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To cite this version:

Serife Topkaya, Cecile Desbiez, Filiz Ertunc. Presence of cucurbit viruses in Ankara and Antalya province and molecular characterization of coat protein gene of zucchini yellow mosaic virus turkish isolates. Fresenius Environmental Bulletin, 2019, 28 (4), pp.2442-2449. ⟨hal-02154657⟩

HAL Id: hal-02154657
https://hal.science/hal-02154657v1

Submitted on 12 Jun 2019

PRESENCE OF CUCURBIT VIRUSES IN ANKARA AND ANTALYA PROVINCE AND MOLECULAR CHARACTERIZATION OF COAT PROTEIN GENE OF *ZUCCHINI YELLOW MOSAIC VIRUS* TURKISH ISOLATES

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ABSTRACT

Cucurbits are widely grown vegetables in different provinces of Turkey. However their production is restricted by pests and pathogens including different plant viruses, among which Z*ucchini yellow mosaic virus* (ZYMV) is particularly damaging. Infections by this virus during the early stages of plant development causes deformation of fruits and significant yield losses. During 2009-2014, 221 cucurbits samples were collected from fields and greenhouses from different provinces of Turkey. Serological testing was performed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for presence of *Watermelon mosaic virus* (WMV), ZYMV, *Cucumber mosaic virus* (CMV), *Papaya ring spot virus* (PRSV), *cucumber green mottle mosaic virus* (CGMMV) and *Squash mosaic virus* (SqMV). For molecular analaysis of ZYMV, full-length CP nucleotide sequences were obtained for 45 ZYMV isolates and compared with sequences from worldwide isolates available in databases. The CP coding regions of Turkish ZYMV were 837 nt long and encoded 279 amino acids (aa). Forty two isolates from different regions were classified in the molecular subgroup A1, the most frequent in the Mediterranean Basin, Europe and Africa. Three isolates collected from Antalya region were classified in subgroup A4 and showed 99 % sequence identity with isolates from the same subgroup that have emerged recently in France.

KEYWORDS:
Coat protein, Cucurbit viruses, Molecular variability, *Zucchini yellow mosaic virus*

INTRODUCTION

Vegetable production has a great economic importance in Turkey. According to the FAO (2014), vegetable cultivated area is 808.000 hectares with a production of 28.448.118 tons [1]. The southern part of Turkey, especially Antalya province, is the main and most intensive vegetable-growing area. However cucurbit production is restricted by different plant virus infections. *Zucchini yellow mosaic virus* (ZYMV) is one of the most economically important viruses of cucurbit crops worldwide, including in Turkey [2, 3, 4]. ZYMV was first isolated from a zucchini squash plant in Northern Italy in 1973 [2, 5] and was later reported as a major cucurbit virus in the world. The virus causes very severe mosaic, mottling, deformations on leaves and fruits of the infected cucurbit plants [2, 6] and affects the yield and quality of the crops: infected plants are reduced in-size and their fruits are often deformed and unmarketable. One of the main features of ZYMV is the great biological variability among strains whereby differences in host range as well as symptoms severity can be expected [7].

ZYMV is a member of the genus *Potyvirus* in the family *Potyviridae*. It has a positive sense-single stranded RNA of about 9.5 kb [8]. So far, more than 500 ZYMV sequences are available in GenBank, mostly CP-coding regions. Three major molecular groups of ZYMV have been defined in the World, namely Group A, Group B, and Group C. Group C contains isolates from Vietnam and China, Group B was observed in Reunion Island and in Australia, whereas Group A is present worldwide [7,9]. Within Group A, three clusters (clusters 1–3) were originally defined [10], and three others, namely clusters 4–6, containing mostly isolates from China and Korea, were distinguished later when the number of sequences available increased [9]. In the Mediterranean Basin, cluster A1 was observed in France since 1979, as well as in Spain, Portugal, Tunisia, Turkey, Israel, Syria, and Jordan [7, 10]; cluster A2 was observed in Italy since 1973 [10],Tunisia [11], Jordan and France since 1979; cluster A3 was detected in Spain and South-eastern France. In addition to these three clusters, surveys have revealed the presence of clusters 4 and 5 insouth-eastern France since 2004, probably in relation to recent introductions, and their current emergence in that area [7].

In Turkey, Turkish ZYMV isolates cluster A1 was reported from Adana and Mersin provinces [10, 12, 13]. Whether other clusters are also present in Turkey remains unknown. The aim of this research was thus to characterize the molecular variability of ZYMV Turkish isolates and investigate the presence of emerging strains.

MATERIALS AND METHODS

Collection of virus isolates. During the years 2010-2014, a total of 221 samples were collected from cucurbit plants (squash, pumpkin, cucumber, melon and watermelon) from Ankara (Nallıhan, Beypazarı, Cubuk, Sereflikochisar, Gölbası, Kazan, Ayaş, Ankara University (AUZF) and Antalya (Kumluca, Demre, Elmalı, Serik, Aksu) provinces of Turkey. Surveys were conducted in Ankara province at open fields during the summer seasons in 2010-2014 and the samples from Demre, Kumluca, Aksu, Serik districts were collected from greenhouse- grown cucurbits in winter in 2011-2012. Antalya-Elmalı samples were collected from open fields in 2010-2014 summer time.

Samples were collected from plants showing severe systemic mosaics, vein banding, stunting, blister, and deformations on fruits and leafs of infected plants. Major simptoms of melon were mottling, leaf deformation and samples collected from greenhouses in Demre, Aksu, Serik and Kumluca districts showed mosaic, stunting and yellowing symptoms on leaf and mottling on fruit, blistering of leaf and mottling on fruits on squash.

DAS-ELISA test. All collected samples were tested by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) [14] for the presence of ZYMV and other viruses: *Cucumber mosaic virus* (CMV, *Cucumovirus*), *Watermelon mosaic virus* (WMV, *Potyvirus*), *Papaya ringspot virus* (PRSV, *Potyvirus*), *Cucumber green mottle mosaic virus* (CGMMV, *Tobamovirus*) and *Squash mosaic virus* (SqMV, *Comovirus*). The test was applied according to manufacturer's instructions (Loewe Biochemica GmbH-GERMANY). Absorbance values were measured at 405 nm using an ELISA reader. The examples used as a positive control were provided from antisera manufacturer's and healthy cucurbit plants were used as negative control. All samples were tested in duplicates. The samples with absorbance value two to three times higher than the negative control were considered to be infected.

Molecular characterization of ZYMV isolates. RNA extraction and RT-PCR amplification. Total RNAs were extracted from samples of ZYMV-infected plants modified the protocol described by Astruc et al. [15]. Forty-five samples were selected from different hosts and geo-graphic locations. Thirty–three samples were cho-sen from collected samples during the surveys and nine ZYMV isolates added from Konya, Karaman and Aksaray, collected in 2009-2010, were supplied by Dr. Serkan Yesil (Selcuk University, Agricultural Faculty, Plant Protection Department). Three samples collected from only one field in Burdur province for moleculer analysis in 2014.

Total RNA extraction was made according to Astruct et al. [15]. For the complementary DNA synthesis (cDNA), a mix of 2 µl total RNA, 1µl random hexamer primer (5'-d(NNNNNN)-3'N = G, A, T or C)(10µ) and 8 µl distilled water were heated at 65°C for 5 min then stored on ice for 3 min before being added to the reverse transcription mix: 4µl MMLV buffer (5X), 0,2 mM dNTP (25 mM), 0,25 µl RNAse inhibitor (10u/µl), 0,25 µl MMLV (Reverse transcriptase- (Thermo Fisher Scientific)(20-20u/µl) and distilled water in a final volume of 20µl. The mix was incubated for 5 minute at 25 ºC, 1 h at 37 ºC and 10 minute at 72 ºC in a Biometra T1 Thermocycler.

PCR reactions were carried out in a volume of 25 µl containing 2,5 µl of cDNA, 10 pmol each primer ZYCP-F 5'-AGAGGCTATYTGCGCTGC-3' and ZYCP-R 5'- TAAAGCTTCCGACAGG-ACTAC-3' [16] 10 mm dNTPs, 1,5mM $MgCl_2$ and 2,5u Taq DNA polymerase (Promega). The primer pair amplifies a nucleotide segment of 1260 bp including partial NIb gene, full CP gene and 3′-NCR. PCR conditions are a first denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 45sec and 72°C for 30 sec, and a final elongation step at 72°C for 7 [16]. The PCR products were visualized by electrophoresis on 1,2% agarose gel and investigated by gel imaging system of Sygene (UK), before being sent for direct sequencing with forward and reverse primers to GENOKS company (Turkey).

Sequence comparisons and phylogenetic analysis. The sequences obtained with forward and reverse primers were assembled with CAP3 software [17]. Complete CP-coding sequences of ZYMV isolates were compared to those of reference isolates belonging to the main ZYMV molecular groups as defined by Lecoq and Desbiez [7]. CP nucleotide sequences were aligned with those of reference isolates (Table 2) with ClustalW included in MEGA 6 [18]. Pairwise distances between isolates (p-distances) were calculated with MEGA 6. CP nucleotide sequences were submitted to GenBank (Table 1).

Host, isolate name, georaphical origin, year of collected samples and accession number of ZYMV isolates are shown in the Table 1.

TABLE 1
List and accession numbers of GenBank ZYMV isolates selected for molecular study

Number	Isolate name	Host	Year collected	Geographical origin	Acsession number
Seq1	KZN1	*Cucurbita sp.*	2010	Ankara-Kazan	KP872538
Seq2	KZ1	*Cucurbita sp.*	2010	Ankara-Kazan	KP872539
Seq3	AKS2-5	C. moschata	2010	Aksaray	KP872540
Seq4	AKS5-7	*Cucurbita pepo L*	2009	Aksaray	KP872541
Seq5 q5	AKS6-2	*Cucurbita pepo L*	2009	Aksaray	KP872542
Seq6	ER6-8	*Cucurbita pepo L*	2009	Konya-Ereğli	KP872543
Seq7	YUN8-4	*Cucurbita pepo L*	2010	Konya-Yunak	KP872544
Seq8	A3-1	*Cucurbita pepo L*	2010	Konya	KP872545
Seq9	KAR12-4	*Cucurbita pepo L*	2010	Karaman	KP872546
Seq10	KAR15-1	*C. moschata*	2010	Karaman	KP872547
Seq11	AS1	*Cucurbita sp.*	2012	Antalya-Aksu	KP872548
Seq12	AS5	*Cucurbita sp.*	2012	Antalya-Aksu	KP872549
Seq13	AS6	*Cucurbita sp.*	2012	Antalya-Aksu	KP872550
Seq14	AS8	*Cucurbita sp.*	2012	Antalya-Aksu	KP872551
Seq15	AS11	*Cucurbita sp.*	2012	Antalya-Aksu	KP872552
Seq16	BE5	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872553
Seq17	BE6	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872554
Seq18	BE7	*C. moschata*	2014	Ankara-Beypazarı	KP872555
Seq19	BE10	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872556
Seq20	BE13 m	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872557
Seq21	BE15	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872558
Seq22	BE18	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872559
Seq23	BE22 m	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872560
Seq24	BE26 m	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872561
Seq25	BE27 m	*Cucurbita sp.*	2014	Ankara-Beypazarı	KP872562
Seq26	BRD1	*C. moschata*	2014	Burdur	KP872563
Seq27	BRD2	*C. moschata*	2014	Burdur	KP872564
Seq28	BRD4	*C. moschata*	2014	Burdur	KP872565
Seq29	C5	*C. moschata*	2011	Ankara- Çubuk	KP872566
Seq30	C11	*C. moschata*	2011	Ankara- Çubuk	KP872567
Seq31	C13	*C. moschata*	2011	Ankara- Çubuk	KP872568
Seq32	C17	*Cucumis melo L.*	2011	Ankara- Çubuk	KP872569
Seq33	H1M m	*Cucumber*	2012	Antalya- Demre	KP872570
Seq34	D14	*Cucumber*	2014	Antalya- Demre	KP872571
Seq35	AYS7	*Cucurbita sp.*	2013	Ankara-Ayaş	KP872572
Seq36	G1 m	*Cucurbita sp.*	2011	Ankara-Gölpazarı	KP872573
Seq37	G2	*Cucurbita sp.*	2011	Ankara-Gölpazarı	KP872574
Seq38	G3	*Cucurbita sp.*	2011	Ankara-Gölpazarı	KP872575
Seq39	K3 m	*Cucurbita sp.*	2012	Antalya-Kumluca	KP872576
Seq40	K17	*Cucurbita sp.*	2012	Antalya-Kumluca	KP872577
Seq41	E-7	*Cucumis melo L.*	2012	Antalya-Elmalı	KP872578
Seq42	Y23	*Cucurbita sp.*	2012	Antalya-Elmalı	KP872579
Seq43	Y4	*Cucumis melo L.*	2012	Antalya-Elmalı	KP872580
Seq44	S5	*Cucurbita sp.*	2012	Ankara-Şereflikoçhisar	KP872581
45	Y21 m	*Cucurbita sp.*	2012	Antalya- Elmalı	MG214089

m: Fruit samples

TABLE 2
Accession number of reference isolates

Referance name	Acsession number	Group	Country
ZYMV KUCHYNA	DQ124239	A1	Slovakia
ZYMV-NAT	EF062582	A1	Israel
ZYMV-FARS	JN183062	A1	Iran
ZYMV- H	KF976712	A1	Czech Republic
ZYMV-ADANA	JF317296	A1	Turkey
VE10-160	JX310115	A1	Venezuela
VE10-129	JX310103	A1	Venezuela
ZYMV –AHLAT	JF317297	A1	Turkey
TN TNV CUCU 2	KJ866939	A1	India
ZYMV-Iraq	JQ026020.1	A1	Iraq
ZYMV-California	L31350	A2	California
ZYMV_PA_2006	JQ716413	A2	USA
MAL	HM005308	A2	Mali
Florida	D13914.1	A3	USA
ZYMV C-16	DQ645729.1	A3	Spain
ZYMV-Liaocheng	EF122498	A4	China
ZYMV-CO5-205	JN861008	A4	France
ZYMV-Shangyu	AF513550	A4	China
ZYMV-CH99/116	AY611021	A4	China
ZYMV-KR-PE	AY278999	A5	Korea
ZYMV-KR-PS	AY279000	A5	Korea
ZYM-KR-PA	AY278998	A5	Korea
ZYMV-RDA	AB369279	A5	South Korea
TW-TN3	AF127929	A6	Taiwan
Strain A	AJ429071	A6	Korea

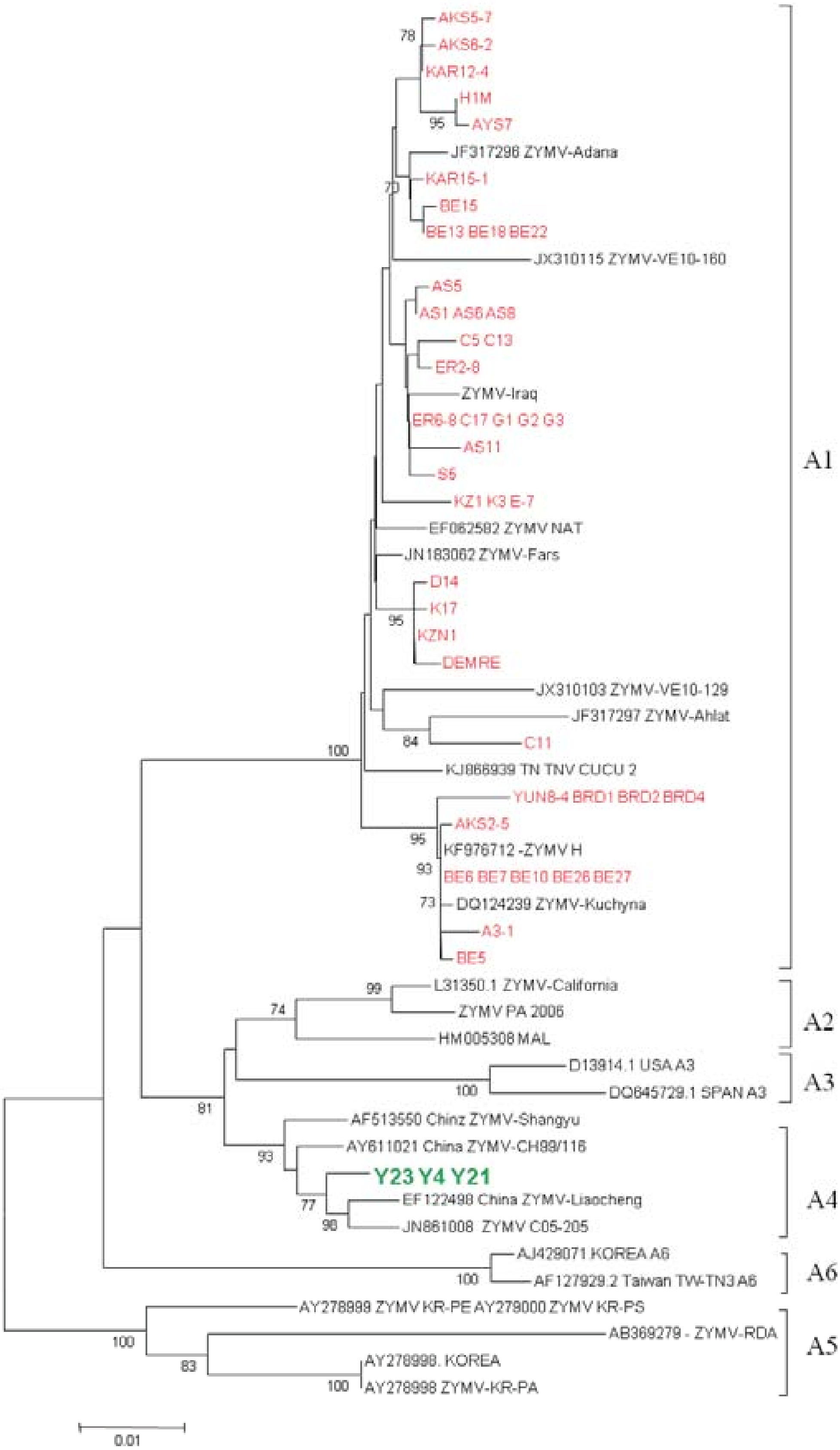

FIGURE 1

Neighbor-joining tree obtained from CP-coding region (837 nt)of the 45 Turkish ZYMV isolates from this study and 15 ZYMV sequences from worldwide origins belonging to molecular group A. Bootstrap support (1000 bootstraps) above 70% are indicated for each node. The scale bar represents a genetic distance of 0.01.

RESULTS

Presence of cucurbit viruses. A total of 217 samples were collected in Ankara (117 samples) and Antalya (100 samples) province and samples tested with DAS-ELISA for presence cucurbit viruses. According to ELISA results, 38% of the 217 samples collected between 2010 and 2014 were infected with ZYMV. Most samples were also infected with other viruses: 46% WMV, 15 % CMV, 9% PRSV, 8% CGMMV, 3% SqMV. In Antalya province, the collected samples were infected with WMV (39%), ZYMV (27%), CMV (17%), CGMMV (%2). SqMV and PRSV were not detected in samples from the Antalya province. The most occurence virus was WMV (39) %followed by ZYMV (27%) and CMV (17%).

In Ankara province, 117 samples were tested and 96 were found to be infected with the investigated viruses (82%). All tested viruses were detected in single or in mixed infection in Ankara province. All viruses were detected with different percentages on collected samples: the collected samples were infected with WMV (53%), ZYMV (50%), CMV (14%), SqMV (5%), PRSV (15%), CGMMV (15%). The most infection rate of WMV was determined in AUZF followed by Cubuk and Beypazari districts. WMV was the most prevalent virus detected in samples (46%) followed by ZYMV in 82 samples (38%). The most prevalent viruses in Antalya and Ankara provinces are WMV and ZYMV.

Molecular diversity and phylogenetic analysis. RT-PCR amplification of 45 isolates from different Turkish provinces yielded a fragment of 1260 nt, encompassing part of the Nib coding region, the coat protein and part of the 3' non coding region. The CP coding regions of all Turkish ZYMV isolates were 837 nt long and encoded 279 amino acids (aa) (GenBank accessions KP872538- KP872581; Table 1). When ZYMV CP aminoacid sequences were analysed, the highly conserved amino acid triplet Asp-Ala-Gly (DAG), essential for aphid transmission [19] was found at the N-terminal part of the coat protein of all Turkish isolates.

Sequence comparisons among the 45 isolates revealed the presence of two clusters of sequences. The first cluster contained CP sequences from 42 isolates from Ankara, Burdur, Aksaray and Konya provinces that shared 98-99% identity between each other, as well as 99% identity with isolates from Iran, Iraq, Venezuela and Germany.Three isolates (Y4, Y21 and Y23) collected from Elmalı-Antalya region in 2011 shared 96% identity between each other, as well as 99 % identity with ZYMV C05-205 isolate from France and ZYMV-Liaocheng and ZYMV-Shangyu from China (Figure 1).

According to phylogenetic tree, the isolates collected from different province clustered same subcluster. For example two isolates (D14 and DEMRE) from Antalya and one isolates (KZN) from Ankara province clustered same subcluster. The H1M isolate from Antalya clustered with ZYMV-Ahlat isolates from Adana province. Also three isolates collected from different region (KZ1 from Ankara-Kazan, K3 from Antalya-Kumluca and E-7 from Antalya-Elmalı) (Table 1) share same nucleotide sequences. On the other hand, the C11 and C13 isolates collected the same field from Ankara province but they are clustered different subcluster in the phylogenetic tree (Figure 1). Isolates collected in the same field were not always more closely related than isolates from different areas of Turkey.

DISCUSSIONS

ZYMV is one of the most destructive viruses of cucurbit crops worldwide. We aimed to estimate the prevalence of ZYMV in Ankara and Antalya province of Turkey and differentiate ZYMV isolates by DAS-ELISA and sequence analysis of CP coding region. For these reasons, the surveys were conducted for detection of some major viruses, ZYMV, WMV, CMV, PRSV, SqMV, and CGMMV in cucurbit plantations (squash, cucumber, melon and watermelon fields) in Antalya and Ankara provinces by using DAS-ELISA method. All of the samples in this study were infected by at least one virus. DAS-ELISA results showed that ZYMV and WMV are prevalent in Turkey, as reported before [20,21,22,23,24,25]. WMV was the most widespread virus followed by ZYMV, CMV, SqMV, PRSV and CGMMV. The five viruses were detected with different prevalences on collected samples from Ankara provinces and WMV, ZYMV and CMV were detected in Antalya province. Our results, the most prevalent virus is WMV in collected sample from open fields but ZYMV was predominant in greenhouse cultivation areas (data not shown). ZYMV was the most widespread virus in Thrace region, as observed before [26], Konya and Karaman province [25,27] WMV infection was the most common virus (53.9%) in Samsun province [28].

In Turkey ZYMV was first isolated from diseased squash from the Adana province by Yılmaz and Davis [3]. After that ZYMV has been reported from other regions: CMV, ZYMV and WMV have been reported in the Black Sea region [29], CMV and ZYMV in Gaziantep province [28], CMV, ZYMV, PRSV, SqMV and WMV in Thrace region [26], CMV, ZYMV and WMV in Samsun [21], ZYMV and CMV in Hatay province [30]. In recent years, ZYMV, CMV, WMV, PRSV, SqMV and CGMMV (*Cucumber green mottle mosaic Tobamovirus*) were detected in Ankara and ZYMV, CMV and WMV in Antalya [31], CMV in lakes region [32], ZYMV, CMV, WMV, PRSV and SqMV were reported in Konya province on seed-pumpkins [27].

Although there are numerous serological and biological studies about the presence of ZYMV, there have been few molecular studies in Turkey. Ozer et al. [12] studied two severe Turkish isolates of ZYMV from squash and muskmelon [ZYMV-Adana (Ad) and ZYMV-Ahlat (Ah), respectively]. Comparison with the nucleotide sequences of 23 isolates from different geographical regions worldwide revealed both isolates belonged to molecular cluster A1, the most common molecular cluster of ZYMV worldwide, including isolates from Europe, Africa, the Middle East, the Indian subcontinent and South America. In another study, Kamberoğlu et al. [13] reported that 3 Turkish ZYMV isolates from Adana and Mersin provinces of Cukurova region (ZYMV-TR3, ZYMV-TR15 and ZYMV-TR17) also belonged to subcluster A1.

On the other hand, in our study, molecular diversity of Turkish isolates of ZYMV was determined from Antalya, Burdur, Ankara, Konya and Aksaray provinces. Sequence analyses based on CP sequences revealed that most of the sequenced isolates (42/45) are closely related molecularly and belong to subcluster A1. Sequence analysis of ZYMV isolates showed that the presence of one major group of strains, and a few highly divergent isolates related to same geographic origins.

Three of the Turkish isolates did not fit to subcluster A1, but belonged to subcluster A4 containing isolates from China as well as from Poland and South-eastern France. Lecoq and Desbiez [7] reported the presence of clusters 4 and 5 in south-eastern France since 2004, probably in relation to recent introductions, and their current emergence in that area [33].

To our knowledge this is the first report about of presence of molecular group (A4) of ZYMV in Turkey. But due to the scarcity of molecular information on ZYMV in Turkey, we do not know if: this group has been present in the country for a long time. Kamberoglu et al. [13] reported the presence of an isolate of “emerging” type of WMV in Turkey. The situation may be similar between ZYMV and WMV, with recent introduction of isolates belonging to divergent molecular subgroups. However the reasons for this almost simultaneous emergence of new molecular groups of the two viruses, similar to what was observed in France in the 2000’, remains incompletely understood province [34]. Further surveys are required to explain the genetic diversity of ZYMV and to determine if group A4 is now emerging in Turkey.

ACKNOWLEDGEMENTS

This research was funded by Ankara University Scientific Research Projects Division (BAP) coded 12B4347002. We are grateful for Dr. Serkan Yeşil for providing us some ZYMV isolates of Konya, Karaman and Aksaray.

This research did not involve any human participants and/or animals. The authors declare that they have no conflict of interest.

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Received: **12.05.2018**
Accepted: **14.02.2019**

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