences of the triple-gene block (TGB) and the coat protein (CP) gene. Sequences were determined from reverse-transcription polymerase chain reaction products amplified using primers PepRecB-D and PepRec-R. Phylogenetically incompatible positions according to Jacobsen and Eastel (11) are represented by black dots. Limits among the TGB and CP genes are indicated.

Isolates of the Peruvian and U.S. strains always were found in mixed infection with the European tomato strain. Recombinant isolates between the European tomato and the U.S. strains occurred in the population, which exchanged portions of the TGB and CP genes. For other RNA plant virus populations analyzed at the local scale (2,24), recombinants within a gene seem not to be favored in the virus population. Future analyses of the PepMV population in Murcia should show if this is also the case for PepMV.

The genetic structure of the tomato population of PepMV has not been analyzed in other countries and it is not known if the Peruvian and U.S. strains also are present in other regions experiencing PepMV epidemics in tomato. If this were not the case, our data would suggest that PepMV has been introduced into Spain from different sources. This possibility is highly relevant for PepMV control, indicating that sanitary measures implemented to minimize dispersal through plant material should be extreme, and should not be terminated simply because PepMV is already present in a region. As shown in this work, the introduction of new strains and the appearance of new genetic types by interstrain recombination represent a high potential risk, and should be considered in the design of efficient control strategies.

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