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Short Communication

Genetic variation of populations of *Citrus psorosis virus*

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Citrus psorosis virus (CPsV), the type species of genus *Ophiovirus*, has a segmented, negative-stranded RNA genome. We examined the population structure and genetic variation of CPsV in three coding regions located in RNAs 1, 2 and 3, analysing 22 isolates from Argentina, California, Florida, Italy and Spain. Most isolates contained a predominant sequence and some minor variants. Estimations of the genetic diversity and phylogenetic clustering of isolates disclosed two populations, one comprising isolates from Spain, Italy, Florida and California and the other including the Argentinean isolates. Isolate CPV-4 (from Texas) included for comparison was distant from both groups, suggesting that it belongs to a third group. The low ratio between non-synonymous and synonymous nucleotide substitutions indicated strong selection for amino acid sequence conservation, particularly in the coat protein gene. Incongruent phylogenetic relationships in different genomic regions suggested that exchange of genomic segments may have contributed to CPsV evolution.

Citrus psorosis virus (CPsV), the type species of the genus *Ophiovirus* (Vaira *et al.*, 2004), is the presumed causal agent of psorosis (Martín *et al.*, 2004), an economically important graft-transmissible disease of citrus. CPsV virions are kinked filaments, 3–4 nm in diameter, that, in a sucrose gradient, are separated into top and bottom components (García *et al.*, 1994). They are formed by three single-stranded, negative-sense RNAs (Fig. 1a) and a coat protein (CP) of ~48 kDa (Derrick *et al.*, 1988; Martín *et al.*, 2005). RNA 1 contains ~8184 nt and its complementary strand (vcRNA) has two ORFs potentially encoding a 24 kDa protein of unknown function and a 280 kDa protein with motifs characteristic of RNA-dependent RNA polymerases (RdRp). vcRNA 2 (~1644 nt) encodes a 54 kDa protein without similarities to other known proteins and vcRNA 3 (~1454 nt) encodes the CP (Barthe *et al.*, 1998; Sánchez de la Torre *et al.*, 1998, 2002; Naum-Onganía *et al.*, 2003; Martín *et al.*, 2005).

Plant RNA viruses have great potential for genetic variation because of the lack of a proof-reading correction mechanism in RdRp (Domingo & Holland, 1994), but other evolutionary factors such as genetic exchange (recombination and reassortment of genomic segments) and selection also contribute to shaping the structure of viral populations. Interestingly, populations of most positive-stranded RNA plant viruses studied are genetically stable and show limited diversity (reviewed by García-Arenal *et al.*, 2001), although a high spontaneous mutation frequency was estimated for *Tobacco mosaic virus* (Malpica *et al.*, 2002).

Knowledge of the structure and variation of viral populations is often necessary for diagnostic, epidemiological and control purposes; particularly, the durability of genetic resistance is highly dependent on the evolutionary potential of the virus (García-Arenal & McDonald, 2003). Data on the population structure and genetic variability of positive-stranded RNA plant viruses are available (García-Arenal

The GenBank/EMBL/DDBJ accession numbers of the CPsV sequences determined in this study are AM268273–AM268314 (region R1), AM235891–AM235946 (region R2) and AM235947–AM236001 (region R3).

Supplementary material is available in JGV Online.

et al., 2001; Rubio *et al.*, 2001; Vives *et al.*, 2002; Vigne *et al.*, 2004; Marco & Aranda, 2005; Teycheney *et al.*, 2005; Turturo *et al.*, 2005), but genetic variation of negative-stranded RNA plant viruses has been less studied and only nucleotide distances and clustering of sequence variants have been reported (Miranda *et al.*, 2000; Callaghan & Dietzgen, 2005; Navarro *et al.*, 2005; Revill *et al.*, 2005). Their population structure and the evolutionary forces that shape these populations have barely been examined. Previously, by using single-strand conformation polymorphism analyses and sequencing, we observed low genetic diversity within Spanish and Argentinean CPsV isolates in the CP gene (our unpublished results) and between isolates from Campania, Italy (Alioto *et al.*, 2003). To assess whether genetic homogeneity also existed in other genome regions and between isolates of different geographical origins, and to gain insight into the potential factors involved in CPsV evolution, we analysed the population structure and genetic variation of 22 isolates from Argentina, California, Florida, Italy and Spain in three genomic regions.

Isolates from Spain (here coded S1–S5) and from California (C1–C3) belong to a collection kept at the Instituto Valenciano de Investigaciones Agrarias. The Italian isolates (IT1–IT5) were collected from infected field trees in Campania, the Argentinean isolates (A1–A5) were from Concordia and isolates F1–F4 were from several locations in Florida. The sequence of isolate CPV-4 (Barthe *et al.*, 1998; Sánchez de la Torre *et al.*, 1998, 2002; Naum-Onganía *et al.*, 2003), probably of Texan origin (Garnsey *et al.*, 1976), was included for comparisons.

Sequence variability was studied using cDNA of regions R1, R2 and R3, located on genomic RNAs 1, 2 and 3, respectively (Fig. 1a), obtained by RT-PCR using total RNA as template and appropriate primers (see Supplementary Table S1 available in JGV Online). Primers Ps-44 and Ps-45, which flank the R1 region delimited by primers Ps1 and Ps7, were used when amplification with this latter set failed, but only the R1 region was considered for sequence analysis.

Total RNA extracted with Trizol (Invitrogen) from 100 mg fresh tissue was resuspended in 50 µl diethyl pyrocarbonate-treated distilled water and 1 µl was used for reverse transcription with Superscript reverse transcriptase (Invitrogen) at 42 °C for 45 min. A 5 µl aliquot was subjected to PCR with Platinum *Pfx* DNA polymerase (Invitrogen) using the following conditions: 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 42 °C for 25 s and 68 °C for 45 s and a final step of 5 min at 68 °C. PCR products were purified using Ultrafree-DA filters (Millipore), ligated into the pUC18 plasmid vector (Pharmacia) and cloned by standard procedures (Sambrook *et al.*, 1989). The nucleotide sequences of three to five clones of each cDNA product were determined using an ABI PRISM DNA Sequencer 3100 (Applied Biosystems).

Multiple sequence alignments were performed using CLUSTAL W (Thompson *et al.*, 1994). Nucleotide distances,

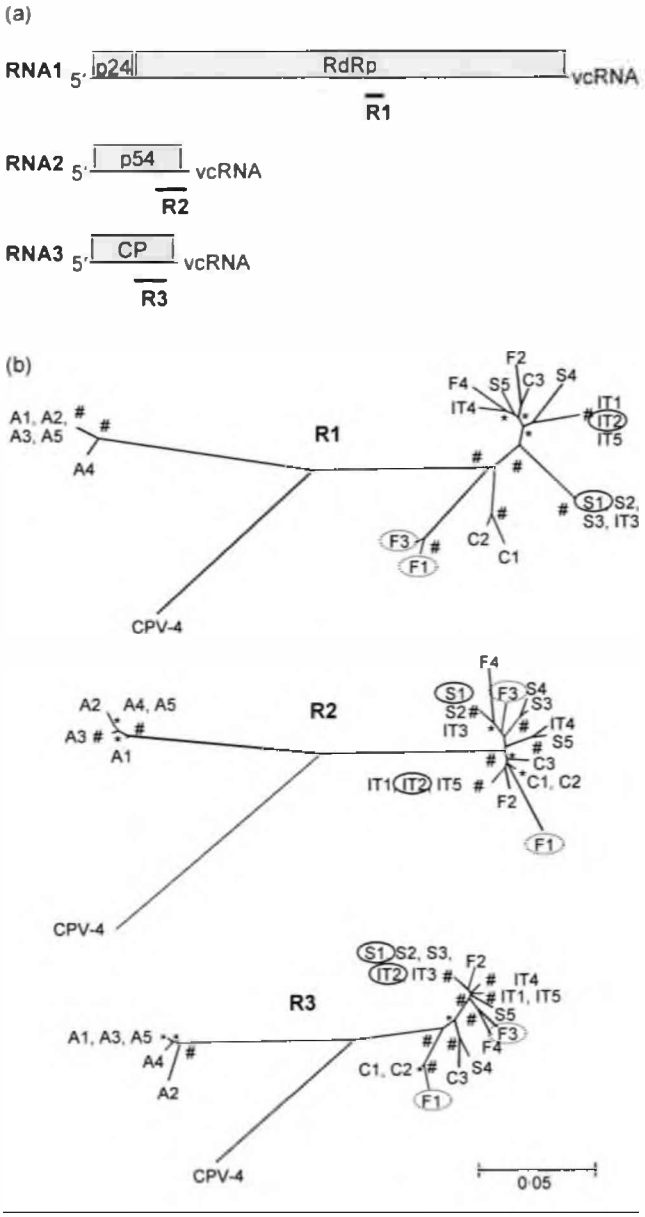

Fig. 1. (a) Schematic representation of the CPsV genome. Boxes indicate the protein products encoded by the vcRNA. The locations of the segments analysed (R1, R2 and R3) are indicated. (b) Phylogenetic trees of the genomic regions R1, R2 and R3, calculated by the neighbour-joining method with nucleotide distances estimated by the Jukes and Cantor method. Significance of the nodes was estimated with 1000 bootstrap repetitions: *, bootstrap values of 60–80 %; #, bootstrap values >80 %. Branch lengths are proportional to genetic distances. Branches that group isolates with distances under 0·01 are not shown. Phylogenetic incongruence in different regions is marked by continuous or dotted ellipses.

nucleotide diversity (mean nucleotide distance between two randomly selected sequence variants), numbers of synonymous substitutions per synonymous site and of non-synonymous substitutions per non-synonymous site and

phylogenetic relationships were estimated using the MEGA program, version 2.1 (Kumar *et al.*, 2001). Standard errors were calculated by bootstrap with 1000 repetitions.

The three regions selected represented about 12 % of the genome and their within-isolate nucleotide diversity ranged from 0 to 0·0120, except for region R3 of isolate A2, the diversity of which was 0·0280. These low values are consistent with a population formed by a predominant sequence and some minor variants that are genetically close. This population structure, probably resulting from the combined effects of high replication rates and natural selection (Domingo & Holland, 1994), has been reported for different positive-stranded RNA plant viruses (García-Arenal *et al.*, 2001; Vigne *et al.*, 2004; Turturo *et al.*, 2005), including the citrus-infecting viruses *Citrus tristeza virus* (CTV) and *Citrus leaf blotch virus* (CLBV) (Rubio *et al.*, 2001; Vives *et al.*, 2002). Because of the low within-isolate diversity, the major sequence variant of each isolate and region was used in subsequent analyses.

Considering the isolates from each country as a distinct population, the within-population diversities for the three regions were low in Argentina ($\leqslant$0·0138) and higher ($\leqslant$0·0743) in California, Florida, Italy and Spain (Table 1). The diversity values of the dataset of 22 isolates were higher than the corresponding within-population values (0·1300$\pm$0·0137, 0·1034$\pm$0·0085 and 0·0829$\pm$0·0078 for regions R1, R2 and R3, respectively) (Table 2), suggesting genetic variation between populations of different geographical origin. These global values are similar to the highest calculated for plant viruses with positive-stranded RNA or DNA genomes (García-Arenal *et al.*, 2001; Chare & Holmes, 2004). Estimations of genetic diversity in negative-stranded RNA plant viruses are not available, although different levels of sequence variation have been described. Thus, while variability of the nucleorhabdovirus Taro vein chlorosis virus (TaVCV) or the cytorhabdovirus *Lettuce necrotic yellows virus* (LNYV) was high, with nucleotide distances up to 0·2740 and 0·1930 for TaVCV genes L and N, respectively, and up to 0·2000 for the N gene of LNYV (Revill *et al.*, 2005; Callaghan & Dietzgen, 2005), the CP gene of the varicosavirus *Lettuce big-vein virus* (LBVaV) showed nucleotide distances $\leqslant$0·0202 (Navarro *et al.*, 2005). In ophioviruses, the CP gene of 17 Mirafiori

Table 2. Genetic diversity (D), mean numbers of synonymous and non-synonymous nucleotide substitutions per synonymous and non-synonymous site (dS and dN, respectively) and codon usage bias in three genomic regions of 22 CPsV isolates from different geographical origins

Protein products potentially encoded by the genes comprising segments R1, R2 and R3 are indicated in parentheses as RdRp (RNA-dependent RNA polymerase), p54 (54 kDa protein with unknown function) and CP (coat protein). Standard errors for D are given in parentheses.

Region	D	dN	dS	dN/dS	ENC
R1 (RdRp)	0·1300 (0·0137)	0·0203	0·6024	0·0483	41·3
R2 (p54)	0·1034 (0·0085)	0·0220	0·4825	0·0739	47·8
R3 (CP)	0·0829 (0·0078)	0·0055	0·3600	0·0220	51·9

Table 1. Genetic diversity of CPsV isolates grouped by geographical origin

Genetic diversity (mean nucleotide distance between pairs of sequences) is shown within populations (on the diagonal, in bold) or between populations (above the diagonal). Standard errors are indicated in parentheses.

Population	Spain	Italy	Florida	California	Argentina
R1					
Spain	**0·0438 (0·0161)**	0·0071 (0·0161)	0·0765 (0·0108)	0·0200 (0·0218)	0·1988 (0·0342)
Italy		**0·0448 (0·0150)**	0·0731 (0·0101)	0·0216 (0·0201)	0·2072 (0·0325)
Florida			**0·0743 (0·0122)**	0·0738 (0·0264)	0·2300 (0·0291)
California				**0·0503 (0·0220)**	0·1921 (0·0336)
Argentina					**0·0103 (0·0058)**
R2					
Spain	**0·0319 (0·0081)**	0·0053 (0·0089)	0·0435 (0·0053)	0·0137 (0·0083)	0·1728 (0·0212)
Italy		**0·0312 (0·0100)**	0·0434 (0·0057)	0·0069 (0·0066)	0·1719 (0·0214)
Florida			**0·0514 (0·0073)**	0·0373 (0·0053)	0·1952 (0·0193)
California				**0·0157 (0·0067)**	0·1742 (0·0220)
Argentina					**0·0070 (0·0031)**
R3					
Spain	**0·0231 (0·0091)**	0·0001 (0·0050)	0·0018 (0·0014)	0·0138 (0·0135)	0·1306 (0·0176)
Italy		**0·0160 (0·0045)**	0·0020 (0·0012)	0·0203 (0·0121)	0·1410 (0·0174)
Florida			**0·0470 (0·0064)**	0·0049 (0·0022)	0·1178 (0·0146)
California				**0·0371 (0·0178)**	0·1178 (0·0183)
Argentina					**0·0138 (0·0054)**

lettuce big vein virus (MLBVV) isolates from eight countries showed nucleotide distances up to 0·1199 (Navarro *et al.*, 2005), whereas the maximum nucleotide distances between CPsV isolates estimated here were about twice as great (0·2466, 0·2174 and 0·1725 for regions R1, R2 and R3, respectively).

Diversities between the populations of California, Florida, Italy and Spain (0·0071–0·0765, 0·0053–0·0435 and 0·0001–0·0203 for R1, R2 and R3, respectively) were of the same order or even smaller than the corresponding within-population values, whereas diversities between each of these populations and the Argentinean population ranged from 0·1178 to 0·2300 (Table 1), more than twice the between-population diversities of the first group and about ten times the Argentinean within-population values. Differentiation of the Argentinean population was evaluated with the D statistic according to Lynch & Crease (1990). The D value calculated for isolates from California, Florida, Italy and Spain was below 1·5, suggesting that these isolates formed a genetically undifferentiated population. Inclusion of the Argentinean isolates resulted in a D value greater than 108, indicating that the latter isolates formed a genetically different population.

Neighbour-joining trees inferred for regions R1, R2 and R3 of the 22 isolates analysed here and of the Texas isolate CPV-4 (GenBank accession numbers AY224663, AF218572 and AF060855) showed three main groups supported by bootstrap values greater than 99% (Fig. 1b). Group I comprised isolates from California, Florida, Italy and Spain, consistent with the hypothesis that these isolates, irrespective of their geographical origin, are genetically members of the same population. CTV populations from California and Spain were also genetically indistinguishable (Rubio *et al.*, 2001), further supporting the idea that gene flow via movement of infected citrus buds contributed to shaping populations of citrus viruses in these areas. Group II included the Argentinean isolates, which formed a genetically homogeneous population (Table 2) clearly separated from the other isolates. Finally, isolate CPV-4 from Texas appeared separated from the other two groups (between-group nucleotide distances greater than 0·14). Genetically distant groups of isolates have been also observed in the cytorhabdovirus LNYV and in MLBVV, a fungus-borne ophiovirus. However, they were not associated with specific geographical areas and isolates of different groups were found in the same country (Callaghan & Dietzgen, 2005; Navarro *et al.*, 2005).

Although clustering of isolates was identical for R1, R2 and R3, some isolates within group I showed incongruent relationships in different regions, suggesting recombination or reassortment of genomic segments. Isolates F1 and F3 were almost identical in the R1 region, but relatively distant in R2 and R3; also, isolate IT2 was close to isolates IT1 and IT5 and more distant from isolates S1, S2 and IT3 in regions R1 and R2, whereas the opposite was true in R3 (Fig. 1b). The different groupings were found to be statistically significant when applying the RETICULATE program (Sneath *et al.*, 1975; Jakobsen & Easteal, 1996) (see supplementary material available in JGV Online). A conversion analysis of the concatenated R1–R2–R3 regions using GENECONV (Sawyer, 1989) (see supplementary material available in JGV Online) gave significant global P-sim values ($<0·05$) for isolates F1 and F3 in region R1 (0·0014) and for isolates IT2 and S1 in region R3 (0·0129). This suggests that (i) the R1 regions of isolates F1 and F3 came from a common ancestor by genetic exchange and (ii) isolate IT2 may have acquired its R3 region from an S1-like ancestor.

Previous experiments had shown that combinations of the top and bottom components of isolate CPV-4 with the complementary component from the Argentinean isolate 90-1-1 were infectious in *Chenopodium quinoa*, indicating that reassortment of genomic segments did not abolish CPsV replication, at least in this host (García *et al.*, 1993). Since natural dispersal of CPsV has not been observed in the areas of origin of the group I isolates, genetic exchange between co-infecting sequence variants might have occurred after top-grafting of trees infected with one of the ancestors using buds infected with the other. Reassortment of genomic segments has been described in positive- and negative-stranded RNA plant viruses (Miranda *et al.*, 2000; Vigne *et al.*, 2004), although reassortants occur at low frequency with some viruses, suggesting that they may be selected against (Bonnet *et al.*, 2005).

To assess the selective constraints on each CPsV genomic region, the mean numbers of non-synonymous substitutions per non-synonymous site (dN) and of synonymous substitutions per synonymous site (dS) were estimated separately (Table 2). The dN/dS ratio was 0·0483, 0·0739 and 0·0220 for regions R1, R2 and R3, respectively, indicating that, although wide nucleotide variation was observed, this was mostly restricted to synonymous positions. These low dN/dS ratios suggest strong selection for amino acid conservation, particularly in R3. In addition to constraints of maintaining virion structure and stability, others related to virus movement or different interactions with host factors may act upon the CP amino acid sequence, as described for other viruses (Forster *et al.*, 1992; Lu *et al.*, 2004). The dN/dS value calculated for R3 is similar to those estimated for the CP of CTV and CLBV, two other citrus-infecting viruses (Rubio *et al.*, 2001; Vives *et al.*, 2002), and lower than those found for CP genes of most positive-stranded RNA viruses (García-Arenal *et al.*, 2001; Chare & Holmes, 2004) and of the negative-stranded plant viruses MLBVV, LBVaV and *Tomato spotted wilt virus* (Chare & Holmes, 2004; Navarro *et al.*, 2005).

The dS value of the R3 region was also significantly lower than those estimated for regions R1 and R2 (Table 2) as determined by the Wilcoxon test ($P<0·0001$), suggesting different selective pressures for nucleotide conservation. To test whether these differences were due to synonymous codon usage bias, the effective number of codons (ENC) was calculated for each region (see supplementary material

available in JGV Online). ENC values were 41·3, 47·8 and 51·9 for R1, R2 and R3, respectively, and they did not correlate with the corresponding dS values (Table 2), indicating that other factors are probably responsible for the lower frequency of synonymous changes observed in R3. Evolutionary constraints at the nucleotide level may be related to the need to maintain secondary structures and/or sequence motifs of functional importance, such as sites for interaction with proteins.

In summary, our analyses show that CPsV has evolved three genetically differentiated populations and that these populations have been shaped by the combined effects of selection for amino acid conservation, genetic exchange between sequence variants and gene flow between countries.

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