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ANNOTATED SEQUENCE RECORD

Molecular characterization and sequence analysis of four Brazilian rice stripe necrosis virus isolates

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Abstract

Rice (*Oryza sativa* L.) is an important food crop for humanity, being cultivated in tropical and temperate regions of the world. This study reports the nearly complete genome sequences of four Brazilian rice stripe necrosis virus (RSNV) isolates. The nucleotide sequences of the RNA1 and RNA2 genome segments of these Brazilian isolates were 96.5 to 99.9% identical, indicating their close phylogenetic relationship to each other. Phylogeny and recombination analysis indicated that the genome of one of the isolates consisted of RNA segments of different origins, suggesting that a reassortment event had occurred.

Rice stripe necrosis virus (RSNV) belongs to the family *Benyviridae,* which comprises one single genus, *Benyvirus*, whose members are rod-shaped, multipartite, positive-sense single-stranded RNA plant viruses. The genus includes four recognized species: *Beet necrotic yellow vein virus*, *Beet soil-borne mosaic virus*, *Burdock mottle virus*, and *Rice stripe necrosis virus*. RSNV is vectored by *Polymyxa graminis* (Cercozoa, Plasmodiophoridae), a root obligate biotrophic plant parasite [1]. RSNV has two positive, polyadenylated segments of RNAs, which are encapsidated individually into separate particles [2]. Three complete genome sequences of RSNV are publicly available, two from Mali and one from Colombia (MK170452, MK170453, MK170454, MK170455, EU099844, and EU099845). These genomes contain 6,617 to 6,634 nucleotides (nt) in RNA1 and 4,657 to 4,871 nt in RNA2. RNA1 contains a single ORF encoding the replication-associated protein, with conserved methyltransferase, helicase, papain-like protease, and RNA-dependent RNA polymerase (RdRP) motifs. RNA2 contains six ORFs encoding: the capsid protein (CP), the readthrough (RT) protein, three movement proteins, called "triple gene block (TGB)" (TGB1, TGB2 and TGB3), and a cysteine-rich protein (ORF6) [2, 3].

The first report of RSNV associated with a rice disease was in 1977 in Côte d´Ivoire [4, 5], although the disease had been known to affect upland rice for many years in West Africa [6]. In 1991, RSNV was first observed in Colombia [7], followed by reports in Ecuador, Panama, Argentina, and Southern Brazil [3, 8–10]. Over the past few years, there have been many reports of the presence of RSNV in African countries, including Burkina Faso, Benin, Mali, and Sierra Leone [11–14], suggesting a re-emergence of the virus [14].

In 2018/2019, yield losses caused by RSNV-like symptoms were observed in southern and, for the first time, central Brazil [15]. In order to identify and characterize viruses associated with those symptoms, leaf and stem samples of rice plants showing stunting and chlorotic stripes (Fig. S1) were collected in rice fields in those regions and stored at -80°C. Samples were collected from Lexus hybrid

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in the municipality of Jacinto Machado (29°05'25.3''S; 49°50'46.8''W; 13 m altitude) and Titan hybrid in the municipality of Imaruí (28°21'27.3''S; 48°53'43.1''W; 8 m altitude) in the state of Santa Catarina, SCS 122 Miura cultivar in the municipality of Brazabrantes (16°25'50"S; 48°23'21"W; 762 m altitude) in the state of Goiás, and BRS Catiana cultivar in the municipality of Lagoa da Confusão (10°47'37"S; 49°37'25"W; 200 m altitude) in the state of Tocantins, hereafter named the SCL, SCT, GO, and UFT-2019 isolate, respectively. For molecular characterization, double-stranded RNA (dsRNA) was extracted as described [16]. Recombination analysis was performed using Recombination Detection Program version 4 (RDP4) [17]. Only recombination events detected by at least four of the methods available in the program were considered reliable. A multiple-comparison-corrected *P*-value cutoff of 0.05 was used throughout. Phylogenetic relationships based on the codon-aligned nt sequences of the RSNV RNA1 and RNA2 genome segments and helicase motif were performed using the maximum-likelihood method implemented in the MEGA X program. For detailed information on the methods, see Supplementary Material. The sequences have been deposited in the NCBI database with the accession numbers MT270127 (SCL RNA1), MT270128 (SCL RNA2), MT270129 (SCT RNA1), MT270130 (SCT RNA2), MT507288 (GO RNA1), MT507289 (GO RNA2), MT027255 (UFT-2019 RNA1), and MT027256 (UFT-2019 RNA2).

Using Illumina HiSeq X Ten and HiSeq 2500 platforms, 21,213,075 (SCT), 22,744,026 (SCL), 27,478,903 (GO), and 53,798,106 (UFT-2019) reads were generated, and, after assembly, two contigs of each isolate corresponding to RSNV RNA1 and RNA2 segments were obtained. From these contigs, four nearly complete viral genome sequences were assembled, each composed of two RNA segments (RNA1 and RNA2). The amino acid sequences of the capsid proteins of the four isolates were found to be over than 90% identical to that of rice stripe necrosis virus (genus *Benyvirus*, family *Benyviridae*) [2, 3]. No other viral sequences were detected in the four samples. Conventional Sanger sequencing with primers specific for RNA segments 1 and 2 confirmed the presence of RSNV in the samples used for HTS (data not shown).

The Brazilian isolates have a genomic organization consistent with that of previously characterized RSNV isolates (Table S1). Although we did not use a specific method to determine the ends of the genomes, the sequences of the 5'- and 3'-UTR regions of both RNA segments obtained by HTS were very similar to those of the 5'- and 3'-UTR regions of a Colombian RSNV isolate characterized previously [3]. A 243-nt insertion was found in the TGB1 coding region of the GO isolate. This insertion is present in both Malian isolates as well, but not in the Colombian isolate or the other three Brazilian isolates. The presence of the 243-nt insertion in the GO isolate was confirmed by conventional Sanger sequencing using specific primers (Fig. S2).

Overall nucleotide sequence identity over 94% for RNA1 and RNA2 was found among Brazilian isolates and isolates from Mali and Colombia (Fig. 1A). In RNA1, the sequences of the Brazilian isolates were over 99% identical, and the Brazilian isolates showed higher similarity to the Malian isolates (97% identity) than to the Colombian isolates (94%) (Fig. 1A). Likewise, the sequence of RNA 2 was more similar to those of the Malian isolates, except in the case of SCL RNA2, which showed 99.9% identity (only three nucleotide differences) to the Colombian isolate.

A phylogenetic tree based on RNA1 sequences joined the Brazilian isolates, closer to Malian isolates and separate from the Colombian isolate (Fig. 1B). On the other hand, the RNA2 sequences grouped SCL with the Colombian isolate, along with the Malian isolates, and separate from the other Brazilian isolates, with strong bootstrap support. Additionally, analysis of the helicase motif coding region of RNA1 of 17 isolates indicated a strong phylogenetic relationship of the Brazilian isolates to Argentinian isolates, close to African isolates and more distant from the Colombian isolate (Fig. 1C).

Despite the few sequences available, recombination analysis revealed four putative recombination events in RNA1 and RNA2 (Table 1). Recombination events in RNA1 were detected at the beginning and end of ORF1, including the RdRp and methyl transferase motifs in the Colombian and Brazilian isolates, respectively; these events had a Malian isolate as possible parent (Table 1). All of the recombination events detected in RNA2 had Malian isolates as possible recipients and Brazilian isolates as putative donors. Recombination events in RNA2 were detected mainly in TGB, ORF6, and the N-terminal part of CP (Table 1).

All descriptors of molecular variability (S, K, π, H, and Hd) indicated higher genetic variability in the expanded dataset (four Brazilian, two Malian, and one Colombian sequences) in all seven ORFs than in the dataset containing only Brazilian sequences (Table S2). The value of θ-W was on the order of 10^{-2}, except for RdRp (10^{-3}) in the dataset comprising Brazilian isolates (Table S2). In general, nucleotide diversity (π) was lower than 0.028 for all of the ORFs in both datasets (Table S2). The genomic organization and genome size of the Brazilian isolates are very similar to those of RSNV isolates from Colombia and Mali. Phylogenetic analysis based on the sequences of RNA1 and RNA2 revealed close relationships among RSNV isolates and corroborated the sequence analysis data. A phylogenetic tree based on the entire sequence of RNA1 clustered the seven isolates according to their geographic origin, separated into three clades: Brazil, Mali, and the Colombian isolate detached from both groups. Similarly, phylogenetic analysis of the coding region of the helicase motif confirmed

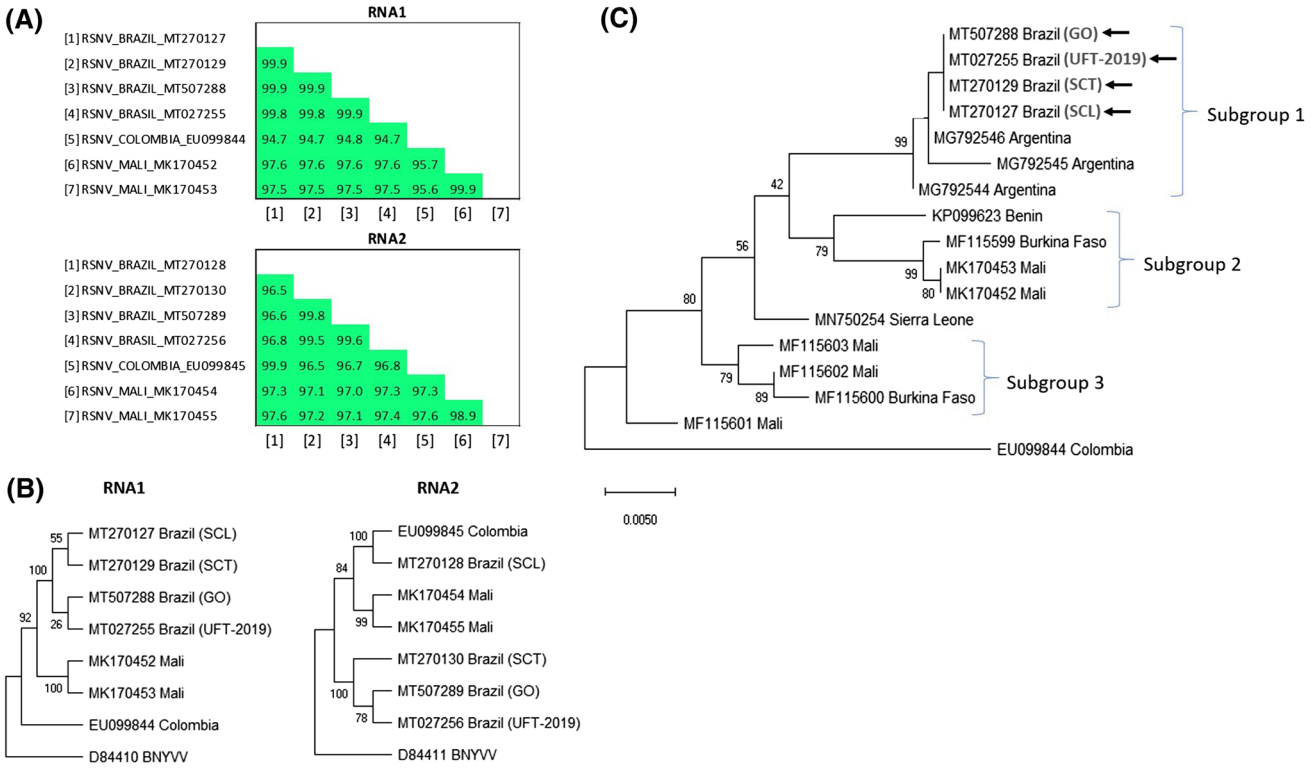

Fig. 1 (A) Two-dimensional plot representing the percentage of nucleotide sequence identity in RNA1 and RNA2 among isolates of rice stripe necrosis virus (RSNV) from Goiás (GO), Santa Catarina (SCL and SCT) and Tocantins (UFT-2019). (B) and (C) Phylogenetic relationships based on the codon-aligned nucleotide sequences of the RSNV RNA1 and RNA2 genome segments, and helicase motif**,** respectively. Numbers on branches indicate bootstrap values. Bars indicate the number of substitutions per site. The arrows indicate the Brazilian RSNV isolates. GenBank accession numbers are indicated.

Table 1 Recombination events detected by RDP in the analyzed rice stripe necrosis virus (RSNV) sequences

Segment	Event	Recombinant (accession number – country)	Recombination breakpoints		Parental isolates		Methods[#]	*P*[##]
			Initial	Final	Major	**Minor**		
RNA1	1	EU099844_Colombia	4156	5100	Unknown	MK170453_Mali	**R**GBMC3	3.965×10^{-33}
	2	MT270127_Brazil	6517-6659	394	MK170453_Mali	Unknown	GB**M**C3	7.840×10^{-03}
RNA2	1	MK170454_Mali	3117	334	MT270130_Brazil	MT270128_Brazil	RG**B**MCS3	9.943×10^{-13}
	2	MK170455_Mali	3117	360	MT270130_Brazil	MT270128_Brazil	RG**B**MCS3	9.943×10^{-13}

[#]R, RDP; G, Genecov; B, Bootscan; M, Maximum x^2; C, Chimaera; S, Sister Scan; 3, 3-Seq

[##]Only the smallest *P*- value is indicated, with the method and underlined in bold.

the geographic clustering pattern of RNA1: Brazilian and Argentinian isolates separated from African isolates and the Colombian isolate in an independent and external branch. Intriguingly, based on the RNA2 sequence, the Brazilian isolate SCL grouped with the Colombian and Malian isolates, and the other three Brazilian isolates occupied a separate clade. In fact, nucleotide and amino acid sequence comparisons revealed that RNA2 of SCL is almost identical to that of the Colombian isolate and more similar to those of the Malian isolates than to those of other Brazilian isolates, suggesting that the SCL genome consists of RNA segments from different origins, which indicates that a reassortment event has occurred. Reassortment is a shared feature of all segmented RNA viruses [18].

Frequent questions arise in plant pathosystems concerning the origin of plant viruses and the way they spread between geographic regions. In the case of RSNV, based on the first reports of this virus from Africa and Colombia, it is likely that the virus originated in Africa and was then introduced into the Americas through Colombia by rice

seed contamination, and from there to other countries in South and Central America. RSNV is not seed-transmitted; however, its vector *P. graminis* can be spread contaminating seeds as resistance spores. Our results do not confirm this pathway for the spread of RSNV. The genetic distance between the Colombian isolate and other South and Central American isolates (but RNA2 of SCL) suggests that there was more than one RSNV introduction event in the Americas. Indeed, the presence of a 243-nt insertion in the TGB1 coding region of the GO isolate, which is shared with the Malian isolates but not with the Colombian isolate or the other three Brazilian isolates, strengthens the multiple-introduction hypothesis. However, the divergence between the Colombian isolate and other American isolates might also be related to the ages of the isolates. Whereas the Colombian isolate was collected and sequenced before 2009, the Brazilian and Malian isolates were collected and sequenced after 2017, giving them more time to accumulate mutations. Unfortunately, the limited number of RSNV sequences currently available does not allow a more detailed analysis.

Regarding the origin of RSNV, Asian rice (*Oryza sativa*) was introduced into West Africa in the 16th century, whereas African rice (*Oryza glaberrima*) was first domesticated and grown in West Africa around 3,000 years ago [19, 20]. The only gene for resistance to RSNV identified so far was mapped in *O. glaberrima*, suggesting a long co-existence of this species with RSNV. Interestingly, Africa and South America share two benyviruses that are transmitted by *P. graminis* to grasses of economic importance (RSNV in rice and wheat stripe mosaic virus [WhSMV] in wheat) [16, 21, 22]. Since neither *O. sativa* (from Asia) nor wheat (*Triticum aestivum*, from the Middle East) are native to these regions, it may be that these viruses share a common ancestor in some native grass and that, once rice and wheat were introduced, modifying the natural landscape, the virus infected these new hosts. Undoubtedly, this is an intriguing possibility that deserves to be investigated in future studies.

To our knowledge, this is the first report of nearly complete genome sequences of RSNV from Brazil, and the results presented here are novel and advance our understanding of the molecular diversity of RSNV isolates infecting rice. The Brazilian RSNV isolates show close phylogenetic relationship to each other and are prone to recombination and reassortment.

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Declarations

Conflict of interest None of the authors have a conflict of interest.

Human and animal rights This article does not contain any studies with animals performed by any of the authors.

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