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Molecular Characterization of Cylindrical Inclusion Protein Gene Regions of Turkish *Zucchini yellow mosaic virus* (ZYMV) Isolates

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Abstract: *Zucchini yellow mosaic virus* (ZYMV) is an economically important viral pathogen that causes intense mosaic symptoms and disfigurement in cucurbits. The aim of this study is to characterize ZYMV isolates obtained from Ankara, Antalya, Burdur, Konya, Karaman, Aksaray provinces of Turkey according to cylindrical inclusion (CI) protein sequences and to determine conserved areas on protein in Potyviruses. For this purpose, molecular studies and sequence analyzes were performed with primers specific to the CI protein region of collected cucurbit samples during 2019-2014 years. At the end of the study, the N terminus of Turkish ZYMV's CI protein 888 nucleotides long and 296 amino acids (aa) was amplified. Phylogenetic analysis of the nucleotide sequences of the CI region showed that the majority of isolates (40) belonged to a large molecular subgroup (A1) most common in Europe and the world, and three isolates (Y4, Y21, Y23) belonged to subgroup A5. Moreover, according to the coat protein nucleotide analysis, these three isolates (Y4, Y21, Y23) were grouped with the molecular subgroup A4, the group that emerged recently in Europe. The CI ZYMV nucleotide binding motif (NTBM) and RNA helicase activity site (five motifs) were conserved among isolates according to amino acid analysis.

Keywords: Cucurbites, cylindrical inclusion, helicase activity, Phylogenetic analysis, Sequence analyzes, ZYMV

Türkiye ZYMV İzolatlarının CI Protein Gen Bölgelerinin Moleküler Karakterizasyonu

Öz: Kabak sarı mozaik virüsü (ZYMV), kabakgillerde yoğun mozaik semptomlarına ve şekil bozukluğuna neden olan ekonomik açıdan önemli bir viral etmendir. Bu çalışmanın amacı, Türkiye'de farklı illerden (Ankara, Antalya, Burdur, Konya, Karaman, Aksaray) elde edilen ZYMV izolatlarının silindirik inklüzyon (CI) protein dizilerine göre karakterize edilmesi ve Potyvirüslerde protein üzerinde korunan alanların belirlenmesidir. Bu amaçla CI protein bölgesine spesifik primerler ile moleküler çalışmalar ve dizi analizleri yapılmıştır. Çalışma sonunda, Türk ZYMV'nin CI proteini 888 nükleotid uzunluğunda ve 296 amino asit (aa) olan N uç kısmı çoğaltılmıştır. CI bölgesinin nükleotid dizilerinin filogenetik analizi, izolatların (40) çoğunluğunun Avrupa ve dünyada en yaygın olan büyük bir moleküler alt grubuna (A1) ve üç izolatın (Y4, Y21, Y23) A5 alt grubuna ait olduğunu göstermiştir. Ayrıca, kılıf proteini nükleotid analizine göre, bu üç izolat (Y4, Y21, Y23), Avrupa'da son zamanlarda ortaya çıkan grup olan A4 moleküler alt grubu ile gruplanmıştır. CI ZYMV nükleotid bağlama motifi (NTBM) ve RNA helikaz aktivite bölgesinin (beş motif) aa analizine göre izolatlar arasında korunduğu görülmüştür.

Anahtar Kelimeler: Filogenetik analiz, Sekans analizi, Kabakgiller,

1. Introduction

Zucchini yellow mosaic virus (ZYMV) causing intense mosaic symptoms and abnormalities in infected in cucurbit crops, causes significant economic losses and is included in the Potyviridae family, the Potyvirus genus (Choi et al., 2007; Desbiez & Lecoq, 1997; Hull, 2002). The genus *Potyvirus* is the largest genus of plant viruses. There are more than 200 species and more uncertain species in this group. Viruses belonging to this genus cause great economic losses by decreasing the quality and quantity of cucurbit products (melon, watermelon, cucumber, squash). The symptoms caused by ZYMV in plants are usually yellow mosaic on the leaves, severe deformation, swelling, significant shrinkage of the leaf blades, and severe stunting. In fruits, it causes swellings such as tubers and causes deformities on them. Infected melon and watermelon fruits also develop misshapen and longitudinal deep cracks. Seed formation is significantly reduced in infected plants and seeds are often deformed. In the tropic regions, ZYMV is often found as a complex with PRSV-W and WMV. Like other strains of Potyviruses, ZYMV is transmitted non-persistently by aphid species such as *Aphis gossypii* and *Myzus persicae* (Desbiez & Lecoq, 1997).

ZYMV particles are a 750 nm long, filamentous polyprotein consisting of a positive-sense RNA molecule (Hull, 2002; Lisa & Lecoq, 1984). At the 5th end of the genome, there is the genome linked protein (VPg) and at the 3rd end there is the polyA tail. The

virus genome encodes 10 functional proteins (Glasa & Pittnerova, 2006; Revers et al., 1999, Urcuqui-Inchima et al., 2001). These are P1 protein, which acts as a protease in opening the genome, auxiliary component protein (helper component protein, HC-Pro), which plays a role in systemic transport of the virus by insects and suppresses gene silencing, cylindrical inclusion protein (CI) that plays a role in cell-to-cell transport and has RNA helicase activity; nuclear inclusion b protein (NIb), which plays a role in genome replication, and cover protein (CP), which plays a role in the systemic transport of the virus from cell to cell. CI plays a role in different stages of viral infection. It has been reported that the CI protein plays a role in virus replication, cell-to-cell, and long-distance transport through interactions with the viral P3N-PIPO protein. CI protein acts as an avirulence factor in gene-for-gene interactions with dominant-resistance host genes and as a recessive-resistance overcoming factor (Sorel et al., 2014). Many viral and plant factors in plant cells have been shown to interact with this protein (Figure 1).

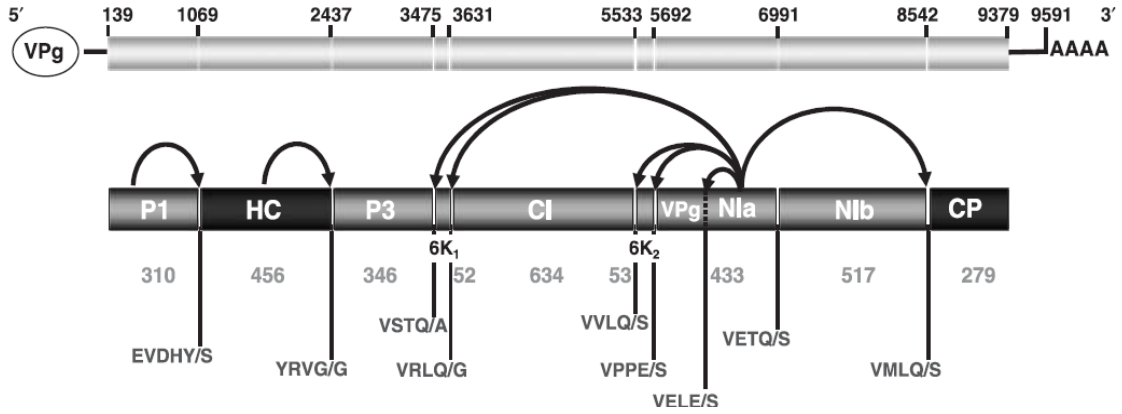


Figure 1: Schematic representation of the ZYMV-RNA genome and processing of proteins (Gal-On, 2007)
Şekil 1*: ZYMV-RNA genomunun şematik gösterimi ve proteinlerin işlenmesi*

In recent years, many studies on the biological and molecular variability of ZYMV have been published in the World (Coutts et al., 2011; Glasa et al., 2007; Maina et al., 2017; Novakova et al., 2014; Yakoubi et al., 2008). Most molecular studies have been based on the analysis of CP and/or partial NIb-CP sequences. It has also been reported that in the absence of the complete genomic sequence, the cylindrical inclusion (CI) coding region can be used for diagnostic and taxonomic purposes (Adams et al., 2005; Ha et al., 2008; Lee et al., 1997).

The presence of ZYMV in various studies has been reported in Turkey such as in Samsun (Şevik & Arlı-Sökmen, 2003), İzmir, Aydın, Manisa ve Balıkesir (Kaya & Erkan, 2011), Adana ve Mersin (Kamberoğlu et al., 2016), Kıbrıs (Helvacı et al., 2019, Nacar et al., 2021), Aksaray (Yeşil, 2019a), Yozgat (Yeşil, 2019b), Nevşehir (Yeşil 2020), Hatay (Sertkaya et al., 2004). In Turkey, there are molecular studies about CP region of ZYMV, but no information about the CI region (Özer et al., 2012; Topkaya et al., 2019; Topkaya, 2020; Yesil & Ertunc, 2012; Yeşil, 2014). In this recent research, it is aimed the molecular characterization of CI region of ZYMV isolates collected from different provinces of Turkey and to investigate the possibilities of use in phylogenetic classification.

2. Material method

2.1. RNA isolation

In this study, total RNA isolation studies from samples (Tablo 1) collected from Konya, Karaman, Aksaray, Burdur, Ankara, and Antalya provinces were performed by Astruc et al. (1996) according to the method proposed.

RT-PCR was performed using RNAs obtained after total RNA isolation from samples taken from different provinces and different cucurbit plants. In the two-step RT-PCR process, firstly, cDNA was obtained from RNAs obtained from RNA isolation by using random hexamer primer and MMLV-RT enzyme. In the second step, these cDNAs were used as templates and PCR was carried out with primers synthesized specifically for CI protein regions.

2.2. cDNA synthesis and RT-PCR methods.

Using 2 µl of total RNA for reverse transcription (RT), complementary DNA (cDNA) synthesis was performed in a 20 µl volume containing 4 µl 5X MMLV buffer (5X), 0,2 mM dNTP (25 mM), 1 µl random

hexamer primer (10 µmol), 0,25 µl RNAse inhibitor (10u/µl), 1 µl Reverse transcriptase (200u/µl) and 11.65 µl distill water for 25ºC for 10 min, followed by 42ºC for one hour and 72ºC for 10 min.

Table 1: The isolates used in the study, location, collected year, and symptoms
Çizelge 1: *Çalışmada kullanılan izolatlar, toplanma yeri, toplanma yılı ve semptomlar*

Province	**District**	**Collected**	**Isolate**	**Host**	Symptoms
Ankara	Şereflikoçhisar	2011	Ş1	Melon	D
			Ş3	Melon	D
			Ş5	Melon	D
			Ş8	Pumpkin	D
	Gölbaşı	2011	G1(m)	Squash	Mo
			G2	Squash	D, Mo
			G3	Squash	D
	Kazan	2010	Kz1	Squash	M,D
			Ahm1	Squash	M, D
	Ayaş	2013	AYS7	Squash	K,
	Çubuk	2011	C5	Pumpkin	M, C
			C11	Pumpkin	D
			C13	Pumpkin	D
	Beypazarı	2014	C17	Melon	D
			Be5	Squash	M
			Be6	Squash	M
			Be13 (m)	Squash	C
			Be15	Squash	C;
			Be18	Squash	M, Mo
			Be22 (m)	Squash	Mo
Antalya	Kumluca	2012	K3 (m)	Squash	D
			K13	Squash	C, M, V
			K17	Squash	C, M, V
	Aksu	2012	AS1	Squash	C, M, V
			AS5	Squash	V
			AS6	Squash	V
			AS8 (m)	Squash	D
			AS11	Squash	C, M, V
	Demre	2013	Demre	Cucumber	D
		2014	D14 (m)	Cucumber	Mo
	Elmalı	2013	Y4	Melon	M
			Y21(m)	Squash	Mo
			Y23(m)	Squash	Mo
			E-7	Melon	D
Burdur		2014	Brd1	Pumpkin	M, D
			Brd2	Pumpkin	M, D
			Brd4	Pumpkin	M, D
*Aksaray	Merkez	2010	AKS 2/5	Pumpkin	M,
	Ortaköy	2009	AK 5/7	Pumpkin	M,D
	Ortaköy	2009	AK 6/2	Pumpkin	M,
*Karaman	Kazımkarabekir	2010	KAR15/1	Pumpkin	M
			KAR12/4	Pumpkin	M,D
*Konya	Merkez	2010	A 3/1	Pumpkin	M
	Ereğli	2009	ER 2/8	Cucumber	M
	Yunak	2010	YUN8/4	Pumpkin	M,D
	Ereğli	2009	ER 6/8	Pumpkin	M,D

M: Mosaic, D: Deformation, Mo: Mottling, V: vein banding, C: Clorosis
*Provided by Dr. Serkan Yeşil

2.3. Phylogenetic analysis

Protein regions of 43 plants from different provinces and districts that gave positive results at the end of RT-PCR were studied. The PCR products with positive bands at the end of the RT-PCR process were sent for DNA sequence analysis by Sanger method. The data obtained at the end of the sequencing were cleaned using the Chromas program and saved as a single file. Sequencing studies were carried out bidirectionally, and the obtained forward and reverse sequences were combined using the "CAP3 Sequence Assembly" computer program and consensus sequences were

obtained. Based on the results obtained, the amplified parts of the isolates were compared with the isolates previously reported in the NCBI gene bank, and similarity rates were determined. For this purpose, the data were sequenced using the MEGAX (Kumar et al., 2018) program and a phylogenetic tree was created. Phylogenetic analysis was obtained using the "Neighbors joining tree" analysis method, 1000 boostrap and Kimura 2 parameters.

3. Results and Discussion

In the study, samples were taken collected from cucurbit plants showing virus symptoms from Ankara, Antalya, and surrounding provinces (Konya, Karaman, Aksaray, Burdur) where cucurbit cultivation is intensively performed. After the diagnosis of ZYMV positive isolates with serological tests (Topkaya et al., 2019), sequence analysis of the cylindrical inclusion protein gene regions encoded by the positive ZYMV isolates was performed, and phylogenetic analysis was performed by comparing the protein gene regions of the ZYMV isolates available in the literature.

3.1. RT-PCR results

In the amplification of the CI protein gene region of the ZYMV isolates used in the study, ZY-debCI-5' and ZY-milCI-3' primers, which amplify the partial region (N-terminal part) of the CI region (approximately 1000 bp), were used and at the end of the RT-PCR process, approximately 1000 bp bands were obtained in 1% agarose gel (Figure 2).

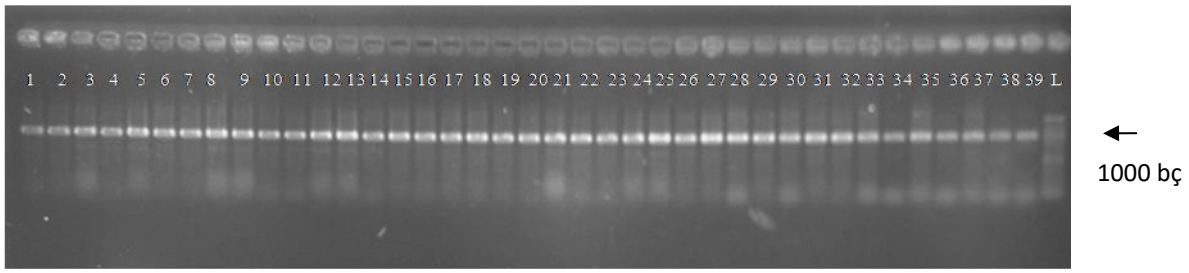


Figure 2: RT-PCR results of CI region
Şekil 2: *CI bölgesinin RT-PCR sonuçları*

L:100 bp ladder (NEB), K: Negatif kontrol, 1: C3, 2:C5, 3:C11, 4:C13, 5:C17, 6:C24, 7:BE5, 8:BE6, 9:BE7, 10:BE10, 11:BE13, 12:BE15, 13:BE18, 14:BE22, 15:BE26, 16:BE27, 17:S1, 18:S3, 19:S5, 20:S8, 21:G1, 22:G2, 23:G3, 24:KZ1, 25:AHM1, 26:AYS7, 27:K3, 28:K17, 29:K13, 31:AS5, 32:AS6, 33:AS8, 34:AS11, 35:H1M, 36 Y4, 37: BRD1, 38: BRD2, 39: AK 5/7

2.4. Phylogenetic analysis

Sequence informations of the ZYMV CI protein region from Turkish ZYMV isolates were successfully obtained and submitted to the GenBank, with accession numbers (KP828388, KP828423, KP828389, KP828390, KP828391, KP828414, KP828427, KP828421, KP828422, KP828393, KP828394, KP828392, KP828401, KP828402, KP828403, KP828395, KP828396, KP828397, KP828398, KP828399, KP828400, KP828404, KP828405, KP828406, KP828410, KP828407, KP828408, KP828409, KP828418, KP828411, KP828412, KP828415, KP828416, KP828417, KP828420, KP828419, KP828413, KP828425, KP828426, KP828424)

Analyses were performed using 43 nucleotide sequences obtained in this work and 12 obtained from GenBank. The CI nucleotide sequences were translated to amino acids using MEGAX computer software programs. Phylogenetic trees were done by neighbor-joining (NJ) methods implemented in MEGAX (Kumar et al., 2018), with 1000 bootstrap replicates.

When we examine the phylogenetic tree obtained by the nucleotide sequence of the CI protein region, it is seen that most of the isolates we used in the study are in the A1 subgroup, which is common in the world and Europe. BE5, BE6, BE7, BE10, BRD1, BRD2, BRD4, C17, AKS2-5, BE26 and YUN8-4 isolates with Kuchyna, the Slovak isolate in the A1 group; C11, AYS7 and H1 isolates showed similar branching as ZYMV-Fars isolate. KZN1, D14, K17, DEMRE isolates were included in the A1 subgroup, but formed a different cluster from the reference and other isolates. AS1, AS5, G1, G2, G3, ER6-8, AS11, S3, C13, C5, ER2-8, KAR15-1, KAR12-4, AKS5-7, AKS6-2 isolates showed similar branching with ZYMV-AG and ZYMV-NAT isolate (Figure 3).

In the DAMBE program, isolates according to CI region, G1, 2, G3 isolates; BE13, BE15, BE18, BE22 isolates; BE5 and BE6 isolates; BE7, BE10, and C17 isolates; BRD1, BRD4 and YUN8-4 isolates; KZN1 and

D14 isolates showed similar base sequences among themselves. While Y23 and Y4 in the A5 subgroup showed similar base sequences, Y21 showed a different sequence from these two isolates (Figure 3). The genetic distance between Turkish ZYMV isolates and with isolates from different parts of the world was 0.001-0.1 and genetic identity between groups was 90–99% (Table 2). Genetic identity indicated a very distant genetic relationship between the groups.

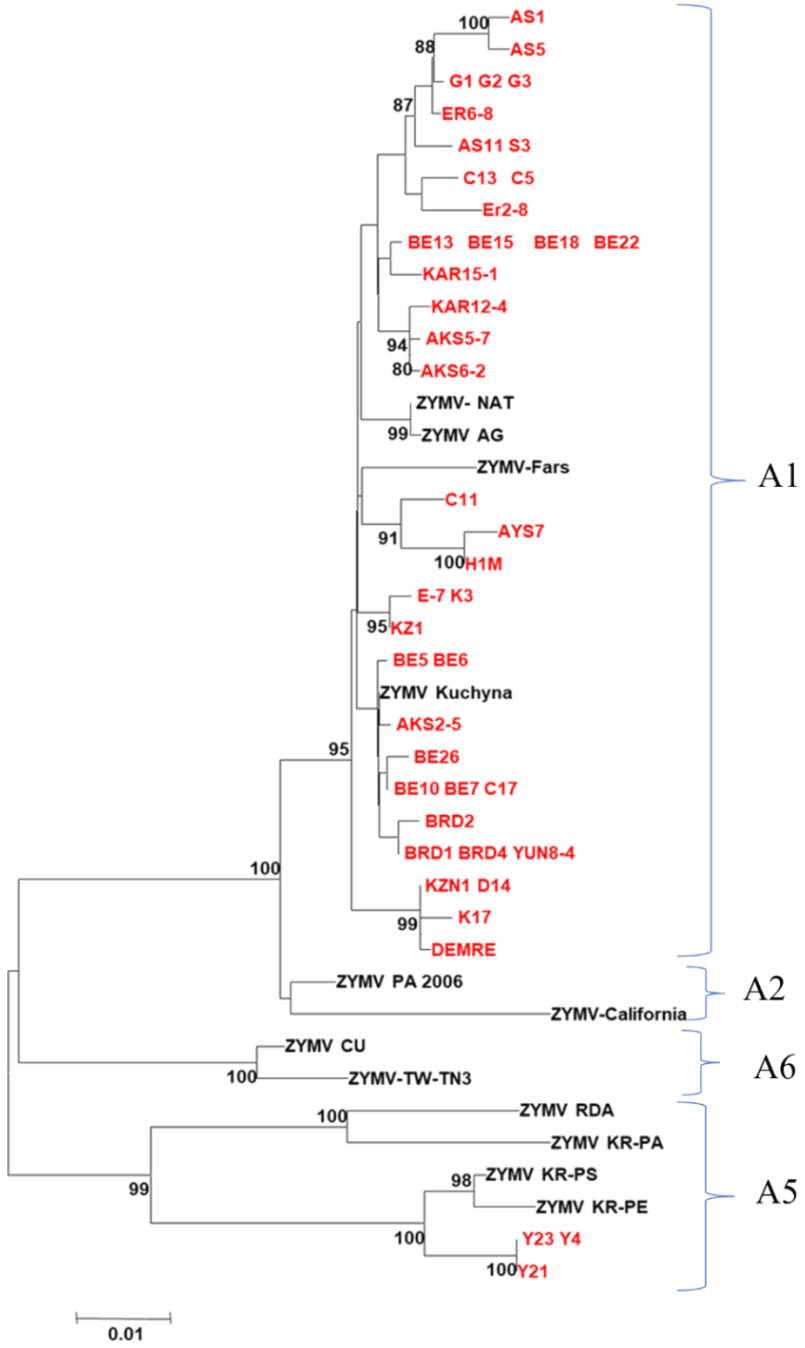


Figure 3: Phylogenetic tree of CI protein region
Şekil 3. *CI protein bölgesinin filogenetik ağacı*

The isolates obtained in this study are shown in red, and the isolates from the GenBank database are shown in black. Boostrap values above 80% are shown.

Table 2: The genetic distance between Turkish ZYMV isolates and with isolates from different references.
Çizelge 2: *Türkiye ZYMV izolatları ile farklı referans izolatlar arasındaki genetik uzaklık*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	DQ124239 ZYMV_Kuchyna																																								
2	EF062582_ZYMV-_NAT	99																																							
3	EF062583 _AG	99	10																																						
4	JN183062 _ZYMV-Fars	99	98	98																																					
5	JQ716413_ZYMV_PA_2006	98	98	98	97																																				
6	L31350 ZYMV-FLORIDA	96	96	96	95	97																																			
7	AJ307036.2_ZYMV_CU	93	93	93	92	94	92																																		
8	AF127929.2_ZYMV-TW-TN3	93	92	92	92	93	91	99																																	
9	AB369279_ZYMV_RDA	90	91	91	90	91	88	92	91																																
10	AY279000_ZYMV_KR-PS	91	91	91	90	91	89	92	91	92																															
11	AY278999_ZYMV_KR-PE	90	90	90	90	91	88	91	91	92	99																														
12	AY278998_ZYMV_KR-PA	90	90	90	89	91	88	91	91	96	92	92																													
13	AKS2-5	10	99	99	98	98	96	93	92	90	91	90	90																												
14	AKS5-7	99	99	99	98	98	95	93	92	90	91	90	90	99																											
15	AKS6-2	99	99	99	98	98	95	93	92	90	91	90	90	99	10																										
16	AS1	98	98	98	97	97	95	92	91	89	90	89	89	98	98	98																									
17	AS5	98	98	98	97	97	95	92	91	89	90	89	89	98	98	98	10																								
18	BE13_BE15_BE18_BE22	99	99	99	98	98	96	93	92	90	91	90	90	99	99	99	98	98																							
19	BE26	10	99	99	98	98	96	93	92	90	91	90	90	10	99	99	98	98	99																						
20	BE5_BE6	10	99	99	98	98	96	93	93	90	91	90	90	10	99	99	98	98	99	10																					
21	BRD2	10	99	99	98	98	96	93	92	90	91	90	90	99	99	99	98	98	99	99	99																				
22	C11	99	99	98	98	98	95	93	92	90	90	90	89	99	98	98	97	97	99	99	99	98																			
23	BE10_BE7_C17	10	99	99	98	98	96	93	93	91	91	90	90	10	99	99	98	98	99	10	10	99	99																		
24	C13_C5	99	98	98	98	98	95	93	92	90	90	90	90	99	99	99	98	98	99	98	99	98	98	99																	
25	KZN1_D14	99	99	99	98	98	95	93	93	90	90	90	90	99	99	99	98	98	99	99	99	99	98	99	98																
26	AYS7	98	98	98	97	97	95	92	92	90	90	90	89	98	98	98	97	97	98	98	98	98	99	98	98	98															
27	ER6-8	99	99	99	98	98	95	92	92	90	90	90	90	99	99	99	99	99	99	99	99	98	98	99	99	98	98														
28	G1_G2_G3	99	99	98	98	98	95	92	92	90	90	90	90	99	99	99	99	99	99	99	99	98	98	99	99	98	98	10													
29	H1M	99	98	98	98	97	95	93	92	91	91	90	90	99	98	98	97	97	98	98	99	98	99	99	98	98	10	98	98												
30	K17	99	98	98	98	97	95	93	92	90	90	90	89	99	98	98	97	97	98	98	99	98	98	99	98	10	97	98	98	98											
31	E-7_K3	99	99	99	98	98	96	93	93	90	91	90	90	99	99	99	98	98	99	99	99	99	99	99	98	99	98	99	99	98	98										
32	KAR12-4	99	99	99	98	98	95	93	92	90	91	91	90	99	10	10	98	98	99	99	99	99	98	99	98	98	98	99	99	98	98	99									
33	KAR15-1	99	99	99	98	98	95	93	92	90	90	90	89	99	99	99	98	98	10	99	99	99	98	99	99	99	98	99	99	98	98	99	99								
34	KZ1	99	99	99	98	98	96	93	93	91	91	90	90	99	99	99	98	98	99	99	99	99	99	99	99	99	98	99	99	99	99	10	98,	99							
35	AS11_S3	99	99	99	98	98	95	92	92	90	90	90	90	99	99	99	99	99	99	98	99	98	98	99	99	98	97	99	99	98	98	98	98,	99	99						
36	Y23_Y4	90	90	90	90	91	88	92	91	92	98	98	92	90	91	91	89	89	90	90	90	90	90	91	90	90	90	90	90	90	90	90	90,	90	90	90					
37	BRD1_BRD4_YUN8-4	10	99	99	98	98	96	93	93	90	91	90	90	10	99	99	98	98	99	99	10	10	99	10	99	99	98	99	99	98	98	99	98,	98,	99	99	91				
38	DEMRE	99	99	98	98	98	95	93	93	90	90	90	90	99	98	98	97	97	99	99	99	98	98	99	98	10	98	98	98	98	10	99	98,	98,	99	98	90	99			
39	Y21	90	90	90	90	91	88	92	91	92	98	98	92	90	91	91	89	89	90	90	90	90	90	91	90	90	90	90	90	90	90	90	90,	90	90	90	10	91	90		
40	Er2-8	98	98	98	97	97	94	93	92	90	90	90	90	98	98	98	98	98	99	98	98	98	98	98	99	98	97	99	99	98	97	98	98,	98,	98	99	90	98	98	90	

Table 3: Protein sequence comparisons of the CI protein region with reference isolates
Çizelge 3: *CI protein bölgesinin referans izolatları ile protein sekans karşılaştırmaları*

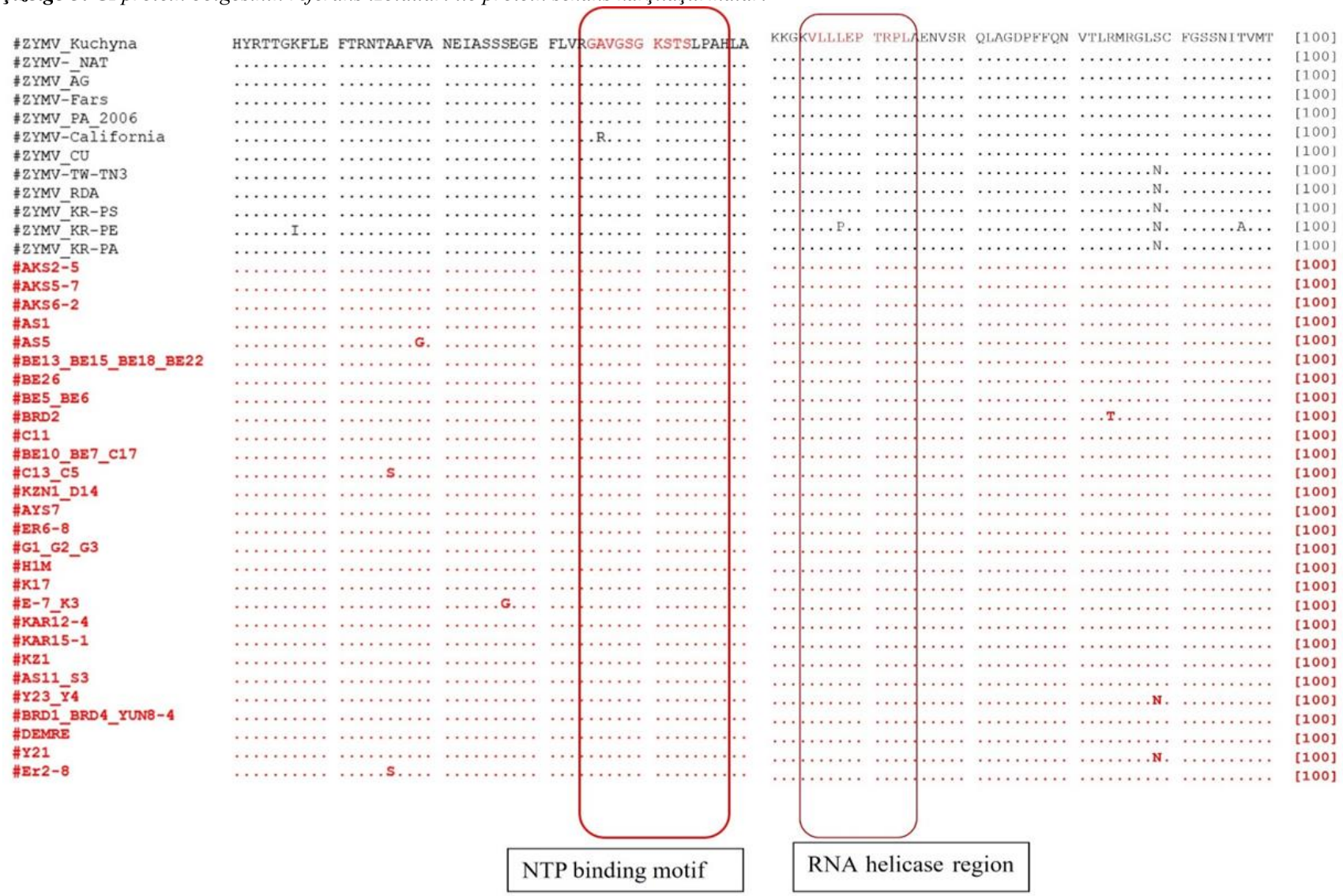
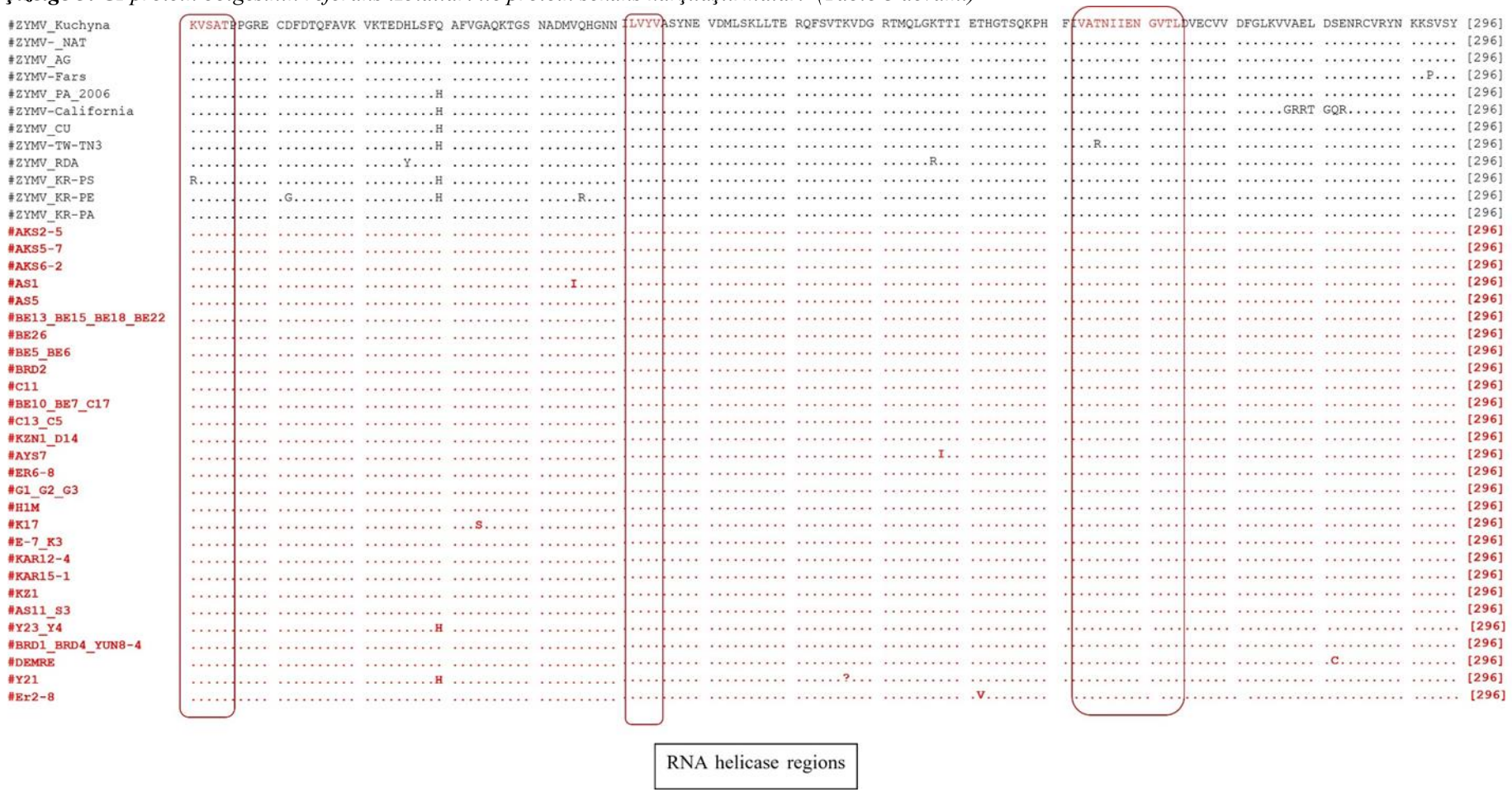


Table 3: Protein sequence comparisons of the CI protein region with reference isolates (Table 3. cont.)

Çizelge 3: *CI protein bölgesinin referans izolatları ile protein sekans karşılaştırmaları (Tablo 3 devamı)*



The protein sequence generated using the nucleotide sequence of the CI protein region and the protein comparison with reference isolates were made using the MEGA6 computer program. The results obtained are shown in Table 1. The 296 amino acid partial protein sequence consisting of 888 nucleotides of the CI protein region was compared with reference isolates selected from available sequences in the NCBI gene bank. When the amino acid regions were evaluated at the end of the comparisons, it was found that the CI protein region was not very variable and the previously reported NTP binding motif "GAVGSGKST" (26-35. aa) and RNA helicase function "VLLLEPTRPL", "KVSAT", "LVYV" and "VATNIIENGVTL" motifs appear to be preserved (Table 3).

According to different researchers, the ZYMV CP region is divided into three main groups (Desbiez et al., 2002; Ha et al., 2008; Zhao et al., 2003). Firstly, ZYMV isolate were classified into two main groups based on the analyses of partial nt sequences of CP gene by Desbiez et al. (2002). Later Zhao et al. (2003) and Ha et al. (2008) reported three groups: I, worldwide; II, containing isolates only from Asia; and III, containing isolates only from China.

In previous studies, Lee et al. (1997) compared the CI protein gene regions of 14 ZYMV Singapore isolates and found five more conserved regions in addition to the sequence of the nucleotide binding (GAVGSGKST) motive, which is seen as the membrane binding component of RNA helicase complexes. In addition, as a result of the phylogenetic tree they made with the sequences of the CI and CP gene regions, similar branches were found, and they showed that the phylogenetic relationship between Potyviruses could be determined using the CI gene region. Researchers have suggested that the CI gene region can be used as an alternative approach in the evolution studies of Potyviruses.

The sequences of the (CP) and (CI) proteins of ZYMV isolates from Austria, Germany, Italy and Slovenia have been reported. As a result of DNA sequence comparison of 30 ZYMV isolates from different geographical regions around the world, it was seen that the Austrian isolates showed a high level of similarity with the Slovenian and Hungarian isolates. The isolates from Germany and Italy turned out to be distantly related and clustered with isolates from other parts of the world. The results of the study showed that a specific isolate can spread rapidly to geographically adjacent areas, but may not be related to isolates found in other neighboring countries (Pfosser & Baumann, 2002)

4. Conclusion

This is the first report of CI protein sequence information of the ZYMV in Turkey. Analysis with the CI region yielded results similar to the results obtained from the CP region and showed that the CI region could be used to classify ZYMV isolates. This study helps us to further understand the genetic diversity of ZYMV isolates infecting cucurbit plants collected from different provinces of Turkey.

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