Genetic diversity of rice tungro spherical virus in tungro-endemic provinces of the Philippines and Indonesia*

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Summary. The two adjacent genes of coat protein 1 and 2 of rice tungro spherical virus (RTSV) were amplifie from total RNA extracts of serologically indistinguishable fiel isolates from the Philippines and Indonesia, using reverse transcriptase polymerase chain reaction (RT-PCR). Digestion with *Hin*dIII and *Bst*YI restriction endonucleases differentiated the amplifie DNA products into eight distinct coat protein genotypes. These genotypes were then used as indicators of virus diversity in the field Inter- and intra-site diversities were determined over three cropping seasons. At each of the sites surveyed, one or two main genotypes prevailed together with other related minor or mixed genotypes that did not replace the main genotype over the sampling time. The cluster of genotypes found at the Philippines sites was significantl different from the one at the Indonesia sites, suggesting geographic isolation for virus populations. Phylogenetic studies based on the nucleotide sequences of 38 selected isolates confir the spatial distribution of RTSV virus populations but show that gene fl w may occur between populations. Under the present conditions, rice varieties do not seem to exert selective pressure on the virus populations. Based on the selective constraints in the coat protein amino acid sequences and the virus genetic composition per site, a negative selection model followed by random-sampling events due to vector transmissions is proposed to explain the inter-site diversity observed.

Introduction

The fact that populations of RNA viral genomes are genetically heterogeneous and have potential for rapid adaptability and evolution is well studied and reviewed

*GenBank accession numbers of the sequences reported in this paper are AF113796-AF113812 for RTSV-CP1 and AF113813-AF113829 for RTSV-CP2.

[6, 7, 15–17, 27] but, there are few studies on how these populations have evolved and how they are related to natural selection. Fraile et al. [11] analyzed a collection of 53 isolates of tobacco mild green mosaic tobamovirus (TMGMV) from *Nicotiana glauca* from four countries and showed that evolutionary mechanisms, such as isolation and geographic-specifi selection, were the main forces driving the variability of TMGMV isolates. Their work also showed that recent colonization of new areas or host plants might have different effects on the TMGMV geographic subpopulations. On the other hand, analysis of the temporal variation in pepper mild mottle virus (PMMV), another tobamovirus, showed that, in the areas newly colonized by the virus, there was strong selection for a single variant that was genetically stable for 4 years [19].

After high-yielding rice varieties were introduced in the early 1960s throughout South and Southeast Asian countries and double-rice cropping and staggered planting became more common in irrigated areas, tungro became an increasingly important disease [13]. Until the mid-1970s, it was believed that this disease was associated with only spherical particles. Saito et al. [24], however, showed evidence that it is associated with two viruses: rice tungro spherical waikavirus (RTSV): family *Sequiviridae*; and rice tungro bacilliform virus (RTBV): family *Caulimoviridae* [18]. Rice tungro has become endemic in most intensive rice-producing areas of the Philippines and Indonesia. Occasional large-scale outbreaks occur, but they are difficul to predict and farmers face difficul management decisions.

Rice varieties with resistance to the vector have been deployed to control the disease, although many of them sustained severe damage within a few years of release [14]. Reliable management strategies vary among producing areas. We were interested in studying the temporal and spatial genetic diversity of both tungro viruses in intensive rice irrigated ecosystems in an attempt to understand the evolutionary mechanisms that favor a more durable resistance and aid in the design of more effective management strategies in such ecosystems. In this study, we shall discuss only RTSV.

In the tungro complex, RTSV is semi-persistently transmitted by green leafhopper vectors and facilitates plant-to-plant transmission of RTBV. RTSV has a polyadenylated single-stranded RNA genome of about 12 kb encapsidated within isometric particles [25]. The capsid comprises three coat protein species that are expressed as a polyprotein at the 5′ end of viral RNA genome, and they are cleaved at glutamine carboxy termini that are recognized by 3C-type proteases [8, 33]. Isolates of RTSV in the Philippines vary in their virulence on specifi rice varieties, and two variants (RTSV-A and RTSV-Vt6) show differential virulence on rice variety TKM6 [3]. In the field plants infected with RTSV alone show no apparent symptoms, except for occasional stunting [5]. In addition, all RTSV isolates described so far are indistinguishable serologically using polyclonal antisera against purifie virus preparations.

Using various molecular tools, several studies have been performed to differentiate tungro viruses. Cabauatan et al. [2] differentiated four biological variants of RTBV by DNA fingerprintin using three restriction enzymes. Arboleda and

Azzam [1], using this method, attempted a preliminary mapping of RTBV fiel populations in tungro-endemic provinces of the Philippines. Fan et al. [9] described two distinct strains of RTBV, one from the Indian subcontinent and the other from Southeast Asia, using Southern blot hybridization and sequencing.

Similarly, reverse transcriptase polymerase chain reaction (RT-PCR), Southern blot hybridization, and sequencing were used to differentiate the Philippine and Malaysian RTSV isolates from the Bangladesh and Indian isolates [32]. The Indian RTSV isolate was also differentiated from the Philippine, Malaysian, and Thailand isolates by differential electrophoretic mobility of its CP3 and the differential response of this protein to cellulolytic enzymes [8]. The two biological variants of RTSV maintained at IRRI were differentiated using RT-PCR, Southern blot hybridization, and restriction analysis of the coat protein species 1 and 2 (CP1 and CP2) [31]. This methodology was used to examine the genetic diversity of RTSV fiel populations in the Philippines and Indonesia during three cropping seasons and to determine the intra- and inter-site diversities. In this paper, we show that RTSV populations exist as groups of variants each with one or two dominant genotypes, and other related minor or mixed genotypes do not replace the main genotypes over three seasons. For that period, IRRI or other varieties had no effect on the genetic composition of the virus population per site. Instead, the virus populations in the Philippines and Indonesia seem to be geographically isolated although migration may occur. Sequence analysis of the coat protein and the stable virus genetic composition per site imply a negative selection evolutionary model followed by random-sampling events for the inter-site diversity of RTSV populations.

Materials and methods

Collection of tungro-infected plants

In the Philippines, three main rice-producing provinces (Nueva Ecija, Bicol, and North Cotabato) were selected and they are >200 km apart. In Indonesia, the main rice-producing provinces in West Java (Subang and Bogor) and Bali island where rice is grown throughout the year, were selected. Bogor is 200 km from Subang while Bali is >2000 km from the other two provinces. Tungro is endemic at all the sites. Randomly chosen sites were sampled in Nueva Ecija, Bicol, and Bogor during the 1996 and 1997 wet seasons, 96 W and 97 W, respectively (Table 1). Three sites were sampled in North Cotabato in the dry and wet seasons of 1997, 97 D, and 97 W, respectively. Also, three sites were sampled in Bali in 97 W and 97 D and one site in Subang in 97 W, 97 D, and the wet season of 1998 (98 W). Samples were collected mainly from the tillering and early booting stages of the rice crop and most exhibited tungro-like symptoms. Each sample was catalogued and tested serologically against RTBV and RTSV antisera by enzyme-linked immunosorbent assay (ELISA) [4]. Because of limited leaf size, all samples collected from Bali in 97 W were directly analyzed by RT-PCR without any serological test. Approximately 0.1 g of leaf sample was used in serological testing and those samples that were ELISA-positive for RTSV (whether they were RTBV-positive or-negative) were subsequently processed for total RNA extraction and RT-PCR. An isolate represents a single field-infecte plant.

Table 5. Distribution of rice tungro spherical virus (RTSV) coat protein genotypes per site

Location	Site	Season[a]	Variety	N[b]	ELISA results[c] RTBV	RTSV	I[d]	II	III	IV	V	VI	VII	VIII	Mix	RT-PCR[e]
Philippines																
Nueva Ecija	6 random fields	96W	IR64	30	0	29	–	13(56)	2(9)	–	–	8(35)	–	–	–	23 [23A]
Bicol	6 random fields	96W	Mixture	12	0	12	–	8(67)	1(8)	–	–	1(8)	–	–	2(17)	12 [10abcefh]
North Cotabato	M'Lang	97D	IR74	30	24	29	–	17(74)	3(13)	–	–	1(4)	–	–	2(9)	23[21bc]
	Tulunan		Unknown	30	27	30	–	11(58)	–	–	2(13)	2(10)	–	–	4(21)	19[15ace]
	Kabacan		Selection 55	30	29	29	–	7(44)	1(6)	–	2(12)	3(19)	–	–	3(19)	16[13acgh]
	M'Lang	97W	IR64	35	32	32	1(4)	17(71)	1(4)	–	–	4(17)	–	–	1(4)	24[23ach]
	Tulunan		Masipag	30	24	26	–	6(43)	6(43)	–	1(7)	1(7)	–	–	–	14[14bg]
	Pigcawayan		Malagkit	30	29	29	3(23)	6(46)	–	–	–	2(15)	–	–	2(15)	13[11eh]
Indonesia																
Bali	P. Galak A	97W	IR64	30	ND	ND	–	4(14)	–	–	24(86)	–	–	–	–	28[28di]
	P. Galak B		IR64	30	ND	ND	–	2(8)	–	3(12)	12(48)	–	1(4)	–	7(28)	25[18ik]
	Gianyar		Krueng Aceh	15	ND	ND	–	1(7)	–	1(7)	6(43)	–	–	2(14)	4(28)	14[10jkln]
	P. Galak A	97D	IR64	28	21	24	1(6)	–	–	–	10(59)	–	–	5(29)	1(6)	17[16djmp]
	P. Galak B		IR64	27	20	21	1(6)	3(18)	–	1(6)	8(47)	–	2(12)	1(6)	1(6)	17[16filmop]
	Gianyar		IR64	27	18	17	1(8)	1(8)	–	1(8)	8(61)	–	1(8)	1(8)	–	13[13dkr]
Subang	Tj. Siang A	97W	IR64	32	27	32	–	–	–	15(47)	1(3)	–	2(6)	8(25)	6(19)	32[26os]
	Tj. Siang A	97D	IR64	28	14	14	–	1(8)	–	1(8)	1(8)	–	2(15)	3(23)	5(38)	13[8fnorst]
	Tj. Siang A	98W	IR64	45	15	30	–	1(4)	–	1(32)	–	–	–	9(41)	5(23)	22[17qs]
Bogor	3 random fields	97W	IR64	35	25	32	–	–	–	–	–	–	13(59)	3(14)	6(27)	22[16t]

[a]96W = 1996 wet season, 97D = 1997 dry season

[b]N = number of samples collected per site

[c]RTBV and RTSV columns show the number of samples that were positive for rice tungro bacilliform virus (RTBV) or rice tungro spherical virus (RTSV) by enzyme-linked immunosorbent assay (ELISA). ND means not done. The RT-PCR column shows the numbers of samples that were amplified using reverse transcriptase polymerase chain reaction (RT-PCR)

[d]Numbers in parentheses are the percentage of coat protein genotypes over the total number of samples analyzed per variety per location

[e]The numbers used for the Chi-square test and the results of the test are in brackets. The same letter indicates that there were no significant differences in the genetic composition of RTSV coat protein genotypes between two populations within sites or between sites over time. Because more than 60% of the cells have expected counts of less than 5, the Chi-square test might not be a valid test

Total RNA extraction, RT-PCR, and restriction analysis

Total RNA was extracted from each of the RTSV isolates and a complementary DNA to the two adjacent coat protein genes 1 and 2 was synthesized using RSCP2C3607 primer (RSCP2 = RTSV coat protein 2, V or C = viral or complementary sense, and number = nucleotide position according to the published sequence by Shen et al. [25]). Amplificatio of one region containing both CP1 and CP2 was done using RSCP1V2453 and RSCP2C3607 primers [31]. About 7.5 μl of RT-PCR products were separated on 1% agarose gels with 1×TBE (Tris-Borate-EDTA) at 100 volts for 3 h. Gels were stained with ethidium bromide for 15–20 min, viewed under UV light, and photographed. RT-PCR products (7–12 μl) were digested with 10–20 U of *Hin*dIII and *Bst*YI (*Xho*II) restriction endonucleases in a volume of 20 μl. These enzymes were selected because they showed polymorphism for the two RTSV biological variants, A and Vt6. The mixture was incubated at 37° C overnight. Undigested and digested products were loaded in 1% agarose gels along with Biomarker high and low (BioVentures, Inc.) with 1× TBE buffer, and run at 100 volts. Each gel was then stained with ethidium bromide for 5 min and photographed, and the sizes of digested DNA fragments were estimated. To eliminate confusion resulting from digestions, selected samples per site were processed 2–3 times and digestion patterns were confirmed

Sequence analysis

To verify the classificatio of coat protein genotypes, four RT-PCR products for each of genotypes I, II, and VIII, two products for genotypes III, IV, and VII, seven products for genotype V, and another four products representing Mix genotypes were selected for further sequence analysis (Fig. 2). Sequencing reactions were performed directly on the PCR products using the following four primers: RSCP1V2453, RSCP1V2872, RSCP2C3607, and RSCP2C3067. Sequencing protocols were those of Tracy and Mulcahy [30] and Rosenthal and Charnock-Jones [23]. Data were analyzed using the PE-Biosystems version 3.0 of Sequencing Analysis. In addition, the following sequences were included: PgA (RTSV-A, an isolate from IRRI greenhouse, Philippines, that was cloned and sequenced in 1989, EMBL accession number M95497 [25]), Pg09VI and Pg05V (two RTSV variants from the same IRRI greenhouse isolate that were purifie in 1995 and 1996, respectively), Pg08V and Pg16VI (the current RTSV isolates at IRRI), PgVt6III (RTSV-Vt6, a resistance-breaking isolate representing genotype III, from Mindanao, Philippines), M70III (a Malaysian RTSV isolate, EMBL accession number U70989 [32]), and P71III (another RTSV isolate from IRRI, Philippines, EMBL accession number U71440 [33]).

Phylogenetic analysis

Protein sequences of the CP1 and CP2 species of the 38 RTSV isolates and maize chlorotic dwarf waikavirus (MCDV = EMBL accession number U67839 [21]): family *Sequiviridae* were aligned using CLUSTALW [29], followed by manual refinemen of the alignments using SeqPup 0.6f [12]. The alignment for CP1 consisted of 196 amino acids (aa) (the firs 12 aa were missing) while that for CP2 had 169 aa (the last 35 aa were missing). Using the protein alignment as a framework, nucleotide sequences were arranged such that each codon was aligned to the corresponding amino acid. Phylogenetic reconstruction was accomplished by neighbor-joining and parsimony methods using programs from PHYLIP 3.5c [10]. SEQBOOT was used to create 500 bootstrap-resampled data sets for each alignment. For the nucleotide alignments, DNADIST was used to calculate matrices using the maximum likelihood method while, for protein alignments, PROTDIST was used with the Dayhoff PAM model for substitution rates. Each set of distance matrices was subjected to neighbor-joining tree construction using NEIGHBOR, with the input order of sequences randomized 10 times.

DNAPARS was used to construct trees from the bootstrap-resampled nucleotide data based on the ordinary parsimony model with input orders randomized 10 times. Using the universal genetic code with input orders randomized 10 times, bootstrap-resampled protein alignments were analyzed by the ordinary parsimony model of PROTPARS. Maximum likelihood analysis was accomplished for the nucleotide alignments of a subset of 24 isolates and MCDV by the quartet puzzling method of PUZZLE 4.0 [28], using default parameters. A PERL script was used to automate the process: a random seed was generated and a parameter fil created for SEQBOOT; one bootstrap-resampled alignment was generated by SEQBOOT; this data fil was used to calculate the quartet tree. Output file were appended and the process was repeated 500 times. For each method, the set of 500 trees (or more if tied trees were found) served as an input for determining the majority rule consensus tree by CONSENSE. For the neighbor-joining and parsimony analyses, branch lengths were estimated by FITCH, supplying the consensus tree as a user-define tree along with the appropriate distance matrix calculated from the non-bootstrap-resampled alignment. For the quartet puzzling method, branch lengths for consensus trees were determined by supplying the consensus tree along with the non-bootstrap-resampled alignment to PUZZLE. Graphics of the trees were drawn using TREEVIEW 1.5 [20]. For all analyses, the MCDV sequence served as an outgroup to root the tree, but was pruned from the tree for fina display.

Insect transmission

To evaluate the biological properties of RTSV fiel populations, the 90 collected samples from North Cotabato in 97D were insect-transmitted individually on the susceptible rice variety, TN1. Three green leafhopper adults (virus-free), which were maintained under greenhouse conditions, were fed on the detached leaf sample (inoculum) overnight and transferred to a healthy 10-day-old TN1 seedling for overnight inoculation. The inoculated plants were then sprayed, kept in separate cages, and monitored by ELISA, Southern blot hybridization and RT-PCR for the presence or absence of tungro viruses at 3 weeks post-inoculation (wpi). Recovered isolates were re-transmitted on TN1 to increase the amount of inoculum and then transmitted on 5 different RTSV-resistant rice hosts: Balimau Putih (IRGC accesssion number 17204, tolerant of tungro infection), Adday Selection (IRGC accession number 177, resistant to RTSV-A and Vt6), TKM6 (resistant to RTSV-A), TW5 (a near-isogenic line derived from Utri Merah IRGC accession number 16680 and resistant to RTSV-A), and TW16 (a near-isogenic line derived from Utri Merah IRGC accession number 16682 and resistant to the two strains of RTSV). Virus infection was checked also by ELISA and RT-PCR at 3 wpi.

Results

RTSV isolates based on the restriction enzyme patterns of RT-PCR products

Three hundred fift RTSV isolates were differentiated by restriction enzyme analysis of amplifie RT-PCR products from the virus coat protein species 1 and 2. Eight coat protein genotypes were identifie in the total population of the Philippines and Indonesia based on the size of the uncut RT-PCR product and the sizes of the *Hin*dIII and *Bst*YI-digested fragments (Fig. 1 and Table 2). A visual code from I to VIII was assigned to each distinct genotype. A Mix code was given to those genotypes that showed variable and/or mixed enzyme restriction patterns.

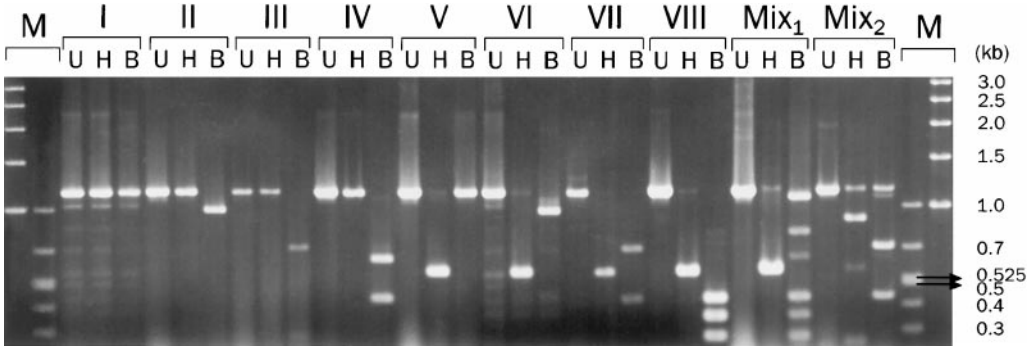

Fig. 1. Coat protein genotypes in natural rice tungro spherical virus (RTSV) populations in the Philippines and Indonesia during three cropping seasons. Genotypes were identifie based on the estimated size fragments of their RT-PCR products when digested with *Hin*dIII and *Bst*YI (*Xho*II) restriction enzymes (see Table 2) and later confirme by sequence analysis. *M* Biomarker high and low (BioVentures, Inc.), *I-VIII* distinct genotypes, *U* undigested RT-PCR product (1.15 kb), *H Hin*dIII-digested product, *B Bst*YI-digested product. The Mix genotype is a genotype with a variable and/or a mixed restriction pattern

Table 2. Estimated size fragments of RT-PCR products for the rice tungro spherical virus coat protein genotypes when digested with *Hin*dIII and *Bst*YI (*Xho*II) restriction enzymes and confirme by sequence analysis

Coat protein genotype	*Hin*dlll (kb)	*Bst*YI (*Xho*ll) (kb)
I	1.15 (no site)	1.1 (1092)
II	1.15 (no site)	1.00 (66, 1057)
III (RTSV-Vt6)[a,b]	1.15 (no site)	0.70, 0.30, 0.20 (108, 815, 1099)
IV	1.15 (no site)	0.68, 0.45 (416, 1065)
V	0.58 (578 or 579)	1.15 (no site)
VI	0.58 (535 or 480)	1.00 (991 or 56 and 1047)
VII	0.58	0.70, 0.45
VIII	0.58 (550 or 579)	0.45, 0.38, 0.25 (440, 806, 1090)
Mix	Mix	Mix

[a]RTSV-Vt6 is a resistance-breaking strain on TKM6 and sequence analysis confirme its restriction pattern [31]

[b]Numbers in parentheses are the mapped positions of the restriction enzyme based on the sequence data of coat protein species 1 and 2 from the different RT-PCR products. If the site was not found, it is still indicated between parentheses

Diversity of RTSV in the Philippines and Indonesia

The frequency of each coat protein genotype per site was calculated and used as an indicator for the genetic structure and diversity of the virus at that site. Results showed that three to six genotypes could occur at one site, but only one or two genotypes were dominant (Table 1). Distinct genotypes could be identifie in the two countries. In the Philippines, genotypes II, III, and VI were

detected in Nueva Ecija and Bicol provinces in 96 W, while genotypes I-III, V, and VI were detected at North Cotabato sites during 97 D and 97 W. Genotypes IV, VII, and VIII were not detected at any site. Genotype II accounted for 56% of the genotypes in the Philippines, followed by genotype VI (15%), during three seasons. Minor or mixed genotypes were also present but at a lower frequency. In Indonesia, the population structure was different. Genotypes I, II, IV, V, VII, and VIII were detected in Bali and Subang, but only genotypes VII and VIII were detected in Bogor and genotypes III and VI were not detected at any site. Based on the frequency distribution and Chi-square analysis, the genotypes and their frequencies vary more by location than by variety or time. In Bali, genotype V accounted for 60% of the genotypes during both the 97 D and 97 W seasons. In Subang, genotypes IV and VIII were dominant (30% and 28%) during three seasons, while in Bogor genotype VII was the most frequent (59%). The presence of mixed infections was seen during both the dry and wet seasons and at most sites and the mixtures consisted mainly of the predominant genotypes (data not shown).

Within M'Lang, P. Galak, Gianyar, and Tj. Siang sites, there were no significant differences in the composition of the virus population over time (Table 1). Only within Tulunan were there significan differences between 97D and 97 W. At the other sites, no sampling was done over time to verify the existence of a temporal pattern. The composition of the virus population per variety, mainly IR64, was compared across sites and time. Results showed that virus populations do not differ according to rice variety. IR64, which was planted in the Nueva Ecija 96 W and M'Lang 97 W, Philippines, had a significantl different virus population than IR64 at the Indonesia sites. Similar to IR64, each of the other varieties was infected by at least two viral genotypes.

Nucleotide sequence analysis of coat protein species 1 and 2

The two coat protein sequences of the selected 38 RTSV isolates that represent most of the coat protein genotype groups were aligned separately and percent nucleotide (nt) and aa sequence dissimilarity for both were calculated using the maximum likelihood distances (PUZZLE 4.0) of the phylogenetic analysis. Sequence data from genotype VII were not clear, and therefore, this group was not included in the sequence analysis. For both CP1 and CP2 sequences, the percent average nt divergence between the genotype groups was significantl higher than among members of the same genotype group except for genotype groups I (Table 3). The most divergent genotype groups were groups I and IV (18.39% sequence divergence in CP1 and 17.34% in CP2) whereas the most closely related genotype groups were groups VIII and IV (4.30% and 4.27% for CP1 and CP2, respectively). The aa divergence was lower and not consistent with the nt divergence except for the aa divergence between IV-IV comparisons for CP1, which was higher than the nt divergence. For CP2, the aa divergence was low and similar between the different genotype groups and among members of the same group.

Table 3. Percent average nucleotide (nt) and amino acid (aa) sequence dissimilarity values based on the pairwise maximum likelihood distances from PUZZLE 4.0 of the phylogenetic analysis for rice tungro spherical virus coat protein genotypes CP1 and CP2 identifie from Indonesia, the Philippines, and Malaysia

Genotype groups	Pairwise Comb.	CP1		CP2	
		nt	aa	nt	aa
I to I	6	10.64	0.26	9.66	1.99
II to II	10	1.64	0.88	1.84	1.34
III to III	10	2.66	1.18	2.12	0.48
IV to IV	1	3.02	4.92	1.22	0.00
V to V	36	3.60	2.45	5.34	0.59
VI to VI	6	5.36	0.34	0.40	0.30
VIII to VIII	6	0.97	0.85	1.64	0.59
All[a]	703	9.33	1.95	9.58	1.50
I to II	20	10.24	0.95	8.29	2.10
I to III	20	12.13	0.75	11.69	2.09
I to IV	8	18.39	5.50	17.34	2.30
I to V	36	10.51	1.49	10.79	1.43
I to VI	16	11.66	0.26	11.50	1.64
I to VIII	16	11.05	1.15	11.11	1.80
I to Rest[b]	136	11.43	1.36	11.10	1.77
II to III	25	12.28	1.43	9.43	3.50
II to IV	10	14.43	4.50	12.30	3.00
II to V	45	12.67	1.97	13.60	3.27
II to VI	20	12.68	1.08	10.07	3.04
II to VIII	20	12.25	1.34	14.59	3.80
II to Rest	165	12.28	1.70	11.86	3.21
III to IV	10	12.02	4.66	10.71	0.62
III to V	45	10.56	2.29	10.59	1.06
III to VI	20	6.77	0.87	4.80	0.74
III to VIII	20	9.45	1.44	10.44	1.31
III to Rest	165	10.84	1.83	10.25	1.56
IV to V	18	9.40	4.87	8.81	0.48
IV to VI	16	5.44	2.13	5.59	0.08
IV to VIII	16	4.30	2.34	4.27	0.46
IV to Rest	72	10.51	4.61	9.69	1.00
V to VI	32	8.49	2.15	10.37	0.69
V to VIII	32	7.74	2.01	9.58	0.78
V to Rest	252	9.41	2.20	10.23	1.28
VI to VIII	16	10.98	1.28	12.41	1.04
VI to Rest	136	9.49	1.48	9.71	1.15
VIII to Rest	136	9.22	1.72	10.41	1.43

[a]All includes PgA and Mix isolates
[b]Rest implies All except the current group

Phylogenies of RTSV coat protein species 1 and 2

Phylogenetic groupings were evaluated for protein sequences of all the 38 RTSV isolates representing Indonesia, Philippines, and Malaysia, using MCDV as an outgroup with neighbor-joining and parsimony phylogenetic methods. Neither method clustered the coat protein sequences of both CP1 and CP2 into well-define groupings. This is not surprising because there was relatively little divergence between isolates at the protein level (Table 3). Neighbor-joining distance method, parsimony, and the maximum likelihood method of quartets were used for phylogenetic reconstruction and for determining the relatedness of RTSV coat protein species 1 and 2. The three methods yielded similar results and showed a consistent tree topology for both CP1 and CP2. Figure 2 shows the parsimony

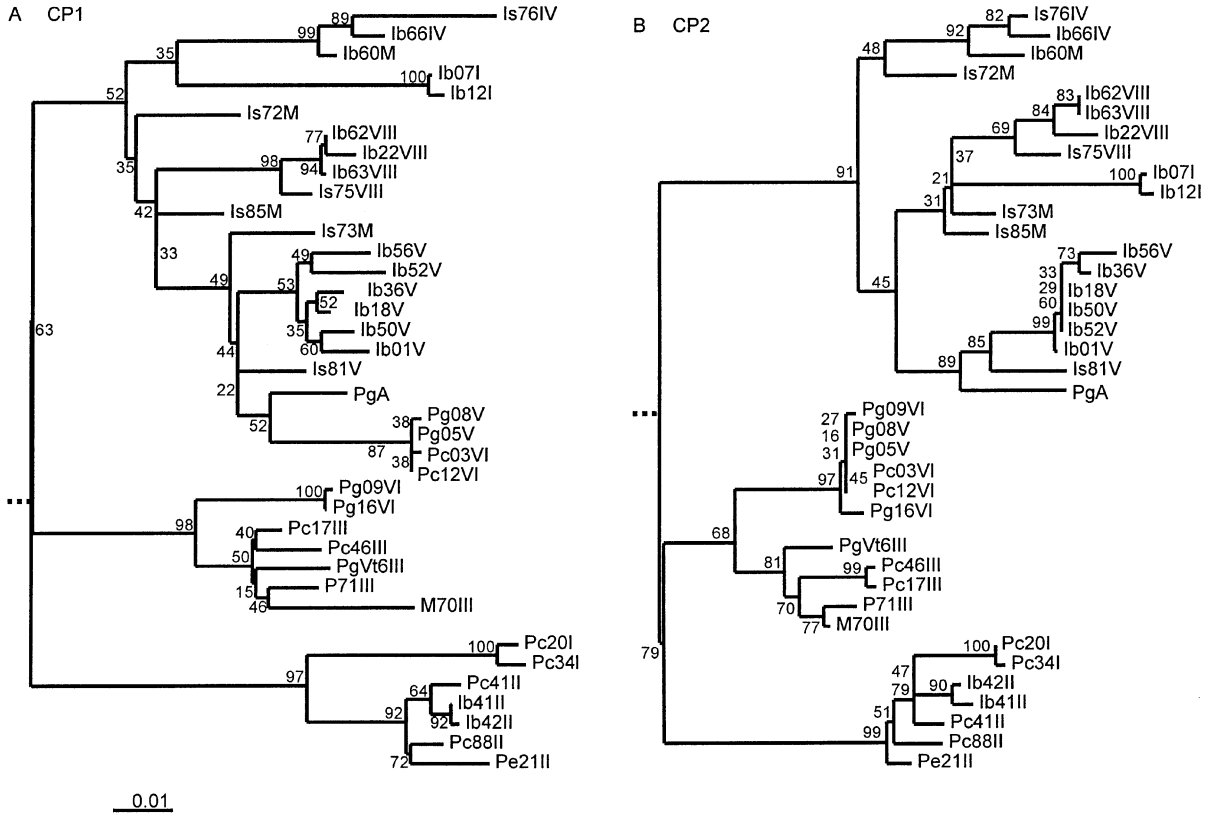

Fig. 2. Parsimony trees for nucleotide sequence alignments for coat protein 1 (*A* CP1) and coat protein 2 (*B* CP2) for 38 RTSV isolates. The numbers above an internal branch are percentages of times that a grouping occurred in 500 bootstrap replicates of the data. Isolate name includes the country (I for Indonesia and P for Philippines), the province (Ib for Bali or Is for Subang or Pc for North Cotabato, Pe for Nueva Ecija, Pg for IRRI-Philippines greenhouse isolates), the isolate fiel number, if any, and the coat protein genotype of that isolate. See text for more details. The bar indicates the branch length corresponding to a maximum likelihood distance of 0.01

tree. For CP1 (Fig. 2A), RTSV isolates were clustered together with their respective genotype class except for genotypes I, VI and PgA. The Indonesia isolates of genotype group I clustered together on a separate branch from the Philippines isolates of the same genotype group. Similarly, Philippines fiel isolates of genotype group VI clustered on a separate branch from the greenhouse isolates of the same genotype group. Isolate PgA (the 1989 IRRI isolate that does not belong to any genotype group) clustered with members of genotype group V. For CP2 (Fig. 2B), the same three major lineages were observed but with minor differences that occurred in the placement of Indonesia isolates from genotype group I and of Philippines isolates from genotype groups V and VI clustered now together on the second major branch of CP2 tree.

Biological properties of RTSV field isolates from North Cotabato

Out of 90 attempted insect-transmissions, only fi e isolates were recovered on TN1 plants based on their positive reactions to both tungro viruses by ELISA (Table 4). Insect mortality rates were high when fed on detached leaves and transmission rates, therefore, were low. The transmission on larger populations of TN1 showed that variable symptoms could be induced by the different isolates. Isolates derived from the Kabacan site could induce mild, typical tungro, or severe symptoms. Because most isolates were also infected with RTBV, it was difficul to assess the effect of RTSV on symptom expression. Further transmissions on the different rice hosts, which carry sources of resistance to RTSV, were performed. Results showed that the recovered isolates had a different RTSV pathogenicity spectrum based on their differential reactions to the selected hosts as measured by positive ELISA reactions and RT-PCR (Table 4). Isolate 17 (genotype III) was confirme after two transmission experiments on TN1, Balimau Putih, and TKM6 plants (Fig. 3). Isolates 32, 37, 38, and 83 (mix genotypes) were retained as mixed genotypes, a single genotype, like genotype VI from isolate 37 that was recovered from Balimau Putih plants, or no amplificatio was obtained.

Table 4. ELISA and RT-PCR results for the recovered RTSV isolates from North Cotabato when insect-transmitted to TN1, the susceptible host (firs transmission), and to Balimau Putih (B. P.), Adday Selection (A. S.), TKM6, TW5, and TW16, the resistant hosts to RTSV (second transmission)

Field isolate	Original CP-genotype	% infection on TN1[a]	% infection on					Recovered CP-genotype
			B.P.	A.S.	TKM6	TW5	TW16	
17	III	91	37	0	5	0	0	III
32	Mix	89	58	0	10	0	0	Mix
37	Mix	20	9	0	0	0	0	VI
38	Mix	100	47	0	0	0	0	No amp.
83	Mix	100	30	0	0	0	0	No amp.

[a]% infection by rice tungro spherical virus using ELISA

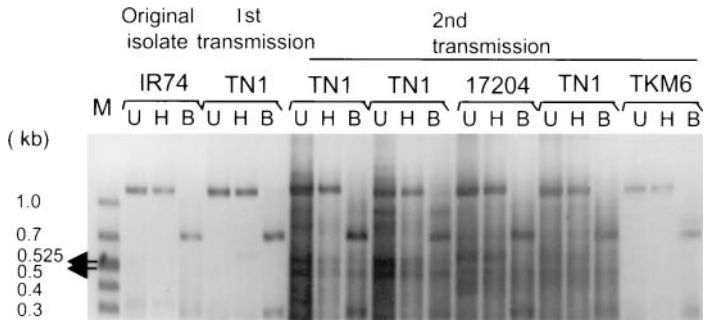

Fig. 3. The distinct coat protein genotype III, as recovered from TN1, TKM6, and Balimau Putih (accession number 17204) infected plants with isolate 17 of M'Lang after three transmission experiments. M Biomarker high and low (BioVentures, Inc.), U undigested RT-PCR product, H *Hin*dIII-digested product, B *Bst*YI-digested product. 17204 = accession number for Balimau Putih, a rice variety

Discussion

In this study, we have characterized the genetic diversity of RTSV fiel populations using two approaches: (1) restriction analysis of a PCR-amplifie region for virus coat protein 1 and 2 species to examine the distribution of different genotypes in tungro endemic sites, and (2) sequence analysis of representative isolates from the different coat protein genotype groups to determine virus population structure. Based on the restriction analysis, three to six RTSV coat protein genotypes with different frequencies can be identifie per site and based on the sequence analysis, genotype groups may have an average nt sequence divergence between 4 to 18% for both CP1 and CP2 regions. Isolates within most of the genotype groups are closely related. In addition, as shown by insect transmission experiments, isolates from a single site may have different biological properties. Therefore, our data show that RTSV populations in the tungro-endemic regions of the Philippines and Indonesia are diverse and exist as sub-populations of related but not identical genetic and biological variants.

Phylogenetic analyses of both CP1 and CP2 sequences show that RTSV virus populations have three major lineages. Most of the Indonesia isolates are clustered in the firs lineage with high significance found for the nodes in the bootstrap analysis of parsimony tree (52% for CP1 tree and 91% for CP2 tree). The other two lineages have also high significance for nodes clustering mostly Philippines isolates (98% and 97% for CP1 and 68% and 99% for CP2). These results confir the geographical distribution of RTSV populations. However, the spatial pattern may not be discrete because two Indonesia isolates from genotype group II clustered in the third lineage along with the Philippines isolates from the same group. Similarly, the 1989 IRRI isolate, PgA, clustered with the Indonesia isolates in the firs lineage along with genotype group V. Migration or gene fl w seems to occur between the populations in addition to the presence of location specifi isolates such as the isolates from genotype groups I, IV, VII, and VIII in Indonesia, and groups I and III in the Philippines.

At most of sites within each country, the genetic composition of the virus population was not significantl different over the two or three cropping seasons. This result suggest that the geographically isolated populations are genetically stable over the sampling time. The silent mutations in most of the CP1 and CP2 sequences show that a purifying negative selection mechanism may be an important factor for the observed genetic stability. Negative selection eliminates deleterious variants from the population and even may defin the original founder population. The inter-site virus diversity could then result from negative selection followed by founder events such as vector transmissions that are the only means of spread for the virus. The semi-persistent mode of transmission, the size and biotype of active green leafhopper transmitters, and the fligh behavior of the vector [22] may impose constraints on the transmissibility and diversity of virus populations.

In intensive rice ecosystems, where rapid temporal and spatial fluctuation in the relative populations of different rice varieties and/or active transmitters occur, it is reasonable to predict that RTSV has to undergo continuous adaptation and is forced through several bottlenecks. Although rice varieties, such as the vector resistant IR64, do not differ from other deployed varieties and do not seem to exert a selective pressure on the virus populations, the short duration of the study makes it difficul to observe an effect, if there is any. However, because the evolution of a virus population is rarely independent of the host, it is essential to keep monitoring virus population structure and its virulence after the deployment of new resistance genes (to the vector or to the virus).

Sequence comparisons of coat protein genotypes and the inconsistent clustering of the Philippines fiel and greenhouse genotypes V and VI or the Indonesia genotypes I and Mix in the CP1 and CP2 phylogenetic trees imply that other evolutionary forces, such as recombination, may also be involved in the inter-site virus diversity and slow that the two coat protein genes evolve at different rates. Indeed, mixed infections were high, ranged between 0–38%, and consisted of mixtures of two or three RTSV variants within a single plant, as shown by our restriction analysis and transmission experiments. Therefore, recombination among variants can easily occur and if the recombinants are transmitted, they can also change the inter-site diversity of local virus populations. Further work is needed to confir that natural RTSV recombinants exist, but, in general, recombination has been demonstrated earlier for many plants RNA viruses [26].

The geographic isolation of RTSV populations has implications for the deployment of resistance to tungro disease. Although our data suggest that the deployed resistance has to take into account local virus variability, the appearance of resistance-breaking strains in one geographic location may not mean that they will easily spread to other locations. Localized outbreaks could then be managed by targeted deployment of relevant resistance genes to that particular environment. In summary, our data support the presence of heterogeneous RTSV populations in the fiel and a less discrete spatial structure of these populations. Moreover, the results imply a purifying negative selection evolutionary model followed by founder effects, mainly due to vector transmissions, to account for the inter-site variability observed.

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