Collection of Rice Tungro-Infected Plants from Hotspots in the Philippines

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ABSTRACT

Tungro is one of the major rice pests causing as much 100% yield loss when unaddressed. Due to the onset of climate change and reports of breakdown of resistance, a study was designed to collect plants infected by tungro in selected tungro hotspots in the Philippines. Collection of tungro-infected plants were conducted in nine provinces: Isabela, Nueva Ecija, Laguna, Albay, Camarines Sur, Camarines Norte, Negros Occidental, Bukidnon, and North Cotabato. A total of 400 suspected RTD-plants were collected. A total of 311 nucleic acid sequences (201 RTBV and 110 RTSV) were obtained and BLAST data yielded 83% to 100% identity with existing databases. Some sequences matched with RTBV strain RTBV-Ic, complete genome, RTBV isolate TB P194 gene, partial cds and some foreign isolates. Most of the samples were collected in Luzon while Mindanao had the least number of samples in terms of collected tungro-infected plants and the RNA extracted since it is highly dependent on the freshness of the plants. In the long run, knowledge on the genetic variation present in these organisms will help in disease management and development of rice tungro disease-resistant varieties.

Keywords: breakdown, crop protection, nucleotide sequence, rice tungro bacciliform virus, rice tungro spherical virus

INTRODUCTION

Rice production is devastated with rice tungro disease (RTD) which effectively affects rice plants in all growth stages and could cause as much as 100% yield loss and occurrence has been recorded worldwide. Azzam and Chancellor (2002) reported occurrence of severe epidemics of RTD about 50 years ago which resulted to major production losses in India, Malaysia, Philippines and Thailand. However, it was only in 1963 when the first reported occurrence of rice tungro disease in the experimental farm of the International Rice Research Institute (IRRI), Los Baños, Laguna and after two years the transmission vector was identified as the green leafhopper which is known as *Nephotettix virescens* (Rivera and Ou, 1965). Since then, the disease has since been recognized as a significant negative factor in rice production. It spreads rapidly and occurs all over the country where major outbreaks of tungro and heavy losses of grain yield have been reported repeatedly. Two viruses that differ in morphology and genome are the causal agents of RTD: rice tungro bacilliform virus (RTBV) with circular double-stranded DNA (Hull, 1996) and rice tungro spherical virus (RTSV) with a positive sense polyadenylated single-stranded RNA (Saito et al., 1976). RTBV causes the symptoms on a susceptible rice cultivar, and its transmission is dependent on the help of RTSV while their combined infection causes the typical disease symptoms such as stunting, discoloration of the

leaves, reduced tillering and small and/or sterile panicles (Hibino et al., 1978; Dasgupta et al., 1991).

Knowledge on the genetic variation present in the two RTD viruses in the Philippines could provide relevant information that could be used in disease management. It was observed that the composition and structure of virus population is not stable, and its genotype differ significantly over time, so by continuously monitoring virus populations, we can better understand and identify the factors why there is tungro outbreak or extinction of the current prevailing tungro virus populations and by this, it may lead for us to achieve durable virus resistance genes and varieties of rice.

Previous researches in rice tungro disease studies had used two approaches in differentiating isolates from each other: (1) characteristic symptoms produced in differential varieties, and (2) enzyme restriction patterns detected from the virus itself. These were the technologies that were available during their execution. In contrast, the proposed work will use a different approach that is more relevant to today's capabilities in genomics: direct comparison of nucleotide and protein sequences of the virus. Comparing the DNA sequences at a given locus affords a more powerful and informative method of reconstructing the phylogenetic relationships of different test entries. Secondly, it is important to monitor populations of the pathogen periodically to determine if they change over time. The reports enumerated above were made in the 1990s, so it is high time to sample the different populations again and make comparisons with previous findings. In addition, the proposed work aims to sample from more provinces in the Philippines to provide a more extensive picture in the end. Whereas Arboleda et al. (1999) sampled RTBV isolates from two provinces (Isabela and North Cotabato, and Umadhay et al. (1999) sampled RTSV isolates from three places (Nueva Ecija, Bicol, and North Cotabato), the proposed work will sample both RTBV and RTSV populations from six to ten provinces representing five or more regions such as: Isabela (Region II), Nueva Ecija (Region III), Albay (Region V), Negros Occidental (Region VIII), Zamboanga del Sur (Region IX), and North Cotabato (Region XII) among others.

The study aimed to collect tungro-infected rice plants from tungro hotspots in the Philippines. Specifically, the study aimed to: a) obtain insights in rice tungro disease incidence in tungro hotspots in the Philippines and b) confirm using PCR-based method the presence of coat protein genes of RTBV and
RTSV.

MATERIALS AND METHODS

Plant Collection

Rice tungro-infected plants were collected in several rice growing regions with recorded high incidence of rice tungro disease in the past years. Common symptoms of tungro disease (i.e., stunted growth with leaf yellowing) were used to select putatively infected plants in the field. Plants that were collected were mostly at tillering to booting stages in at least one municipality of nine major rice growing provinces in the Philippines, namely: Isabela (II), Nueva Ecija (III), Laguna (IVA), Albay (V), Camarines Sur (V), Camarines Norte (V), Negros Occidental (VI), Bukidnon (X), and North Cotabato

(XII). The global coordinates of the sampling sites were recorded with Sony SLT-A65V digital camera (Sony Corp., Tokyo, Japan). Collected plants were put in a double-walled glass box with cover and cleaned before sealing and transport following the recommendation from quarantine procedures.

Detection of RTBV and RTSV Homologous Sequence

Genomic DNA was extracted from leaf tissue showing rice tungro disease symptoms using CTAB method. Total RNA was isolated using Qiagen RNeasy Plant Mini Kit following the manufacturer's instruction (Qiagen, Valencia, CA). Copy DNA (cDNA) of coat protein genes of RTSV was synthesized using gene-specific primers aided by Qiagen One-Step Reverse Transcription-PCR kit (Qiagen, Valencia, CA). Diluted genomic DNA was used for RTBV detection while the total RNA the detection of RTSV with both using gene-specific primers. Publicly available gene-specific primers were used that corresponded to genomic regions of viral coat protein. PCR components included 6.5 µL sterile distilled water, 1.5 µL 10X PCR buffer, 1.0 µL $MgCl_2$, 1.0 µL dNTPs, 1.0 µL each primer, 0.1 µL i*Taq* (Intron) and 3.0 µL DNA template. PCR of DNA template-based was carried out using T100 PCR (Biorad) under the following conditions: 1) initial denaturation at 94°C for 5 minutes; 2) 36 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 2 minutes, and elongation at 72°C for 3 minutes; 3) final extension at 72°C for 7 minutes. For RTSV detection, the PCR components included 6.25 µL RNAse-free water, 2.5 µL 5X PCR buffer, 0.5 µL dNTPs, 0.75 µL each primer, 0.5 µL Qiagen RT-PCR Enzyme and 2.5 µL RNA template. PCR of RNA template-based was carried out using T100 PCR (Biorad) under the following conditions: 1) heating up at 50°C; 2) initial denaturation at 94°C for 5 minutes; 3) 36 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 2 minutes, and elongation at 72°C for 3 minutes; 4) final extension at 72°C for 7 minutes. PCR products with positive RTBV- and RTSV coat protein gene amplicons were purified using QIAquick® Purification Kit following the manufacture's specification (Qiagen, Valencia, CA). PCR amplification products will be resolved by horizontal gel electrophoresis using mini-vertical electrophoresis system (CBS Scientific). Five µL of each amplified DNA product will be separated on 1.5% agarose gels in 1X Tris-acetate EDTA buffer for 2-3 hours at 150-160V. It will be stained with ethidium bromide and will be viewed using gel documentation machine in the laboratory to check the quantity and quality if the PCR product. Purified PCR products were sent for sequencing in 1st Base for single pass sequencing (First Base, Selangor, Malaysia). Raw sequence trace files of forward and reverse primer reads were processed with the Pregap4 module of the Staden Package (Bonfield and Staden, 1996) to clip base calls with poor quality at the ends of sequence reads, and to infer the consensus sequence. The consensus sequences from different genotypes were aligned using the MAFFT (Multiple Alignment using Fast Fourier Transform) alignment procedure (Katoh et al., 2005) in Jalview (Waterhouse et al., 2009).

BLAST Analysis

Representative samples from each province was queried against the GenBank on-redundant nucleotide collection (nr/nt) using nucleotide BLAST (basic local alignment search tool). This validated the sequences were indeed amplified from the CP1 gene of RTBV and CP3 gene of RTSV. Default search parameters on the standard nucleotide BLAST (blastn) web interface were used (NCBI, USA).

RESULTS and DISCUSSION

The collection of rice tungro-infected plants was conducted in several tungro hotspots in the Philippines based on historical occurrence. Moderate to severe rice tungro disease occurrence for

Murcia, Negros Occidental; Calabanga and Pili in Camarines Sur; and in Quezon, Isabela (Figure 1). Symptoms ranged from typical yellow-orange leaves coupled with stunted growth to yellowing. Most of the collection were timed at the maximum tillering stage and each visited municipalities practice asynchronous planting. This observation could have been the cause of continuous cycle of tungro disease. Convenience sampling with representative plants per plot in the infected sites were followed.

High tungro disease incidence was observed in Isabela for Luzon, Negros Occidental for Visayas and in North Cotabato for Mindanao. Based on RT-PCR analysis, most of the plants contained RTBV and very few contained RTSV. The quantity of detected RTBV is almost double of the RTSV (Table 1).

Figure 1 showed a representative agarose gel electrophoresis with lanes confirming presence of RTBV and those lanes without amplifications. The presence of RTBV and RTSV coat protein homologous were detected using PCR-based detection method and later on confirmed by single-pass sequencing result showed.

In 1990, there was a study by Bottenberg on the use of a line transect survey method for rice tungro disease. They investigated the potential of a new sampling method that is sensitive to low incidence levels of tungro, the major virus disease of rice in South-east Asia, yet can cover large rice areas in a relatively short period. At low incidence levels, spatial distribution of tungro was assumed not to be related to proximity to rice bunds. More reliable results can be obtained with longer transect lines and precise measurements of distance. In compliance to the basic assumptions of line transect theory, scanning effort should be intense along the rice bund and decrease with distance into the field. In this paper, we reported a larger coverage of survey by selecting the most severe tungro disease incidence in a municipality. The idea is to collect a wide range of isolates coming from severely-infested areas.

Some challenges were met in the collection which include non-reported tungro disease incidence in the local technicians which could have been the source of preliminary intervention. Other challenges include planting of same varieties for more than two years and the use of susceptible varieties. Main concern was the timing of planting due to availability of water. Even if farmers know that they should practice synchronous planting, the availability of the irrigation water is the main limiting factor. Most of the collection sites were irrigated lowland areas where tungro is primarily observed. However, in some rainfed areas in Camarines Norte and North Cotabato, tungro disease were also observed and infected plants collected.

Plants supposed to be infected with tungro disease with yellowing only symptom were observed to contain only the RTSV homologues and no RTBV homologues based on the presence and absence of bands corresponding to the coat protein primer for each virus. While in severely infected plants, both RTBV and RTSV homologues were present from the amplification of coat protein. These observations follow the established information on the co-infection of the two viruses which produce the yellow-orange and stunted growth that leads to senescence of the plants.

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BIBLIOGRAPHY

Arboleda, M., Sta. Cruz, F. and Azzam, O. (1999). Preliminary analysis of genetic variation if rice tungro bacilliform virus in two provinces of the Philippines. Pages 11-16 in Chancellor TCB, Azzam O, Heong KL (Eds.). *Rice Tungro Disease Management*. International Rice Research Insititute, Los Baños, Philippines.

Azzam, O. and Chancellor, T.C.B. (2002). The biology, epidemiology and management of rice tungro disease in Asia. *Plant Disease* 86:88-100.

Bonfield, J.K. and Staden, R. (1996). Experiment files and their application during large-scale sequencing projects. DNA Sequence 6:109-117.

Cabauatan, P.Q., Cabunagan, R.C. and Koganezawa, H. (1995). Biological variants of rice tungro viruses in the Philippines. *Phytopathology* 85:77-81.

Cabauatan, P.Q. and Choi, I.R. (2008). The genetic and biological diversity of rice tungro viruses. Pages 81-91 in Tiongco ER, Angeles ER, Sebastian LS (Eds.). Rice Tungro Virus Disease: A Paradigm in Disease Management. Philippine Rice Research Institute, Munoz, Philippines and Honda Research Institute, Chiba, Japan.

Cabunagan, R.C., Angeles, E., Tiongco, E.R., Villareal, S., Truong, X.H., Astika, I.G.N., Muis, A., Chowdhury, A.K., Ganapathy, T., Chancellor, T.C.b, Teng, P.S., and Khush, G.S. (1996). Evaluation of rice germplasm for resistance to tungro disease. Pages 92-99 Chancellor TCB, Teng PS, and Heong KL (Eds.). Rice Tungro Disease Epidemilogy and Vector Ecology: the development of sustainable and cost-effective pest management practices to reduce yield losses in intensive rice-cropping systems. IRRI Discussion Paper Series No. 19. International Rice Research Institute, P.O. Box 933, Manila, Philippines.

Choi, I.R., Cabauatan, P.Q. and Cabunagan, R.C. (2009). Rice Fact Sheets:Rice Tungro Disease. International Rice Research Institute. Retrieved from http://www.knowledgebank.irri.org

Dasgupta, I., Hull, R., Eastop, S., Poggi, P.C., Blakebrough, M.I. and Davies, J.W. (1991). Rice tungro bacilliform virus DNA independently infects rice after Agorbacterium mediated transfer. *J Gen. Virol.* 72:1215-1221.

De Los Reyes, J. 2000. Molecular Variability of Rice TUngro Bacilliform Virus Genomes in Tungro-outbreak Areas in the Philippines. Unpub. Thesis.

IRRI. (2009). Rice fact sheets: Rice tungro disease. International Rice Research Institute, Los Baños, Philippines.

Katoh, K., Kuma, K.I.m Toh, H. and Miyata, T. (2005). MAFFT version 5: Improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* 33:511-518.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). "ClustalW and ClustalX version 2". Bioinformatics. 23 (21): 2947–2948. PMID 17846036. doi:10.1093/bioinformatics/btm404.

Lee, J.H., Muhsin, M., Atienza, G.A., Kwak, D.Y., Kim, S.M., De Leon, T.B., Angeles, E.R., Coloquio, E., Kondoh, H., Satoh, K., Cabunagan, R.C., Kikutchi, P.Q., Leung, S. and I.R Choi. 2010. Single nucleotide polymorphism in a gene for translation initiation factor (eIF4G) of rice (*Oryza sativa*) associated with resistance to rice tungro spherical virus. Mol. Plant-Microbe Interact. 23:29-38.

Hibino, H., Roechan, M. and Sudarisman, S. (1978). Association of two types of virus particles with penyakit habang (tungro disease) of rice in Indonesia. *Phytopathology* 68:1412-1416.

Hull, R. (1996). Molecular biology of rice tungro viruses. *Ann. Rev. of Phytopathology* 34:275-297.

Jena, K.K. and Mackill, D.J. (2008). Molecular markers and their use in marker-assisted selection in rice. Crop Science 48:1266-1276.

Librado, P. and Rozas, J. (2009). DNAsp v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25:1451-1452.

Ling, K.C., Tiongco, E.R. and Cabunagan,R.C. (1983). Insect vectors of rice virus and MLO-associated disease. Pages 415-437 in *Proc. First Intl. Workshop on Leafhoppers and Plant hoppers on Economic Importance*. London, UK: Commonwealth Inst. Entomology.

Mangrauthia, S.K., Malathi, P., Agarwal, S., Ramkumar, G., Krishnaveni, D., Neeraja, C.N., Madhav, M.S., Ladhalakshmi, D., Balachandran, S.M., and Viraktamath, B.C. (2012). Genetic variation of coat protein gene among the isolates of Rice tungro spherical virus from tungro-endemic states of the India. *Virus Genes* 44:482-487.

Morrison, D.A.2009. A framework for phylogenetic sequence alignment. Plant Syst Evol 282:127-149.

NCBI, USA. https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

Okonechnikov K, Golosova O, Fursov M, the UGENE team. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 2012 28: 1166-1167. doi:10.1093/bioinformatics/bts091

Ou, S.H. (1985). Rice disease. Second ed. Commonwealth Agricultural Bureau, Wallingford, UK. 33-47 pp.

Rivera, C.T. and Ou, S.H. (1965). Leaf hopper transmission of tungro disease of rice. *Pl. Dis. Reptr*. 49:127-131.

Saito, Y., Iwaki, M. and Usugi, T. (1976). Association of two types of particles of tungro-group disease of rice. *Ann. Phytopathol. Soc. Jpn*. 43:375.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. and Evol.* 4:406-425.

Sebastian, L.S., Tiongco, E.R., Tabanao, D.A., Maramara, G.V., Abdula, S. and Tabelin, E.B. (1999). Breeding for rice tungro disease resistance at PhilRice. Pages 23-29 in Chancellor TCB, Azzam O, Heong KL (Eds.). *Rice Tungro Disease Management*. International Rice Research Institute, Los Baños, Philippines.

Tagubase, J. 2004. Molecular Dynamics of Rice Tungro Bacilliform Virus (RTBV) Temporal Population in Response to Host Resistance. Unpub. Thesis.

Tamura, K., Nei, M. and Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)* 101:11030-11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Mol. Biol. and Evol*. 28:2731-2739.

Umadhay, K.M.L., Yambao, M.L.M. and Azzam, O. (1999). Preliminary analysis of genetic variation of rice tungro spherical virus in the Philippines. Pages 17-21 in Chancellor TCB, Azzam O, Heong KL (Eds.). *Rice Tungro Virus Disease Management.* International Rice Research Institute, Los Baños, Philippines.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M. and Barton, G.J. (2009). Jalview v2-a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189-1191.

Yambao, M.L.M., Cabauatan, P.Q. and Azzam, O. (1998). Differentiation of rice tungro spherical virus variants by RT-PCR and RFLP. *International Rice Research Notes* 23:22-24

TABLES AND FIGURES

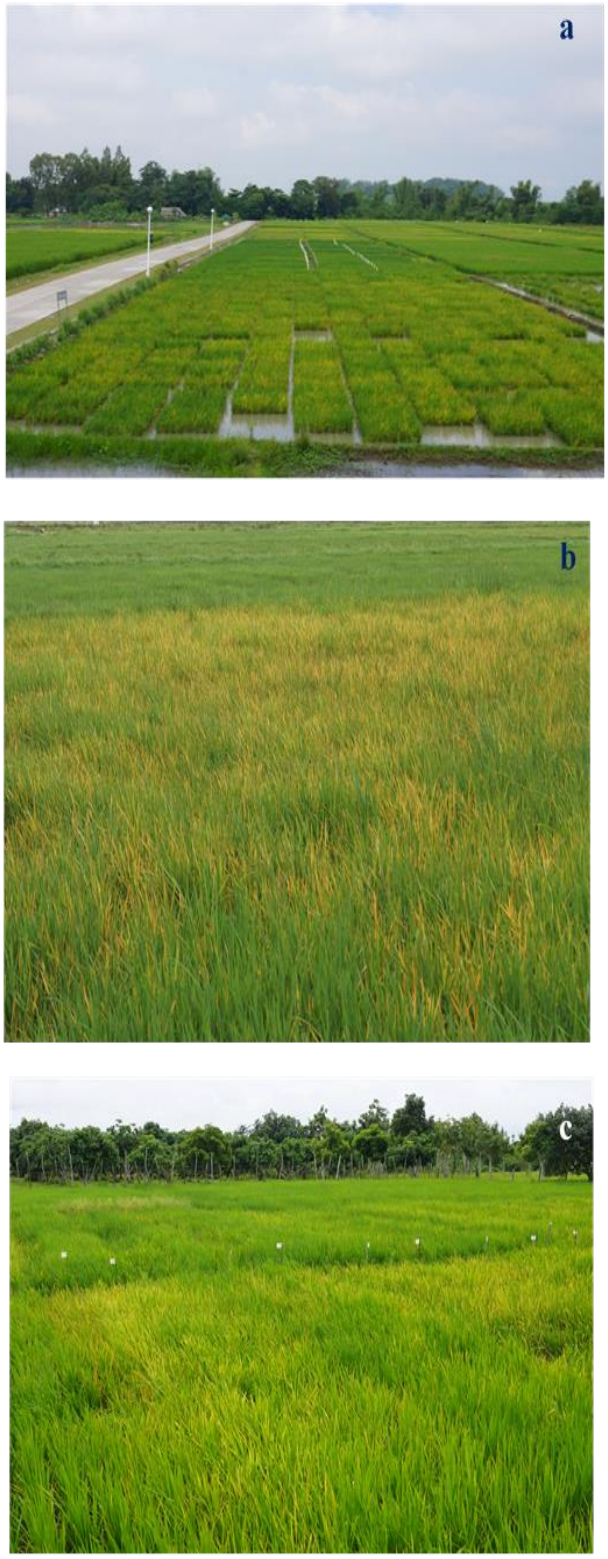

Figure 1. Representative tungro infected field in a) Murcia, Negros Occidental, b) Calabanga, Camarines Sur and c) Quezon, Isabela showing severity of rice tungro disease.

Table 1. Tungro-infected plants collection from nine provinces in the Philippines showing the presence of RTBV and RTSV sequences as confirmation of the causal organism

Province/Region	Source	Latitude	Longitude	Plants Collected	(+) with RTBV*	(+) with RTSV**
Isabela (II)	Cauayan City	N 16° 55' 44"	E 121° 45' 8"	20	19	4
	Mallig	N 17º 11' 5.59"	E 121º 34' 59.7"	30	18	0
	Quezon	N 17º 19' 12"	E 121º 37' 12"	20	17	3
	Roxas City	N 17º 9' 40"	E 121º 35' 12.2"	30	23	1
	San Manuel	N 17º 2' 38.9"	E 121º 37' 42.7"	10	8	5
Nueva Ecija (III)	Gabaldon	N 15º 27' 15.6"	E 121º 20' 31.5"	20	0	7
	Laur	N 15º 34' 34.1"	E 121º 11' 56.6"	10	0	2
	Muñoz	N 15º 40' 15.1"	E 120º 53' 30.7"	10	0	2
	Sto. Domingo	N 15º 38' 52"	E 120º 53' 54.2"	10	0	0
Laguna (IVA)	Los Baños	N 14° 10′ 12″	E 121° 13′ 12″	10	0	10
Albay (V)	Libon	N 13º 17' 25.7"	E 123º 26' 59.2"	10	4	0
	Polangui	N 13º 17' 25.7"	E 123º 26' 59.2"	10	4	0
Camarines Norte (V)	Daet	N 14° 6' 14"	E 122° 57' 28"	10	0	3
	Jose Panganiban	N 14º 18'	E 122º 72'	30	20	5
Camarines Sur (V)	Basud	N 14º 06' 61"	E 122º 96' 48"	10	0	5
	Calabanga	N 14° 06' 60.00"	E 122° 56' 59.99"	10	8	3
	Ocampo	N 40° 26' 46"	E 79° 58' 56"	10	5	5
	Pili	N 13º33'56.4"	E 123º16'5.39"	20	12	10
Negros Occidental (VI)	Bago City	N 10º 32' 31"	E 122º 55' 19.7"	30	11	4
	Hinigiran	N 10º 14' 17.6"	E 122º 55' 50.7"	10	8	8
	Murcia	N 10º 33' 59.2"	E 122º 59' 46.8"	30	10	8
	Sipalay City	N 9º 44' 32.5"	E 122º 23' 50.09"	10	11	8
Bukidnon (X)	Maramag	N 7° 45' 46.80"	E 125° 00' 18.00"	10	10	2
North Cotabato (XII)	Kabacan	N 7º 10' 15.5"	E 124º 49' 31.5"	10	3	3
	Midsayap	N 7º 10' 43.7"	E 124º 29' 49.3"	10	5	5
	Pigcawayan	N 7º 18' 49.6"	E 124º 25' 31.1"	10	5	7
TOTAL				400	201	110

** using RTBV CP1 primers*

***using RTSV CP3 primers*

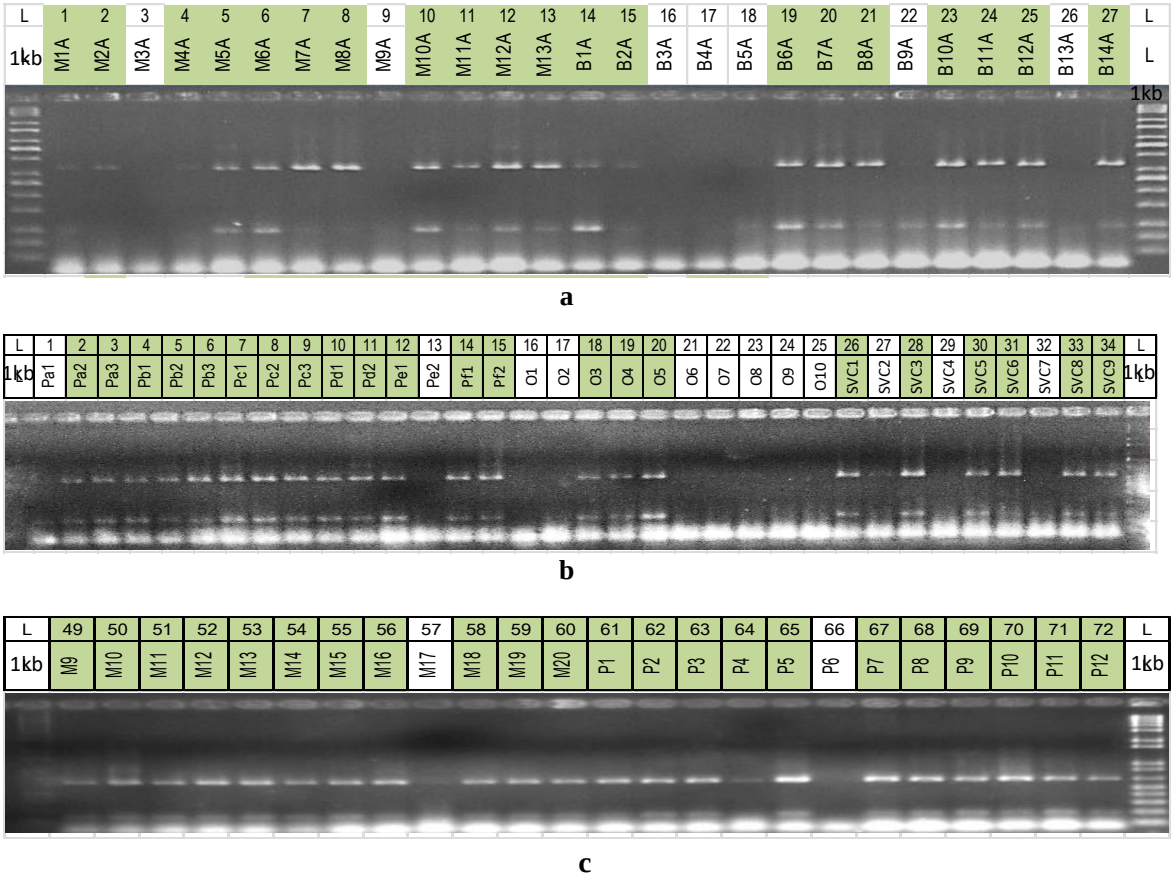

Figure 2. Representative electrophoresis gel showing CP1-gene specific primer for RTBV confirmation resolved with 1.5% agarose gel (highlighted are the confirmed RTVB-infected plants). Collected from a) Negros Occidental; b) Camarines Sur, and; c) Isabela.

https://www.scribblemaps.com/

Figure 3. Geotag of the collection of putative rice tungro-infected plants from Luzon, Visayas and Mindanao.