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Molecular analysis of the coat protein gene of peanut stripe virus from China

S.-S. HOU[1$], Y.-C. CHI[2$], Y.-Y. LIU[1], X.D. LI[3*], S.-L. YU[2], X.-D. LI[1*]

[1]Department of Plant Pathology, College of Plant Protection, Shandong Agricultural University, Tai'an, Shandong 271018, P.R. China; [2]Shandong Peanut Research Institute, Qingdao, Shandong 266100, P.R. China; [3]State Key Laboratory of Crop Biology, College of Agronomy, Shandong Agricultural University, Tai'an, Shandong 271018, P.R. China

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Summary. – Peanut stripe virus (PStV) is one of the most common viruses infecting peanut that causes great economic losses every year. The 3′-terminal 1082 bp of 74 PStV isolates collected from 12 districts of Shandong province, China were sequenced. Their coat protein (CP) genes were 864 bp in length and shared identities of 98.0%~100% and 98.3% ~100% at nt and aa levels. The identities between the CP genes of these isolates and other 36 isolates from the GenBank were 93.5%~100% and 92.0%~100% at nt and aa levels, respectively. PStV isolates can be clustered into two phylogenetic groups. The isolates from United States, mainland China, and Indonesia formed group I and those from Viet Nam, Thailand, and Taiwan formed group II. The PStV isolates in group I can be further classified to two subgroups. The gene flow of PStV populations within a country was frequent, but that between countries was infrequent.

Keywords: peanut stripe virus; molecular variability; phylogenetic analysis; gene flow

Introduction

Peanut (*Arachis hypogaea* L.) is one of the most important oilseed crops and food legumes in the world. In China, peanuts are grown on 3.5 million hectares each year. However, viral diseases are important limiting factors constraining the production of peanuts. So far, there have been 28 viruses belonging to the 7 families and 12 genera reported to infect peanut (Xu and Chen, 2008).

Peanut stripe virus (PStV; the family *Potyviridae*, the genus *Potyvirus*) was first described in USA in 1984 and initially regarded as a strain of bean common mosaic virus (BCMV) or peanut mottle virus (Xu *et al*., 1983; Saleh *et al*., 1989). PStV is one of the most widely distributed peanut viruses that can be detected in China, Indonesia, Korea, Malaysia, Philippines, Thailand, USA, Viet Nam, and other countries (Xu *et al*., 1983; Demski and Lovell, 1985; Reddy *et al*., 1988; Saleh *et al*., 1989; Choi *et al*., 2006). PStV can be transmitted by aphids in a non-persistent manner. Its incidence was 10–100% in the fields and 1–50% in peanut seeds (Chen *et al*., 1990; Xu *et al*., 1991; Bi *et al*., 1999; Xu, 2002). The infested peanut seeds serve as a primary infection source in the field. PStV causes various symptoms on peanut including stripe, mild mottle, and blotch sometimes surrounded by necrotic or chlorotic ringspot (Middleton and Saleh, 1988). There are many biologically distinct PStV strains reported. Wongkaew and Dollet (1990) categorized 24 PStV isolates from 8 countries into 8 strains based on their symptoms on hosts of specific genotypes. Two PStV strains, Ts and Tc, were detected in Taiwan and induced severe mosaic and systemic necrosis or stripe and vein-banding, respectively (Chang *et al*., 1990). The PStV isolates from mainland China are classified into three groups – mild mottle, blotch, and necrosis – according to their symptoms on the peanut (Chen *et al*., 1999).

The genomic characteristics of some PStV isolates have been reported (Cassidy *et al*., 1993; Gunasinghe *et al*., 1994; Flasinski *et al*., 1996; Chen *et al*., 1999). However, these studies analyzed the sequences of a small number of isolates. So

*Corresponding authors. E-mail: xdongli@sdau.edu.cn, lixdong@sdau.edu.cn; fax: +86-538 8226399. $These authors contributed equally to this research.

Abbreviations: AzBMV = azuki bean mosaic virus; BCMV = bean common mosaic virus; CP = coat protein; d_N = average number of non-synonymous substitutions per non-synonymous site; d_S = average number of synonymous substitutions per synonymous site; NTR = nontranslated region; PStV = peanut stripe virus

far, there has been no report about the genetic structure of PStV populations available. Shandong is one of the biggest peanut production areas in China and exports 60 million tons of peanuts every year (Ma *et al.*, 2007).

In this study, we determined the 3′-terminal 1082 bp sequence of 74 PStV isolates coming from Shandong. Further, we analyzed molecular diversity and evolutionary relationships of PStV isolates from different regions.

Materials and Methods

Virus isolates and RNA extraction. During the seasons 2008 and 2009, peanut leaves showing typical viral symptoms were collected from 12 districts of Shandong province. The samples were used to extract total RNA directly or stored at -80°C for further use. Peanut leaves (200 mg) were ground with liquid nitrogen, mixed with 1 ml of Transzol (TransGen Biotech) and incubated for 5 mins at room temperature. After addition of 200 µl chloroform, the mixture was vortexed for 15 secs and extracted for 3 mins. Then, the mixture was centrifuged at 12,000 rpm for 15 mins. The supernatant was precipitated with 500 µl isopropanol for 10 mins and centrifuged at 12,000 x g for 10 mins. The pellet was washed with 75% ethanol, air-dried, and resuspended in 50 µl RNase-free water.

RT-PCR. The first strand cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) according to the instructions with the initial primer CP-R (5′-CCACACTGAAACTAAAGAGA-3′, complementary to nt 9891–9872 of the sequence Acc. No. U05771) (Gunasinghe *et al.*, 1994). Primary mixture (7 µl of total RNA, 1 µl of 25 mmol/l CP-R, 8.5 µl RNase free water) was incubated at 70°C for 5 mins, on ice for 5 mins, and then 5 µl 5× M-MLV buffer, 2 µl dNTP (10 mmol/l each), 0.5 µl RNase inhibitor, and 200 U of M-MLV reverse transcriptase were added and incubated at 37°C for 1 hr. Then the cDNA was amplified by PCR using primers CP-R and CP-F (5′-TACATAGCAGAATCAGCACT-3′, identical to nt 8810–8829 of Acc. No. U05771) (Higgins *et al.*, 1998). PCR reaction mixture contained 2 µl template cDNA, 1 µl of 10 mmol/l of each primer, 2 µl dNTP (2.5mmol/l each), 1 U *Taq* DNA polymerase (Takara). PCR program included an initial denaturation step at 94°C for 3 mins followed by 5 cycles of 45 secs at 94°C, 45 secs at 37°C, 1.5 min at 72°C, then 32 cycles of 45 secs at 94°C, 45 secs at 52°C, 1.5 min at 72°C with a final extension of 10 mins at 72°C. The PCR products were examined by 0.8% agarose gel electrophoresis.

Cloning, sequencing, and sequence analysis. Target fragments were cloned into pMD18-T vector (Takara Dalian). Recombinant plasmids were screened by electrophoresis and PCR, and sequenced by Biosune Biotechnology Company. For each isolate, two clones from independent PCR reactions were sequenced twice. If there was discrepancy at any position, at least two more clones were sequenced to determine the consensus base (Yu *et al.*, 2007; Liu *et al.*, 2009).

The CP gene sequences of PStV isolates obtained in this study and those available in the GenBank were aligned using Clustal W program (Thompson *et al.*, 1994). Their nt and aa identities were calculated by DNAstar (Lasergene). Phylogenetic analyses were performed using the neighbor-joining (NJ) method, minimum evolution (ME) method, and unweighted pair-group method using arithmetic averages (UPGMA) packaged in the MEGA4.0 software (Tamura *et al.*, 2007). All branches with bootstrap values support <50% were collapsed. Only bootstrap values >80% were listed. The CP genes of one BCMV isolate and one azuki bean mosaic virus (AzBMV) isolate were used as outgroup. MEGA4.0 was also used to calculate the ratio of non-synonymous (d_N, amino acid-altering) to synonymous (d_S, silent) substitutions for the CP gene of PStV. A value of $d_N/d_S = 1$ means that neutral evolution has occurred. When $d_N/d_S < 1$ or > 1, it means that negative (purifying) or positive (diversifying) selection, respectively, has occurred.

Analyses of population demography and gene flow. DnaSP version 4.10 was used to estimate the values for Tajima′s D, Fu & Li's D and F statistical tests and population genetic parameters including haplotype diversity and nucleotide diversity (Tajima, 1989; Fu and Li, 1993; Rozas *et al.*, 2003). Tajima′s D test compares the nucleotide diversity with the proportion of polymorphic sites. Fu & Li's *D test* is based on the differences between the number of singletons (mutations appearing only once among the sequences) and the total number of mutations. Fu & Li's F test is based on the differences between the number of singletons and the average number of nucleotide differences between pairs of sequences. The negative values of Tajima′s D, Fu & Li′s D and F indicated that a population maintained low frequency polymorphism and demographic forces were acting on the population, while positive values implied a balancing selection and decrease of the population size. Mismatch distribution among the CP gene of PStV was used to evaluate a population with a star-like phylogeny due to the accumulation of low frequency mutations during recent expansion. The level of gene flow between populations was measured using DnaSP 4.10 by estimating F_{st}, the standardized variance in allele frequencies across populations (Rozas *et al.*, 2003). $F_{st} = 0$ indicated undifferentiated populations, while $F_{st} = 1$ indicated fully differentiated populations. The absolute value of $F_{st} > 0.33$ suggested an infrequent gene flow.

Results and Discussion

Nucleotide and amino acid identities between PStV isolates

The 3′-terminal 1082 bp genomic sequences of 74 PStV isolates were obtained. The resultant sequences included 129 bp encoding partial NIb, 864 bp encoding CP, and 89 bp 3′-nontranslated region (NTR). These sequences were deposited in the GenBank and allocated Acc. Nos. of HM776057–HM776130.

The CP genes of these 74 PStV isolates showed identities of 98.0%~100% and 98.3%~100% at nt and aa levels, respectively. They shared 93.5%~100% nt identities and

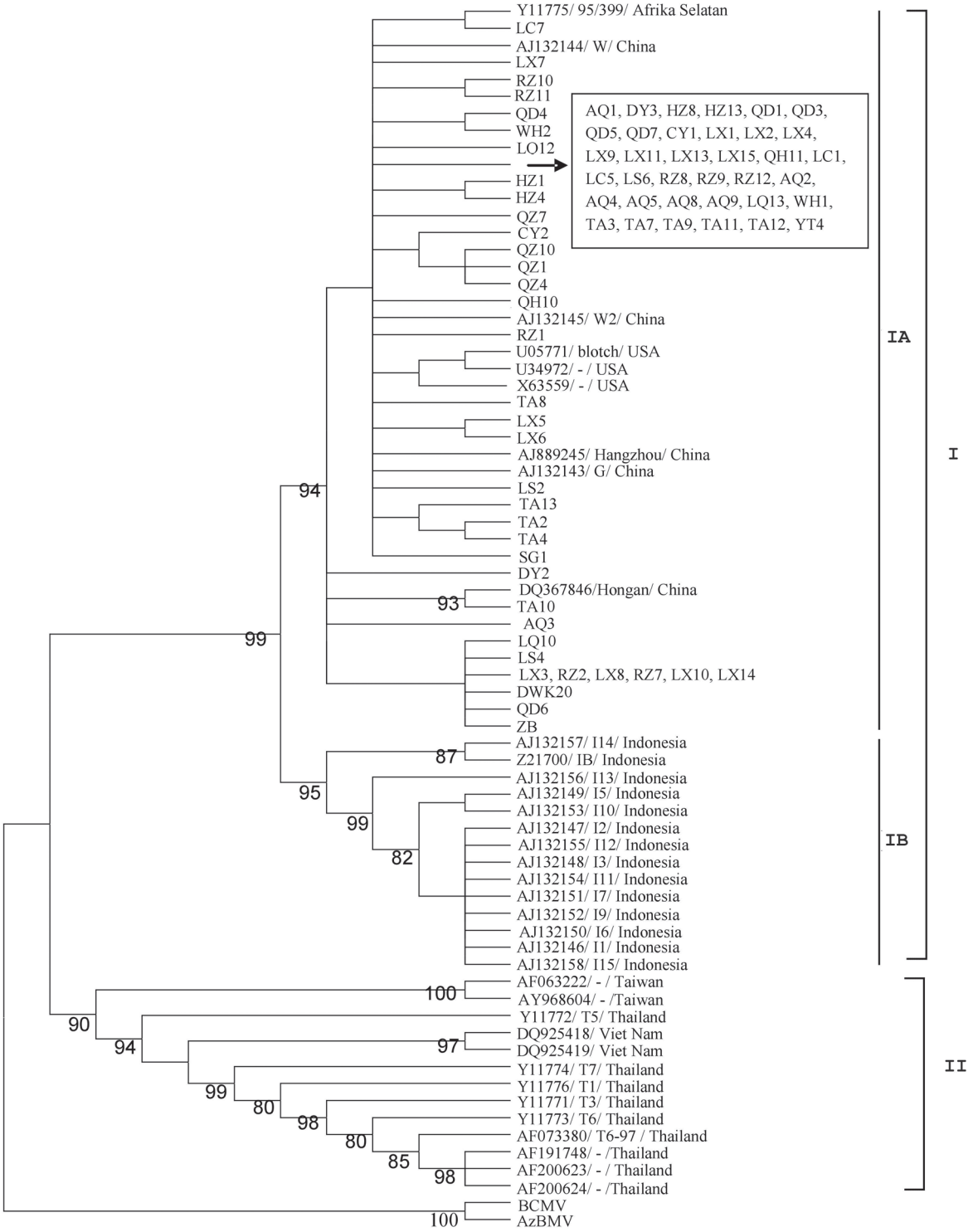

Fig. 1

Phylogenetic tree based on the nucleotide sequences of CP gene of PStV isolates

Isolates characterized in this research are indicated only by the isolate names, while those from the GenBank are indicated by the Acc. No./isolate name/ geographical origin. Roman numerals on the right show groups and subgroups.

92.0%~100% aa identities with other 36 PStV isolates available in the GenBank. The isolates characterized in this research were most similar to those from USA with identities of 98.4%~99.8% at nt level and 98.6%~100% at aa level. Their nt and aa identities with isolates from Taiwan, Indonesia, and Thailand were 93.8%~94.9% and 94.4%~96.2%, 96.5%~97.9% and 96.5%~98.6%, 93.5%~96.0% and 92.7%~96.9%, respectively.

Phylogenetic relationship of PStV isolates

The phylogenetic relationship of these PStV isolates was calculated with the CP genes using NJ, ME, and UPMGA methods assembled in the program MEGA 4.0 with corresponding sequences of BCMV and AzBMV as outgroup. The phylogenetic trees were identical in topology, but only the NJ tree was presented here (Fig. 1). The 110 isolates were clustered into two geographical origin-specific groups. Group I consisted of 97 isolates from mainland China, Indonesia, South Africa, and USA, while group II contained 13 isolates from Taiwan, Thailand, and Viet Nam. Group I could be further divided into two subgroups. The isolates from mainland China, USA, and one isolate from Africa formed the subgroup IA and those from Indonesia formed the subgroup IB.

The genetic distance between groups I and II (0.054 ± 0.006) was two or five times bigger than the distance within groups, e.g. 0.011 ± 0.002 for group I and 0.026 ± 0.003 for group II. The genetic distance between subgroups IA and IB (0.026 ± 0.005) was also bigger than that within subgroups (both 0.006 ± 0.001). These results indicated that the classification of PStV isolates into groups I and II and further classification of the group I into subgroups IA and IB was reasonable.

Table 1. Nucleotide diversities for CP gene of PStV isolates from different regions

		Mainland China	Thailand	Indonesia
Mainland China	d_N	0.002 ± 0.000	0.016 ± 0.004	0.008 ± 0.003
	d_S	0.016 ± 0.003	0.138 ± 0.024	0.063 ± 0.016
	d_N / d_S	0.125	0.114	0.127
Thailand	d_N		0.007 ± 0.002	0.019 ± 0.005
	d_S		0.029 ± 0.007	0.156 ± 0.027
	d_N / d_S		0.233	0.120
Indonesia	d_N			0.002 ± 0.001
	d_S			0.018 ± 0.005
	d_N / d_S			0.111

Subpopulation is considered as a set of isolates collected from a given geographical region. Nucleotide diversities are computed separately for d_N and d_S positions in CP genes by Pamilo-Bianchi-Li method assembled in MEGA 4.0.

Selection pressure operating on PStV populations from different countries

We grouped all these PStV isolates according to their geographical origins and calculated the mutation frequency of non-synonymous and synonymous (d_N/d_S ratio) among CP genes (Table 1). The d_N/d_S ratios of PStV CP genes within and between groups were less than 1.0 indicating that the CP gene of PStV was under negative (or purifying) selection. The d_N/d_S ratios for groups of mainland China and Indonesia were similar (0.125 and 0.111), but that for isolates from Thailand (0.233) was higher implying that the PStV isolates from Thailand were under a higher selection pressure. The number of isolates from USA, Viet Nam, and South Africa were not sufficient enough and therefore, these isolates were not included in the analysis.

Population demography

The values of Tajima′s D, Fu and Li′s D and Fu and Li′s F for the whole PStV population were significantly negative (P <0.02; Table 2), what meant that PStV population was in a state of expanding. The values of Tajima′s D, Fu and Li′s D, and Fu and Li′s F for populations of Thailand and Indonesia were also negative, but the results were not significant. The values for PStV population of mainland China were less than 0 with P <0.02 implying that this population was in a state of expansion (Table 2). The situation of PStV subpopulation of Shandong was identical to that of mainland China. The haplotype diversity of all populations was close to 1 with the smallest one for PStV population from Indonesia. The nucleotide diversity of all populations was low and the lowest one was for the population of the mainland China (0.00606) indicating that PStV population was in a low frequency polymorphism. The mismatch distribution for PStV population of mainland China showed a smooth unimodal peak and fit into the expected model of sudden expansion after a bottle neck (Fig. 2a). This result suggested that PStV was relatively new emergent and still intact in the mainland China. The mismatch distribution of PStV populations of Indonesia and Thailand showed ragged multimodal peak, implying that PStV had existed for a long time in these regions and the population had been in a balancing state (Fig. 2b, c).

Gene flow analysis

The absolute values of F_{st} between populations of the mainland China and those from Thailand and Indonesia were >0.33 (0.81887 and 0.75452, respectively) indicating that the gene flow between China and these two countries was infrequent. The gene flow of PStV between populations from Thailand and Indonesia was infrequent either (the absolute value of F_{st} was 0.83180). However, the absolute

Table 2. Neutrality tests, haplotype, and nucleotide diversity of PStV

Group	Tajima′s D	Fu & Li′s D	Fu & Li′s F	Haplotype diversity	Nucleotide diversity
All	-1.84223*	-3.79338**	-3.52001**	0.986±0.005	0.01963±0.00223
Thailand	-1.15261	-1.41927	-1.52032	0.917±0.092	0.01196±0.00302
Indonesia	-1.55503	-1.38460	-1.63512	0.795±0.109	0.00625±0.00255
Mainland China	-2.51253***	-5.95737**	-5.46540**	0.979±0.009	0.00606±0.00052
Shandong	-2.49436**	-5.81500**	-5.37501**	0.981±0.008	0.00605±0.00052

*P <0.05; **P <0.02; ***P <0.01.

values of F_{st} within populations of mainland China, Thailand, and Indonesia were all <0.33 (0.01282, 0.12500 and 0.08333, respectively) indicating that the gene flow of PStV within a country was frequent.

The molecular characterization of several PStV isolates has been reported (Higgins *et al.*, 1998; Chen *et al.*, 1999). The PStV isolates from Thailand were closely related to each other, but not to the isolates from USA or Indonesia (Higgins *et al.*, 1998). Chen *et al.* (1999) showed that the PStV isolates from China and USA belonged to one group, while those from Thailand and Indonesia belonged to the different groups. Our assessment of more than 100 isolates from several countries showed that the Chinese and American PStV isolates were clustered to the one well-defined subclade (subgroup IA), the Indonesian PStV isolates formed a sister subclade (subgroup IB), while the PStV isolates from Thailand, Taiwan, and Viet Nam belonged to the different clade (group II). All these PStV isolates shared high identities at nt and aa levels and therefore had the same origin (Higgins *et al.*, 1998; Chen *et al.*, 1999). However, the geographical origin-specific phylogenetic results indicated that PStV isolates may evolve separately in the different geographical locations. The PStV isolates from mainland China and USA were closely related that was consistent with the hypothesis that PStV was introduced to the USA from China (Demski and Lovell, 1985). Our results also showed that PStV isolates from Thailand were more diverse and under a higher selection pressure. Considering these results together with the shorter length of branch of group II in the phylogenetic tree (Fig. 1), we hypothesized that PStV originated in Thailand.

The frequent gene flow of PStV isolates within a country and infrequent gene flow of PStV between countries reflected the fact that seed transportation and exchanging of peanut resources was far more frequent within a country than between countries. Though the origin remained unknown, the isolate 95/399 Afrika Selatan was closely related to the isolates of subgroup 1A and probably was introduced to Africa through the seed exchange. The supervision and constraint of peanut seed transportation and resources exchange should be always emphasized.

PStV isolates from one region are highly variable in the severity of symptoms and efficiency of aphid transmission. However, their identities of CP gene at nt and aa levels were higher than 98% and even reached 100% for some isolates (Wongkaew and Dollet, 1990; Higgins *et al.*, 1998; Chen *et al.*, 1999) suggesting that the symptom expression and ef-

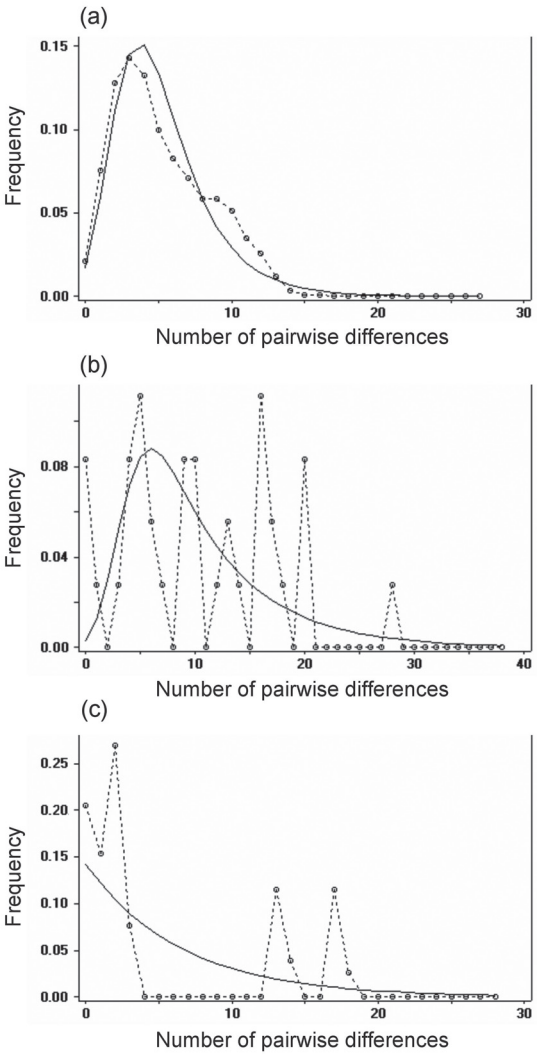

Fig. 2

The mismatch distribution of PStV populations from mainland China (a), Thailand (b), and Indonesia (c)

Broken line represents the observed data and unbroken line represents the data expected under the sudden population expansion model.

ficiency of aphid transmission of PStV may be regulated by genes other than CP.

Mismatch distribution analysis was often used to evaluate the state of existing viruses. The turnip mosaic virus isolates of group basal-BR were proved to be new emergents and were under a state of expansion (Tomitaka *et al*. 2006, 2007). Our results indicated that the PStV population as a whole was in the state of expansion and that from mainland China was new emergent and still intact.

Recombination of potyviruses is common and exists not only between isolates of a virus species, but also between virus species of the same genus or different genera (Silbernagel *et al*., 2001; Valli *et al*., 2007). The blotch isolate IB from Indonesia was reported to be a recombinant of PStV and blackeye cowpea mosaic virus with the recombination joint located at CP/3'NTR (Revers *et al*., 1996), while the isolate T3 from Thailand was reported to be a recombinant of different PStV isolates (Higgins *et al*., 1998). We detected possible recombination event in the CP genes of these 110 PStV isolates with several recombination detection programs, however, we failed to detect any "clear" recombinant event. One reason for this outcome was that we just analyzed the CP genes of isolates and therefore, we could not detect recombinant events between different genes or regions like CP/3'NTR. Another reason was that our criteria were much stricter, e.g. only those isolates supported by four softwares and showing the *P* values $<10^{-6}$ were regarded as "the clear recombinants". According to the previous studies, the recombination among CP genes of PStV was less frequent than among other genes (Silbernagel *et al*., 2001; Valli *et al*., 2007) .

The most effective measure to control PStV is the planting of resistant peanut cultivars, and evidently, genetic engineering is an important tool for breeding such cultivars. The data presented here provide a clue for the preparation of efficient RNA interfering vectors needed for the engineering of resistant peanut plants and for the design of suitable PCR primers for PStV detection.

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