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Molecular characterization of an almond isolate of *Prune dwarf virus* in Tunisia: putative recombination breakpoints in the partial sequences of the coat protein-encoding gene in isolates from different geographic origin

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Summary. Recombination is an important driving force in evolution. To investigate this evolutionary process in *Prune dwarf virus* (PDV), 31 accessions retrieved from international databases, supplemented by one Tunisian isolate described here, were analyzed. Two recombination detection programs were used: RDP v3.31β and RECCO. While the first program did not detect any significant recombination events, RECCO detected several breakpoints in partial sequences of the coat protein gene (CP) of fifteen isolates. The Tajima neutrality test implemented in the MEGA4.1β program indicated that there were numerous deletion/insertion events in the sequences. The strongest signal was found in Portuguese isolate 3.12N.14 (accession number AY646846) which had position nt 296–523 in the CP gene. The evolutionary historical relationships between all analyzed isolates were determined by constructing a dendrogram using Neighbor joining (NJ), Minimum evolution (ME), Maximum parsimony (MP), Maximum likelihood (ML), and the Unweighted pair group method with arithmetic mean (UPGMA); all gave similar results. Two main clusters were delineated, one representing recombinant and one nonrecombinant isolates. The recombinant isolates were mostly collected from Portugal, whereas the nonrecombinant isolates originated in eastern Europe.

Key words: PDV, sequence, polymorphism, evolution.

Introduction

Plant viruses are genetically heterogeneous. As with all living entities, reproduction results in individuals that may differ genetically from their parents; such individuals are called mutants or, more vaguely, variants. The frequency distribution of genetic variants in the population of an organism (i.e. the genetic structure of the population) may change over time, and this process is called evolution. Genetic variation is generated by errors occurring during the replication of genomes due to a failure in the proofreading ability of the RNA replicase. Genetic variations are a key factor in RNA virus pathogenicity, where adaptation to changing situations must maintain genetic robustness and fitness despite the occurrence of mutations in the genome (Drake and Holland, 1999; Elena, 2002; Garcia-Arenal *et al*., 2003).

Plant viruses frequently use recombination and reassortment as driving forces in evolution (Roossinck, 1997). Recombination, defined as the exchange of genetic information between two nucleotide sequences, is an important process that influences biological evolution at many levels. Recombination explains a considerable amount of genetic diversity in natural populations, and in general, genes located in regions of the genome with low levels of recombination have low levels of polymorphism (Posada and Crandall, 2001). Recombination reshuffles existing variation, and even creates new variants. A single virus isolate does not consist of a single RNA sequence: it consists of a population of related sequence variants, often referred to as quasispecies (Holland *et al*., 1992; Domingo *et al*., 1995; Eigen, 1996). The quasispecies nature of RNA viruses implies a high adaptive potential, allowing

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for the rapid selection of biologically distinct sequence variants with the highest fitness in a new environment. It may result in dramatic changes in the biological properties of the virus, with major epidemiological consequences, including the appearance of resistance-breaking strains, or the acquisition of broader host ranges (Legg and Thresh, 2000; Monci *et al*., 2002).

Recombination in natural populations has been reported for several plant viruses (Fraile *et al*., 1997; Bousalem *et al*., 2000; Rubio *et al*., 2001; Ohshima *et al*., 2002; Glasa *et al*., 2004; Tomitaka and Ohshima, 2006). *Prune dwarf virus* (PDV), on the other hand, has an evolutionary history that seems to be but little known, and no information on recombination events in the CP gene of this virus is available. It should be noted, however, that Codoner and Elena (2008) have recently reported that, based solely on RNA-2 genomic region analysis, PDV is potentially a minor parent of *Alfalfa mosaic virus*.

Prune dwarf virus (genus *Ilarvirus*, family *Bromoviridae*) is an economically important virus infecting stone fruits worldwide. It occurs in single or in mixed infections with other ilarviruses: *Prunus necrotic ringspot virus* (PNRSV), *Apple mosaic virus* (ApMV) and *Plum line pattern virus* (PLPV). In temperate regions where *Prunus* spp. are cultivated (Uyemoto *et al*., 1992), PDV causes dwarf symptoms in prune and interferes with the vegetative growth of infected trees, but leaves are typically symptomless on mature trees (Helguera *et al*., 2002). In the USA, PDV is the cause of sour cherry yellows (Waterworth and Fulton, 1964, Fridlund, 1965; Halk and Fulton, 1978). Nemeth (1986) reported that on sweet cherry, PDV causes symptoms that vary greatly depending on the cultivar and the climate. In some sweet cherry cultivars and rootstocks, the infection is latent, and even when symptoms occur they are not always displayed every year. On the leaves of several sweet cherry cultivars, chlorotic spots and rings as well as varying degrees of necrosis and shot-holing may occur. PDV causes considerable damage in many hosts in single or mixed infections, and is common in the Mediterranean, especially in Tunisia, where it has a role in causing almond mosaic, a disease with a number of field symptoms, the most frequent of which are line pattern, yellow mottle, vein banding and yellow speckling (Boulila and Marrakchi, 2001; Boulila, 2002). PDV is transmitted by pollen and seed, is readily graft-transmissible and may infect healthy mother plants during pollination (Spiegel *et al*., 1998). PDV belongs to subgroup 4 of the genus *Ilarvirus*. It has a tripartite, positive-sense, single-stranded RNA genome. The two larger genomic RNAs (1 and 2) are monocistronic and encode nonstructural proteins involved in viral replication. In contrast, RNA 3 is bicistronic, encoding a polypeptide required for cell-to-cell movement (MP) at the 5' proximal end, and the viral coat protein at the 5' distal end. The putative MP is translated directly from RNA 3, while the coat protein is expressed from a fourth, subgenomic RNA, termed RNA 4. RNA 4 is collinear with the 3' end of RNA 3 and is encapsidated (Bachman *et al*., 1994; Bol, 2005). Viral coat proteins are presumed to have evolved more rapidly than proteins involved in the replication and expression of virus genomes. For this reason, the coat protein genes have often been used in phylogenetic comparisons (Zimmern, 1988; Koonin and Gorbalenya, 1989), and they are also used in this work.

The objective of the present paper was to demonstrate that PDV is subject to recombination in its CP gene, and to determine the evolutionary history of this virus. This was achieved by analyzing 31 accessions retrieved from databanks supplemented by one Tunisian isolate described in this study.

Materials and methods

Virus source

Surveys were carried out in early spring 2007, and samples were collected from trees of one almond variety (Jemmal 1) growing in the Sousse region (Tunisia) and exhibiting severe stunting. The leaves of the trees were also reduced in size and showed ringspotting and tatter leaf. The sample collected consisted of 25 shoots bearing leaves with virus symptoms and taken from the four compass points as well as from the inner portion of the crown.

Mechanical transmission

Cucumis sativus L. cv. Marketer was inoculated with sap from PDV-infected almonds. The virus was transmitted to cucumber by crushing leaf tissues in 0.1 M phosphate buffer, pH 7.4, containing 0.1% 2-mercaptoethanol and rubbing the leaf extract onto celite-dusted cotyledons. Inoculated plants were kept in a glasshouse at a temperature of 18–24°C.

Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR)

A single step immunocapture-RT-PCR was carried out following Wetzel *et al*. (1992).

Sample preparation

Using a rolling grinder (Homex 6, Bioreba, Basel, Switzerland), the leaf samples were ground (1:5, w:v) in a PBS-T buffer (1.44 g Na_2HPO_4 $2H_2O$, 8 g NaCl, 0.2 g KH_2PO_4, 0.2 g KCl, 0.2 g NaN_3, 0.5 mL Tween 20) routinely used for ELISA assays, supplemented with

2% polyvinylpyrrolidone (PVP) K25 and 0.2% sodium diethyldithiocarbamate (DIECA) in individual plastic bags containing gauze (Bioreba). After grinding, the extracts were rapidly clarified by centrifugation (5 min, 825 g, 4°C) using a Sigma 1-15K centrifuge (Sigma, MO, USA)

IC-RT-PCR assay

The selected primers spanned the genomic portion overlapping part of the coat protein gene and part of the 3' untranslated region (3' UTR) of the RNA-3. They were located between nucleotides 1398 and 2010 (L28145, Bachman *et al*., 1994) with the sequences: 5'-CCGGTATGATATCTCGTACCGAG-3', for the sense primer and 5'-TAGTGCAGGTTAACCAAAAGGAT-3', for the antisense primer, to amplify specifically a 613 bp product.

One hundred µL of clarified plant extract was subjected to immunocapture performed directly in the tubes used for the reverse transcription PCR. The immunocapture tubes were coated with 2 µg of immunoglobulins per mL. The tubes were incubated overnight at +4°C in both steps. Following three washes with PBS-Tween buffer, the tubes were dried and further processed for amplification. Fifty µL of RT-PCR mix [6 µL 1% Triton X100, 5 µL 5× Green *GoTaq* DNA polymerase buffer (Promega, Madison, WI, USA), 1.5 µL 25 mM $MgCl_2$, 2.5 µL formamide 5%, 1 µL 10 mM dNTPs (2.5 mM each of dGTP, dATP, dCTP, and dTTP), 15 pmol of each of the two primers, 2.5 U AMV reverse transcriptase (Promega), 1 U *GoTaq* DNA polymerase (Promega), 10 U RNAsin (Promega), 31 µL sterile water] were added to each tube. The cycler PTC 100 (MJ Research, Inc, Watertown, MA, USA) was used with the following cycling scheme: 30 min at 46°C (RT-reaction), 3 min at 95°C to denature the templates and the reverse transcriptase followed by 35 cycles of amplification: 30 s at 94°C (denaturation), 30 s at 52°C (annealing) and 45 s at 72°C (DNA synthesis). A final elongation step of 7 min at 72°C was performed at the end of the 35 cycles. Amplification products were analyzed by electrophoresis of 10 µl aliquots on 1.5% agarose gel, in 1× Tris-Borate-EDTA buffer (Sambrook *et al*., 1989). Bands were visualized by ethidium bromide staining (5 µg mL^{-1}) and photographed using a UV transilluminator (ETX 20.M) at a wavelength of 312 nm and with a Vilber Lourmat photo-print system (Model DP-001.FDC, Proxylab, Quevaucamps, Belgium).

Restriction fragment length polymorphism (RFLP)

The amplified product was purified according to manufacturer's instructions (Promega). An aliquot of the amplicon was subjected to RFLP with 2 selected endonucleases dispatched depending on the virus genometargeted. An aliquot equivalent to 12 µL of the PCR product was digested with restriction enzymes as outlined in the following scheme: The mix used was: (i) *Rsa* I (Promega): 5 µL PCR product, 1 µL *Rsa* I (10 $U.\mu L^{-1}$), 1 µL 10× buffer C [(R003A), 100 mM Tris-HCl (pH 7.9), 500 mM NaCl, 100 mM $MgCl_2$, 10 mM DTT], 1 µL acetylated BSA (0.1 $mg.mL^{-1}$) and 4 µL sterile water. (ii) *Ava* II (Q-Biogene, Montreuil, France): 5 µL amplicon, 2 µL *Ava* II (2.5 $U.\mu L^{-1}$), 2 µL blue buffer I (1×C) (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM NaCl, 10 mM 2-mercaptoethanol, 0.1 $mg.mL^{-1}$ BSA), and 3 µL sterile water.

Cloning and sequencing of PCR products

The cDNA obtained by RT-PCR was cloned into the pMOS*Blue* plasmidic vector (Amersham, Buckinghamshire, UK). Briefly, the plasmid was linearized by digestion with E*coR* V endonuclease, then ligated using T4 DNA ligase (Amersham) according to manufacturer's instructions, followed by transformation into *Escherichia coli* DH5α. Four clones obtained from the cDNA product were selected for sequencing.

The PCR product was subjected to nucleotide sequencing by the dideoxy chain termination method (Sanger *et al*., 1977) using the Big dye terminator ready reaction mix (Applied Biosystems, Foster City, CA, USA) in an automated capillary sequencer 3130. Sequencing was done in both directions, using the upstream and downstream universal primers T7 and U-19mer. The resulting sequences were analyzed with CHROMASLITE (Technelysium Pty. Ltd, Helenvale, Australia). No differences among clones were observed.

Computer-assisted sequence analyses

Databank searches for homologies to PDV were performed using FASTA (Pearson and Lipman, 1988) and WU-BLAST 2, based on the Basic local alignment search tool algorithm (Altschul *et al*., 1990; 1994).

The nucleotide sequences of the 32 accessions were aligned using CLUSTALW 2.0.9., and CLUSTALX 2.0.9. (Larkin *et al*., 2007) with default parameters, and visualized with the SEEVIEW graphic tool (Galtier *et al*., 1996). The phylogenetic relationships of the accessions were determined by five methods: neighbor-joining (NJ), minimum evolution (ME), maximum parsimony (MP), and the unweighted pair group method with arithmetic mean (UPGMA) implemented in the MEGA4.1β program (Kumar *et al*., 2008), and Maximum-likelihood (ML) using the PHYLO_WIN graphic tool (Galtier *et al*., 1996). Bootstrap analyses were performed with 500 replicates to assess the robustness of the branches. Amino acid residues were obtained using the ExPASy translate tool.

Recombination events between diverged nucleotide sequences were explored with two programs: RDP v3.31β (Martin *et al*., 2005b) and RECCO (Maydt and Lengauer, 2006). RDP incorporates several published recombination detection methods into a single set of tools: RDP (Martin and Rybicki, 2000), GENECONV (Padidam *et al*., 1999), BOOTSCAN (Martin *et al*., 2005a), MAXCHI (Smith, 1992), CHIMAERA (Posada and Crandall, 2001), SISCAN (Gibbs *et al*., 2000), 3SEQ (Boni *et al*., 2007) and LARD (Holmes *et al*., 1999). Default parameters were used in all cases. Only events predicted by half the methods were considered significant. The algorithm developed and described by Maydt and Lengauer (2006) as a fast, simple and sensitive method for detecting recombination in a set of sequences and locating putative recombination breakpoints was based on cost minimization. This method has only two tunable parameters, recombination and mutation cost. In practice the only parameter considered is α, representing the cost of mutation relative to recombination. When α changes from 0 to 1, the cost of mutation weighted by α increases, and the cost for recombination weighted by 1-α decreases. In other words, the parameter α controls the ambiguity between mutation and recombination.

Results

Mechanical transmission

Chlorotic and necrotic lesions were observed on cotyledons of *Cucumis sativus* cv. Marketer. Systemic symptoms consisted of conspicuous mosaic and stunted growth.

PCR product and RFLP

Immunocapture-RT-PCR successfully amplified the targeted genome portion of the PDV isolate collected. The expected size of 613 bp was obtained (Figure 1a).

In assessing the diversity in PDV, only the amplicon digested with *Rsa* I endonuclease (Figure 1b) gave rise to the following sizes: 357, 134, 115 and 7 bp (not shown), according to the 613 bp fragment sequence mentioned in Figure 2.

Detection of recombination events in PDV genome

To identify recombination events within each CP partial sequence of the 32 analyzed accessions, we used the RDP v 3.31β and RECCO programs. Although the first program did not detect any significant recombination events, the second program detected recombination signals in the set of PDV sequences. The graph in Figure 3 displays the breakpoints represented by the downward peaks in the dataset. The p-value for a recombination event is shown if that recombination event was the strongest in all the data. The analysis yielded several recombination signals in the partially sequenced CP genes. The assigned positions in the sequences are shown in Table 1.

It is noteworthy that the length of the recombination sites varied from one single point recombination, with isolate Jemmal 1 and isolate 1.8.A3 (nt 436 and nt 83 respectively) to much greater lengths (all remaining isolates). Furthermore, the recombination sites differed from one another by one location (cp1.2, cp4.3, B.11.15, 3.19.A1.2, 3.12N.14, 3.17.A1, 3.20.1, RS 38.1, Algarve, 2/16, Skierniewice), by two locations (N1.2, 26B), or by four locations (Jemmal1, 1.8.A3). Interestingly, the strongest recombination signal was detected with isolate 3.12N.14 (AY646846) from Portugal, extending from nt 115 to nt 342 in the CP gene partial sequence (corresponding to position 296-523 in the entire CP gene) (Figure 3).

Nucleotide sequence analysis

Multiple alignments of the nucleotide showed a divergence of sequences ranging roughly from 86 to 99%. The maximum composite likelihood estimate of the pattern of nucleotide substitution (Table 2) was conducted in MEGA4.1β. The rates of the different transitional substitutions varied from 18.75 to 23.69, and those of the transversional substitutions varied from 2.13 to 2.69. The nucleotide frequencies were as follows: 0.244 (A), 0.288 (T/U), 0.228 (C), and 0.241 (G). The transition/transversion rate ratios were k_1=8.562 (purines) and k_2=8.794 (pyrimidines). The overall transition/transversion bias was R=4.25, where R=[AGk_1 + TCk_2]/[(A+G)(T+C)]. There was a total of 465 positions in the final dataset.

The MEGA4.1β program also incorporated Tajima's neutrality test. The purpose of this test was to identify sequences which did not fit the neutral theory model in equilibrium between mutation and genetic drift. Tajima's test compared a standardized measure of the total number of segregating sites (these are DNA sites that are polymorphic) in the sampled DNA with the average number of mutations between pairs in the sample. The Tajima's D was determined for the 32 sequences analyzed (D=-0.709314).

Evolutionary relationships

The phylogenetic relationships between the PDV isolates were inferred using NJ, ME, MP, ML, and UPGMA. The constructed trees basically gave a somewhat similar phylogenetic grouping for the five methods used in the study. The 32 accessions split into four distinct clades (Figure 4). The clustering was mostly correlated with both the geographic

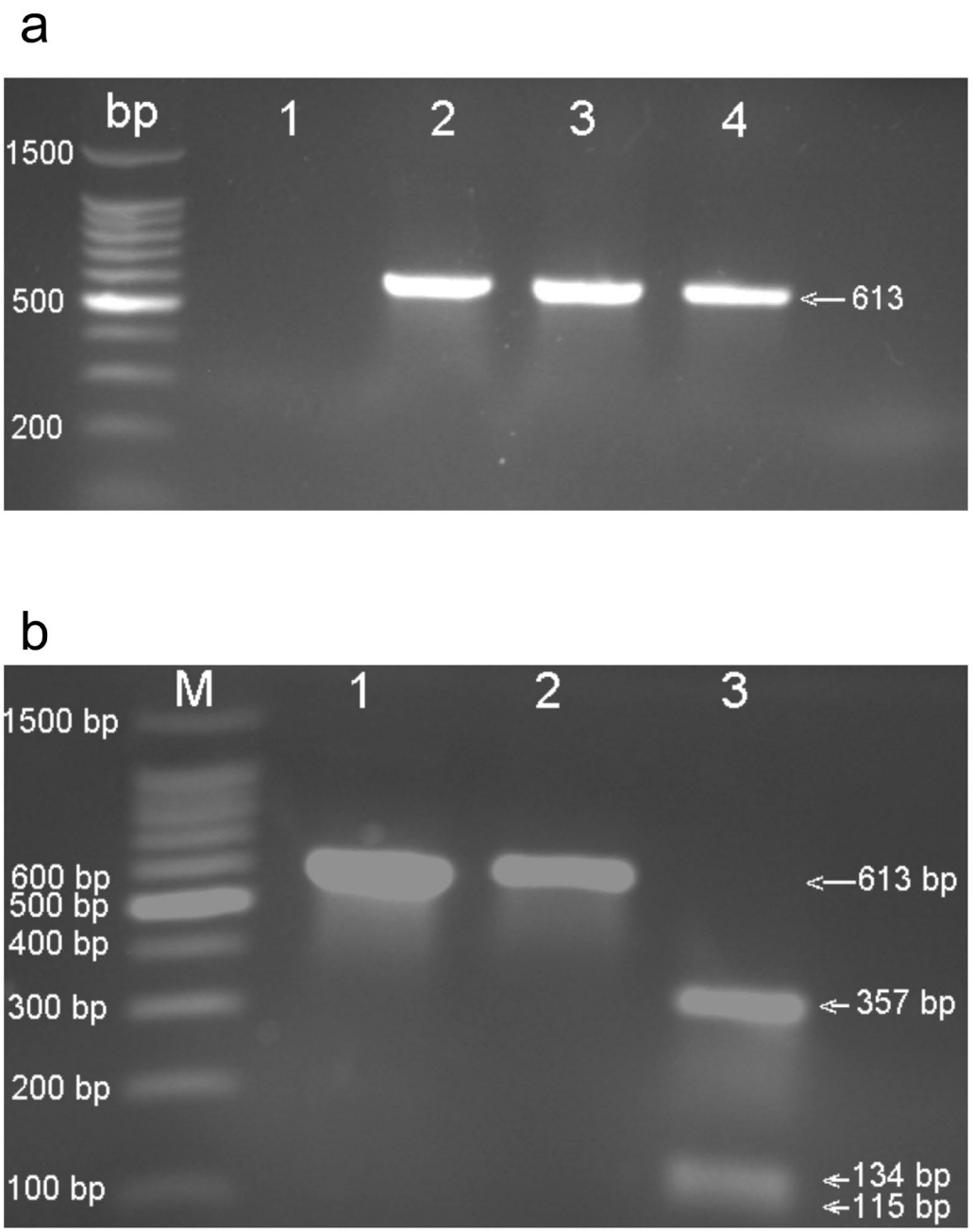

Figure 1. (a) Agarose gel of products amplified from *Prune dwarf ilarvirus*. M, Marker; lane 1, negative control; lane 2, positive control (provided by Bio-Rad, France), lane 3, fragment amplified of PDV isolate Jemmal1 from the natural host, almond; lane 4, fragment amplified from *Cucumis sativus* cv. Marketer. The arrow shows the fragment size obtained (613 bp). (b) RFLP analysis of PCR products. Lane 1, uncleaved amplicon; lane 2, PCR product subjected to *Ava* II endonuclease (no digestion); lane 3, amplicon digested by *Rsa* I restriction enzyme. The arrows show the uncleaved PCR product (613 bp), and the digested fragment having the following sizes: 357, 134, and 115 bp.

Table 1. Detected recombinant isolates of *Prune dwarf virus*, their positions in the coat protein gene, accession numbers and geographic origins.

Isolate	Putative recombination events in various PDV isolates			
	Recombination site position in partial CP gene	Corresponding position in entire CP gene.	Accession No.	Country
N1.2	329-336 388-400	513-520 572-584	AY646838	Portugal
cp1.2	115-181	299-365	AY646839	Portugal
cp4.3	73-81	257-265	AY646840	Portugal
B.11.15	443-444	627-628	AY646841	Portugal
26B	127-144 147-181	307-324 327-361	AY646842	Portugal
1.8.A3	51-60 83 115-181 189-195	235-244 267 299-365 373-379	AY646844	Portugal
3.19.A1.2	21-26	211-216	AY646845	Portugal
3.12N.14	115-342	296-523	AY646846	Portugal
3.17.A1	279-342	469-532	AY646847	Portugal
3.20.1	115-123	305-313	AY646849	Portugal
RS 38.1	442-450	629-637	AY554274	Hungary
Algarve	136-181	320-365	AF202117	Portugal
2/16	316-330	499-513	AF208737	Czech Republic
Skierniewice	68-73	255-260	U31310	Poland
Jemmal1	79-82 127-129 145-195 436	- - -	This study	Tunisia

Table 2. Maximum composite likelihood estimate of the pattern of nucleotide substitution in *Prune dwarf virus*. Rates of different transitional substitutions are shown in bold and those of transversional substitutions in italics.

Probability of substitution from one base (row) to another base (column)				
	A	T	C	G
A	-	*2.69*	*2.13*	**19.3**
T	*2.28*	-	**18.75**	*2.25*
C	*2.28*	**23.69**	-	*2.25*
G	**19.54**	*2.69*	*2.13*	-

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ccggttagattctcggaccgagattctgcggtcaattcgaccgcttccgggtgtttatta
 P  V  R  F  S  D  R  D  S  A  V  N  S  T  A  S  G  C  L  L
caccatgaaagtccgagagttcttcaagacttctctgttgataccaaggtatacggcatt
 H  H  E  S  P  R  V  L  Q  D  F  S  V  D  T  K  V  Y  G  I
gttttccgttattgccttgatgtttctaatggtgtctacggactcattaaaggtttcgat
 V  F  R  Y  C  L  D  V  S  N  G  V  Y  G  L  I  K  G  F  D
gtgaatgcgcctgtggcgccaaatcccctacaacgtaggaagttcacggcgaaacaagct
 V  N  A  P  V  A  P  N  P  L  Q  R  R  K  F  T  A  K  Q  A
agtggggtgcaaattcttgcccctactggaatgagcgttggggatataccagatgatctc
 S  G  V  Q  I  L  A  P  T  G  M  S  V  G  D  I  P  D  D  L
tggtttgtcataaaatatgacagtgcttttcagcccaacgttccggtgtggtttt***gtac***t
 W  F  V  I  K  Y  D  S  A  F  Q  P  N  V  P  V  W  F  C  T
ca***gtac***ctccaacactcgatgcctaagagagtcgagatccctgactcagtgttatatgct
 Q  Y  L  Q  H  S  M  P  K  R  V  E  I  P  D  S  V  L  Y  A
gaacgggatactaccgctctcatggatgcaatggatagaatagtcagtgga**tga**ctatat
 E  R  D  T  T  A  L  M  D  A  M  D  R  I  V  S  G  -
gatccattgtttgatt***gtac***ttccactatgagtattcctagaaatattcatagttggaaa

tgctgcttttgcaatagaatccaccattcagattttgtcacgcaaaatcccttcggtaaa

tttgccccggccta
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Figure 2. Nucleotide and deduced amino acid sequences of the 613 bp fragment of PDV isolate Jemmal1. The *Rsa* I restriction sites are indicated in bold and italics. The stop codon is in bold. The recombination sites are shaded.

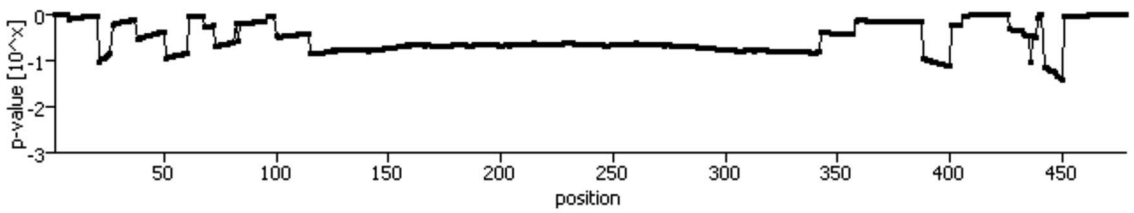

Figure 3. Graph displaying putative recombination breakpoints represented by downward peaks in the coat protein gene partial sequences of 32 accessions, and detected by the RECCO method based on cost optimization.

origin and the recombination events. The major recombinant isolates were from Portugal and grouped in cluster 3. In contrast, cluster 1 gathered nonrecombinant isolates from eastern Europe (Hungary and the Czech Republic). Clusters 2 and 4 were distantly related. Cluster 2 consisted of two isolates from Hungary and Jemmal 1 isolate from Tunisia, whereas cluster 3 had two isolates, one from Hungary and one from Poland.

Discussion

The study of the molecular evolutionary history of viruses helps to understand important features of their biology such as changes in their virulence and geographical range and their emergence in new epidemics. This information is essential to design strategies for controlling these viruses. Nonetheless, among evolutionary driving mechanisms, recombination can falsify the phylogenetic estimation procedure (Posada and Crandall, 2002) and distort subsequent inferences based on inferred phylogenies (Schierup and Hein, 2000a,b). Consequently, an essential step in any phylogeny-based analysis is to screen for and quantify the evidence for recombination (Kosakovsky Pond *et al*., 2006). In recent years, there has been a growing interest in the role of recombination in the evolution of

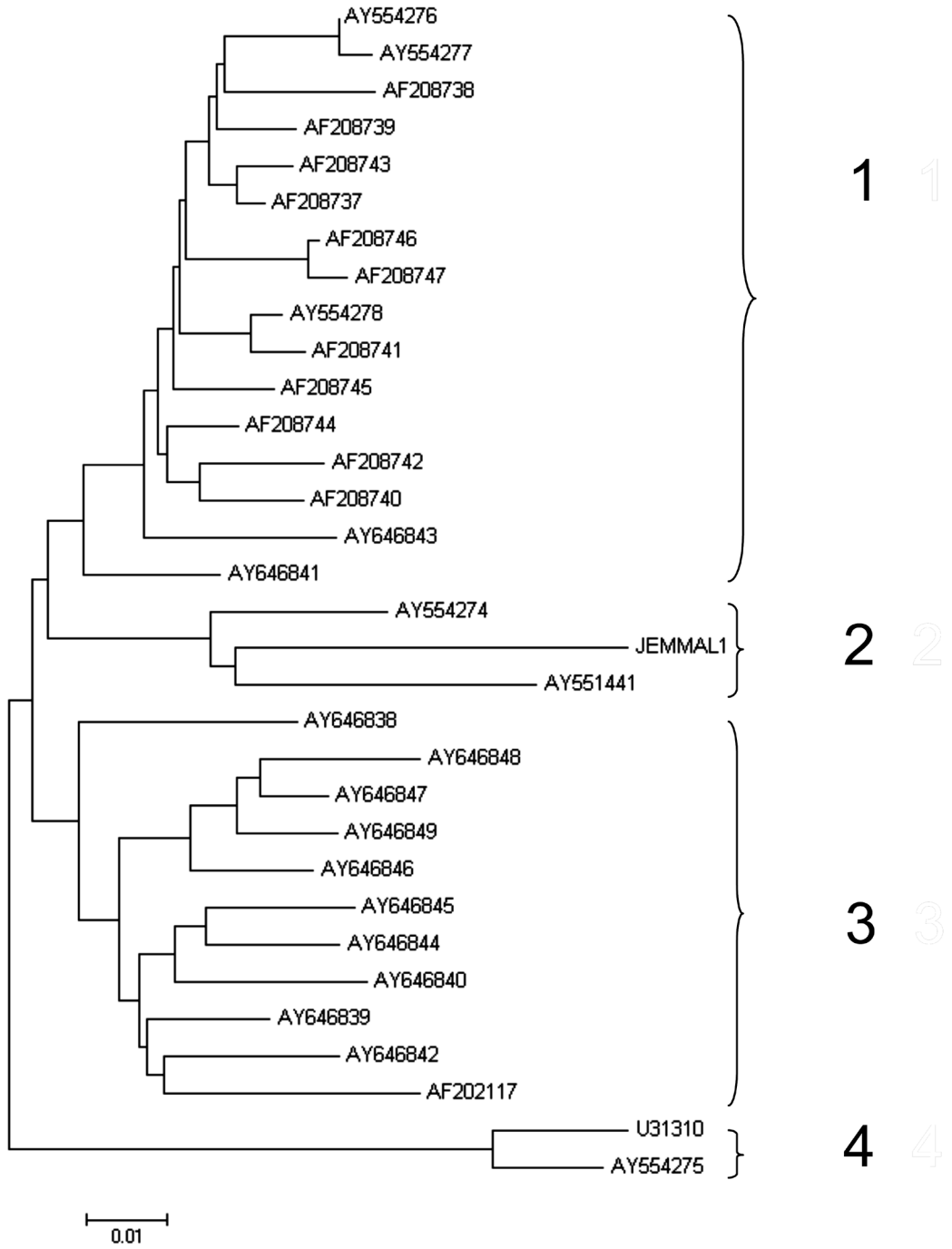

Figure 4. Dendrogram depicting phylogenetic relationships among Tunisian isolate Jemmal1, and other isolates available in the databanks. Four clades were delineated and indicated by Arabic numerals. The tree was produced using the NJ algorithm option of MEGA4.1β (Kumar *et al*., 2008). Bootstrap analysis of 500 replicates was performed. The scale bar shows the number of substitutions per nucleotide.

field populations of RNA plant viruses. Recombination may result in the exchange of long nucleotide sequences and it could have a more phenotypic effect than most mutations. This could jeopardize the effectiveness of current control methods, particularly so for crop varieties with inbred resistance to viruses (Garcia-Arenal and McDonald, 2003). Recombination has been extensively studied for numerous viruses in the *Bromoviridae*. However, to our knowledge, this is first report of recombination events in part of the coat protein-encoding gene of PDV. Fifteen out of 32 PDV isolates of different geographic origin were recombinants. Recombination resulted in a considerable insertion/deletion event. This was supported by the negative D value of Tajima's neutrality test (D= -0.709314) which tested the distribution of pairwise differences in the 32 PDV sequences. A negative D value is also obtained when the population has recently experienced a bottleneck (Tajima, 1989). The RECCO method used in this work was more sensitive, precise and accurate than the RDP v3.31 method, which did not detect any recombination signal. In spite of these interesting features, RECCO had one disadvantage, in that a long sequence of a given recombination site masked another, shorter sequence, so that both sequences appeared as one in the dataset graph. For instance, the strongest signal (extending from nt 115 to nt 342) detected in isolate 3.12N.14 (AY646846) from Portugal masked other signals (N1.2, cp1.2, 26B, 1.8A3, 3.17.A1, 3.20.1, Algarve, 2/16) (Figure 3). It should be noted that a similar situation was seen in a previous work dealing with *Grapevine fanleaf virus* (Boulila, 2007). As mentioned above, the recombination events potentially influence the evolutionary history of PDV. The 32 isolates fell into two main distinct and coherent groups (Figure 4). The first group included the nonrecombinant isolates from eastern Europe (subgroup 1), the second group contained the recombinant isolates from Portugal (subgroup 3). The recombination breakpoints corresponded to the areas of low phylogenetic correlation.

Studies conducted on viruses during the last 40 years indicate that RNA-RNA recombinations occur between the RNAs of the same or different strains of a single species, of different species or between the RNAs of a virus and its host. In our case, it is plausible that the recombination events detected in PDV occurred within the same virus, or combined to a varying extent with other viruses such as *Prunus necrotic ringspot* and *Apple mosaic ilarviruses*.

It is worth noting that besides recombination, genetic exchange can also be brought about by reassortment (Pressing and Reaney, 1984). Reassortment and recombination are distinct but not mutually exclusive types of genetic exchange. Reassortment occurs only in multipartite viruses and involves the viruses swapping one or more of the discrete RNA molecules that make up the segmented viral genome. Recombination occurs in either segmented or unsegmented viruses when the donor nucleotide sequence is introduced into a single, contiguous acceptor RNA molecule to produce a new RNA containing genetic information from more than one source. Masuta *et al*. (1998) reported an interspecific hybrid of two *cucumoviruses* that arose by both reassortment and recombination. Codoner and Elena (2008) stated that reassortment occurred during the genesis of PDV. The question that remains is: can an interspecific hybrid arise in PDV by both recombination and reassortment? This question can provide a fertile area for future research.

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