International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (4), pp. 001-009, April, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Molecular characterization of *CITRUS TRISTEZA* virus strains in Peninsular Malaysia

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Accepted 11 March, 2019

Sixty Malaysian *CITRUS TRISTEZA* virus (CTV) isolates were characterized by bi-directional polymerase chain reaction (BD-PCR) and restriction fragment length polymorphism (RFLP) analysis of their coat protein (CP) gene. In BD-PCR analysis, 392-bp fragments were amplified from seven isolates. The other 53 isolates produced only 320-bp fragments. RFLP patterns of RT-PCR products of CP gene digested with HinfI restriction enzyme were similar to I-IV, VI-VII and two new groups. Isolates AMK1, AMJ12, AMT38, AMT39 and AMT43 could not be classified when they were compared to any standard CTV digest pattern. These isolates produced a unique restriction pattern with two fragments of 210 and 300 bp and isolate AMI61 produced different restriction pattern with three fragments of about 100, 270 and 300 bp. Therefore these isolates were designated as Groups IX and X. These results suggest that CTV populations in Malaysia contain new genetic variants.

Key words: *Citrus tristeza* virus (CTV), strains, coat protein (CP) gene, restriction fragment length polymorphism (RFLP), bi-directional PCR, Malaysia.

INTRODUCTION

Citrus is a very ancient crop that occurs since over 4000 years ago (Mukhopadhyay, 2004). It is an important commercial fruit crop worldwide with a total production of 105.4 million tons, which is grown in tropical and subtropical regions of the world (Olivares-Fuster et al., 2003; Mohan Jains and Priyadarshan, 2009). The origin and diversity center of citrus and its related genera is considered to be Southeast Asia, possibly ranging from Northeastern India eastward through the Malay Archipelago (Mohan Jains and Priyadarshan, 2009).

Citrus tristeza virus (CTV) is a worldwide distributed Closterovirus (family Closteroviridae), which causes one of the most economically important diseases of citrus in the world (Bar-Joseph et al., 1989; Che et al., 2001; Gowda et al., 2009; Lair et al., 1994; Narvaez et al., 2000; Satyanarayana et al., 2001). CTV particles are flexuous and threadlike with a size of 2000×10-12 nm (Huang et al., 2004; Jiang et al., 2008; Ruiz-Ruiz et al., 2007) and are composed of a positive sense, single stranded genomic RNA about 20 kb in size (Che, et al., 2002; Hilf, et al., 1995; Huang, et al., 2004; Ruiz-Ruiz, et al., 2007) that contains 12 open reading frame (ORFs) (Che et al., 2002; Fagoaga et al., 2005; Narvaez et al., 2000; Satyanarayana et al., 2001) and encode at least 19 proteins (Che et al., 2002; Che et al., 2001; Huang et al., 2004; Satyanarayana et al., 2001). Two of these proteins are capsid proteins of 25 and 27 kDa which comprise about 95 and 5% of the virus coat, respectively (Bar-Joseph et al., 1989; Jiang, et al., 2008; Roy, et al., 2005; Ruiz-Ruiz et al., 2007). This closterovirus is a phloem limited virus and is transmitted by aphids in a semi-persistent manner (Bar-Joseph et al., 1989; Brown et al., 1988; Genc 2005; Gottwald et al., 1999). *Toxoptera citricida* and *Aphis gossypii* are the most efficient vectors (Bar-Joseph et al., 1989; Brlansky et al., 2003; Brown et al., 1988; Gottwald et al., 1999; Roy et al., 2005).

The virus is genetically and biologically diverse and the virus isolate, citrus cultivar, rootstock, time of infection and environmental conditions can affect symptoms (Huang et al., 2004; Satyanarayana et al., 2001). A complex range of symptoms are produced under field

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conditions. There are three economically devastating field symptoms caused by CTV, including death and decline, stem pitting (Broadbent et al., 1996; Garnsey et al., 1987; Genc, 2005; Gmitter et al., 1996; Satyanarayana et al., 2001) and seedling yellows (Ruiz-Ruiz et al., 2007). Decline and death of most citrus species grafted on sour orange (*Citrus aurantium* L.) which are caused by most isolates of CTV can be avoided by using CTV resistant or tolerant rootstocks (Dominguez et al., 2000). In citrus growing areas, where sever isolates of CTV are common, coat protein (CP) mediated resistant transgenic plants (Dominguez et al., 2002; Febres et al., 2008) and cross protection with mild strains (Lin et al., 2002) can reduce yield losses (Dominguez et al., 2000). This needs quick and sensitive methods for differentiation of mild strains from virulent strains. It is possible to detect both severe and mild strains of CTV in the same plant simultaneously using bi-directional PCR (Jiang et al., 2008). CTV isolates were first classified into seven p25/HinfI groups. Groups IV and V produced mild symptoms while others produced sever symptoms in the indicator plants (Gilling et al., 1993). Later another group was found in china and defined as Group VIII. This isolate produced no symptoms in indicator plants (Jiang et al., 2008). We reported the detection of CTV in the asymptomatic and symptomatic citrus samples by molecular techniques and the association of CTV with diseased citrus in Peninsular Malaysia (Ayazpour et al., 2011). However, there was not enough information about CTV and its p25/HinfI groups in Malaysia, so this research was performed to characterize CTV isolates in Malaysia.

MATERIALS AND METHODS

Sampling

Samples were randomly collected from 340 asymptomatic and symptomatic citrus trees throughout peninsular Malaysia. Mature shoots and leaves of citrus plants were collected from eastern, western, southern and northern branches of each tree and mixed for the test. Their infection with CTV was checked by ELISA. For BD-PCR and RFLP analysis, sixty positive samples including *Citrus aurantifolia, Citrus sinensis, Citrus maxima, Citrus reticulata, Citrus hystrix, Citrus microcarpa and Fortunella sp*. were selected from Selangor, Pahang, Johor, Terengganu, Perak and Kedah states (Table 1).

Nucleic acid extraction from citrus tissues

Total RNA was extracted from shoot barks, midribs and petioles. About 0.2 g of tissues was pulverized in liquid nitrogen with mortar and pestle and collected in a 1.5 ml sterile microtube. Each sample was suspended in 400 µl TES buffer (100 mM Tris-HCl pH 8.0, 2 mM EDTA, 2% w/v SDS) and 400 µl phenol/chloroform/isopropanol (25/24/1) and was shaken vigorously for ten minutes. After centrifugation (14000 rpm) for ten minutes, the supernatant was treated with 200 µl ethanol (99.8%) in a new tube and used for total RNA extraction by RNeasy mini kit (Qiagene) according to the manufacturer's instructions. The extracted RNA was used as a template for amplification of the CP gene of CTV.

Primers

Primer pair CP1 (5′-ATG-GAC-GAC-GAA-ACA-AAG-AA-3′)/ CP2 (5′-TCA-ACG-TGT-GTT-GAA-TTT-CC-3′) were used for amplification of the complete CP cistron (672 bp) of CTV (Jiang et al., 2008). The internal sense primer CP3 (5′-TTTGGACTGACGTCGTGTT-3′) and the internal anti sense primer CP4 (5′-TTACCAATACCCTTAGAATTAT-3′) were used for differentiation of CTV severe and mild strains (Huang et al., 2004). The expected sizes of PCR products with primer sets CP2/CP3 and CP1/CP4 were 320 and 392 bp, respectively.

cDNA synthesis and polymerase chain reaction amplification

cDNA was synthesized using RNA extracted from citrus tissues as template and CP2 as primer. The total reaction volume was 40 µl, which contained 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 0.2 mM each of the four dNTPs, 1 µm CP2, 1 µm CP4, 20 U reverse transcriptase and 18.75 µl extracted RNA. First, RNA and primer were mixed gently and heated for 10 min at 65°C and then immediately cooled on ice. Then other materials were added and the contents were mixed gently and incubated at 25°C for 10 min, 42°C for 60 min and 72°C for 10 min, respectively. Preparation of cDNA for BD-PCR was done as above, but CP2 and CP4 were used as primers. PCR amplification was performed in 25 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.05 mM each of the four dNTPs, 2 mM $MgCl_2$, 0.3 µM of each primer (CP1, CP2), 1.25 U Taq DNA polymerase (iNtRON Biotechnology) and 1 to 4 µl of RT mixture. For BD-PCR, the mixture was the same, except that 0.3 µM of each primer, CP1, CP2, CP3 and CP4, was added and RT mixture has been made with CP2 and CP4 as primers. The PCR cycling profile was one cycle at 94°C for five min, followed by 35 cycles of 94°C for 30 s, 56°C for one min, and 72°C for one min, with a final extension step at 72°C for 10 min. PCR amplified fragments were separated in 1.2% agarose gel in Tris-borate (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide and analyzed using BIO imaging system (Syngene). A 100 bp DNA Ladder (Fermentas) was used as a nucleic acid marker.

Restriction fragment length polymorphism of CP gene

The PCR amplified products of the CP gene of different CTV isolates were digested with the restriction enzyme HinfI to differentiate CTV strains and evaluate their variation in peninsular Malaysia. Each digestion reaction was composed of 20 µl of PCR product, 20 U of the restriction enzyme, and 2 µl of 10 × digestion buffers in a total volume of 32 µl. The digestion reaction was performed at 37°C for 90 min and the products were separated by electrophoresis in a 3% agarose gel and visualized after staining with ethidium bromide.

Sequencing and Phylogenetic analysis of the CP gene of CTV

Fifty six isolates of CTV were chosen for sequencing of their CP genes. The amplified products of approximately 672 bp for the complete CP gene were sequenced commercially (NHK BIOSCIENCE SOLUTIONS, Korea). A multiple sequence alignment was performed by using Clustal W 1.6 (Thompson et al., 1994) and

Table 1. Host and GenBank accessions of CTV isolates in peninsular Malaysia.

Isolates	Host	Accession number
AMC2	*Citromelo*	HQ012375
AMC13	*Citrus reticulata*	HQ012378
AMC18	*Citrus sinensis*	HQ012380
AMI61	*Citrus microcarpa*	HQ012381
AMI62	*C. microcarpa*	HQ012382
AMj1	*C. microcarpa*	HQ012383
AMJ12	*C. microcarpa*	HQ012384
AMJ31	*Citrus aurantifolia*	HM131219
AMK1	*C. aurantifolia*	HQ012385
AMK8	*C. microcarpa*	HQ012386
AMK10	*C. aurantifolia*	HQ012387
AMK11	*C. aurantifolia*	HQ012388
AMK17	*C. aurantifolia*	HQ012389
AMK19	*C. aurantifolia*	HQ012390
AMK22	*Citrus hystrix*	HQ012391
AMK25	*C. hystrix*	HQ012392
AMK27	*C. hystrix*	HQ012393
AMK30	*C. hystrix*	HQ012394
AMK35	*C. microcarpa*	HQ012395
AMK42	*C. aurantifolia*	HQ012396
AMKu2	*C. microcarpa*	HQ012397
AMM11	*C. reticulate*	HQ012398
AMM14	Lemon	HQ012399
AMM18	*C. aurantifolia*	HQ012400
AMM20	*C. aurantifolia*	HQ012401
AMM22	*C. aurantifolia*	HQ012402
AMM28	Lemon	HQ012403
AMM29	*C. aurantifolia*	HQ012404
AMSB2	*C. microcarpa*	HQ012405
AMSB3	*C. hystrix*	HQ012406
AMSB7	*C. microcarpa*	HQ012407
AMT1	*C. aurantifolia*	HQ012408
AMT3	*C. aurantifolia*	HQ012409
AMT4	*C. aurantifolia*	HQ012410
AMT5	*C. aurantifolia*	HQ012411
AMT7	*C. aurantifolia*	HQ012412
AMT8	*C. aurantifolia*	HQ012413
AMT9	*C. aurantifolia*	HQ012414
AMT12	*C. microcarpa*	HQ012415
AMT14	*C. hystrix*	HQ012416
AMT15	*C. microcarpa*	HQ012417
AMT21	*C. hystrix*	HQ012418
AMT27	*C. aurantifolia*	HQ012419
AMT28	*C. hystrix*	HQ012420
AMT31	*C. hystrix*	HQ012421
AMT32	*C. hystrix*	HQ012422
AMT35	*C. microcarpa*	HQ012423
AMT36	*C. aurantifolia*	HQ012424
AMT37	*C. aurantifolia*	HQ012425
AMT38	*C. aurantifolia*	HQ012426
AMT39	*C. aurantifolia*	HQ012427

Table 1. Contd

AMT40	*C. aurantifolia*	HQ012428
AMT41	*C. microcarpa*	HQ012429
AMT42	*C. aurantifolia*	HQ012430
AMT43	*C. aurantifolia*	HQ012431

a phylogenetic tree was constructed by MEGA software Version 4 (Tamura et al., 2007), using neighbor-joining method with 1000 bootstrap replications. GenBank accessions and the sequence sources of the CTV CP genes used for RFLP and construction of Phylogenetic tree are listed in Table 1.

RESULTS

CTV strains differentiated by bi-directional PCR

Bi-directional (BD) PCR was done based on two sets of strain-specific primers for CP encoding region to identify mild and sever strains in the samples. The 392-bp fragment was amplified only from AMK19, AMK27, AMC18, AMM11, AMM20, AMM28 and AMJ12. This means that only 11.67% of total samples produced the 392-bp fragment. Of these seven samples, only AMM28 produced a 392-bp fragment, while the other six samples produced both 320 and 392-bp fragments. The other 53 samples produced only the 320-bp fragment, accounting for 88.33% of total samples. These results suggest that 88.33% of the analyzed plants were infected with sever strains, 1.67% with mild strain and 10% were infected with both sever and mild strains (Figure 1).

RFLP profiles of the CP gene from different CTV isolates

672 bp amplified fragments of CTV isolates using CP1 and CP2 primers of the CP gene were digested with the restriction enzyme HinfI. Digestion of the PCR products of CTV isolates revealed a high sequence divergence between CTV isolates (Figure 2). According to the previous defined RFLP groups (Gilling et al., 1993; Jiang et al., 2008), the restriction enzyme HinfI created digestion patterns of Groups I to IV, VI to VII and two other new groups. Isolates AMK1, AMJ12, AMT38, AMT39 and AMT43 produced a unique restriction pattern with two fragments of about 220 and 300 bp. These results were confirmed by virtual digesting of their sequences in computer software (Bikandi et al., 2004). These isolates were designated as Group IX. Isolate AMI61 alone produced another restriction pattern with three fragments of about 100, 270 and 300 bp. This isolate was designated as Group X. AMT31 and AMT36 were placed in RFLP Group I, AMK25 in Group II, AMC2, AMJ31, AMSB2 and AMSB7 in Group III and AMM28 in Group IV. AMT1, AMT3, AMT5, AMT7, AMT9, AMT21, AMT27, AMT32, AMT34, AMT35, AMT42, AMT44, AMM14, AMM18, AMM22 and AMM29 indicated the same restriction sites and were placed in RFLP Group VI. Isolate AMT41 did not produce any restriction pattern. None of the CTV isolates produced the restriction pattern of groups V or VIII. The remaining 34 CTV isolates had mixed infections. Among these, the restriction pattern of Group VI was the most frequent (67.24%) followed by Group III (38.33%), Group IV (25%), Group II (15.52%), Group I (12.07%), Group VII (8.62%), Group IX (8.62%) and Group X (1.72%), respectively (Table 2).

Phylogenetic analysis

Phylogenetic tree of these isolates in comparison with seven isolates from other countries showed that these isolates were clustered into two separate clades (Figure 3). Most of the Malaysian CTV isolates were placed in Clade 1. Clade 1 was separated into two subclades, comprising all Malaysian isolates in Subclade 1-1 and the isolates Bangalore, NUagA and ML12 in Subclade 1-2. Considering that these tree samples are from South Asia, can conclude that most isolates are close to another CTV isolates of Sought Asia. Some CTV isolates of Malaysia were clustered in Clade 2. Isolates T36 and NZRB and five isolates from Malaysia were placed in Subclade 2-1; while isolates T30 and T385 and other five Malaysian isolates were close together and placed in Subclade 2-2.

DISCUSSION

Based on the BD-PCR results, seven isolates of CTV comprising AMK19, AMK27, AMC18, AMM11, AMM20, AMM28 and AMJ12 were characterized as mild strains. According to Table 1, these mild strains were found in all citrus species; therefore there is no differentiation between hosts of mild and severe strains. The HinfI restriction fragment patterns revealed that the Malaysian CTV population structure is complex. By using the HinfI restriction fragment patterns for separating mild strains from sever strains, the Group IV was identified as mild strain (Gilling et al., 1993), so the 15 isolates recognized as Group IV (AMT8, AMT15, AMT28, AMK1, AMK8, AMK17, AMK19, AMK22, AMK27, AMK42, AMC18, AMM11, AMM20, AMM28 AND AMJ12), were either mild strain or complexes of mild and severe strains. By

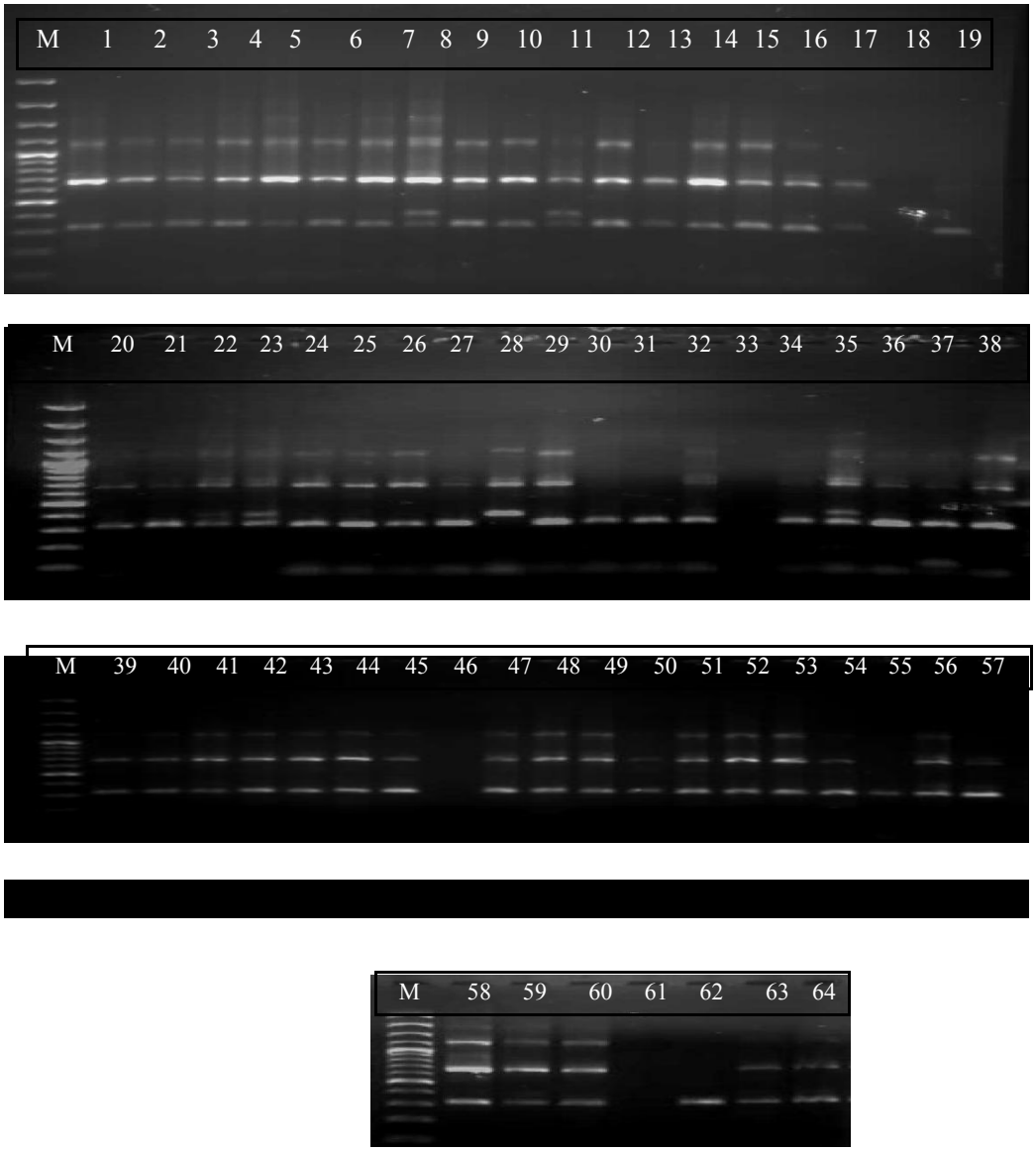

Figure 1. Amplification of partial CP gene from CTV Malaysian isolates with CP_1, CP_2, CP_3 and CP_4 primer pairs). M, molecular marker 100bp, 1- AMK1, 2-AMK5, 3-AMK8, 4-AMK10, 5-AMK11, 6-AMK16, 7-AMK17, 8-AMK19, 9-AMK22, 10-AMK25, 11-AMK27, 12-AMK30, 13-AMK35, 14-AMK42, 15-AMSB2, 16-AMSB3, 17-AMSB7, 18-WATER, 19-L, 20-AMC2, 21-AMC13, 22-AMC18, 23-AMM11, 24-AMM14, 25-AMM18, 26-AMM20, 27-AMM22, 28-AMM28, 29-AMM29, 30-L, 31-AMKu1, 32-AMKu2, 33-water, 34-AMJ1, 35-AMJ12, 36-AMJ31, 37-AMI61, 38-AMI62, 39-AMT1, 40-AMT3, 41-AMT4, 42-AMT5, 43-AMT7, 44-AMT8, 45-AMT9, 46-water, 47-AMT12, 48-AMT14, 49-AMT15, 50-AMT21, 51-AMT27, 52-AMT28, 53-AMT31, 54-AMT32, 55-AMT36, 56-AMT37, 57-AMT38, 58-AMT35, 59-AMT39, 60-AMT40, 61-water, 62-AMT41, 63-AMT42, 64-AMT43.

comparing these two methods for separating mild strains from severe strains, it is perceived that BD-PCR cannot recognize all mild strains. On the other hand, the HinfIrestriction fragment patterns method verified the results of BD-PCR, so we recognize this method more efficient. In China Jiang et al. (2008) also detected only two mild isolates of CTV by BD-PCR method and seven mild isolates with RFLP method. These results are consistent with our observations in Malaysia.

Isolates AMK1, AMJ12, AMT38, AMT39 and AMT43 produced a unique restriction pattern with fragments of 210 and 300 bp. On the other hand, isolate AMI61 produced another restriction pattern with three fragments of about 100, 270 and 300 bp. These patterns appeared neither in the seven groups defined by Gilling et al. (1993), nor in the group defined by Jiang et al. (2008). To our knowledge, this is the first report on the presence of these restriction sites in a CP gene of any CTV isolate.

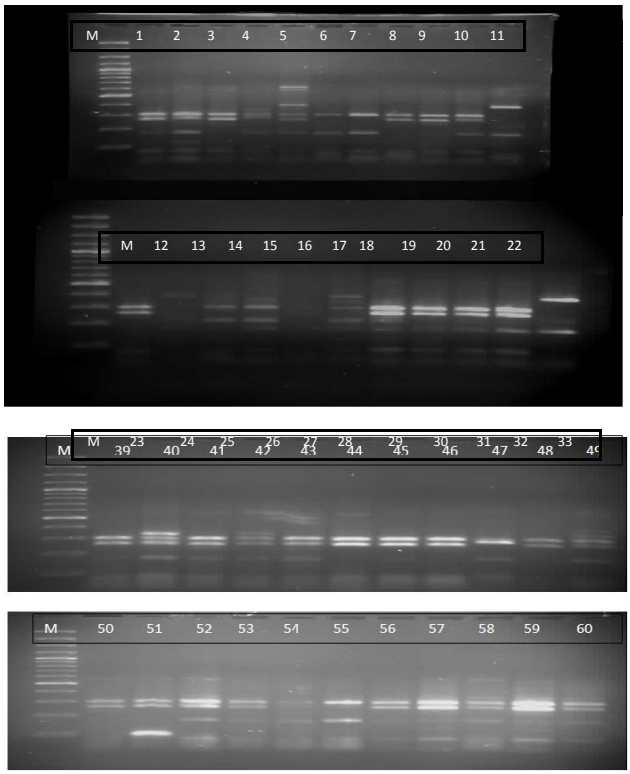

Figure 2. RFLP profiles of CP gene of 60 CTV isolates from Peninsular Malaysia. M-Molecular marker 100bp, 1-AMT1, 2-AMT14, 3-AMT21, 4-AMT28, 5-AMT32, 6-AMT34, 7-AMT36, 8-AMT37, 9-AMT40, 10-AMT42, 11-AMT44, 12-AMK8, 13-AMK19, 14-AMK22, 15-AMK27, 16-AMK30, 17-AMC2, 18-AMM20, 19-AMM22, 20-AMT8, 21-AMT15, 22-AMT31, 23-AMT7, 24-AMT36, 25-AMT38, 26-AMT39, 27-AMT41, 28-AMT43, 29-AMK1, 30-AMK5, 31-AMK10, 32-AMK11, 33-AMK25, 34-AMM11, 35-AMJ12, 36-AMSB2, 37-AMSB3, 38-AMSB7, 39-AMT3, 40-AMT4, 41-AMT5, 42-AMT12, 43-AMT27, 44-AMT35, 45-AMM14, 46-AMM18, 47-AMM28, 48-AMM29, 49-AMK12, 50-AMT9, 51-AMI61, 52-AMI62, 53-AMC13, 54-AMJ1, 55-AMJ31, 56-AMK16, 57-AMK17, 58-AMK35, 59-AMK42, 60-AMC18.

Table 2. Restriction patterns of the CP gene from 60 isolates created by HinfI digestion and frequency of each pattern.

Isolate	I	II	III	IV	VI	VII	VIII	IX	X
AMT1					+				
AMT3					+				
AMT4		+			+				
AMT5					+				
AMT7					+				
AMT8				+	+				
AMT9					+				
AMT12		+			+				

Table 2. Contd.

AMT14	+								
AMT15			+	+	+				
AMT21					+				
AMT27					+				
AMT28	+			+	+	+			
AMT31	+								
AMT32					+				
AMT34					+				
AMT35					+				
AMT36	+								
AMT37		+				+			
AMT38								+	
AMT39		+						+	
AMT40		+				+			
AMT41									
AMT42					+				
AMT43	+		+					+	
AMT44					+				
AMK1			+	+	+			+	
AMK5			+		+				
AMK8			+	+	+				
AMK10			+		+				
AMK11			+		+				
AMK12			+		+	+			
AMK16			+		+				
AMK17				+	+				
AMK19			+	+	+				
AMK22				+	+				
AMK25		+							
AMK27		+		+	+				
AMK30	+		+		+				
AMK35		+	+		+				
AMK42				+	+				
AMC2			+						
AMC13			+		+				
AMC18			+	+	+				
AMM11			+	+		+			
AMM14					+				
AMM18					+				
AMM20			+	+					
AMM22					+				
AMM28				+					
AMM29					+				
AMJ1			+		+				
AMJ12	+	+	+	+				+	
AMJ31			+						
AMSB2			+						
AMSB7			+						
AMI61					+				+
AMI62			+		+				
Frequency and % of each group	7 (12.07)	9(15.52)	23 (38.33)	15 (25)	39 (67.24)	5(8.62)	0(0)	5 (8.62)	1 (1.72)

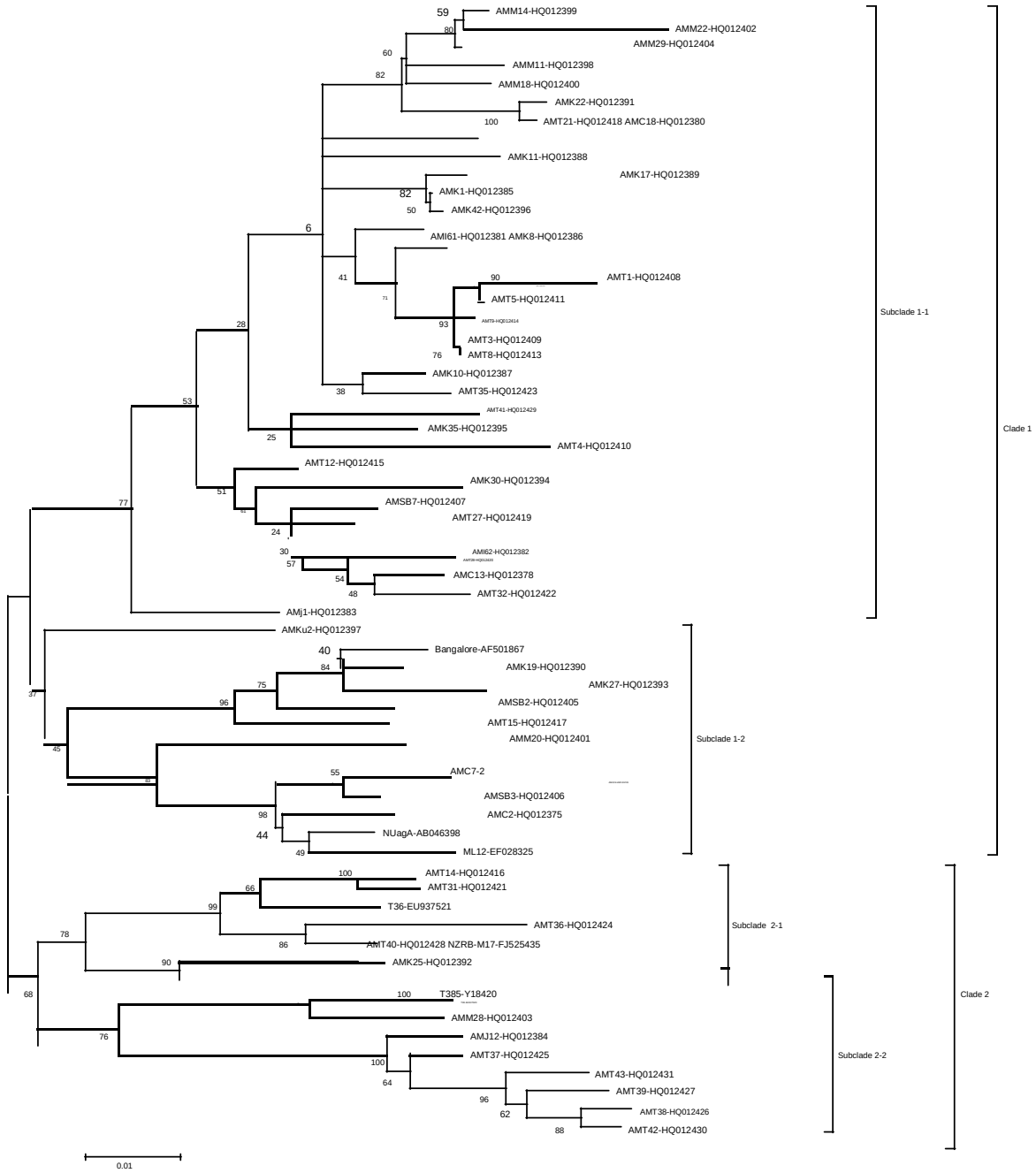

Figure 3. Neighbor-joining tree with bootstrap values of 63 isolates, 56 samples in this work and 7 isolates published in the GenBank.

According to these results we can conclude that CTV populations in Malaysia contain new genetic variants.

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