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# *Pepper chat fruit viroid*: Biological and molecular properties of a proposed new species of the genus *Pospiviroid*

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ABSTRACT

In autumn 2006, a new disease was observed in a glasshouse-grown crop of sweet pepper (*Capsicum annuum* L.) in the Netherlands. Fruit size of the infected plants was reduced up to 50%, and plant growth was also slightly reduced. Here we show that the disease is caused by a previously non-described viroid. The pepper viroid is transmitted by both mechanical inoculation and pepper seeds and, when inoculated experimentally, it infects several solanaceous plant species inducing vein necrosis and reduced fruit and tuber size in tomato and potato, respectively. The viroid RNA genome consists of 348 nucleotides and, with minor modifications, it has the central conserved and the terminal conserved regions characteristic of members of the genus *Pospiviroid*. Classification of the pepper viroid within the genus *Pospiviroid* is further supported by the presence and structure of hairpins I and II, the presence of internal and external RY motifs, and phylogenetic analyses. The primary structure of the pepper viroid only showed a maximum of 66% nucleotide sequence identity with other viroids, which is far below the main species demarcation limit of 90%. According to its biological and molecular properties, we propose to assign the pepper viroid to a new species within the genus *Pospiviroid*, and to name this new species *Pepper chat fruit viroid*.


## 1. Introduction

Viroids are small circular single-stranded RNAs of approximately 245–400 nucleotides (nt) that are able to infect plants (Diener, 1999; Flores et al., 2005a). They share structural similarities with certain satellite RNAs; however, viroids replicate autonomously whereas the replication of satellite RNAs depends on their helper viruses. Viroids do not code for any protein, and thus they lack the characteristic capsid of viruses. Taxonomically, viroids are divided into the families *Pospiviroidae* and *Avsunviroidae*, consisting of five and three genera, respectively. Within the family *Pospiviroidae*, the nucleotide sequence of the central conserved region (CCR), in combination with the nucleotide sequence of the terminal conserved region (TCR) or the terminal conserved hairpin (TCH), have been used for genus discrimination (Flores et al., 1997; Koltunow and Rezaian, 1988). Species demarcation criteria include sequence identity below 90% and different biological properties, particularly host range and symptom expression (Flores et al., 2005a). Currently, 29 viroid species have been admitted by the International Committee on Taxonomy of Viruses (ICTV), six are listed as tentative viroid species (Flores et al., 2005b), and another two have been characterized recently (Nakaune and Nakano, 2008; Serra et al., 2008).

In the autumn of 2006, a new disease was observed in a glasshouse-grown crop of sweet pepper (*Capsicum annuum* L.) in the Netherlands. The type of symptoms and the distribution pattern in the glasshouse raised the suspicion of a virus or viroid infection. However, the symptoms differed not only from those incited by known viruses, but also from *Potato spindle tuber viroid* (PSTVd), the only known viroid reported from *C. annuum* (Lebas et al., 2005). Therefore, studies were started to detect and identify the viruses and viroids in the diseased pepper plants. Here we report results showing that the disease is caused by a viroid. Biological and molecular properties of the pepper viroid support that it should be considered a new species of the genus *Pospiviroid*, for which we propose the name *Pepper chat fruit viroid* (PCFVd) on the basis of symptom analogy (reduced fruit size) to apple chat fruit disease (Luckwill, 1963).

## 2. Materials and methods

### 2.1. Mechanical inoculation

For biological studies, the plant leaves were mechanically inoculated according to Verhoeven and Roenhorst (2000). Unless otherwise indicated, inoculated plants were grown at 25 °C with

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**Table 1**
Primers used for RT-PCR amplification of PCFVd.

| Primer | Nucleotide sequence | Positions[a] |
|---|---|---|
| Pospi1-FW[b] | 5′-GGG ATC CCC GGG GAA AC-3′ | 85–101 |
| Pospi1-RE[b] | 5′-AGC TTC AGT TGT (T/A)TC CAC CGG GT-3′ | 277–255 |
| AP-FW1 | 5′-ACC CTT CCT TTC TTC GGG TTT CC-3′ | 178–200 |
| AP-RE2 | 5′-CAG CGG GGA TTA CTC CTG TCA-3′ | 164–144 |
| PCFVd-FW1[c] | 5′-*ggt cta g*AC CCT TCC TTT CTT CGG GTT TCC-3′ | 178–200 |
| PCFVd-RE1 | 5′-GAA AAC CCT GTT TCA GCG GGG AT-3′ | 177–155 |

[a] See Fig. 2.
[b] Specific for RT-PCR amplification of all pospiviroids except CLVd (Verhoeven et al., 2004); the first four nucleotides at the 5′ terminus of Pospi1-RE do not match the PCFVd sequence.
[c] Lower case characters refer to non-PCFVd nucleotides incorporated for cloning purposes.

supplemental daily illumination for 13 h over 6 weeks. To fulfill Koch's postulates, after return-polyacrylamide gel electrophoresis (r-PAGE) (Roenhorst et al., 2000; Schumacher et al., 1986), nucleic acids were eluted from the position of a non-stained polyacrylamide gel where viroid RNA was expected to migrate and then inoculated into pepper plants (*C. annuum* L.) 'Yolo Wonder'. These plants were tested 6 weeks later for the presence of the viroid by RT-PCR using the primer pair AP-FW1/RE2 (Table 1).

For host range studies the plants species listed in Table 2 were mechanically inoculated. Plants were weekly inspected for symptom expression along 8 weeks, and then RT-PCR tested for the pepper viroid using the primer pair AP-FW1/RE2. In addition, for each plant species the RT-PCR product of one plant was sequenced. Potato plants (*Solanum tuberosum* L.) were grown for another 8 weeks at 18–20 °C for tuber production.

To compare the symptoms of the pepper viroid with PSTVd in pepper, plants of 'Yolo Wonder' were inoculated with the pepper viroid and two isolates of PSTVd, i.e., 'Howell' and '20011470' (Verhoeven et al., 2004). Prior to inoculation, the relative viroid concentration of the inocula was estimated by r-PAGE (Roenhorst et al., 2000; Schumacher et al., 1986) at dilutions 1:5, 1:10 and 1:50, with the plants being then inoculated with comparable viroid amounts. After 8 weeks, plants were tested for viroid accumulation by RT-PCR using the primer pair AP-FW1/RE2 (Table 1), and the temperature was decreased to 20–23 °C during night and day, respectively. Symptoms were recorded weekly for 14 weeks.

### 2.2. Transmission through seed

To examine transmission through seed, the mechanically inoculated plants of pepper 'Yolo Wonder' from the experiment to fulfill Koch's postulates were grown at 20–23 °C to induce fruit setting, and 72 seeds from three fruits were collected and stored at ca. 18 °C. After 2 weeks the seeds without any further treatment were sown in potting soil at 25 °C, and from 2 weeks after emergence plants were grown for 10 weeks at 20–23 °C, and then tested in groups of six plants by RT-PCR with the primer pair AP-FW1/RE2 (Table 1). Groups that reacted positively were subsequently tested individually, and finally, RT-PCR products of three plants were sequenced.

**Table 2**
Plant reaction after mechanical inoculation of PCFVd.

| Plant species | Symptoms |
|---|---|
| *Brugmansia suaveolens* 'Geel' | ns[a] |
| *Capsicum annuum* 'Yolo Wonder' | ns |
| *Lycianthes rantonetti*, syn. *Solanum rantonetti* | ns |
| *Lycopersicon esculentum* 'Money-maker' | d, gr, ld, vn |
| *Solanum jasminoides* | ns |
| *Solanum melongena* 'Black Beauty' | ns |
| *Solanum tuberosum* 'Nicola' | nl, td, tgr |

[a] d = discoloration, gr = growth reduction, ld = leaf distortion, nl = necrotic lesions, ns = no symptoms (symptomless infection); td = tuber distortion and tgr = tuber growth reduction, vn = veinal necrosis.

### 2.3. Nucleic acid extraction, RT-PCR amplification and PAGE

For molecular studies, nucleic acids were extracted from 1 g of young leaves with a Homex grinder (BioReba, supplied by Sanbio, Uden, NL) using an extraction bag containing 5 ml of extraction buffer (0.02 M PBS plus 0.05% Tween, 2% PVP and 0.2% ovalbumine). The RNA was purified with the PureScript kit from Gentra (Biozym, Landgraaf, NL) according to the manufacturer's instructions. The final RNA pellets were resuspended in 40 μl of the buffer included in the RT-PCR kit (see below).

For the initial RT-PCR amplifications one set of primers (AP-FW1/RE2) was developed in addition to the Pospi1-FW/RE pair (Verhoeven et al., 2004) (Table 1). RT-PCR was performed in a 25 μl reaction volume (containing 1 μl of the RNA preparation and 1.0 μM of each primer), using the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen, Breda, NL) according to manufacturer's instructions. RT-PCR amplification of the full-length PCFVd was performed with SuperScript™ II and *Pwo* DNA polymerase (Roche Applied Science), using the primer pair PCFVd-FW1/RE1 (Table 1). The resulting products were separated by PAGE in non-denaturing 5% gels, and the DNA of the expected full-length was eluted, digested with Xba*I* and cloned into pUC18 opened with SmaI and XbaI.

The circular forms of PCFVd and the two viroid standards, *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd), were purified by double PAGE (Flores et al., 1985). Their relative mobilities were examined by PAGE in 5% gels containing 0.225 M Tris–borate–EDTA and 8 M urea (Sänger et al., 1977), and silver staining.

### 2.4. Sequence analysis, prediction of secondary RNA structure, and phylogenetic studies

The nucleotide sequences of PCFVd partial-length uncloned RT-PCR products was determined by BlaseClear in Leiden, NL, and that of full-length cloned RT-PCR products by an ABI 3100 Genetic Analyzer (Applied Biosystems) in Valencia, Spain. Multiple sequence alignments were performed using BLASTn (Altschul et al., 1990) and Clustal W (Thompson et al., 1994). The predicted viroid RNA secondary structure was obtained using Mfold, version 3.2 (Mathews et al., 1999; Zuker, 2003) and RnaViz, version 2 (De Rijk et al., 2003). Finally, phylogenetic analyses were conducted using MEGA, version 4 (Tamura et al., 2007). The nucleotide sequence of PCFVd has been deposited in the NCBI GenBank (Accession No FJ409044)

## 3. Results

### 3.1. Description of the disease in pepper

Near the end of the growing season in September 2006, first symptoms of a new disease were recorded in a glasshouse with 4 ha of pepper 'Jaguar'. Fruit size of the affected plants was reduced by as much as half of the normal size (Fig. 1A). Furthermore, plant growth was slightly reduced, and the young leaves of the infected plants were slightly smaller and paler than those of healthy plants. The affected plants were mainly located next to each other, in a few rows near the rear end of the glasshouse. At the end of May 2007, the disease reappeared in plants of the next cultivation of pepper 'Easy', again in a few adjacent rows at the same location in the glasshouse. By taking hygienic measures the disease was contained to a few rows for several months. Only at the end of the growing season did a few plants at other locations in the glasshouse show symptoms.

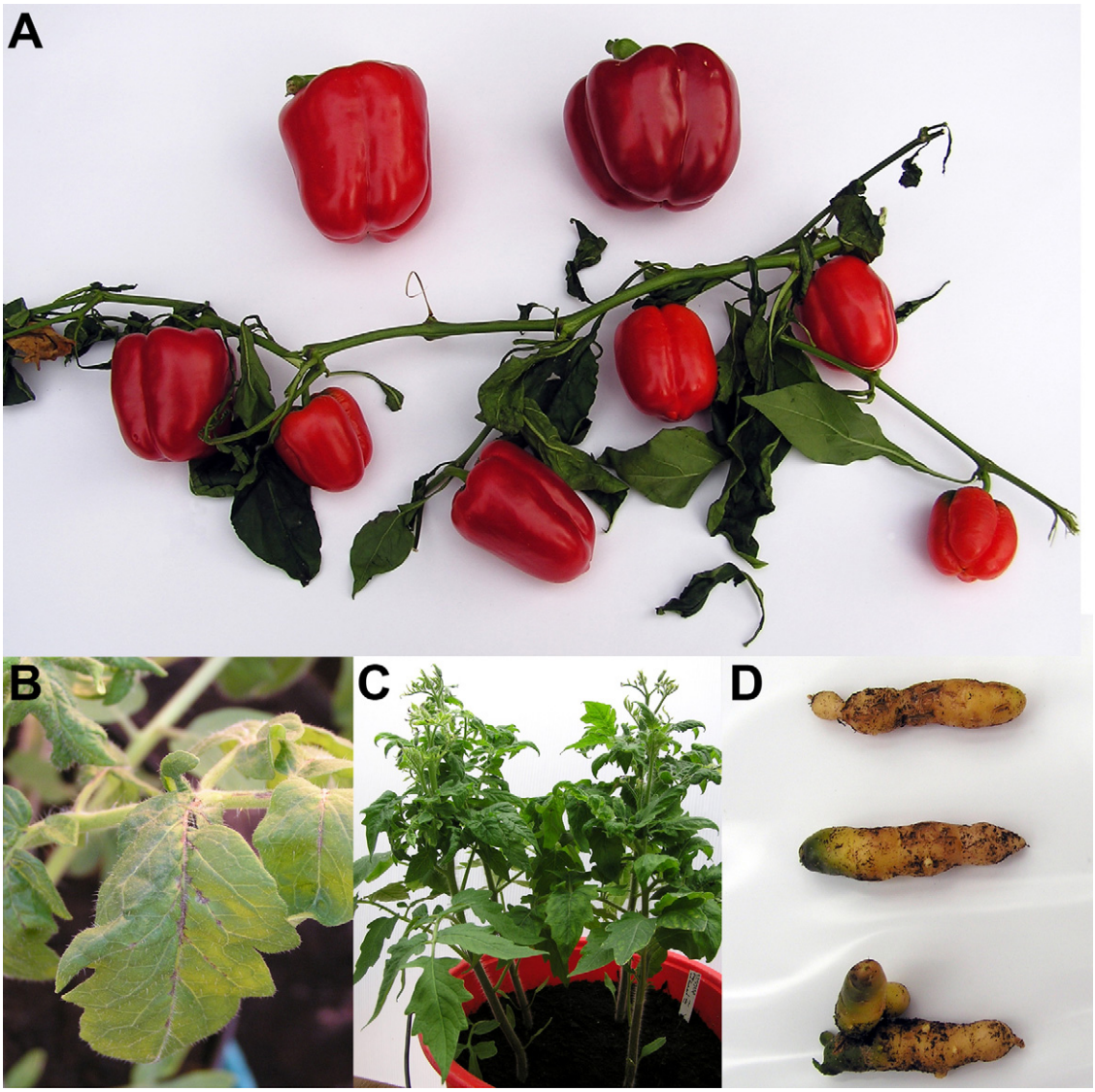


**Fig. 1.** (A) Fruits of pepper 'Jaguar' infected with PCFVd (bottom) compared with fruits from a healthy plant (top). (B) Necrosis along the veins and on the petioles of tomato 'Money-maker' 3 weeks after mechanical inoculation with PCFVd. (C) Stunting of tomato 'Money-maker' 5 weeks after mechanical inoculation with PCFVd. (D) Small, elongated and distorted tubers of potato plants 'Nicola' inoculated with PCFVd.

Stringent hygienic measures were taken during crop rotation at the end of 2007.

### 3.2. Fulfilling Koch's postulates

No viruses were detected by electron microscopy and mechanical inoculation to herbaceous test plants (data not shown); however, using RT-PCR with primers Pospi1-FW/RE for the detection of eight of nine known pospiviroids, a PCR product of the expected half viroid-genome size was obtained. Sequencing and alignment of the PCR product presumed the presence of a viroid species of the genus *Pospiviroid,* although only limited identities with known pospiviroids were found (see below). The presumed viroid RNA was separated from other RNAs and DNAs by r-PAGE and then eluted and mechanically inoculated to plants of pepper 'Yolo Wonder'. Examination of the pepper plants 6 weeks after inoculation by RT-PCR, using the primer pair AP-FW1/RE2, showed the presence of the pepper viroid-like RNA in all inoculated plants. Furthermore, comparison of inoculated and non-inoculated plants after 12 weeks revealed a severe fruit size reduction in the inoculated plants. Hence, the viroid-like RNA isolated from pepper was indeed a viroid, which was then tentatively called *Pepper chat fruit viroid* after its most conspicuous symptoms.

### 3.3. Experimental host range

In PCFVd-inoculated tomato (*Lycopersicon esculentum* L.) 'Money-maker', young leaves showed necrotic spots and streaks along the veins and on the petioles (Fig. 1B) 2–3 weeks after inoculation. Even if these symptoms did not always occur, they appeared characteristic for this viroid because, under similar environmental conditions, we have never observed them in tomato plants infected by other viroids. New leaves that developed 2–3 weeks after inoculation were small and a little distorted; their color was light-green, and later bronze or purple (Fig. 1C). Furthermore, the growth of the tomato plants was severely reduced. The type of discoloration and stunting was similar to the severe type of symptoms incited by other tomato-infecting pospiviroids. In potato (*Solanum tuberosum* L.) 'Nicola', occasionally, PCFVd also evoked necrotic lesions on the leaves and petioles 2–3 weeks after inoculation. No further leaf symptoms were observed on potato. The tubers, however, appeared misshapen: they only measured one fourth to half of the normal

**Table 3**
Symptomatology of PCFVd and PSTVd on mechanically inoculated plants of pepper 'Yolo Wonder'.

| Inoculum[a] | Plant height (cm) | | | Number of fruits per plant | | Maximum fruit size (cm) | |
|---|---|---|---|---|---|---|---|
| | 6 wpi[b] | 10 wpi | 14 wpi | 10 wpi | 14 wpi | 10 wpi | 14 wpi |
| PCFVd (3259237) | 19.2 (±3.6)[c] | 38.4 (±8.3) | 52.4 (±11.1) | 1.4 (0–4) | 3.6 (0–6) | 2.5 (±0.7) | 3.5 (±1.0) |
| PSTVd (Howell) | 23.2 (±6.4) | 42.2 (±4.9) | 51.8 (±8.6) | 3.0 (0–8) | 5.8 (3–11) | 4.0 (±0.0) | 4.4 (±1.3) |
| PSTVd (20011470) | 26.6 (±1.5) | 41.6 (±2.3) | 46.6 (±5.9) | 3.4 (2–5) | 6.0 (4–9) | 3.4 (±0.5) | 4.4 (±0.6) |
| Mock-inoculated[d] | 23.2 (±2.9) | 31.2 (±5.5) | 35.0 (±5.1) | 5.4 (3–10) | 5.6 (3–10) | 5.4 (±0.9) | 6.6 (±0.9) |

[a] The specific isolate is indicated within parenthesis.
[b] Weeks post-inoculation.
[c] Numbers are mean values per five plants, with the standard deviation (in plant height and maximum fruit size) and the variation (in number of fruits per plant) indicated within parenthesis.
[d] A plausible explanation for the reduced height of the mock-inoculate plants is provided in the main text.

size, were elongated, and produced lateral extensions along the main tubers (Fig. 1D).

No symptoms were recorded for the other inoculated plant species during 8 weeks (Table 2). Nevertheless, they were PCFVd-infected as shown by RT-PCR using the primer pair AP-FW1/RE2. Direct sequencing of the PCR product of one plant of each inoculated species revealed that all sequences were identical to the original PCFVd sequence from pepper, showing that no genome mutant had become dominant in the experimental hosts.

### 3.4. Symptoms of PCFVd and PSTVd on pepper

Six weeks after inoculation first flowers appeared on the mock-inoculated plants and fruit setting started, while fruit setting started 2 weeks later on the PSTVd- and PCFVd-inoculated plants. Ten weeks after inoculation, the mock-inoculated plants were both smaller and more chlorotic than the inoculated ones due to the energy and nutrients needed for fruit setting, which not only started earlier at the mock-inoculated plants but also was more successful (Table 3). Furthermore, fruit number was reduced by PCFVd, and maximum fruit size was reduced by both PCFVd and the two PSTVd isolates, although the effects were more pronounced in the case of PCFVd. Nevertheless, the symptoms of PSTVd on pepper were more severe than those reported before (Lebas et al., 2005). Eight weeks after inoculation, PCFVd and PSTVd infections were confirmed by RT-PCR in all inoculated plants.

### 3.5. PCFVd is transmitted through pepper seed

Fifty-nine seedlings were raised from seventy-two seeds from PCFVd-infected pepper fruits. Eleven seedlings appeared infected by PCFVd after testing by RT-PCR using the primer pair AP-FW1/RE2. This is an infection rate of approximately 19%, assuming that the non-emergence of 13 seeds is not correlated to viroid infection. We ignore whether PCFVd is located inside and/or outside the seeds. Sequencing of the RT-PCR products from three infected pepper seedlings demonstrated that the predominant nucleotide sequence of the viroid had not changed during seed transmission.

### 3.6. Molecular characterization and classification of PCFVd

The primary structure of the viroid genome was determined by direct sequencing of the partially overlapping RT-PCR products obtained with the primer pairs Pospi1-FW/RE and AP-FW1/RE2 (Table 1). PCFVd consists of 348 nt: 106 C (30.5%), 100 G (28.7%), 76 U (21.8%), and 66 A (19.0%), which makes a C + G content of 59.2%. The computer-predicted secondary structure of minimum free energy is a rod-like conformation (Fig. 2A) with 69.5% of the nucleotides paired (64.5% G:C, 29.7% A:U, and 5.8% G:U pairs). Accordingly with its size, examination by denaturing PAGE showed that PCFVd migrated with a mobility intermediate between that of CEVd (371 nt) and HSVd (297 nt) (Fig. 3). The primary structure of PCFVd was confirmed by cloning the full-length RT-PCR products obtained with the primer pair PCFVd-FW1/RE1 (Table 1). Analysis of the inserts of seven recombinant plasmids revealed the same sequence.

The primary structure of PCFVd shows the presence of a CCR, which is the main criterion to classify a new viroid as a member of the family *Pospiviroidae*. Furthermore, the type of CCR and the presence of a TCR or a TCH are the main criteria for genus classification within this family (Flores et al., 1997; Flores et al., 2005b). The CCR of PCFVd closely resembles that of members from the genus *Pospiviroid*. Nevertheless, three and two nucleotide differences are observed in the upper and lower CCR strands, respectively, with the latter having a 3′ terminus three nucleotide shorter (Fig. 2B). The classification of PCFVd as a member of the genus *Pospiviroid* is

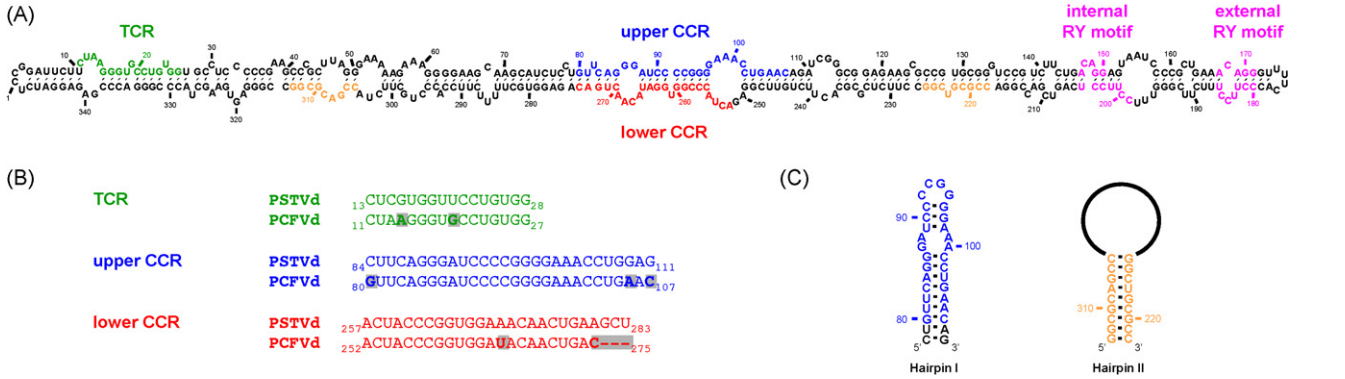


**Fig. 2.** (A) Primary and proposed secondary structure of minimum free energy of PCFVd. Nucleotides forming the terminal conserved region (TCR), upper and lower central conserved region (CCR), hairpins I and II, and internal and external RY motifs are highlighted in green, blue, red, orange and purple letters respectively. (B) Comparison between the TCR and the upper and lower CCR of PCFVd and PSTVd, the type species of the genus *Pospiviroid*. Nucleotides conserved in pospiviroids (Flores et al., 1997), but not in PCFVd, are denoted in bold characters with a grey background. (C) Schematic representation of hairpins I and II that PCFVd and PSTVd can potentially form.

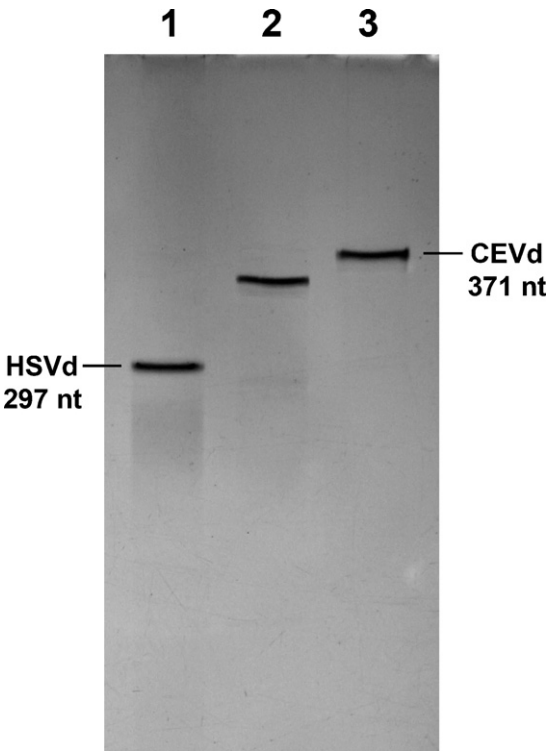


**Fig. 3.** Mobilities of the purified circular forms of HSVd (297 nt), PCFVd and CEVd (371 nt) (lanes 1, 2 and 3, respectively) after PAGE in 5% gels containing 0.225 M Tris–borate–EDTA and 8 M urea, and silver staining.

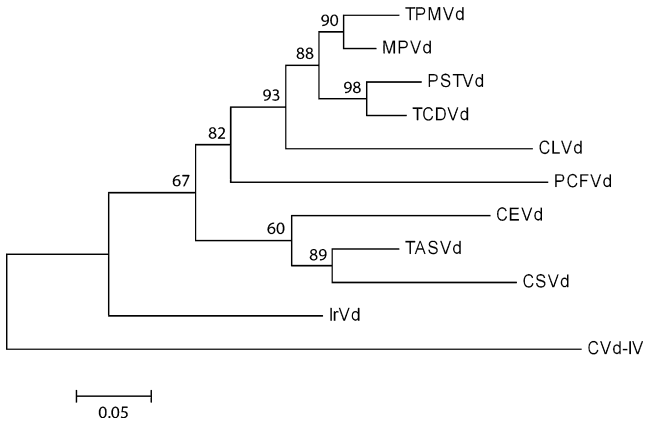


**Fig. 4.** Evolutionary relationships of PCFVd and all current pospiviroids: TPMVd (K00817), MPVd (L78454), PSTVd (V01465), *Tomato chlorotic dwarf viroid*, TCDVd (AF162131), CLVd (X15663), CEVd (M34917), TASVd (NC001553) and *Chrysanthemum stunt viroid*, CSVd (NC003613). CVd-IV (X14638) was added as an outgroup. The phylogenetic reconstruction was performed with the Neighbor-joining method (Saitou and Nei, 1987). The fractions (%) of replicate trees in which the groups clustered together in the bootstrap test (5000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree, which were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are expressed in base substitutions per site. Phylogenetic analyses were conducted with MEGA4 (Tamura et al., 2007).

further supported by the absence of a TCH and the presence of a TCR, in which two nucleotide differences are also found (Fig. 2B). On the whole, PCFVd best matches the molecular characteristics of the genus *Pospiviroid*.

Further evidence for classifying PCFVd within the genus *Pospiviroid* was obtained from the conservation of other structural and sequence motifs. Like all members of the family *Pospiviroidae,* PCFVd can form one or two thermodynamically stable hairpins (Fig. 2C). The structure of hairpin I resembles that of all genera of the family *Pospiviroidae* (Flores et al., 1997; Gas et al., 2007) including the apical palindromic tetraloop, the adjacent 3-bp stem, and the long 9-bp stem; however, it is most similar to that of the genus *Pospiviroid* and, interestingly, the three differences observed between the upper CCR strand of PCFVd and PSTVd (the type species of this genus) do not affect the stability of the stem of hairpin I because two are co-variations and the third transforms a G:U wobble into a G:C canonical base-pair. The lower strand of the rod-like secondary structure can alternatively form a stable hairpin (II) with a GC-rich stem of 9 bp resembling the hairpin II formed in PSTVd during thermal denaturation (Riesner et al., 1979; Loss et al., 1991); again, the sequence differences between PCFVd and PSTVd do not affect the stability of the stem of hairpin II because they are co-variations. In the terminal right domain of the rod-like secondary structure PCFVd also shows an internal and an external RY motif that has been proposed to mediate viroid systemic transport (Maniataki et al., 2003). Both, or at least one of these motifs, are present in all members of the genus *Pospiviroid* (Gozmanova et al., 2003).

A key criterion for viroid species demarcation is based on comparisons of the nucleotide sequence identity of the complete genomes (Flores et al., 2005b). For being considered a new species, a viroid must show less than 90% nucleotide identity with the sequences from all known viroid species. Using BLASTn (Altschul et al., 1990), PCFVd shows the highest nucleotide sequence identities with members of the genus *Pospiviroid*; however, identities are less than 66%. As a consequence, the primary structure of PCFVd differs so much from all other viroids that creating a new species is justified on the basis of its molecular properties. Sequence differences are mainly located in the terminal left, pathogenic, and variable domains of the rod-like secondary structure (Keese and Symons, 1985), while the terminal right domain shows sequence identities even up to 100% with some isolates of *Columnea latent viroid* (CLVd), *Mexican papita viroid* (MPVd), *Tomato apical stunt viroid* (TASVd) and *Tomato planta macho viroid* (TPMVd).

### 3.7. Phylogenetic reconstructions also support the classification of PCFVd as a new species of the genus Pospiviroid

Several phylogenetic analyses were performed including PCFVd, all current members of the genus *Pospiviroid*, *Citrus viroid IV* (CVd-IV, recently renamed *Citrus bark cracking viroid,* CBCVd, genus *Cocaviroid*), *Grapevine yellow speckle viroid 1* (GYSVd-1, genus *Apscaviroid*) and HSVd (genus *Hostuviroid*). Fig. 4 shows a phylogenetic tree based on the Neighbor-joining method (Saitou and Nei, 1987), in which PCFVd groups with the current members of the genus *Pospiviroid* while CVd-IV appears as an outgroup. Similar results were obtained when including GYSVd-1 or HSVd instead of CVd-IV (data not shown). Therefore, the phylogenetic analyses support the classification of PCFVd as a member of the genus *Pospiviroid*. Furthermore, the branch length of PCFVd in the phylogenetic trees is also consistent with considering it as new viroid species.

## 4. Discussion

Our present results show that a new pepper disease is caused by a viroid, PCFVd, which can be discriminated from other viroids by its biological and molecular properties. PCFVd is transmitted by both mechanical inoculation and pepper seeds, which is in line with previous observations with other pospiviroids infecting solanaceous plants (Sing et al., 1988; Singh and Dilworth, 2009), and, it can experimentally infect several solanaceous plants wherein, occasionally, it causes a new type of necrotic symptoms on tomato and

potato leaves. The molecular properties of PCFVd also differ from viroids that have been reported so far, with which it only shows up to 66% sequence identity. However, the CCR and TCR of PCFVd mostly resemble those of the genus *Pospiviroid*, while a TCH is lacking as in other species of this genus. Furthermore, the presence and structure of hairpins I and II, and of internal and external RY motifs, also favor classification of PCFVd within the genus *Pospiviroid*, as well as the phylogenetic analyses showing that PCFVd differs from current members of the genus *Pospiviroid* at the species but not at the genus level.

The origin of the PCFVd infection in pepper 'Jaguar' in 2006 is unclear. Although PCFVd is seed-borne, two reasons make unlikely that infected pepper seeds were the source of infection. First, the disease was noticed only at the end of the growing season whereas, in the case of seed transmission, symptoms would have been expected earlier. Second, the disease was only reported in a single crop of pepper 'Jaguar', whereas more infections would have been expected considering that 40 ha of pepper 'Jaguar' were grown in 2006 and our estimate of a seed-transmission rate of nearly 20%. The infection of pepper 'Easy' by PCFVd in 2007 most probably originated from the infection in the previous crop because the glasshouse had not been cleaned and disinfected thoroughly during crop rotation.

The observation of the first symptoms in the crop of pepper 'Jaguar' only late in the growing season of 2006 suggests another host plant, probably from outside the glasshouse, as the source of infection. In addition to pepper 'Jaguar', other plants in the glasshouse included: a group of 25 pepper plants of different varieties, a few ornamental plants of *Bougainvillea* sp. and *Dipladenia* sp., grown in the rear end of the glasshouse, and some weeds. Testing the 25 pepper plants in groups of five before their removal at the end of the growing season detected PCFVd in two groups (data not shown). Nevertheless, it is unlikely that these plants had acted as sources of infection because the presence of PCFVd in only part of them and their lack of symptoms rather suggest that they became infected later than the plants of pepper 'Jaguar'. Furthermore, testing all ornamental plants as well as some weeds of *Calystegia sepium*, *Equisetum* sp. and a fern did not reveal any PCFVd infection (data not shown). Because our study also shows that PCFVd can infect various solanaceous species, and pepper was the only species of this family present in the glasshouse; we presume that solanaceous plants from outside the glasshouse may have acted as the primary sources of infection in analogy with the situation for CEVd, CLVd and PSTVd infections of tomato (Verhoeven et al., 2004; Verhoeven et al., unpublished results).

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