MOLECULAR CHARACTERIZATION OF RESISTANCE-BREAKING *TOMATO SPOTTED WILT VIRUS* (TSWV) ISOLATE MEDIUM SEGMENT IN TOMATO

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Abstract. *Tomato spotted wilt virus* (TSWV) is an economically important disease causing significant yield and quality loss in tomato. The most efficient method of disease control relied on the use of the *Sw-5* resistant gene. The new resistance-breaking Tswv isolates have severely infected greenhouses grown tomatoes Eastern Mediterranean area, including Antalya, Turkey. The objective of the study was to identify the cause of the resistance-breaking genetic mutations on the virus genome. The plant materials included resistant *Solanum peruvianum* PI126944 and four commercial hybrids, and a susceptible control. The six resistance-breaking strains (RBS) of the virus were collected from greenhouse-grown tomato plants throughout Antalya, Turkey. Beginning at the seedling stage, five consecutive inoculations were carried out at five-day intervals, using both the resistant and susceptible genotypes. The Medium segment of the virus includes the cell-to-cell movement protein (NSm) and a precursor of the surface glycoproteins (GN/GC) that were sequenced with different primer combination. The medium segment of the RBS genomes was amplified and 4825 bp sequence aligned and blasted using NCBI database. On-structural movement (NSM) domain of the M segment consistently carried C118Y mutation in all RBS analyzed. The multiple mutations on Gc-Gn domain were not associated with resistance break down. Phylogenetic analysis indicated that RB strain identified in Turkey was closely related to Spanish RB strains. The C118Ymutation overcame the resistance conferred by the Sw-5 gene. Hence, a new resistance source is needed to protect the tomato from new RB strains.
Keywords: *orthotospoviruses, NSm protein, avrirulence determinant, hypersensitive response, Sw-5b gene*

Introduction

Tomato production is under threat from viral diseases causing significant yield and quality loss all over the world (Pappu et al., 2009). One of the most common viral diseases occurs with *Tomato spotted wilt virus* (TSWV) on tomato. The TSWV is a member of genus *Orthotospovirus,* family Bunyaviridae (Adams, 2017), limits tomato production especially in greenhouses. The TSWV virus is transmitted by thrips (Thysanoptera, *Thripidae),* the most common vector being *Frankliniella occidentalis*in a persistent and circulative manner (Todd et al., 1995; Mandal et al., 2001).

Orthotospoviruses are enveloped viruses including additional protein package on their RNA genomes. The virus genome contains three RNA segments known as a tripartite RNA genome structure (Cortez et al., 2001). The TSWV has similarly three RNA parts described as small (S), medium (M), and large (L) RNAs (de Haan et al., 1991). The whole genome of TSWV codes six proteins via five different open reading frames (ORFs). The M and S RNAs have special structures where genomes are ambisense The ambisense viruses include both parts of positive and part of negative polarities in their genome (Kormelink et al., 1994; Lewandowskia and Adkins, 2005).

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The genome size of TSWV is 16.6 kbp where small, medium and large segments are 2.9 kb, 4.8 kb, and 8.9 kb, respectively (de Haanet al., 1990). The S segment has two ORFs encoding non-structural RNA silencing suppressor (NSS) and nucleocapsid (NC) proteins (Takeda et al., 2002). The M segment produces both non-structural movement protein (NSm) and envelope glycoproteins Gn-Gc (Kormelink et al., 1994). The last L segment encodes putative RNA-dependent RNA polymerases (RdRp), such as replicase, transcriptase, nuclease, helicase, cap-binding and NTPase proteins responsible for several enzymatic functions of TSWV (de Haan et al., 1991).

TSWV is the second most destructive viral pathogen in the list of economic damage causing plant viruses (Scholthof et al., 2011). The prevention and control of the spread of TSWV is very difficult due to its vectors. For TSWV management, resistant tomato varieties carrying *Sw-5* resistance gene derived from *Solanum peruvianum* have extensively been used (Pappu et al., 2009). In recent years, the resistant tomato plants have been infected with TSWV in Spain (Debreczeni, 2011). Similarly, resistant-breaking TSWV isolates have been reported on genetically resistant pepper plants since 2014 in Samsun, Turkey (Deligöz, 2014). Likely, severe TSWV infections are obtained on resistant gene containing tomato varieties indicating TSWV changed its genetic structure in 2016 in Turkey (Fidan et al., 2016; Batuman et al. 2017). In order to understand the genetic cause of virulence, the study has been conducted. Hence, whole genome structure of avirulent non-resistant breaking isolates and virulent resistance-breaking TSWV isolates are compared in nucleotide sequences and possible functional mutation site(s) have been investigated.

Materials and methods

Determination of the Sw-5 gene containing TSWV resistant plants

Total genomic DNAs were extracted by DNA extraction kit (Thermo Scientific, Germany,) from the resistant *Solanum peruvianum* PI126944, a susceptible commercial tomato hybrid variety (Hazera 5656 (Hazera Seed), and four resistant commercial hybrids, namely Torry F1 (Syngenta), Matatu F1 (RijkZwan), TayfunF1 (De Ruiter), Swanson F1 (Seminis). The DNA samples were PCR amplified using Sw5-2 primers F-AATTAGGTTCTTGAAGCCCATCT, and R-TTCCGCATCAGCCAATAGTGT as reported by Dianese et al. (2010) where the 574 bp and 500 bp amplicons representing resistant and susceptible Sw-5 alleles, respectively.

Inoculations of susceptible and resistant tomato plants with TSWVAntRB isolate

The six resistance-breaking isolates (RBS) were collected throughout Antalya, Turkey, from tomato plants grown in greenhouses. Because sequence analysis indicated the presence of single haplotype, all six RBS were mixed to inoculate the resistant and susceptible tomato samples. The resistant and susceptible samples, five plants in each replicate, were inoculated with RBS and a mock inoculation with phosphate buffer was used as a control. The inoculations were repeated five times at 5 intervals. At 15 days' post inoculations (dpi) when susceptible plant showed virus symptoms, leaf tissue was collected from mock and RBS inoculated plants. Systemic viral infection was subsequently determined by RT-PCR analysis as described previously (Dianese et al., 2010).

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Genome analysis of TSWV isolates

The TSWV isolates were collected from greenhouse-grown tomato plants in Antalya province, Turkey. The TSWV isolates originated from tomato hybrids reported to carry resistance against TSWV in 2016. The TSWV isolates were kept in -20 °C as infected leaf material at Virology Laboratory, Plant Protection Department, Akdeniz University, Antalya, Turkey. Total RNAs were extracted from the samples using an RNA extraction Kit (K0731**,** Thermo-Scientific, Germany). The cDNAs of TSWV were obtained with high capacity cDNA Reverse Transcription Kit (Thermo Scientific, Germany) according to the manufacturer's instructions. The M segment specific primers (Hallwass et al., 2014 and Zhong et al., 2011**)** presented in *Table 1* were paired (*Table 2*) to amplify TSWV isolates by RT-PCR analyses.

Table 1. *Primer sequences representing M segment of tomato spotted wilt virus (TSWV) used in RT-PCR studies (Hallwass et al., 2014)*

Segment	Primers' name	The sequence of primers from 5' to 3'	Position in M segment
M	M1 (F)	AGAGCAATCAGTGCATCAGAAATATACCTATTA	1-36
	M2 (F)	GTAGATACAAACCATCATATCTCAAACTGG	365-394
	M3 (R)	TCTTTATCAGCTCTGGGTGAATCAC	771-795
	M4 (F)	CAAGGTGAGACAAATCCATAGGTGGCC	1335-1361
	M5 (R)	TGATGAGTATGCTCATGAAGAACAAC	1638-1663
	M6 (F)	CAGGATCATTCAAGTTTGCAATATTTCCAG	2268-2297
	M7 (R)	CTTATTGGGGATGTGAAGAAGCTTGG	2566-2591
	M8 (F)	GATGTTAACCCTAAAGAGCTTCCTG	3029-3053
	M9 (R)	GTCTCAAATGCCCATGTCTATGGCTC	3348-3373
	M10 (F)	GTTATAGGATAATTATCTTGTGTC	4130- 153
	M11 (R)	CCAGAGGTTTATGATGATTCTGCTGAG	4579 4065
	M12 (R)	AGAGCAATCAGTGCAAACAAAAACCTTAATCC	4790-4821

Table 2. *Primer names and sequences used in RT-PCR studies*

Primers' name	The sequence of primer from 5' to 3'	Expected amplicon size
TSWV-M-1F	AGAGCAATCAGTGCATCAGA	955 bps
TSWV-M-1R	CTTCTTCTTCAACTGATCTCTCAAG	
TSWV-M-2F	GCAAGCTGATAATTCCTAAAGG	1351 bps
TSWV-M-2R	AAGGAGATGACATGTCTTGGG	
TSWV-M-3F	CCGCATAGAAGACAGCC	1276 bps
TSWV-M-3R	GTTATAGAAGGTCCTAATGATTGCA	
TSWV-M-4F	GTTAACCCTAAAGAGCTTCCTG	979 bps
TSWV-M-4R	GAGAAGATCATGGGTTATTTGAT	
TSWV-M-5F	CTTATCCAAGAAAAATTGATGC	1051 bps
TSWV-M-5R	AGAGCAATCAGTGCAAACAAAA	

The amplified RT-PCR products were both run on a 1.5% agarose gel, and stained with ethidium bromide and then visualized under UV (Integrated Biometra Gel Imager, Goettingen/(Germany)), and sequenced from both ends at HibriGen

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(Biotechnology Research, Development Industry and Trade Co. Ltd, Turkey). The M Segment were divided into smaller sequences (700 bp to 1200 bp) using primers (*Tables 1* and *2*) to yield overlapping sequences for alignments. The sequence analysis and multiple sequence alignment were carried out using Chromas (Technelsium DNA Sequencing Software Australia) and CodonCode Corporation (Florida) software. The complete M segment sequences were analyzed in National Center for Biotechnology Information (NCBI) using nucleotide BLAST. The agarose gel electrophoresis of M segment amplifications is shown in *Figure 3*.

Results

The plant materials used in this study were tested using Sw-5 specific molecular marker (Dianese et al., 2010). The codominant SCAR *Sw5-2* marker confirmed the resistant vs susceptible status of the plant materials. The resistant *Solanum peruvianum* was homozygous resistant (574 bp), the four commercial hybrids were heterozygous (574 and 500 bp), and the susceptible one was homozygous (500 bp) as expected (*Fig. 1*).

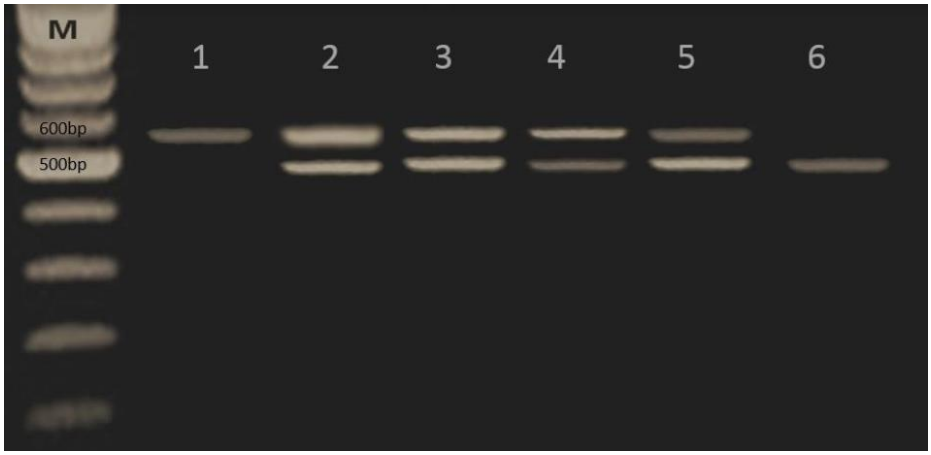

Figure 1. *Agarose gel electrophoresis of Sw-5 primers. M: 100 bpDNA ladder; Lane 1 resistant Solanum peruvianum, is a source of resistance with homozygous alleles (574 bp); Lanes 2 Torry F1 (Syngenta), 3 Matatu F1 (RijkZwan), 4 Tayfun F1 (De Ruiter), and 5 Swanson F1(Seminis). heterozygous resistant alleles known as resistant tomato varieties (574-500 bp); Lane 6 sensitive negative control. Tomato variety (Hazera 5656 (Hazera Seed)) without Sw-5 allele (500 bp)*

Antalya resistance-breaking (AntRB) TSWV isolates are confirmed in mechanical inoculations

After the TSWV mechanical inoculation (Hull, 2014), first typical virus symptoms were observed at 7 dpi on susceptible tomato variety. In resistant *Solanum peruvianum* and 4 commercial tomato varieties, the symptoms were barely evident at 15 dpi, similar in both homozygous and heterozygous plants.

RT-PCRs were performed at 21 dpi for 12 different tomato viruses (*Alfalfa Mosaic Virus* (AMV) (Saleh and Amer, 2013), *Cucumber mosaic virus* (CMV) (Paradies et al., 2000), *Potato virus X* (PVX) (Fidan et al., 2011), *Potato virus Y* (PVY) (Fidan et al., 2011), *Tabaco Etch Virus* (TEV) (Lee et al., 2011), *Tobacco Mosaic Virus* (TMV)

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(Kumar et al., 2011), *Tomato mosaic virus* (ToMV) (Kumar et al., 2011), *Tomato yellow leaf curl virus* TYLCV (Anfoka et al., 2008), *Tomato Chlorosis Virus* (ToCV) (Tiberini et al., 2010), *Tomato ringspot virus* (ToRSV) (Fuchs et al., 2009), *Pepino Mosaic Virus* (PePMV) (Ge et al., 2013) and TSWV (Adkins et al., 2005) (*Fig. 2*) it was confirmed that the samples were infected with only TSWV.

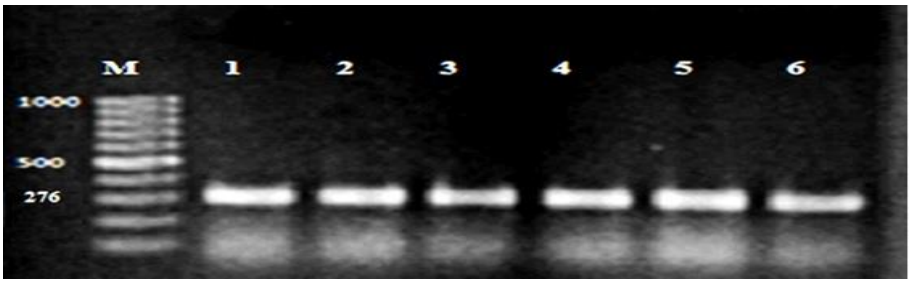

Figure 2. *Agarose gel electrophoresis of PCR products obtained using L1TSWVR and L1TSWVF primers specific to TSWV. The TSWV coat protein specific primers have produced 276 bp amplicon in PCR analyses. Lanes: M: 100 bp Standard Marker; Lane 1 resistant source 'Solanum peruvianum', Lanes 2 'Torry F1' (Syngenta), 3 'Matatu F1' (RijkZwan), 4 'TayfunF1' (De Ruiter), and 5 'Swanson F1' (Seminis). Resistant varieties, Lane 6 ('Hazera 5656' (Hazera Seed)) sensitive varieties*

After confirming that the samples got infected with TSWV (*Fig. 2*), the M domain (Medium segment) were sequenced using different primer combinations. The 4825 bp M segment was amplified at five overlapping pieces (*Fig. 3*). The sequence of M Segment parts was aligned and the consensus sequence was subjected to haplotype analysis, using DnaSP 5 program (DNA Sequence Polymorphism. Universidad de Barcelona). Both sequence and haplotype analysis showed that RBS of TSWV collected in Antalya had a single haplotype (Additional file A4), which was named and deposited into NCBI as TSWVAntRB.

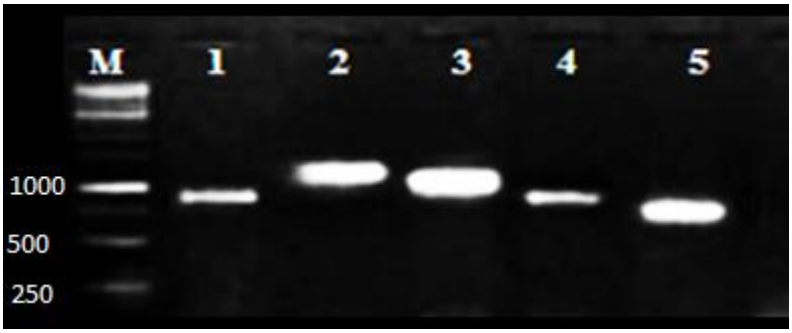

Figure 3. *TSWVAntRB Medium site is belonging to agarose gel electrophoresis. M: marker, Lane 1: M1-M3 primers (759 bp), Lane 2: M2-M5 (1269 bp), Lane 3: M4-M7 primers (1230 bp), Lane 4 primers: M6-M9 primers (1082 bp), Lane 5: M-5F/M-5R- primers (1051 bp)*

Phylogenetic analysis of complete nucleotide sequences of the M segment

The complete nucleotide M segment sequence obtained after assembly was recorded at NCBI database (MH367503). For phylogenetic analyses, the M segment sequences of

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both resistance breaking and non-breaking isolates originating from different parts of the world were retrieved from NCBI to trace the possible origin of resistance-breaking TSWVAntRB isolate. The nucleotide comparison was made in the NCBI BLAST system and the isolates with the highest similarity were selected (*Table 3*). Five of these isolates were RB (Resistance-breaking) and 13 of them were NRB (Non-Resistance-breaking).

Table 3. *The table is nucleotide comparison made in the NCBI BLAST system and the TSWVAntRB (MH367503) with the highest similarity was selected. The first five rows are RB (Resistance-breaking) and 13 of them are NRB (Non-Resistance-breaking) isolates*

	Name of isolate	Region	Host	% identity	Note	Accession No.
1	TSWVAntRB	Turkey	Tomato		RB	MH367503
2	Pujol1TL3	Spain	*Solanum lycopersicum*	99	RB	HM015520
3	Sala1TL3	Spain	*Solanum lycopersicum*	99	RB	HM015521
4	D-191	Australia	Tomato	97	RB	HM015516
5	Borgo1	Italy	*Solanum lycopersicum*	97	RB	MG457158
6	CA SW21	USA	Tomato	94	RB	KX898453
7	D	USA	Tomato and Tobacco	98	NRB	AF208497
8	p202/3RB	Italy	Pepper	98	NRB	HQ830185
9	LK-1	South Africa	Tomato and *Amaranthus*	96	NRB	KY250489
10	GA-1L	Spain	*Solanum lycopersicum*	92	NRB	FM163371
11	ALPA	Spain	*Datura stramonium*	92	NRB	HQ537114
12	LS3	South	*Leonurus sibiricus*	92	NRB	KM076652
13	TSWV-YN	China	Tomato	93	NRB	JF960236
14	YNta	China	Tobacco	93	NRB	KM657118
15	YN5576	China	*Solanum indicum*	93	NRB	KY495608
16	SPAIN-2	Spain	Tomato	93	NRB	AY744493
17	BasC	USA	*Ocimum basilicum*	93	NRB	KU179514
18	PA01	USA:	Pepper	93	NRB	KT160281

NRB: non-resistance-breaking

The phylogenetic analysis of the isolates selected from NCBI according to the Neighboring method was carried out using the Mega 7 program. The phylogenetic trees showed that the isolate TSWVAntRB is originated from Europe (*Fig. 4*). The genome analysis placed the virus with HM15520.1, Spanish Resistance-breaking isolate.

Of the 17 TSWV isolates with the highest similarity to the RB isolate (TSWVAntRB), five belonged to RB isolates originating from tomato reported in Spain, Italy, USA, and Australia (*Table 3*). The RB isolate TSWVAntRB located on the same branch with one of the Spanish RB isolates. The result indicates that the RB isolate TSWVAntRB identified in Antalya, Turkey most probably originates from Spain. TSWV isolates worldwide have been divided into two roots, Asian and European isolates (Lian et al., 2013). It was determined that the isolates divided into two separate groups and TSWVAntRB isolate was found in group II (*Fig. 4*). The Group II consisted of European resistance-breaking isolates. The most of Group I isolates of Asian origin.

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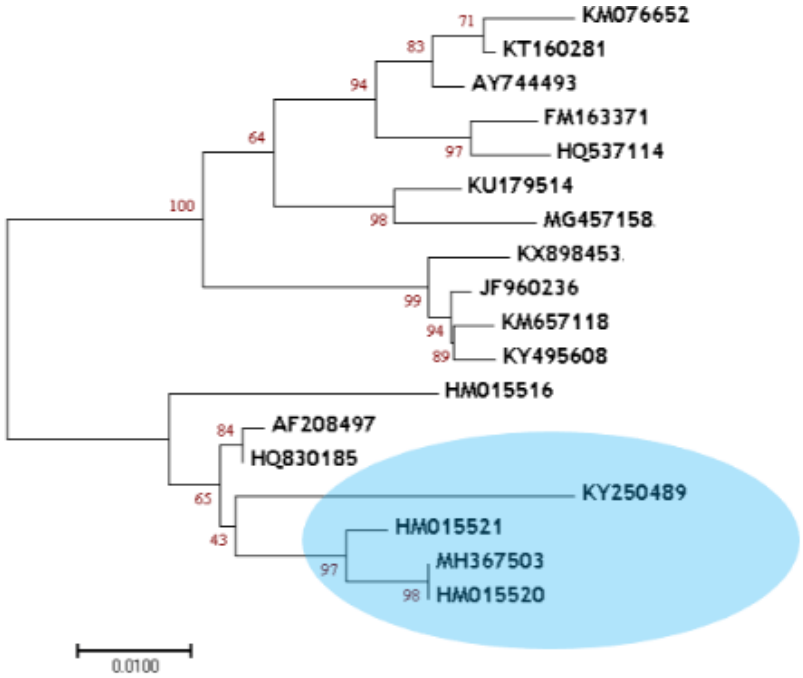

Figure 4. *Phylogenetic analysis showing the relationship between TSWVAntRB (MH367503) isolates with world isolates. The phylogenetic tree MEGA7 program was created using the Neighbor-Joining method. It was determined that the 18 isolates that were taken into consideration were formed by two groups and were calculated using the maximum composite likelihood method, which is the same root as the Spanish isolate (HM015520.1) that breaks down on the same branch with European isolates*

Protein-based comparisons on medium segmentation

Worldwide studies of TSWV indicate that resistance-breaking isolates arise due to a mutation in the NSm protein responsible from cell to cell movement on the Medium segment (Lopez et al., 2011).

The Bioedit and CodonCode Aligner programs were used to process sequence data and ExPASY Tool to obtain the predicted protein sequences of the M segment (*Figs. 5* and *6*). The predicted protein sequences of the TSWVAntRB isolate and the 17 isolates were aligned on which C118Y mutation was highlighted (*Fig. 5*) (Additional files 1, 2). The predicted protein alignment of the M segment further confirmed that a single point mutation on the movement protein located on M segment causes the resistance to break (KY973680.1; KY973679.1, KY973677.1, KT192625.1, KT192624.1, KT192623.1, KM379142.1, KM379141.1, MH367502.1, KX618636.1, KX618635.1, KM407603).

The remaining M segment sequence was also analyzed. The 40 different mutations detected throughout glycoproteins Gn and Gc (1135 aa) were not meaningful for resistance (Additional files 3).

It was previously reported that C118Y point mutation prevents the formation of HR response, causing systemic disease establishment on infected plants (Lopez et al., 2011).

The NSm protein domain on the TSWV's Medium region is the region that allows the virus to move from cell to cell. The resistance provided by the Sw-5 gene on the old isolate both causes rapid deaths in the contaminated tissues through HR reactions and there is no possibility of virus infection and contamination of new tissues in these tissues. However, it is shown that the TSWV resistance-breaking isolates related to

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NSm protein, a point mutation on the cell to cell movement gene, caused resistance breaking on TSWV resistant tomato.

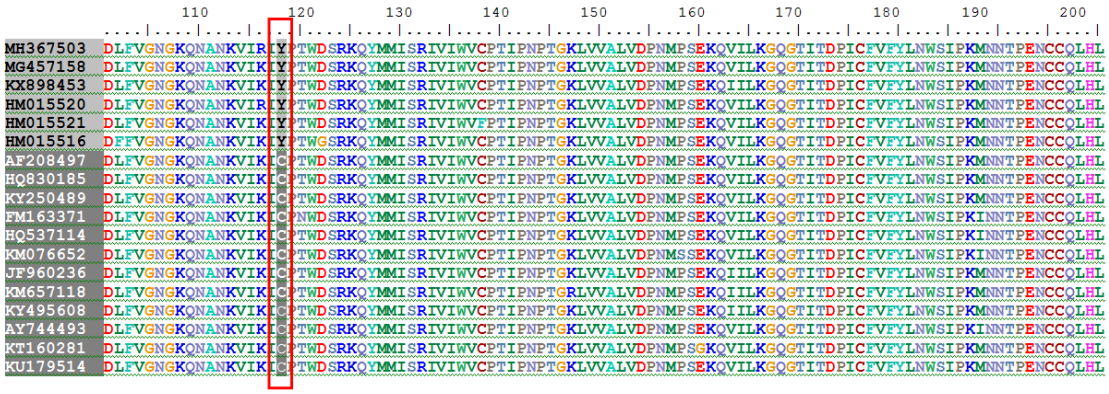

Figure 5. *Sequence alignment of the NSm proteins derived from the six resistance-breaking (RB) and 12 non-resistant-breaking (NRB) strains. The C118Y mutation on predicted amino acids derived from RB and NRB is indicated. The MH367503 (TSWVAntRB Antalya-Turkey RT tomato) line 2 to 6 are resistance-breaking (R.B). Lines 7 to 19 are non-resistance-breaking (N.R.B)*

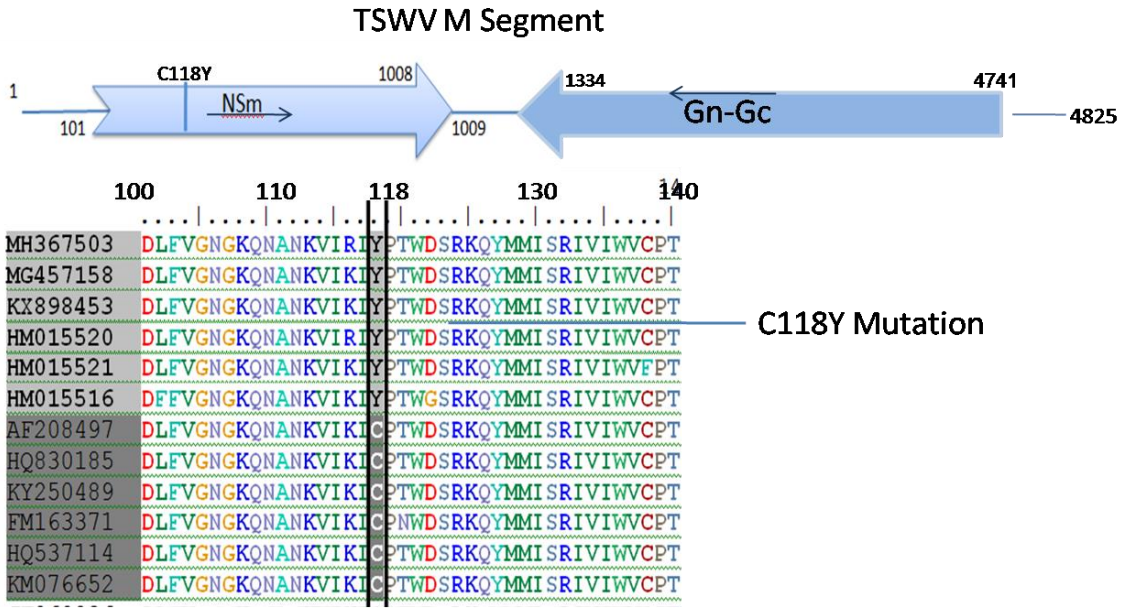

Figure 6. *The mutation site in the NSm protein domain (ORF-open reading frame) of the medium segment of the TSWVAntRB isolate*

Recombination events for single-stranded RNA viruses are thought to be involved in a major evolutionary process. Genetic alterations that occur due to recombination events can be very important for RNA viruses with ambisense character, which has a multi-segmented structure such as TSWV. Earlier studies emphasized the emergence of new isolates due to changes in the form of point mutations on viruses with multi-segmented structures such as TSWV.

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Discussion

Comparison of NCBI isolates and evaluation of the phylogenetic analysis

The TSWV isolates were divided into two origins, namely Asia and Europe (Lian et al., 2013). The sequence comparison of M segment of TSWVAntRB isolates with that of isolates in NCBI returned a similarity ratio of 93-98%, confirming previous reports for two distinct origins (Lopez et al., 2011; Tsompana et al., 2005). However, origins of RB isolates are independent of sequence diversity where the same single point mutation results with resistance break. The extensive use of Sw-5 allele may have caused positive selection, leading to the occurrence of RB isolate (de Ronde et al., 2014). The Sw-5 gene is known to provide resistance to *Orthotospovirus*es through HR response. When infection occurs in plants, cell death occurs rapidly via the Sw-5 gene and the virus is trapped within the dead cells. Recent studies have informed that C118Y mutation on the NSm proteins of *Orthotospovirus*es fails Sw-5 protein to initiate the HR, leading to systemic virus infections in plants (Leastro et al., 2015). The NSm protein is a non-structural protein (Kormelink et al., 1994; Storms et al., 1995) that promotes the generation of *Orthotospovirus*es through plasmodesmata into envelope-free nucleocapsids. Hence, the NSm protein is able to spread to neighboring healthy cells through plasmodesmata at the absence of a functional Sw-5 gene or C118Y mutation that make Sw-5 protein fail to trigger HR.

Lopez et al. (2011) reported that C118Y mutation on the NSm protein in the medium segment is a very important for adaptation on new hosts. This mutation, which is likely to occur on the cysteine, is considered to be a fundamental point in the adaptation of TSWV to the new hosts during the evolutionary process.

The TSWVAntRB may have been transported from Spain via thrips vectors. Persistent propagative transmission of the virus with thrips may explain the long-distance transmission. However, it has been noted that there is no association in the breakdown of the resistance of the TSWV isolate accumulated in thrips body (Debreczeni et al., 2014). It has been argued that the phylogenetic relationship of isolates obtained from different continent may be effective in transporting vector thrips with long-distance migration capability (Lopez et al., 2011). Knowing the factors involved in the evolution of viruses is essential to understanding the processes involved in molecular biology and epidemiology (including the emergence of new viruses), to develop more effective and strong control strategies (Garcia et al., 2001; Moya et al., 2004).

The entire genomic sequences of the Gn-Gc domain in the medium segment were also obtained and compared against the database. The result showed that although this domain carries multiple mutations, none of which plays a role in the breakdown of resistance.

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Conflict of interests. The authors declare that they have no conflict of interests.

Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

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APPENDIX

Appendix 1. *Comparison of MH367503 (TSWVAntRB) other TSWV isolates and difference ratios*

MH367503		**0,00**	**0,01**	**0,04**	**0,02**	**0,02**	**0,05**	**0,08**	**0,09**	**0,08**	**0,08**	**0,08**	**0,08**	**0,08**	**0,09**	**0,09**	**0,09**	**0,08**
HM015520	0,00		0,01	0,04	0,02	0,02	0,05	0,08	0,09	0,08	0,08	0,08	0,08	0,08	0,09	0,09	0,09	0,08
HM015521	0,01	0,01		0,04	0,02	0,01	0,04	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,08	0,09	0,08
HM015516	0,04	0,04	0,04		0,03	0,03	0,06	0,08	0,09	0,08	0,08	0,08	0,08	0,08	0,09	0,09	0,09	0,08
AF208497	0,02	0,02	0,02	0,03		0,00	0,04	0,06	0,07	0,07	0,06	0,07	0,07	0,06	0,07	0,07	0,08	0,07
HQ830185	0,02	0,02	0,01	0,03	0,00		0,04	0,06	0,06	0,06	0,06	0,07	0,07	0,06	0,06	0,07	0,08	0,07
KY250489	0,05	0,05	0,04	0,06	0,04	0,04		0,09	0,10	0,10	0,09	0,10	0,10	0,09	0,10	0,09	0,10	0,10
FM163371	0,08	0,08	0,08	0,08	0,06	0,06	0,09		0,01	0,03	0,06	0,06	0,06	0,02	0,02	0,04	0,05	0,06
HQ537114	0,09	0,09	0,08	0,09	0,07	0,06	0,10	0,01		0,04	0,05	0,05	0,06	0,02	0,03	0,04	0,05	0,06
KM076652	0,08	0,08	0,08	0,08	0,07	0,06	0,10	0,03	0,04		0,05	0,05	0,05	0,02	0,01	0,04	0,05	0,05
JF960236	0,08	0,08	0,08	0,08	0,06	0,06	0,09	0,06	0,05	0,05		0,01	0,01	0,05	0,05	0,04	0,05	0,01
KM657118	0,08	0,08	0,08	0,08	0,07	0,07	0,10	0,06	0,05	0,05	0,01		0,01	0,05	0,05	0,04	0,05	0,01
KY495608	0,08	0,08	0,08	0,08	0,07	0,07	0,10	0,06	0,06	0,05	0,01	0,01		0,05	0,05	0,04	0,05	0,01
AY744493	0,08	0,08	0,08	0,08	0,06	0,06	0,09	0,02	0,02	0,02	0,05	0,05	0,05		0,01	0,03	0,04	0,05
KT160281	0,09	0,09	0,08	0,09	0,07	0,06	0,10	0,02	0,03	0,01	0,05	0,05	0,05	0,01		0,04	0,05	0,06
KU179514	0,09	0,09	0,08	0,09	0,07	0,07	0,09	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,04		0,02	0,04
MG457158.1	0,09	0,09	0,09	0,09	0,08	0,08	0,10	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,05	0,02		0,05
KX898453.1	0,08	0,08	0,08	0,08	0,07	0,07	0,10	0,06	0,06	0,05	0,01	0,01	0,01	0,05	0,06	0,04	0,05	

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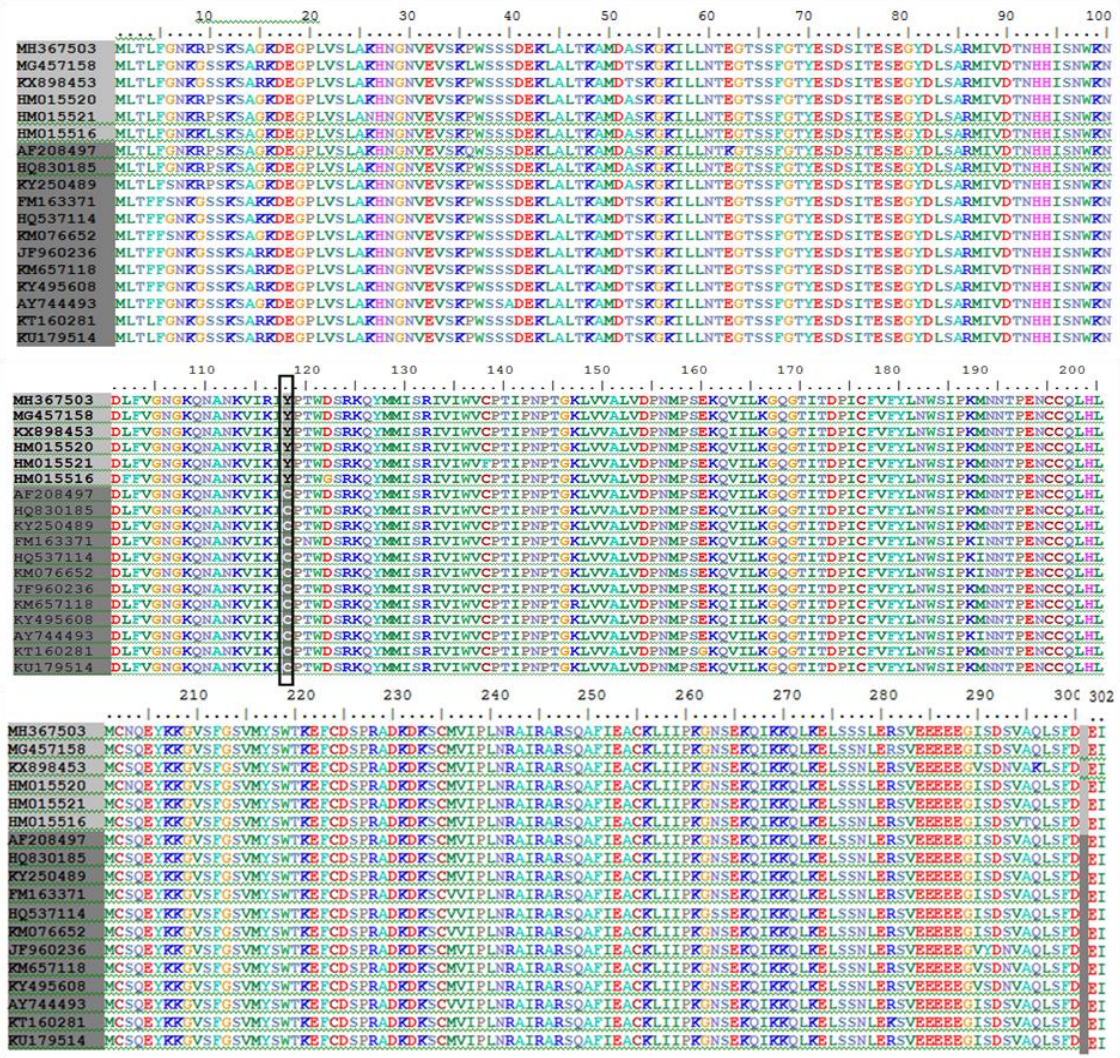
Appendix 2a. *Amino acid sequencing of the NSm open reading region of MH367503 (TSWVAntRB) isolate (302 AA)*



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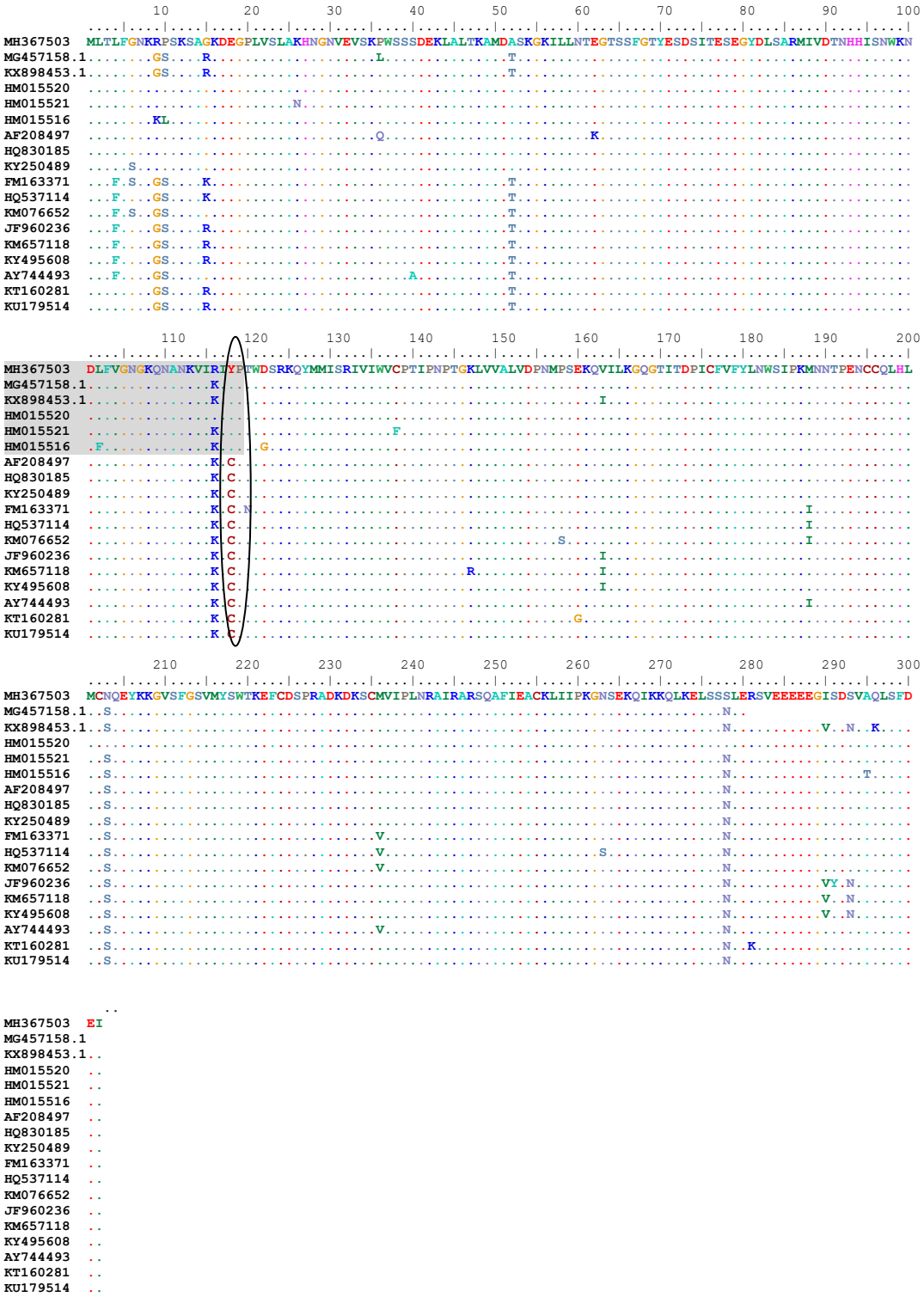
Appendix 2b. *Amino acid sequencing of the NSm open reading region of MH367503 (TSWVAntRB) isolate (302 AA) and consensus points with other isolates*



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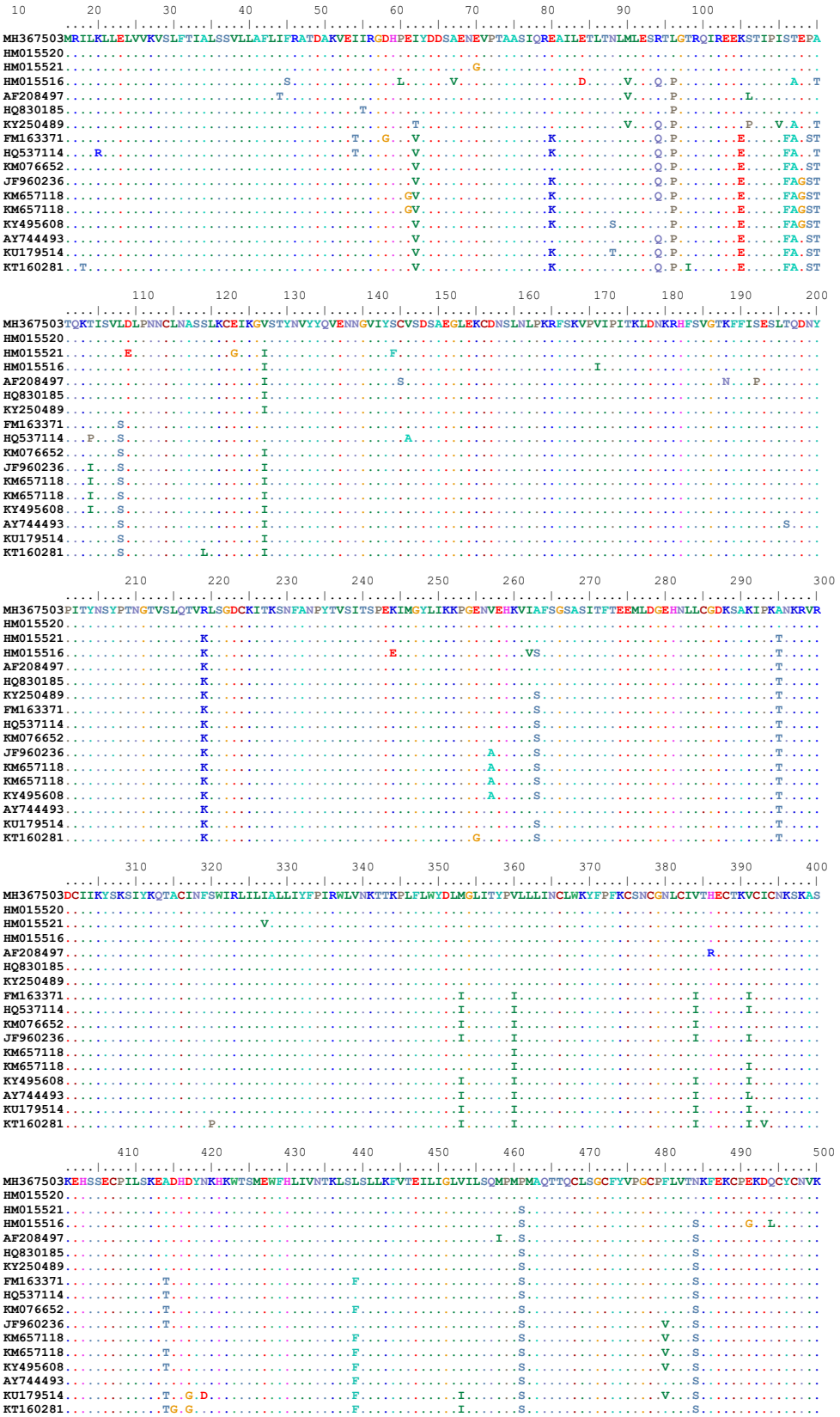
Appendix 3. *Amino acid sequencing of the Gn-Gc domain of MH367503 (TSWVAntRB) isolate*



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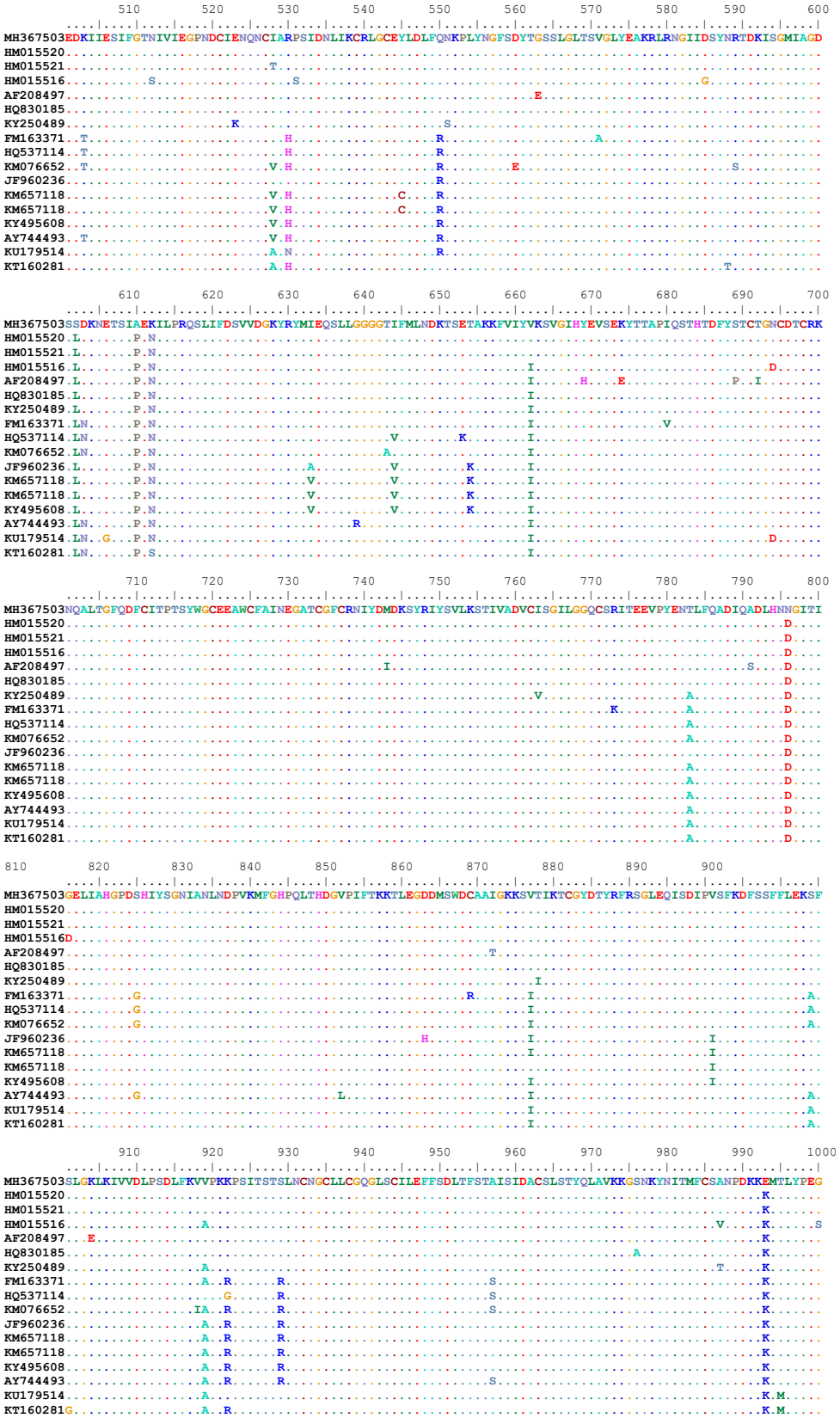

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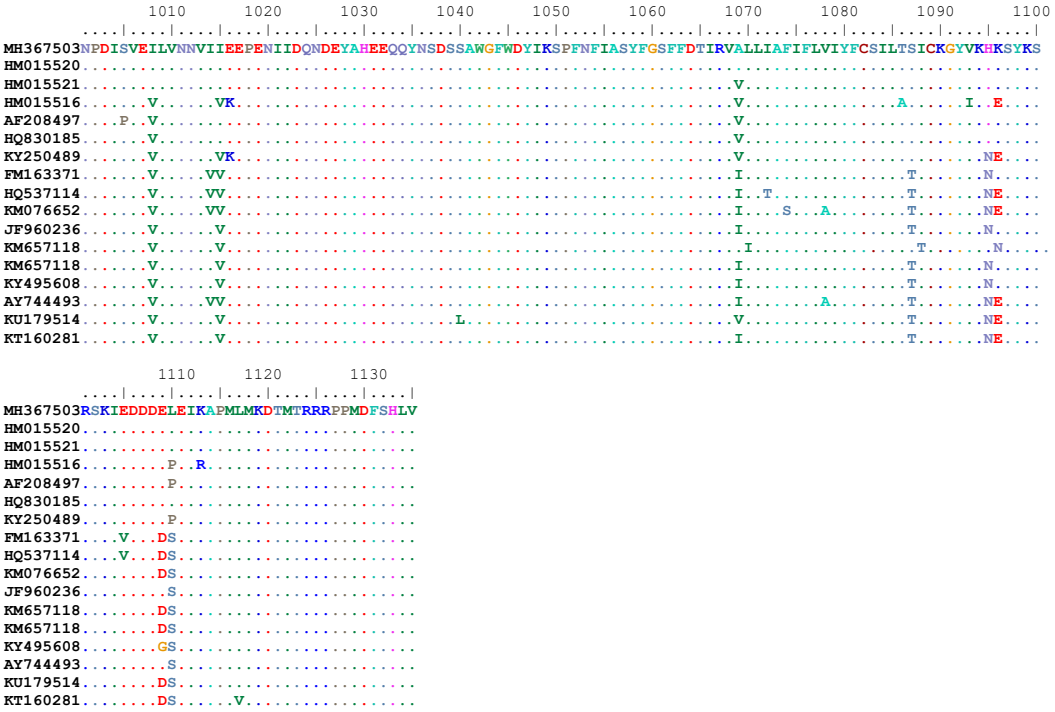

Appendix 4. *Results of haplotype analysis of 6 sequences using DnaSP Ver. 5.10.01*

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[Selected Region: 1-4825]
[Sites with alignment gaps: not considered]
[Invariable sites: included]

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TAXLABELS
Hap_1;
END;

[Hap# Freq. Sequences]
[Hap_1: 6 1-6]

[Hap# Freq. Sequences]
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