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An evolutionary analysis of Rice Tungro bacilliform virus collected from Odisha, India

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Rice tungro is one of the most severe diseases of rice (*Oryza sativa*) and is caused by joint infection of Rice tungro bacilliform virus (RTBV) and Rice tungro spherical virus (RTSV). The RTBV isolate collected from Odisha was analysed based on nucleotide sequence. The results indicated that the Odisha isolate belongs to the "South Asian type" with close nucleotide homology (about 95%) to Kanyakumari isolate and distant relation was found with "South East Asian type". Attempts were taken to detect possible recombination, if any among the Indian and South East Asian types of RTBV both in glasshouse and field samples, which revealed considerable changes in sequence between the two types, might have occurred in the segment of Philippine and Malaysian isolates. It is confirmed from the present analysis that the isolate from Philippines (South East Asian) type [RTBV-(PH) AF113831] would be the actual recombinant and originated from Indian types. The results clearly indicated that the isolate from Malaysia (RTBV-[MY] AF0764700 is the potential recombinant and originated from Philippine types which had close homology with the Thailand type.

Key words: *Oryza sativa*, multiple alignments, RTBV, recombination analysis, phylogenetic tree

INTRODUCTION

Rice (*Oryza sativa*) is the foremost food crop in the world and is the primary source of dietary calories for more than half of the world population. It is, thus, very important that its productivity is enhanced in the light of the increase in population pressure, especially in the areas of the world where rice is the primary food. Several biotic factors operate against rice in limiting its production, which include mainly bacteria, fungi, and viruses.

Rice tungro disease (RTD) is one of the most severe virus diseases of rice (*Oryza sativa* L.) and a significant threat to rice production in South-East Asia. In India, it causes an estimated loss of 2% (Muralidharan *et al.* 2003). RTD is a composite disease caused by joint infection of Rice Tungro Bacilliform Virus (RTBV) and Rice Tungro Spherical Virus (RTSV), which appear in the form of severe yellowing and stunting in susceptible rice cultivars.

Recombination among isolates of RTBV were investigated by Sharma *et al.* (2011), which revealed that the RTBV isolate from Kanyakumari had a high degree of identity to the two previously reported RTBV sequences from India. The study also noted a possible recombination in the Kanyakumari isolate of the virus from the RTBV isolates in the genomic region encompassing the coat protein gene.

Plant viruses make use of several mechanisms to produce large amounts of genetic diversity observed within and between species. RNA viruses and pararetroviruses infecting plants possibly contain highly error-prone replication mechanisms, which produce numerous mutations (Roossinck, 1997). Plant DNA viruses also display multiplicity. But the source of the same is not clear. The plant viruses exploit recombination and reassortment as dynamic forces in evolution, and, sometimes, other mechanisms such as gene copying and overprint-

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ing. The quantity of disparity found in different species of plant viruses is amazingly unusual, even though there is no proof that the mutation rate varies. The tendency for rapid adjustment makes tracing the evolutionary history of viruses intricate, and long-term management of virus disease seems tricky. This paper reports the nucleotide homology Odisha isolate of the rice tungro bacilliform virus (RTBV) has with the Kanyakumari and "South Asian type" and the recombination lineage of the same.

MATERIALS AND METHODS

The surveys were conducted during *kharif* (wet) season of 2014 at farmers' fields of rice-growing districts viz. Burdwan [Bhatar (23.4194^0 N, 87.9167^0 E), Memari (23.2000^0 N, 88.1200^0 E), Rasulpur (23.1840^0 N, 88.0301^0 E)], Hooghly [Panduah (23.0800^0 N, 88.2800^0 E), Mogra (22.97920 N, 88.3748^0 E), Dadpur (22.9600^0 N, 88.3000^0 E), Chinsurah (22.9000^0 N, 88.3900^0 E)], Nadia [Chakdaha (23.0800^0 N, 88.5200^0 E), Bethuadahari (23.6107^0 N, 88.3849^0 E)] and North 24-Parganas [Mondouri (23.6564^0 N, 88.2253^0 E)] of West Bengal. The samples were collected on the basis of tungro-like symptoms. The plant samples with tungro-like symptom were also collected from rice fields of NRRI, Cuttack, Odisha (20.4539^0 N, 85.9349^0 E) and brought to the laboratory at BCKV for indexing of RTBV.

The leaf samples and the stubbles from the suspected plants were indexed using a PCR-based method. All the leaf samples of the regenerated stubbles and the ratoon plants showing RTD-like symptoms were analysed by PCR against a standard DNA marker following standard protocol using kit of GeNei, Bangalore, India. The presence of RTBV was confirmed through Polymerase Chain Reaction (PCR) using viral genome sequence specific primers (Banerjee, 2010; Roy *et al.* 2011). The primers for RTBV were designed from the previously available sequences of both the viruses. The sequences of the West Bengal isolate of both the viruses were collected from NCBI database. For RTBV forward 5'AGATGAATCAGAAGAAGGATGG3' and reverse 5'AGAATCCCCTGAGGAATTCCATATCC3' primers were designed to cover a 1.1 kb region between 5444 to 6553 nucleotides (i.e., covering the parts of RT/RNAse H and ORF IV) of the RTBV genome.

The PCR amplicon fragment was bi-directionally sequenced commercially from Xcelaris Laboratory Ltd., Ahmedabad, India. The DNA sequences of PCR products of ~1.1 kb were evaluated using the BLASTn program from the NCBI website (www.ncbi.nlm.nih.gov). The genomic DNA sequence data of the present isolate was compared only to the complete sequences of published RTBV isolates. The multiple alignments of nucleotide and amino acid sequences were understood using Clustal W 2.1 programme and Darwin 6.0 version software. The sequence was compared with the RTBV nucleotide databases in NCBI blast for sequences producing alignments system.

The amplified PCR product of the RTBV partial codons was sequenced and analysis of the sequence was performed using Clustal W 2.1 and MAFFT 2.2.2.9 programs, and CLUSTAL W 5.2.2 version from MEGA 5 software and the phylogenetic tree was constructed using the neighbour-joining method and bootstrap with 1000 replicates. Six published data of the RTBV of South-Asian type and South East Asian types is provided in Table1. Pair-wise matrix based on percent nucleotide sequence identity of RTBV-Odisha isolate with other RTBV infecting rice were created by Clustal W 2.1 programme from EMBL database and nucleotides were matched. For recombinant analysis of the sequences Recombination Detection Programme version 2.29 and Bio Edit software were used.

RESULTS AND DISCUSSION

The length of the sequenced fragment of RTBV was 1.1kb and the base composition analyzed as A- 415, G- 209, C-196 and T-285. The sequence was compared with previously reported RTBV isolates from India i.e., West Bengal (22.5667^{0}N, 88.3667^{0}E), New Delhi (28.6353^{0}N, 77.2250^{0}E) and Kanyakumari (8.0883^{0}N, 77.5385^{0}E) and those of Thailand, Malaysia, and Philippine isolates. The amplified segment of Kanyakumari (IN_Kan/ HQ385226) isolate showed 95% similarity with WB isolate (IN_WB/ FN377814) at the position 5446 – 6554 nucleotides, encoding the terminal part of ORF III and initial part of the ORF IV along with small inter-genic region between ORF III and ORF IV and with Kanyakumari isolate (HQ385226) from 5444 to 6561 nucleotides. There were approximately 27 substitutions of nucleotides between the catch isolates, but there was no addition or deletion of nucleotide in the sequenced region. After nucleotide position 377 in our sequence, a stretch of 30 nucleotides was unique in both the present and previous WB isolates. These resulted in an

Table 1: List of RTBV isolates

Species/ Strain Name	Host Name	Isolate Name	Country	Abbreviation	GenBank Accession No.
Rice tungro bacilliform virus	*Oryza sativa*	Kanyakumari	India: Kanyakumari	RTBV-[IN:Kan]	HQ385226
Rice tungro bacilliform virus	*Oryza sativa*	Chinsurah, West Bengal	India: Chinsura, West Bengal	RTBV-[IN:WB]	FN377814
Rice tungro bacilliform virus	*Oryza sativa*	New Delhi	India: New Delhi	RTBV [IN:ND]	AJ292232
Rice tungro bacilliform virus	*Oryza sativa*	-	Philippines	RTBV- [PH]	AF113831
Rice tungro bacilliform virus	*Oryza sativa*	Chai Nat	Thailand	RTBV- [TH]	AF220561
Rice tungro bacilliform virus	*Oryza sativa*	-	Malaysia	RTBV- [MY]	AF076470

Table 2: Pair-wise matrix-based on percent nucleotide sequence identity of RTBV-Odisha isolate with other RTBV infecting rice were created by Clustal 2.1 programme from EMBL database

Identity Name / Accession No.	Virus	Odisha isolate	HQ385226	FN377814	AJ292232	AF113831	AF220561	AF076470
Odisha isolate	RTBV-[OD]	**						
HQ385226	RTBV-[IN:Kan]	99.73	**					
FN377814	RTBV-[IN:WB]	95.32	95.48	**				
AJ292232	RTBV-[IN:ND]	95.56	96.97	95.06	**			
AF113831	RTBV-[PH]	76.17	78.04	77.85	78.00	**		
AF220561	RTBV-[TH]	76.36	77.98	78.14	78.07	94.36	**	
AF076470	RTBV-[MY]	77.20	78.52	78.63	78.52	91.92	92.64	**

addition of 10 amino acid residues close to the C-terminus of ORF III in WB isolate.

The Odisha (20.2700^{0}N, 85.5200^{0}E) isolate was more similar to Chai Nat (15.1872^0 N, 100.1283^0 E) isolate of Thailand. Therefore, the comparison was made at nucleotide level between Odisha isolate and previously reported RTBV isolates from India in order to assess the extent of molecular homology of Odisha (20.2700^{0}N, 85.520^0)E) iso-

late. The partial sequence of present isolate showed 94-99% nucleotide sequence identity with the published data of the Indian isolates, where the recovery of the sequence identity was 45-100%. The partial sequence of RTBV of Odisha isolate was found distantly related to the Malaysia, Thailand and other Southeast Asian isolates (Table 2). The partial codons of the RTBV of Odisha isolate

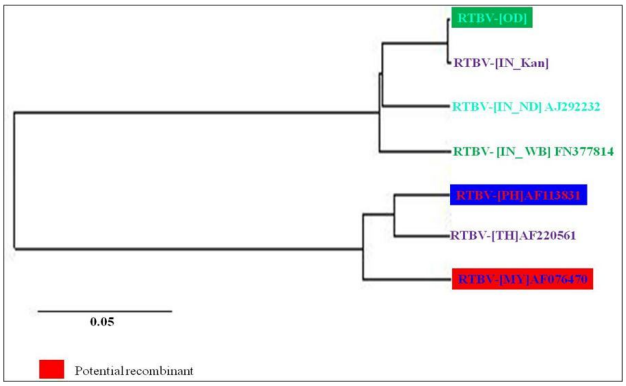

Fig. 1 : Phylogenetic relationship of RTBV-Odisha isolate, other Indian isolates and isolates from Philippines (AF113831)

was checked with BLASTn in NCBI and EMBL, compared with published sequences of RTBV. It was found that the sequence of the present isolate was the partial codons of ORF III of the RTBV genome and showed very close similarity of ORF III of the other isolates. The multiple alignment of

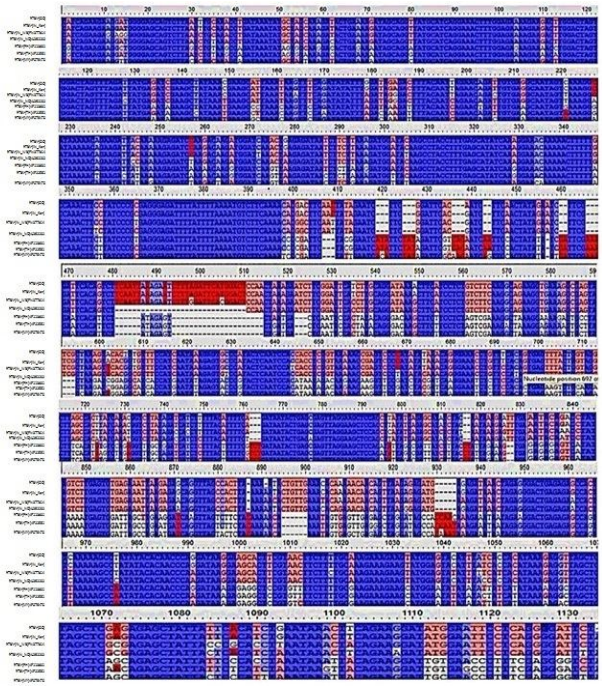

Fig. 2 : Multiple alignment of the RTBV Odisha isolate with South Asian and South-East Asian isolates. The alignment was generated using Recombination Detection Program version 2.29.

the partial sequences of our present isolate from Odisha showed that the nucleic acid sequence had close homology with the isolates of RTBV-[IN_Kan] HQ385226, RTBV-[IN_WB] FN377814 within the

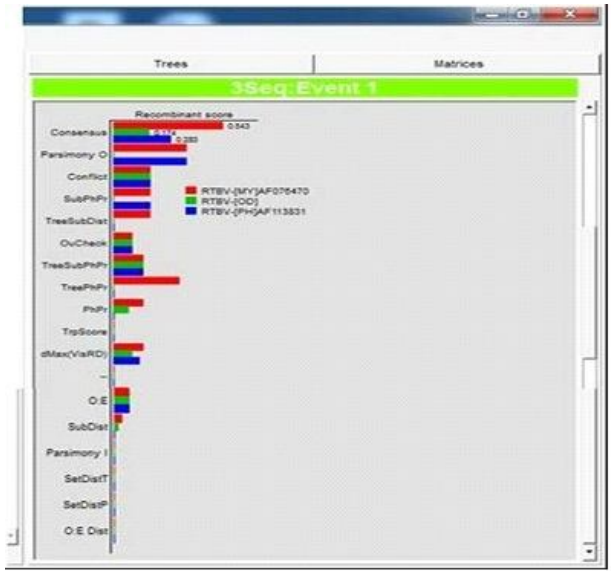

Fig. 3 : Relative distance of the RTBV Odisha isolate and RTBV-[MY] AF076470 (Malaysia) and RTBV-[PH] AF113831 (Philippines) and the recombination score

part of ORF III sequences and distantly related with the isolates viz. RTBV-[PH] AF113831, RTBV-[TH] AF220561 and RTBV-[MY] AF076470. However, it has been confirmed that the present Odisha isolate belongs to the South-Asian type of RTBV (Table 2). Clustal W 2.1 and MAFFT 2.2.2.9 analyses indicated that the RTBV isolate from Cuttack, Odisha was similar to the Kanyakumari (8.0780^{0}N, 77.5410^{0}E) (HQ 385226), AJ 314596, EU684541, FN377814 and AJ292232 isolates of RTBV, where the E-value was 0.0 and had very close similarity. But the nucleotide sequence was distantly related with Malaysia (3.1333^{0}N, 101.7000^{0}E), Thailand, Philippine isolates as found in Clustal W 2.1. The phylogenetic tree was generated with RTBV-Cuttack (Odisha) isolate and Kanyakumari (TN), Chinsurah (West Bengal), the Philippines, Malaysia and Thailand isolates of RTBV for comparison of nucleotides (Fig.1).

The sequences were aligned using CLUSTAL W 5.2.2 version from MEGA 5 software and the phylogenetic tree was constructed using neighbour-joining and bootstrap with 1000 replicates. Vertical distances were arbitrary and horizontal distances were proportional to genetic distances. The bootstrap numbers at each node indicated the percentage at which the grouping occurred (shows

only when >50%). The phylogenetic tree gave two major clads, where the Indian isolates were in one clad and the RTBV isolates from South-East Asia were in another, which were significantly separated from the Indian isolates. It is, however, revealed from the cluster that Indian isolates are South Asian types, which are distantly related to the South-East Asian types of the Philippines, Malaysia, and Thailand. The pair-wise matrix was generated using the Odisha isolate and other South Asian and South-East Asian isolates using Clustal W 2.1 program from the EMBL databases. There was an evidence of only 76.17, 76.36 and 77.20% similarity of Odisha isolate when compared with the Philippines, Thailand and Malaysia isolates whereas the Odisha isolate was very close to the Kanyakumari isolate (99.73%) India, New Delhi isolate (95.56%) India and Chinsurah isolate (95.32%) India. The preliminary investigation of the partial sequence of the RTBV confirmed that the Indian isolates of RTBV have a close homology in nucleotides of ORF III gene regions and conserved in majority of its part rather than the nucleotides of the Southeast Asian isolates.

Multiple Alignment of the Odisha isolate of RTBV

The 1.1kb partial sequence of RTBV, Odisha isolate was compared with the published data of the Kanyakumari, New Delhi, Chinsurah isolates (South Asian type) and the Philippines, Malaysia, and Thailand (South-East Asian) isolates using the Clustal W 2.1 and Recombination Detection Program version 2.29. The sequence alignment showed that a major part of the 5'-end of the sequence was conserved in all the isolates and marked differences were noticed in the 3'-end region. Several deletion and substitution of nucleotides was found between the South-Asian and South-East Asian isolates. It was interesting to note that a small nucleotide position between 15-29, 114-122, 336-347, 778-794, 950-960, 1074-1084 were unique in all the Indian and South-East Asian isolates; the conserved regions of these nucleotides showed a specific feature of the RTBV isolates compared here. The alignment of nucleotides also predicted that a large region (80-104, 306-328, 366-397 nucleotide position) had close homology which could be the specific functional region of RTBV isolates among the South Asian and South-East Asian isolates of RTBV (Fig. 2). It was found that there marked alterations and substitutions of nucleotide between the South Asian (Kanyakumari, New Delhi and Chinsurah, India isolates) and South-East Asian (The Philippines, Malaysia, and Thailand) isolates. The nucleotide regions ranged 481-510, 895-901, where a major deletion of nucleotide in the Thailand, Malaysia and Philippines isolates occurred but interestingly the positions of nucleotides in 425-428, 418-422, 435-438, 929-935 had minor and major deletions in our present Odisha, India and Kanyakumari, New Delhi and Chinsurah, India isolates as compared to South-East Asian isolates. However, the multiple alignment of the RTBV-[OD], RTBV-[IN_Kan], RTBV-[IN_WB]FN377814, RTBV-[IN_ND]AJ292232, RTBV-[PH]AF113831. RTBV-[TH] AF220561, RTBV-[MY] AF076470 showed some unique features of the isolates. The positions of nucleotides ranging 81-103, 306-328, 366-397 and 778-798 were conserved regions among the isolates (Annexure-1). The present findings point out the marked similarity with previously reported Kanyakumari and Chinsurah, India isolates and the important difference with the Philippines, Malaysia and Thailand isolates (Fig.2).

Previously all the RTBV isolates from within the Indian subcontinent were designated as "South Asian" type (Fan *et al*. 1996). But Nath *et al.* (2002) reported variations among the Indian isolates (WB and AP isolates) were primarily due to the presence of a hypervariable region in ORF III. The present findings point out the marked similarity with previously reported WB isolate. The Odisha (20.2700^{0}N, 85.5200^{0}E) isolate was more similar to Chai Nat (15.1872^0 N, 100.1283^0 E) isolate of Thailand. Therefore, the comparison was made at nucleotide level between Odisha isolate and previously reported RTBV isolates from India in order to assess the extent of molecular homology of Odisha (20.2700^{0}N, 85.5200^{0}E) isolate.

Roy *et al.* (2011) sequenced 1.1kb fragment from Chinsurah isolate, and reported that 95% similarity with previously reported WB isolate (EMBL accession No. AJ314596) at the nucleotide position 5444-6554 nucleotide (nt), encoding the terminal part of ORF III and initial part of the ORF IV along with small intergenic region between ORF III and ORF IV. These resulted in an addition of 10 amino acid residues close to the C-terminus of ORF III in the old WB isolate. Previously, all the RTBV isolates from within the Indian subcontinent were des-

ignated as “South Asian” type (Fan *et al*. 1996). But Nath *et al*. (2002) reported variations among the Indian isolates due to the presence of a hypervariable region in ORF III. ORF III (P3) is a large poly protein of 194 kDa. Sequence comparison with retroviral and other para-retroviral proteins suggest that P3 contains domains corresponding to the movement protein (MP), coat protein (CP), as partate protease (PR), reverse transcriptase (RT) and ribonuclease H (RNaseH), ordered from the 5- to the 3-terminus (Hay *et al*., 1991; Qu *et al*., 1991; Laco and Beachy, 1994; Tzafrir *et al*., 1997). Thus, this is a poly-protein containing the analogous of the *gag* and *pol* genes of retroviruses (Hull, 1996). The cell-to-cell movement protein has been suggested from amino acid sequence alignments (Bouhida *et al*. 1993; Hagen *et al*. 1993) and the 5-proximal 300 amino acids of ORF III are characteristically located in the MP regions. But difficulties remain to rationalize a mesophyll cell-to-cell movement function with the phloem limitation of the virus (Hull, 1996).

However, as evidenced from the sequence alignment that there was no addition or deletion of nucleotide in the sequenced region of Kanyakumari isolate but very little substitution was found when compared with other Indian isolates. Previously, all the RTBV isolates from within the Indian subcontinent were designated as “South Asian” type (Fan *et al*. 1996).

Recombination analysis

The isolates of Indian subcontinent and South-East Asia were compared to find out any possible recombination among the isolates *viz*. RTBV-[OD], RTBV-[IN_Kan], RTBV- [IN_ WB] FN377814, RTBV-[IN_ND] AJ292232, RTBV-[PH] AF113831, RTBV-[TH] AF220561, RTBV-[MY] AF076470. Analysis on the basis of the partial sequence of the Odisha isolate using Recombinant Detection program version 2.29 generated a unique feature among the isolates; the highest recombination score (0.543) was in Malaysian isolate which was close to Philippines isolate (0.283) compared to our present isolate (0.174) on the basis of the consensus sequences (Fig.3). The isolate from Philippines has a distinctive molecular character and completely different type but a major part is highly identical with the isolate from Malaysia; it is, therefore, hypothesized that Malaysian type of RTBV might have been originated from the Philippine isolate. The major parental type has been found in Philippine isolate and the Odisha isolate was a minor parental type and highly deviated from Philippine isolate, where high P-value was scored on the basis of the selective positions of the nucleotide sequences. The isolate from Philippines (South East Asian) (RTBV-[PH] AF113831) was confirmed as the actual recombinant that originated from Indian types. The results clearly indicated that the isolate from Malaysia (RTBV-[MY] AF0764700) was the potential recombinant and originated from Philippine types which had close homology with the Thailand type.

In an earlier report, analysis of the nucleotide sequences of six isolates of RTBV carried out at the International Rice Research Institute indicated recombination with a hypothetical parental sequence, which was at that time, uncharacterized (Cabauatan *et al*. 1999). The present study provides more direct evidence of recombination in RTBV, with the precise identification of the parental sequences and cross-over points. Sharma *et al.* (2011) reported that RTBV isolate from Kanyakumari (RTBV- KN) has a high degree of distinctiveness to the two previously reported RTBV sequences from India, RTBV-WB, which had been collected from field locations about 10 years ago and 1000–2000 km away from the collection site of RTBV-KN. Most of the sequence domains reported earlier in other RTBV isolates were found to be conserved in RTBV-KN (Sharma *et al*. 2011). Chen *et al*. (2014) opined that most of the endogenization events of the eRTBVLs were initiated before delineation of the rice progenitor (> 160,000 years ago).

The recombination events reported for the six isolates of RTBV from the Philippines were predicted to be the result of inter-genomic strand-switching during reverse transcription because the cross-over points correlated with the template switch sites (Cabauatan *et al*. 1999). The cross-over points identified for the recombination event of KN did not match with the site of template switching 600 bp upstream of the D1 discontinuity on the negative strand with its 5’ end mapped at nucleotide 1 and the 3’ end between 7980 and 7998 nucleotides for Phi1lipines (Bao and Hull, 1992). Therefore, this recombination event can be hypothesized to be the result of homologous DNA recombination among Indian isolates, as the cross-over points in such cases are found distributed randomly all along the

viral genome. A recent report of the simultaneous presence of both tomato yellow leaf curl Sardinia virus as well as tomato yellow leaf curl virus genomic DNAs in a single nucleus of an infected cell support this hypothesis (Morilla *et al*., 2004). It is important to have recent data as reported by Sharma *et al*. (2011) that on the extent of variability of RTBV in India, as it can help in making decisions regarding the resistance strategies to be used against tungro. Comparison of sequences of RTBV indicated that isolate from Kanyakumari had a high degree of identity to the previously reported RTBV sequences from India, which had been collected from field locations about 10 years ago, 1000–2000 km away from the collection site of Kanyakumari. Most of the sequence domains reported previously in other RTBV isolates were found to be conserved in Kanyakumari isolate. Nucleotide sequence data mentioned in the research article are available in the GenBank sequence database under accession number HQ385226.

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