Tropical Plant Pathology, vol. 39(3):234-241, 2014

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RESEARCH ARTICLE

First report of recombination in *Potato yellow vein virus* (PYVV) in Colombia

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ABSTRACT

Potato yellow vein virus (PYVV) is currently one of the most important viruses that infects potatoes in Colombia and other Andean countries, causing losses in the production of tubers ranging from 25% to 50%. This study analyzed the genetic variability of different viral isolates collected in the department of Nariño, Colombia, through bioinformatics analysis of the sequences of three genes encoding the capsid protein (CP), the heat-shock protein 70 (Hsp70) and the minor capsid protein (CPm). We found that CPm is the gene that shows greater diversity, with higher values of nucleotide substitution and evidence of recombination. Based on an analysis of the haplotype map using nucleotide sequences of the CPm, we propose a model of putative recombination in this genomic region. The non-recombinant segments are supported by the results of the program GARD (Genetic Algorithm for Recombination Detection), phylogenetic trees and the paired values of genetic distances of each non-recombinant segments. The model clearly shows that the amino region of the CPm is prone to recombination. To our knowledge, this is the first report of genetic recombination as an evolutionary strategy in the CPm of PYVV.
Key words: *Crinivirus*, *Solanum tuberosum,* genetic variability, minor coat protein.

INTRODUCTION

Members of the genus *Crinivirus* (family *Closteroviridae*) are emerging plant viruses in many parts of the world. An important factor contributing to the increase in the incidence of these viruses is their association with, and transmission by whitefly vectors that have increased in distribution in the last several decades (Stewart et al., 2010). *Potato yellow vein virus* (PYVV) is a member of the *Crinivirus* genus (Martelli et al., 2012) transmitted by the whitefly *Trialeurodes vaporariorum* (Westwood), responsible for a yellowing disease in potato plants. PYVV was observed for the first time in the Department of Antioquia, Colombia, in the early 1940's (Salazar et al., 2000).

Variability among members of the family *Closteroviridae* was investigated at the molecular level in different genomes (Rubio et al., 2001; Marco & Aranda, 2005; Rodríguez et al., 2010). For PYVV, genetic variability studies have been limited to the gene encoding the major capsid protein (CP) using approaches such as single-strand conformation polymorphism (SSCP) analysis (Offei et al., 2004), restriction fragment length polymorphism (RFLP) (Guzman et al., 2006), and nucleotide sequence analysis (Rodríguez et al., 2010; Chaves-Bedoya et al., 2013).

Nucleotide composition of plant virus populations provide information about their genetic stability, and the comparison of the nucleotide sequence data from plant viruses isolates from different geographic origins helps in the understanding of their biology. For example, changes in virulence, geographical distribution, and their potential to rise as new epidemics as well as evolutionary mechanisms such recombination. The understanding of this information is key for designing strategies for controlling viruses diseases (Moreno et al., 2004; Tan et al., 2004).

RNA-RNA recombination is one of the strongest forces shaping the genetic diversity of plant RNA viruses (Sztuba-Solinska et al., 2011). Each RNA-based virus seems to be capable of recombining. However, the evolutionary implications of such phenomena for different types of viruses remains to be elucidated (Sztuba-Solinska et al., 2011). Currently there is increasing information on natural recombination in several plant viruses, nevertheless reports about recombination among members of the *Closteroviridae* family is scarse. Thus far genetic recombination has been detected in the closterovirus *Citrus tristeza virus* (CTV) (Rubio et al., 2001; Che et al., 2003) and in the criniviruses *Strawberry pallidosis associated virus* (SPaV) (Tzanetakis & Martin, 2004), *Sweet potato chlorotic stunt virus* (SPCSV) (Cuellar et al., 2008) and *Blackberry yellow vein associated virus* (BYVaV) (Poudel et al., 2012). SPaV is also transmitted by *T. vaporarium* (Tzanetakis et al., 2006).

Since there are no reports about recombination as a possible evolutionary force in the PYVV genome, the aim of this work was to search for and to identify putative break points in the regions encoding the CP, HSP70 and CPm

genes. For the first time, molecular evidence of multiple genetic recombination in the CPm coding region of PYVV is reported. The evidence suggests the importance of this genetic region for PYVV evolution.

MATERIAL AND METHODS

Viral isolates and RNA extraction

The PYVV isolates were obtained from potato plants exhibiting symptoms of PYVD harvested in the Department of Nariño (South of Colombia). The leaf samples were collected from 30 *Solanum tuberosum* (group Phureja, or criolla native potato) and 30 *S. tuberosum* (group Andigena, or yearly potato) plants. The double-stranded viral RNA (dsRNA) was extracted from the leaf samples, previously pulverized in liquid nitrogen, using phenol-chloroform (1:1) and purified with Sephadex G50 columns (Pappu et al., 1993; Cevik et al., 1996; Manjunath et al., 1996; Pappu et al., 2000; Lee et al., 2002; Guzman et al., 2006).

Primer Design

In this study we used previously described primers (Offei et al., 2004) as well as new, specific CP, CPm, and Hsp70 primers designed based on the alignment of nucleotide sequences reported in GenBank (Livieratos et al., 2004) using the PRIMER3 (Rozen & Skaletsky, 2000) software. The accession numbers AJ508757 and AJ557129 identifies the sequences used. For the CP gene, the forward and reverse primers were 5' CTC GAG GAT CCT CAT GGA AAT CCG ATC 3' and 5' CTA CTC AAT AGA TCC TGC TA 3', respectively. For the CPm gene, the primers were 5' ATG GAT AAG TCT GTT TTA GAT G 3' and 5' TCA AAA GTT TTG ATT CAC ATT C 3'; for the Hsp70 gene, the primers were 5' TGC CCT CTA TCT TCA ATA CCA G 3' and 5' CAC TTC AAA AAT TAT CCT ACA AAG TGA 3'. To sequence the CPm gene, the internal primer 5' TCT CTC CAG ATC AGG CCA AT 3' was used. The size of the expected fragments was 768 base pairs for the CP gene, 2024 base pairs for the CPm gene, and 1664 base pairs for Hsp70 gene.

Amplification of viral RNA

The RNA was amplified using two-step RT-PCR with 8 µl RNA, 20 U/µl Moloney murine leukemia virus (MMLV) reverse transcripatase (Epicentre), 4 U/µl RNAse (RNAsin; Fermentas), 1X buffer MMLV, 1 mM dNTPs, and 0.24 µM reverse primer in a final volume of 25 µl. The RT reaction was performed at 42ºC for 1 h. For the PCR reaction, 4 µl of the RT-PCR reaction, 3.75 U/µl Accuzyme (Bioline), 1.875 U/µl Biolase (Bioline), 1X Accuzyme buffer, 1 mM $MgCl_2$, 0.4 mM dNTPs, and 0.4 µM of each primer was used. The final volume of the reaction was adjusted to 25 µl. The amplification conditions were as follows: 1 cycle at 94ºC for 3 min, 35 cycles at 94ºC for 1 min, 55ºC for 1 min, and the extension time was 68ºC for 1 min for the Hsp70 and CP genes and 68ºC for 1.30 min for the CPm gene. A final incubation at 68ºC for 10 min was performed. The PCR products were purified using the Wizard SV Gel kit and the PCR Clean-Up System (Promega) and were quantified using the QubitTM dsDNA HS kit (Invitrogen). Accuzyme (Bioline) and Biolase (Bioline) enzymes were used because of their high proof reading capacity.

The 60 field isolates were submitted to RT-PCR and the PCR purified products used for the Single Stranded Chain Polymorphism (SSCP) technique searching for gene variability (data not shown) as already has been shown for other closteroviruses (Rubio et al., 1999; Rubio et al., 2001). Only 34 of the 60 isolates showed variability by SSCP, so these isolates were sent to Macrogen USA for direct nucleotide sequencing of the specific genes: CP gene, 8 isolates; CPm gene, 12 isolates; and Hsp70 gene, 14 isolates (Table 1).

Sequence analysis

The PYVV nucleotide sequences were obtained from a single reaction using the direct strand as the reverse. The sequences correspond to the 100% of the nucleotide sequence of each gene. Sequences of these genes area available in the public database GenBank and are listed in Table 1.

The bioinformatic analysis was performed comparing the 34 nucleotide sequences that were obtained with the only complete genomic sequence of PYVV corresponding to a Peruvian isolate (GenBank accession number AJ557129). So far, there is no strain definition or biological characterization for this virus.

Multiple alignment of nucleotide and deduced amino acids sequences were performed using the ClustalX software

TABLE 1 - Genbank accesion numbers of Colombian PYVV isolates analyzed. In bold, PYVV isolates from *S. tuberosum* group Phureja.

	Gene		
	CP	**CPm**	**Hsp70**
Isolates	**JN701950**, **JN701951**	**JN701972, JN701973**	**JN701958, JN701959**
	JN701952, **JN701953**	**JN701974, JN701975**	**JN701960, JN701961**
	JN701954, JN701955	**JN701976, JN701977**	**JN701962, JN701963**
	JN701956, JN701957	JN701978, JN701979	**JN701964, JN701965**
		JN701980, JN701981	JN701966, JN701967
		JN701982, JN701983	JN701968, JN701969
			JN701970, JN701971

(Thompson et al., 1997) with the default values of multiple alignment parameters, or the Martinez/Needleman-Wunsch algorithms from the MegAlign (version 5.0) program of the DNASTAR software. The Martinez/Needleman-Wunsch algorithms were also used to calculate a pairwise distance matrix between nucleotide sequences. The phylogenetic relationships of the nucleotide sequences of each gene were inferred using the maximum likelihood method. The evolutionary distances were calculated using the Kimura 2-parameter method (Kimura, 1980) using 1000 replications to estimate the confidence of the taxon grouping in tree branches (Felsenstein, 1985). The analyses were performed using the MEGA 5 software (Tamura et al., 2011).

Recombination analysis and recombination model based on a haplotype map

The search for evidence of recombination was performed using the GARD algorithm (Kosakovsky et al., 2006) in the remote server Datamonkey (Delport et al., 2010). GARD scans alignments for evidence of phylogenetic incongruence. The nucleotide substitution model was selected automatically before being applied to the recombination-site analysis. This must be done because the use of an inadequate model of nucleotide substitution may lead to differences in the results (Kosakovsky & Frost, 2005). The putative breakpoints suggested by GARD were further visually located in a haplotype map generated, removing indels and excluding infinite-sites violations using the SNAP program (Price & Carbone, 2005). Recombination model is proposed on the haplotype map.

In the model, viral isolates were grouped with the support of the results of the recombination analysis (GARD), the phylogenetic trees and pair distances between all the isolates in segments 1-80, 79-114, 114-135, 136-317, 319-382, 379-491, 490-546, 541-735, 736-937, 937-1551, 1552-1636 and 1636-2025 (Figure 1).

In the grouping a pattern of gray scale was used in order to facilitate the recognition of the non-recombinant segments and putative break points. For example, in the CPm gene analysis, in the first 80 nucleotides of the CPm the nucleotide similarity between isolates is 100% (with the exception of the isolate JN701979). This segment, in all isolates, was highlighted in dark gray (70%). Subsequently, any segment in which the isolates were sharing nucleotide similarities higher than 93% was highlighted with the same dark gray color. The criterion for selecting nucleotide similarity of 93% for grouping PYVV isolates was the frequency distribution of the percentages of similarity on the basis of 436 nucleotide sequence comparisons (Figure 1). The segments in which the isolates shared nucleotide similarities below 93% were highlighted with a different color.

If two isolates in a given nucleotide region share a different gray scale color it indicates that among them the similarity is close or equal to 100%, but less than 93% with the others, suggesting a possible different ancestor. For instance, in the segment 135-317 (Figure 3) the isolates JN701977 and JN701979 have a similarity of 100%, but with the other isolates the similarity is below 93% (62.1%-90.7%). Therefore this was highlighted in gray to 40%. The isolates JN701974 and JN701978 have 100% similarity, but a similarity below 93% with the other isolates so this was highlighted in gray to 20%. The isolate JN701975 has a nucleotide similarity below 93% with all the other isolates and as such it is highlighted in gray to 10%. Finally, in this segment the other isolates share similarities above 93% and were highlighted in dark gray (70%). The proposed model of recombination in the CPm of PYVV was drawn using the Canvas program for Mac, version 9.0.

RESULTS

Sequence analyses

Nucleotide sequence analyses revealed differences in the genetic variability among the three PYVV genes. The mean evolutionary diversity for the entire population in the CPm is 0.064 (sd=0.003); CP and Hsp70 coding regions have a nucleotide diversity of 0.010 (sd=0.003). Data analysis shows that genes of PYVV isolates obtained from *S. tuberosum* group Andigena (AG) has a higher number of substitutions per site compared with those from *S. tuberosum* group Phureja (PhG) except in the CPm coding region where the nucleotide substitution is 0.046 for AG and 0.084 for PhG. The nucleotide diversity values for gene and host are indicated in Table 2.

Phylogenetic trees and GARD analysis

Phylogenetic trees were built from the alignment of nucleotides and graphed on the same scale to facilitate comparisons. The CPm coding region has the largest nucleotide substitutions per site, i.e., it is the more variable, followed by the Hsp70 and finally the CP. The phylogenetic trees did not allow for the determining of the correlation between viral isolates and the host from which they were obtained, e.g. AG or PhG (Figure 2).

On the other hand GARD analysis found no evidence of recombination in the CP, however, the analysis suggests the presence of a breakpoint in the position 778 of the region coding for Hsp70 (*data not shown*) and at least ten breakpoints in the region coding for the CPm located at positions 80, 135, 317, 476, 541, 735, 927, 1551, 1636 and 1819 according to Kishino-Hasegawa (KH) test (Kishino & Hasegawa, 1989). Breakpoints in positions 541 and 1819 are highly significant.

Recombination model

Based on information from paired values of genetic distances, phylogenetic trees, recombinant segments defined by GARD and haplotype map analysis, a model of the possible evolutionary relationships is proposed among isolates of PYVV (Figure 3). The haplotype map was used to visually identify non-recombinant segments and breakpoints detected by GARD. Haplotype map

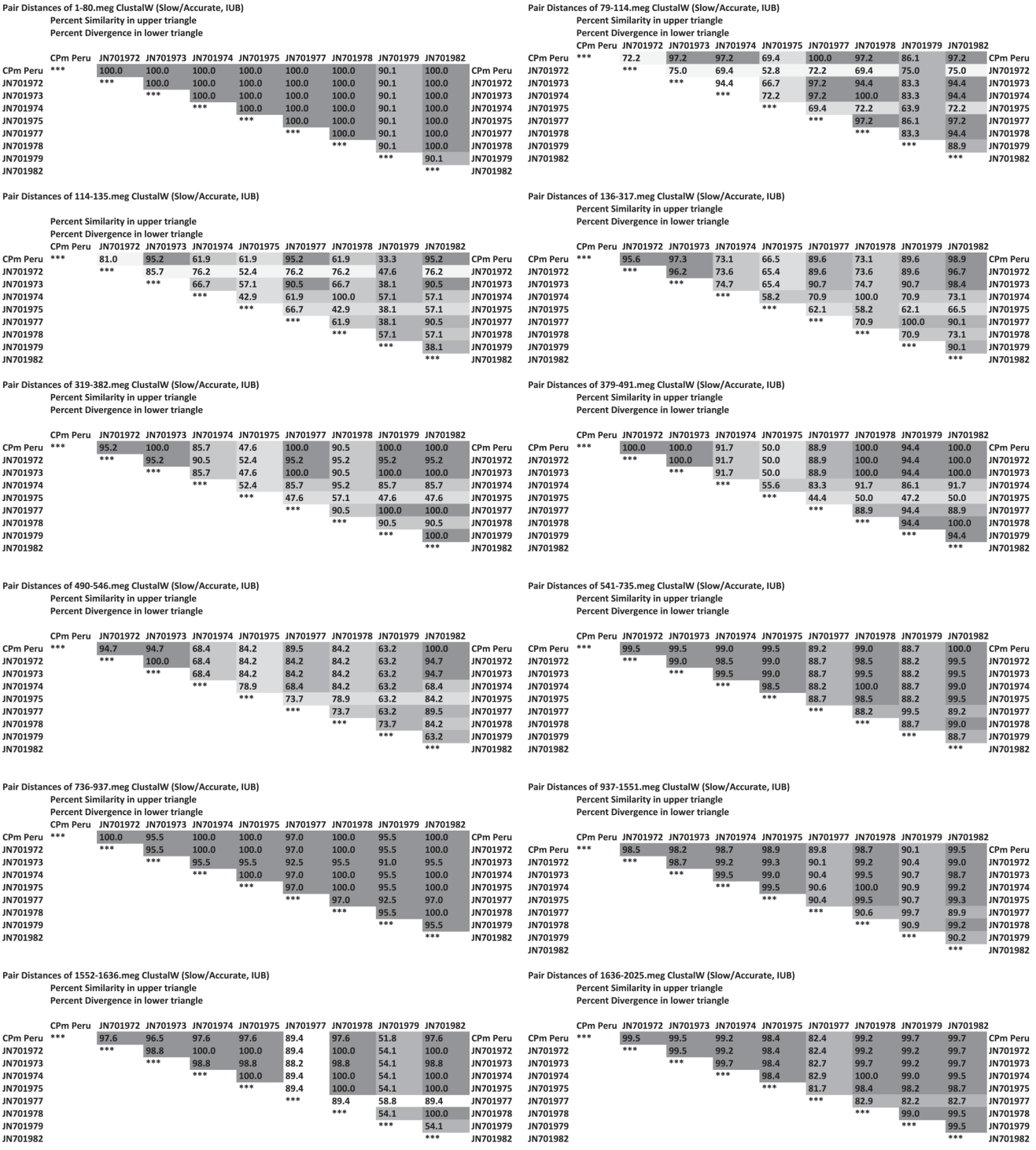

FIGURE 1 - Pairwise distances between different genomic regions of the CPm gene of *Potato yellow vein virus* (PYVV). Grayscale patterns correspond to the segment in the haplotype map depicted in Figure 3.

analysis of CPm reveals clear signals of recombination mainly in the N-terminal and central region. The proposed model of recombination suggests CPm of PYVV is highly recombinant mainly in the first 1/3 of the sequence around positions 80 and 541.

DISCUSION

The genetic variability of PYVV based in three different genes suggests that in Colombia there are few variants of this virus, with high nucleotide conservation in

TABLE 2 - Nucleotide diversity values for different PYVV genes in each host.

Gene	Host[a]	Nucleotide diversity
CP	AG	0.010
	PhG	0.008
Hsp70	AG	0.016
	PhG	0.005
CPm	AG	0.046
	PhG	0.084

[a]AG, *Solanum tuberosum* grop Andigena; PhG, *S. tuberosum* group Phureja

the CP and Hsp70 coding regions indicating a high genetic constrain. On the other hand, the substitution of bases and amino acids in the CPm indicates minor restrictions suggesting the importance of this region in the evolutionary processes of PYVV including genetic recombination.

PYVV presents a high genetic similarity in the studied genes; nevertheless, some isolates have nucleotide similarities below 75% in some regions. According to the criterion of species demarcation among criniviruses (King et al., 2012), these genetic segments could come from a different virus. The generation of new sequences will allow in the discarding or confirmation of interspecific recombination in PYVV.

It is well known that recombination is a very important process for the evolution of plant viruses (Roossinck, 2003) and it is common both intraspecific and interspecific. Recombination is particularly significant in persistent infections in which multiple viral genotypes are simultaneously replicated in a host generating genetic variants, some of which have new characteristics of pathogenicity and some others can colonize new hosts.

Our model of recombination in PYVV proposes that isolates JN701977 (AG host) and JN701979 (PhG host) have a different genetic background in most of the CPm. The isolates JN701975, JN701974 and JN701978 are formed by the contribution of two recombinant ancestors as the isolate JN701972. No evidence of recombination was found in isolates JN701982, JN701973 and the Peruvian accession. In spite of the GARD results, no recombination was visualized in position 927 on the haplotype map. The lack of evidence of recombination in isolates JN701982, JN701973 and the Peruvian strain does not mean that they are not recombinant. This may be possible due to the high genetic similarity among the isolates, or because the possible ancestral recombination event is hidden by subsequent mutations (Posada et al., 2002).

Nucleotide variability and clear and abundant points of recombination detected in the CPm of remaining PYVV isolates suggest that this region is a putative hot spot of recombination and an indicator of a possible recent molecular event (Tomimura et al., 2003), or a recent emergence (Tan et al., 2004). The evidence also suggests that this gene may play a key role in the diversity and evolution of PYVV with a possible role in the adaptation or host specificity. However, there is no evidence for these functions in this gene for PYVV. Nevertheless, recently the CPm of the crinivirus *Lettuce infectious yellows virus* (LIYV) was reported as required for whitefly transmission (Stewart et al., 2010). Although the biological significance of the recombination of the CPm gene in PYVV is not yet understood, it is important to note that within the *Closteroviridae* family, the CPm has been reported as a protein involved in cell-to-cell transportation and assembly (Alzhanova et al., 2001). So the evidence obtained in this study of CPm gene recombination in some PYVV isolates suggest that this re-emergent virus could be trying to improve its performance for transmission. Furthermore, CPm of PYVV could be indispensable in transmission by its vector *T. vaporariorum*, nonetheless more work is needed to test this hypothesis.

Recombination in PYVV occurs in viral isolates from both AG and PhG hosts (Table 1). However, among the sequences analyzed recombination is likely more frequent in PYVV isolates infecting PhG hosts.

In contrast to the CPm, the genetic variability of the Hsp70 and CP of PYVV is low, as has been previously reported (Offei et al., 2004; Guzman et al., 2006; Rodríguez et al., 2010) and coinciding with a recent study based on 69 nucleotide sequences of the CP reported in different years and locations in Colombia (Chaves-Bedoya et al., 2013).

High genetic stability found in the CP and Hsp70 of PYVV isolates from two different hosts could be attributed to negative or purifying selection that allowed it to maintain the functional integrity of the viral genome as proposed for *Cucurbit yellow stunting disorder virus* (Marco & Aranda, 2005). Studies of other members of the *Closteroviridae* family suggest that the genes in the genomic arrangement specific for this family are likely conserved because of the biological implications of the proteins produced (Rubio et al., 1999). It is known that capsid proteins in viruses transmitted by vectors are subject to purifying selection due to the interactions between capsid proteins and cellular receptors in the vector, which are necessary for successful transmission (Chare & Holmes, 2004). Therefore, it is common to find that even though these genes have the potential to mutate, as is suggested by the number of non-synonymous substitutions, they preserve the more stable genomes because of negative selection on the new variants, causing the prevalence of one viral variant in the isolates.

The results obtained in this study are new and important when taking into account the variability of the three genes of PYVV in two potato species analyzed (*S. tuberosum andigena* and *S. phureja*) collected at the Colombian Department of Nariño, which is one of the most important growers of potatoes, and center of genetic diversity. Although we cannot yet correlate the genes variability with each one of the hosts, or the significance of changes within an isolate, the results suggest that mutation and recombination are very important process for CPm evolution. Despite these mechanisms, the genetic polymorphism of PYVV is very

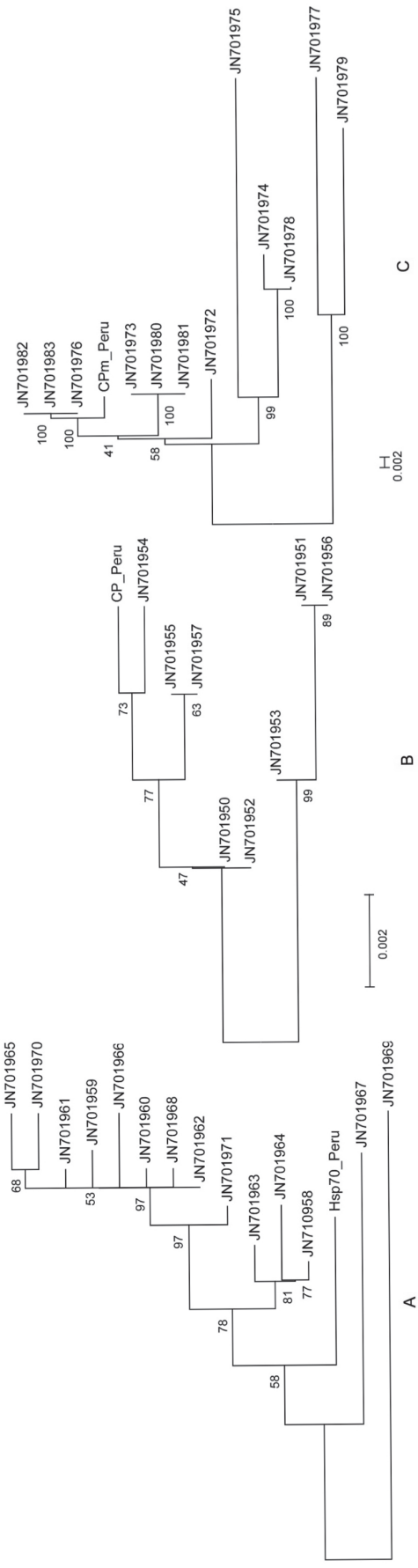

FIGURE 2 - Phylogenetic trees of full-length sequences of **A.** Hsp70; **B.** CP; and **C.** CPm genes of *Potato yellow vein virus* (PYVV).

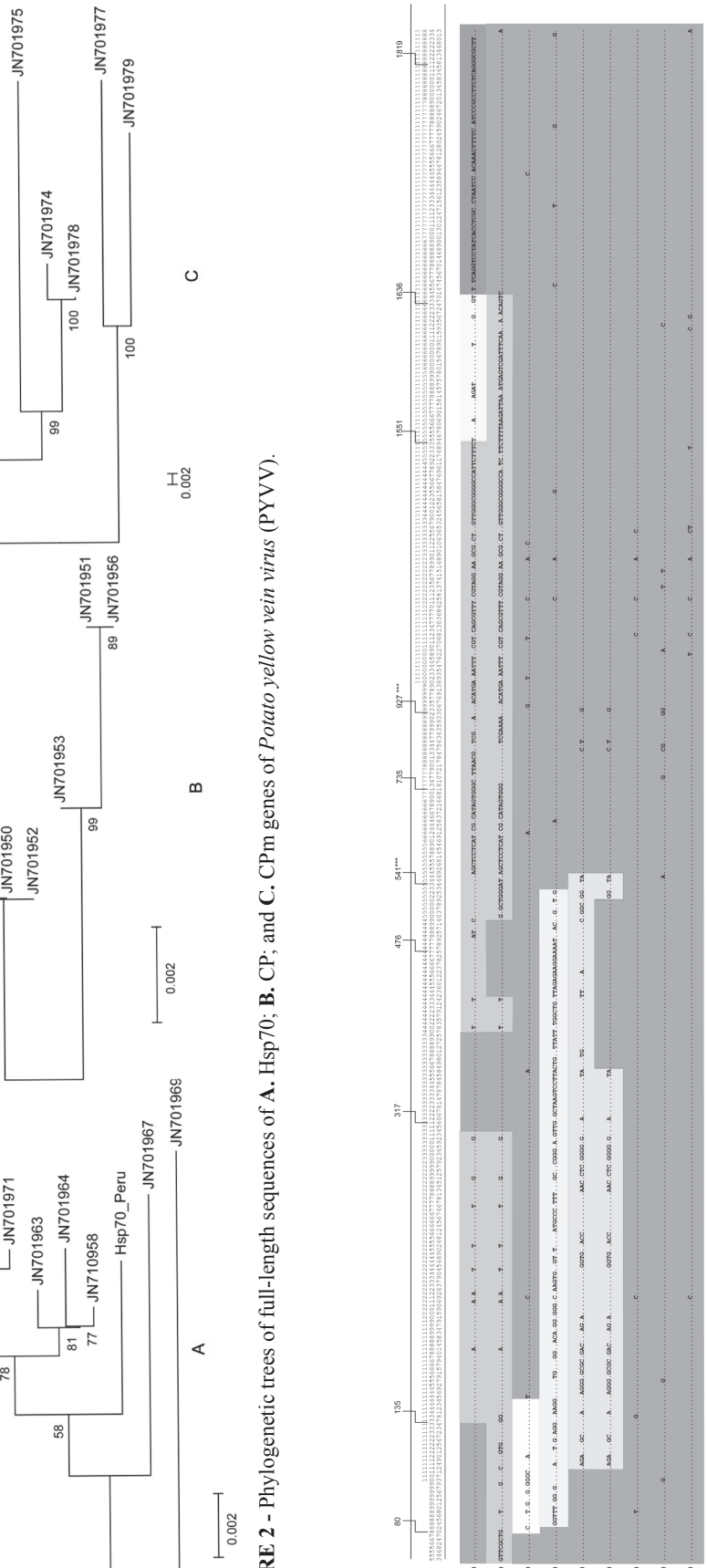

FIGURE 3 - Proposal of the genetic relationships among the different haplotypes of *Potato yellow vein virus* (PYVV). The left side shows the GenBank accession number for each isolate. The top indicates the positions of putative recombination breakpoints detected by GARD. The asterisks at positions 541 and 927 indicate a level of high statistical significance (p= 0.01) according to the Kishino-Hasegawa test (KH) for the topological inconsistencies in the breakpoint. The absence of asterisks indicates no significance. Pattern of gray scale was used in order to facilitate the recognition of the non-recombinant segments and putative breakpoints. The criterion of gray scale change is indicated in the text.

low considering it is a RNA virus. More studies are needed to estimate the prevalence of the recombinant isolates and for their biological characterization.

ACKNOWLEDGEMENTS

We are grateful to Colciencias, project 115-2007, project MARD MARD2007S4654-69, Dr. Liliana Franco (Universidad Militar Nueva Granada), and Patricia Rodríguez (Laboratorio de Vírus de Plantas, IBUN). We are also grateful to the Language Center from Universidad Francisco de Paula Santander in Cúcuta-Colombia for grammar corrections of the manuscript.

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TPP-2013-0143
Submitted: 16 August 2013
Revisions requested: 6 September 2013
Accepted: 13 December 2013
Section Editor: Jorge Alberto Marques Rezende