Arch Virol (2000) 145: 275–289

Archives of Virology

Inter- and intra-site genetic diversity of natural field populations of rice tungro bacilliform virus in the Philippines

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Accepted September 1, 1999

Summary. The genetic structure of rice tungro bacilliform virus (RTBV) populations within and between growing sites was analyzed in a collection of natural field isolates from different rice varieties grown in eight tungro-endemic sites of the Philippines. Total DNA extracts from 345 isolates were digested with *Eco*RV restriction enzyme and hybridized with a full-length probe of RTBV, a procedure shown in preliminary experiments capable of revealing high levels of polymorphism in RTBV field isolates. In the total population, 17 distinct *Eco*RV-based genome profiles (genotypes) were identified and used as indicators for virus diversity. Distinct sets of genotypes occurred in Isabela and North Cotabato provinces suggesting a geographic isolation of virus populations. However, among the sites in each province, there were few significant differences in the genotype compositions of virus populations. The number of genotypes detected at a site varied from two to nine with a few genotypes dominating. In general the isolates at a site persisted from season to season indicating a genetic stability for the local virus population. Over the sampling time, IRRI rice varieties, which have green leafhopper resistance genes, supported similar virus populations to those supported by other varieties, indicating that the variety of the host exerted no apparent selection pressures. Insect transmission experiments on selected RTBV field isolates showed that dramatic shifts in genotype and phenotype distributions can occur in response to host /environmental shifts.

Introduction

Spatial diversity has been studied in many virus systems, especially in viruses with ssRNA genomes [13, 19, 20, 28] or with dsRNA genomes [23] but spatial and temporal diversity within a given location was rarely reported. Rodriguez-Cerezo et al. [26] reported that pepper mild mottle virus populations, collected from epidemic outbreaks in greenhouse-grown peppers, were more homogeneous and stable with slow replacement of genotypes over time. In addition, Nichol [24]

showed that vesicular stomatitis virus populations were homogeneous within successive virus outbreaks but more heterogeneous among different virus outbreaks and in endemic disease areas. Moreover, McNeil et al. [22] showed that the natural populations of wheat streak mosaic virus were geographically homogenous in four counties of Nebraska but the frequency of the circulating genotypes changed in a year. In contrast, Sobrino et al. [27] reported that foot-and-mouth disease virus populations have a fast evolution rate and are heterogeneous in nature at any given location or time.

In the rice tungro disease complex, rice tungro bacilliform virus (RTBV) acts synergistically with rice tungro spherical virus (RTSV) to cause tungro symptoms including yellow orange leaf discoloration, plant stunting, and reduced tillering. In rice, RTBV depends on RTSV for transmission by the green leafhopper, *Nephotettix virescens*, Distant [15] while RTSV infections are symptomless. Like other plant pararetroviruses, RTBV packages its genome as double-stranded (ds) DNA but replicates via an RNA intermediate. The packaged DNA resembles that of the badnaviruses in having one discontinuity in each strand at a specific site [18]. Individually, RTBV is easily distinguished from RTSV by serological methods [4]; however, to discriminate between different strains of RTBV, differential hosts or molecular methods are required.

Field management of tungro in Southeast Asia has relied on the continuous deployment of new resistant rice varieties, mainly varieties with sources of resistance to the green leafhopper [14, 16, 19]. In endemic regions, tungro resistance is short-lived due either to the adaptation of the green leafhopper [8, 9] or to the presence of new strains of tungro viruses [3]. Thus, understanding the local genetic structure of natural populations of tungro viruses and their vector is a prerequisite for resistance breeding and deployment of tungro resistance genes.

The error-prone processes of transcription and reverse transcription could generate RTBV sequence variants. If these variants establish themselves in the plant and are transmitted to other plants in the field, they will become a part of a quasispecies field population, a phenomenon often observed for viruses with RNA genomes [11, 12]. Indeed, complete nucleotide sequences of several RTBV genomes derived from an isolate in the Philippines demonstrated that the isolate was a heterogeneous population of variants [1]. However, Villegas et al. [29] suggested that although RTBV might exhibit microvariation at the field level, the transmission bottleneck should result in strong selection against the quasispecies structure and thus the virus would be expected to maintain a stable population. Here, we report on the frequency distribution of RTBV genotypes in a large population of field isolates collected from eight tungro-endemic sites in the Philippines over two cropping seasons. Based on two restriction enzymes and three virus probes, we devised a restriction fragment length polymorphism (RFLP) assay using a full-length Ic RTBV DNA probe with EcoRV-digests of total DNA extracts from RTBV infected plants that revealed a high level of polymorphism in the experimental and field virus populations. Using this assay, 17 distinct genotypes were identified and used as indicators for field virus diversity. The two provinces

surveyed, Isabela and North Cotabato, had distinct RTBV populations. The genetic composition of these populations was mostly stable with time and does not seem to be affected by the variety of host. However, instability was noted at a few sites and observed during insect transmission of RTBV field isolates in the laboratory. Implications of our results on management of tungro resistance are discussed.

Materials and methods

Collection of tungro-infected plants

Two of the main rice-producing provinces (Isabela and North Cotabato, >2000 km apart) in the Philippines were selected. In Isabela, three sites (Ilagan and Quirino, and San Manuel) were sampled in the 1996 wet season (96W) and the first two sites and Cauayan were sampled in the 1997 wet seasons (97W). Four sites were sampled in North Cotabato in the dry and wet seasons of 1997 (97D, and 97W, respectively). Tungro is endemic at all the sites (Table 1). Rice samples were collected mainly from tillering and early booting stages 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]. Approximately 0.1 g of leaf sample was used in serological testing. Samples that were ELISA-positive for RTBV (whether they were RTSV-positive or -negative) were subsequently processed for total DNA extraction, enzyme digestion and DNA hybridization. An isolate represents a single field-infected plant. During initial experiments, three RTBV variants, RTBV-L, RTBV-G2, and RTBV-BS were used. The first two variants have originated from the RTBV-BS isolate

Table 1. A detailed description for the collected samples

Location	Site	Season[a]	Variety	N[b]	RTBV[c]	RTSV	RFLP/DNA hyb
Philippines							
Isabela	IIagan	96W	PSBRc6	30	29	30	27
	Quirino		IR64	30	26	24	26
	San Manuel		IR64	30	24	28	24
	IIagan	97W	IR60	31	10	03	10
	Quirino		IR64	30	15	16	14
	Cauayan		PSBRc10	30	15	21	15
North Cotabato	M'Lang	97D	IR74	30	24	29	24
	Tulunan		Unknown	30	27	30	27
	Pigcawayan		IR68	30	29	30	29
	Kabacan		Selection 55	30	29	29	29
	M'Lang	97W	IR64	35	32	32	32
	Tulunan		Masipag	30	26	26	25
	Pigcawayan		Malagkit	30	29	29	27
	Kabacan		Farmers'39	33	30	24	28

[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). RFLP/DNA hyb column shows the number of samples that hybridized with the full-length RTBV probe

collected locally and they have been maintained at the International Rice Research Institute (IRRI) greenhouse for several years [1]. Recently, the three variants have been shown to be biologically and genetically different [2, 3].

DNA typing

Total DNA isolation, digestion, hybridization, and detection were done as described earlier [2]. The final pellet was suspended in 50 μl sterile distilled water. During initial experiments, DNA extracts were treated with RNase to remove cellular and viral RNAs. To detect viral DNA, 2–5 μg of total DNA were digested with 20–30 units *Eco*RV or *Eco*RI restriction enzymes (New England Biolabs) overnight at 37 °C and loaded into a 0.8% agarose gel (Boehringer-Mannheim) in 1×TBE. The gel was run for 5 h with appropriate standards. Southern DNA hybridization was performed using a full-length Ic plasmid DNA probe or 651-bp and 451-bp probes derived from the Ic plasmid DNA by *Eco*RV-digestion of the full-length Ic plasmid DNA. The full-length Ic probe represents the 8005 bp genome of RTBV-Ic variant that was cloned (clone number 3) in our laboratory in 1996 [1]. The 651-bp probe covers from nucleotide 3439 to 4090 within RTBV ORF3 and includes the RNA binding site and the aspartate protease functional domains. The 451-bp probe was derived from ORF4 and extends from nucleotide 6727 to 7190. The fragment probes were chosen because of their potential to differentiate a large number of RTBV variants from the field. Hybridized bands were detected via ECL chemiluminescent detection (Amersham, UK).

Total molecular weights of *Eco*RV-based genome profiles (genotypes) revealed by the full-length probe were estimated using the Molecular Analyst program (BioRad, Inc.) for which *Eco*RV-restricted Ic plasmid DNA was used as a standard. Profiles with major bands adding up to more than 16.0 kb were assumed to come from plants infected by more than one RTBV type (mixed infection). Polymorphism was scored by visual comparison of banding patterns. Distinct *Eco*RV-based genome profiles showing consistent patterns over 2–3 enzyme digestion and repeated occurrence in the population were identified and arbitrarily assigned numbers.

Data analysis

A χ^2 test was performed (SAS, Version 6, IML Software usage and reference; SAS/STAT User's guide, SAS Institute, Inc., Cary, NC) to determine whether the frequency of RTBV genotypes differed per site over two cropping seasons. Values for Nei's gene diversity were estimated from equation 2.11 of Chakraborty and Rao [5]. Standard deviations were obtained from variances calculated according to equation 2.12 of the same source.

Insect transmission

To evaluate the biological properties of RTBV field populations, the 30 collected samples from Kabacan, North Cotabato in 97D were individually insect-transmitted to the susceptible rice variety, Taichung Native 1 (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 and kept in separate cages. Three weeks post-inoculation (wpi), transmission was monitored by ELISA and Southern blot hybridization. Transmission was repeated using a larger population of TN1 plants and virus infection was checked in these plants, also at 3 wpi, by symptom severity score, ELISA and Southern blot hybridization.

Results

Polymorphism in RTBV greenhouse isolates assayed by full-length Ic, 651-bp and 463-bp probes

An ideal RFLP assay for polymorphism will, in one assay, reveal multiple polymorphic fragments, but not so many fragments as to make interpretation difficult. Thus, fragment patterns of restricted RTBV DNA probed with full length and partial probes were compared. Total DNA was extracted from 4-week-old plants infected with RTBV-L, RTBV-G2, or RTBV-BS variants, digested with *Eco*RV and *Eco*RI restriction enzymes, and hybridized sequentially with the full-length Ic, 651-bp, and 463-bp probes. A clear polymorphism was observed in the DNA profiles of the three RTBV isolates when hybridized with the full-length Ic probe (Fig. 1A). The three isolates could easily be differentiated using either *Eco*RV or *Eco*RI digests. On the other hand, hybridization with the 651-bp probe showed

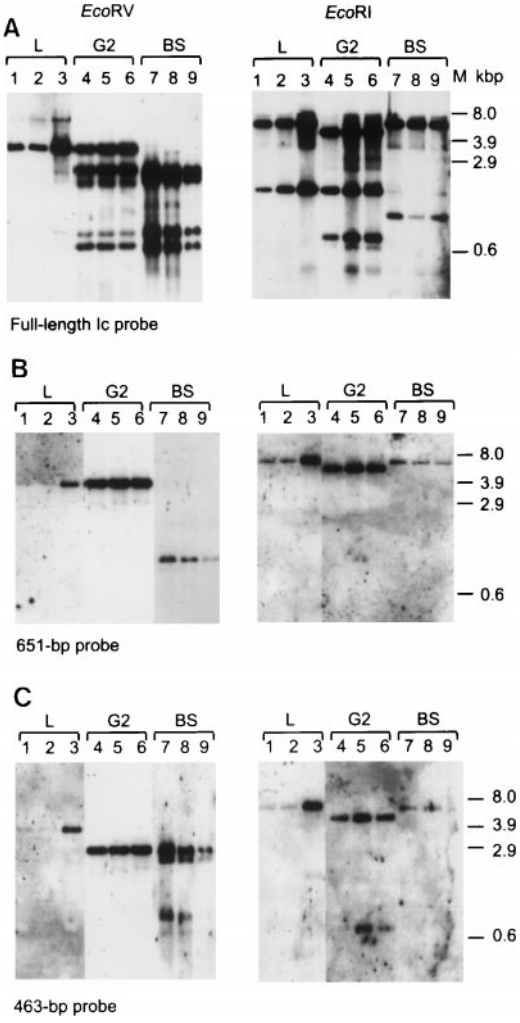

Fig. 1. Polymorphic patterns of restricted RTBV DNA for IRRI greenhouse isolates when hybridized with full-length and partial RTBV probes. Total DNA extracts from three infected plants with either RTBV-L, RTBV-G2, or RTBV-BS variant were digested with *Eco*RV or *Eco*RI restriction enzymes and hybridized sequentially with the full-length Ic (**A**), 651-bp (**B**), and 463-bp (**C**) probes. The approximate location of the composite size markers, which included the full-length and *Eco*RV-digested plasmid Ic DNA, is shown on the right

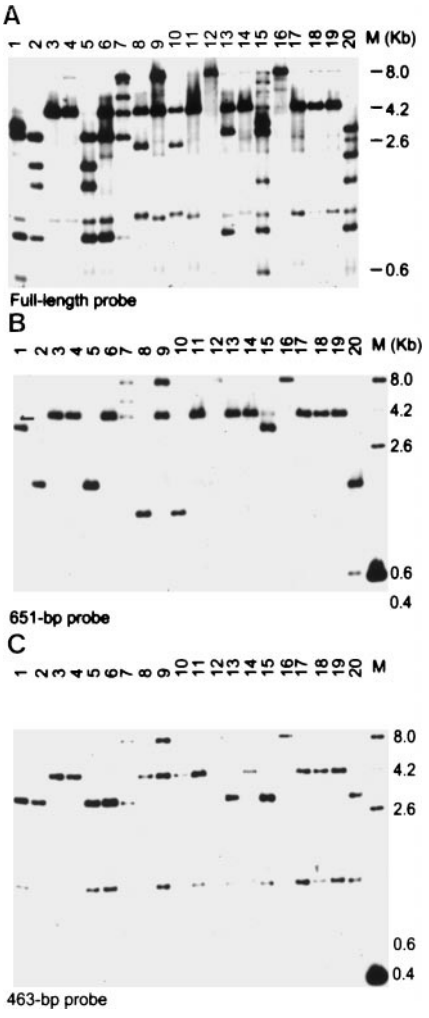

Fig. 2. Polymorphic patterns of restricted RTBV DNA for field isolates when hybridized with full-length and partial RTBV probes. Total DNA extracts from 20 random RTBV-ELISA positive samples were digested with *Eco*RV or *Eco*RI restriction enzymes and hybridized sequentially with the full-length Ic (**A**), 651-bp (**B**), and 463-bp (**C**) probes. The approximate location of the composite size markers, which included the full-length and *Eco*RV-digested plasmid Ic DNA, is shown on the right

only two polymorphic DNA patterns among the three RTBV isolates, for each restriction enzyme used (Fig. 1B). *Eco*RV digestion differentiated RTBV-BS from RTBV-L and RTBV-G2 isolates, and *Eco*RI differentiated RTBV-G2 from the other two isolates. Hybridization with the 463-bp probe showed a clear polymorphism among the three isolates using the *Eco*RV digests only (Fig. 1C). When the *Eco*RI digests were hybridized with the same probe, only RTBV-G2 could be differentiated.

Polymorphism in RTBV field isolates based on Eco*RV digestion and DNA hybridization with the three probes*

The full-length Ic, 651-bp, and 463-bp probes were compared for their ability to differentiate *Eco*RV-digested total DNA extracts from field samples. Initially, twenty random samples collected from Ilagan, Isabela 96W were used. Of the three hybridization probes, the full-length probe revealed the highest level of polymorphism (Fig. 2). Using this probe, samples fell into 9 distinct banding

patterns. For example, samples in lanes 3, 4, 11, 14, 17, 18, and 19 belong to one banding pattern and samples in lanes 6 and 13 belong to another (Fig. 2A). For some samples (e.g. lane 7) the sum of the fragments exceeded the 8-kbp size of the RTBV genome. They were considered to result from mixed infections instead of incomplete digestions because the same pattern was observed after three repeated digestions with *Eco*RV. Hybridization of the same blot with the 651-bp probe produced slightly different groupings, but, consistent with the first probe, the banding pattern within a group was maintained. Six distinct banding patterns were observed and mixed infections and/or partial digests could be identified. For example, the sample in lane 9 could be considered a mixed infection between DNA types from lanes 11 and 16 or a partial digest (Fig. 2B). The results obtained when hybridization with the 463-bp probe was performed agree with the results from the other probes. This probe showed less polymorphism than the first two probes and only 4 banding patterns were identified. Because the full-length Ic probe showed interpretable patterns and a higher level of polymorphism than the other two probes with the *Eco*RV-total DNA extracts, it was used to characterize the RTBV field populations in Isabela and North Cotabato.

RTBV diversity in the Philippines

Of 345 RTBV-ELISA positive samples, 337 could be classified by *Eco*RV digestion and DNA hybridization with the full-length and Ic probe (Table 1). The *Eco*RV-based genome profile (genotype) was defined based on two criteria: consistency of the pattern after 2–3 *Eco*RV digestions and its occurrence more than once in the total population. Genotypes, which met the two criteria, were each given a code number at random. Those that occurred only once in the population, gave inconsistent banding patterns, or represented mixed infections were classified in a "??" group. In the total population, 17 distinct genotypes were identified (Fig. 3). Their frequencies at each site were estimated (Table 2).

To compare their frequencies within and among sites as well as over time, a χ^2 test was performed. Results showed that the RTBV population at Isabela had a genetic structure distinct from the RTBV population at North Cotabato. Between sites within a province, in 96W or 97D, there were no significant differences in virus populations among most of the sites. But in 97W, significant differences were identified between the virus populations at Ilagan and that at Cauayan in Isabela and between M'Lang and Tulunan and between Tulunan and Pigcawayan in North Cotabato. In addition, within a site, except for M'Lang and Ilagan sites, there were no significant differences in the genetic composition of the virus population over the sampling time. Overall, two to nine distinct genotypes occurred at any one site, but at most sites two or three accounted for the majority of genotypes. The most notable exception was the Kabacan site in 97W. At Isabela sites, genotypes 3 and 5 dominated, while in North Cotabato, genotypes 11, 12, and 13 were the most frequent ones identified across the four sites. The province-wide pattern of

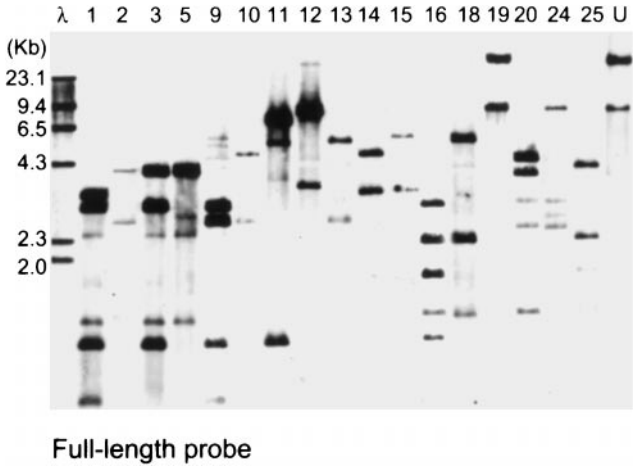

Fig. 3. The 17 distinct RTBV genotypes that were identified in the total population of the Philippines. Total DNA extracts were digested with *Eco*RV restriction enzyme and hybridized with the full-length Ic probe. The genotypes, which showed consistent patterns over 2–3 *Eco*RV digestions and repeated occurrence in the population were identified and assigned random numbers. The location of size markers using (λ-*Hin*dIII digest is shown on the left and U = undigested total DNA extract on the right)

dominant isolates did not change from season to season. The "??" group, which included the mixed infections, accounted for 11–54% of the total samples and were identified in each season across all the sites, except at Ilagan in 97W where fewer infected plants were found and analyzed.

The host variety did not noticeably affect the genetic structure of RTBV populations. Different varieties planted at different sites in the same province had statistically indistinguishable RTBV populations (for example in Table 2, compare PSBRc6 at Ilagan and IR64 at Quirino, Isabela, in 96W). The same variety planted at sites in different provinces (for example, IR64 planted at Quirino, Isabela, and M'Lang, North Cotabato in 97W) harbored statistically distinguishable populations.

Gene diversity values measure the complexity of populations, including the number of genotypes and their frequencies, and range from 0 (no diversity) to 1.0 (all genotypes equally represented). The overall gene diversities for the North Cotabato province population (0.87±0.01 in 97D and 0.85±0.01 in 97W) were significantly higher than those for the Isabela province population (0.73±0.03 in 96W and 0.70±0.04 in 97W), though differences between some pairs of sites from the two provinces were not significant, probably due to the smaller sample size. Gene diversity at the Cauayan site was significantly lower than for all other sites. Populations at M'Lang and Pigcawayan became significantly less complex from 97D to 97W, while that at Kabacan became more complex over the same period, consistent with the lack of dominating isolates at this site in 97W. Taken together, the χ^2 and gene diversity analyses suggest an overall stability in population structure with occasional significant changes at individual sites.

Table 2. Frequency of genotypes of rice tungro bacilliform virus (RTBV) per site over two cropping seasons

	Philippines													
	Isabela-96W			Isabela-97W			North Cotabato-97D				North Cotabato-97W			
Site Variety	Ilagan PSBRc6	Quirino IR64	San Manual IR64	Ilagan IR60	Quirino IR64	Cauayan PSBRc10	M'Lang IR74	Tulunan Unknown	Pigcawayan IR68	Kabacan Sel 55	M'Lang IR64	Tulunan Masipag	Pigcawayan Malagkit	Kabacan F 39
Genotype														
1	3 (11)[a]													
2	2 (7)	5 (19)							1 (3)					
3	2 (7)	4 (15)	5 (21)	3 (30)	3 (21)	2 (13)		1 (4)						
5	10 (37)	12 (46)	7 (29)	2 (20)	4 (29)	10 (67)								
9	1 (4)			3 (30)										
10							3 (12)	1 (4)	3 (10)	1 (3)		1 (4)	3 (11)	2 (7)
11	1 (4)		1 (14)				5 (21)	3 (11)	1 (3)	4 (14)	1 (3)	5 (20)	6 (22)	2 (7)
12	3 (11)	1 (4)	2 (8)				4 (17)	3 (11)	4 (14)	6 (21)	2 (6)	5 (20)	5 (18)	2 (7)
13							1 (4)	5 (18)	4 (14)		12 (37)		7 (26)	2 (7)
14							2 (8)			2 (7)	1 (3)	3 (12)		
15							1 (4)	1 (4)	3 (10)	1 (3)		2 (8)	1 (4)	
16			2 (8)				1 (4)							2 (7)
18							1 (4)	1 (4)	2 (7)	2 (7)		3 (12)		
19			1 (4)		1 (7)			2 (7)	1 (3)		4 (12)	1 (4)	2 (7)	2 (7)
20							1 (4)	3 (11)	2 (7)		1 (3)			1 (4)
24										1 (3)		1 (4)		
25		1 (4)		2 (20)	4 (29)									
??	5 (18)	3 (11)	6 (25)	–	2 (14)	3 (20)	5 (21)	7 (26)	8 (28)	12 (41)	11 (34)	4 (16)	3 (11)	15 (54)
Total[b]	27	26	24	10	14	15	24	27	29	29	32	25	27	28
h[c]	0.77 ± 0.06	0.68 ± 0.06	0.78 ± 0.05	0.82 ± 0.05	0.77 ± 0.05	0.30 ± 0.10	0.88 ± 0.03	0.90 ± 0.03	0.90 ± 0.02	0.83 ± 0.05	0.65 ± 0.07	0.87 ± 0.03	0.82 ± 0.03	0.92 ± 0.03
χ^2-Test	[22 a]	[23 ab]	[18 ac]	[10 c]	[12 bcd]	[12 ad]	[19 eh]	[20 efh]	[21 efh]	[17 egh]	[21 f]	[21 h]	[24 ef]	[13 efh]

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

[b]Total number of samples per variety per location. The numbers used for the Chi-square (χ^2) 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 RTBV genotypes between two populations within sites or between sites over time. Because more than 60% of the cells have expected counts less than 5, the Chi-square test might not be a valid test

[c]h: gene diversity based on equations 2.11 and 2.16 of Chakraborty and Rao [5]

Insect transmission of RTBV field isolates from one field in Kabacan

An attempt to transmit the 30 isolates collected from Kabacan, North Cotabato, during 97D by insects resulted in the recovery of six isolates. When these isolates were passaged by another insect transmission into a larger population of TN1 plants, a variety of symptoms were observed. Figure 4 shows the symptoms, percent infection as determined by ELISA, and RTBV DNA profiles of four such sets of derived populations. Plants inoculated with transmitted isolate 2 (Fig. 4 A), were highly infected with both tungro viruses (88%), however, the symptoms exhibited were mild. When the total DNA was extracted from each plant, digested with *Eco*RV, and probed with the full-length Ic probe, a relatively uniform set of genotype patterns resulted. Most of the *Eco*RV-based genome profiles were identical to those of the original (O) and of the first transmitted (1^{st}) isolate. Similar results were obtained when isolate 3 was examined (Fig. 4B). Although the plants inoculated with this isolate were mainly infected with RTBV alone based on the ELISA results, very few had a RTBV DNA profile different from that of the original isolate. Plants inoculated with transmitted isolate 4 (Fig. 4C), were 100% jointly infected with both tungro viruses but exhibited variation in symptoms. These isolates also showed a large variety of *Eco*RV-based genome profiles. Only plant # 4.2 showed a pattern consistent with that of the original isolate. Similar results were obtained when the genome profiles derived from isolate 8 were examined (Fig. 4D). In this population, the symptoms were so severe that total DNA could only be extracted from 10 out of 31 inoculated plants, digested with *Eco*RV and hybridized. The recovered genome profiles were not identical to those of the original or first transmitted isolate. The proliferation of profiles (Figs. 4C and 4D) is unlikely to be due to incidental contamination. Control plants not exposed to insects did not develop symptoms and did not have RTBV DNA detected by hybridization. Insects from the same source used in the above experiments and given access to plants not infected with RTBV did not, after transfer to further plants, produce plants with disease symptoms or hybridization patterns with the RTBV probe. The experiments shown in Fig. 4 were done side-by-side. These experiments were repeated at least twice with similar results.

Discussion

In this study, three probes and two restriction enzymes were evaluated for their usefulness in detecting polymorphisms among experimental and field RTBV populations. Results showed that the full-length Ic probe detects high levels of polymorphism in RTBV field populations when used with *Eco*RV-restricted total DNA extracts from ELISA-positive plants. In addition, the probe has a broad-spectrum detection capacity because 337 out of the 345 ELISA-positive samples could be detected and classified after *Eco*RV digestion. Based on their repeated occurrence in the total population and their consistent banding profile over 2–3 *Eco*RV digestions, the identified genotypes were used to analyze the genetic diversity of RTBV populations in the Philippines.

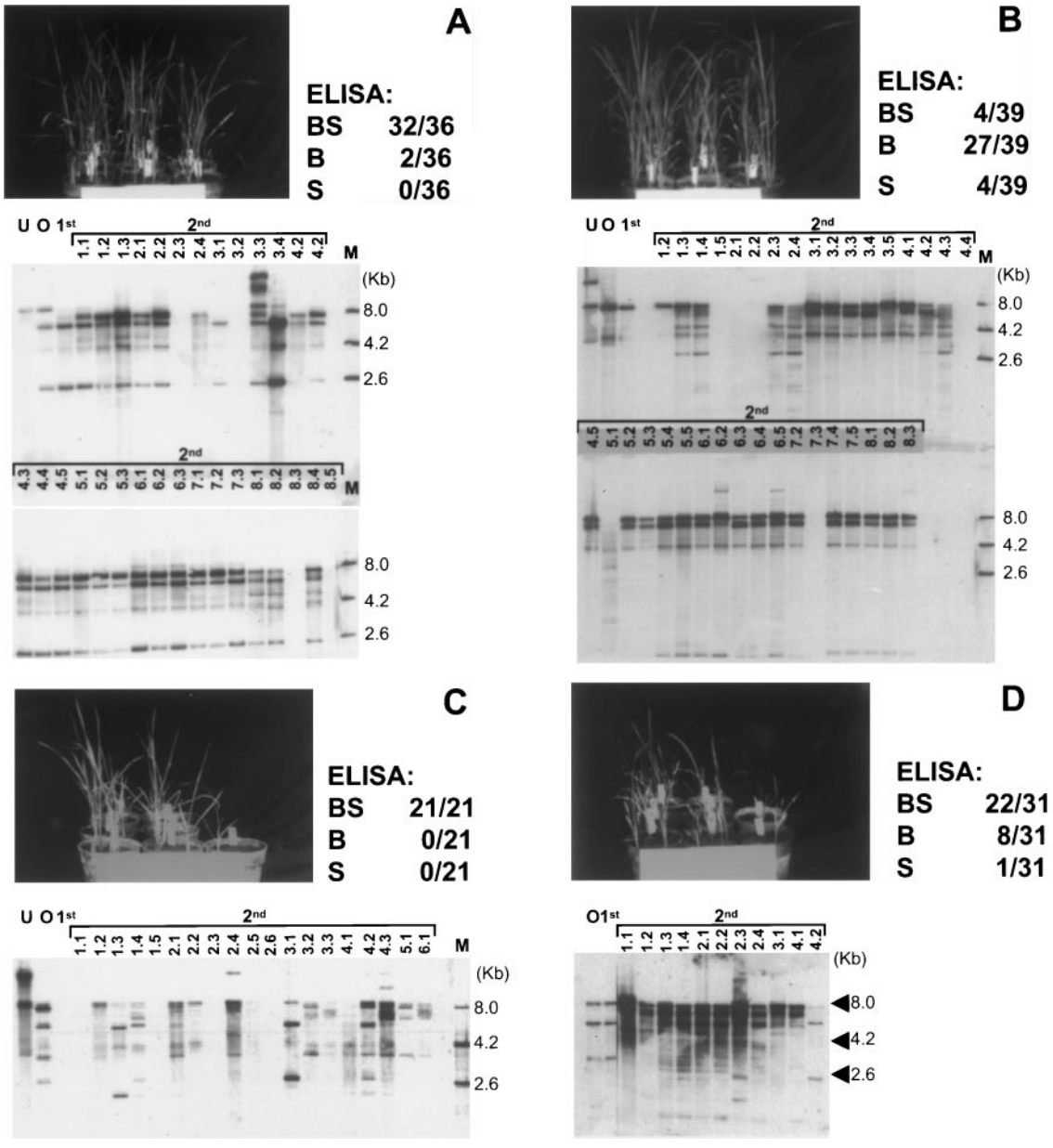

Fig. 4. Symptoms, percent infection by ELISA, and RTBV DNA profiles of derived populations from four transmitted isolates (**A–D**). The isolates were recovered from Kabacan, North Cotabato and passaged twice by insect transmission under greenhouse conditions. At three weeks post inoculation, symptoms were recorded, plant extracts were tested by ELISA, and total DNA extracts were digested by *Eco*RV and hybridized with the full-length Ic probe. *U* Undigested total DNA extract from the original field isolate; *O Eco*RV-genome profile of the original field isolate; *1*st *Eco*RV-genome profile of the same isolate when transmitted for the first time; *2*nd *Eco*RV-genome profile of derived populations from the same isolate after second transmission. Numbers like 1.1 reflect the pot and the plant number within that pot for the same samples shown in the picture on the top

From eight tungro-endemic sites of the Philippines and over two seasons, 17 distinct genotypes were identified in the RTBV populations and a distinct virus genetic structure was identified in Isabela and North Cotabato provinces. At each site within a province, two to nine genotypes were observed with different frequencies but a few genotypes were usually dominant and persisted over time. High proportions of infections were mixed at almost all sites and in both seasons. Among sites within a province, there were few significant differences in virus populations. These results suggest that RTBV populations in the Philippines are genetically diverse, geographically isolated, and generally stable under current conditions.

Recombination can play an important role in generating and maintaining RTBV diversity. At individual sites, 11–54% of samples were infected with more than one RTBV genotype, based on molecular weight estimates. Phylogenetic analyses of IRRI RTBV variants showed that recombination must have generated diversity among RTBV isolates [1]. Recombination by a mechanism of template switching during reverse transcription has also been demonstrated for CaMV viral genomes, a similar pararetrovirus [10]. In addition, several studies have shown that recombination of CaMV viral genomes occurs frequently in its turnip host [7, 17, 21].

The geographical isolation of RTBV populations could be imposed by several barriers. The transmission bottleneck is very critical for RTBV adaptability and pathogenicity in rice. The virus depends on RTSV for transmission and therefore, local RTSV populations might determine the kind and number of transmissible RTBV variants present in the province. At present, we do not have any evidence whether local RTSV populations influence the extent of RTBV diversity, or if RTSV populations are geographically distinct. Another barrier could be the dispersal ability of the vector that could restrict the movement of RTBV to a specific region. Studies on the flight behavior of *N. virescens* in rice fields [6, 25] have shown that the dispersal range of most leafhoppers is limited to short distances. Therefore, limited dispersal of RTBV populations due to a limited dispersal of the green leafhopper becomes plausible.

The RTBV populations studied appear generally to be stable in genetic composition and biological effects. Few significant differences in genotype profiles exist among sites in the same province. For the most part, the genotype profile at a site or in a province does not change significantly from season to season. In general, gene diversity values did not change from site to site within a province from season to season. Despite the existence of dominant genotypes in these populations, equilibrium with minor genotypes appears to exist.

However, occasional instabilities in genetic composition and biological effect were detected against the background of general stability. These instabilities occurred both in field populations and in laboratory propagations. Significant changes in genotype composition between seasons occurred at two of six sites. Gene diversity changed significantly at three sites from one season to the next and was strikingly lower at one site than at all others. In the laboratory, controlled transmission by leafhoppers resulted in two isolates that produced an explosion

of new genotype patterns, correlated with a diversity of tungro symptoms. The new patterns may have been due to minor variants present in the initial isolate at concentrations too low to be detected or to extensive recombination among genotypes present in the isolates. Further work is needed to confirm the identity of these variants. Because further transmission of isolates 4 and 8 showed the recovery of stable RTBV populations with milder symptoms from these isolates (data not shown), the proliferation of genotypes may have been transient. The pattern of occasional dramatic shifts in virus populations super-imposed on a general stability of genetic composition suggests chaotic behaviour in which populations are at a delicate equilibrium easily shifted by small perturbations to new equilibria.

The geographic isolation of RTBV populations in Isabela and North Cotabato show that deployment of targeted resistance genes might be an effective strategy in managing tungro outbreaks in these provinces. Although several of the varieties studied have vector resistance genes, they were similar in the RTBV populations they supported to the other varieties and do not seem to exert selection pressure on the local virus population under the current conditions. However, since the time scale of our study was very short (less than two years), further experiments are still needed to better understand the dynamics of RTBV field-populations and their response to the deployment of virus resistance genes.

In summary, our results show that RTBV field populations are diverse, geographically isolated and relatively stable. The genetic structure of RTBV populations in Isabela was significantly different from that in North Cotabato. Over the sampling time within a site, there were no significant differences in the genetic composition of virus populations indicating an inter-site genetic stability. Under the current conditions, IRRI rice varieties are similar to other varieties and do not seem to exert selection pressure on the virus population. However, the transmission experiments show that the RTBV isolates from a single site may change dramatically resulting in different biological properties.

Acknowledgements

The authors wish to thank Lani Quintana for the analysis; Ulrich Melcher for the gene diversity calculations, helpful suggestions, and editing of the manuscript; Ed Coloquio, Panfilo Domingo, and Esquirion A. Baguioso for their technical help; and Roger Cabunagan, Jessie Fernandez, and Truong Hoai Xuan for their assistance in collecting samples.

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Received June 25, 1999