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BRIEF REPORT

Characterization of a new potyvirus infecting pepper crops in Ecuador

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Abstract Sequencing 2,951 nucleotides of the 3′ proximal region of the genome of a potyvirus isolate collected from *Capsicum pubescens* (rocoto) pepper in Ecuador revealed that this was the first representative of a new species tentatively named Ecuadorian rocoto virus (ERV). Phylogeny reconstruction showed that this isolate clustered with potato virus V (PVV), Peru tomato virus and wild potato mosaic virus into a monophyletic group, and was closest to PVV. The isolate was shown to be infectious in tobacco, tomato and, contrary to PVV, in pepper. The *pvr2*^{1}, *pvr2*^{2}, and *Pvr4* genes present in many pepper cultivars conferred resistance toward this isolate and could help control ERV.

Among plant viruses, the genus *Potyvirus* is one of the largest, with over 200 species differing in their biological properties such as the range of host plants. As a whole, potyviruses have a worldwide distribution and have been reported to infect over 500 plants species in more than 60 plant families [11]. Members of the genus *Potyvirus* are characterized by long (700–900 nm) flexuous particles composed of subunits of the coat protein (CP) arranged in a helical structure which entwines a positive-sense single-strand RNA molecule of approximately 10 kb. These RNA molecules encode a large polyprotein which is cleaved into ten functional proteins [1]. Potyviruses are transmitted nonpersistently by a large number of aphid species, and many of them cause important yield losses in solanaceous crops (potato, tomato, pepper, tobacco) throughout the world. Among these crops, pepper can be infected by members of nine different potyvirus species (Table 1). Potato virus Y (PVY) is distributed worldwide and is the only potyvirus affecting pepper crops in Europe [16]. Other pepper potyviruses have narrower geographical distributions corresponding to the different continents. Thus, tobacco etch virus (TEV) and pepper mottle virus (PepMoV) are prevalent in America [17, 20] and have been detected sporadically on other continents [3, 16], pepper yellow mosaic virus (PepYMV), Peru tomato virus (PTV) and pepper severe mosaic virus (PepSMV) have been described only in South America [5, 12, 23], chilli veinal mottle virus (ChiVMV) and chilli ringspot virus in Asia [9, 16] and pepper veinal mottle virus (PVMV) in Africa [16].

Unambiguous identification of potyviruses is not easy because they often induce similar symptoms, but it is essential to understand their epidemiology and to choose appropriate resistant plant cultivars. The range of plant hosts and serological properties have long been the essential tools used to identify members of different virus species [1]. Since close serological relationships can exist between members of distinct potyvirus species [22, 23], genome sequence analysis has become indispensable for reliable virus species and strain identification [1].

In this work, genomic and biological properties (host range and serology) of an original potyvirus isolate collected in 2006 in Riobamba (Ecuador) from fruit samples of *Capsicum pubescens* Ruiz and Pavon (called “rocoto” in

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Table 1 Potyvirus isolates used for biological and/or sequence analyses

Virus species[a]	Isolate	Country	GenBank accession no.
ChiRSV	**VN/C8**	**Vietnam**	DQ925438
ChiVMV	**India**	**India**	AJ237843
ERV	**Rocoto**	**Ecuador**	EU495234
PepMoV	**C**	**USA (California)**	M96425
	FL	**USA (Florida)**	AF501591
PepSMV	…	**Argentina**	AM181350
PepYMV	…	**Brazil**	AF348610
PTV	**PPK13**	**Peru**	AJ437280
	PPK11	**Peru**	AJ516010
	Cuzqueño 2	**Chile**	EU495235
PVMV	**CAC2**	**Senegal**	AJ780966
PVY	**SON41p**	**France**	AJ439544
	N605	**Switzerland**	X97895
	PO7	**Canada**	U09509
TEV	**HAT**	…	M11458
PVV	Dv42	Scotland	AJ243766
	…	Ireland	X61279
	PV0318 (DSMZ)	Germany	…
PVA	B11	Hungary	NC004039
WPMV	…	Peru	AJ437279

[a] Potyvirus species in bold are those whose members infect pepper

PVY Potato virus *Y*, *PTV* Peru tomato virus, *PVV* Potato virus V, *WPMV* Wild potato mosaic virus, *PepYMV* Pepper yellow mosaic virus, *PepMoV* Pepper mottle virus, *PepSMV* Pepper severe mosaic virus, *PVA* Potato virus A, *TEV* Tobacco etch virus, *ChiVMV* Chilli veinal mottle virus, *PVMV* Pepper veinal mottle virus, *ERV* Ecuadorian rocoto virus, *ChiRSV* Chilli ringspot virus, *DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen

the Andean region of South America) are described and compared to those of other potyviruses infecting solanaceous crops. This isolate, hereafter named Rocoto isolate, was originally identified as a member of the genus potyvirus based on its reaction, in ACP (antigen-coated plate)-ELISA, with the potyvirus-group antiserum (Agdia, Elkhart, USA). In order to confirm that the Rocoto isolate belongs to the genus *Potyvirus* and to identify its taxonomic position, the 3′ end of its genome was sequenced and compared to those of other potyviruses. Total RNAs were first extracted from infected pepper or tobacco plants using a Tri Reagent Kit (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions. Then, complementary DNA was obtained by a reverse transcription step with the poly-T primer (5′-CACGGATCCTTTTTTTTTTTTTTTTV-3′) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, USA). Primer pairs A (5′-GGBAAYAAYAGYG GDCARCC-3′) and poly-T [8] and B (5′-CCNGAYAM WGTKYTRTGGGG-3′) and C (5′-ATRCACCANACCA TNARNCC-3′) were used for PCR amplification of the CP-coding and 3′ non-translated regions (NTRs), and of the Nib-coding region, respectively. Primers B and C were designed, respectively, in regions of the Nia protease and CP of PVY, PTV, PepMoV, PepSMV, and TEV that are conserved at the amino acid level. RT-PCR products were purified and cloned into pGEM-T Easy Vector (Promega) and used to transform *Escherichia coli* DH5α competent cells according to standard transformation protocols. Sequencing of the clones was performed by Genome Express (Grenoble, France). At the same time, the 3′ end of the genome of a pepper isolate of PTV (Cuzqueño2) collected in Chile in 2006 (Table 1) was obtained and sequenced using the same strategy. Sequences obtained for these two isolates were deposited in GenBank (accession numbers EU495234 and EU495235). The sequence of the Rocoto isolate was first compared with virus sequences available in databases using the Blastn application (http://www.ncbi.nlm.nih.gov/BLAST/blastn). This research revealed that potato virus V (PVV), PTV, and wild potato mosaic virus (WPMV) were the viral species whose members are most closely related to the Rocoto isolate. With the aim of correctly identifying the taxonomic position of the Rocoto isolate, the 3′-proximal genome sequences of potyviruses infecting pepper or other solanaceous crops were retrieved from GenBank (Table 1) and aligned with those of the Rocoto and PTV Cuzqueño2 isolates. These sequence alignments were performed with ClustalW [25] with the default parameters and then checked by eye. Before calculating genetic distances between the aligned potyviruses, a detection of potential recombination events between sequences was performed with RDP version 3.22 [14], and this did not reveal any evidence of recombination. The genetic distance between the different potyviruses was then calculated from the multiple alignments. The Kimura 2-parameter substitution model and the Poisson-correction model in MEGA version 4 [24] were used to calculate nucleotide and amino acid distances, respectively. Excluding the 3′NTR, where the alignment showed numerous gaps, the nucleotide sequence of the Rocoto isolate was 77–78% identical to the homologous regions of PVV, PTV, and WPMV, and their encoded amino acid sequences were 85–87% identical. In this part of the genome (comprising about one-third of the total length), the Rocoto isolate was exactly as distant from PVV, PTV, and WPMV as these three potyviruses were from each other, both at the nucleotide and the amino acid level. Other potyviruses showed identity percentages lower than 70% at the nucleotide level and 75% at the amino acid level with the Rocoto isolate. Consequently, the Rocoto isolate should be considered a member of a new potyvirus species for which we propose the name Ecuadorian rocoto virus (ERV).

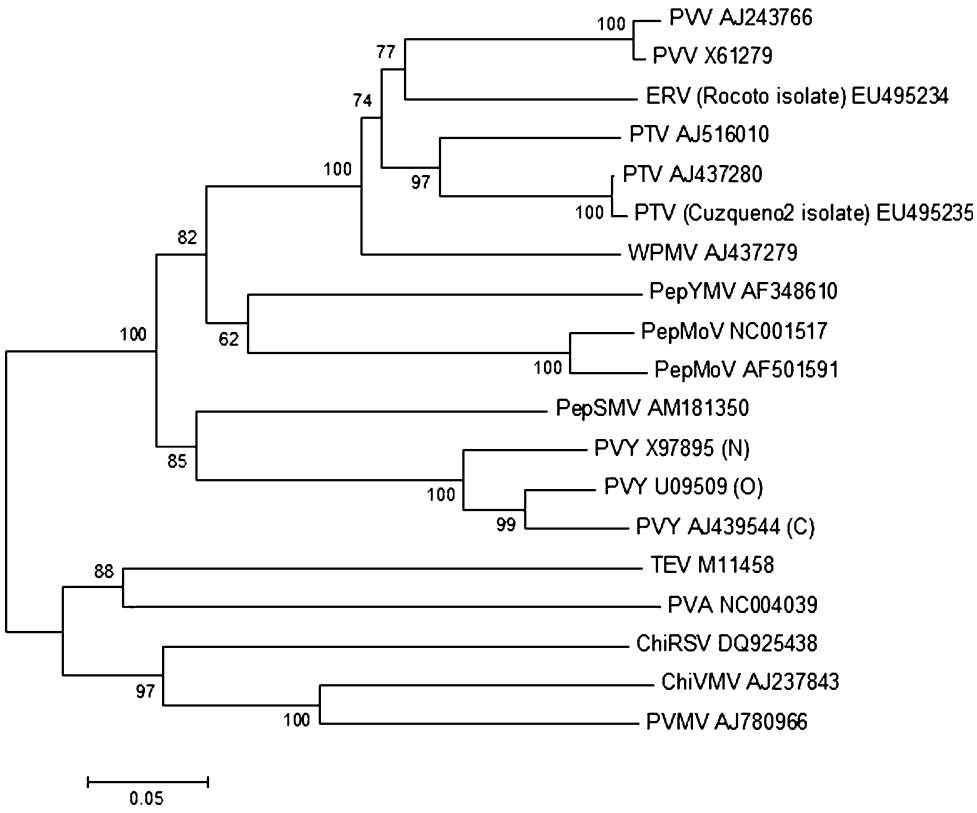

Fig. 1 Phylogenetic tree of potyviruses infecting solanaceous crops, obtained from a nucleotide alignment covering the 3′ end of the genome using the neighbor-joining method implemented in the MEGA 4 software. The reliability of the tree topology was assessed with 1,000 bootstrap samples (percentage values are indicated). The *scale bar* represents the relative genetic distance (number of substitutions per nucleotide). GenBank accession numbers of the sequence are reported

Nucleotide and amino acid sequence alignments of potyviruses were also used to reconstruct phylogenetic trees. Unrooted phylogenetic trees were constructed by several methods available in MEGA (neighbor-joining, maximum parsimony, minimum evolution, and UPGMA), and their robustness was evaluated with 1,000 bootstrap replicates. All of these methods consistently provided the same topology of trees, whatever the sequence alignments (nucleotides or amino acids). For the nucleotide alignment, the neighbor-joining tree was almost fully resolved, with bootstrap values greater than 70% except for PepYMV (62%) (Fig. 1). The topology of the phylogenetic trees revealed that the Rocoto isolate clustered with PVV into a monophyletic group (Fig. 1). Similarly, an alignment of the 3′NTRs showed that the Rocoto isolate was closer to PVV than to PTV or WPMV. Most of gap positions in the 3′NTR were shared by PVV and the Rocoto isolate but not with WPMV or PTV. Notably, a 25-nucleotide-long segment immediately upstream from the polyadenylated tail of PTV and WPMV was absent from the Rocoto isolate and PVV (data not show).

In order to accurately characterize this new potyvirus, the serological properties and host range of the Rocoto isolate were determined and compared to those of members of the two genetically closest potyvirus species, which are PVV and PTV (Fig. 1) using PV0318 and Cuzqueño2 as representative isolates (Table 1). DAS (double antibody sandwich)-ELISAs with seven specific antisera raised against PVY, PVV, PTV, PepMoV, TEV, ChiVMV, and PVMV were used to evaluate the serological properties of the isolates. Some of these antisera (PVY, ChiVMV, PVMV, and TEV) were described previously [16, 18]. The PepMoV antiserum was provided by V. Vance, and PVV and PTV antisera were obtained from the DSMZ. All of the isolates tested were first multiplied in *Nicotiana benthamiana* plants before DAS-ELISA. For all antisera tested, homologous reactions were always strong (10–30 times the average absorbance values at 405 nm [A_{405}] of healthy controls). The Rocoto isolate was not detected by any of the antisera tested, and no serological cross-reactions were observed between the PTV and PVV isolates and heterologous antisera (A_{405} values were below twice those of healthy controls). Aliquots of the same virus extracts were readily detected by the potyvirus-group antiserum in ACP-ELISAs. Although other studies have shown serological relationships between PTV and PVY [6], PVV and PVY [7] and, more weakly, reaction between PTV and PVV [23], these reactions could depend on the virus isolates or on antisera [22].

To characterize the host spectrum of the Rocoto isolate, a range of 45 plant genotypes was inoculated, and infectivity and symptoms were recorded and compared to PVV and PTV (Table 2). Plants were inoculated by rubbing the cotyledons or the first expanded leaves with the inoculum

Table 2 Infections and symptoms induced by three viruses: PTV (Cuzqueño2 isolate), ERV (Rocoto isolate) and PVV (PV 0318 isolate)

Plant				Virus					
Family	Genus	Species and cultivar	Resistance genes	ERV (Rocoto)		PTV (Cuzqueño2)		PVV (PV0318)	
				Symptom[a]	ELISA[b]	Symptom[a]	ELISA[b]	Symptom[a]	ELISA[b]
Amaranthaceae	Gomphrena	*G. globosa* 'Buddy'	…	Ø	−	Ø	−	Ø	−
Asteraceae	Cichorium	*C. endivia*	…	Ø	−	Ø	−	Ø	−
Chenopodiaceae	Chenopodium	*C. quinoa*	…	CLL	−	CLL	−	Ø	−
		C. amaranticolor	…	CLL	−	CLL	−	Ø	−
Cucurbitaceae	Cucumis	*C. melo* 'Védrantais'	…	Ø	−	Ø	−	Ø	−
		C. melo 'Charentais'	…	Ø	−	Ø	−	Ø	−
		C. sativus 'Beit Alpha'	…	Ø	−	Ø	−	Ø	−
	Cucurbita	*C. pepo* 'Black-Beauty'	…	Ø	−	Ø	−	Ø	−
	Citrullus	*C. vulgaris*	…	Ø	−	Ø	−	Ø	−
Fabaceae	Trifolium	*T. pratense*	…	Ø	−	Ø	−	Ø	−
	Phaseolus	*P. vulgaris* 'Pinto'	…	Ø	−	Ø	−	Ø	−
	Glycine	*G. max*	…	Ø	−	Ø	−	Ø	−
Lamiaceae	Ocimum	*O. basilicum*	…	Ø	−	Ø	−	Ø	−
Solanaceae	Nicotiana	*N. benthamiana*	…	SM, LD	+	SM, LD	+	SM	+
		N. clevelandii	…	SM	+	SM	+	SM	+
		N. glutinosa	…	MM	+	MM	+	SM	+
		N. sylvestris	…	MM	+	SM	+	MM	+
		N. debneyi	…	SM	+	SM	+	SM	+
		N. tabacum 'Xanthi'	…	CLL, MM	+	MM	+	MM	+
		N. tabacum 'Samsun'	…	MM	+	MM	+	CLL, MM	+
	Physalis	*P. pubescens*	…	SM	+	SM	+	MM	+
		P. pubescens 'Floridana'	…	SM	+	SM	+	SM	+
		P. peruviana	…	MM, VC	+	SM	+	Ø	−
	Petunia	*P. hybrida*	…	MM	+	MM	+	MM, LD	+
	Datura	*D. stramonium*	…	Ø	−	Ø	−	Ø	−
	Solanum	*S. sessiliflorum* (944750064, 'Costa Rica')[c]	…	NLL, SNL	+	SM	+	Ø	+
		S. sessiliflorum (A04750018, 'Peru')[c]	…	SM	+	SM	+	MM	+
		S. nigrum	…	Ø	+	Ø	+	Ø	+
		S. melongena 'Violette de Barbentane'	…	Ø	+	Ø	+	Ø	−
		S. tuberosum 'Spunta'	…	Ø	−	Ø	−	NLL	−
		S. tuberosum 'Safrane'	…	NLL	−	NLL	−	NLL	−
		S. lycopersicum 'Monalbo'	…	SM	+	SM	+	Ø	+
		S. lycopersicum 'Cervil'	…	MM	+	MM	+	Ø	+
		S. lycopersicum 'Microtom'	…	MM	+	MM	+	Ø	+
		S. pimpinellifolium 'West Virginia 700'	…	MM	+	MM	+	Ø	+
		S. habrochaites 'PI 134417'	*pot-1*$^{+}$	MM	+	MM	+	Ø	+
		S. habrochaites 'PI 247087'	*pot-1* [19]	MM	+	NT	NT	NT	NT
	Capsicum	*C. annuum* 'Yolo Y'	*pvr2*^{1} [21]	Ø	−	NT	NT	NT	NT
		C. annuum 'Florida VR2'	*pvr2*^{2} [21]	Ø	−	NT	NT	NT	NT
		C. annuum 'HD285'	*pvr2*^{3} [2]	SM	+	NT	NT	NT	NT
		C. annuum 'CM334'	*pvr2*^{3} + *Pvr4* [4]	Ø	−	NT	NT	NT	NT
		C. annuum 'Yolo Wonder'	*pvr2*$^{+}$	SM	+	SM	+	Ø	−
		C. frutescens 'Boca vieja'	…	SM	+	SM	+	Ø	−

[a] *Ø* no symptoms, *CLL* chlorotic local lesions, *MM* mild mosaic, *SM* severe mosaic, *NLL* necrotic local lesions, *SNL* systemic necrotic lesions, *LD* leaf deformations, *VC* vein clearing, and *NT* not tested

[b] ACP-ELISA performed with the Potyvirus-Group antiserum [Agdia]. Virus detection was considered negative when $A_{405} < 2$ times the healthy controls and positive when $A_{405} > 3$ times the healthy controls

[c] Seeds of *S. sessiliflorum* 'Costa Rica' [944750064] and 'Peru' [A04750018] were provided by the Botanical and Experimental Garden of the Radboud University, Nijmegen, the Netherlands

prepared as described by Moury et al. [16]. The inoculated plants belonged to seven families and 17 genera (Table 2). Four to ten seedlings, three to five weeks old, were inoculated and then placed in insect-proof greenhouses where temperature varied between 18 and 25°C and the experiment was reproduced two or three times, depending

on the plant genotype. Symptoms were recorded from 10 to 30 days after inoculation, and ELISAs were performed on non-inoculated apical leaves 30 days post-inoculation. Only solanaceous plants showed systemic infections and/or symptoms with these three isolates. Among solanaceous plants, only *Datura stramonium* remained uninfected at the systemic level with all three isolates (Table 2). The most important differences between the Rocoto isolate and PTV or PVV were observed in pepper and in potato. PVV was not infectious in the three pepper genotypes tested but induced necrotic local lesions on all *Solanum tuberosum* genotypes tested, whereas PTV and ERV were able to infect *Capsicum annuum* and *Capsicum frutescens* and induced necrotic local lesions only on *Solanum tuberosum* 'Safrane' (Table 2). Although these three virus isolates did not infect the *S. tuberosum* genotypes tested, PVV was previously shown to be infectious in some potato cultivars [7]. These results corroborate previous observations [6, 7] which showed that PTV was infectious in pepper while PVV was not. Plants in the other families (Amaranthaceae, Asteraceae, Chenopodiaceae, Cucurbitaceae, Lamiaceae, and Fabaceae) were not susceptible to the Rocoto isolate (no symptoms in apical leaves and no virus detected by ACP-ELISA). However, in plants of the genus *Chenopodium*, chlorotic and necrotic lesions were observed in leaves inoculated with PTV or with the Rocoto isolate (Table 2). In the plant genotypes tested, no species allowed the Rocoto isolate and the PTV isolate to be distinguished. Dissimilar symptoms were observed between these two isolates on one genotype of *Solanum sessiliflorum*, but some tomato isolates of PTV were shown to induce systemic necrotic symptoms similar to those induced by the Rocoto isolate in *S. sessiliflorum* 'Costa Rica' [15].

Pepper and *Solanum* spp. genotypes carrying a particular potyvirus resistance gene were also checked for their susceptibility to the Rocoto isolate (Table 2). Thus, recessive resistance alleles at the *pvr2* locus and the dominant *Pvr4* gene, which have been introgressed into many pepper cultivars to control PVY and other potyviruses [4, 21], and the *pot-1* resistance gene, an orthologue of the *pvr2* gene which confers resistance to PVY and which was introgressed in tomato [13, 19], were tested with the Rocoto isolate. Three independent inoculation experiments of ten plants per genotype were carried out. The results showed that the $pvr2^1$ and $pvr2^2$ (but not $pvr2^3$) alleles and the *Pvr4* gene in pepper controlled strong resistance against the Rocoto isolate, since no virus could be detected in apical leaves one month post-inoculation (Table 2), and these could be used to efficiently control ERV. In contrast, the *pot-1* resistance gene present in *Solanum habrochaites* 'PI247087' did not confer resistance to the Rocoto isolate (Table 2).

The fact that the Rocoto isolate represents a new South American potyvirus infecting solanaceous plants reinforces the hypothesis that this area corresponds to a hotspot of viral diversity for this group of plants. Since it is known to be the centre of origin for the domestication of many solanaceous crops (potato, tomato, and pepper) [10], it is plausible that the abundance and diversity of solanaceous plants in that area, both wild and cultivated, could have increased the diversification and differentiation of their viruses [23].

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