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ORIGINAL ARTICLE

A black-streaked dwarf disease on rice in China is caused by a novel fijivirus

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Abstract An isolate of a plant reovirus causing severe stunting and dark leaf symptoms on rice from Guangdong, China, was similar in virion morphology and serologically related to rice black-streaked dwarf virus (RBSDV). The electrophoretic profiles of genome segments of the two viruses in agarose or polyacrylamide gel were indistinguishable. The four genome segments of the new isolate corresponding to RBSDV S7–S10 were amplified by ligation RT-PCR and sequenced. The size and organization of each genome segment was very similar to its counterparts in RBSDV, maize rough dwarf virus (MRDV), and mal de Rio Cuarto virus (MRCV). Sequence identity was greatest to RBSDV and MRDV (ranging from about 60–85% depending on the protein), but identities were always much lower than those between RBSDV and MRDV. These comparisons and phylogenetic analyses suggested that the virus represents a new species in genus *Fijivirus* group 2, tentatively named Rice black-streaked dwarf virus-2.

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Introduction

Plant-infecting reoviruses are classified into three genera, *Oryzavirus*, *Phytoreovirus*, and *Fijivirus* on the basis of particle morphology, number of genome segments, serology and insect vector specificity [18, 22]. All known fijiviruses contain ten linear genomic segments (S1–Sl0) 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. Based on vectors, plant hosts, and serological and nucleotide sequence similarities, the genus *Fijivirus* is subdivided into five 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) while group 2 has 4 members: rice black-streaked dwarf virus (RBSDV), maize rough dwarf virus (MRDV), mal de Rio Cuarto virus (MRCV) and pangola stunt virus (PaSV). The four members of group 2 are closely related [3] and this is especially so for the better studied members, MRDV, MRCV and RBSDV. These three viruses have similar particle morphology, genomic profile, and serological relationships [14] and cause similar symptoms on maize. There have been suggestions that they should be considered geographical races of the same virus [15] but there are no reports that MRDV (in Europe) or MRCV (in Argentina) can naturally infect rice, whereas RBSDV can be found on both rice and maize in China, Japan, and Korea [3, 24]. Hybridization experiments also suggest that they are distinct viruses [5, 10]. The complete genomes of at least one isolate of MRCV and RBSDV have been determined, but the sequences of only four segments of MRDV are known. Comparisons show that there are 44–85% identical amino acids between MRCV and the corresponding RBSDV or MRDV proteins (depending on the

protein used for comparison), further supporting the suggestion that MRCV is a member of a species distinct from RBSDV and MRDV [5–7, 9]. This view is reflected in the latest taxonomic treatment [18].

Rice black-streaked dwarf virus is transmitted by the planthopper *Laodelphax striatellus* Fallen in a persistent manner and can be experimentally transmitted to rice, maize, wheat and other cereal and grass hosts. The symptoms on all hosts typically include pronounced stunting, darkening of leaves, and white waxy or black-streaked swellings along the veins on the underside of the leaf blades, sheaths and culms. Diseased plants often produce poor heads or none at all. RBSDV was first reported as an important disease on rice in Japan, and when an isometric virus producing symptoms of severe stunting and dark leaf on rice, and transmitted by the planthopper *L. striatellus*, was discovered to be causing severe damage on rice in eastern China in the 1960s and on maize in Hebei province in the 1970s, it was identified as RBSDV [23, 24]. In China, there have been several serious disease outbreaks causing serious yield losses on rice and maize [23, 24]. After some years when it was rarely found, the disease re-occurred in the late 1990s on rice in most parts of eastern China and on maize in northern, northwestern and north-eastern China. Damage was very severe, with disease incidence exceeding 90% in some areas due to widespread release of susceptible cultivars, to the abundance of the virus vector in fields, and to cultivation patterns that favoured infection [8, 13]. The virus overwinters in wheat, in which it causes some symptoms but much less damage.

Recently, a viral disease occurred on rice and maize in Hainan and Guangdong provinces in southern China. The infected plants displayed all the typical symptoms of black-streaked dwarf disease, and the causal agent was transmitted efficiently between maize and rice plants by the planthopper *L. striatellus*. The virus isolated was indistinguishable in shape and serological properties from RBSDV and was therefore initially considered an isolate of RBSDV [25]. In some fields, more than 80% of plants were infected, causing serious yield losses. We now report the complete sequences of the genome segments S7–S10 of this virus and present molecular evidence that the virus should be considered a new member of the fijivirus group 2.

Materials and methods

Infected rice plants with typical dwarf symptoms were collected from Yangxi County, Guangdong province, in October 2005, and the infected leaves were frozen and stored at −80°C. The virus was purified, and viral dsRNAs were extracted from purified particles, or directly extracted from naturally infected leaves, using the methods described by Uyeda et al. [21]. The dsRNA preparation was loaded onto a 1% agarose or 10% polyacrylamide gel. After electrophoresis, the gel was stained with ethidium bromide and observed under UV light.

cDNA libraries for segments S7–S10 were constructed essentially as described previously [23, 24] 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 nine-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 (2 U/μl) 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 for 24 h in the presence of 0.5 M NaCl, purified using the PCR Gel Extraction Kit (QIAGEN), 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 transformants were screened using the ampicillin resistance and α-complementation methods [19]. Recombinant plasmid DNA was isolated by the alkaline lysis method [19], analyzed by agarose gel electrophoresis, and 5–20 clones for each segment, each containing insertions of about 200–1,000 bp, were used for sequencing.

After sequence assembly and analysis, it was evident that fragments of approximately 1.8–2.0 kb had been obtained from each segment. To further confirm and extend the sequences, eight primers were designed on the basis of sequences determined (Table 1) and used to amplify the internal regions of S7–10 of the virus isolate by RT-PCR. To ensure that complete terminal sequences were obtained, primer zhm-1 (Table 1) was first ligated to both 3′-ends of the viral RNA. To prevent concatenation of primer zhm-1 in subsequent dsRNA/DNA ligation reactions, the 3′-terminus of the primer was blocked by amine. Ligation reactions and cDNA synthesis were performed as previously described [23, 24]. The resulting cDNA was purified and used as a template for PCR with primer zhm-2 (Table 1, complementary to zhm-1) and one of the eight segment-specific internal primers (Table 1). PCR was done in a Mastercycler thermal cycler (Eppendorf): one cycle of 3 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 60°C and 1–2 min at 72°C; and a final extension at 72°C for 10 min. The fragments amplified were purified using the PCR Gel Extraction Kit (Qiagen) and cloned as previously described.

Recombinant plasmid DNA used for sequencing was prepared using the QIAprep spin mini prep kit (Qiagen Ltd), and the inserts were sequenced entirely on both

Table 1 Primers used for sequencing genome segments S7–10 of the new Guangdong rice virus isolate

Primer name	Position	Direction	Sequence (5′–3′)	*Tm* (°C)
pS7-1	188–208	Forward	ATG GCT CTT TCC GAC TAC TTC	62
pS7-2	1985–1965	Reverse	ATA CTT GCG TTC TGC TTC TAC	60
pS7-3	321–299	Reverse	AGA TCT AAA TCT ACG TCA TTA GT	60
pS7-4	1839–1860	Forward	TAG ATC ATT GGT TTC CTA CAG A	60
pS8-1	285–305	Forward	AAC GAT CTT TAA CCG AAC AGG	60
pS8-2	1683–1662	Reverse	GTG TAA CAT CTG ATT CGC AAT G	62
pS8-3	319–299	Reverse	AAT CAG GAA AAT GGC CTG TTC	60
pS8-4	1548–1568	Forward	ATT AGC GTT CGT ACC TCA TTC	60
pS9-1	104–125	Forward	CAA TTA GAA ACG ACC AAC CAAC	62
pS9-2	1765–1744	Reverse	CAG TAC CTC CAT TGA ACA CTT G	64
pS9-3	313–292	Reverse	CGA TGT TAA AGT CAC GTT CTA G	62
pS9-4	1471–1491	Forward	CGG TGT GTA TGT TGT AAT CCT	60
pS10-1	271–292	Forward	ACT CTA CCG AAC ATT ACT CAA C	62
pS10-2	1588–1566	Reverse	CTT CAA TAA ATT GTT GGC TCT G	60
pS10-3	477–457	Reverse	GTT TCC TTC AGT TAG GGT CTC	60
pS10-4	1492–1513	Forward	GTT TTG CCA AAC ATC ATT CAA G	60
zhm-1			PO4-CTC TTC CCC TCC CTC CTC-NH2	60
zhm-2			GAG GAG GGA GGG GAA GAG	60

strands using the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster, USA) on an ABI PRISM 3730 DNA Sequencer (Perkin Elmer Applied Biosystems, Foster, USA) with universal primers T7 and SP6. Sequence assembly and analysis was performed using the DNAman version 4.0 program (Lynnon BioSoft, Quebec, Canada). Search for proteins homologous to the predicted proteins was performed with the BLAST program [1]. To construct a phylogenetic tree, amino acid sequences were obtained from Swissprot databases (http://www.sanbi.ac.za/mrc/Databases/SWISSPROT.htm) or deduced from EMBL/NCBI/DDBJ DNA databases and aligned using the progress Clustal W, version 1.6 computer program [20] and Multalin, version 5.4.1 program [4]. Aligned sequences were used to build an evolutionary tree according to the neighbor-joining method using MEGA version 3.1 [12].

Results and discussion

The viral isolate had ten segments of dsRNA with a genome profile indistinguishable from RBSDV in both 1% agarose gel and 10% polyacrylamide gel electrophoresis (data not shown). However, expected fragments were not amplified by RT-PCR from infected plants or purified virions using RBSDV segment-specific primers [24]. Using the strategy described above, a consensus sequence for each of the segments S7–10 was obtained from 3 to 14 clones in each region. The full lengths of these segments were 2176, 1928, 1899, and 1797 nucleotides (nt), respectively. The size and sequence of these segments was then confirmed by designing two terminal primers for each segment (based on the sequences determined) and using these to amplify, clone, and sequence each of the four full-length genome segments. The extreme 5′- and 3′-ends of the sense strand of each segment had the same sequence 5′-AAGTTTTT….CAGCTGATGTC-3′ (Table 2), not only conforming to the consensus terminal sequences of members of the genus *Fijivirus* proposed by McMahon et al. [16], but also nearly identical to the terminal sequences of RBSDV, MRDV, MRCV, and FDV, which have the consensus termini 5′-AAGTTTTT………. CAGCNNNNGTC-3′. A perfect or imperfect 6–9-bp inverted repeat was identified immediately adjacent to the conserved terminal sequences in each genomic segment (see Table 2). Conserved 5′- and 3′-terminal sequences and inverted repeats are a feature of all reoviruses sequenced to date. The conserved terminal sequences are thought to be genus-specific and to act as a packaging signal for viral rather than host RNA, while the inverted repeats are thought to be segment-specific signals [2]. Some properties of the genome segments S7–S10 are summarized in Table 3. The genome segments S8 and S10 each contained one major open reading frame (ORF), while S7 and S9 were bicistronic. In this, their organization resembles that of other fijiviruses, especially RBSDV, MRDV, and MRCV.

Searches within the Swiss-Prot, GenPept, PIR and PDB databases were performed for the overall deduced amino acid sequences of each ORF on the four genome segments

Table 2 The terminal sequences of genome segments S7–10 of the new virus isolate

	5′	3′
S7	AAGTTTTTTTCGACCT	AGGTCGAAATGCAGCTGATGTC
S8	AAGTTTTTTTCGCAC	GTGCGAAAATTCAGCTGATGTC
S9	AAGTTTTTAAGCC	GGCTTACAGCTGATGTC
S10	AAGTTTTTTTCCTC	GGGGAAAGCAGCTGATGTC
Consensus	AAGTTTTT……….	………CAGCTGATGTC

Inverted repeats are underlined

Table 3 Genome organization of segments S7–10 of the new virus isolate

	Accession number	Total size	GC content	5′-UTR	ORF1		Intergenic region	ORF2		3′-UTR
		nts	%	nts	nts	kDa	nts	nts	kDa	nts
S7	EU784841	2176	32.5	40	1073	40.5	52	930	36.4	81
S8	EU784842	1928	35.5	24	1776	67.9	–	–	–	128
S9	EU784843	1899	34.6	51	1044	39.9	63	630	24.2	111
S10	EU784840	1797	35.6	21	1674	62.6	–	–	–	102

– not present on this segment

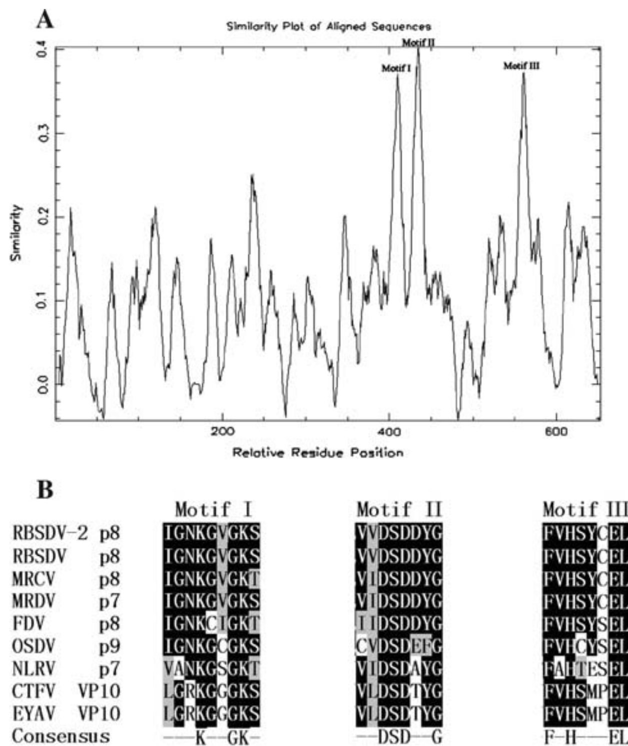

Fig. 1 Comparisons between the new virus (RBSDV-2) p8 protein sequence and the corresponding sequences of other fijiviruses (*FDV* Fiji disease virus, *MRCV* mal de Rio Cuarto virus, *MRDV* maize rough dwarf virus, *OSDV* oat sterile dwarf virus, *NLRV* Nilaparvata lugens virus, *RBSDV* rice black-streaked dwarf virus) and two members of the genus *Coltivirus* (*CTFV* Colorado tick fever virus, *EYAV Eyach virus*). **a** Similarity plot of the aligned sequences. **b** Sequences of the three conserved motifs identified

of the virus isolate using protein–protein BLAST (BLASTP) [1]. The results showed that, in addition to homology with those of other fijiviruses, the deduced amino acid sequence p8 (encoded on S8) was related (32% identical and 45% similar amino acids) in certain regions to the VP10 proteins of Colorado tick fever virus (CTFV) and Eyach virus (EYAV), two members of the genus *Coltivirus*. In alignments of these corresponding sequences, the two motifs described previously [17] could be identified together with a third not previously reported. The motif I '[I/V/L][G/A][R/N]K[G/C][G/V/I/C]GK[S/T]' is thought to be an NTP-binding motif [5, 11, 17] but the function(s) of motif II '[V/I/C][V/I/L]DSD[D/E/A/T][Y/F]' and the novel motif III 'F[V/A]H[S/T/C][Y/E/M][C/S/P]EL' remain to be studied (Fig. 1).

A complete set of comparisons using the available sequences of the corresponding proteins in other fijiviruses is summarized in Table 4. The data show that the new virus isolate is about equidistant from MRDV and RBSDV and slightly more distantly related to MRCV in each of its proteins. These distances are considerably greater than those between MRDV and RBSDV sequences. In general, sequence diversity between members of different species in the genus is remarkable and is a general feature of the family *Reoviridae*. No doubt these differences are related to differences in biology, but the functions of many of these proteins are not yet understood, so further discussion is premature.

A set of phylogenetic trees for each of the six proteins encoded on segments S7–10 and the corresponding proteins in other members of the genus is presented in Fig. 2. For each protein, the new isolate occupied a well-supported branch that was placed between MRCV on the one hand and a MRDV-RBSDV cluster on the other. The major branches of each tree were well supported in bootstrap analysis, and their topology showed that each of the currently recognized members was localized independently

Table 4 Amino acid identity between the proteins of the new isolate and the cognate proteins of other fijiviruses

Virus	Protein[a]					
	Non-structural p7a	Non-structural p7b	Core NTP-binding p8	Non-structural p9a	Non-structural p9b	Major outer shell p10
FDV	53.1	26.4	35.6	35.6	38.5	46.0
NLRV	17.6	–[b]	9.3	8.6	14.7	17.0
OSDV	34.6	10.4	23.9	23.8	17.7	32.8
MRCV	62.1	42.7	55.8	65.0	60.3–61.7	71.8
MRDV	82.0	61.5	71.0	78.1	73.2	83.1
RBSDV	79.5–81.2	59.6–60.8	67.7–71.9	77.5–78.4	71.3–72.7	83.1–85.1
MRDV v. RBSDV	91.4–92.8	85.4–86.7	87.3–92.2	91.1–93.7	93.3–95.2	90.9–92.8

Comparisons between MRDV and RBSDV are also shown

[a] Labeled according to their position on the new isolate (and RBSDV). The cognate proteins of other viruses are sometimes located on differently numbered segments

[b] NLRV does not have a protein related to 7b of the new isolate

FDV Fiji disease virus, *MRCV* mal de Rio Cuarto virus, *MRDV* maize rough dwarf virus, *OSDV* oat sterile dwarf virus, *NLRV* Nilaparvata lugens virus, *RBSDV* rice black-streaked dwarf virus

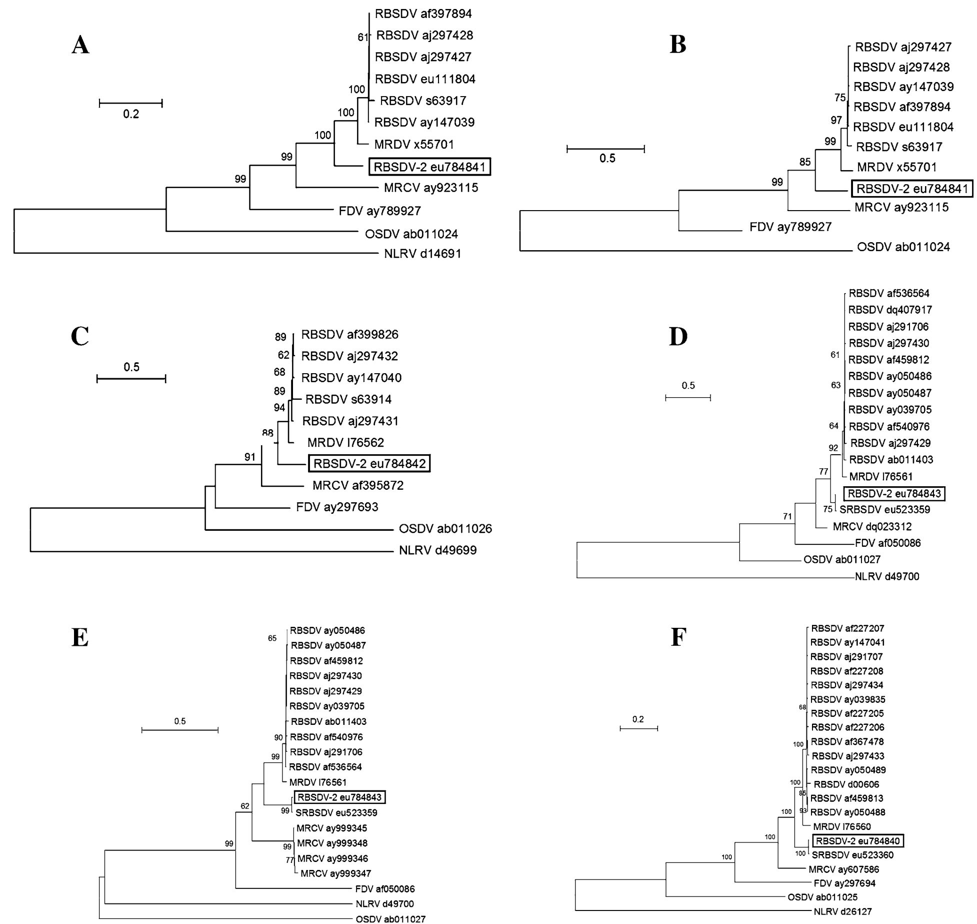

Fig. 2 Phylogenetic (neighbor-joining) trees based on the amino acid sequences of RBSDV-2 proteins p7a (**a**), p7b (**b**), p8 (**c**), p9a (**d**), p9b (**e**), p10 (**f**) and the corresponding sequences of other fijiviruses. The values at the *forks* indicate the percentage of trees in which this grouping occurred after bootstrapping (1,000 replicates; shown only when >60%). The *scale bar* shows the number of substitutions per base

(despite the high level of similarity between RBSDV and MRDV), supporting their taxonomic status as members of independent species in the genus. Our new virus isolate, which infects rice, appeared to be very slightly more closely related to MRDV (natural host maize) than to RBSDV (which infects both maize and rice). Using the current

molecular criteria, the results suggest that our virus isolate represents a new species within group 2 of the genus *Fijivirus*, which we have tentatively named Rice black-streaked dwarf virus-2. If RBSDV-2 is not to be considered a new species, a re-evaluation of the taxonomy of this group, and in particular the status of MRDV and RBSDV, is certainly required.

During the preparation of this paper, two sequences corresponding to genome segments S9 and S10 of a virus isolate (named by the authors southern black streaked dwarf virus, SBSDV) from Hainan Province, China, were made public (accession numbers EU523359 and EU523360). The Hainan sequences are 98–99% identical to our Guangdong RBSDV-2 ones, suggesting that they are isolates of the same species. We prefer the name RBSDV-2, as we think it unlikely that RBSDV is absent from southern China and because there is little information yet available on the distribution of the new virus.

The complete nucleotide sequences of genome segments S7–10 have been deposited in the GenBank/EMBL/DDBJ databases with the accession numbers EU78480–EU784843.

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