# APPLE DIMPLE FRUIT VIROID: SEQUENCE VARIABILITY AND ITS SPECIFIC DETECTION BY MULTIPLEX FLUORESCENT RT-PCR IN THE PRESENCE OF APPLE SCAR SKIN VIROID

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**SUMMARY**

*Apple dimple fruit viroid* (ADFVd) causes a severe fruit disorder in several apple cultivars. The viroid was discovered a few years ago, and only a limited number of sequence variants has been reported so far. In this work, the sequence variability of two ADFVd field isolates from two commercial apple cultivars was studied. Sequencing of 18 full-length cDNA clones revealed five new sequence variants, all adopting a similar quasi-rod-like secondary structure of lowest free energy. Sequence comparison showed nine polymorphic positions distributed in different regions of the ADFVd molecule. Since symptoms of apple dimple fruit disease resemble those of dapple apple induced by *Apple scar skin viroid* (ASSVd), and it has been experimentally demonstrated that these two viroids may co-exist in the same plant, a fast and sensitive method for their rapid detection and discrimination is needed. We report here a method that meets such needs. Total nucleic acid minipreparations from symptomatic fruits, obtained by extraction with buffer-saturated phenol and further treatment with a silica-gel capture system, were used as templates for the simultaneous detection of ADFVd and ASSVd by multiplex fluorescent RT-PCR amplification. The incorporation in the PCR reaction of two viroid-specific primers, each labeled with a different fluorescent dye, simplifies and facilitates distinction of the amplified products, and avoids the use of the mutagenic and cancer inducing agent ethidium bromide for gel staining.

*Key words:* viroids, apple, *Malus,* multiplex fluorescent RT-PCR.

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## INTRODUCTION

In 1996, apple plants of cv. 'Starking Delicious' with depressed, yellow-green, rounded spots on the fruit skin, were observed in Campania (southern Italy) (Di Serio *et al.,* 1996). Subsequently, similar symptoms were found on fruits of other commercial apple cultivars grown in the same area (Di Serio *et al.,* 1998; 2000). This disease, denoted apple dimple fruit, was reminiscent of dapple apple induced by *Apple scar skin viroid* (ASSVd) (Koganezawa, 1989). However, symptomatic plants were not infected with ASSVd but by another viroid, *Apple dimple fruit viroid* (ADFVd) (Di Serio *et al.,* 1996), which further studies proved to be the causal agent of the disease (Di Serio *et al.,* 2001).

ADFVd is a circular RNA of 306-307 nucleotide residues (nt) that adopts a quasi-rod like structure of minimum free energy with the central conserved region (CCR) typical of members of genus *Apscaviroid* (Flores *et al.,* 2000). ADFVd shows the highest sequence similarity (62.9%) with ASSVd (Hashimoto and Koganezawa, 1987), the type species of this genus. Because of the high similarity between ADFVd and ASSVd sequences, as well as between the fruit symptoms they induce, diagnostic methods for specific detection and clear discrimination of the two viroids are needed, especially because both can co-exist within the same plant (Di Serio *et al.,* 2001). Methods based on molecular hybridization with full-length cRNA probes of both viroids have been previously described, but their use is limited because of cross-hybridization (Di Serio *et al.,* 1996; 2000).

Detection of viroids by RT-PCR is well-documented (Hadidi and Yang, 1990; Rezaian *et al.,* 1992; Wan Chow Wah and Symons, 1997; Faggioli *et al.,* 2001; Nie and Singh, 2001). One of the advantages of this technology is the possibility of using specific primers for the simultaneous detection of more than one viroid in a multiplex format (Levy *et al.,* 1992; Faggioli *et al.,* 2001; Nie and Singh, 2001). A method for the simultaneous detection of ADFVd, ASSVd and *Pear blister canker viroid* (PBCVd) using a common pair of primers has been

recently reported (Faggioli *et al.,* 2001), but the close size of the resulting cDNAs, which differ only by 6 to 12 nt, makes it difficult a clear electrophoretic discrimination even with resolving polyacrylamide gels. When the amplified cDNAs from the co-infecting pathogens differ sufficiently in size, they can be easily discriminated by agarose gel electrophoresis and ethidium bromide staining but, ideally, it should be desirable to label specifically each of the amplified products. An additional aspect that must be taken into account in this context is that viroids exhibit sequence heterogeneity as a consequence of the quasi-species nature of their populations. There are numerous examples of this, including members of genus *Apscaviroid, i.e.* ASSVd (Puchta *et al.,* 1990; Yang *et al.,* 1992; Desvignes *et al.,* 1999), PBCVd (Ambrós *et al.,* 1995), *Grapevine yellow speckle viroid 1* (GYSVd 1) (Polivka *et al.,* 1996), and *Citrus viroid III* (CVd III) (Owens *et al.,* 1999). However, the available information for sequence heterogeneity of ADFVd is very limited (Di Serio *et al.,* 1998), and this needs to be considered when appropriate primers for RT-PCR amplification are designed.

An RT-PCR method for discriminating strains of *Potato virus Y* (PVY), which uses strain-specific primers tagged with different fluorescent labels in conjunction with a universal PVY primer, has recently been developed (Walsh *et al.,* 2001). Discrimination is conferred by the fluorescent labels. We reasoned that a similar approach could be adapted for the sensitive and specific detection of ADFVd and ASSVd. Here we report a study of the sequence heterogeneity of ADFVd and, based on this information and on that available for ASSVd, we describe a RT-PCR procedure with fluorescent primers that permits the simultaneous and unequivocal detection of ASSVd and ADFVd.

## MATERIALS AND METHODS

**Plant material.** Two apple trees, one of cv. 'Annurca' (Di Serio *et al.,* 2000) and the other of cv. 'Red Delicious', grown in the Caserta area (Southern Italy), were used as ADFVd sources (ADFVd-an and ADFVd-rd isolates, respectively) for the study of the sequence variabilty of this viroid. 'Red Delicious' plants naturally infected by ADFVd were detected for the first time in 2001. One of these plants, showing typical fruit symptoms, was selected as the source of 'Red Delicious' isolate. The ASSVd isolate PK13 (Desvignes *et al.,* 1999) was also used to set up the detection method based on fluorescent RT-PCR.

**Nucleic acid extraction and viroid purification.** Preparations enriched in RNAs with high secondary structure were obtained from symptomatic fruits (20 g) by extraction with buffer-saturated phenol and chromatography on non-ionic cellulose (Pallás *et al.,* 1987). ADFVd circular forms were purified by two consecutive steps of polyacrylamide gel electrophoresis (PAGE) (Di Serio *et al.,* 2001), a routine procedure adopted in our laboratory to clone viroid cDNAs. For detection and cloning aims, total nucleic acid minipreparations were obtained with an alternative extraction method (Dalmay *et al.,* 1993). Briefly, small pieces corresponding to one or two lesions of the fruit skin (100-200 mg), were rapidly macerated with a pestle in an ice-cooled mortar, resuspended in 600 µl of extraction buffer (0.1 M glycine-NaOH, pH 9, containing 2% SDS and 1% sodium lauroylsarcosine), and mixed with an equal volume of water-saturated phenol. Following a second extraction with phenol-chloroform, total nucleic acids in the final aqueous phase were precipitated with ethanol and resuspended in 50 µl of RNase-free water. To improve reproducibility of RT-PCR amplifications, total nucleic acids were further purified by a modified silica gel capture system (Foissac *et al.,* 2001). Briefly, RNase-free water (up to 300 µl) was added to the nucleic acid preparations and mixed with the silica-capture mixture (300 µl of 6 M NaI, 150 µl of 95% ethanol and 25 µl of autoclaved pH 2 silica at a concentration of 1 g $ml^{-1}$). After incubation for 10 min at room temperature with intermittent shaking, the silica was washed twice with a solution containing 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, and 50% ethanol and, finally, nucleic acids were eluted with RNase-free water.

**Cloning and sequencing of ADFVd.** Purified circular forms of ADFVd and total nucleic acid minipreparations were used to clone ADFVd cDNAs from ADFV-dan and ADFVd-rd isolates, respectively. First-strand cDNA was synthesized with the reverse transcriptase from the Moloney murine leukemia virus (MoMLV-RT, Gibco-BRL) using primers AD-5 or AD-34, complementary to positions 69 to 93 and 135 to 155 of the ADFVd reference sequence (Di Serio *et al.,* 1996), respectively. PCR was performed with the same primer used in RT reaction and an adjacent primer AD-6, homologous to positions 94 to 112, or AD-35, homologous to positions 156 to 176. The sequences of the ADFVd primer pairs and the sizes of the expected amplified products are shown in Table 1. *Vent* DNA polymerase (New England BioLabs) or *Taq* DNA polymerase (Roche Molecular Biochemicals) were used for PCR amplifications adopting a cycling profile (94°C for 40 s, 55°C for 40 s and 72°C for 1 min) that was repeated for 30 cycles with a final extension step at 72°C for 7 min. *Vent*-amplified cDNAs were electrophoresed in

**Table 1.** Primers used for RT-PCR amplification of ADFVd and ASSVd, and sizes of the resulting cDNAs. Primers of complementary polarity are marked with an asterisk, and bold FL and RH indicate 5'-terminal labeling with fluoresceine and rhodamine, respectively.

| Primer | Sequence | Expected cDNA (bp) |
|---|---|---|
| AD-5* | 5'-GACGACGACAGGTAAGTCTCTTCA-3' | |
| AD-6 | 5'-GACGAAGGCTGGTAAGCCG-3' | 306-307 |
| AD-34* | 5'-GGTTACCCCCAAGAGCGCGAC-3' | |
| AD-35 | 5'-CCTTTGAGACTTGACCGGTTC-3' | 306-307 |
| ADAS-36* | 5'-GCCTTCGTCGACGACGACAG-3' | |
| AS-37fl | 5'-**FL**-CGGTGACAAAGGAGCTGCCAG-3' | 330 |
| AD-38rh | 5'-**RH**-CCTTTGAGACTTGACCGGTTCCTC-3' | 254 |

agarose gels and the expected full-length product was eluted and cloned into pUC18 (Amersham Pharmacia Biotech.) linearized in the *Sma*I site. The corresponding *Taq*-amplified cDNAs were directly cloned in pGEM-T Easy vector using the improved 2X rapid ligation kit (Promega). Inserts were automatically sequenced with an ABI PRISM DNA-377 apparatus (Perkin-Elmer).

**Sequence analysis.** ADFVd and ASSVd reference sequences were aligned using the GAP program (gap weight 5, length weight 0.3) of the GCG package from the University of Wisconsin (Devereux *et al.,* 1984). The secondary structures of lowest free energy were obtained with the MFOLD program (Zuker, 1989).

**Fluorescent RT-PCR.** Total nucleic acid minipreparations from symptomatic fruits were reverse transcribed with MoMLV-RT and primer ADAS-36, complementary to positions 83 to 102 of the ADFVd reference sequence corresponding to the upper strand of the central domain conserved in ADFVd and ASSVd. The amplification with *Taq* DNA polymerase was performed using primer ADAS-36 and primer AS-37fl, homologous to positions 98 to 118 of the ASSVd reference sequence (Hashimoto and Koganezawa, 1987), and/or primer AD-38rh, homologous to positions 156-179 of the ADFVd reference sequence (Di Serio *et al.,* 1996). Primer AS-37fl, specific for ASSVd, was labeled with fluoresceine at its 5'-end and when used in combination with primer ADAS-36 should generate in ASSVd-infected samples a PCR-amplified full-lenght (330 bp) cDNA emitting green light when excited by UV irradiation. Primer AD-38rh, specific for ADFVd, was labeled with rhodamine at its 5'-end and when used in combination with primer ADAS-36 should generate in ADFVd-infected samples a PCR-amplified cDNA of 254 bp emitting red light when excited by UV irradiation. PCR cycling profile consisted of 30 cycles of 40 s at 94°C, 40 s at 58°C and 50 s at 72°C, followed by a final extension at 72°C for 7 min. PCR-amplified products were analysed by electrophoresis in 1.3% agarose gels and revealed by ethidium bromide staining or irradiation with a UV lamp.

## RESULTS

**Cloning and molecular characterization of ADFVd isolates.** RT-PCR amplification with *Vent* DNA polymerase and primers AD-5 and AD-6, using as template purified viroid circular RNA from the ADFVd-an isolate, led to a cDNA of the expected size that was subsequently cloned. Sequencing of ten clones confirmed a size of 307 nt, in accordance with that of ADFVd sequence variants from other isolates characterized previously (Table 2). A common feature of all ADFVd-an variants with respect to the reference sequence (Di Serio *et al.,* 1996), was the insertion of one residue (U or C) between U225 and A226 (Fig. 1). Variant ADFVd-06 showed only this polymorphic position (insertion of U, Table 2). Insertion of a C residue at the same position and two nucleotide substitutions at positions 46 and 222 characterized ADFVd-07 variant that was the most frequently recovered in this cloning experiment (Table 2). Variant ADFVd-08, showing the substitution of G163 by U and the insertion of a U between U 225 and A 226, was found just once.

The possible existence of sequence variability in the region covered by primers AD-5 and AD-6 was also checked. To this aim, another primer pair (AD-34 and AD-35) designed on a different region of the molecule was used for cloning purposes. Sequencing of four full-

**Table 2.** Polymorphic positions observed in ADFVd sequence variants with respect to the reference sequence variant ADFVd-01 from 'Starking Delicious' (Di Serio *et al.,* 1996). Nucleotide insertions are indicated by + and nucleotide changes by ⇒.

| Sequence variant | Isolate | Independent clones | Primer pair used for cloning | Size (nt) | Polymorphic positions | Reference |
|---|---|---|---|---|---|---|
| ADFVd-06 | Annurca | 3 | AD-5/AD-6 | 307 | +U (225-226) | this work |
| | Red Delicious | 2 | AD-5/AD-6 | | | this work |
| ADFVd-07 | Annurca | 6 | AD-5/AD-6 | 307 | +C (225-226); A46⇒C; G222⇒A | this work |
| | Annurca | 2 | AD-34/AD-35 | | | this work |
| | Red Delicious | 2 | AD-5/AD-6 | | | this work |
| ADFVd-08 | Annurca | 1 | AD-5/AD-6 | 307 | +U(225-226); G163⇒U | this work |
| ADFVd-09 | Annurca | 1 | AD-34/AD-35 | 307 | +U(225-226); A46⇒C | this work |
| ADFVd-10 | Annurca | 1 | AD-34/AD-35 | 307 | +U(225-226); G42⇒A | this work |
| ADFVd-01 | Starking Delicious | 3 | AD-5/AD-6 | 306 | | Di Serio *et al.,* 1996 |
| | Royal Gala | 2 | AD-5/AD-6 | | | Di Serio *et al.,* 1998 |
| ADFVd-02 | Golden Delicious | 1 | AD-5/AD-6 | 306 | U175⇒G | Di Serio *et al.,* 1998 |
| ADFVd-03 | Golden Delicious | 1 | AD-5/AD-6 | 306 | U175⇒G; C274⇒U | Di Serio *et al.,* 1998 |
| ADFVd-04 | Golden Delicious | 1 | AD-5/AD-6 | 307 | +U (225-226); A128⇒G | Di Serio *et al.,* 1998 |
| ADFVd-05 | Royal Gala | 1 | AD-5/AD-6 | 306 | C116⇒U | Di Serio *et al.,* 1998 |

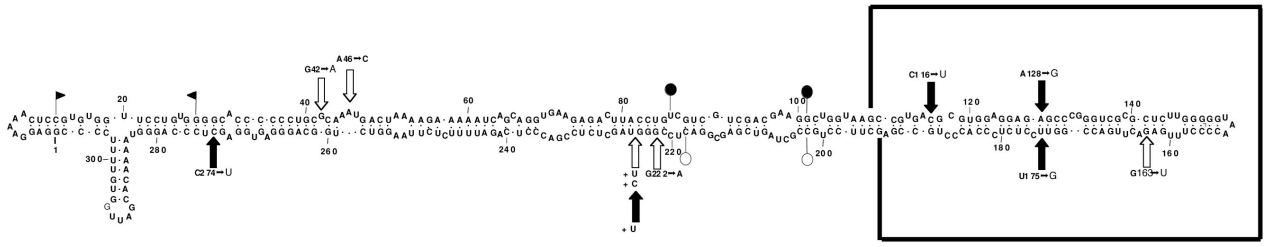


**Fig. 1.** Sequence variability of ADFVd. Changes found in the sequence variants characterized in this work are indicated by white arrows on the secondary structure of lowest free energy predicted for the reference sequence (Di Serio *et al.,* 1996), and other changes reported previously are indicated by black arrows. The region between positions 109 and 194, characterized by a high sequence divergence with respect to ASSVd, is boxed (see Fig. 2).

length cDNA clones did not reveal any variability in the region corresponding to the first pair of primers. However, two new sequence variants were found. Variant ADFVd-10 (Table 2) had the insertion of a U between U225 and A226 already found in other variants, together with a new polymorphic position, the substitution of G42 by A (Fig. 1 and Table 2). Variant ADFVd-09 showed only two of the three changes observed in ADFVd-07 variant, the U insertion between positions 225 and 226, and the substitution of A46 by C (Table 2). The other two cloned cDNAs had sequences identical to ADFVd-07 variant.

RT-PCR amplification with *Taq* DNA polymerase and primers AD-5 and AD-6, using as template total nucleic acid preparations from the ADFVd-rd isolate, also led to the expected cDNA. Sequencing of four clones showed that they had sequences identical to the two most frequent variants (ADFVd-06 and ADFVd-07) of the ADFVd-an isolate (Table 2). Altogether these results showed that ADFVd is a viroid with limited sequence variability.

**Primer design for multiplex fluorescent RT-PCR detection of ADFVd and ASSVd.** An alignment of the reference sequences of ASSVd and ADFVd showed that regions delimited by positions 109 and 194 of ADFVd, and 104 and 216 of ASSVd, are characterized by a high sequence divergence (Fig. 2). Taking this into account, as well as the known sequence variability of ADFVd (Di Serio *et al.,* 1996; 1998; this work) and ASSVd (Hadidi and Yang, 1990; Puchta *et al.,* 1990; Desvignes *et al.,* 1999), the fluorescent primers AD-38rh and AS-37fl (Fig. 2 and Table 1) for RT-PCR amplification of specific cDNAs from both viroids were designed. Although two polymorphic positions (163 and 175) had been observed in the region covered by primer AD-38rh (Fig. 1), they are located internally and far from its 3' terminus, making it unlikely that cDNA

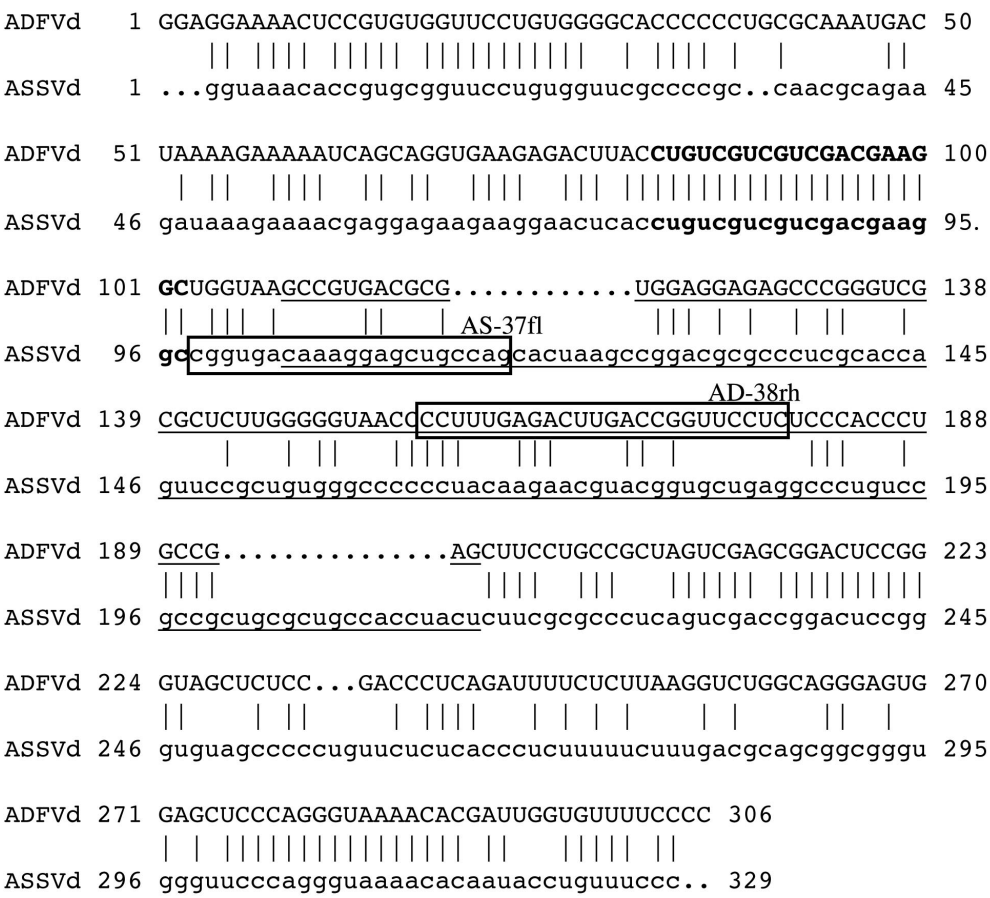


**Fig. 2.** Alignment of ADFVd and ASSVd sequences represented with capital and lower case letters, respectively. Regions of high sequence diversity between both viroids are underlined. The sequence covered by ADAS-36 primer, complementary to a fragment conserved in ADFVd and ASSVd, is in bold. Sequences corresponding to AS-37fl and AD-38rh primers are boxed.

amplification could be negatively influenced by changes at these positions in the target RNA. The AS-37fl primer is partially similar to a specific ASSVd primer previously designed by Hadidi and Yang (1990).

**Detection of ADFVd and ASSVd by singular and multiplex fluorescent RT-PCR.** In a first RT-PCR experiment, ASSVd and ADFVd primers were tested for their specificity. Nucleic acid preparations from equivalent weights of ASSVd- and ADFVd-infected tissues were mixed, and first-strand cDNAs were synthesized with the universal primer ADAS-36 derived from the upper strand of the central domain conserved in ADFVd and ASSVd (Fig. 2). Aliquots of this reaction mixture were used for two separated PCR amplifications with primers ADAS-36 and AS-37fl, or ADAS-36 and AD-38rh. Following agarose gel electrophoresis and examination under UV (without staining with ethidium bromide), primer pair ADAS-36/AD-38rh amplified only a red fluorescent product of the expected lenght for ADFVd cDNA (Fig. 3A, lane 1). The PCR reaction carried out with the primer pair ADAS-36/AS-37fl generated a green fluorescent product of the expected size for the ASSVd cDNA (Fig. 3A, lane 2). In the negative controls, in which no first-strand cDNA was added, only the fluorescent primers were visible (Fig. 3A, lanes 3 and 4). Therefore, the designed primers were specific for each viroid and the presence in the PCR reaction mixture of both cDNAs caused neither interference nor generation of unexpected products.

In a second experiment, the simultaneous detection of both viroids in a multiplex format was checked. Reverse transcription with primer ADAS-36 was followed by a single PCR reaction with this primer and the two viroid-specific primers, AD-38rd and AS-37fl. The resulting products were analyzed by agarose gel electrophoresis, and examined under UV. A cDNA of the expected size and fluorescence (254 bp and red, and

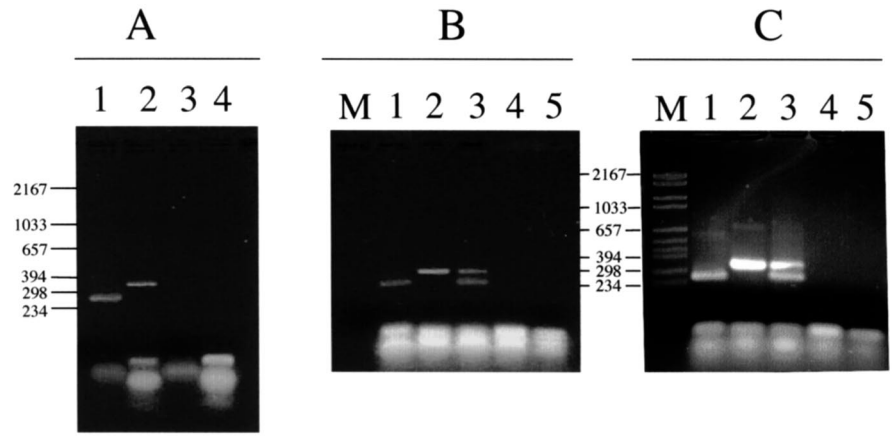


**Fig. 3.** Detection of ADFVd and ASSVd by multiplex RT-PCR with viroid-specific fluorescent primers and agarose gel electrophoresis. **A**. Amplification products obtained using for reverse transcription primer ADAS-36 and mixtures of total nucleic acid minipreparations from equivalent weights of ADFVd- and ASSVd-infected material. Following electrophoresis the gel was visualized by UV irradiation. Lanes 1 and 2, ADFVd- and ASSVd-specific products synthesized with the primer pairs ADAS-36 and AD-38rh, and ADAS-36 and AS-37fl, respectively. Lanes 3 and 4, negative controls in which no cDNA was incorporated into the PCR reaction. **B** and **C**. Amplification products obtained using for reverse transcription primer ADAS-36 and mixtures of total nucleic acid minipreparations from ADFVd- and ASSVd-infected material. Following electrophoresis the gel was visualized by UV irradiation (B) or stained with ethidium bromide (C). Lane M: DNA molecular weight markers. Lanes 1 and 2: ADFVd- and ASSVd-specific products amplified separately with primer pairs ADAS-36 and AD-38rh, and ADAS-36 and AS-37fl, respectively. Lane 3: ADFVd- and ASSVd-specific products amplified simultaneously with primers ADAS-36, AD-38rh and AS-37fl. Lane 4: total nucleic acid minipreparation from healthy material. Lane 5: negative control in which no cDNA was incorporated into the PCR reaction.

330 bp and green) was amplified with nucleic acid preparations from tissues infected by ADFVd and by ASSVd, respectively (Fig. 3B, lanes 1 and 2). When PCR was done with first-strand cDNAs from a reverse transcription reaction with aliquots of nucleic acid preparations of equivalent weights of ASSVd- and ADFVd-infected tissues, the same two viroid-specific bands were detected simultaneously (Fig. 3B, lane 3). No cDNA was amplified in the negative controls, *i.e.* a healthy apple plant (Fig. 3B, lane 4) and a blank (Fig. 3B, lane 5). When the same gel was stained with ethidium bromide, the same two bands corresponding to the fluorescent viroid cDNAs were observed (Fig. 3C). As ADFVd and ASSVd accumulate to similar levels in co-infected plants (Di Serio *et al.,* 2001), the experiment was carried out by adding equivalent amounts of nucleic acid preparations from ASSVd and ADFVd-infected tissues to the reverse transcription reaction. The same results were obtained when nucleic acid preparations from ASSVd- and ADFVd-infected tissues were added to the reverse transcription reaction in a 5:1 ratio (data not shown), indicating that the method can also detect double infections in which one of the two viroids is more concentrated than the other.

## DISCUSSION

Sequencing of 18 independent cDNA clones from two ADFVd field isolates showed five previously undescribed ADFVd sequence variants (Table 2, variants 06 to 10). Two of them, ADFVd-06 and 07, were recovered several times with variant 07 representing ≥ 50% of the ADFVd population in both isolates. This is not surprising considering that the 'Annurca' and 'Red Delicious' plants used as ADFVd sources in the present study were grown in nearby fields. These data may also suggest that the type of cultivar does not influence viroid populations.

Among the five polymorphic positions presently observed (Fig. 1), only the insertion between U225 and A226 was reported in a previously characterized variant of a different isolate (Table 2, variant 04). Polymorphic positions were distributed along the viroid molecule and no major variable region was found (Fig. 1). However, two polymorphic positions, corresponding to the insertion between 225 and 226, and the substitution at 222, were located in the lower strand of the central domain. Polymorphic positions in this same region have been found in other members of genus *Apscaviroid*

(Ambrós *et al.,* 1995; Desvignes *et al.,* 1999; Owens *et al.,* 1999).

Several arguments support the view that the changes observed in the ADFVd sequence variants likely represent actual mutations in the viroid RNA population. A DNA polymerase (*Vent* DNA polymerase) with proof-reading activity was used in most experiments to minimize introduction of artefactual changes during PCR amplification. Moreover, some variants (Table 2, ADFVd-07) were recovered in two independent experiments in which two different pairs of primers were used. Even the changes observed in variants from the 'Red Delicious' source, which were obtained with *Taq* DNA polymerase that does not have proof-reading activity, likely reflect actual mutations because the same variants were found in more than one experiment and also because the same changes were observed in variants cloned from the 'Annurca' source (Table 2).

The predicted quasi-rod like secondary structure of lowest free energy is preserved in all known ADFVd sequence variants (data not shown). In fact, most of the nucleotide substitutions are located in unpaired regions (Fig. 1, G42⇒A; A46⇒C) or do not affect the base pairing (Fig. 1, C116⇒U; A128⇒G; G222⇒A; C274⇒U). These data, together with the relatively low sequence variability observed in ADFVd so far, suggest that this viroid has strong structural constrains limiting the genetic divergence of its nucleotide sequence. Alignment of ASSVd and ADFVd sequences has shown a long region of high sequence heterogeneity between both viroids (Fig. 2). It is interesting to note that this region, which has been used to design viroid-specific primers for RT-PCR detection, maps in both viroids to the right terminal domain of their proposed secondary structures (Fig. 1; see also Fig. 1 in Hashimoto and Koganezawa, 1987). Furthermore, the nucleotide residues delimiting this region are located very close in the rod-like structure. Notwithstanding the sequence heterogeneity between ADFVd and ASSVd, both regions are composed by sequences with high self-complementarity that contribute to their respective secondary structure. This suggests the existence of strong selective pressures favoring the conservation of the rod-like or quasi rod-like structures.

We have used the sequence conservation existing between certain regions of ADFVd and ASSVd, as well as their sequence divergence in other regions, to design viroid-specific primers, each labeled with a different fluorescent dye, for a quick detection and discrimination of the two viroids by RT-PCR amplification. Although mixed infections of ASSVd and ADFVd have not been detected in nature so far, experimental inoculations have demonstrated that both viroids can co-exist in the same plant (Di Serio *et al.,* 2001). The specificity of the proposed method is supported by its ability to detect both viroids in artificially prepared mixtures. As already reported by others (Walsh *et al.,* 2001), fluorescent primers provide the advantage that hazardous chemicals, such as the powerful mutagen and cancer inducing agent ethidium bromide, are not needed for gel staining. Moreover, although the ADFVd- and ASSVd-amplified cDNAs have different size, the use of specifically labeled primers facilitates their discrimination, avoiding possible misinterpretation of the results.

Apple dimple fruit is an emerging disease found with increasing frequency in the southern part of Italy in the last few years, whereas ASSVd infecting apple has never been reported from Italy. Fruit symptoms induced by ADFVd on several cultivars are very similar to those of dapple apple disease caused by ASSVd, making it difficult a reliable differential diagnosis based on field symptoms. The method here presented is a rapid, specific and sensitive system for the simultaneous detection of both viroids, useful for establishing on firm grounds the presence of ADFVd in plants showing typical fruit symptoms, for monitoring the spread of this viroid, and for identifying possible non symptomatic hosts.

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