Identification, Characterization, and Distribution of Southern rice black-streaked dwarf virus in Vietnam

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Abstract

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A novel dwarf and twisting syndrome first observed on rice in Nghe An Province, Vietnam, in 2009 has spread rapidly to the other 19 provinces of North and Central Vietnam. Infected rice plants showed stunting, darkening of leaves, twisting of leaf tips, and splitting of leaf margins. At a later stage, white waxy enations that eventually turned black were observed on the underside of leaf blades, leaf sheaths, and culms. The disease also infected maize after rice was harvested. Infected maize plants were stunted and dark green with small enations along the minor veins on the back of leaves. The disease agent has now been identified as Southern rice black-streaked dwarf virus (SRBSDV) recently reported from Southern China. Typical fijivirus viroplasms containing crystalline arrayed spherical virions approximately 70 to 75 nm

in diameter were observed under the electron microscope in ultrathin sections of infected rice leaves. The virus was transmitted to rice and maize seedlings by the white-backed planthopper (*Sogatella furcimer*). A one-step reverse transcription-polymerase chain reaction (RT-PCR) protocol was used to confirm the presence of SRBSDV in 477 samples of rice or maize from 29 provinces among 5 agroecological regions in North and Central Vietnam. *Rice black-streaked dwarf virus* was not detected in these samples. Partial sequences of RNA segments 4 and 10 from several isolates showed very low genetic divergences between isolates from Vietnam and China, suggesting a common origin, and phylogenetic analysis confirmed the placement of SRBSDV as a distinct virus within subgroup 2 of the genus *Fijivirus*.

In recent years, rice production in Vietnam has suffered from two emerging viruses, *Rice grassy stunt virus* (RGSV; genus *Tenuivirus*) and *Rice ragged stunt virus* (RRSV; genus *Oryzavirus*), both of which are transmitted by the brown planthopper (BPH; *Nilaparvata lugens* Stal). The outbreak has seriously damaged rice production in 22 provinces in the Mekong Delta and Southeastern region since winter–spring 2005–2006 (17). These diseases have been restricted to the southern part of the country and have not threatened rice production in the Red River Delta region. However, in the summer 2009 crop, a novel dwarf and leaf-twisting syndrome was first observed on rice in Nghe An Province in North Vietnam. The disease spread rapidly among diverse agroecological regions in North and Central Vietnam and also infected maize after rice was harvested. Although the disease was at first thought to be caused by RRSV, further studies have allowed us to identify the causal agent as Southern rice black-streaked dwarf virus (SRBSDV), a virus only recently described from the Southeastern region of China. This virus is also named in the literature as Rice black-streaked dwarf virus-2.

SRBSDV was first observed in the Chinese provinces of Hainan and Guangdong in 2001 and was first considered to be a new strain of *Rice black-streaked dwarf virus* (RBSDV; genus *Fijivirus*, family *Reoviridae*) (21). However, its major vector was identified as the whitebacked planthopper (WBPH; *Sogatella furcimer* Horváth), and it was only poorly transmitted by the small plant-

hopper (*Laodelphax striatellus* Fallen), the main vector of RBSDV. Further studies included the determination of the complete sequences of all 10 double-stranded RNA (dsRNA) genome segments of two isolates and suggested that the causal agent was a new virus in the genus *Fijivirus* (18,21). The genus *Fijivirus* is one of three plant-infecting genera in the large family *Reoviridae*, characterized by a segmented genome of 10 to 12 linear dsRNA segments embedded in an icosahedral particle. Fijiviruses have 10 genome segments and are currently classified into eight species: *Fiji disease virus* (FDV), *Oat sterile dwarf virus* (OSDV), *Garlic dwarf virus* (GDV), *Nilaparvata lugens reovirus* (NLRV), *Mal de Río Cuarto virus* (MRCV), *Pangola stunt virus* (PaSV), *Maize rough dwarf virus* (MRDV), and *Rice black-streaked dwarf virus* (RBSDV) (8). Based on their vectors, host plants, and serological and nucleotide similarities, the genus has been divided into five groups, of which MRCV, PaSV, MRDV, and RBSDV constitute group 2. SRBSDV appears to be an additional member of group 2.

In this paper, we report the identification, characterization, and distribution of SRBSDV in Vietnam and present some partial nucleotide sequence data on the genetic diversity of the virus in Vietnam in comparison with Chinese isolates and other fijiviruses.

Materials and Methods

Sources of plant and insect materials. Field surveys were done between September 2009 and March 2010 in most of provinces in North and Central Vietnam as far South as Quang Ngai Province. Samples were collected and sent to Plant Protection Research Institute (PPRI), and some additional samples were collected by the provincial Plant Protection Sub-Department (Sub-PPD). Leaves of rice and maize with different symptoms were collected from diseased fields, dried, and stored over silica gels at room temperature for reverse transcription-polymerase chain reaction (RT-PCR) tests. Some diseased rice plants with typical symptoms were maintained in insect-proof cages in the greenhouse at PPRI as a virus source for inoculation experiments. BPH and WBPH were also collected

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from diseased rice fields and stored in absolute alcohol for RT-PCR tests.

Electron microscopy. Leaf pieces with typical virus symptoms were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated in an alcohol series of 50, 70, 80, 90, 95, 100% (15 min each) and 100% acetone (20 min), and embedded in Spurr resin. Ultrathin sections were cut transversely from samples using a Leica UC6 microtome, stained with 2% uranyl acetate and 1% lead citrate, and examined with a Hitachi H7650 or a JEOL 1010 transmission electron microscope.

Vector transmission experiments. Virus-free BPH and WBPH were reared in cages on healthy rice cultivar Taichung native 1 (TN1). Third to fourth instar nymphs of virus-free BPH and WBPH were placed on infected rice or maize plants collected from the field for at least 10 days to acquire the virus. These infected plants had been checked by symptomatology and RT-PCR. Viruliferous insects were transferred onto 10-day-old healthy rice cv. TN1 and maize seedlings. Single inoculations (1 insect per seedling) were done on rice only, and mass inoculations (10 insects) were done on groups of either 10 rice or 3 maize seedlings in separate insect-proof cages. Inoculations were done for 24 h in the laboratory at $25 \pm 3^\circ\text{C}$ and 16/8 h light/dark. After inoculation, seedlings were maintained inside insect-proof cages in the greenhouse, and the insects were collected and preserved in absolute alcohol for further RT-PCR tests. Healthy rice or maize seedlings of the same source were also maintained in similar cages as controls.

RT-PCR. Total RNA was extracted from infected leaves of rice plants using an RNeasy kit (Qiagen) according to manufacturer's instructions or using Trizol as described previously (13). Viral genomic dsRNAs were extracted and purified using the modified methods of Uyeda et al (16). The purified genomic dsRNAs were electrophoresed on agarose or polyacrylamide gel and quantified on a NanoVue Plus Spectrophotometer (GE Healthcare) according to the commercial manual.

Primers for segments S4 and S10 of SRBSDV and for S3 and S9 of RRSV (Table 1) were designed from published genomic sequences (13,18–21) and were used to amplify fragments of a gene of unknown function from S4 or the entire major outer capsid protein ORF (1,674 bp, SRBSDV). cDNAs were synthesized and amplified according to commercial instructions. In brief, 1 μg of total RNA or 100 ng of viral genomic dsRNAs were used as a template for first-strand cDNA synthesis by Superscript Reverse Transcriptase (MBI) in the presence of primers. After incubation at 42°C for 60 min, the reaction was stopped by heating at 70°C for 15 min, and then placing on ice. The resulting cDNA was purified using the PCR Purification Kit (Qiagen) and used as templates for PCR. The cycling program included a denaturation step at 94°C for 3 min, 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min, and a final extension step at 72°C for 10 min.

In one-step RT-PCR for virus detection in fresh and dried plant samples and in individual insects, each reaction tube contained 1 $\times$ *Taq*-buffer, 2.5 mM MgCl_2 , 200 μM dNTP mix, 200 pM each primer, 100 U RevertAid Reverse Transcriptase (Fermentas), 2.5 U *Taq*-DNA Polymerase (Fermentas), and 0.5 to 2 μg total RNA in a total volume of 25 μL . The reverse transcription was done at 42°C for 45 min, and the PCR was initiated with 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1

min, terminated at 72°C for 10 min, and then held at 15°C . The amplified fragments were visualized under the Biorad image-system after electrophoresis on 1% agarose containing ethidium bromide.

Cloning and sequencing. Amplified DNA fragments were separated on a 1% agarose gel, purified using the PCR Gel Extraction Kit (Qiagen), ligated into pGEM T easy vector (Promega), and finally transformed into competent *Escherichia coli* TG1 cells for cloning. Plasmids were isolated using the QIAprep spin mini prep kit (Qiagen), and the inserts were sequenced entirely on both strands using the BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) on an ABI 3730 DNA Sequencer with universal primers T7 and SP6.

Sequence analysis. Sequence assembly and analysis was performed using DNAMAN version 6.0 (Lynnon BioSoft, Quebec, Canada). Molecular diversity among the isolates was estimated by calculating the p-distance values for nucleotide and amino-acid comparisons in MEGA5 (14) using the Kimura 2-parameter (6). To determine the relationships between SRBSDV and other fijiviruses, a phylogenetic tree was constructed using MEGA5 (14) by the neighbor-joining method (3).

Results

Identification of SRBSDV in Vietnam. A novel dwarf syndrome was observed for the first time on rice fields in North Vietnam during the summer crop season in August 2009, and by the end of December 2009 similar symptoms had been observed in 19 provinces (report of Plant Protection Department, *data not shown*). In many fields of Nghe An, Thai Binh, Nam Dinh, and Quang Ninh provinces (Fig. 1), incidence was more than 80% (up to 100% in some cases) and there was almost no grain yield. In other provinces, the infected rice plants were distributed randomly with a low incidence in many fields, although some fields with 50% plants infected (or more) were reported.

Symptoms on infected plants in fields. At the early stage, infected rice plants are stunted and dark green with twisting of leaf tips and/or leaf blades, splitting of leaf margins, and white waxy enations along veinlets on the underside of leaf blades and/or on the leaf sheath. At the end of the tillering stage, there is profuse secondary branching. At maturity, infected rice plants remain moderately or severely stunted; the flag leaves are usually malformed or twisted leading to incompletely opened panicles with a high rate of unfilled grains or even no panicle at all. Yellowing and brownish blotches occur on old leaves; these are caused by a secondary unidentified fungal infection but were restricted to virus-infected plants. The most dominant symptom at this stage is the white waxy galls along culms (Fig. 2A to D). On the other hand, infected maize plants are stunted and dark green with very typical white waxy galls along veinlets on the underside of leaf blades (Fig. 2E and F).

Virus particle morphology. Many spherical particles about 70 to 75 nm in diameter and aggregated into crystalline arrays in viroplasms were observed by electron microscopy of ultrathin sections of infected rice leaves collected from several provinces (Fig. 3). The particle morphology and cytopathology are typical of fijivirus infections (19).

Vector transmission of the virus. Transmission experiments showed that WBPH, but not BPH, can transmit the virus from in-

Table 1. Primers used for reverse transcription-polymerase chain reaction (RT-PCR) detection of rice viruses

Name of primer	Sequence	Genome segment	Size of product
SRBSDV-S10-1	5'-ATGGCTGACATAAGACTTGACATA-3'	S10	1,674 bp
SRBSDV-S10-2	5'-TCATCTGGTGACTTATTTAAACAC-3'		
SRBSDV-S4-16	5'-CAAGACAATCTCGGTACTAATCG-3'	S4	533 bp
SRBSDV-S4-18	5'-TGCTCTGAAACTTCAAACAATTTG-3'		
RRSV-S3F	5'-GTAAGTGGTCTGCCCCGCC-3'	S3	825 bp
RRSV-S3R	5'-TCGCATTAAAGAATTGCCCTC-3'		
RRSV-S9F	5'-GCCTTTGCCAGAGATCCTTTTACA-3'	S9	1,110 bp
RRSV-S9R	5'-GCACCATGGTCTCGCAGTTTTC-3'

Fig. 1. Distribution of Southern rice black-streaked dwarf virus (SRBSDV) in 28 provinces in Vietnam (gray shadow). Locations where *Rice ragged stunt virus* (RRSV) was found are marked as black dots. Isolates selected for studying the molecular diversity of SRBSDV are marked with a black square. The eight agroecological zones are shown in uppercase letters.

fecting rice plants to rice and maize seedlings (Table 2). However, attempts to transmit the virus from maize to rice were not successful. WBPH can be reared on maize, and the insects remain alive for 3 or 4 weeks, but no eggs or newly hatched nymphs are produced. After inoculation, symptom development followed the pattern observed in the field. The first symptoms observed on rice seedlings were dark greening of leaves and stunting of plants. The later symptoms were twisting of the leaf tips and/or leaf blades, splitting of leaf margins, and very typical and clear white waxy galls along veinlets on the underside of leaf blades and leaf sheaths. Several symptoms were usually observed in the same inoculated plant (Fig. 4A to C). At flowering stage, white waxy galls were observed on culms. The symptoms on some inoculated maize seedling were stunting, dark greening of the leaf with very typical white tiny enation along veinlets on the underside of leaf blades (Fig. 4D). Presence of the virus in the vector and in some seedlings showing early symptoms was confirmed by RT-PCR and sequencing.

RT-PCR for virus detection. Rice samples were collected from heavily diseased fields from Nghe An, Nam Dinh, Thai Binh, and Quang Ninh provinces and maintained in the greenhouse. RT-PCR using several specific primers described previously for RBSDV (7) or RRSV (13) gave negative results in many experiments (data not shown). By contrast, the primers SRBSDV-S4-16/18 (Table 1) gave a single band of the expected size on all samples in those tests. As additional confirmation, some positive RT-PCR products were sent for direct sequencing, and the results showed them all to be SRBSDV.

Distribution of SRBSDV in Vietnam. Vietnam is divided into eight agroecological regions: North-western, North-eastern, Red River Delta, North-midlands, South-midlands, Tay-Nguyen (also called highlands of the South-midlands), South-eastern and South-western (also called Mekong River Delta). Field surveys were done during the summer 2009 crop and the 2009–2010 winter-spring crop in 30 of the 37 provinces of North and Central Vietnam, as far

as Quang Ngai Province. The provinces not investigated were Hoa Binh (North-western); Cao Bang, Bac Kan, and Ha Giang (North-eastern), and Binh Dinh, Phu Yen, Khanh Hoa (South-midland) (Fig. 3). SRBSDV was detected by RT-PCR in samples from each of the 29 provinces except Hung Yen (Red River Delta), where all samples collected were negative. From 477 rice and maize fields sampled, 214 (44.9%) were positive for SRBSDV. Most of the negative results on rice were from samples with unusual twisted symptoms that were probably caused by herbicides or other factors. Similarly, most of negative results on maize were from plants with malformed top leaves, with twisted and broken leaves or which were stunted with profuse shooting. In contrast, most positive results were derived from samples with at least one or more symptoms as described above.

No infection by RRSV was found in any of the 181 samples collected during summer 2009, but in the winter-spring 2009–2010, 15 samples contained RRSV. Five of these were co-infections among the 85 SRBSDV-positive samples, and 10 were found in the 194 SRBSDV-negative samples (Table 3).

Molecular diversity of the virus. A 533-bp fragment from S4 and the entire major outer capsid protein ORF (1,674 bp) from S10

Fig. 3. Spherical particles of 70 to 75 nm in diameter and aggregated into crystalline array in the viroplasm of infected rice leaf. Bars are 200 nm.

Fig. 2. Field symptoms of Southern rice black-streaked dwarf virus (SRBSDV) on rice (A to D) and maize (E and F).

were sequenced from isolates originating from nine provinces (Hunan, HaTinh, NamDinh, NgheAn, NinhBinh, QuangNam, ThaiBinh, VinhPhuc, and Haiduong). Respective GenBank accession numbers for these sequences are HM583657-65 (S4) and HM583666-74 (S10). The diversity among all available 11 SRBSDV isolates was very low in both genomic regions and slightly lower on S10 than on S4 (p-distances of $0.8 \pm 0.3\%$ and $1.4 \pm 0.3\%$, respectively). These values are much lower than those among the homologous S10 region of the 11 available RBSDV isolates ($3.9 \pm 0.3\%$). There was no obvious correlation between geographic distribution and genetic diversity on either S4 or S10.

In a phylogenetic analysis using the corresponding regions of all other plant-infecting fijiviruses, all the SRBSDV isolates clustered in one distinct clade well-separated from isolates of other species in the genus (Fig. 5).

Discussion

The dwarf syndrome on rice in North and Central Vietnam was first suspected to be caused by RRSV. Some of the symptoms of the two diseases are similar, and it is not always possible to provide an accurate diagnosis from field symptoms alone. However, it is clear from the results presented here that the causal agent of the emerging rice disease in North and Central Vietnam is the new fijivirus described from China as Southern rice black-streaked dwarf virus. RT-PCR results, vector transmission by WBPH, disease symptoms, and virus particle morphology all support this conclusion.

Vector transmission appears to be species-specific in fijiviruses (2,19). In our experiments, WBPH was shown to be an efficient vector, transmitting SRBSDV between rice seedlings and from rice to maize. Field investigation confirmed that the most severely affected areas were those with a large WBPH population in rice fields at the beginning of the crop, but WBPH was not observed in diseased maize fields during the survey. A change in the relative

populations of different plant hoppers has recently been reported in Vietnam. Between 1981 and 2007, the balance has changed from about 70% BPH / 30% WBPH to about 30% BPH / 70% WBPH (15). BPH is the only known vector of RRSV but did not transmit SRBSDV in our experiments. The origins of the WBPH population surge have not been confirmed, but typhoons could carry migrating WBPH from Hainan to Vietnam, and three typhoons in 2009 (Data from Department of Meteorology and Hydrograph, Ministry of Agricultural and Rural Development, Vietnam) could link the outbreak of SRBSDV in Hainan Province in early 2009 to the Vietnam outbreak starting in Nghe An Province in the 2009 summer crop. On the other hand, there is also evidence that WBPH moves from the Red River Delta in North Vietnam to the Southern provinces of China like Guangxi, Southern Hunan, Jiangxi, Northern Guangdong, and northwestern Fujian starting from late April to early May every year (10). There is good reason to believe that the movement of WBPH has been the main factor in the spread of SRBSDV between provinces in North Vietnam and South China. WBPH is a strong-flying insect (1,12) that moves over long distances, and the very low genetic diversity among the virus isolates also supports the idea of a common origin of isolates dispersed by the vector.

SRBSDV is a new virus only recently reported from the South of China (18,20,21). More needs to be known about its epidemiology, the virus-vector-host relationships, and sequence diversity in other segments of the SRBSDV genome. In this study, we investigated the molecular diversity of RNA segment S10 because it encodes the outer capsid protein known to be involved in vector specificity and because interactions with the vector play an important role in fijivirus evolution (4,9,11). The partial sequence of segment S4, which has an unknown function, showed a slightly higher diversity. In the future, the molecular diversity of segments S5 and S6, the most divergent between the two Chinese isolates (18), should also be analyzed.

Table 2. Transmission of Southern rice black-streaked dwarf virus (SRBSDV) to rice or maize using either brown planthoppers (BPH, *Nilaparvata lugens*) or white-backed planthoppers (WBPH, *Sogatella furcifera*) previously fed on SRBSDV-infected rice or maize seedlings

Virus source	Type of inoculation	Vector insect	No. of inoculated seedlings	Symptoms observed		Latent period in inoculated plants (DAI)
				No.	%	
Rice	1 insect/1 rice	BPH	383	0	0	na ^a
		WBPH	395	217	54.9	7-23
	10 insects/10 rice	BPH	148	0	0	na
		WBPH	274	60	25.9	7-22
	10 insects/3 maize	BPH	12	0	0	na
		WBPH	21	13	61.9	12-24
Maize	1 insect/1 rice	WBPH	50	0	0	na
	10 insects/10 rice	WBPH	30	0	0	na
	10 insects/3 maize	WBPH	15	0	0	na

^a na: not available.

Fig. 4. Early symptoms on inoculated rice (A to C) and maize (D) plants.

Although it was not the major cause of disease in the north and midlands of Vietnam, RRSV was also detected in some fields and sometimes occurred as a co-infection with SRBSDV. Field investigations in the south of Vietnam during an outbreak of RRSV and RGSV in recent years showed that the proportion of mixed infection of the two viruses was high. The co-infected plants usually died before maturity, showing a higher pathogenicity in the co-infected plants (17). Co-infections between RBSDV and *Rice stripe virus* (RSV, genus *Tenuivirus*) have also been reported, although the incidence of co-infection was low in the field (5). In greenhouse experiments, the latent period of RBSDV increased

when the virus was inoculated on RSV-infected rice seedlings and co-infected plants survive until maturity (5). RRSV/SRBSDV co-infections are an interesting biological model to investigate the relationships between two rice viral species at an intra-family level. The nature of the interactions inside the family *Reoviridae* could be compared to those between the genera *Oryzavirus* or *Fijivirus* (belonging to family *Reoviridae*) and genus *Tenuivirus*.

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Table 3. Reverse transcription-polymerase chain reaction (RT-PCR) detection of Southern rice black-streaked dwarf virus (SRBSDV) and *Rice ragged stunt virus* (RRSV) in samples collected from different sites in Vietnam

Time of sample collection	Sample origin	SRBSDV		RRSV		Co-infection
		Samples	Positive	Samples ^a	Positive	
Summer crop 2009	North-eastern	35	28	35	0	0
	North-western	18	15	18	0	0
	Red River Delta	63	33	63	0	0
	North-midlands	57	45	57	0	0
	South-midlands	8	8	8	0	0
	2009 total	181	129	181 ^a	0	0
Winter-spring crop 2009-2010	North-eastern	72	15	68	1	0
	North-western	12	4	12	0	0
	Red River Delta	109	25	44	4	0
	North-midlands	45	18	28	1	1
	South-midlands	58	23	42	9	4
	2010 total	296	85	194 ^a	15	5
Total samples tested (%)		477	214 (44.86)	375 ^a	15 (4.00)	5 (1.33)

^a In summer 2009, all samples were tested for both viruses. In the 2009–2010 winter–spring crop, all samples were tested for SRBSDV and then only SRBSDV-positive samples and other samples with RRSV-like symptoms were tested for RRSV (total 194 samples).

Fig. 5. Molecular phylogenetic analyses of the partial sequences of Southern rice black-streaked dwarf virus (SRBSDV) S4 (left) and S10 (right) and the corresponding regions of all other available plant-infecting fijiviruses. Trees were prepared from codon-aligned nucleotide sequences using MEGA5 by the Neighbor-joining Maximum Composite Likelihood method and 10,000 bootstrap replicates (values shown where >60%). Bars indicate numbers of substitutions per site.

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