DOI: 10.14601/Phytopathol_Mediterr-11711

RESEARCH PAPERS

Detection of Maize rough dwarf virus in Spain: a survey of susceptible host genotypes and molecular characterization of two genomic segments of the virus

Vilma ORTIZ[1], Josep BETBESE LUCAS[2], Antonio LOPEZ QUEROL[2] and Javier ROMERO[1]

[1] Departamento de Protección Vegetal, INIA. Carretera de La Coruña km. 7.0, 28040 Madrid Spain
[2] Unitat Operativa de Cultius Extensius, IRTA, Av. Rovira Roure 191, 25198 Lleida, Spain

Summary. An increase in the number of plants infected with Maize rough dwarf disease (MRDD) has been reported recently in Spain. The disease was presumed to be caused by *Maize rough dwarf virus* (MRDV), but there was no conclusive evidence for this assumption. Two viruses have been associated with MRDD: *Rice black streaked dwarf virus (*RBSDV) and MRDV. In this study, maize plants showing MRDD in the Ebro zone province of Lerida, Spain were assessed using common primers for MRDV and RBSDV. Molecular characterization of one isolate and phylogenetic analysis were also carried out. Polyacrylamide electrophoretic profiles of genome segments of dsRNA, the size of PCR amplified fragments and the nucleotide sequence comparison matched closely with *Maize rough dwarf virus* Italian isolate (MRDV accession no. L76561), confirming that MRDV is present in maize plants showing MRDD in Spain. The phylogenetic analysis made with segments S9 and S10 from *Fijivirus* and the MRDV-Sp isolate obtained in the present study, showed that: i) MRDV is closely related to RBSDV; ii) there is high variability within isolates clustering as RBSDV in S9, especially in the ORF1 at the amino acid level, which allowed grouping one isolate close to MRDV; and iii) the grouping of RBSDV isolates at the 3'NCR of S9 was correlated with the host. The incidence of MRDD varied between two locations sampled, probably associated with sowing date, the presence of the winged form of the MRDV vector *Laodelphax striatellus* and differences in the abundance of virus reservoir plants.

Key words: Fijivirus, etiology, resistance, maize, sequences.

Introduction

Dwarfing diseases in cereals crops, including Rice black streaked dwarf disease (RBSDD), Maize rough dwarf disease (MRDD) and, more recently, Mal del Rio Cuarto disease (MRCD), are caused by members of the virus genus *Fijivirus*. They are responsible for important losses in grain yield around the world (Distéfano *et al*., 2002; Wang *et al*., 2006; Wang *et al*., 2011). Causal agents associated with dwarfing diseases of cereals are: i) *Rice black streaked dwarf virus (*RBSDV) responsible for RBSDD of rice (in China, Japan and Korea), wheat and sorghum (in China) and MRDD on maize (in China) (Isogai *et al*., 1995; Fang *et al*., 2001; Bai *et al*., 2002; Lee *et al*., 2005); ii) *Maize rough dwarf virus* (MRDV) which is associated with MRDD in maize (in Italy and Greece) (Marzachi *et al*., 1995; Dovas *et al*., 2003); and iii) *Mal del Rio Cuarto virus* (MRCV) responsible for MRCD in maize (in South America) (Distéfano *et al*., 2002, 2005). Recently, a novel *Fijivirus* species, *Southern rice black streaked dwarf virus* (SRBSDV), also known as *Rice black-streaked dwarf virus*-2 (RBSDV2), has been described infecting rice (in China) and maize (in Vietnam) (Zhang *et al*., 2008; Hoang *et al*., 2011; Yin *et al*., 2011).

MRDD was first reported in Asia in 1950, described from field symptoms on rice in China (Wang

Corresponding author: J. Romero
Fax: +34 91 3572293
E-mail: romero@inia.es

ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm
© Firenze University Press

et al., 2006); the pathogen associated with this disease was MRDV. The disease was later found to be caused by RBSDV as determined from sequence analysis. In Europe, MRDD was first reported in Italy and the causative pathogen was identified as MRDV (Marzachi *et al*., 1996). MRDD is not caused by the same virus in Europe as in Asia; in Europe MRDD is caused by MRDV (Lovisolo, 1971; Marzachi *et al*., 1996; Dovas *et al*., 2003), while in Asia the disease is caused by RBSDV (Lovisolo, 1971; Fang *et al.*, 2001; Isogai *et al*., 2001; Bai *et al.*, 2002; Lee *et al.*, 2005).

MRDV is a double-stranded RNA (dsRNA) virus, in the genus *Fijivirus* and family *Reoviridae*. Three genera including plant-infecting species are found within *Reoviridae*: *Fijivirus*, and *Oryzavirus* (Sub-family *Spinareovirinae)* and *Phytoreovirus* (Sub-family *Sedoreovirinae*). The genus *Fijivirus* includes eight species: *Fiji disease virus* (FDV), *Oat sterile dwarf virus* (OSDV), *Garlic dwarf virus* (GDV), *Nilaparvata lugens reovirus* (NLRV), MRDV, MRCV, *Pangola stunt virus* (PSV) and RBSDV (King *et al*., 2011). The genome organization of *Fijiviruses* consists of 10 dsRNA segments, two of which are bicistronic (Guzman *et al*., 2007).

In Spain, an increasing number of maize plants showing MRDD symptoms have been observed in the Ebro Valley, province of Lerida, during recent growing seasons (A. Lopez, Personal communication). These symptoms are similar to those described in maize plants infected by two members of the genus *Fijivirus,* RBSDV and MRDV (Marzachi *et al.*, 1996; Fang *et al*., 2001; Isogai *et al.*, 2001; Bai *et al*., 2002; Dovas *et al*., 2003; Lee *et al*., 2005). MRDV is the only *Fijivirus* described in Spain that is associated with dwarfed maize plants. However, MRDV detection is based on common characteristics shared by MRDV, RSBDV, and MRCV. These include dsRNA profiles, field symptoms on maize, and serological properties (Luisoni *et al.,* 1973; Milne and Luisoni, 1977; Achon and Alonso-Dueñas, 2009). It is still unknown, therefore, whether MRDV or RBSDV are infecting maize in Spain. The present study provides molecular evidences supporting MRDV as the only species in the genus *Fijivirus* isolated from maize plants showing MRDD symptoms in the Ebro Valley.

Materials and methods

Field plots

Two field experiments were conducted during 2010 in the region of Catalonia, Spain, to determine the susceptibility of 48 commercial maize genotypes to MRDD, and to investigate whether MRDV or other members of the *Fijivirus* genus were present in plants showing MRDD symptoms. The trials were sown on different dates and locations in the Ebro Valley: Poal and Gimenells.

The Gimenells site was in the west irrigation zone, where high incidence of MRDD has been observed during the last 10 years. The experiment at Gimenells was sown on June 3th (2010) and in Poal 13 days later. Both experiments were sown late in the growing season so that high numbers of vectors were present during the first developmental stages of the crops. No insecticide treatments were applied during the growing season, and all plots received the standard cultural practices used in the region. In both of the experiments, 48 different commercial maize cultivars were evaluated, with a range of 41 to 68 maize plants in each plot. The experimental design consisted of an augmented randomized complete block (Besag and Kempton, 1986). To determine the incidence of MRDD among different genotypes, the number of diseased plants showing symptoms was recorded for each cultivar. The plants were categorized according to the percentages of plants with MRDD (Tables 1 and 2) and compared to the control cv Helen and Eleonora. Additionally, two leaf samples from mature plants showing MRDD symptoms were collected for each genotype and at each location, and stored at -80°C until processing.

Virus detection

The extractions dsRNA from all samples were performed using a commercial kit (Favorgen), and also by CF-11 cellulose differential adsorption as described by Dodds *et al*. (1984). For each sample, extracted dsRNA was loaded on a 1% agarose gel, stained with Red Safe (iNtRON Biotechnology) and visualized under UV light. Because the symptoms observed in the field were similar to those caused by MRDV in Greece (Dovas *et al.,* 2003), a set of primers (MRDV-F1; 5`-AGCGGAGAACGTTTGGATC-3´ and MRDV F2; TTAACAACAGCAGCTTCACC-3´) were designed for MRDV and RBSDV detection by RT-PCR. The expected RT-PCR amplifies highly conserved regions (568 bp) in segment S9 of both viruses. For primer annealing prior to cDNA synthesis, 2 μL of RNA extraction and 1 μL of MRDV F2 primer (5 pmol) in a total volume of 12.5 μL in nuclease-free

Table 1. Incidence of maize genotypes plants showing Maize rough dwarf disease (MRDD) in the locality of Gimenells, Spain.

N. Var	Cultivar	N. Plants/ plot.	N. Plants MRDD +	% Plants MRDD +
C1	Helen	59	53	89.8
48	Eleonora2	62	47	75.8
25	LG 37.11	59	44	74.6
23	LG 36.27	63	30	47.6
40	PR33P67	62	21	33.9
7	CARELLA YG	61	37	60.7
9	DKC6451YG	62	47	75.8
C2	Eleonora	63	18	28.6
38	PR32T83	64	37	57.8
41	PR33W82	68	22	32.4
1	AGN 717	66	46	69.7
12	DKC6677	67	51	76.1
20	KERMESS	67	48	71.6
43	PR33Y74	63	43	68.3
C1	Helen	48	25	52.1
18	HELEN Bt	61	46	75.4
2	ANTISS	54	44	81.5
32	NKVITORINO	56	32	57.1
28	MAS58M	55	46	83.6
36	PR32G49	58	36	62.1
37	PR32T16	61	29	47.5
C2	Eleonora	56	29	51.8
3	BELES SUR	60	49	81.7
10	DKC6666	58	30	51.7
31	NEPAL	52	38	73.1
33	NOAH	58	35	60.3
11	DKC6667YG	59	32	54.2
19	KARTER YG	58	38	65.5
C1	Helen	58	27	46.6
46	VIVANI CS	68	59	86.8
30	MAS70F	60	56	93.3
35	PR32B41	61	51	83.6
24	LG 37.10	56	35	62.5
14	ELIOSO	56	27	48.2
13	ELEONORA	61	21	34.4
C2	Eleonora	65	25	38.5
26	LYNXX	57	42	73.7
4	BENAZIR	58	48	82.8
5	BENGALI	54	37	68.5
27	LYNXX YG	55	49	89.1
15	ES CALIENTE	58	33	56.9
16	GUADIANA	54	29	53.7
C1	Helen	50	46	92.0
47	VIVANI YG	59	55	93.2
42	PR33Y72	64	29	45.3
22	KORIMBOS	56	40	71.4
34	PR31N28	62	34	54.8
6	CARELLA	53	53	100.0
17	HELEN	54	40	74.1
C2	Eleonora	59	34	57.6
21	KLIMT	57	43	75.4
45	SANCIA	60	47	78.3
8	DKC6450	58	48	82.8
44	PR34N43	55	49	89.1
29	MAS59P	58	40	69.0
39	PR32W86	61	36	59.0
C1	Helen	51	43	84.3
	Average			66.8

Table 2. Incidence of maize genotypes plants showing Maize rough dwarf disease (MRDD) in the locality of Poal, Spain.

N. Var	Cultivar	N. Plants/ plot	N. Plants MRDD +	% Plants MRDD +
C1	Helen	45	1	2.2
26	LYNXX	52	0	0.0
41	PR33W82	55	0	0.0
1	AGN 717	52	1	1.9
2	ANTISS	67	0	0.0
3	BELES SUR	67	0	0.0
34	PR31N28	59	0	0.0
33	NOAH	54	0	0.0
12	DKC6677	58	1	1.7
C2	Eleonora	53	0	0.0
C1	Helen	58	0	0.0
45	SANCIA	61	0	0.0
44	PR34N43	57	0	0.0
38	PR32T83	50	0	0.0
8	DKC6450	50	0	0.0
16	GUADIANA	51	0	0.0
15	ES CALIENTE	55	0	0.0
6	CARELLA	55	0	0.0
19	KARTER YG	47	1	2.1
C2	Eleonora	53	0	0.0
C1	Helen	58	1	1.7
31	NEPAL	62	1	1.6
4	BENAZIR	57	1	1.8
24	LG 37.10	57	0	0.0
39	PR32W86	61	1	1.6
35	PR32B41	60	0	0.0
37	PR32T16	56	0	0.0
20	KERMESS	48	0	0.0
11	DKC6667YG	57	2	3.5
C2	Eleonora	52	0	0.0
C1	Helen	57	0	0.0
29	MAS59P	53	2	3.8
17	HELEN	53	1	1.9
7	CARELLA YG	58	1	1.7
42	PR33Y72	54	0	0.0
32	NKVITORINO	54	0	0.0
43	PR33Y74	59	0	0.0
23	LG 36.27	46	1	2.2
36	PR32G49	51	0	0.0
C2	Eleonora	49	0	0.0
C1	Helen	44	0	0.0
40	PR33P67	41	0	0.0
14	ELIOSO	57	1	1.8
21	KLIMT	49	1	2.0
18	HELEN Bt	57	0	0.0
5	BENGALI	45	0	0.0
47	VIVANI YG	54	0	0.0
27	LYNXX YG	57	1	1.8
48	Eleonora2	50	1	2.0
C2	Eleonora	56	0	0.0
C1	Helen	52	0	0.0
13	ELEONORA	62	0	0.0
22	KORIMBOS	59	0	0.0
46	VIVANI CS	61	1	1.6
10	DKC6666	42	0	0.0
30	MAS70F	63	2	3.2
28	MAS58M	52	3	5.8
25	LG 37.11	53	0	0.0
9	DKC6451YG	58	1	1.7
C2	Eleonora	53	2	3.8
		Average		0.9

water was heated for 5 min at 95°C, incubated at 40°C for 10 min and chilled on ice. For the cDNA, an equal volume of 2× reaction buffer (12.5 μL) containing 200 units of Maloney *Murine leukemia virus* reverse transcriptase (M-MLV-RT; Promega), 2.5 μL reaction buffer provided with the enzyme [5× buffer contains 250 mM Tris-HCl (pH 8.3 at 25°C), 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT] and 1 μL dNTP mix 10 mM each (dATP, dCTP, dGTP and TTC) was added before incubating the mixture (25 μL) at 40°C for 60 min. The volume of the mixture was increased to 50 μL with nuclease-free water which was heated at 65°C for 10 min. DNA amplification was carried out in a 50 μL reaction containing 5 μL of 10× PCR buffer (Tris HCl 750 mM (pH 9.0), KCl 500 mM, $(NH_4)_2SO_4$ 200 mM), 2.5 μL of $MgCl_2$ 50 mM, 1μL of each one forward and reverse primer (5 pmol), 1 μL of dNTPs mix containing 10 mM each (dATP, dCTP, dGTP and TTC), 2.5 units *Taq* polymerase (Biotools), 15 μL of the cDNA preparation and made up to the final volume with water. The thermal cycler was programmed for one cycle at 95°C (1 min), 50°C (2 min) and 72°C (20 min), followed by 40 cycles at 94°C (1 min), 50°C (1 min), and 72°C (2 min), with a final cycle of 72°C (10 min). The obtained RT-PCR fragments were sequenced and compared to sequences deposited in GenBank using BLASTn program (Altschul *et al*., 1990).

Molecular characterization of S9 and S10 segments

Virus isolate "Lerida 21" was used for the characterization of the S9 and S10 segments. Three sets of primers were designed for MRDV-S9: (S91) 5`-GGATCCAAGTTTTDDAGCCTGG-3; (S92) 5`-GGTACCGGCACCACGTAGGGTG-3′; (S93) 5`-AGAGAATGGCAGACCAAGAGCGG-3′; (S94) 5´-GGCACCACGTAGGGTGTTCGA-3′; (S95) 5´-GGTACCCTTCAAAACAGACCCCTC-3′; and (S96) 5´-ACGCGTGACATCAGCTGTTAGCC-3′ and two for MRDV-10: (S101) 5´-GGATCCAAGTTTTDDDDCCTCACCC-3′; (S102) 5´-CATATGCAACGAATGACGCTACTGCGC-3′; (S103) 5´GGATCCAACTTGGAGCGCAGTAGCGT-3′; (S104) and 5´-CATATGGACAATAGCTGCATTTCCCC-3′. These were designed using the nucleotide sequences of the MRDV Italian isolate. The exact position of the primers is indicated in Figure 1. The RT and posteriori PCR conditions were the same as described above for virus detection. The products were separated by electrophoresis in a 1% agarose gel. The expected fragment was purified using the PCR gel Extraction Kit (Qiagen), then inserted into a pGEM-T easy vector (Promega) and cloned in *E. coli* DH5α competent cells. Plasmids were isolated using the Mini-Prep Kit (Qiagen).

DNA sequencing and phylogenetic analysis

Both strands of the fragments inserted into pGEM-T from S9 and S10 were sequenced (Secugen S.L.). The Genbank database was searched using BLASTn. Alignments of the S9 and S10 and corresponding sequences from GenBank were performed using Clustal IX 2.1 (Thompson *et al*., 1997). The percent of similarity between the MRDV isolate obtained in Spain and the rest of maize-infecting *Fijiviruses* (RBSDV, MRCV, SRBSDV and MRDV) was calculated using the MegAlign program (DNASTART). Phylogenetic trees were constructed using the Neighbour Joining (NJ) method with the best fit substitution model using MEGA v5.10. Statistical significance of the nodes was tested by 1000 bootstrap. FDV was used as outgroup for comparisons.

Nucleotide sequence accession numbers.

The S9 and S10 sequences of the Spanish isolate reported in this paper are deposited in GenBank under the accession numbers: (JQ975000 and JQ975001).

Results and discussion

Incidence of MRDD and RT-PCR detection

The survey made in Catalonia show greater numbers of MRDD symptomatic plants at Gimenells (an average of 67% for all cultivars) compared to Poal (1%) (Tables 1 and 2). At Gimenells, all of the genotypes showed MRDD symptoms, and in general the incidence was over 50%, reaching 100% in the cultivar Carella. In contrast, at Poal, only 19 out of the 48 cultivars showed MRDD symptoms, and the greatest incidence was 6% in one cultivar (MAS58 M). A yield comparison obtained in both surveyed sites, indicated that at Gimenells MRDD was severe and the crop unproductive, whereas in Poal, symptomatic plants were productive in infected genotypes (data not shown).

DsRNA profiles showing ten genome segments were observed from dsRNA extractions from leaf

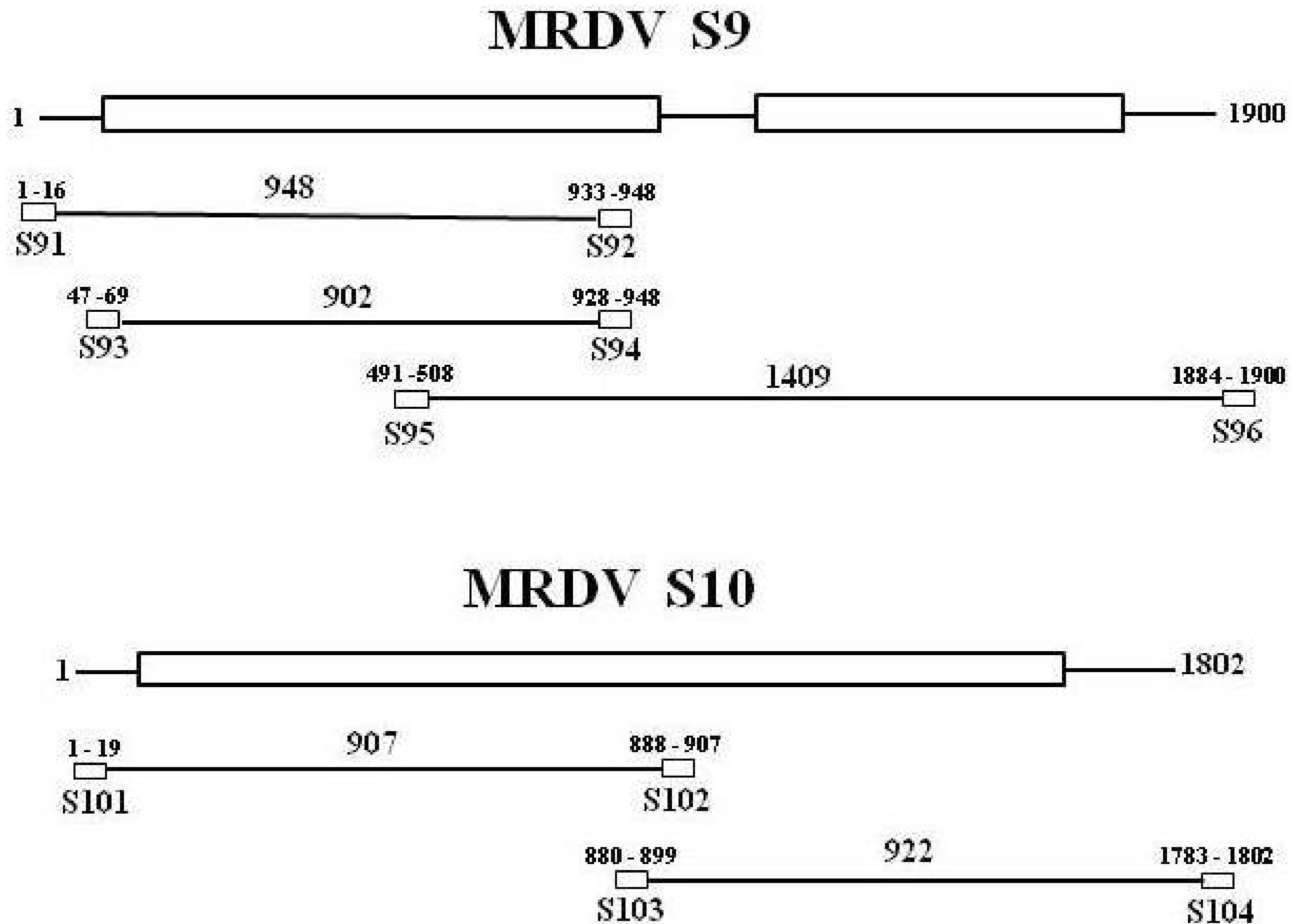

Figure 1. Schematic diagram of primer design for Reverse transcription-polymerase chain reaction (RT-PCR) and subsequent identification of the full length sequence of the segments 9 and 10 of *Maize rough dwarf virus* Spain isolate.

samples of all collected plants showing MRDD symptoms (data not shown). All profiles match the profile of *Fijivirus* (Guzman *et al*., 2007). Additionally, RT-PCR amplified a fragment within the expected size (568 bp) (Dovas *et al*., 2003) in all MRDV isolates, which were purified, cloned and sequenced. Comparison of the obtained sequences with those deposited in GenBank showed that the fragments sequenced had homology of 98% with the Italian MRDV isolate.

MRDD was first reported in Spain, in 1971 (Lovisolo, 1971), but only during the last 10 years has the incidence of this disease increased, particularly in the regions of Catalonia and Aragon (Northeastern Spain). MRDD has been associated with MRDV based on common characteristics between species of *Fijivirus* (Fang *et al.*, 2001; Wang *et al.*, 2006; Achon and Alonso-Dueñas, 2009) including RBSDV, which is also associated with MRDD in Asia. Therefore, there is no conclusive evidence indicating that MRDV is associated with the maize plants showing MRDD symptoms in Spain. Considering the high homology between the sequences obtained from samples showing MRDD symptoms and the sequences of MRDV in the GenBank, this indicates that MRDV is the only detected species of *Fijivirus* associated with maize plants showing MRDD symptoms at the Ebro Valley.

The survey also revealed that Gimenells has the greatest incidence of MRDD. This may have been associated with abundance and distribution of virus reservoirs in the region, as suggested by Achon and Alonso-Dueñas (2009). This locality is closer than

Poal to the Huesca province, where high incidence of maize plants showing MRDD symptoms was reported during recent growing seasons compared to other localities in northeastern Spain (Achon, and Dueñas, 2009). Moreover, it is possible that late establishment of the crop during the sowing season favoured an overlapping of initial development stages of the crop with the appearance of winged forms of the plant hopper *Laodelphax striatellus* Fallen, favouring the spread of the virus. However, this is speculation, because plant hoppers in both of the experimental field plots were observed but not recorded.

The occurrence of MRCD in Argentina, RBSDD in Korea, Japan, and China and MRDD in Spain has been described as sporadic and limited to small patches in fields (Zhang *et al.*, 2001; Bai *et al.*, 2002; Distefano *et al.*, 2002; Achon and Alonso-Dueñas, 2009). It is important to highlight that major outbreaks of this disease are usually associated with sowing date, environmental conditions during the growing season, the introduction of susceptible maize cultivars, virus reservoirs and large numbers of vectors (Zhang *et al.*, 2001; Distefano *et al.*, 2005; Achon and Alonso-Dueñas, 2009).

Complete nucleotide sequence of MRDV S9 segment

The sequence analysis of the two segments of the isolate "Lerida 21" showed that segment S9 of the Spanish MRDV isolate comprises 1899 bp and contains two non-overlapping ORFs. The S9 ORF1 and ORF2 are 1044 and 630 bp respectively, and encode two polypeptides of 39,947 kDa and 24,251 kDa, respectively, similar to segment 8 of the Italian isolate of MRDV (Marzachi *et al*., 1996). The calculated sequence homologies between Spanish and Italian isolates revealed that both share a high percentage of identity: 97.6% for the complete segment. For ORF1, the Spanish and Italian isolates had 97.7% nucleotide and 98.6% amino acid similarity, while for ORF2 these similarities were 98.4 and 98.6% (Table 3). The phylogenetic analysis clustered both isolates in a distinct clade separated from the rest of the *Fijivirus* species that also infect maize plants (Figure 2). A comparison between MRDV S9 from Spain and Italy and other *Fijivirus* sequences of the same fragment showed that the greatest sequence similarity was with RBSDV isolates: ranging from 84.7 to 86.7 % for the complete segment; 84.2 to 85.4 %/88.4 to 90.1 % for ORF1 and 86.2 to 89.0 %/93.4 to 95.3 % for ORF2 at the nucleotide and amino acid levels, respectively (Table 3). Thus, the topology of the tree obtained by neighbour joining using the ORF1 and ORF2 at the amino acid level showed that MRDV and RBSDV are closely related (Figure 2A, 2B).

As reported in previous studies (Azuhata *et al.*, 1993; Zhang *et al.*, 2001), there is evidence that ORF2 of S9 is more conserved between MRDV and RBSDV isolates than ORF1, both at nucleotide and amino acid levels (Table 3), but there are still differences that allow distinction between the two species (Figure 2B). On the other hand, considering the grade of homology within species, ORF1 is more divergent than ORF2, especially in RBSDV, reaching divergence values of 11.9 to 10.5 % at the nucleotide and amino acid levels, respectively (Table 3). The values obtained for MRDV isolates ORF2 were 2.3–1.4 %, and for RBSDV isolates 8.6–4.2 % at nucleotide and amino acid levels, respectively (Table 3).

The topology of the phylogenetic trees yielded further information, especially at the amino acid level. ORF1 of isolate RBSDV (LJ-M, HQ394209) clustered more closely to MRDV than RBSDV (Figure 2A). ORF2 from all the RBSDV isolates clustered apart from MRDV (Figure 2B). This may indicate recombination in ORF1 of segment S9. Recombination in isolate LJ-M at segment S10 (HQ394210) has already been reported (Chen *et al*., 2008; Li *et al.*, 2012), but this was recombination between hybrids of RBSDV isolates from different geographical regions and hosts, and not recombination between species (RBSDV and MRDV), as occurred in segment S9. MRDV and RBSDV may have the same ancestral origin and may have evolved separately, as suggested by Marzachi *et al*. (1995), considering both species have not been reported in the same geographic area. Therefore, it is unlikely that one species may be a variant of the other, or that an inter-species recombination may have occurred through co-infection of a single host. It is also possible that changes in the amino acid level of MRDV and RBSDV are not the same in S9.

The five non-coding regions (5`NCR) of S9 were found to be more conserved between species: MRDV, RBSDV, MRCV and SRBSDV isolates (88.2 to 100%), compared with the 3'NCR (75.4 to 93.0%) (Table 4). Although the number of sequences in *Fijivirus* is limited, the 3'NCR phylogram showed that there are two distinct groups of RBSDV associated with the two studied host species (Figure 3). All isolates collected from maize clustered together and diverged

Table 3. Sequence identity (%) among species of the *Fijivirus* genus that infect maize at the S9 full length and ORFs at nucleotide and amino acids level.

Virus	Segment/ Sequence	RBSDV		MRDV-Sp		MRDV-It		SRBSDV		MRCV	
		nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
RBSDV[a]	Full length	99.1–89.6									
	ORF1	99.2–88.1	98.6–89.5								
	ORF2	99.2–91.4	99.1–95.8								
MRDV-Sp	Full length	85.9–84.7									
	ORF1	84.7–84.2	89.2–88.4								
	ORF2	88.9–86.2	94.8–93.4								
MRDV-It	Full length	86.7–85.2		97.6							
	ORF1	85.4–84.8	90.1–89.2	97.7	98.6						
	ORF2	89.0–86.5	95.3–92.5	98.4	98.6						
SRBSDV	Full length	74.4–74.1		75.1		75.1					
	ORF1	74.7–74.2	78.5–76.2	75.1	77.6	75.3	78.2				
	ORF2	73.9–73.6	72.2–71.7	74.0	77.6	73.7	77.6				
MRCV	Full length	68.5–68		67.4		68.0		67.8			
	ORF1	68.2–67	64–62.9	66.4	61.2	66.7	61.2	66.0	63.5		
	ORF2	67.3–66.7	62.7–63.2	67.6	64.2	67.1	63.7	66.8	61.8		
FDV	Full length	49.0–48.7		49.2		49.4		48.9		50.2	
	ORF1	47.9–46.4	34.8–34.6	48.6	34.6	48.9	34.6	47.8	34.3	48.2	36.5
	ORF2	52.7–52.0	38.7–39.2	52.3	38.7	51.8	39.2	50.5	37.7	50.5	34.0

[a] Accession numbers of sequences included in the analysis: *Southern rice black streaked dwarf virus*-SRBSDV (HQ394211.1); *Rice black streaked dwarf virus*-RBSDV (AY039705, AF540976, HQ394209, AF536564, AJ291706); *Mal del rio cuarto virus-MRCV* (DQ023312); *Maize rough dwarf virus*-MRDV (L76561, JQ975000) and *Fiji disease virus*-FDV (AF050086) as outgroup.

from isolate AF540976 that also infects rice. A possible role of the 3'NCR in host specific adaptation was proposed by Parrella and Lanave (2009) in reference to *Bean yellow mosaic virus*. Although the genomic functions in the *Fijivirus* genus are not completely known, this result suggests that 3'NCR of S9 may be involved in host adaptation of the genus.

Complete nucleotide sequence of MRDV S10 segment

The S10 segment of the Spanish MRDV isolate was 1802 bp long and contained a single non-overlapping ORF of 1677 bp which encodes a polypeptide of 63.098 KDa. It also had 3' and 5' non-coding regions of 103 and 21 bp, respectively. The similarity between Spanish and Italian MRDV isolates was 97.4% for the complete segment and ORF at nucleotide level. At the amino acid level, the similarity was of 97%. The two MRDV isolates (from Spain and Italy) were clustered separately from the rest of the *Fijivirus* isolates (Figure 4). A summary of the maximum and minimum percentage of similarity between all the sequences of the *Fijivirus* species is also presented in Table 5. RBSDV and MRDV from Spain and Italy presented the greatest similarity from 87.8 to 88.8% in the complete S10 segment and 87.2 to 88.3% at the nucleotide level and 91.9 to 95.9% at the amino acid level, respectively. Additionally, a phylogenetic tree based on the ORF1 at the amino acid level of the S10 segment also showed that MRDV and RBSDV

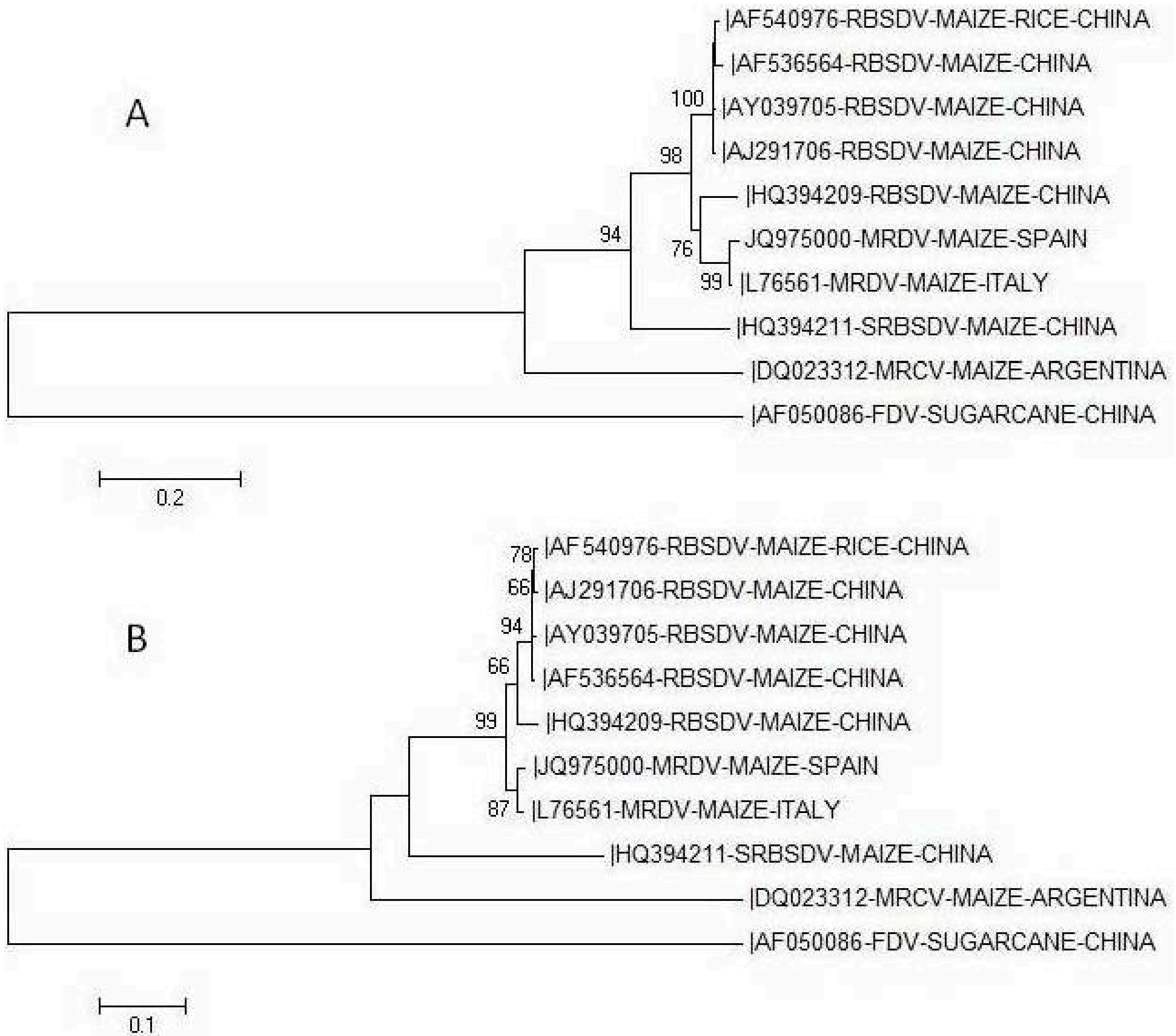

Figure 2. Phylogenetic trees constructed with amino acid sequences of (A) ORF1 and (B) ORF2 of segment 9, from *Fijivirus* that infect maize using the neighbour joining method. The numbers above the branches indicate the frequency of the cluster after bootstrap analysis (1000 replicates). Branches with bootstrap values <50% were collapsed, and only branches with bootstrap values ≥60% are shown.

isolates were more closely related to each other than to the rest of the species (Figure 4).

The S10 segment (HQ394210) of the LJ-M RBSDV isolate grouped in a separated clade, but closer to RBSDV isolates when included in the analysis of other *Fijivirus* genus sequences. This was also observed with ORF2 S9 segment (HQ394209) at the amino acid level (Figure 2B). In contrast, in ORF1 of S9, the LJ-M RBSDV isolate clade grouped closer to MRDV isolates. These results show the relationship of the LJ-M RBSDV isolate with MRDV or RBSDV depends not only of the ORF but also on the segment analyzed, and that segments S9 and S10 evolved differently. Wylie *et al*. (2008) also suggested that CP evolves differently to VPg in *Bean yellow mosaic virus* isolates.

Table 4. Sequence identity (%) between species of the *Fijivirus* genus that infect maize at the 3′ and 5′ noncoding region of the segment 9.

Virus/Species	Terminal	MRDV-Sp	MRDV-It	RBSDV	SRBSDV	MRCV
MRDV-It[a]	3′NCR	95.6				
	5′NCR	96.1				
RBSDV	3′NCR	86–92.1	86.8–93	93.9–99.1		
	5′NCR	90.2–96.1	88.2–100	88.2–100		
SRBSDV	3′NCR	82.5	81.6	78.1–82.5		
	5′NCR	92.2	90.2	90.2–94.1		
MRCV	3′NCR	75.4	76.3	76.3–78.9	84.2	
	5′NCR	92.2	94.1	90.2–96.1	95.2	
FDV	3′NCR	47.4	46.5	43–44.7	49.1	44.7
	5′NCR	72.5	74.5	68.6–76.5	70.6	74.5

[a] Accession numbers of sequences included in the analysis: *Southern rice black streaked dwarf virus*-SRBSDV (HQ394211.1); *Rice black streaked dwarf virus*-RBSDV (AY039705, AF540976, HQ394209, AF536564, AJ291706); *Mal del Rio Cuarto*-MRCV (DQ023312); *Maize rough dwarf virus*-MRDV (L76561, JQ975000) and *Fiji disease virus*-FDV (AF050086) as outgroup.

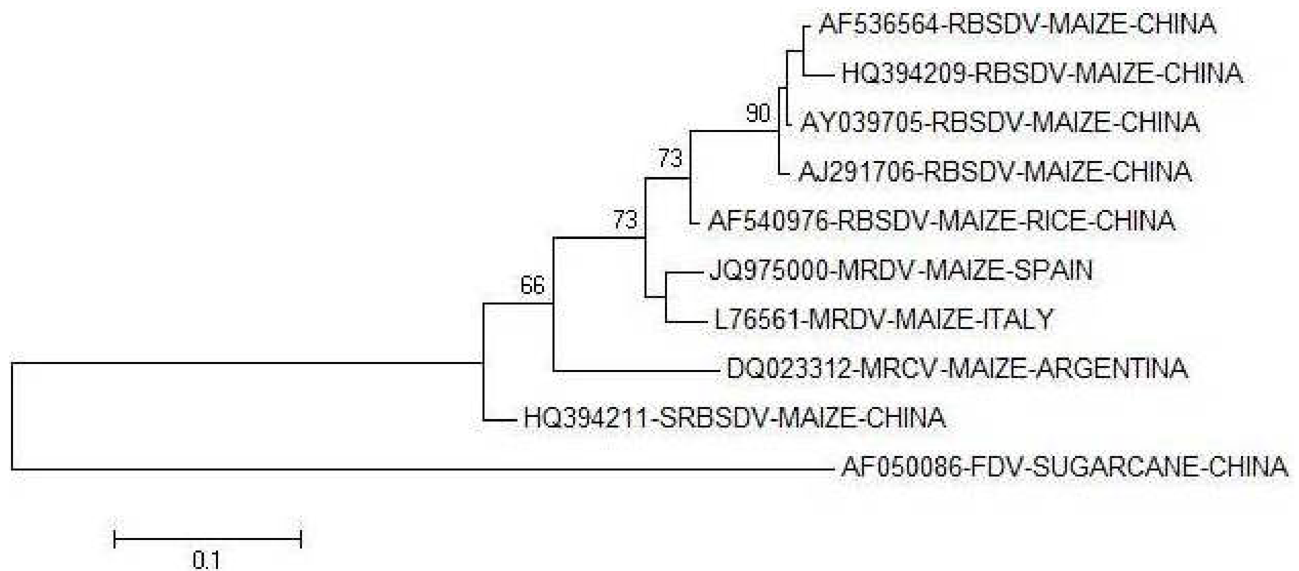

Figure 3. Phylogenetic tree constructed with nucleotide sequences of 3′ non-coding region of segment 9 from *Fijivirus* that infect maize using the neighbour joining method. The numbers above the branches indicate the frequency of the cluster after bootstrap analysis (1000 replicates). Branches with bootstrap values <50% were collapsed, and only branches with bootstrap values ≥60% are shown.

Regarding the non-coding region in segments S9 and S10, it was observed: i) that high levels of nucleotide sequence homology existed at the 5′ and 3′ non-coding regions (NCRs) between homologous segments of species (Table 4 and 6), also reported for different strains in *Rotavirus* (Desselberger and Mc-

Table 5. Sequence identity (%) among species of the *Fijivirus* genus that infect maize at the S10 full length and ORFs at nucleotide and amino acids level.

Virus	Segment/ Sequence	RBSDV		MRDV Spain		MRDV Italy		SRBSDV		MRCV	
		nt	aa	Nt	aa	Nt	aa	Nt	Aa	Nt	aa
RBSDV[a]	Full length	99.4–91.1									
	ORF1	99.5–90.4	99.8–97.3								
MRDV Spain	Full length	88.8–88.0									
	ORF1	88.3–87.4	95.9–94.1								
NMRDV Italy	Full length	88.7–87.8		97.4							
	ORF1	88.2–87.2	93.4–91.9	97.4	97.0						
SRBSDV	Full length	79.5–78.5		79.1		79.0					
	ORF1	78.6–77.4	84.4–83.5	78.2	84.6	78.1	83.5				
MRCV	Full length	73.3–72.4		73.2		73.3		72.4			
	ORF1	72.2–71.2	72.0–71.3	71.8	71.9	71.7	71.0	71.0	71.3		
FDV	Full length	58.2–57.7		57.0		56.8		55.6		57.0	
	ORF1	58.2–57.9	48–47.1	56.8	48.4	57.2	48.2	55.9	46.2	57.3	47.3

[a] Accession numbers of sequences included in the analysis: *Southern rice black streaked dwarf virus*-SRBSDV (HQ394212); *Rice black streaked dwarf virus*-RBSDV (AF227208, AF227205, AJ291707; EU267108; EU267107, EU267106.1; EU267105, AF227207, HQ394210); *Mal del Rio Cuarto*-MRCV (AY607586); *Maize rough dwarf virus*-MRDV (L76560, Spanish isolate) and *Fiji disease virus*-FDV (AY297694) as outgroup.

Crae, 1994); and ii) that a conserved sequence (CS) of seven nucleotides in the 5′NCR (AAGTTTT) and three other nucleotides (**GTC**) in the 3′ NCR was found to be common among both segments (S9 and S10) and between all isolates including the outgroup FDV isolate. Comparison of the CS of the S10 segment but only the 3′NCRs for each segment showed seven nucleotides (TAT**TGTC**) were present in all of the isolates with the exception of the FDV isolate (AGA**TGTC**), in which there were four common nucleotides. In the case of the S9 segment, the CS was the same for MRDV, SRBSDV, MRCV and one isolate (AF540976) of RBSDV (TGAT**GTC**), but different for the rest of the RBSDV isolates (TATC**GTC**) and the FDV isolate (AGAT**GTC**). It was also observed that only three 3′NCR nucleotides were common among these two segments in contrast to the 5′NCR where the CS was longer and conserved. CS at the 5′ and 3′ termini have been reported among 11 segments of dsRNA in *Rotavirus* (Desselberger and McCrae, 1994) and are associated with a stable stem-loop that contains important functional elements for assortment, packaging and replication (Tortorici *et al*., 2006; McDonald and Patton, 2011), and the 3′ CS is proposed as a critical polymerase recognition element in *Rotavirus* (Tortorici *et al*., 2003; Silvestri *et al*., 2004).

MRDV have been reported in Israel and several European countries (Lovisolo, 1971), and until now, molecular evidence of the presence of this virus is available only in Mediterranean countries. These include from Italy, with full length segments S7, S8 and S10 defined (Marzachi *et al*., 1996), Spain, with full length segments S9 and S10 defined (the present study) and Greece, the partial length of S9 segment outlined (Dovas *et al*., 2003). Is possible that the isolates found in these three countries have a common origin. The presence of MRDV in these countries could relate to the movement of the vector, considering that until now there has been no evidence that this virus is seed-borne. The vector and spreading of SRBSDV has also been discussed by Hoang *et al*. (2011). Recent research has also suggested that *L. striatellus* has the potential to migrate from China to Japan and cause outbreaks of rice stripe disease (Otuka *et al*., 2010; Sanada-Morimura *et al*., 2011).

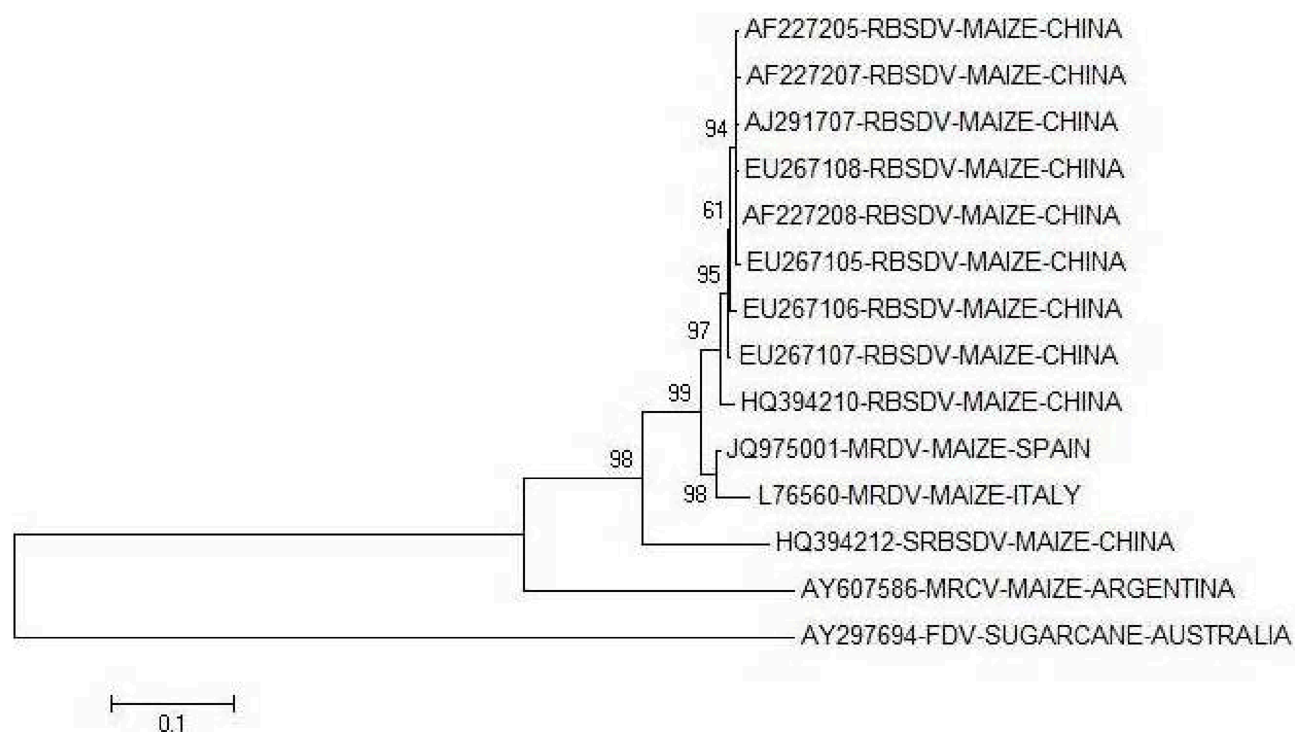

Figure 4. Phylogenetic tree constructed with amino acid sequences of ORF1 of segment 10 from *Fijivirus* that infect maize, using the neighbour joining method. The numbers above the branches indicate the frequency of the clusters after bootstrap analysis (1000 replicates). Branches with bootstrap values <50% were collapsed, and only branches with bootstrap values ≥80% are shown.

Table 6. Sequence identity (%) between species of the *Fijivirus* genus that infect maize at the 3′ and 5′ noncoding region of the segment 10.

Virus	Terminal	MRDV-Sp	MRDV-It	RBSDV	SRBSDV	MRCV
MRDV-It[a]	3′NCR	98.4				
	5′NCR	87.0				
RBSDV	3′NCR	95.3–97.7	93.8–96.1	95.3–100		
	5′NCR	87.0	95.7	100		
SRBSDV	3′NCR	91.4	90.6	89.1–91.4		
	5′NCR	82.6	91.3	95.7		
MRCV	3′NCR	91.4	91.4	86.7–89.1	89.1	
	5′NCR	87	100	95.7	91.3	
FDV	3′NCR	41.2	41.4	40.6–43.0	40.6	39.8
	5′NCR	52.2	60.9	60.9	65.2	60.9

[a] Accession numbers of sequences included in the analysis : *Southern rice black streaked dwarf virus*-SRBSDV (HQ394212); *Rice black streaked dwarf virus*-RBSDV (AF227208, AF227205, AJ291707; EU267108; EU267107, EU267106.1; EU267105, AF227207, HQ394210); *Mal del Rio Cuarto*-MRCV (AY607586); *Maize rough dwarf virus*-MRDV (L76560, JQ975001) and *Fiji disease virus*-FDV (AY297694) as outgroup.

Acknowledgements

The authors thank Dr I. Pagan for critical review of the manuscript of this paper. This research was supported by the Ministerio de Ciencia e Innovación (Grant RTA2009-00081), co-financed by FEDER (European Fund for Regional Development) and the Institute of Agrifood Research and Technology (IRTA). We also thank anonymous referees for their useful comments.

Note: The nucleotide sequence data reported in this manuscript have been deposited at the DDBJ/EMBL/GenBank under the accession No. JQ975000 and JQ9755001.

Literature cited

Achon M., N. Alonso-Dueñas, 2009. Impact of 9 years of Bt-maize cultivation on the distribution of maize viruses. *Transgenic Research* 18, 387–397.

Altschul S.F., W. Gish, W. Miller, E.W. Myers, D.J. Lipman, 1990. Basic local alignment search tool. *Journal Molecular Biology* 215, 403–410.

Azuhata F., I. Uyeda, I. Kimura, E. Shikata, 1993. Close similarity between genome structures of Rice black-streaked dwarf and Maize dwarf viruses. *Journal of General Virology* 74, 1227–1232.

Bai F.W., J. Yan, Z.C. Qu, H.W. Zhang, J. Xu, M.M. Ye, D.L. Shen, 2002. Phylogenetic analysis reveals that a dwarfing disease on different cereal crops in china is due to Rice Black Streaked Dwarf Virus (RBSDV). *Virus Genes* 25, 201–206.

Besag J., R. Kempton, 1986. Statistical analysis of field experiments using neighboring plots. *Biometrics* 42, 231–251.

Chen J., Q. Zhu, C. Yuan, Z. Sun, X. Li, T. Zhou, Z. Fan, 2008. Molecular Characterization of Rice black-streaked dwarf virus isolates causing maize rough dwarf diseases in China. *Acta Phytopathological Sin*ca 38, 540–543.

Desselberger U., M.A. McCrae, 1994. The rotavirus genome. *Current Topics in Microbiology and Immunology* 185, 31–66.

Distefano A., L. Conci, M. Muñoz, F. Guzman, H. Hopp, M. del Vas, 2002. Sequence analysis of genome segments S4 and S8 of Mal de Río Cuarto virus (MRVC): evidence that the virus should be a separate Fijivirus species. *Archives of Virology* 147, 1699–1709.

Distéfano A., H. Hopp, M. Del Vas, 2005. Sequence analysis of genome segments S5 and S10 of Mal de Río Cuarto Virus (Fijivirus, *Reoviridae*). *Archives of Virology* 150, 1241–1248.

Dodds J., T. Morris, R. Jordan, 1984. Plant viral double stranded RNA. *Annual Review of Phytopathology* 22, 151–168.

Dovas C., K. Eythymiou, N. Katis, 2003. First report of Maize rough dwarf virus (MRDV) on maize crops in Greece. *Diseases Reports* 8, 17.

Fang S., J. Yu, J. Feng, C. Han, D. Li, Y. Liu, 2001. Identification of rice black-streaked dwarf Fijivirus in maize with rough dwarf disease in China. *Archives of Virology* 146, 167–170.

Guzmán F.A., A. J. Distéfano, J.D. Arneodo, H.E. Hopp, S.L. Lenardon, M. del Vas, L.R. Conci. 2007. Sequencing of the bicistronic genome segments S7 and S9 of Mal de Rio Cuarto virus (Fijivirus, Reoviridae) completes the genome of this virus. *Archives of Virology* 152, 565–573.

Hoang A., H.M. Zhang, J. Yang, J.P. Chen, E. Hébrard, G.H. Zhou, V. Vingh, J.A. Cheng, 2011. Identification, characterization and distribution of Southern rice black-streaked dwarf virus in Vietnam. *Plant Disease* 95, 1063–1069.

Isogai M., F. Azuhata, I. Uyeda, E. Shikata, I. Kimura, 1995. Genomic relationships between *Rice black-streaked dwarf* and *Maize rough dwarf Fijiviruses* detected by nucleic acid hybridization. *Annual Phytopathology Society of Japan* 61, 513–518.

Isogai M., I. Uyeda, J.K. Choi, 2001. Molecular diagnostic of *Rice Black-Streaked Dwarf Virus* in Japan and Korea. *Plant Pathology* 17, 164–168.

King A.M.Q., M.J. Adams, E.B. Carstens, E. Lefkowitz, 2011. Virus taxonomy. Classification and nomenclature of viruses. *Ninth Report of the International Committee on Taxonomy of Viruses*. Elsevier Academic Press, London, UK, 1338 pp.

Lee B-C., Y.K. Hong, S.J. Hong, S.T. Park, K.W. Lee, 2005. Occurrence and detection of Rice black streaked dwarf virus in Korea. *Plant Patholo*gy *Journal* 21, 172–173.

Li Y., M. Jia, Z. Jiang, 2012. Molecular variation and recombination in RNA segment 10 of rice black-streaked dwarf virus isolated from China during 2007–2010. *Archives of Virology* 157, 1351–1356.

Lovisolo O., 1971. *Maize rough dwarf virus*. *CMI/AAB descriptions of plant viruses* no. 72. Commonwealth Mycological, Kew, Surrey, England.

Luisoni E., O. Lovisolo, Y. Kitawa, E. Shikata, 1973. Serological relationship between maize rough dwarf virus and rice black streaked dwarf virus. *Virology* 52, 81–283.

Marzachi C., G. Boccardo, R. Milne, M. Isogai, I. Uyeda, 1995. Genome structure and variability of *Fijivirus*. *Seminars in Virology* 6, 103–108.

Marzachi C., S. Antoniazzi, M. d´Aquilo, G. Boccardo, 1996. The double-stranded RNA genome of maize rough dwarf Fijivirus contains both mono and dicistronic segments. *European Journal of Plant Pathology* 102, 601–605.

McDonald S., J. Patton, 2011. Assortment and packaging of the segmented rotavirus genome. *Trends in Microbiology* 19, 136–144.

Milne R., E. Luisoni, 1977. Serological relationship among maize rough dwarf–like viruses. *Virology* 80, 12–20.

Otuka A., M. Matsumura, S. Sanada-Morimura, H. Takeuchi, T. Watanabe, R. Ohtsu,, H. Inoue, 2010. The 2008 overseas mass migration of the small Brown planthopper, *Laodelphax striatellus*, and subsequent outbreak of rice stripe disease in western Japan. *Applied Entomology and Zoology* 45, 259–266.

Parrella G., C. Lanave, 2009. Identification of a new pathotype of Bean yellow mosaic virus (BYMV) infecting blue passion flower and some evolutionary characteristics of BYMV. *Archives of Virology* 154, 1689–1694.

Sanada-Morimura, S., S. Sakumoto, R. Ohtsu, A. Otuka, S.H. Huang, D. Van Thang and M. Matsumura, 2011. Current

status of insecticide resistance in the small brown planthopper, *Laodelphax striatellus*, in Japan, Taiwan and Vietnam. *Applied Entomology and Zoology* 46, 65–73.

Silvestri L.S., Z.F. Taraporewala, J.T. Patton, 2004. Rotavirus replication: plus-sense templates for double-stranded RNA synthesis are made in viroplasms. *Journal of Virology* 78, 7763–7774

Thompson, J., T. Gibson, F. Plewniak, F. Jeanmougin, D.G Higgins, 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25, 4876–4882.

Tortorici M.A., T.J. Broering, M.L. Nibert, J.T. Patton, 2003. Template recognition and formation of initiation complexes by the replicase of a segmented double-stranded RNA virus. *Journal of Biological Chemistry*, 278, 32673–32682.

Tortorici M.A., B.A. Shapiro, J. Patton, 2006. A base-specific recognition signal in the 5′ consensus sequence of rotavirus plus-strand RNAs promotes replication of the double-stranded RNA genome segments. *RNA* 12, 133–146.

Wang F., G. Qin, Z. Wang, J. Yu, J. Zhang, 2006. Improved method for assaying maize plant resistance to maize rough dwarf disease by artificial inoculation and real-time RT-PCR. *European Journal of Plant Pathology* 116, 289–300.

Wang Q., T. Tao, Y. Zhang, W. Wu, D. Li, J. Yu, C. Han, 2011. Rice black-streaked dwarf virus P6 self-interacts to form punctuate viroplasm-like structures in the cytoplasm and recruits viroplasm-associated protein P9-1. *Virology Journal* 8, 1–24.

Wylie S.J., B.A. Coutts, M.G.K. Jones, R.A.C. Jones, 2008. Phylogenetic analysis of Bean Yellow mosaic virus isolates from four continents: Relationship between the seven groups found and their hosts and origins. *Plant Disease* 92, 1596–1603.

Yin X., F. Xu, F. Zheng, X. Li, B. Liu, C. Zhang, 2011. Molecular characterization of segments S7 to S10 of a Southern Rice Black-Streaked Dwarf Virus isolate from Maize in Northern China. *Virologica Sinica* 26, 47–53

Zhang, H., J. Chen, J. Lei, M. Adams, 2001. Sequence analysis shows that a dwarfing disease on rice, wheat and maize in China is caused by Rice black-streaked dwarf virus. *European Journal of Plant Pathology* 157, 563–567.

Zhang H.M., J. Yang, J.P. Chen, M. Adams, 2008. A black-streaked dwarf disease on rice in China is caused by a novel Fijivirus. *Archives of Virology* 153, 1893–1898.

Accepted for publication: May 27, 2013