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The complete nucleotide sequence of a Spanish isolate of *Citrus psorosis virus*: comparative analysis with other ophioviruses

Brief Report

S. Martín[1], **C. López**[2], **M. L. García**[3], **G. Naum-Onganía**[3], **O. Grau**[3], **R. Flores**[2], **P. Moreno**[1], and **J. Guerri**[1]

[1]Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain
[2]Instituto de Biología Molecular y Celular de Plantas (UPV and CSIC), Valencia, Spain
[3]Instituto de Bioquímica y Biología Molecular, Universidad Nacional de La Plata, Argentina

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Summary. The complete genomic sequence (11278 nt) of *Citrus psorosis virus* (CPsV), isolate P-121 from Spain, was determined and compared with those from isolate CPV-4 and from other ophioviruses. The three RNAs of P-121 had similar size and identical organization as those of CPV-4. The 24K and the RdRp proteins were potentially encoded in the viral complementary (vc) strand of RNA 1, the 54K protein potentially encoded in vcRNA 2 and the coat protein encoded in vcRNA 3. These four proteins from P-121 and CPV-4 had 87, 92, 93 and 94% amino acid identity, respectively, but only 22, 38, 25 and 33% identity with their homologous proteins from *Mirafiori lettuce big vein virus* (MLBVV), the only other ophiovirus completely sequenced. Biological and genetic differences between CPsV and MLBVV (and the other ophioviruses), would support their future allocation in different genera within a tentative family *Ophioviridae*.

*

Citrus psorosis virus (CPsV), the type species of the genus *Ophiovirus* [16], is the presumed causal agent of citrus psorosis [15], a damaging graft-transmissible disease of citrus. CPsV virions are kinked filaments 3–4 nm in diameter of at least

Note: Nucleotide sequence data reported are available in the GenBank database under accession numbers: AY654892; AY654893 and AY654894.

two sizes [11], containing a single-stranded RNA [10] and a coat protein (CP) of 46–50 kDa, as estimated by electrophoretic mobility [5, 6, 10, 16, 23]. The viral RNA (vRNA) of isolate CPV-4 from Florida (formerly called citrus ringspot virus CRSV-4) has been sequenced and found to be composed of three molecules of negative polarity. RNA 1 is 8184 nucleotides (nt) in size and its complementary strand (vcRNA) contains two ORFs potentially encoding a 24 kDa protein (24K) of unknown function and an RNA-dependent RNA-polymerase (RdRp) [20]. RNA 2, of 1644 nt, encodes in its vcRNA a 54 kDa protein (54K) without similarities with other known proteins [29]. The vcRNA 3 (1454 nt) encodes the CP (48.6 kDa) [3, 28]. Presently, sequence data available from other ophioviruses include the full genome of *Mirafiori lettuce big vein virus* (MLBVV, formerly Mirafiori lettuce virus), which has four RNAs of 7.8, 1.7, 1.5, and 1.4 kb and seven ORFs [33], and partial sequences from the RNA 1 of *Ranunculus white mottle virus* (RWMV), *Tulip mild mottle mosaic virus* (TMMMV), Freesia ophiovirus (FOV) [32] and an ophiovirus associated with lettuce ring necrosis (LRNV) [30].

Psorosis isolates differing by symptoms induced in diverse hosts species [21, 26], CP size [11, 23] or reactivity with monoclonal antibodies against the CP [1, 2, 9] have been reported, suggesting a wide genetic variability of CPsV. However, sequence comparisons have been restricted to segments of RNA 1 from some Argentinean isolates [12] and of RNA 3 from Italian isolates [2]. Supporting this view, preliminary comparisons between partial RNA segments of CPV-4 and several Spanish CPsV isolates showed nucleotide identities of only 80.8 to 86.5% (unpublished data). The complete sequencing of CPsV isolate P-121 from Spain, here reported, has made possible a genome-wide comparison with the available sequences from isolate CPV-4 and from other members of genus *Ophiovirus.*

To prepare cDNA, P-121 virions were partially purified in a sucrose gradient as previously described [5, 22] and their RNA was extracted with TRIZOL® (Life Technologies). Using this RNA and primers based on the CPV-4 sequence (Table 1), a first group of cDNA clones (regions 1a, 1b, 1c, 1d, 2a and 3a, Fig. 1) was obtained by reverse transcription (RT) and PCR amplification with Thermoscript™ reverse transcriptase (Invitrogen) and the Advantage cDNA PCR kit (Clontech). The PCR products were electrophoresed in a 2% agarose gel, and cDNAs of the expected size were eluted from the excised bands using ultrafree-DA tubes (Millipore), ligated into the pGem-T vector (Invitrogen) and cloned [27]. The nucleotide sequences of at least two clones from each region were determined with an ABI PRISM DNA Sequencer 377 (PE Biosystems). To confirm that the amplified sequences were predominant in the P-121 isolate, digoxigenin-labeled probes of several clones were prepared by PCR amplification and hybridized in a dot-blot format with total RNA extracts of P-121 or CPV-4 infected tissues using the DIG-DNA Labeling and Detection Kit (Roche Diagnostics). Prehybridization and hybridization were at 42 °C or at 60 °C in 50% formamide, and washing and detection steps as described in Narváez et al. [19]. To fill the gaps, overlapping clones (regions 1f, 1g and 1h, Fig. 1) were synthesized by RT-PCR using the same RNA template and primers derived from the previously obtained P-121

Table 1. Primers used for reverse transcription and PCR amplification of viral RNA from CPsV isolate P-121

Region*	Primers (sense)	Nucleotide sequence 5′–3′	Positions[a]
1a	Ps10 (forward)	CATCATGAAAACATCCAGAATAATCGI	154–180
	Ps11 (reverse)	ATCCAGAATTATCATTGTGCTGTATCCI	626–599
1b	Ps12 (forward)	GAAACTGGACAAAGAAATAGGAGAAGAI	2029–2053
	Ps13 (reverse)	GTGTTCCGAATGATATGATTGTTGTAAI	2370–2344
1c	Ps6 (forward)	GAGGAAGGTATTTCCATAGG	4818–4837
	Ps7 (reverse)	CCTATAAATGATAATTGCAC	5020–4991
1d	Ps14 (forward)	CAGATGCTATAAAAACAGGGACAATGGI	7328–7355
	Ps17 (reverse)	CCCTTGTCCTATCTTTCTAAAI	7576–7555
1f	Ps20 (forward)	AATTTGGATGTTTATGCCTGAAATCGTTCAAGAATCACTG	181–220
	Ps21 (reverse)	AGATGCCATTAAATTCAGTTCCTGTTCTAGCAACCCTGA	2335–2297
1g	Ps33 (forward)	ACATATGCTGAGGTCAATGAGCTAGAGGGTCTAAG	2126–2160
	Ps34 (reverse)	GATTTGATCAAGCATGAATGTCACATCTCTGGCAA	5103–5064
1h	Ps24 (forward)	CAGGAGACTTATCATCCAACATGTTAAGTTCAAGAG	4866–4901
	Ps23 (reverse)	AGGTTGCTTGATTGTATTATGATGTTCCTTTCTTCTG	7520–7485
1i	Ps50 (forward)	GATACTTTTTTTCAAGAAAAAGTGATCTAGCTGAAACATG	1–40
	Ps51(reverse)	TGAGTCATCAGAGTATGTGGTTCCTATATATTTTTCTATTCC	3196–3155
1j	Ps52 (forward)	GCAGAAATATAAAATATGTCGATTAATAGAAAAGGAAAAAGA	2640–2679
	Ps53 (reverse)	CTTGTCCTCAAATATTGTGATTTTCAATGTATTCCC	5683–5647
1k	Ps54 (forward)	CAGTTAATGAAGACATAGCAGGCAAGTTCATAGATCA	5214–5250
	Ps59 (reverse)	TCATTTTCCTCCAAAATACTTATCAATTTATCATCAC	6773–6758
1l	Ps58 (forward)	ATAGAATCACCTGGTTATATATATTTGTTATTTATTGG	6524–6564
	Ps56 (reverse)	TAATTAGGATTATCGTCTTGTTAGACAATTGATAGCGC	8180–8143
1n	RF287 (reverse)	CACTGTCACACCTAGATATCATGAAGAATTCTGAGTCC	380–343
1m	RF288 (forward)	GAACAGGATGCTGGTGTTATCAGAAGAAAGGAACATC	7465–7501
2a	PM55 (forward)	TTGACAATGATGGACACTGG	1052–1071
	Ps25 (reverse)	AGGGGCACAATGCTTTGACC	1605–1586
2d	Ps69 (forward)	GATACTTTTTTTTGTGATTAAAGCATCACGTTGAC	1–35
	Ps70 (reverse)	GTCATCCTGGTGACATTTCCACGCC	1642–1618
2b	RF259 (reverse)	GTTTGGCCCGCTCAACTTCTAGTTGGGCTACTCCTCC	1169–1133
2c	RF258 (forward)	GGATGATGTTTGGGCTGAGGTGACTGAGCATGGCTCG	1384–1420
3a	CPV-3b (forward)	ATGTCGATTCCTATTAAAG	59–77
	CPV-4b (reverse)	TTAAAGCATAACATGCAAGC	1423–1403
3d	Ps71 (forward)	GATACTTTTTTTTGTGGAAAAAGCATCACTTTGTCA	1–36
	Ps72 (reverse)	GCGAATTTGGGAACTTTTGTTTATTTAAAACGAT	1447–1414
3b	Ps19 (reverse)	AATGACTCTTTTGTCCTTTGATAACCCGGACAGAAAGGCTG	244–204
3c	Ps18 (forward)	GGCCGAGAGGATAATAACCGACAAGAACAAGGGGTTTCA	1204–1242
	PM1	CCGGATCCTTCTAGAGCGGCCGC$(T)_{17}$V; (V = A,C or G)	

*See Figure 1 for location of these regions in the different genomic RNAs
[a]Referred to the sequence of the CPsV isolate P-121

sequences (Table 1). To prepare cDNA clones of the 5′- and 3′-termini (regions 1n, 2b, 3b, 1m, 2c, and 3c, Fig. 1), virion RNA was denatured, polyadenylated and reverse transcribed using primer PM-1, with a $(dT)_{17}$ 3′-tail, and the cDNA was PCR-amplified with PM-1 and specific internal primers (Table 1), following

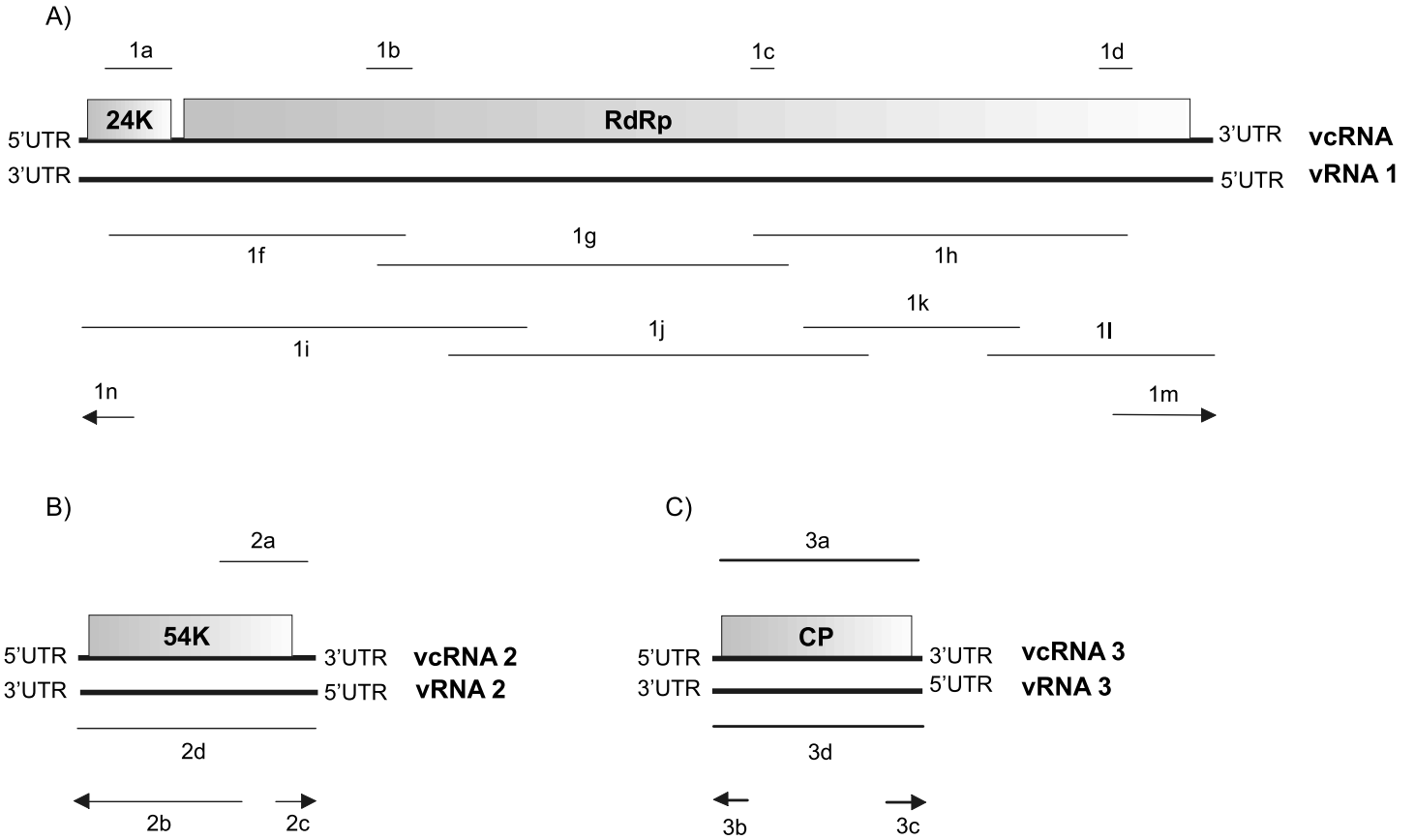

Fig. 1. Schematic representation of the genome of *Citrus psorosis virus* (CPsV) and the strategy for cloning and sequencing the genomic RNAs 1 (**A**), 2 (**B**) and 3 (**C**) of the CPsV isolate P-121. Boxes represent ORFs, with the putative protein products indicated inside. Clones indicated above the boxes were prepared by RT-PCR using primers based on the sequence of the isolate CPV-4, and those below the boxes by RT-PCR with primers derived from known P-121 sequences or from sequences conserved in P-121 and CPV-4. Clones of the 5′ and 3′ ends (arrows) were obtained by polyadenylation of viral RNA, reverse transcription using primer PM-1, with a $(dT)_{17}$ tail in its 3′ end, and PCR amplification using PM-1 and specific primers derived from the P-121 sequence (Table 1)

a previous protocol [13]. To further confirm the sequence, fragments 1i, 1j, 1k, 1l, 2d and 3d (Fig. 1) were RT-PCR amplified using virion RNA and primers based on sequences conserved in CPV-4 and P-121 isolates (Table 1), and then cloned and sequenced. Sequence comparisons were performed with the GAP program and ORFs were predicted with the FRAMES program, both of the GCG package [7]. Searches for protein similarities in non-redundant GenBank CDS translations, PDB, SWISSPROT, PIR, PRF and conserved domains (CDD) data bases were done with the BLAST program.

The sequence of the first cDNA clones of isolate P-121 (regions 1a, 1b, 1c, 1d, 2a and 3a of the Fig. 1) showed identities as low as 80.3% with isolate CPV-4, raising the question of whether they represented minor variants of the P-121 population. However, P-121 probes derived from these cDNA clones hybridized intensely with RNA extracts from both P-121 and CPV-4 isolates at 42 °C, whereas hybridization at 60 °C only gave an intense signal with the P-121 extract, suggesting that the PCR-amplified DNAs represented major components of the viral population.

The full genomic sequence (11278 nt) of isolate P-121 was determined from at least two independent overlapping cDNA clones, with identity between overlapping regions being always over 99%. A total of 86 mismatches were observed: four were located in untranslated regions (UTRs), 29 were silent, and 18 caused conservative amino acid substitutions.

Table 2. Nucleotide (*nt*) identity and aminoacid (*aa*) identity and similarity (%) between the different ORFs and the untranslated regions (UTR) of the CPsV isolate P-121 compared to the corresponding regions of CPV-4 isolate and MLBVV

	Region (vcRNA)	CPsV isolate CPV-4			MLBVV		
		Identity			Identity		
		nt	aa	aa similarity	nt	aa	aa similarity
RNA 1	5′-UTR	86.5			48.6		
	24K	82.9	86.9	92.2	38.3	22.4	35.8
	IgR	45.3			49.1		
	RdRp	81.8	91.6	95	50.7	37.7	50.5
	3′-UTR	64.6			40.9		
	Average	81.1			49.9		
RNA 2	5′-UTR	89.1			57.1		
	54K	82.4	92.8	96.4	42.6	24.9	37.4
	3′-UTR	89.8			40.1		
	Average	83.3			41.9		
RNA 3	5′-UTR	86.2			46.3		
	CP	85.5	94.1	95.9	45.5	33.3	45.5
	3′-UTR	82.6			39.1		
	Average	85.5			45.5		

The genomic organization of P-121 was identical to that of CPV-4 isolate (Fig. 1). Its RNA 1 was 8186 nt in size, two nt longer than CPV-4 RNA 1 (GenBank accession AY224663), due to minor (2–5 nt) deletions and insertions in the intergenic region (IgR) and in the 3′-UTR. The overall nucleotide identity between P-121 and CPV-4 RNA 1 was 81%, with the lowest values in the IgR and 3′-UTR (45.3% and 64.6%, respectively), and the highest in the 5′ UTR an in the ORFs encoding the RdRp and the 24K protein (86.5, 81.8 and 82.9%, respectively) (Table 2). The IgR of P121 did not contain either the sequence $(UUAAAA)_3$ or the putative polyadenylation signal (AAUAAA) found in isolate CPV-4 [20].

The two ORFs predicted in the P-121 vcRNA 1 had the same size as those potentially encoding the RdRp and the 24K protein in isolate CPV-4 (Fig. 1). ORFs potentially encoding polypeptides up to 11 kDa were also found in the P-121 vRNA 1, but they differed in size from those reported in CPV-4, suggesting that they may not be expressed *in vivo*.

The 24K protein of P-121 showed significant similarity with that of CPV-4 isolate, but not with the homologous 25K protein of MLBVV (GenBank accession NC_004779). More specifically, the amino acid identity (similarity) between the P-121 and the CPV-4 24K proteins was 86.9% (92.2%), whereas the identity (similarity) between the P-121 or CPV-4 24K proteins and the 25K protein of MLBVV was only 22% (36%) (Table 2).

The predicted amino acid sequence of the P-121 RdRp showed 91.6% (95%) identity (similarity) with that of the CPV-4 isolate, whereas the RdRp of both CPsV isolates had only 38% (50%) identity (similarity) with the RdRp of MLBVV. Identity (similarity) between the P-121 and the CPV-4 RdRp increased up to 99.1% (99.6%) in the core module, comprising the five conserved motifs proposed to be part of the polymerase active site [18, 24]. The polymerase core module of CPsV P-121 showed 63% (70%) and 61% (73%) identity (similarity) with the core modules of MLBVV and RWMV (GenBank accession AF335429) ophioviruses, respectively, whereas the identity (similarity) between the core modules of MLBVV and RWMV was 84% (89%), suggesting that these viruses are more closely related to each other than to CPsV.

The RNA 2 of P-121 had 1645 nt, 1 nt more than the RNA 2 of isolate CPV-4 (GenBank accession AF218572), due to an extra C at its 3′ terminus, whose presence in CPV-4 could not be ruled out [29]. Nucleotide identity between RNA 2 of the two CPsV isolates was 83.3%. The vcRNA 2 of isolate P121 contained a single ORF potentially encoding a 54 kDa protein, as reported for isolate CPV-4 [29] but no other ORF of significant size was shared by vRNA 2 of both isolates. This is an important difference with vRNA 2 of MLBVV (GenBank accession NC_004781) in which van der Wilk et al. [33] found an additional ORF potentially encoding a 10 kDa protein. Database search showed that the 54K protein of P-121 isolate only had similarity with the 54K protein of isolate CPV-4 and with the 55K protein of MLBVV. Alignment of the predicted amino acid sequences showed that the 54K proteins of P121 and CPV-4 isolates had 92.8% (96.4%) identity (similarity), whereas identity (similarity) between these proteins and the 55K protein of MLBVV was only 24% (38%).

The RNA 3 of P-121 was 1447 nt in size, 7 nt shorter than the CPV-4 RNA 3 (GenBank accession AF060855), due to an internal deletion in the 5′-UTR. The nucleotide identity between the RNA 3 of both isolates (85.5%) was higher than that found for the two other RNAs. The P-121 vcRNA 3 also had a single ORF encoding a 48.7 kDa protein, whose predicted amino acid sequence showed 94.1% (95.9%) identity (similarity) with the CP of the CPV-4 isolate and 33.3% (45.5%) identity (similarity) with the CP of MLBVV (Table 2) (GenBank accession NC_004782). Similarity between LNRV CP and the CP of CPsV and MLBVV were 53.8% and 69.7%, respectively [30].

Comparison between the 5′-UTRs of CPsV isolates P-121 and CPV-4 showed 86.5%, 89.1% and 86.2% nucleotide identities in vcRNAs 1, 2 and 3, respectively (Table 2). The 5′-terminal sequence 5′-GAUAC$(U)_7$-3′, conserved in the three vcRNAs of CPV-4, was also present in P-121. This 5′-terminal sequence resembles, but is not identical to, that found in MLBVV (5′-GAUUA$(U)_4$-3′), contrasting with observations in other negative-stranded RNA viruses with segmented genome, in which species of the same genus have the 5′ termini of their vcRNAs conserved.

The nucleotide identities between the 3′-UTRs of vcRNAs 1, 2 and 3 from P-121 and CPV-4 isolates were 64.6, 89.8 and 82.6%, respectively (Table 2). However, the 3′-terminal sequences of these regions were more conserved, with identity values of 90% in the 3′-terminal 68 nt of vcRNA 1, 96% in the 3′-terminal 148 nt of vcRNA2, and 92% in the 3′-terminal 60 nt of vcRNA 3, suggesting that these sequences might be involved in recognition by the polymerase during RNA replication. The 5′-UCAAAGCAUUG-3′ motif was found close to the terminus of the 3′-UTR of vcRNA 1 and 2 of both P-121 and CPV-4 isolates. As reported for CPV-4 [29], no complementarity was found between the 5′- and 3′-terminal sequences of P-121 RNAs 1, 2 or 3, in spite of the circular structures observed for CPsV virions by electron microscopy [11, 15].

Sequencing of the genomic RNAs of P-121 has allowed examination of the variation along the full genome of two CPsV isolates. Although the genomic organization of P-121 and CPV-4 was identical, their overall nucleotide identity was 82%. Sequence comparisons of the CP gene of 19 CPsV isolates from Campania (Italy), showed 97–100% identity between them [2] and with the CP gene of P-121 (this work), but only 85–86% with the CP gene of CPV-4, suggesting that CPV-4 might be an atypical isolate and that the Italian and the Spanish isolates have a common origin.

In spite of genetic variation between isolates, CPsV shows broader biological and molecular differences from the other ophioviruses. First, CPsV is presently the only ophiovirus whose natural hosts are woody plants (species of *Citrus* and related genera) and in most areas it is dispersed only by propagation of infected buds. Natural spread of psorosis based on symptom observation has been reported in limited citrus areas [4, 26], but spatial patterns suggested an aerial vector [8]. In contrast, MLBVV, LRNV and TMMMV are transmitted by *Olpidium brassicae* [14, 17, 25, 30], a root-infecting fungus not reported colonizing citrus. Second, Western-blot assays have revealed serological relationship between the

CPs of MLBVV, RWMV, TMMMV and LRNV, but not with the CP of CPsV [25, 30, 31]. Third, virions of CPsV isolates CPV-4 and P-121 have three RNAs, whereas MLBVV and LRNV have four [30, 33]. The genomic nature of the fourth RNA detected in an isolate of RWMV [31] was not established. Fourth, CPsV and MLBVV also differ: i) by the 5′-terminal sequence conserved between their genomic RNAs, ii) by the presence of 5′- and 3′-terminal complementary sequences in MLBVV RNAs, but not in CPsV RNAs, and iii) by the 10 kDa polypeptide potentially encoded in the MLBVV vRNA 2 not found in CPsV. And fifth, sequence similarity between proteins encoded by CPsV and MLBVV is weak or non-significant, whereas similarity between proteins encoded by MLBVV, RWMV and LRNV ophioviruses is much higher.

Based on RdRp phylogenetic relationships between ophioviruses and other negative-stranded RNA viruses, Naum-Onganía et al. [20] proposed the creation of a new family (*Ophioviridae*). Biological and molecular differences between CPsV and MLBVV (and the other ophioviruses), would support their future allocation in different genera within this tentative family. However, additional ophiovirus sequences will be necessary for such taxonomic revision.

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Author's address: Dr. José Guerri, Instituto Valenciano de Investigaciones Agrarias Ctra. Moncada-Náquera Km. 4.5, 46113 Moncada, Valencia, Spain; e-mail: jguerri@ivia.es