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Molecular characterisation of *Rice stripe necrosis virus* as a new species of the genus *Benyvirus*

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Abstract The complete nucleotide sequences of RNAs 1 and 2 of *Rice stripe necrosis virus* (RSNV) were determined and compared to the corresponding genomes of all sequenced, rod-shaped plant viruses. The genome organisation of RSNV RNA1 and RNA2 is nearly identical to that of *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV), definitive species of the genus *Benyvirus*. As demonstrated for BNYVV and BSBMV, the RNA1 of RSNV also encodes a single ORF with putative replicase-associated motifs, which distinguishes benyviruses from all other viruses possessing rod-shaped particles. As described for BNYVV, RNSV RNA-2 also contains six ORFs: the capsid protein gene, the read-through protein gene, a triple gene block gene that codes for three different proteins, and a 17 kDa cysteine-rich protein. RNAs 3 and 4 (or 5 in the case of BNYVV), identified in natural infections of BNYVV and BSBMV, were not detected in any of the 44 RSNV cDNA clones obtained in this investigation. Nevertheless, phylogenetic and amino comparative acid sequence analyses demonstrated that RSNV is more closely related to BNYVV and BSBMV than to any other rod-shaped plant virus characterised to date.

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Introduction

Rice stripe necrosis was first described in 1977 as a new disease of rice (*Oryza sativa*) in the Ivory Coast, west Africa (Louvel and Bidaux 1977). This disease may cause early seedling death and a characteristic, severe plant malformation (Fig. 1). Diseased rice plants usually show conspicuous chlorotic stripes and systemic necrosis on their leaves, stunting and, in mature plants, panicles show malformation and poor seed production. The causal agent of rice stripe necrosis was originally characterised as a rod-shaped virus with a bi-modal particle length (110–160 nm and 270–380 nm). The occurrence of the disease was consistently associated with the presence of the plasmodiophorid fungus *Polymyxa graminis* in the soil of affected rice fields (Fauquet and Thouvenel 1983). These researchers named the pathogen '*Rice stripe necrosis virus*' (RSNV), and partially characterised it as a multi-partite virus consisting of at least three classes of particles (380 nm, 270 nm and 110–160 nm long, and 20 nm in width). RSNV became a tentative species of the genus *Furovirus* after the revision of the genus in 1997 (Torrance and Mayo 1997), as shown in the seventh report of the International Committee on Taxonomy of Viruses (ICTV) in 2000 (Van Regenmortel et al. 2000). No further research on RSNV was conducted until its

Fig. 1 Characteristic plant malformation induced by *Rice stripe necrosis virus*

emergence in the Americas in 1991 (Morales et al. 1995, 1999). The latter investigations pointed out notable differences in particle length between RSNV and the known species of the genus *Furovirus*, as well as close similarities in the number and size of RNA molecules detected in the case of RSNV, and those reported for the two definitive species, *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV), of the recently created *Benyvirus* genus (Fauquet et al. 2005). Additionally, as reported for BNYVV and BSBMV, RSNV was shown to have a polyadenylated 3′ end, not present in furoviruses or in any other plant virus with rod-shaped particles (Morales et al. 1999; Van Regenmortel et al. 2000; Fauquet et al. 2005). Based on those preliminary findings, RSNV was classified as a tentative species of the genus *Benyvirus* (Fauquet et al. 2005). This investigation provides further support for the classification of RSNV as a new species of the genus *Benyvirus*.

Materials and methods

Rice plants showing foliar malformation symptoms characteristic of the 'crinkling' or 'entorchamiento' (Spanish) disease of rice, were originally collected near the city of Villavicencio, Meta, in the eastern plains of Colombia. The roots were washed, dried under vacuum, and subsequently ground to a powder, to be used as inoculum. Rice 'Orizica 3' plants were grown under glasshouse conditions in a mixture of RSNV-infected root powder and soil at CIAT, Palmira, Colombia. For cDNA synthesis, an oligo $(dT)_{12-18}$ and random primers were used with the SuperScript Choice System kit (Gibco BRL, Rockville, MD), using viral RNA extracted from purified RSNV virions (Morales et al. 1999). The resulting cDNA was ligated into the *SmaI* site of pBlue-Script II (Stratagene). A total of 44 cDNA clones of varying sizes were selected and then sequenced by a combination of sub-cloning and internally-primed sequencing, using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA) and an ABI 373 automated sequencer. Forward specific primers: F3216-1(5′-CCCAGAAGGAGATAGACG-3′), F3216-2: (5′-TTGCAAGAATCAAACCTTC-3′), F3216-3(5′-TGAATTTGGTGCTCTCTTG-3′), and reverse specific primers: R2688-4(5′-CTTCAGGCGGCATGACAAG-3′), R2688-5(5′-TGTGGCGTTTCCAGACCTAAA-3′), were designed based on the sequences obtained, to complete the full RSNV RNA1 sequence. The 3′ terminal end (870 nucleotides) of RSNV RNA2 was amplified by 3′ Rapid Amplification of cDNA Ends (3′ RACE), using a System Kit (3′RACE version E) according to the supplier's instructions (Invitrogen, Carlsbad, CA ,USA). Total RNA was extracted from leaf and roots of RSNV-infected rice plants using RNeasy plant mini-kit (Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer. 'Nested' PCR was performed using sense, gene-specific primers RNA2: 5′-GGTGGCATTGTCGTAGTT-3′ (position 3625–3642) and 5′-TCTTATGGTCCACTGTTCA-3′ (position 3653–3671). PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). Sequencing of cDNA clones was performed at the DNA Facility of Iowa State University, Ames, IA, USA.

Sequence data were analysed using SEQUENCHER version 4.1.2 for Macintosh. Comparative analyses, including multiple alignments of amino acid sequences, and calculations of the genetic distances between sequences, were performed using NCBI BLASTX, and DNAMAN version 4.13 (Lynnon Biosoft, Vaudreuil, Quebec).

Results

Of the 44 cDNA clones initially obtained in this investigation, 29 clones produced partial nucleotide (nt) and amino acid (aa) sequences possessing homology to the RNA1 of the definitive benyviruses, BNYVV and BSBMV. The use of the specific primers F3216-1,-2 or -3/ R2688-4 and F3216-1, -2 or-3/ R2688-5, described above, yielded PCR products of the expected size (850 nt and 900 nt, respectively) that included the entire RNA1 genome sequence of RSNV. The RNA 1 nucleotide sequence and genomic organisation of RSNV were determined and compared to the same characteristics of the RNA1 genomes of BNYVV, BSBMV, and other rod-shaped plant viruses (Table 1).

RSNV RNA1 is 6,614 nt in length (accession number EU099844), excluding the 3′ poly(A) tail, and contains a single ORF with the potential to encode a 236 kDa polypeptide with a putative replicase function. Similarly, BNYVV RNA1 (6,746 nt) and BSBMV RNA1 (6,683 nt) contain a single ORF encoding a 237 kDa and 239 kDa polypeptide, respectively (Fauquet et al. 2005). RSNV RNA1 shares a 50% nt sequence identity to the respective RNA 1 of BNYVV and BSBMV. The putative NTP-binding helicase motif (Gly-X-X-Gly-X-Gly-Lys-Ser), apparently involved in duplex unwinding during viral RNA replication and translation (Hall and Matson 1999), is located at aa positions 955–962 in the RSNV RNA1 ORF. An RdRp motif (Gly-Asp-Asp), known as the 'GDD' motif (Poch et al. 1989), was also present in the

Table 1 Comparative amino acid sequence identity analyses of the viral proteins of *Rice stripe necrosis virus* (RSNV) and selected plant viruses possessing rod-shaped virions (RSPV)[abc]

Genus	RSPV	RNA1 236 K		RNA2				
		MeT/H[c]	RdRp	CP (20.6K)	TGB1 (38.4K)	TGB2 (12.3K)	TGB3 (15K)	Cys-R (17K)
Benyvirus	BNYVV	39	67	27	37	61	58	20
	BSBMV	38	67	25	37	60	31	19
Pomovirus	PMTV	11	10	24	24	31	25	26
	BSBV	10	12	18	23	39	17	na
	BBNV	10	12	14	26	32	15	35
Pecluvirus	IPCV	8	8	20	27	34	19	17
	PCV	7	14	19	27	32	8	17
Furovirus	SBWMV	9	8	19	na[d]	na	na	15
	SBRMV	8	10	8	na	na	na	18
Hordeivirus	BSMV	9	12	12	28	23	15	14
Tobamovirus	TMV	8	17	19	na	na	na	na
Tobravirus	TRV	7	8	12	na	na	na	na

[a] Using DNAMAN version 4.13

[b] The sequences of the RSPVs selected for these analyses are available in the Genbank under the following accession numbers: *Beet necrotic yellow vein virus* (BNYVV) D84410, D84411; *Beet soil-borne mosaic virus* (BSBMV) NC003506, AF061869; *Potato mop top virus* (PMTV) CAM35543, AJ238607, AJ277556; *Beet soil-borne virus* (BSBV) Z97873,Z66493,U64512; *Broad bean necrosis virus* (BBNV) D86636, D86637, D86638; *Indian peanut clump virus* (IPCV) X99149,AF239729; *Peanut clump virus* (PCV) NC004729, AF447398; *Soil-borne wheat mosaic virus* (SBWMV) AB033689, AAA48494; *Soil-borne rye mosaic virus* (SBRMV) AF146280, AF146283; *Barley stripe mosaic virus* (BSMV) NC003469, NC003481; *Tobacco mosaic virus* (TMV) EF392659; *Tobacco rattle virus* (TRV) X03686, XO6172. The nucleotide sequences data for RSNV were deposited as GenBank accession numbers EU099844 (RNA1), EU099845 (RNA2).

[c] The predicted N-terminal and C-terminal amino acids identified in the cDNA sequence analyses, were used to compare the methyltransferase/helicase (Met/H) and RNA-dependent RNA polymerase (RdRp) regions; the coat protein (CP); and triple gene block (TGB) of RSNV and selected RSPVs

[d] Not applicable or not present. Data shown are expressed as percentages of aminoacid identity

RNA-dependent RNA polymerase located at aa positions 1922–1924, in the C-terminal region of the 236 kDa protein of RSNV. The sequence motifs of a putative, type 1 methyl-transferase domain, were located at aa positions 222–231 and 414–426 (Fig. 2).

RSNV RNA2 is 4,631 nt in length (accession number EU099845), and shows nt identities of 40% and 39% when compared to the 4,609 nt and 4,616 nt long RNA2s of BNYVV and BSBMV, respectively. Six putative ORFs were identified on the RSNV RNA2 genome obtained (Fig. 2), as reported for the RNA2 molecules of BNYVV and BSBMV (Putz, 2001; Lee et al. 2001). RSNV RNA2 has a 5′proximal cistron (ORF1) that encodes the 20.6 kDa viral capsid protein (CP), followed by a leaky UAG stop codon and ORF 2, which codes for a 52 kDa protein. Both ORFs produce a read-through (RT) protein with a predicted size of 74 kDa. The Tyr (Y), Arg-Phe (RF) and Phe-Glu (FE) motifs, characteristic of the CPs encoded by rod-shaped viruses (Koonin and Dolja 1993), were identified in the N-central and C-terminal regions of the RSNV CP ORF. A KTER-encoding domain found in the RT region of BNYVV and BSBMV (Tamada et al. 1996), is also present in the RT region (nt 1178–1186) of RSNV. ORF 2 is followed by three overlapping ORFs that code for polypeptides 38.4 kDa, 12.3 kDa and 15 kDa in size, possessing motifs typical of triple gene block proteins (TGBp1–3). These proteins have been shown to be involved in cell-to-cell movement of the virus in infected plants (Gilmer et al. 1992). RSNV TGB shares aa sequence identities of 37%, 61% and 58% with the corresponding TGB proteins of BNYVV, and 37%, 60% and 31% with the TGBp1–3 of BSBMV (Table 1). Finally, ORF6 encodes a 17 kDa cysteine-rich protein that shares aa sequence identities of 20% and 19%, with the corresponding proteins of BNYVV and BSBMV, respectively. Pomoviruses were not included in the Cys-R sequence comparisons because the size of their corresponding ORF is very different from those of other genera of rod-shaped viruses (Table 1).

The 5′-UTRs of BNYVV RNAs 1 and 2, share 46% and 47% nt sequence identities with the corresponding 5′-UTRs of RSNV RNAs 1 and 2. Similarly, the 5′-UTRs of BSBMV RNAs 1 and 2, share 51% and 45% nt sequence identities with the respective 5′-UTRs of RSNV RNA 1 and 2. In the case of the 3′-UTRs, BNYVV and BSBMV RNA1s share 42% and 48% nt sequence identities, respectively, when compared to the RNA1 3′-UTR of RSNV. The RNA1 3′-UTRs of these three viruses, possess a highly conserved region of 72 residues (Fig. 3). None of the 44 true clones obtained and

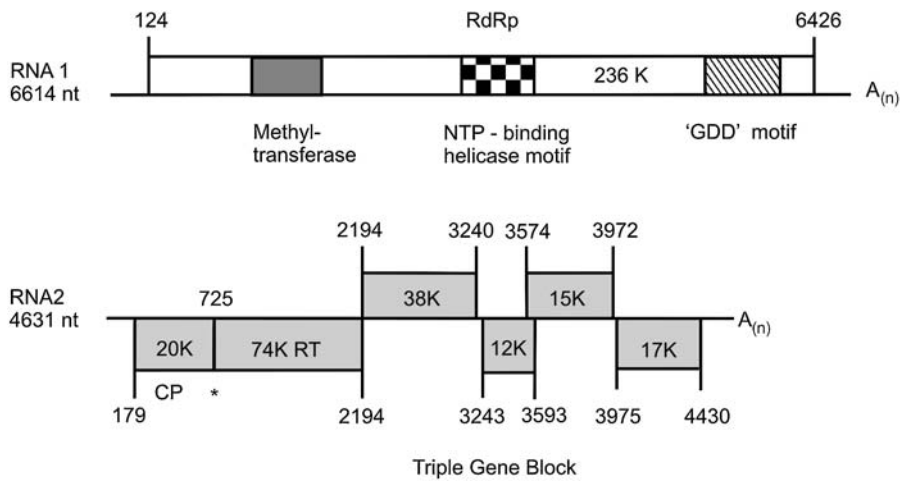

Fig. 2 Genome organisation of RSNV RNAs 1 and 2, and their open-reading frames (ORFs, shown as rectangles), as deduced by cDNA sequence analysis. The methyl-transferase (shaded); helicase (cheked area); and GDD (hatched area) motifs are indicated on the 236 kDa RNA1 polymerase. An asterisk (*) at nucleotide 725 on RNA2 represents the leaky UAG amber codon that permits expression of the 74 kDa read-through protein (RT). The respective size of each predicted ORF is indicated inside each rectangle, and the nt position of the start and stop codons are shown for each ORF. RNA2 encodes the 20 kDa capsid protein (CP); the triple gene block (TGB) ORFs (38K, 12K and 15K); and the 17 K cysteine-rich protein ORF

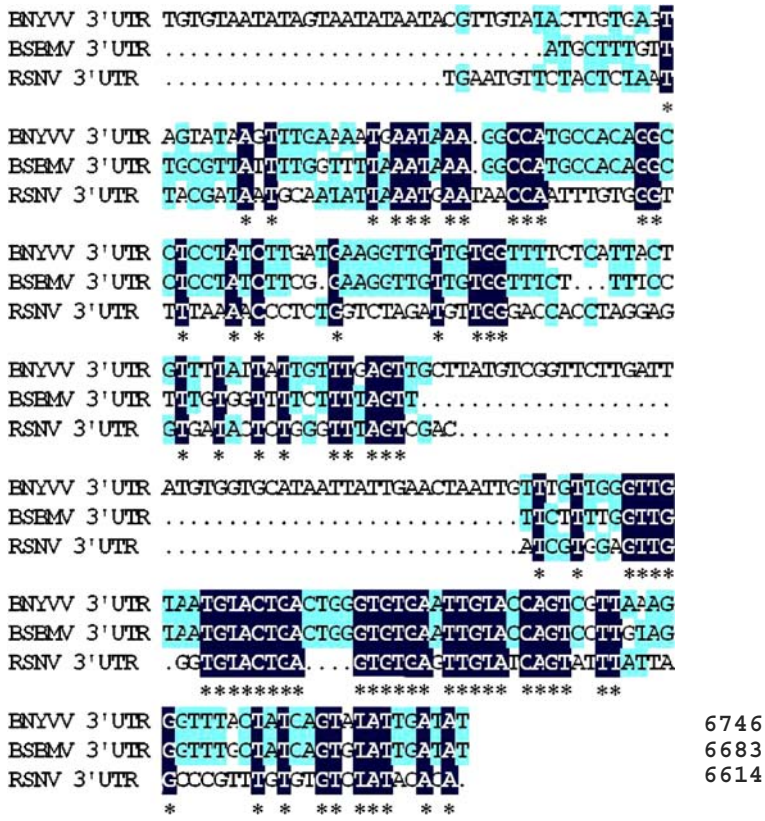

Fig. 3 Alignment of the 3′- untranslated terminal regions (UTRs) of BNYVV, BSBMV and RSNV RNAs 1. *Asterisks* indicate conserved nucleotides

sequenced in this investigation, produced sequences matching those reported for RNAs 3 or 4, found in plants naturally infected by either BNYVV or BSBMV; or RNA5, described for some BNYVV isolates (Lemaire et al. 1988; Quillet et al. 1989; Lee et al. 2001) .

Comparative phylogenetic analyses of the CP (Fig. 4a) and replicase (Fig. 4b) regions of RSNV and the corresponding ORFs of representative species of seven different genera of rod-shaped plant viruses, clearly show that RSNV is in a sister taxon located in the same node as the *Benyvirus* taxon conformed by the definitive benyvirus species BNYVV and BSNMV.

Discussion

The presence of a single ORF encoding putative replication-associated proteins (RdRp), as observed in BNYVV, BSBMV and RSNV, is a unique property of benyviruses (Fauquet et al. 2005). In the case of other rod-shaped viruses, the replicase is encoded in two ORFs (Koonin & Dolja 1993). The putative replicase motifs of RSNV and those of BNYVV and BSBMV, share a 67% aa sequence identity within the 500 C-terminal aa's surrounding the GDD region, but only a 39% identity in the 1100 aa's encompassing the methyl-transferase region. The RNA1 ORFs of these three viruses did not share any significant aa sequence identity with the same ORF or motifs of any other rod-shaped plant virus known (Table 1). The single RNA1 ORF of RSNV, as deduced by cDNA sequence analyses, initiates at the first AUG codon (position124) to produce a 236 kDa protein. This polypeptide contains the above-mentioned motifs, including the putative protease motif (Hehn et al. 1997). The six potential ORFs identified in the RNA2 molecule of RSNV, match the corresponding six RNA2 ORFs of the recognised benyviruses BNYVV and BSBMV.

Besides RNAs 1 and 2, both BNYVV and BSBMV have two or up to three (BNYVV) additional RNAs: RNA3–5 (Tamada et al. 1996; Lee et al. 2001). In the original report on the emergence of RSNV in Colombia, four distinct RNA molecules of approximate size 6,300 bp, 4,600 bp, 2,700 bp and 1,800 bp, were observed in 5% acrylamide gels of ds-RNA

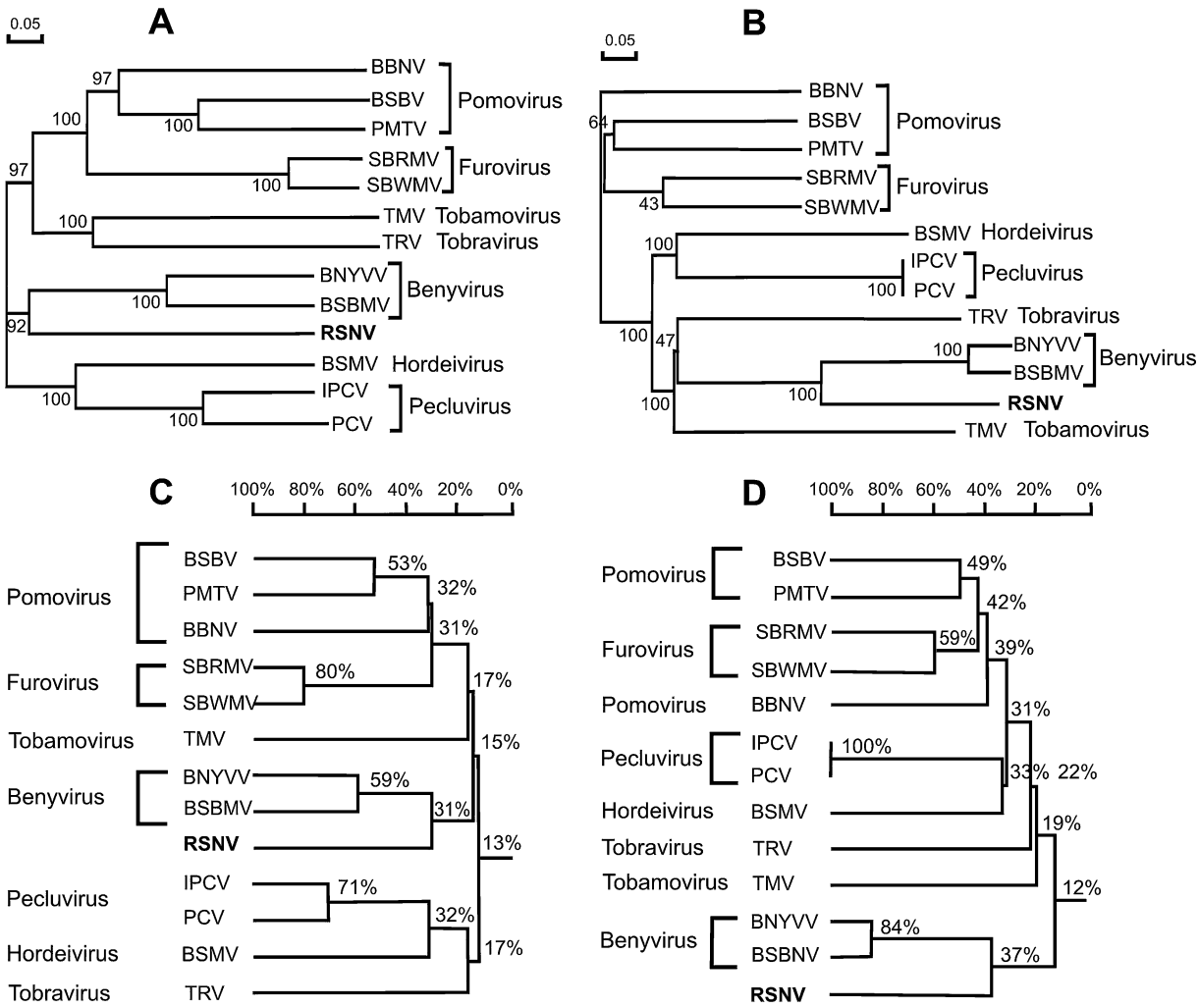

Fig. 4 Phylogenetic trees of the capsid proteins (**a**) and putative polymerases (**b**) derived from analyses of **a** the coat protein, and **b** the polymerases of rod-shaped plant virus genera, and percentage amino acid sequence identities between their respective capsid proteins (**c**) and polymerases (**d**). **Pomovirus:** *Potato mop top virus* (PMTV), *Beet soil-borne virus* (BSBV), *Broad bean necrosis virus* (BBNV); **Furovirus:** *Soil-borne rye mosaic virus* (SBRMV), *Soil-borne wheat mosaic virus* (SBWMV); **Tobamovirus:***Tobacco mosaic virus* (TMV); **Tobravirus:** *Tobacco rattle virus* (TRV); **Benyvirus:** *Beet necrotic yelloe vein virus* (BNYYV), *Beet soil-borne mosaic virus* (BSBMV); **Hordeivirus:** *Barley stripe mosaic virus* (BSMV); **Pecluvirus:** *Indian peanut clump virus* (IPCV), *Peanut clump virus* (PCV). Strict consensu trees were derived from the programme DNAMAN (Lynnon Bio/Scott). Values were obtained by bootstrap analysis (1000 replicates)

extracts obtained from RSNV-infected rice plants (Morales et al. 1999). Although the possible presence of sub-genomic RNAs in these gels cannot be ruled out, the third and fourth ds-RNA species observed for RSNV are within the size range expected for RNAs 3 and 4 of recognised benyviruses (Fauquet et al. 2005). However, none of the RSNV cDNA clones obtained in this investigation produced sequences showing any degree of similarity to the RNAs 3, 4 or 5 of BSBMV or BNYVV. Perhaps, as reported for the RNAs 3 and 4 of BNYVV, these molecules may be partially deleted or lost completely under certain experimental conditions (Lemaire et al. 1988; Bouzoubaa et al. 1991). Although BNYVV RNAs 3–5 have been shown to regulate the biological transmission and pathogenicity of this benyvirus, the lack of these RNAs does not seem to impede virus replication (Tamada et al. 1989; Rahim et al. 2007).

The phylogenetic and other comparative analyses of the CP and replicase of RSNV and other rod-

shaped plant viruses, conducted in this investigation, clearly demonstrate that RSNV is more closely related to the definitive benyviruses BNYVV and BSBMV than to any other rod-shaped plant virus, including those viruses associated with a plasmodiophorid vector (Fauquet et al. 2005). The relatively low aa sequence identities observed between RSNV and the two benyviruses compared at the CP (31%) and replicase (37%) levels (Fig. 4c and d), might reflect the different nature of these viruses (different primary hosts, specific plasmodiophorid vectors, and tropical vs temperate conditions), and the significant differences that also exist between BNYVV and BSBMV, particularly at the CP level (<60%). Additionally, the aa sequence identities between non-structural proteins of BNYVV and BSBMV range between 38–84% for the cysteine-rich and replication-associated proteins, respectively (Fauquet et al. 2005). A similar situation can be observed in the case of *Broad bean necrosis virus* (BBNV), considered a definitive species of the genus *Pomovirus* despite exhibiting significant differences at the CP and polymerase levels when compared to the type species of the genus, *Potato mop top virus* (PMTV), and another definitive pomovirus, *Beet soil borne virus* (BSBV), also shown in Fig. 4. Thus, based on the experimental evidence obtained in this investigation and the existing criteria for species demarcation in the different genera of rod-shaped plant viruses (Fauquet et al. 2005), we propose that RSNV should be considered as a distinct species of the genus *Benyvirus*.

Rice stripe necrosis continues to spread in Latin America due to the international trade of rice seed produced in fields contaminated with RSNV-carrying cystosori of the fungus vector, *P. graminis*. Besides Colombia, the Virology Research Unit of CIAT has also detected the presence of RSNV and its vector in Ecuador, Panama and Brazil. Whereas RSNV has not caused severe disease outbreaks in these rice-producing countries, as it occurred in 1991 in the eastern plains of Colombia (Morales et al. 1999), rice stripe necrosis remains a potential threat to rice production in the Americas. A search for sources of resistance to RSNV conducted at CIAT, has revealed the existence of few rice genotypes possessing moderate levels of disease resistance, and has confirmed the high levels of RSNV resistance found in the west African rice species *Oryza glaberrima.* Interspecific hybrids obtained from crosses between *O. sativa* x *O. glaberrima* have shown adequate levels of resistance to RSNV (Morales 2004). In the meantime, affected rice growers practice higher sowing densities to offset the loss of seedlings and mature rice plants to RSNV.

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