

Genomic and phylogenetic evidence that Maize rough dwarf and Rice black-streaked dwarf fijiviruses should be classified as different geographic strains of a single species

L. XIE, M.-F. LV, J. YANG, J.-P. CHEN*, H.-M. ZHANG*

State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control (Key Laboratory of Plant Protection and Biotechnology, Ministry of Agriculture / Zhejiang Provincial Key Laboratory of Plant Virology), Institute of Virology and Biotechnology, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, P. R. China

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Summary. – Maize rough dwarf disease (MRDD) has long been known as one of the most devastating viral diseases of maize worldwide and is caused by single or complex infection by four fijiviruses: Maize rough dwarf virus (MRDV) in Europe and the Middle East, Mal de Rio Cuarto virus (MRCV) in South America, rice black-streaked dwarf virus (RBSDV), and Southern rice black-streaked dwarf virus (SRBSDV or Rice black-streaked dwarf virus 2, RBSDV-2) in East Asia. These are currently classified as four distinct species in the genus *Fijivirus*, family *Reoviridae*, but their taxonomic status has been questioned. To help resolve this, the nucleotide sequences of the ten genomic segments of an Italian isolate of MRDV have been determined, providing the first complete genomic sequence of this virus. Its genome has 29144 nucleotides and is similar in organization to those of RBSDV, SRBSDV, and MRCV. The 13 ORFs always share highest identities (81.3–97.2%) with the corresponding ORFs of RBSDV and phylogenetic analyses of the different genome segments and ORFs all confirm that MRDV clusters most closely with RBSDV and that MRCV and SRBSDV are slightly more distantly related. The results suggest that MRDV and RBSDV should be classified as different geographic strains of the same virus species and we suggest the name cereal black-streaked dwarf fijivirus (CBSDV) for consideration.

Keywords: Maize rough dwarf virus (MRDV); Rice black-streaked dwarf virus (RBSDV); Fijivirus; Cereal black-streaked dwarf fijivirus (CBSDV)

Introduction

Maize (*Zea mays* L.) is one of the most important food, forage, and energy crop plants in the world but a variety of viral diseases cause significant agronomic losses each year

(Ali and Yan, 2012). Maize rough dwarf disease (MRDD) is one of the most destructive viral diseases worldwide and has threatened crop production and food supplies in maize-growing regions of the world in recent years (Lenardon *et al.*, 1998, 2001; Zhang *et al.*, 2001b, 2008; Dovas *et al.*, 2004; Hoang *et al.*, 2011; Achon *et al.*, 2013, 2015; Ortiz *et al.*, 2014). Diseased maize plants are typically stunted with white waxy swellings or enations along the veins on the abaxial surfaces of the sheath and underside of the leaf blade, malformed tassels, and poor or no ears (or nubbins), thus resulting in substantial yield losses (Boccardo and Milne, 1984). MRDD is caused by single or complex infections of four viruses, including Maize rough dwarf virus (MRDV), Mal de Rio Cuarto virus (MRCV), Rice black-streaked dwarf virus (RBSDV), and Southern Rice black-streaked dwarf

*Corresponding authors. E-mail: zhhengmu@tsinghua.org.cn; jpchen2001@yahoo.com.cn; phone: +86-571-8641-6056; +86-571-8640-4003.

Abbreviations: FDV = Fiji disease virus; GDV = Garlic dwarf virus; ICTV = International Committee on Taxonomy of Viruses; MRDD = Maize rough dwarf disease; MRDV = MRD virus; MRCV = Mal de Rio Cuarto virus; NLRV = Nilaparvata lugens reovirus; OSDV = Oat sterile dwarf virus; RBSDV-2 = Rice black-streaked dwarf virus 2; SRBSDV = Southern rice black-streaked dwarf virus

virus (SRBSDV) (also known as Rice black-streaked dwarf virus 2 (RBSDV-2)) (Zhang *et al.*, 2001a,b; 2008; Zhou *et al.*, 2008; Wu *et al.*, 2013). These four viruses together with the little-studied Pangola stunt virus (PaSV), form group 2 of the genus *Fijivirus*, the family *Reoviridae*, based on insect vector specificity, plant hosts, and serological and nucleotide sequence similarities (Attoui *et al.*, 2012). The other groups (Groups 1 and 3–5) each have a single member, respectively, Fiji disease virus (FDV), Oat sterile dwarf virus (OSDV), Garlic dwarf virus (GDV) and Nilaparvata lugens reovirus (NLRV) (Attoui *et al.*, 2012). All known fijiviruses contain ten linear genomic segments of double-stranded RNA (dsRNA), ranging in size from approximately 1.4 to 4.5 kb and named S1-10 according to their migration in PAGE, and most of them replicate in their plant hosts, where they induce growth abnormalities. Previous extensive studies have shown that the four viral pathogens of MRDD are closely related to each other and similar in particle morphology, genomic profile, and serological relationships (Luisoni *et al.*, 1973; Milne *et al.*, 1973, 1977a,b; Reddy *et al.*, 1975, 1983; Isogai *et al.*, 1995; Zhang *et al.*, 2001a,b; Distéfano *et al.*, 2002, 2003, 2005, 2009; Guzmán *et al.*, 2007; Wang *et al.*, 2010). All these viruses naturally infect maize and cause similar symptoms on maize or other host plants (Milne *et al.*, 1977a; Attoui *et al.*, 2012).

There have been suggestions that these four viruses should be considered as geographical races of same species (Azuhata *et al.*, 1993), but there are some clear differences: (1) There are no reports that MRDV (in Europe) or MRCV (in Argentina) can naturally infect rice, whereas RBSDV and SRBSDV can be found naturally occurring on both rice and maize in China, Japan, Korea, and Vietnam (Milne *et al.*, 1977a; Boccardo and Milne, 1984; Lenardon *et al.*, 1998; Zhang *et al.*, 2001b, 2008; Distéfano *et al.*, 2003; Zhou *et al.*, 2008; Hoang *et al.*, 2011; Yin *et al.*, 2011; Achon *et al.*, 2013; Matsukura *et al.*, 2013; Ortiz *et al.*, 2014). (2) MRDV and RBSDV are transmitted almost exclusively by the planthopper, *Laodelphax striatellus* Fallén, whereas SRBSDV is transmitted by the white-backed planthopper (*Sogatella furcifera* Horváth) and MRCV by the planthopper *Delphacodes kuscheli* Fennah. (3) MRDV is transmitted through the eggs of its vector insect but RBSDV is not (Harpaz and Klein 1969; Harpaz, 1972; Milne *et al.*, 1977a). (4) Serological comparisons and hybridization experiments using cDNA probes also suggest that they are distinct viruses (Isogai *et al.*, 1995; Marzachi *et al.*, 1995). (5) The complete genomes of at least one isolate of MRCV, RBSDV, and SRBSDV have been determined and comparisons support the view that these three fijiviruses, at least, should continue to be members of distinct species (Zhang *et al.*, 2001a; Guzmán *et al.*, 2007; Wang *et al.*, 2010; Xue *et al.*, 2014). This view is reflected in the latest taxonomy (Attoui *et al.*, 2012).

MRDV was first described in Italy in the late 1940s (Boccardo and Milne, 1984) and now causes a severe and economically important disease in several European and Mediterranean countries (Boccardo and Milne, 1984; Dovas *et al.*, 2004; Achon *et al.*, 2013; 2015). However, although Maize rough dwarf virus is recognised as an independent species in the latest ICTV report (Attoui *et al.*, 2012), there has been only limited sequence data to help inform debates about its taxonomic status (Marzachi *et al.*, 1991; 1995; Azuhata *et al.*, 1993). We now report the complete sequence of an Italian isolate of MRDV and genomic comparisons indicating that MRDV and RBSDV can best be classified within a single virus species.

Materials and Methods

Plant materials. Infected maize plants with typical symptoms were generously provided by Dr. Piero Caciagli, Istituto di Virologia Vegetale – CNR, Torino, Italy, in July 2008. Infected leaves were frozen and stored at -80°C until use.

Extraction and purification of viral genomic dsRNA. The viral genomic double-stranded RNAs were extracted directly from infected maize plants as previously described (Uyeda *et al.*, 1998; Zhang *et al.*, 2007a, b). In brief, total nucleic acids were extracted with phenol-chloroform and then ethanol-precipitated. After treatment with DNase I (TaKaRa) and RNase A (Boehringer Mannheim), the dsRNAs were further purified with CC41 cellulose powder (Whatman). The genomic dsRNA segments were then separated on polyacrylamide gels, individual bands were excised from the gel, dissolved in 2-mercaptoethanol (2-ME) and further purified from the solubilized fractions by specific adsorption on CC41 cellulose powder as previously described by Dulieu and Bar-Joseph (1989).

Construction and sequencing of viral cDNA library. The purified genomic dsRNAs were suspended in 30 μl of H_2O and then denatured by adding 20 μl DMSO (dimethyl sulfoxide), incubating at 95°C for 5 min and quickly transferring to ice for 10 min. The denatured dsRNA was precipitated with 3 volumes of ethanol and 1/10 volume of sodium acetate, pH5.2 and used for cDNA synthesis. cDNA libraries were constructed essentially as described previously (Zhang *et al.*, 2001a, 2007a) with minor modifications. In brief, each purified genomic segment was denatured and used as a template for first-strand cDNA synthesis by Superscript Reverse Transcriptase (MBI) in the presence of 100 ng of 9-nucleotide random primers. After incubation at 42°C for 60 min, the reaction was stopped by heating at 70°C for 15 min, and the mixture was placed onto ice for 3 min. Thereafter, 1 μl of RNase H (2U) was added, and the mixture was incubated at 37°C for 20 min. The resulting cDNA was purified using the PCR Gel Extraction Kit (QIAGEN), and then annealed at 65°C and repaired using exTaq DNA polymerase (TaKaRa). It was then purified using the PCR Gel Extraction Kit (QIAGEN), and finally ligated into pGEM T easy vector (Promega), and transformed

into competent *Escherichia coli* TG1 cells for cloning. The recombinant plasmid DNAs were isolated by the alkaline lysis method or using the QIAprep spin mini prep kit (Qiagen Ltd., Crawley, UK) and analyzed by agarose gel electrophoresis (Sambrook *et al.*, 1989), and the clones for each of the genomic segments containing insertions of more than 200 bp were used for sequencing on an ABI Prism 3730 DNA sequencer (PerkinElmer Applied Biosystems) with the BigDye Terminator v3.1 cycle sequencing kit according to the manufacturer's instructions.

Sequence assembly, validation and analysis. Sequences of each genomic segment were assembled using the DNAMAN version 6.0 program (Lynnon Corporation, Canada). On the basis of assembled contigs, the internal and terminal sequences of each genomic segment were amplified by RT-PCR or ligation RT-PCR as previously described (Zhang *et al.*, 2007a, b) and were purified using a PCR Gel Extraction Kit (QIAGEN) for validation by sequencing as above. Searches for protein homology were performed with the BLAST program (Altschul *et al.*, 1997). Other related viral sequences were downloaded from EMBL/NCBI/DDBJ databases and aligned using ClustalW, version 2.0 (Thompson *et al.*, 1994). Phylogenetic and molecular evolutionary analyses were done using MEGA version 6.0 (Tamura *et al.*, 2007).

Results

Genomic profile and organization

The viral dsRNAs of an Italian MRDV isolate were extracted from infected maize leaves and then analyzed on a 12.5% polyacrylamide gel overnight, which yielded ten independent bands that were named S1-10 according to their migration in PAGE (Fig. 1). Individual dsRNA bands



Fig. 1

Ten genomic segments of MRDV on polyacrylamide gel electrophoresis (PAGE)

Approximate sizes (kbp) are shown on the left.

were excised from the gel and used to construct cDNA libraries for sequencing. The full-length sequences of each genomic segment have been determined and deposited in the GenBank/EMBL/DDBJ databases with the accession numbers HQ637550-HQ637559. The chief characteristics of the segments are shown in Table 1. The total ten-segment genome of MRDV comprised of 29144 nucleotides (nt), 3 nt longer than the RBSDV genome, making it the largest

Table 1. Summary of the genomic segments of MRDV

No.	Acc. No.	Size		GC content		ORFs		IR ^a	5'/3'-UTRs ^b
		nts	%	nts	kDa	nts	nts		
S1	HQ637550	4501	32.0	4395	168.4	-	35/71		
S2	HQ637551	3813	33.2	3681	141.2	-	46/86		
S3	HQ637552	3573	34.6	3441	131.8	-	14/118		
S4	HQ637553	3617	30.6	3510	135.0	-	33/74		
S5	HQ637554	3164	37	2814 612	106.8 23.5	-	15/91		
S6	HQ637555	2645	37.7	2379	89.5	-	81/185		
S7	HQ637556	2193	34.4	1089 930	40.9 36.5	52	41/81		
S8	HQ637557	1936	34.7	1776	67.8	-	24/136		
S9	HQ637558	1900	34.3	1044 630	39.8 24.1	64	51/111		
S10	HQ637559	1802	37.0	1677	62.9	-	22/103		

^aIR: intergenic regions; ^bUTR: untranslated regions.

S1 AAGTTTTTTTTGCGGACCTACGTATCTCAC. . . AGTGTCACGGCGGAAGCAGCTGATGTC
 S2 AAGTTTTTTTACCGGACCCTAGAGATTTCAC. . . TCCACAACCGGTAATTCAGCTATTGTC
 S3 AAGTTTTTTTCAGGATGTTGAAAGTAAAC. . . CGGCTCAAACCTGAAAAACAGCTATTGTC
 S4 AAGTTTTTTTCCATGTCTTTAAATTGAAC. . . TTTGACATACATGGGAACAGCTATCGTC
 S5 AAGTTTTTTTCACTCATGACATATTCGAA. . . GCGGGGGGAGTGAATACAGCTGATGTC
 S6 AAGTTTTTTGAGTCTGAGATACTGTTACC. . . CGGCAAGAGACTCAAATCAGCTGATGTC
 S7 AAGTTTTTTTCGACCTGTCTGGACCAGTA. . . GTGTCAAGGTCGAAATGCAGCTGATGTC
 S8 AAGTTTTTTTCGCACTGTCTAAAGATGAC. . . TAGAGTGTGCGAGAATTCAGCTATTGTC
 S9 AAGTTTTTTAGCCTGGAAGTACACATTC. . . GTTCTCAAGCCGGCTAACAGCTGATGTC
 S10 AAGTTTTTTTCCCTACCCATAATGGCTG. . . ACGGCTAGGGGAAATGCAGCTATTGTC
 CON AAGTTTTTTD-----DWDCAGCTRWYGTC

Fig. 2

Multiple alignment of both terminal sequences from each segment of MRDV
 CON: consensus sequences (D=A, T or G; R=A or G; W=A or T; Y=T or C).

reovirus genome so far reported. Segments ranged in size from approximately 1.8 to 4.5 kb. Each of the genome segments S1-4, S6, S8 and S10, encoded a single major protein, while S5, S7, and S9 each harbored two non-overlapping or partially overlapping ORFs with different lengths of untranslated regions (UTRs) or intergenic regions (IRs) (Table 1). Thus the MRDV genome encodes at least 13 viral proteins, ranging in calculated molecular weight from approximately 23.5 to 168.4 kDa (Table 1). This is similar to the genomes of other group 2 fijiviruses. All segments had a low GC content (30.6–37.7%) (Table 1), slightly different from those

of other fijiviruses (Zhang *et al.*, 2001; Xue *et al.*, 2014). The extreme 5'- and 3'-ends of the sense strand of each segment had a conserved sequence 5'-AAGTTTTTTD. . . DWD CAGCTRWYGTC-3' (D=A, T or G; R=A or G; W=A or T; Y=T or C) (Figure 2), which is similar to the conserved terminal sequences reported from RBSDV, MRCV, and SRBSDV segments (Zhang *et al.*, 2001a; Guzmán *et al.*, 2007; Wang *et al.*, 2010; Xue *et al.*, 2014). A perfect or imperfect 7–9 bp inverted repeat was identified immediately adjacent to the conserved terminal sequences in each genomic segment (Fig. 2). The conserved terminal sequences together

Table 2. Identities between the proteins encoded by genomic segments of MRDV and other fijiviruses

Genome segment	Viral protein	Amino acid sequence identities (%) with other fijiviruses ^a					Putative function	
		Group 2			Group 1	Group 3		Group 5
		RBSDV	SRBSDV	MRCV	FDV	OSDV		NLRV
S1	P1	93.65-94.74	84.34-86.82	78.73	64.08	—	32.64	RdRp
S2	P2	97.15-97.23	88.17-89.56	84.83	58.27	—	22.16	Major core
S3	P3	94.68-95.11	84.82-86.82	74.30	55.94	—	20.83	Minor core
S4	P4	91.36-91.87	72.71-73.65	57.40	38.40	—	17.69	Major core
S5	P5-1	89.86-90.50	58.80-59.33	65.45	32.90	—	18.90	Viroplasm
	P5-2	81.28-82.27	68.41-69.05	52.71	—	—	—	Nonstructural
S6	P6	84.72-85.61	63.26-64.39	44.20	25.03	—	17.03	Viroplasm
S7	P7-1	91.99-92.27	82.63-83.19	59.56	53.33	32.32	20.00	Tubular
	P7-2	85.76-87.38	61.81-61.81	44.01	27.05	14.98	—	Nonstructural
S8	P8	91.54-92.05	70.90-71.40	57.87	37.59	25.84	18.72	Minor core
S9	P9	89.05-90.49	76.95-77.81	62.31	35.98	25.10	15.47	Viroplasm
		91.87-94.74	72.73-72.73	60.67	39.11	21.43	21.39	Nonstructural
S10	P10	93.91-94.80	84.92-85.28	72.17	48.38	32.25	20.08	Major capsid

^aAmino acid sequence identity of MRDV with other fijiviruses, including RBSDV-ZJ (AJ294757, AJ409145-8, AJ297427, AJ297430-1, AJ297433, AJ293984), -AH (HF954985-94), -HeB (KC134289-98), -Hub (AY144568-70, AF521806, AF432355, AY160687, AF397894, AF399826, AF536564, AF227206), -JS (KM921673-82) isolates; SRBSDV-GD (FN563983-8, EU784840-3), -HN (FN563989-96, EU523359-60), -YN (JQ773420-9), -HB (HM585270-9) and -HuN (JQ034348-57) isolates; MRCV (AF499925-8, AF395872-3, AY607586-7, AY923115, DQ023312), FDV (AF049704-5, AF050086, AF356083, AF359556, AY029520-1, AY297693-4, and AY789927), OSDV (AJ583250-1 and AB011026-7), and NLRV (D49693-700, D26127, and D14691). '—' denote no available sequences or no corresponding proteins.

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MRDV AIRFQIDRRARVI---VGKQIGNLLDAR---SGDMKGMDAH---FQTGFFATSAQHTL---VMGDDVL---SLSRMYGVFLQQAALLGVYVYPYPSRMSLFCDESD
RBSDV AIRFQIDRRARVI---VGKQIGNLLDAR---SGDMKGMDAH---FQTGFFATSAQHTL---VMGDDVL---SLSRMYGVFLQQAALLGVYVYPYPSRMSLFCDESD
SRBSDV AIRFQIDRRARVI---VGKQIGNLLDAR---SGDMKGMDAH---FQTGFFATSAQHTL---VMGDDVL---SLSRMYGVFLQQAALLGVYVYPYPSRMSLFCDESD
MRCV AIRFQIDRRARVI---VGKQIGNLLDAR---SGDMKGMDAH---FQSGFFATSAQHTL---VMGDDVL---SLSRIFGVFLQQAAILGVYVYPYPSRMSLFCDESD
FDV AIRFQIDRRARVI---VGKQIGNLLDAR---SSDMKGMDAH---FQSGFFATSAQHTL---VMGDDVFL---SLSRIFGVFLQQAAILGVYVYPYPSRMSLFCDESD
NLRV WVRHQIDRRGRII---VGKQVGNILDAN---SSDMKGMDAH---FMSGLFATSGQHTM---VMGDDIF---NYSKYSAQFLQQAALNGVYVYPLMRVGVFCDEKSQ
RRSV VGRVQIGRRQRAI---SGKQMGNYLDLL---SADVDAMDAS---FPGQFPFTVHHTF---VQGDTR---ETSSHTSEYLQQRVSCGTFVGYVYPSRMSLFAAERP
CPV-1 VERQSDRRQRRAI---QKQAGNAFDIG---SIDVAGMDAS---YPSGRADTSTHHTV---ILGDDIM---ELSQNSIVLLQQLVNVGTFWGFADRI SLWTRREDTK
CPV-14 VERIQIDRRQRRAI---QKQAGNALDIQ---SIDVAGMDAS---YPSGRADTSTHHTV---ILGDDVVR---EYSKNSVTLQQLVNVGTFWGFADRI SLWTRERQK
CPV-5 VERQIDRRQRMI---QKQSGSALDIA---SMDVKGMDDA---FSSGRADTSAHHTA---AMGDDAN---ELSRSMVFLQQAALLGVYVYPYPSRMSLFAAERP
CPV-15 VQRQIDRRQRGI---QKQSGSALDIA---SMDVKGMDDA---FSSGRADTSAHHTA---AMGDDLI---EASRSMVFLQQAALLGVYVYPYPSRMSLFAAERP
MyRV-1 GMRVQQRPRVI---SGKNIIDIRDMQ---SVDIIGMDAS---FRSGWYNTSSQHTM---VLGDDIL---AFSDVMCEFLKQKQVGFAGPHMFPDRVLVYTSERGN
MyRV-3 GSRTQQRRRII---SGKNVGDIDRMV---SDDVAGMDAT---FRSGKFTSSQHTT---VLGDDLQ---FMSEVMCEFLKQKQVGFAGPHMFPDRVLVYTSERGD
CTFV GERHQVGRPRVI---SGKNSGDIRDMN---SLDVKGMDDA---FRSGLLNTADQHTF---VLGDDQV---EISEYSAEFLKQKQVGFAGPHMFPDRVLVYTSERGD
EYAV GERHQVGRPRVI---SGKNSGDIRDMN---SLDVKGMDDA---FRSGLLNTADQHTF---VLGDDQV---EISEYSAEFLKQKQVGFAGPHMFPDRVLVYTSERGD
APRV VVRKQIDRRQRGI---HGKQSGNASDIH---SADVKGMDTH---FPSGLITTSNHHTQ---ILGDDVS---DESRNFVFLQQAALLGVYVYPYPSRMSLFAAERP
AARV VKREQIDRRQRRAI---SGKQGHLDGDIS---GLDVAGFDAS---FPSGLAYTTVHHTI---VQGDDMF---EASRSTAEFLQQAALLGVYVYPYPSRMSLFAAERP
OpBRV GERKQVDRRGRVI---SKKNSGTINDMM---CADVKGMDAA---FPSGKFTNAQHSF---ISGDDLTL---VLSKYSATFLQQAALLGVYVYPYPSRMSLFAAERP
RLV VGRDQISRRQRRAI---SGKQGTGFLDLL---SSDVEGMDAS---FPSGLPFTGAHHSF---VQGDDEL---ETSKNTCEFLQQAALLGVYVYPYPSRMSLFAAERP
SFRV VERTQIDRRARAV---SGKQKGDMSDML---SMDVSGFDAS---FPSGLYTTAHTTI---VQGDDIS---ESSVTMEFLQQAALLGVYVYPYPSRMSLFAAERP
CON R Q RR R GK G D D G D G T H GDD S L Q G R E

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Fig. 3

Similarities between MRDV and other reoviruses within the central regions of their RNA-dependent RNA polymerases (RdRPs)

The sequences used for comparison were: Rice black-streaked dwarf virus (RBSDV) (AJ294757), SRBSDV (FN563983), Mal de Rio Cuarto virus (MRCV) (AF499925), Fiji disease virus (FDV) (AY029520), and Nilaparvata lugens reovirus (NLRV) (D49693); Rice ragged stunt virus (RRSV) (U66714); Lymantria dispar cypovirus 1 (CPV-1) (AF389463); Lymantria dispar cypovirus 14 (CPV-14), (AF389452); Trichoplusia ni cytoplasmic polyhedrosis virus 15 (CPV-15) (AF291683), Heliothis armigera cypovirus 5 (CPV-5) (EF634323); Cryphonectria parasitica mycoreovirus 1/9B21 (MyRV-1) (AF277888), Rosellinia anti-rot virus (MyRV-3) (AB102674); Colorado tick fever virus Florio N-7180 strain (CTFV) (AF133428), Eycha virus (EYAV) (AF282467); Aedes pseudoscutellaris reovirus (APRV) (DQ087277); Acinotopus angulatus reovirus (AARV) (JN792199); Operophtera brumata reovirus (OpBRV) (DQ192235), Raspberry latent virus (RLV) (HQ012655), Spissistilus festinus reovirus (SFRV) (JF773383); CON: consensus sequences.

with the inverted repeats immediately adjacent to them are thought to be genus-specific and segment-specific signals, respectively, to act as recognition signals for replication and packaging of viral RNAs (Anzola *et al.*, 1987). The similarities amongst conserved terminal sequences of the group 2 fijiviruses suggests that they have similar mechanisms for their replication and packaging.

Sequence comparisons

Comparisons with the corresponding segments of other fijiviruses showed that MRDV shared 84.3–88.8%, 67.7–79.0%, and 57.2–76.5% nucleotide identity with RBSDV, SRBSDV, and MRCV, the other three members of group 2 that have been sequenced. The 13 predicted viral proteins were not only similar in position and size to those found in RBSDV, SRBSDV, and MRCV, but also shared highest identities (81.3–97.2%, 58.8–89.6%, 44.0–84.8%) with them (depending on the protein used for comparison). As expected, MRDV segments were more distantly related (15.0–64.1%) to the other fijiviruses, including FDV, OSDV, and NLRV (Table 2). The results therefore confirm that MRDV is a member of *Fijivirus* group 2.

Protein comparisons indicated that the protein P1 with predicted molecular weight of 168 kDa contained the major motifs characteristic for a reoviral RNA-dependent RNA polymerase (RdRp) (Zhang *et al.*, 2007), which can be aligned well with those from other fijiviruses and reoviruses more generally (Fig. 3). Within the second group of the genus *Fijivirus*, these motifs appeared to be almost identical (Fig. 3), sug-

gesting that these viruses have a similar mechanism for viral RNA replication and transcription. In the family *Reoviridae*, the RdRp is believed to be a minor component of the core particle. This is likely to be the case for MRDV, as an earlier study showed that RdRp activity was associated with purified MRDV particles (Marzachi *et al.*, 1990).

The P1 protein of MRDV was significantly related (22–40% identity and 39–58% similarity) to those of Diaphorina citri reovirus (DCRV), an unclassified reovirus (Marutani-Hert *et al.*, 2009), Bloomfield virus, a novel unclassified virus (Webster *et al.*, 2015), Cimodo virus, a novel unclassified virus (Hermanns *et al.*, 2014), Torrey Pines virus, an unclassified *Cypovirus* (Webster *et al.*, 2015) and Lutzomyia reovirus, a novel unclassified virus (Aguar *et al.*, 2015). Interestingly, the P8 protein was significantly related to the VP5 of Inachis io cypovirus 2 (KC588370) and VP6 of Mycoreovirus 3 (AB073277) (Fig. 4) and contained the motifs I and II that may be involved in ATP-binding

	Motif I	Motif II
MRDV P8	I I G N K G V G K S E I G A M L	V V D S D D Y G R F L
MyRV-3 P6	M V G K K N S G K G M I G K L I	V I D S D D Y G R V L
IiCPV-2 VP5	I I A N K G T G K P T L S R M L	V I D S D Y G R W L
CON	K G K	V D S D Y G R L

Fig. 4

Conserved motifs that could be involved in ATP-binding activity

The sequences used are the VP5 of Inachis io cypovirus 2 (KC588370) and VP6 of Mycoreovirus 3 (AB073277).

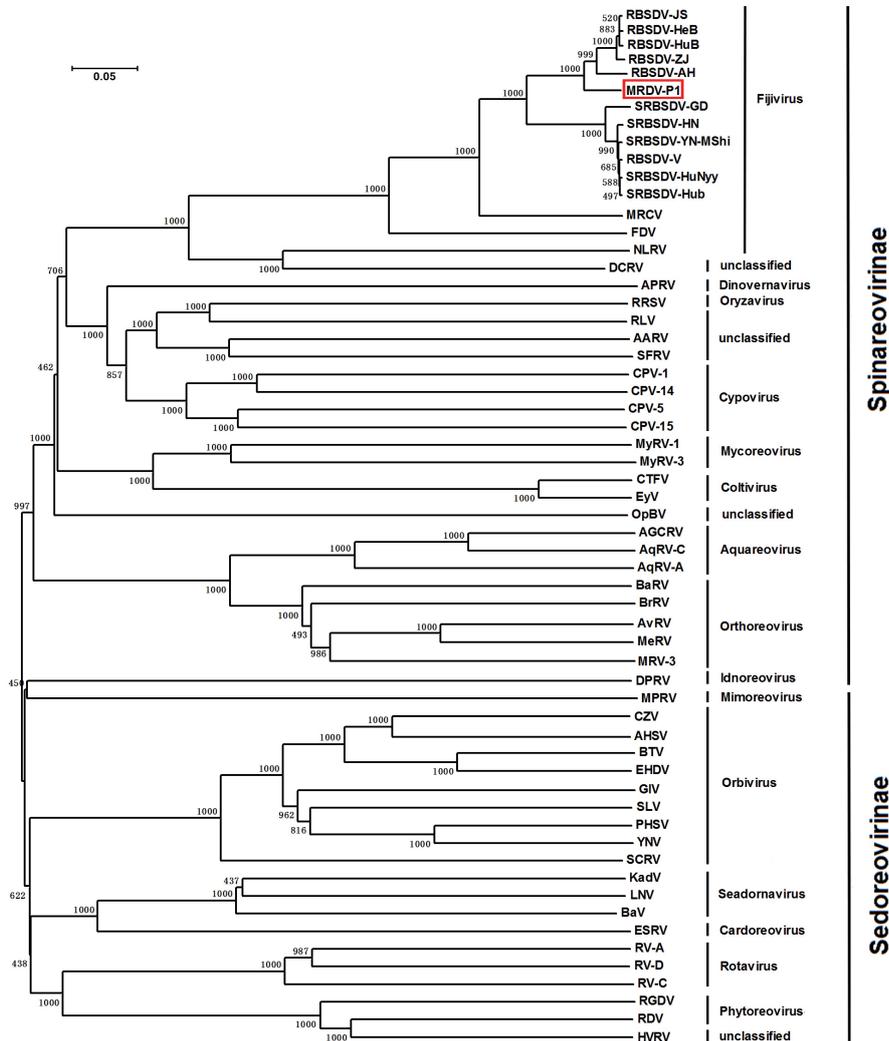


Fig. 5

Phylogenetic tree constructed with MRDV and other reoviral RdRPs

The sequences used for phylogenetic analysis are listed below:

Subfamily Spinareovirinae

Genus *Fijivirus*: MRDV (in this study), RBSDV-ZJ (AJ294757), -JS (KM921673), -HeB (KC134289), -HuB (AY144568), -AH (HF954985), SRBSDV-GD (FN563983), -HN (FN563989), -YN-MShi (JQ773420), -V (HQ731492), -HuNyy (JQ034348), and -HuB (HM585279), Mal de Rio Cuarto virus (MRCV) (AF499925), Fiji disease virus (FDV) (AY029520), and Nilaparvata lugens reovirus (NLRV) (D49693); genus *Oryzavirus*: Rice ragged stunt virus (RRSV) (U66714); genus *Dinovernavirus*: *Aedes pseudoscutellaris* reovirus (APRV) (DQ087277); genus *Cyporeovirus*: *Lymantria dispar* cypovirus 1 (CPV-1) (AF389463), *Lymantria dispar* cypovirus 14 (CPV-14) (AF389452), *Heliothis armigera* cypovirus 5 (CPV-5) (EF634323), *Trichoplusia ni* cytoplasmic polyhedrosis virus 15 (CPV-15) (AF291683); genus *Mycoreovirus*: *Cryphonectria parasitica* mycoreovirus-1/9B21 (MyRV-1) (AF277888), *Rosellinia anti-rot* virus (MyRV-3) (AB102674); genus *Coltivirus*: Colorado tick fever virus Florio N-7180 strain (CTFV) (AF133428), Eyach virus (EYAV) (AF282467); genus *Aquareovirus*: American-grass-carp-reovirus (AGCRV) (EF589099), Aquareovirus C (AqRV-C) (AF403399), Aquareovirus A (AqRV-A) (AF418295); genus *Orthoreovirus*: Baboon orthoreovirus (BaRV) (HQ847904), Broome virus (BrRV) (GQ258979), Avian orthoreovirus (AvRV) (FR694192), Melaka orthoreovirus (MeRV) (JF342661), Mammalian orthoreovirus 3-1 (MRV-3-1) (GQ468266); genus *Idnoreovirus*: *Diadromus pulchellus* reovirus (DpRV) (X80481).

Subfamily Sedoreovirinae

Genus *Mimoreovirus*: *Micromonas pusilla* reovirus (MPRV) (DQ126102); genus *Orbivirus*: Chuzan virus (CZV) (AB018086), African horse sickness virus (AHSV) (U94887), Blue tongue virus (BTV) (X12819), Epizootic hemorrhagic disease virus (EHDV) (AM744977), Great Island virus (GIV) (HM543465), Stretch Lagoon orbivirus (SLV) (EU718676), Peruvian horse sickness virus (PHSV) (DQ248057), Yunnan orbivirus (YNV) (AY701509), St Croix River virus (SCRV) (AF133431); genus *Seadornavirus*: Kadapiro virus (KadV) (AF133429), Liao ning virus (LNV) (AY701339), Banna virus (BaV) (AF133430); genus *Cardoreovirus*: Eriocheir sinensis reovirus (ESRV) (AY542965); genus *Rotavirus*: Rotavirus A (RV-A) (DQ838640), Rotavirus D (RV-D) (GU733443), Rotavirus C (RV-C) (AJ304859); genus *Phytoreovirus*: Rice gall dwarf virus (RGDV) (AB254451), Rice dwarf virus (RDV) (U73201).

Unassigned Reoviridae

Diaphorina citri reovirus (DCRV) (AB458510), Raspberry latent virus (RLV) (HQ012655), Acinopterus angulatus reovirus (AARV) (JN792199), Spissistilus festinus reovirus (SFRV) (JF773383), Operophtera brumata reovirus (OpBRV) (DQ192235), Homalodisca vitripennis reovirus (HVRV) (FJ497789).

activity (Zhang *et al.*, 2008). The P2 protein encoded by S2 of MRDV, a putative major core capsid protein, appeared to be the most conserved viral protein among the members of group 2 fijiviruses (Table 2), suggesting that the conservation provides the structural basis for similar morphology and replication mechanism of this group of viruses. However, the viral proteins encoded on S2-S7 and S9-S10 did not exhibit any significant sequence similarity to other reoviral (or other viral) proteins except those of fijiviruses. On the basis of these similarities, putative functions and structures can be assigned to the predicted MRDV proteins (Table 2).

Phylogenetic analysis

To determine the appropriate taxonomic position of MRDV, the new sequences were further used in phylogenetic analysis. The protein P1 (RdRp) is the only protein that can be used for establishing substantial (if relatively distant) relationships between different genera within the family *Reoviridae*. In the phylogenetic tree (Fig. 5) constructed with reoviral RdRps by the UPGMA method, two sub-families, fifteen genera and some unclassified members of the family *Reoviridae* were separated by branches supporting the virus taxonomy in the Ninth report of ICTV (Attoui *et al.*, 2012). The known members of the genus *Fijivirus* form a mono-

phyletic branch, within which MRDV, RBSDV, SRBSDV, and MRCV cluster closely together. Of the four viruses causing rough dwarf disease, MRCV is known only from Argentina, South America, and appears to be somewhat distant from other three viruses, reflecting its geographic isolation, but MRDV (from Europe) appeared to be closer to RBSDV than was SRBSDV, although RBSDV and SRBSDV are both known from East Asia. This suggests that MRDV and RBSDV might best be considered geographic strains of a single species.

A phylogenetic tree of the highly conserved major capsid proteins of fijiviruses (Fig. 6) confirmed this pattern of grouping within the genus. Group 2 fijiviruses formed a distinct group, within which all known MRDV and RBSDV isolates were clustered in a distinct clade well-separated from all known MRCV and SRBSDV isolates. Phylogenetic trees using the other corresponding segments of all known fijiviruses had a similar pattern of branching and relationships (data not shown).

The conserved terminal sequences of reoviral genome segments also have taxonomic significance (McMahon *et al.*, 1999). The terminal sequences of MRDV are almost identical to those of RBSDV, slightly different from those from SRBSDV, MRCV, and FDV, and obviously distant from those of OSDV and NLRV, although all fijiviruses share the consensus (5'-A.....GTC-3') (Fig. 7a). When the terminal sequences were used to construct a phylogenetic

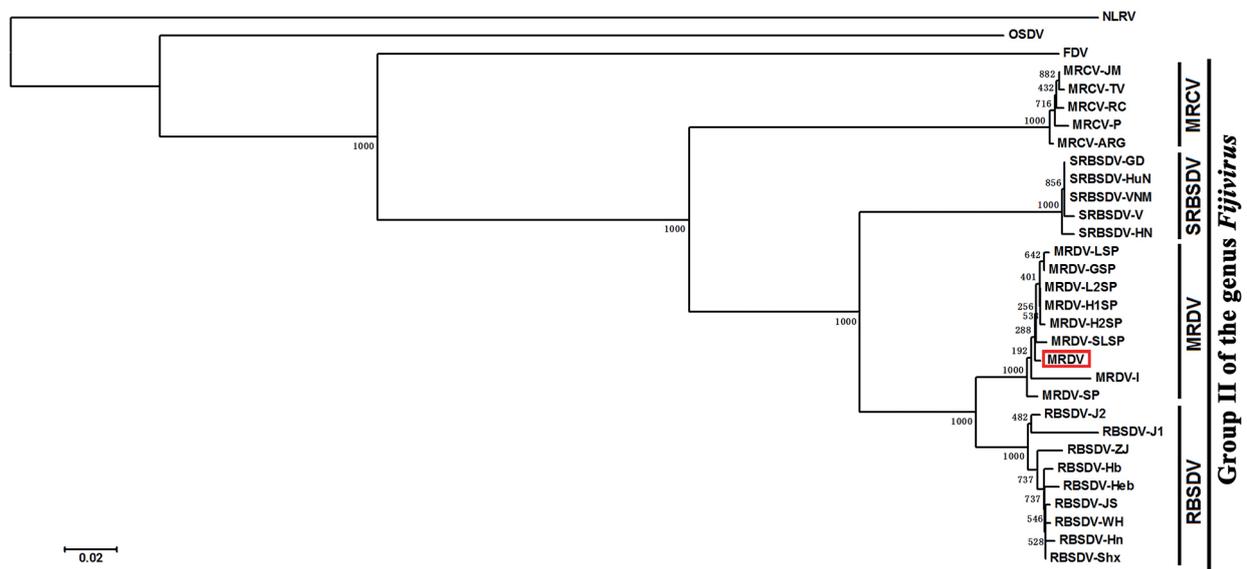


Fig. 6

Phylogenetic tree constructed with MRDV (from this study) and other known fijiviral major capsid proteins

The sequences used for phylogenetic analysis were: NLRV (D26127); OSDV (AB011025); FDV (AY297694); MRCV-JM (AY999338), -TV (AY999339), -RC (AY999337), -P (AY999340), -ARG (AY607586); SRBSDV-GD (EU784840), -HuN (JQ034357), -VNM (JQ692581), -V (HQ731501), -HN (EU523360); MRDV-LSP (LK392325), -GSP (LK392328), -L2SP (LK392330), -H1SP (LK392326), -H2SP (LK392327), -SLSP (LK392329), -I (L76560), -Sp (JQ975001); RBSDV-J1 (D00606), -J2 (GU322365), -ZJ (AJ297433), -Hb (AF227205), -Heb (AJ297434), -JS (AY039835), -WH (AJ291707), -Hn (AF227207), -Shx (AF227208).

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