Malaysian Journal of Microbiology, Vol 14(4) Special Issue 2018, pp. 309-312
DOI: http://dx.doi.org/10.21161/mjm.144182

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Molecular characterisation of rice tungro bacilliform virus isolated from Bario, Sarawak

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ABSTRACT

Aims: Rice tungro disease is one of the most damaging and destructive diseases of rice in South and Southeast Asia. The disease is caused by the co-infection of two viruses, the Rice tungro bacilliform virus (RTBV) and Rice tungro spherical virus (RTSV). The symptoms and severity of the disease depend on these two viral agents, if rice is co-infected by both viruses, it will show the typical severe symptoms of yellow-orange leaf discoloration, plant stunting and reduced in yield. On the other hand, if rice is infected only with RTBV, it shows milder symptoms and in contrast, rice plants will show no symptoms if they are infected only with RTSV. The disease had been detected in Malaysia since the 1930s. However, the first incursion of the disease was only reported in Sarawak in 2012. Since the disease was not seen in the Sarawak until recently, very little information on local virus isolate is available. This study was conducted to obtain and record the nucleotide sequence of partial coat protein gene of two primary isolates of RTBV collected from Bario, Sarawak in 2012 and 2013.
Methodology and results: Based on the phylogenetic analysis, the isolates cluster with the Southeast Asia group with sequence identity at nucleotide and amino acid level of 91.1 to 95.1% and 98.6 to 99.5% respectively.
Conclusion, significance and impact of study: This study provide the first genetic information on RTBV isolates from Sarawak. This data is important for future reference of the virus variants and diversity for epidemiological and diagnosis purposes.

Keywords: Tungro, RTBV, isolate, Sarawak

INTRODUCTION

Rice tungro bacilliform virus (RTBV) is a plant *pararterovirus* in the genus *Tungrovirus* of the family *Caulimoviridae* (Bousalem *et al*., 2008). RTBV together with Rice tungro spherical virus (RTSV) caused rice tungro disease (RTD) (Hibino *et al*., 1978) in rice plants grown in South and Southeast Asia (Ling, 1972; Azzam and Chancellor, 2002). Symptoms of RTD are caused by RTBV (Hibino *et al*., 1978) but RTBV can only be transmitted into host plant by green leafhopper (*Nephotettix* spp.) in the presence of RTSV (Hibino, 1983).

RTBV has a circular double-stranded deoxyribonucleic acid (dsDNA) of about 8 kb and one site-specific discontinuity in each strand (Bao and Hull, 1993). The genome has four open reading frames (ORFs) namely ORF1, ORF2, ORF3 and ORF4 (Hay *et al*., 1991). Only ORF2 and ORF3 have known functions. ORF2 encodes a protein that is involved in capsid protein assembly (Herzog *et al*., 2000) while ORF3 encodes movement protein (MP), coat protein (CP), aspartate transferase (AT) and reverse transcriptase/ ribonuclease H (RT/ RNase H) which are involved in virus replication and assembling (Hay *et al*., 1991; Qu *et al*., 1991; Laco *et al*., 1995; Marmey *et al*., 1999).

Rice tungro disease (RTD) was first reported in Sarawak in 2012 (Yee and Eng, 2013) but no nucleotide sequence of RTBV circulating in this region is available. Although RTD had been detected in other parts of Malaysia as early as 1930s, not much genetic information of the viruses is available. The sole RTBV complete genome from Malaysia deposited in NCBI GenBank is of the isolate from Serdang which was reported in the 90s (Marmey *et al*., 1999). Therefore, this study was conducted to obtain and record the partial CP gene sequence of RTBV from Sarawak to study the virus diversity in East Malaysia.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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DOI: http://dx.doi.org/10.21161/mjm.144182

MATERIALS AND METHODS

Samples

Leaves of rice plants infected with RTBV circulating in Bario, Sarawak collected during the main planting seasons of 2012 and 2013 were used in this study. Two samples, one from each planting season, tested positive for RTBV using modified methods by Dasgupta *et al.* (1996) were selected for sequencing. Partial CP gene generated from RTBV primary isolates in this study were designated as MQ 2012-11-Bario (sample collected in 2012) and MQ 2013-5-Bario (sample collected in 2013).

Nucleic acids extraction and RTBV CP gene amplification

Three pieces of 1 cm^2 of infected rice leaf were ground with liquid nitrogen and homogenised in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). Total nucleic acids were then extracted from the homogenized leaf sap using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany). Partial RTBV CP gene was amplified in a reaction containing 1X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTPs mix (Thermo Scientific), 2.5 units of *Taq* DNA polymerase (Thermo Scientific), 20 pmol of forward primer (5´-TCAGAAATAAGGCCAACCAAACGAC-3´) and 20 pmol of reverse primers (5´-AGCCTGATTGGTATATCTTCTAGGGCATCT-3´) with 2 µl of extracted nucleic acids. Amplification was done with initial denaturation at 94 °C for 5 minutes followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 54 °C for 1 minute and extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes.

Sequencing and data analysis

Amplified products were purified using QIAquick gel extraction Kit (Qiagen, Germany). Fifty to one hundred nanograms of purified templates were then subjected to sequencing amplification using Big Dye Terminator v3.1 (Applied Biosystem, CA, USA). The obtained sequencing products were electrophoresed in a DNA sequences, ABI 3130 Genetic Analyzer (Applied Biosystem, Hitachi, Tokyo, Japan) after removing unincorporated dye terminator by precipitation using Dynabeads® Magnetic Beads (Life Technologies, UK) and BigDye® Sequencing Clean Kit (MCLAB, USA). Nucleotide sequences generated were assembled and analysed using EditSeq, MegAlign and SeqMan programs in Lasergene software from DNASTAR Inc. (Madison, WI, USA). Assembled RTBV CP nucleotide sequences were aligned with CP gene sequences of RTBV isolates and a badnavirus retrieved from NCBI GenBank database for construction of a phylogenetic tree of partial CP gene and on the basis of alignment with 1000 bootstrap replicates using the Molecular Evolutionary Genetics Analysis software (MEGA) version 5.05 (Tamura *et al*., 2011).

RESULTS AND DISCUSSION

RTBV isolates are grouped according to their geographical distribution based on their nucleotide sequences into South Asia (SA) and Southeast Asia (SEA) clusters (Fan *et al*., 1996; Banerjee *et al*., 2011). For the purpose of this study, nucleotide sequences of CP gene of RTBV from 25 isolates representing both clusters were retrieved from NCBI GenBank for the phylogenetic analysis. Five isolates from India represent the SA cluster and the remaining 20 isolates from Malaysia (n = 1), Thailand (n = 3), the Philippines (n = 6) and Indonesia (n = 10) represent the SEA cluster. All partial CP gene sequences were first aligned using ClustalW method and the phylogenetic tree was constructed using the neighbour-joining tree algorithm (Saitou and Nei, 1987) based on bootstrap resampling of 1000 replicates (Felsenstein, 1985). CP gene of *Banana streak OL virus* (BSOLV) from the genus *Badnavirus (*Bousalem *et al.,* 2008*)* was used to out root the phylogenetic tree constructed.

Table 1: Comparison of the nucleotide and amino acid of the Bario isolate to the isolates of South and Southeast Asia clusters.

Sample	Nucleotide identity (percentage)		Amino acid identity (percentage)	
	South Asia	Southeast Asia	South Asia	Southeast Asia
MQ 2012-11-Bario	83.7-84.9	92.1-95.1	88.3-89.2	98.2-99.1
MQ 2013-5-Bario	82.9-84.7	91.9-94.8	88.3-89.2	98.6-99.5

Based on the analysis both of the isolates from Bario were identified as members in the SEA cluster (Figure. 1). The primary isolates shared 91.1 to 95.1% similarities at nucleotide level, and 98.2 to 99.5% similarities at amino acid level with SEA isolates. These sequence identities were almost ten percent higher compared to SA isolates (Table 1). However, it is interesting to note that the two primary isolates are grouped into two different sub-clusters within the SEA cluster. The isolate MQ 2013-5-Bario, is grouped in the similar sub-clustering with the isolates from the Philippines (AF 113830 Phi 1994 Phi-G1 and AF113832 Phi 1994 Pbi Ic), while the isolate MQ 2012-11-Bario, sub-cluster with the isolate from Serdang as well as isolates from Thailand. The two isolates from Bario, only share 93.9 % similarities at nucleotide levels which may explain the different grouping of the two isolates in the SEA cluster.

It has been reported that the RTBV populations in fields may comprised of multiple variants and the diversity increases in an endemic area. One variant may be dominant and easily detected compared to another. A slight change in the dynamic of RTBV populations due to pressure in the environment and green leafhopper populations can lead to an outbreak (Arboleda and Azzam, 2000; Azzam *et al.,* 2000).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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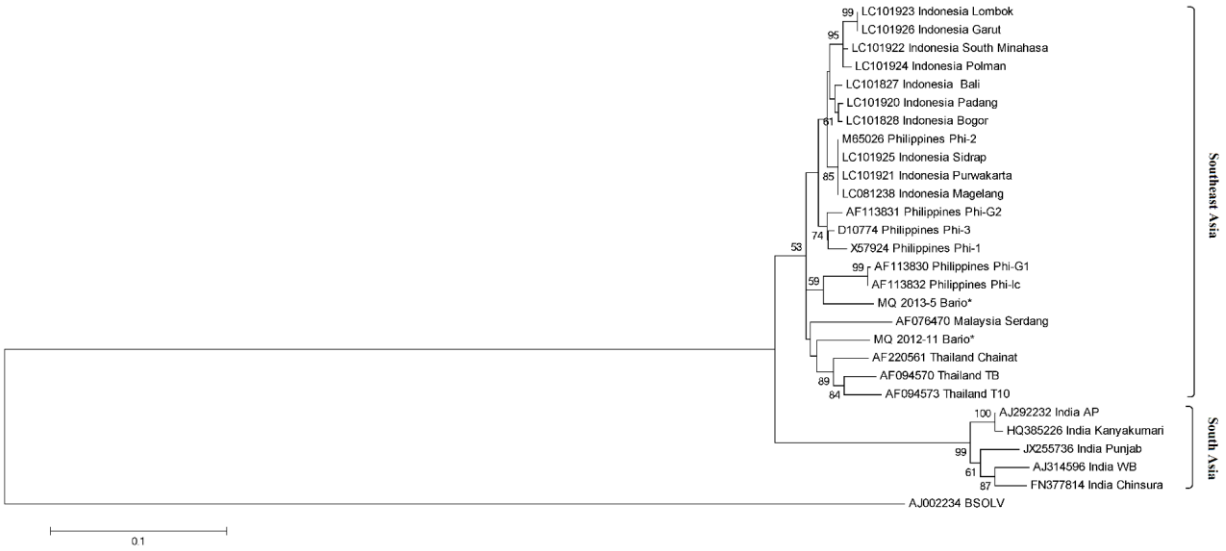

Figure 1: Phylogenetic tree of Bario ioslates. Phylogenetic analysis showing the genetic relationships between RTBV primary isolates from Bario, Sarawak and other RTBV isolates from SA and SEA available from NCBI GenBank database. The tree was constructed based on partial CP gene and on the basis of alignment with 1000 bootstrap replicates. The RTBV CP tree was rooted with Banana streak OL virus (BSOLV), a badnavirus in the same family with RTBV. Bootstrap numbers at each node indicate the percentage at which the clustering occurred (shown only when more than 50%). Scale bar represents genetic distance (substitution per nucleotide). Sequences generated in this study indicated by *.

To date, there are no information on how and when the tungro inoculum was introduced in Bario. It may have been recent or was introduced much earlier but was unnoticed because no major damages were reported. There may even be more than one variants of RTBV circulating in Bario as implied by the results of this study. However, the data available to date is insufficient to determine the occurrence of dynamic changes of the RTBV populations in Bario. Therefore, continued collection of isolates and further genetic analysis of RTBV genome from East Malaysia is important to understand the diversity of the virus populations in order to formulate ways to prevent and manage RTD outbreak, especially in important rice growing area, such as Bario.

CONCLUSION

In conclusion, to date, this is the first molecular characterization study of RTBV isolates from Sarawak. Since tungro disease was only recently detected in this state, genetic data on the viruses is important for epidemiological, evolutionary point study and diagnosis purposes.

ACKNOWLEDGEMENTS

This study was funded by Universiti Malaysia Sarawak (MyRA special Grant: I02/SpGS/1402/16/3) and supported by DOA Sarawak. We would like to thank the staffs of Agrobiotechnology Branch and Plant Pathology Section, DOA Sarawak for their assistance in collecting the field samples.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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