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Phylogenetic analysis and recombination events in full genome sequences of apple stem grooving virus

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Summary. – Apple stem grooving virus (ASGV) is one of the most important viral pathogens infecting pome and stone fruit trees worldwide. In this study, with the complete nucleotide sequence of isolate ASGV-T47, which we generated, molecular variation and recombination in ASGV full genomic sequences worldwide were analyzed. ASGV-T47 shared 79.7–97.6% nucleotide identity with the other isolates worldwide and had the highest identity with an isolate from Japan. Phylogenetic analysis based on whole genome clustered all 16 isolates from around the world into two groups with no correlation to host or geographical origin. Four isolates were detected to be recombinants. Selection pressure estimation indicated that the two codons at positions 1756 and 1798 are under positive selection, while purifying selection is the primary evolutionary dynamics for ASGV.

Keywords: capillovirus; full genome sequence; recombination; variation; evolution; selection

Introduction

Apple stem grooving virus (ASGV) is the type member of the genus *Capillovirus* in the family *Betaflexiviridae* (Adams *et al*., 2012) and has a single-stranded RNA genome consisting of approximately 6,500 nucleotides (nt), with particles of 600–700 nm in length. The genome contains two overlapping open reading frames (ORFs). ORF1 encodes a polyprotein (241 kDa) containing the replication-associated proteins and a coat protein (CP), while ORF2 encodes a 36 kDa movement protein (MP) located within the sequence of ORF1, but having a different reading frame (Yoshikawa *et al*., 1992; Ohira *et al*., 1995; Hirata *et al*., 2003). Recent studies revealed that the CP is expressed from a subgenomic RNA (Tatineni *et al*., 2009a) and it is essential for infection (Komatsu *et al*., 2012). ASGV has a wide host range including pome fruits, stone fruits, citrus, and lily (Sawamura *et al*., 1988; Takahashi *et al*., 1990; Ohira *et al*., 1994; Adams *et al*., 2012; Hailstones *et al*., 2000), and it is one of the most economically important viruses infecting pome and stone fruit trees worldwide (Khan and Dijkstra, 2006). ASGV is transmitted by grafting and no vectors are known (Adams *et al*., 2012). Associated with latent infections in many commercial apple and pear cultivars (Lister 1970), ASGV dramatically reduces plant growth and productivity (Pleše *et al*., 1975; Yanase, 1982; Cembali *et al*., 2003). In China, 33.4–100% of apple trees were infected by ASGV in different production regions (Liu and Wang, 1989, and our unpublished data).

Abundant genetic variations exist in RNA virus populations owing to the error-prone replication caused by the lack of proofreading activity of RNA-dependent RNA polymerase (RdRp), small genomes, extremely short generation times, and large population sizes (Domingo and Holland, 1997; Drake and Holland, 1999). Moreover, recombination plays an important role in the evolution of RNA viruses. The recombination in RNA viruses can cause a selective advantage over parental viruses in certain instances and act as a major factor in deleterious mutation clearance (Simon and Bujarski

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Abbreviations: ASGV = apple stem grooving virus; CP = coat protein; ORF(s) = open reading frame(s); SPMMV = sweet potato mild mottle virust

1994), which could facilitate host adaptation and contribute to RNA virus evolution (Holmes, 2009).

For ASGV and other capilloviruses, there are few reports on analyzing the molecular variation and recombination. A recent study described genomic evolution based on the two variable regions of ASGV genome (Liebenberg *et al.*, 2012), however, there is no genetic analysis, recombination test, or investigation of natural selection based on the whole genome. In this study, to gain insights into the evolutionary mechanism of ASGV, the complete sequence of an isolate ASGV-T47 was first determined, and it was compared with 15 other isolates worldwide to investigate their genetic variation, recombination and phylogenetic relationships on whole genome level.

Materials and Methods

Virus and plant material. The isolate ASGV-T47 was propagated on apple *in vitro* plantlets, which were generated from one bud on an ASGV-infected apple tree (*Malus domestica* cv. Fuji) growing at a garden in Changping district, Beijing, China, in a growth chamber at a temperature of 25±1°C under a 16-hr light/8-hr dark photoperiod with a light intensity of 3600Lx by cool-white fluorescent tubes.

RNA isolation, reverse transcription PCR, cloning and sequencing. Total RNA was extracted from leaves of apple *in vitro* plantlets by an EASYspin plant total RNA extraction kit (Biomed, China) according to the manufacturer's instructions. First-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, USA) with a random primers. Polymerase chain reaction (PCR) was performed with high-fidelity Golden *Taq* DNA polymerase (FunGenome, China) using specific primers (Table 1) designed according to the reference ASGV genomic sequence (NC_001749). To amplify the 3′-terminal region, a modified oligo d(T) primer R4 and an ASGV-specific primer F4 were used (Table 1). The 5′-terminal region was determined using the classic 5′-RACE method (Scotto-Lavino *et al.*, 2006). PCR products were purified using EasyPure Quick gel extraction kit (TIANGEN, China) according to the manufacturer's instructions. The purified PCR products were then ligated to vector pMD18-T (TaKaRa, China) and transformed into *E. coli* strain DH5α competent cells. Consistent sequences were obtained from at least three independent positive clones.

Sequence identity and phylogenetic analysis. The full-length genome sequence of ASGV-T47 was assembled using the SeqMan program in the software Lasergene DNASTAR (DNASTAR Inc., USA). Identities at the nt and putative amino acid (aa) levels between sequences were evaluated with the MegAlign program in DNASTAR (http://www.dnastar.com). Phylogenetic analysis was performed with the neighbor-joining algorithm using the Maximum composite likelihood method with 1,000 bootstrap replicates as implemented in the Molecular Evolutionary Genetics Analysis tool (MEGA v.5.0) (Tamura *et al.*, 2011).

Recombination analysis and selection pressure analysis. Recombination was tested by seven different programs, RDP (Martin and Rybicki, 2000), GENECONV (Padidam *et al.*, 1999), BootScan (Martin *et al.*, 2005), MaxChi (Smith, 1992), Chimaera (Posada and Crandall, 2001), SiScan (Gibbs *et al.*, 2000), and 3Seq (Boni *et al.*, 2007) within the RDP3 software package (Martin *et al.*, 2010). *P*-value was estimated according to the default parameters, except for the options of linear sequence and of disentangling overlapping signals (Cordin *et al.*, 2012; Li *et al.*, 2013a,b). Natural selection was investigated by the dN (the rate of non-synonymous substitutions per non-synonymous sites) to dS (the rate of synonymous substitutions per synonymous sites) ratio (ω) using the Pamilo-Bianchi-Li method implemented in MEGA5 (Pamilo and Bianchi 1993; Tamura *et al.*, 2011). For each codon, the value of ω was estimated to identify selection using SLAC, FEL, and IFEL within the HYPHY software package on the Datamonkey server (www.datamonkey.org) (Pond and Frost, 2005) by setting the significance levels at $P = 0.05$ (Cordin *et al.*, 2012)

Results

The full genome sequence of isolate ASGV-T47 was assembled from 5 fragments including 3′-terminal and 5′-terminal regions (Table 1), and was assigned to GenBank Acc. No. KF434636. It consists of 6,496 nt, excluding the poly(A) tail, and contains three ORFs, encoding a 241 kDa protein, which is involved in replication, a 36 kDa MP (ORF2), and a 27 kDa CP (ORF3), as is characteristic for ASGV. To compare the identity of ASGV-T47 genome sequence to other isolates from around the world, totally 15 isolates fully sequenced worldwide were retrieved from GenBank (Table 2). ASGV-T47 shared a 79.7–97.6% identity with the other 15 isolates, and had the highest identity to a Japanese isolate (NC_0011749) not only for the whole genome sequence (97.6%) but also for each ORF in both nt (97.2–98.5%) and aa (96.9–99.6%) sequence (Table 2). Among the 5 isolates from China, ASGV-T47 has the highest identity to a citrus isolate (KC588948) on whole genome sequence (90.9%), ORF1 (90.7%), ORF2 (91.5%) and ORF3 (92.2%) on nt level, while on aa level, ORF1 of ASGV-T47 has the highest identity (93.9%) with a citrus isolate (KC588948), while ORF2 and ORF3 have the highest identities (95.0% and 97.1%, respectively) with sand pear isolate (JN701424) (Table 2).

All 16 isolates shared 78.8% to 98.5% identities with each other (Table 3). For particular ORFs, the identities at the nt and aa levels between the 16 isolates were 79.2–97.5% (nt) and 85.6–98.2% (aa ORF1), 84.2–97.2% (nt) and 92.2–96.9% (aa ORF2), and 89.1–98.5% (nt) and 94.1–99.6% (aa ORF3) (data not shown), respectively, indicating that the CP, as reported previously (Magome *et al.*, 1997), is the most conserved. Moreover, the alignment of the ORF1 aa sequences

Table 1. Primers used to amplify the full-length genome of ASGV

Primers	Sequences (5'-3')	Annealing temperature	Nucleotide positions
F1	ACCTTAGAAGTGACCAACC	54°C	138–3633
R1	GAACACCTGCCCAGAAAGTAAC		
F2	GATCCGACGCTTGCGGAGAT	62°C	3438–4965
R2	AGGCCGTCACCAGATCGAGA		
F3	GGGTCCAAATGGCTATCGT	55°C	4779–5874
R3	CGGGAAACTGGGTCTTGTCAG		
F4	AAGAGAGGATTTAGGTCCCTC	56°C	5592–3'end
R4	CAGGATCCAAGCTTTTTTTTTTTTTTTTT		
Q_{total}	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT		
Q_{outer}	CCAGTGAGCAGAGTGACG	58°C	5'end–240
GSP1	GGATACTGGATGGGAGTGTGA		
Q_{inner}	GAGGACTCGAGCTCAAGC	56°C	5'end–201
GSP2	GTGTCAGACCATGTTTGGTC		

GSP1: gene-specific primer 1; GSP2: gene-specific primer 2.

Table 2. Percentage of nucleotide and predicted amino acid sequence identities of ASGV-T47 to other complete ASGV genomes available in GenBank

GenBank No.	Host	Location of virus isolation (year)	Genome identity (%)	Nucleotide identity (%)			Amimo acid identity (%)		
				ORF1	ORF2	CP	ORF1	ORF2	CP
AB004063	Lily	Japan (1997)	82.9	82.5	85.4	92.2	88.0	94.4	98.3
AY596172	Pear	South Korea (2004)	79.7	79.2	84.2	89.9	85.6	92.2	95.0
FJ355920	Citrus	Taiwan (2004)	81.7	81.2	85.5	89.5	87.9	94.4	96.6
JX416228	Citrus	Taiwan (2012)	81.8	81.3	85.0	89.6	87.7	94.4	94.1
AY646511	Kumquat	Taiwan (2004)	81.8	81.4	85.7	89.8	87.8	94.1	95.4
D16681	Lily	Japan (1993)	82.9	82.5	84.9	91.9	88.4	94.4	98.3
EU553489	Meyer lemon	USA (2008)	81.8	81.5	84.6	90.6	87.1	95.0	94.5
JQ765412	Citrus	China (2011)	81.6	81.2	85.0	89.6	87.7	93.5	95.0
JN701424	Sand pear	China (2009)	87.1	86.8	86.0	91.7	90.5	95.0	97.1
JQ308181	Apple	China (2011)	86.4	86.1	86.2	91.3	90.7	93.5	96.6
JX080201	Apple	Germany (2009)	82.5	82.1	85.0	91.5	88.0	95.3	97.9
NC_001749[a]	**Apple**	**Japan (1993)**	**97.6**	**97.5**	**97.2**	**98.5**	**98.2**	**96.9**	**99.6**
HE978837	Apple	India (2012)	81.3	80.8	84.2	90.9	85.1	93.1	97.5
KC588947	Citrus	China (2013)	81.2	80.8	84.8	89.1	87.2	93.4	94.5
KC588948	Citrus	China (2013)	90.9	90.7	91.5	92.2	93.9	94.7	96.2

[a]Japanese isolate (NC_001749) which shares the highest identity to ASGV-T47.

of the 16 ASGV isolates revealed two highly variable regions, one from aa 532 to 570 and another from aa 1,583 to 1,868, which were the same as in previous reports (Tatineni *et al.*, 2009b; Liebenberg *et al.*, 2012).

To investigate the genetic relationship of ASGV isolates worldwide based on whole genome sequences, a phylogenetic analysis was performed with the neighbor-joining algorithm (Fig. 1). It indicated that the ASGV clusters had no correlation to host or geographical origins.

Though two recombinants were reported based on the *cp* genes alone (Negi *et al.*, 2010), the profile of recombination on complete ASGV genome sequences remains unknown. In this study, the 16 full genomic sequences worldwide were processed and tested for recombination. Four recombinants were detected to be "clear" recombinants by five different methods (Table 4). The major parent, minor parent, beginning breakpoint, ending breakpoint, programs detecting the event and the corresponding *P*-value for all recombinants are listed in Table 4. Three recombinants, two lily isolates from Japan (AB004063 and D16681) and one pear isolate from China (JN701424), originated from the same parents; their major parent was an apple isolate from Germany (JX080201) and their minor parent was a pear isolate from South Korea (AY596172). The two recombinants, AB004063 and D16681, shared the beginning and ending breakpoints located at nt 5094 and nt 5557, while recombinant JN701424 had its beginning and

ending breakpoints located at nt 4888 and nt 5519. The fourth recombinant event was observed between two citrus isolates, JX416228 from Taiwan as the major parent, and JQ765412 from China as the minor parent, leading to the recombinant citrus isolate (FJ355920) from Taiwan. Given the fact that ASGV can be transmitted by grafting with no known vectors (Adams *et al.*, 2012), the recombination between isolates from different host species and different countries was likely to be the result of long-distance transportation of infected propagation materials between countries and multiple grafting on a tree.

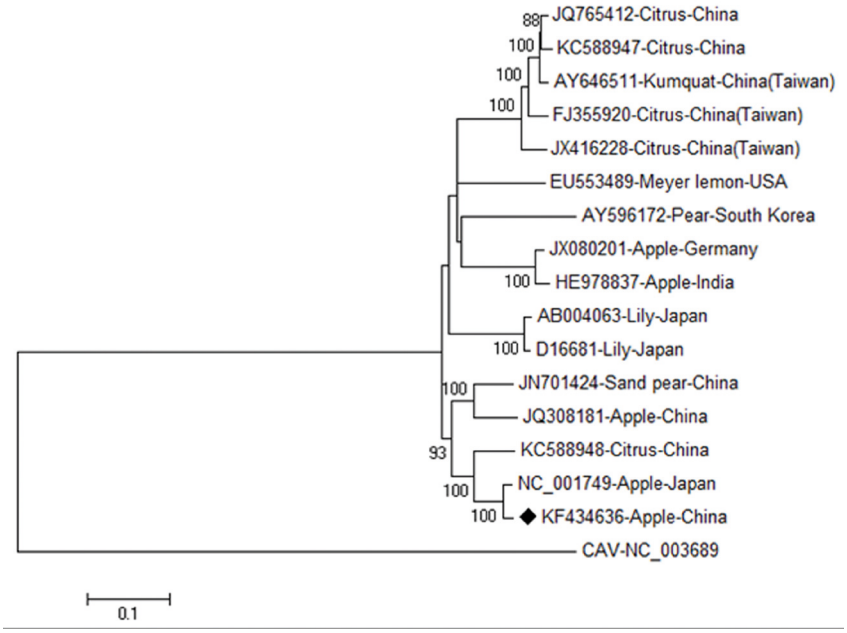

Fig. 1
Neighbor-joining tree depicting the phylogenetic relationships based on whole genomic sequences of ASGV isolates available in GenBank

The bootstrapping replicates = 1,000. Only branches with bootstrap values of ≥70% are shown. Cherry virus A (CVA) was used as the outgroup. The scale bar represents the genetic distance (substitutions per nucleotide). ASGV-T47 is marked with a black diamond.

Table 3. Percentage of nucleotide sequence identities of 16 full-length genomes of ASGV worldwide

	NC001749	KC588948	KC588947	JX416228	JX080201	JQ765412	JQ308181	JN701424	HE078837	FJ355920	EU553489	D16681	AY646511	AY596172	AB004063	**KF434636**
KF434636	97.6	90.9	81.2	81.8	82.5	81.6	86.4	87.1	81.6	81.7	81.8	82.9	81.8	79.7	82.9	100
AB004063	83.0	83.3	80.9	81.6	81.6	81.2	83.0	84.1	80.9	81.3	81.3	98.5	81.3	80.4	100	
AY596172	79.8	79.9	78.8	79.0	80.1	79.0	80.0	79.8	79.9	79.0	79.4	80.5	79.0	100		
AY646511	81.9	82.4	97.6	94.0	81.8	98.0	81.2	81.6	81.6	95.1	81.9	81.4	100			
D16681	83.0	83.5	80.9	81.6	81.7	81.2	83.1	84.1	80.9	81.3	81.4	100				
EU553489	81.7	82.1	81.4	81.8	81.8	81.7	81.5	81.1	81.5	81.9	100					
FJ355920	81.7	82.0	95.2	93.9	82.1	95.4	81.4	81.5	81.7	100						
HE078837	81.8	81.5	81.5	81.6	97.3	81.6	80.9	81.2	100							
JN701424	87.1	86.5	81.1	82.0	81.9	81.7	90.5	100								
JQ308181	86.3	85.8	80.9	81.6	81.5	81.4	100									
JQ765412	81.7	82.3	98.0	94.1	81.9	100										
JX080201	82.7	82.1	81.8	81.9	100											
JX416228	81.7	82.1	93.7	100												
KC588947	81.3	81.9	100													
KC588948	91.1	100														
NC001749	100															

This highlighted isolate (KF434636) is ASGV-T47.

Table 4. Recombination events detected in ASGV isolates worldwide

Recombinant (origin)	AB004063 (Lily, Japan)	D16681 (Lily, Japan)	JN701424 (Sand pear, China)	FJ355920 (Citrus, Taiwan)
Major parent (origin)	JX080201 (Apple, Germany)	JX080201 (Apple,Germany)	JX080201 (Apple,Germany)	JX416228[a] (Citrus, China)
Minor parent (origin)	AY596172[a] (Pear, South Korea)	AY596172[a] (Pear, South Korea)	AY596172[a] (Pear, South Korea)	JQ765412 (Citrus, China)
P-values determined using seven different programs				
RDP	3.602×10^{-5}	3.602×10^{-5}	3.602×10^{-5}	ND
GENECONV	ND	ND	ND	7.485×10^{-4}
BootScan	1.764×10^{-5}	1.764×10^{-5}	1.764×10^{-5}	9.653×10^{-5}
MaxChi	2.357×10^{-5}	2.357×10^{-5}	2.357×10^{-5}	5.431×10^{-3}
Chimaera	4.448×10^{-3}	4.448×10^{-3}	4.448×10^{-3}	4.405×10^{-2}
SiScan	7.615×10^{-6}	7.615×10^{-6}	7.615×10^{-6}	1.082×10^{-2}
3Seq	ND	ND	ND	ND
Beginning breakpoint (nt)	5094	5094	4888[b]	4417
Ending breakpoint (nt)	5557	5557	5519	4909

ND, recombination was not detected by this program. [a]The parent was tentative, because the parental isolates are 'unknown'. [b]The breakpoint was tentative, because the breakpoint is 'unknown'.

Finally, natural selection was examined by estimating the selection pressure on ORF1 and each of its codons using the dN to dS ratio (ω). ORF1 was chosen for the analysis, because it covers almost the entire ASGV genome and encodes proteins for replication and encapsidation. The values of dN and dS for ORF1 were separately estimated to be 0.1738 and 0.2825, respectively. Consequently, the dN/dS ratio was 0.615, indicating predominant purifying selection on ORF1. For each codon of ORF1, the value of ω was estimated to identify selection using SLAC, FEL, and IFEL within the HYPHY software package on the Datamonkey server (www.datamonkey.org) (Pond and Frost, 2005). All three methods concluded that the codons at positions 1756 and 1798 were under positive selection (Table 5), while the other codons were under purifying selection or neutral evolution (data not shown).

Table 5. The positive selection estimation results for ASGV ORF1 by three methods: SLAC, FEL, and IFEL

Codon	SLAC		FEL		IFEL	
	dN[a]/dS[b]	P-value	dN/dS	P-value	dN/dS	P-value
1756	3.982	0.043	1.523	0.004	1.803	0.011
1798	4.371	0.024	1.870	0.003	1.137	0.041

[a]dN, the rate of non-synonymous substitutions per non-synonymous sites.
[b]dS, the rate of synonymous substitutions per synonymous site.

Discussion

In this study, with the newly determined full genome sequence of an isolate, ASGV-T47, from China, two phylogenetic groups and four recombinants were detected among ASGV isolates worldwide. To our knowledge, this is the first analysis of recombination based on complete sequences of ASGV isolates. Though no recombination hotspot was found, recombination events play an important role in ASGV evolution as reported recombination in *cp* gene (Negi *et al.*, 2010). Moreover, recombination might be common in members of *Betaflexiviridae* as detected in a few reports (Singh *et al.*, 2007, 2008, 2012; Komorowska *et al.*, 2011; Singh *et al.*, 2012; Dhir *et al.*, 2013; Pramesh and Baranwal, 2013; Alabi *et al.*, 2014; Chen *et al.*, 2014). As no vector is known for ASGV and it can be transmitted by grafting, recombinant events among isolates from different host species and countries, and phylogenetic clusters with no correlation to host or geographical origins are likely to be the result of vegetative propagation and exchanges of propagation materials between countries. At the same time, recombinant events could benefit the survival of ASGV between host species, as recombination contributes to the RNA virus´ genetic diversity and ability to adapt to changing environments, including new hosts (Holmes, 2009).

On whole genome, ASGV-T47 has the highest identity (97.6%) with an isolate (NC_0011749) from Japan and both clustered into the same sub-clade in the phylogenetic tree (Fig. 1), while among all 5 isolates from China, ASGV-T47 shares the highest identity (only 90.9%) with KC588948. Conclusively, though ASGV-T47 was cloned in China, it might have come from Japan by the transport of vegetative propagation material.

Though previous reports revealed the evolution of ASGV genomic RNA (Liebenberg *et al.*, 2012), our result for the

first time identified the two codons, at positions 1756 and 1798, under positive selection. Similar results were observed for the *cp* genes of apple stem pitting virus (Komorowska *et al.*, 2011), grapevine rupestris stem pitting-associated virus (Alabi *et al.*, 2010), sweet potato mild mottle virus (SPMMV) (Tugume *et al.*, 2010), and the P1 proteinase of SPMMV (Tugume *et al.*, 2010), where several codons were under positive selection whereas purifying selection acted on the other sites. Purifying selection could occur by functional constraints during the virus life cycle when interacting with hosts (Schneider and Roossinck, 2000; French and Stenger, 2003), and it is the main evolutionary force (García-Arenal *et al.*, 2001). Further studies will be conducted to test whether the two codons are involved in reactions with host factors.

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