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SUMMARY

Potato virus Y (PVY) is one of the rare plant viruses for which some biological traits (host range and symptomatology) are highly correlated to phylogeny, allowing the reconstruction of evolutionary history of these traits. Here, a new lineage of PVY isolates from Chile is described, showing unique genome and biological properties. This lineage was found to be the sister group of all other PVY isolates and helped reconstructing ancestral traits and evolutionary history of PVY, suggesting that veinal necrosis in tobacco was an ancestral state and that adaptation to pepper (*Capsicum* spp.) and potato (*Solanum tuberosum*) was modified several times during PVY history.

Potato virus Y (PVY), the type member of the genus *Potyvirus*, is a major pathogen of solanaceous crops such as potato, tobacco and pepper. Isolates of PVY largely differ by their pathogenicity properties in differential host species and cultivars (De Bokx and Huttinga, 1981; Gooding and Tolin, 1973; Gebre Selassie et al., 1985). These biological properties are partly correlated to PVY phylogeny. Based on genome sequences, three major lineages can be distinguished among PVY, named O, C and N (Moury et al., 2002). Only the isolates from the O lineage induce hypersensitive reactions associated to resistance in potato cultivars carrying the Ny_{tbr} resistance gene, while only the isolates from the C lineage induce similar reactions in potato cultivars carrying the Nc_{tbr} gene and only the isolates from the N lineage induce systemic veinal necrosis in a set of tobacco cultivars (Kerlan *et al.* 1999). The C group was further divided into two phylogenetic subgroups, isolates from the C1 subgroup being able to infect pepper (*Capsicum annuum*) contrarily to those from the C2 subgroup (Blanco-Urgoiti et al., 1998). In addition, many inter- and intra-lineage recombinant isolates have been characterized (Revers et al., 1996; Moury et al., 2002; Glais et al., 2002; Fanigliulo et al., 2005; Ogawa et al., 2008). The O, C and N letters have also been used to classify PVY isolates according to symptomatology or serological properties (Singh et al., 2008). In this article they designate phylogenetic groups which correspond to some biological traits shared by non-recombinant PVY isolates. More than forty complete genomic sequences and more than 240 coat protein (CP) cistron sequences of PVY are available in databanks, providing a quite exhaustive image of its diversity. Almost all of them fall into the O, N or C lineages or are recombinants between these lineages. A tobacco isolate from Chile was suspected to belong to another PVY lineage (Sudarsono et al., 1993), but only a small part of its genome has been sequenced (GenBank accession number X68221) and

no phylogenetic analyses were provided to support that assumption. Like isolates from the N group, this Chilean isolate induced veinal necrosis in tobacco (Sudarsono et al., 1993).

Three PVY isolates (Chile1, Chile2 and Chile3) were obtained from distinct plants of pepper *Capsicum baccatum* L. cv. Crystal, familiarly termed "ají" throughout South America, collected in Chile in 2005. They were inoculated once to *Nicotiana tabacum* cv. Xanthi plants to obtain high-titer inocula for tests on different solanaceous plants and for genome analyses. The symptoms induced by these three Chilean isolates in reference tobacco, potato and pepper genotypes were investigated and compared to those induced by isolates N605, O139, C Adgen and SON41p, representative of PVY groups N, O, C2 and C1, respectively (Table 1). The Chilean isolates exhibited symptoms which are typical of two different groups of PVY. Like isolates of the C1 group they were infectious in pepper *C. annuum* cv. Yolo Wonder and induced mosaic symptoms at the systemic level in these plants and like isolates of the N group they induced necrotic symptoms in leaves of tobacco Xanthi at the systemic level. Due to their peculiar host range and symptom traits, the Chilean isolates could be helpful to unravel the evolutionary history of PVY. Therefore, I determined the full-length genome sequence of one of these isolates (Chile3; GenBank accession no. FJ214726) and partial genome sequences of the other two (GenBank accession nos. FJ951642 to FJ951647).

Total RNAs from leaves of systemically-infected Xanthi plants were purified with the Tri Reagent kit (Molecular Research Center, Cincinatti, USA) and used for reverse transcription-polymerase chain reaction (RT-PCR) with Avian myeloblastosis virus reverse transcriptase (Promega, Madison, USA.) and *Taq* DNA polymerase (Promega). PVY-polyvalent primers (see

Supporting Table S1) were used to amplify and sequence parts of the helper component-proteinase (HcPro) and viral protein genome-linked (VPg) cistrons and the total CP cistron and 3' untranslated region (UTR). These sequences allowed the design of specific primers to amplify and sequence the remaining parts of the genome of the Chile3 isolate. Sequencing reactions were performed directly on RT-PCR products by Genome Express (Grenoble, France). The three Chilean isolates were 97.1 to 99.5% identical based on a total of 1331 sequenced nucleotides. Compared to sequences available in the GenBank database, the Chilean isolates clearly belonged to the species *Potato virus Y*, sharing 92.7 to 94.3% nucleotide identity (based on complete genome alignments with the Chile3 isolate) with other PVY isolates, but had unique genome properties. The most obvious difference was located in the 3' untranslated region (UTR). The 3' UTR of all three Chilean isolates was 79 nucleotides longer than that of other PVY isolates (excluding the poly-adenylated tail), which corresponds probably to the tandem duplication of a 68-nucleotide-long segment in the 3' UTR, including a 53-nucleotide-long stem-loop structure which was consistently predicted by the use of the mFOLD version 2.3 program (Zucker, 1989). As a result, the three Chilean isolates were predicted to possess two stem-loop structures corresponding to that genome region while isolates from the N, O or C groups were predicted to possess only one of these structures (Fig. 1). The fact that the boundaries of the sequence duplicated in the Chilean isolates roughly correspond to a predicted stem-loop structure strongly suggests an important biological function for that structure. Haldeman-Cahill et al. (1998) showed that secondary structures in the 3' UTR of another potyvirus were involved in genome amplification. Short insertion/deletion polymorphisms specific of the Chilean PVY isolates were also observed in the 5' UTR and in the P3 cistron (data not shown). The unique genome properties of the Chilean PVY isolates were confirmed by phylogenetic analyses.

To root the PVY tree, an outgroup, as close as possible to PVY, must be chosen. Bidens mosaic virus and Sunflower chlorotic mottle virus are the viruses closest to PVY and are considered as distant PVY isolates by some authors (Inoue-Nagata et al., 2006; Dujovny et al., 2000). However, only a small part of the genome of these two viruses, at the 3' end, is available. *Pepper severe mosaic virus* (PepSMV) is more distant to PVY, but the sequence of its whole genome has been determined (Ahn et al., 2006), giving access to more information. Consequently, two separate analyses were conducted, one with the CP cistron of PVY and Bidens mosaic virus, Sunflower chlorotic mottle virus or PepSMV as outgroups, and the other with full-length genomes of PVY and PepSMV as outgroup. Nucleotide sequences were aligned with PVY sequences available in GenBank (in May 2008) using the ClustalW program (Thompson et al., 1994) and analyzed with the RDP version 2 software (Martin et al., 2005) implementing several algorithms to detect putative recombinant sequences. Only recombination sites detected by more than two out of six independent methods with the default probability threshold were considered. A large number of full-length genome sequences of PVY were found to be recombinants in this and/or previous studies (Revers et al., 1996; Moury et al., 2002; Glais et al., 2002; Fanigliulo et al., 2005; Ogawa et al., 2008) (see a list of accession numbers of recombinant isolates in Supporting Information). In further analyses, only non-recombinant PVY sequences were included and all the nucleotide positions that contained insertion/deletion polymorphisms in the alignments were excluded.

Analysis of the CP cistron revealed that the three pepper Chilean isolates clustered together and with the tobacco isolate collected in Chile (GenBank accession no. X68221) that was previously suspected to belong to a new PVY lineage (Sudarsono et al., 1993) (a 100%

bootstrap value supported the clade composed of these four isolates, see Supporting Fig. S1). The other 88 PVY isolates included in the analysis belonged to the four PVY groups N, O, C1 and C2. The precise topological position of the Chilean group relative to the other PVY groups could not be reliably established due to insufficient information in the CP cistron. Indeed, the quartet puzzling maximum likelihood (ML) method implemented in TREE-PUZZLE version 5.2 (Strimmer and Von Haeseler, 1997) did not support a privileged tree topology between the N, O+C1+C2 and Chilean groups of PVY and Bidens mosaic virus as an outgroup (22 to 47% support for the three possible topologies between these four clades). This is illustrated by the low bootstrap values that supported the internal branches linking groups C1, C2, Chile, O and N (see Supporting Fig. S1). Using Sunflower chlorotic mottle virus or PepSMV as outgroups for this genome region provided similarly ambiguous results (data not shown).

Applied to the full-length genome dataset, the quartet puzzling method supported unambiguously the clustering of the N and O+C1+C2 groups of PVY separate from the Chilean group of PVY and PepSMV (100% probability support for this topology against the two alternative ones). This was confirmed by the ML method implemented in PhyML version 3.0 (Guindon and Gascuel, 2003), incorporating the Tamura-Nei+Γ+I nucleotide substitution model which was selected by the MODELTEST program (Posada and Crandall 1998) as the most appropriate for this nucleotide sequence alignment. With this method, the clustering of the N, O and C groups of PVY was supported both at the nucleotide and amino acid levels by a 92% bootstrap value (Fig. 2). These results indicate that the Chilean group of PVY isolates diverged earliest during PVY evolution, *i.e.* it is the sister group of all other PVY groups of isolates.

Diversity in the VPg of the Chilean PVY isolates was shown to correlate with their adaptation to *pvr2* recessive resistance alleles in pepper. The Chile1 and Chile3 isolates were shown to belong to pathotype (0,3), *i.e.* they were able to infect pepper plants homozygous at the $pvr2^3$ resistance allele or devoid of resistance allele ($pvr2^+/pvr2^+$), while Chile2 belongs to pathotype (0,1,3), *i.e.* it is additionally able to infect pepper plants homozygous at the $pvr2^1$ resistance allele (Table 1). The amino acid sequence of the VPg virulence factor towards the *pvr2* resistance alleles is identical for Chile1 and Chile3, whereas it differs at positions 117 and 120 for Chile2 (Fig. 3). As the VPg cistron was previously demonstrated to be the virulence determinant of PVY towards *pvr2* (Moury et al., 2004; Ayme et al., 2006, 2007), amino acid substitutions at one or both of these sites are likely to be responsible for this difference. During the tests, two and three *C. annuum* cv. Yolo Y plants ($pvr2^1/pvr2^1$) showed late systemic infections after inoculation with the Chile1 and Chile3 isolates, respectively (Table 1). Further analyses revealed that PVY variants virulent towards the $pvr2^1$ resistance allele were selected in these five plants since (i) 100% of Yolo Y plants were infected after back-inoculation by isolates from these five plants and (ii) a single nucleotide substitution was observed in the VPg cistron of the PVY populations in these five plants compared to the original isolates (causing a serine to glycine substitution at amino acid position 105 of the VPg; Fig. 3). It is however unknown if these virulent variants pre-existed at low frequency in the original inocula or if they appeared by mutation in the inoculated Yolo Y plants. Together, these results indicate that amino acid substitutions at positions 105 and 117 and/or 120 of the VPg affected the virulence properties of the Chilean isolates towards the *pvr2* resistance alleles of pepper. Positions 105 and 120 were already shown to determine virulence changes towards the *pvr2* resistance alleles of pepper in a PVY isolate which belonged to the C1 group (Ayme et al., 2006, 2007).

Combining biological traits of the members of the major PVY clades and the topology of their phylogenetic tree allows inferences to be made about their evolutionary history and about their ancestral and derived traits. In addition, identification of the new 'Chilean' clade helped discriminate between various evolutionary scenarios. Systemic veinal necrosis in a number of tobacco cultivars is one of the traits that have long been used to discriminate between the different groups of PVY, defining the N group. The three pepper Chilean isolates together with the previously identified tobacco Chilean isolate (Sudarsono et al., 1993) induce necrosis in tobacco, while isolates belonging to the O and C groups do not (Table 1). Before the characterization of the Chilean group of PVY isolates, the two evolutionary scenarios considering that tobacco necrosis was either an ancestral or a derived trait were equally parsimonious and both could be reconstructed with only one phenotypic evolution step (Fig. 4A). Including the Chilean group of PVY now suggests that the ancestral state was more probably "necrotic", since one evolutionary step (versus two steps when necrosis is considered a derived trait) is enough to reconstruct PVY history (Fig. 4B). Mutations at amino acid positions 400 and 419 of the HcPro of PVY were shown to determine veinal necrosis in tobacco (Tribodet et al., 2005). Confirming the above evolutionary hypothesis, the three pepper Chilean isolates were shown to possess a lysine and a glutamic acid at positions 400 and 419, respectively, of their HcPro, similarly to the necrotic isolates from the N group of PVY. In contrast, almost all non-necrotic PVY isolates in the O and C groups possess an arginine and an asparagin at positions 400 and 419, respectively, of their HcPro. Consequently, the scenario where veinal necrosis is the ancestral state of PVY requires only two amino acid substitutions whereas the alternative scenario requires four amino acid substitutions. Note that analysing codon evolution instead of

amino acid evolution at positions 400 and 419 of HcPro did not help discriminate further between these scenarios (data not shown).

The scenario where tobacco necrosis evolved twice from non-necrotic PVY isolates through the fixation, in parallel, of the same two amino acid substitutions in the HcPro (Fig. 4B) would suggest that these substitutions conferred a strong fitness advantage to the virus. However, recent results indicate instead that the amino acid substitutions which confer necrosis in tobacco are costly to the virus (Rolland *et al.*, 2009). Consequently, both the phylogenetic parsimony analyses and the fitness data converge towards the same scenario, *i.e.* that veinal necrosis is an ancestral trait for PVY.

Correlation between PVY phylogeny and host range is established on several grounds: (i) Based on the phylogeny of all PVY sequences available in databanks, no potato isolate belongs to the C1 group, while no pepper isolate belongs to the N, O or C2 groups (nor are they recombinants among these three groups); (ii) Epidemiological studies in regions where potato and pepper crops coexist and are heavily infected by PVY confirm the existence of a host barrier between the distinct phylogenetic groups (see for example Bouhachem *et al*. (2008) and Ben Khalifa *et al*. (2009) for northern Tunisia); (iii) Most recombination events in PVY occurred between the N and O groups while very few recombination events involved isolates from the C1 group, which could be explained by the fact that more host species are shared between the O and N groups than between the N/O and the C1 groups; (iv) Finally, manual inoculations showed that PVY isolates from groups C1 and Chile are infectious in pepper while isolates from groups N, O and C2 are not (Gebre-Selassie et al., 1983; d'Aquino et al., 1995; Blanco-Urgoiti et al., 1998; Table 1). In contrast, pepper isolates of PVY, either from group C1 or Chile, were not infectious

in potato cultivars after manual inoculation (Gebre-Selassie et al., 1983; Table 1). Such correlation between phylogeny and host range suggest that evolution of PVY host range could be reconstructed with a limited number of phenotypic changes.

Since neither the potato nor the pepper groups of PVY isolates are monophyletic, changes of host adaptation occurred at least twice during PVY history (Fig. 5). Considering adaptation to pepper, the two most parsimonious scenarios involve two changes of host species adaptation, the ancestral state for PVY being either “adapted” or “not adapted” to pepper (Fig. 5). These two scenarios are very similar, since in both cases the putative ancestor of the clade comprising the C1, C2, O and N groups of PVY was not infectious in pepper and a later adaptation to pepper occurred after the divergence of groups C1 and C2 but before the diversification of group C1 (Fig. 5). The only difference between these two scenarios concerns the history of pepper adaptation of isolates belonging to the Chilean clade. Since adaptation to pepper corresponds to maladaptation to potato and *vice versa*, similar evolutionary scenarios could be drawn for adaptation to potato (data not shown). To discriminate between these scenarios, knowledge of the genome regions and mutations involved in PVY adaptation to pepper and potato would be required.

For several reasons, the evolutionary history of plant viruses remains difficult to unravel. Some of these reasons are (i) the lack of fossils or ancient historical records, (ii) the frequent lack of correlation between phylogenetic trees and biological traits, which suggests complex histories and/or that other events (*e.g.* recombination, strong geographic differentiation of isolates, demography…) have obscured these histories, (iii) the lack of many clear-cut viral pathogenicity traits and/or the lack of knowledge of their genetic determinism, (iv) the lack of genome data to

build reliable phylogenies or to place reliably the root of the phylogenetic trees. The fact that PVY has been extensively studied, providing a relatively exhaustive image of its diversity, together with the relative simplicity of its phylogeny and the knowledge of the genetic bases of some of its major biological traits made this kind of reconstruction easier. Similar studies could certainly be performed with other plant viruses that show a certain level of correlation between phylogeny and pathogenicity or host range traits such as TuMV (Ohshima *et al*., 2002) or *Plum pox virus* (Bodin *et al*., 2003) for potyviruses.

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Table 1: Pathogenicity of isolates representative of the different PVY phylogenetic groups in different solanaceous plant genotypes. The resistance alleles at the *pvr2* resistance locus of *Capsicum annuum* and at the Ny_{tbr} and Nc_{tbr} loci in *Solanum tuberosum* are described in Ayme et al. (2007) and Kerlan et al. (1999), respectively. Test plants grown in greenhouse conditions with one fully expanded leaf (pepper and *Nicotiana* spp.) or with four expanded leaves (potato) were inoculated manually two to three weeks after sowing (or tuber planting for potato) as in Moury et al. (2004). Symptoms were recorded between 14 and 60 days post inoculation (dpi) and evaluation of virus infections in inoculated or apical leaves was performed by DAS-ELISA as in Moury et al. (2004).

PVY isolate (clade)	Plant species and genotype and known resistance alleles							
	Nicotiana tabacum	*Solanum tuberosum*			*Capsicum annuum*			
	Xanthi	Bintje	King Edwards (Nc_{tbr})	Désirée (Ny_{tbr})	Yolo Wonder	Yolo Y ($pvr2^1$)	Florida VR2 ($pvr2^2$)	HD285 ($pvr2^3$)
N605 (N)	Nec.[a]	Mo.	Mo.	Mo.	Ø	nt	nt	nt
O139 (O)	Mo.	Mo.	Mo.	HR	Ø	nt	nt	nt
SON41p (C1)	Mo.	Ø	Ø	Ø	Mo.	nt	nt	nt
C Adgen (C2)	Mo.	Nec.	HR	Nec.	Ø	nt	nt	nt
Chile1	Nec.	Ø	Ø	Ø	Mo.	Ø (38/40) Mo. (2/40)	Ø	Mo.
Chile2	Nec.	Ø	Ø	Ø	Mo.	Mo. (40/40)	Ø	Mo.
Chile3	Nec.	Ø	Ø	Ø	Mo.	Ø (37/40) Mo. (3/40)	Ø	Mo.

[a] A total of twenty plants in two independent experiments were inoculated for each virus and each plant genotype, except when figures are indicated (number of plants with the indicated phenotype/total number of inoculated plants)

Nec: necrosis in uninoculated upper leaves; Mo: mosaic in uninoculated upper leaves; Ø: no infection, *i.e.* no symptoms and no virus detected in uninoculated upper leaves; nt: not tested; HR: hypersensitive reactions observed in inoculated leaves and no symptoms nor virus detected in uninoculated upper leaves.

Figure legends

Fig. 1. Comparison of the RNA secondary structures of the 3' untranslated regions (UTR) of PVY isolates SON41p and Chile3 predicted by the use of mFOLD version 2.3 program (Zucker 1989) with the temperature parameter set at 25 or 30°C. The predicted stem-loop structure duplicated in the sequence of the 3' UTR of Chile3 is boxed. RNA secondary structures obtained with different isolates of the N, O, C1 or C2 groups of PVY were very similar to that obtained with SON41p (data not shown)

Fig. 2. Phylogenetic tree obtained with full-length genome sequences of non-recombinant PVY isolates and PepSMV as outgroup using the maximum likelihood method implemented into PhyML with the Tamura-Nei+Γ+I nucleotide substitution model. Bootstrap analysis was applied using 1,000 bootstrap samples. The scale bar represents the relative genetic distance (number of substitutions per nucleotide)

Fig. 3. Amino acid sequences of the viral protein genome-linked (VPg) of pepper-infecting PVY isolates from the C1 group (SON41p) or from the Chilean group. The sequence of five variants of the Chile1 and Chile3 isolates which became virulent toward the *pvr2*^{1} allele and presented the same VPg cistron is indicated. Polymorphic sites among sequences of the Chilean isolates are boxed. Arrows indicate amino acid sites involved in PVY adaptation to resistance alleles at the *pvr2* locus (Ayme et al., 2006, 2007)

Fig. 4. Most parsimonious scenarios of evolution of symptom traits in tobacco cv. Xanthi (mo: systemic leaf mosaic; nec: systemic veinal necrosis) (A) and (B) show the most parsimonious evolutionary scenarios before and after the characterization of the Chilean group of PVY isolates, respectively. Alternative ancestral traits (boxed) and evolutionary steps are indicated in

black and grey. Most parsimonious evolutionary scenarios of the amino acid substitutions in the HcPro critical for systemic veinal necrosis (Tribodet et al., 2005) are also indicated.

Fig. 5. Most parsimonious scenarios of evolution of infectivity in pepper (*Capsicum annuum* cv. Yolo Wonder) (pep: infectious in pepper; non pep: not infectious in pepper *i.e.* no virus detected in inoculated or upper leaves). Alternative ancestral traits (boxed) and evolutionary steps are indicated in black and grey.

Supporting Information:

Table S1. Primers used for reverse transcription, PCR amplifications and/or sequencing of parts of the genome of the Chilean PVY isolates.

Genome region	Polarity	Primer sequence (5' to 3')[a]	Binding site [b]
HcPro cistron	+	TTYTAYCCICCIACNAARAARC	1950 to 2001
P3 cistron	-	GCTGCTGACTCAGACATTATG	2468 to 2488
VPg cistron	+	GAATYCAAGCHYTRAAGTTTCG	5734 to 5755
VPg cistron	-	GCTTCATGYTCYACHTCCTG	6261 to 6280
CP cistron	+	GCTGAACACAGGCTCGAAG	8289 to 8307
3' UTR	-	CACGGATCCTTTTTTTTTTTTTTTV	9700 to 9717

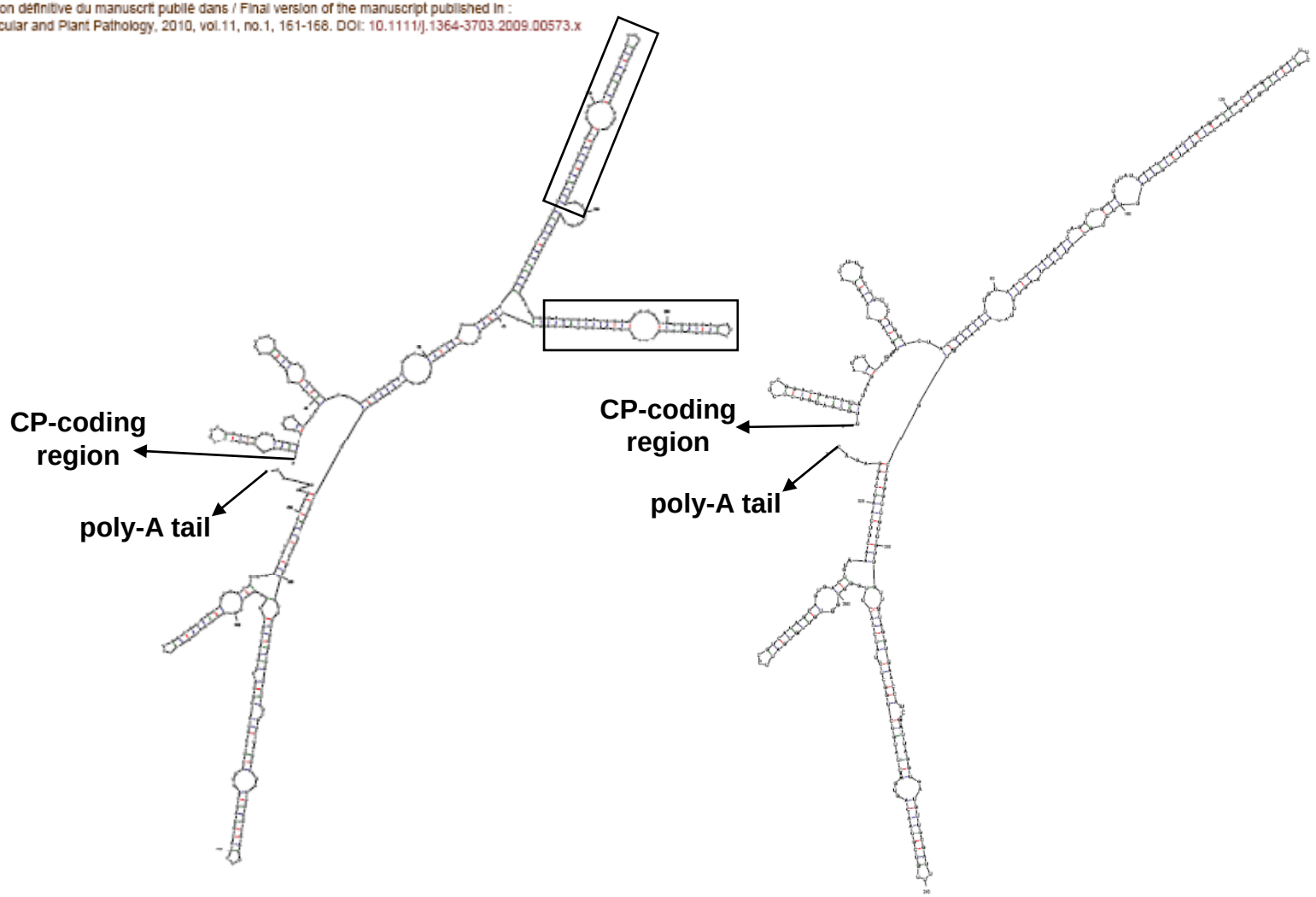
[a] Y: C or T; I: inosine; N: A, C, G or T; R: A or G; H: A, C or T; V: A, C or G.
[b] nucleotide positions referring to PVY strain SON41p (accession number AJ439544). Positions 9701 to 9717 correspond to the poly-adenylated tail.

List of accession numbers of full-length PVY genome sequences showing evidence of recombination (May 2008)

AB270705, AF237963, AF522296, AJ584851, AJ585197, AJ585342, AJ889866, AJ889867, AJ889868, AJ890342, AJ890343, AJ890344, AJ890345, AJ890346, AJ890347, AJ890349, AJ890350, AM113988, AM113988, AY745491, AY745492, AY884982, AY884985, D00441, DQ008213, DQ157178, DQ157179, DQ157180, DQ309028, EF016294, EF026075, EF026076, EF558545, EU182576, M95491, NC_001616.

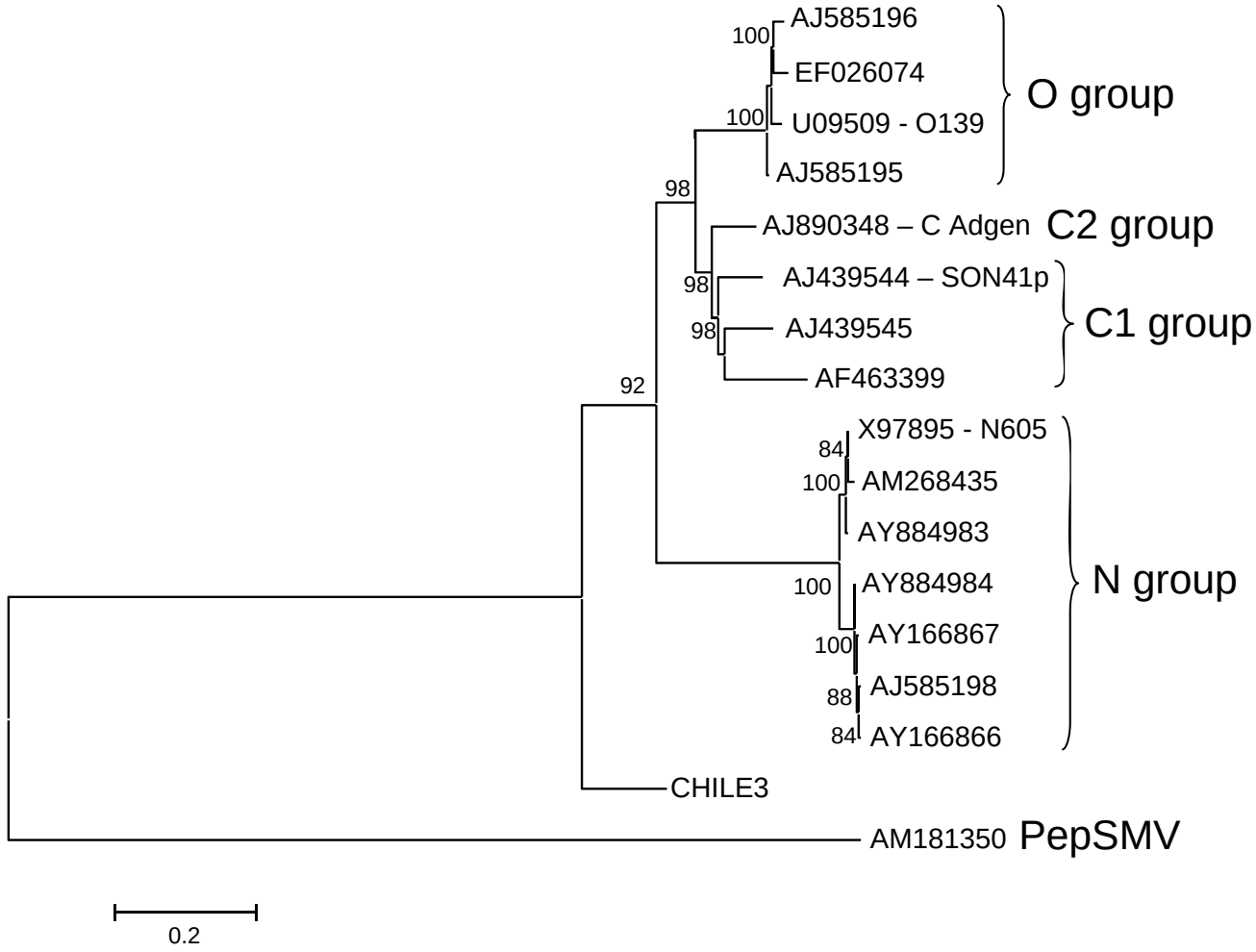
Fig. S1. Phylogenetic tree obtained with sequences of the coat protein (CP) cistron of PVY isolates and Bidens mosaic virus as outgroup using the maximum likelihood method implemented into PhyML with the Tamura-Nei+Γ+I nucleotide substitution model. Sequences showing evidence of recombination within the CP cistron were excluded. Bootstrap analysis was

applied using 1,000 samples. All bootstrap support values above 70% are shown and bootstrap support values below 70% are shown for internal branches linking the main PVY groups (circled) The scale bar represents the relative genetic distance (number of substitutions per nucleotide).



Chile3

SON41p



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                                                                      50
SON41p          GKNKSKRIQALKFRHARDKRAGFEIDNNDDTIEEFFGSAYRKKGKGKGTT
Chile2          ......................Q....E.....Y.....T.........
Chile1 & 3      ......................Q....E.....Y.....T.........
Chile1 & 3 -pvr2¹ ....................Q....E.....Y.....T.........

                                                                     100
SON41p          VGMGKSSRRFINMYGFDPTEYSFIQFVDPLTGAQIEENVYADIRDIQERF
Chile2          .....A....V.....................................L
Chile1 & 3      .....A....V.....................................L
Chile1 & 3 -pvr2¹ .....A....V.....................................L

                ↓   ↓           ↓   ↓↓↓                           150
SON41p          SEVRRKMVEDDEIETQALDSHTSIHAYFRKDWSDKALKIDLMPHNPLKVC
Chile2          G...S...I....DPA..R.N.T..............V...........
Chile1 & 3      G...S...I....DPAT.RGN.T..............V...........
Chile1 & 3 - pvr2¹ G...G...I....DPAT.RGN.T..............V...........

                                                                  188
SON41p          DKTNGIAKFPEREFELRQTGPAVEVNVKDIPKQEVEHE
Chile2          ........................D............
Chile1 & 3      ........................D............
Chile1 & 3 -pvr2¹ ......................D............
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