Short communication

A new lineage sheds light on the evolutionary history of Potato virus Y

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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 with phylogeny, allowing the reconstruction of the evolutionary history of these traits. In this article, a new lineage of PVY isolates from Chile is described, showing unique genomic and biological properties. This lineage was found to be the sister group of all other PVY isolates and helped in the reconstruction of the ancestral traits and evolutionary history of PVY, suggesting that vein necrosis in tobacco is an ancestral state and that adaptation to pepper (*Capsicum* spp.) and potato (*Solanum tuberosum*) has been 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 different host species and cultivars (De Bokx and Huttinga, 1981; Gebre Selassie *et al.*, 1985; Gooding and Tolín, 1973). These biological properties are partly correlated with PVY phylogeny. Based on genome sequences, three major lineages can be distinguished among PVY, namely O, C and N (Moury *et al.*, 2002). Only isolates from the O lineage induce hypersensitive reactions associated with resistance in potato cultivars carrying the *Ny_{tblr}* resistance gene, only the isolates from the C lineage induce similar reactions in potato cultivars carrying the *Nc_{tblr}* gene, and only isolates from the N lineage induce systemic vein necrosis in a set of tobacco cultivars (Jones, 1990; Kerlan *et al.*, 1999). The C group has been 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 (Fanigliulo *et al.*, 2005; Glais *et al.*, 2002; Moury *et al.*, 2002; Ogawa *et al.*, 2008; Revers *et al.*, 1996). The letters O, C and N 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 certain biological traits shared by non-recombinant PVY isolates. More than 40 complete genomic sequences and more than 240 coat protein (CP) cistron sequences of PVY are available in databanks, providing an exhaustive image of its diversity. Almost all 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 this assumption. Like isolates from the N group, this Chilean isolate induced vein necrosis in tobacco (Sudarsono *et al.*, 1993).

Three PVY isolates (Chile1, Chile2 and Chile3) were obtained from distinct plants of *Capsicum baccatum* L. cv. Crystal, familiarly termed 'aji' throughout South America, collected in Chile in 2005. They were inoculated once into *Nicotiana tabacum* cv. Xanthi plants to obtain high-titre inocula for tests on different solanaceous plants and for genomic analyses. The symptoms induced by these three Chilean isolates in reference tobacco, potato and pepper genotypes were investigated and compared with 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 typical of two different groups of PVY. Like isolates of the C1 group, they were infectious in *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. Because of their peculiar host range and symptom traits, the Chilean isolates could be helpful in unravelling the evolutionary history of PVY. Therefore, 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–FJ951647) were determined.

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Table 1 Pathogenicity of isolates representative of the different *Potato virus Y* (PVY) phylogenetic groups in different solanaceous plant genotypes.

PVY isolate (clade)	Plant species and genotype and known resistance alleles							
	<i>Nicotiana tabacum</i>	<i>Solanum tuberosum</i>			<i>Capsicum annuum</i>			
	Xanthi	Bintje	King Edward (<i>N_{C_{tr}}</i>)	Désirée (<i>N_{Y_{tr}}</i>)	Yolo Wonder	Yolo Y (<i>pvr2¹</i>)	Florida VR2 (<i>pvr2²</i>)	HD285 (<i>pvr2³</i>)
N605 (N)	Nec.*	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.

Ø, no infection, i.e. no symptoms and no virus detected in uninoculated upper leaves; HR, hypersensitive reactions observed in inoculated leaves and no symptoms or virus detected in uninoculated upper leaves; Mo, mosaic in uninoculated upper leaves; Nec, necrosis in uninoculated upper leaves; nt, not tested.

The resistance alleles at the *pvr2* resistance locus of *Capsicum annuum* and at the *N_{Y_{tr}}* and *N_{C_{tr}}* loci of *Solanum tuberosum* are described in Ayme *et al.* (2007) and Kerlan *et al.* (1999), respectively. Test plants grown in glasshouse conditions with one fully expanded leaf (pepper and *Nicotiana* spp.) or with four expanded leaves (potato) were inoculated manually 2–3 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 the evaluation of virus infections in inoculated or apical leaves was performed by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) as in Moury *et al.* (2004).

*A total of 20 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).

Total RNAs from leaves of systemically infected Xanthi plants were purified with the Tri Reagent kit (Molecular Research Center, Cincinnati, OH, USA) and used for reverse transcription-polymerase chain reaction (RT-PCR) with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) and *Taq* DNA polymerase (Promega). PVY-polyvalent primers (Table S1, see Supporting Information) 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%–99.5% identical based on a total of 1331 sequenced nucleotides. Compared with sequences available in the GENBANK database, the Chilean isolates clearly belonged to PVY, sharing 92.7%–94.3% nucleotide identity (based on complete genome alignments with the Chile3 isolate) with other PVY isolates, but had unique genomic properties. The most obvious difference was located in the 3' 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 this genomic region, and 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 corresponded 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 to the Chilean PVY isolates were also observed in the 5' UTR and in the P3 cistron (data not shown). The unique genomic 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 to be distant PVY isolates by some authors (Dujovny *et al.*, 2000; Inoue-Nagata *et al.*, 2006). 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 and PepSMV as outgroups, and the other with full-length

Fig. 1 Comparison of the RNA secondary structures of the 3' untranslated regions (UTRs) of *Potato virus Y* (PVY) isolates SON41p and Chile3 predicted by the use of the 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).

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 analysed with RDP version 2 software (Martin *et al.*, 2005), implementing several algorithms to detect putative recombinant sequences. Only recombination sites detected by more than two of six independent methods with the default probability threshold were considered. A large number of full-length genomic sequences of PVY were found to be recombinants in this and/or previous studies (Fanigliulo *et al.*, 2005; Glais *et al.*, 2002; Moury *et al.*, 2002; Ogawa *et al.*, 2008; Revers *et al.*, 1996) (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 Chilean pepper isolates clustered together 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; Fig. S1, see Supporting Information). 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 because of 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 out-

group (22%–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 Fig. S1). Using sunflower chlorotic mottle virus or PepSMV as outgroups for this genomic 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 Chile3 isolate of PVY and PepSMV (100% probability support for this topology against the two alternatives). 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 groups of PVY isolates.

Diversity in 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 the resistance allele (*pvr2*⁺/*pvr2*⁺), whereas 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

Fig. 2 Phylogenetic tree obtained with full-length genomic sequences of non-recombinant *Potato virus Y* (PVY) isolates and *Pepper severe mosaic virus* (PepSMV) as outgroup using the maximum likelihood method implemented into PhyML with the Tamura-Nei + Γ + I nucleotide substitution model. Figures shown near branches are bootstrap percentages obtained with 1000 bootstrap samples. The scale bar represents the relative genetic distance (number of substitutions per nucleotide).

		50
SON41p	GKNKSKRIQALKFRHARDKRA GFEIDNNDTIEEFFGSAYRKKGKGGTT	
Chile2	.....Q.....E.....Y.....T.....	
Chile1 & 3	.....Q.....E.....Y.....T.....	
Chile1 & 3 - <i>pvr2</i> ¹	.....Q.....E.....Y.....T.....	
		100
SON41p	VGMKSSRRFINMYGFDPT EYSFIQFVDPLTGAQIEENVYADIRDIQERF	
Chile2	.....A.....V.....	L
Chile1 & 3	.....A.....V.....	L
Chile1 & 3 - <i>pvr2</i> ¹	.....A.....V.....	L
		150
SON41p	↓ ↓ ↓ ↓ SEVERKIMVEDDEIETQALDSHTSIHAYFRKOWSDKALKIDLMPHNPLKVC	
Chile2	G...S...I...DPA...R...N...T.....	V
Chile1 & 3	G...S...I...DPAT...RGN...T.....	V
Chile1 & 3 - <i>pvr2</i> ¹	G...G...I...DPAT...RGN...T.....	V
		188
SON41p	DKTNGIAKFFEREFELRQTGP AVEVNVKDIPKQEV EHE	
Chile2	.....D.....	
Chile1 & 3	.....D.....	
Chile1 & 3 - <i>pvr2</i> ¹	.....D.....	

Fig. 3 Amino acid sequences of the viral protein genome-linked (VPg) of pepper-infecting *Potato virus Y* (PVY) isolates from the C1 group (SON41p) or the Chilean group. The sequences of five variants of the Chile1 and Chile3 isolates, which became virulent towards the *pvr2*¹ allele and presented the same VPg cistron, are 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).

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 has been demonstrated previously to be the virulence determinant of PVY towards *pvr2* (Ayme *et al.*, 2006, 2007; Moury *et al.*, 2004), 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, as (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 with the original isolates (causing a serine to glycine substitution at amino acid position 105 of VPg; Fig. 3). However, it is unknown whether these virulent variants pre-existed at low frequency in the original inocula or whether 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 VPg affect the virulence properties of

the Chilean isolates towards the *pvr2* resistance alleles of pepper. Positions 105 and 120 have already been 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 the biological traits of the members of the major PVY clades and the topology of their phylogenetic tree allowed inferences to be made about their evolutionary history and their ancestral and derived traits. In addition, the identification of the new 'Chilean' clade helped in the discrimination between various evolutionary scenarios. Systemic vein necrosis in a number of tobacco cultivars is one of the traits that has long been used to discriminate between the different groups of PVY, defining the N group. The three Chilean pepper isolates, together with the previously identified Chilean tobacco isolate (Sudarsono *et al.*, 1993), induce necrosis in tobacco, whereas isolates belonging to the O and C groups do not (Table 1). Before characterization of the Chilean group of PVY isolates, the two evolutionary scenarios considering that tobacco necrosis is either an ancestral or a derived trait were equally parsimonious, and both could be reconstructed with only one phenotypic evolutionary step (Fig. 4A). Including the Chilean group of PVY now suggests that the ancestral state was more probably 'necrotic', as one evolutionary step (vs. two steps when necrosis is considered to be a derived trait) is sufficient to reconstruct PVY history (Fig. 4B). Mutations at amino acid positions 400 and 419 of HcPro of PVY were shown to determine vein necrosis in tobacco (Tribodet *et al.*, 2005). Confirming the above evolutionary hypothesis, the three Chilean pepper isolates were shown to possess a lysine and a glutamic acid at positions 400 and 419, respectively, of HcPro, similar 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 asparagine at positions 400 and 419, respectively, of HcPro. Consequently, the scenario in which vein necrosis is the ancestral state of PVY requires only two amino acid substitutions, whereas the alternative scenario requires four amino acid substitutions. It should be noted that the analysis of codon evolution instead of amino acid evolution at positions 400 and 419 of HcPro did not help in the further discrimination between these scenarios (data not shown).

The scenario in which tobacco necrosis evolved twice from non-necrotic PVY isolates through the fixation, in parallel, of the same two amino acid substitutions in HcPro (Fig. 4B) would suggest that these substitutions conferred a strong fitness advantage to the virus. However, recent results have indicated instead that the amino acid substitutions that 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 vein necrosis is an ancestral trait for PVY.

Fig. 4 Most parsimonious scenarios of evolution of symptom traits in tobacco cv. Xanthi (mo, systemic leaf mosaic; nec, systemic vein 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 helper component-proteinase (HcPro) critical for systemic vein necrosis (Tribodet *et al.*, 2005) are also indicated.

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, whereas no pepper isolate belongs to the N, O or C2 groups (nor are they recombinants among these three groups); (ii) epidemiological studies in regions in which 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); Ben Khalifa *et al.* (2009) for northern Tunisia]; (iii) most recombination events in PVY occurred between the N and O groups,

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.

whereas 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, whereas isolates from groups N, O and C2 are not (d'Aquino *et al.*, 1995; Blanco-Urgoiti *et al.*, 1998; Gebre Selassie *et al.*, 1985; 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.*, 1985; Table 1). Such a correlation between phylogeny and host range suggests that the evolution of the PVY host range could be reconstructed with a limited number of phenotypic changes.

As neither potato nor 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 as, 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. As 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, a knowledge of the genomic 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 include: (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 geographical differentiation of isolates, demography, etc.) have obscured these histories; (iii) the lack of many clear-cut viral pathogenicity traits and/or the lack of knowledge of their genetic determinism; and (iv) the lack of genomic 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, make 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 *Turnip mosaic virus* (Ohshima *et al.*, 2002) or *Plum pox virus* (Bodin *et al.*, 2003) for potyviruses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Phylogenetic tree obtained with sequences of the coat protein (CP) cistron of *Potato virus Y* (PVY) isolates and *Bidens* mosaic virus as outgroups 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 1000 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).

Table S1 Primers used for reverse transcription, polymerase chain reaction (PCR) amplifications and/or sequencing of parts of the genome of the Chilean *Potato virus Y* (PVY) isolates.

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

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