

Characterization and Phylogenetic Analysis of Zucchini Yellow Mosaic Virus Infecting Cucurbita pepo in Egypt

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Abstract: *Zucchini yellow mosaic virus* (ZYMV) was isolated from naturally infected squash plants exhibiting systemic vein-banding, yellowing, mosaic, leaf deformation and stunting symptoms. In this study, the virus isolate was identified using biological, serological and molecular techniques as ZYMV. In biological analysis, the isolate produced severe symptoms on susceptible cucurbit hosts and local lesions on leaves of indicator plants. Direct enzyme linked immunosorbent assay and direct tissue blot immunosorbent assay (DTBIA) were successfully used to detect ZYMV isolate in squash plants. Electron microscopy of leaf dip preparation of infected squash leaves showed long flexuous filamentous virus particles of size (750X13 nm). Reverse transcription polymerase chain reaction (RT-PCR) was carried out using ZYMV-specific primers, designed to amplify a 1005-bp fragment covering the entire coat protein (CP) gene and part of 3'-untranslated region (3'-UTR) from infected squash plants. The partial nucleotide sequence of the CP gene of ZYMV-EG isolate has been deposited in NCBI GenBank under accession number KU127244. According to the sequence analysis the Egyptian isolate has specific amino acids sequences that are different from other ZYMV isolates. Comparison with 30 ZYMV sequences retrieved from GenBank presented nucleotide identities in the range of 95-97%, amino acids sequences similarities ranged from 92.23-96.12%. Phylogenetic analysis revealed that ZYMV-EG isolate was grouped into a distinct clade comprising Taiwan isolates (TW-TN3 and TW-NT1), Chinese isolate (Hangzhou) and Brazilian isolate (ZYMV-DF) in the cluster A which is apparently the most widespread throughout the world.

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1. Introduction

Zucchini yellow mosaic virus (ZYMV) is one of the major viruses of cucurbit crops causing yield losses of up to 100% (Desbiez and Lecoq, 1997). ZYMV belongs to the genus potyviruses in the Potyviridae, a group of plant viruses characterized by a monoparticle, positive-sense, single stranded RNA genome encapsidated in elongated and flexuous particles that are approximately 750 nm long and 12 nm in diameter. Viral RNA consists of about 9600 nucleotides, with a 5' viral protein genome linked (VPG) and a poly (A) tail (Dougherty and Semler, 1993). The viral RNA encodes a single polyprotein cleaved by virus-encoded proteases into eleven proteins. The coat protein (CP) has a molecular weight of 36 kDa (Adams *et al.*, 2012).

ZYMV was first isolated in Italy in 1973 and 10 years later was found in Egypt (Provvidenti *et al.*, 1984b; Desbiez and Lecoq, 1997). The virus was

reported in other countries, including France, Japan, Central Europe, China, Brazil, Iran, Pakistan, Australia, Canada and USA (Desbiez and Lecoq, 1997; Coutts *et al.*, 2011; Ashfaq *et al.*, 2015).

ZYMV- infected plants exhibit severe mosaic, yellowing and leaf deformation. The fruits are stunted, twisted and deformed, resulting in reduced yield making the plants unmarketable; especially zucchini squash (Spadotti *et al.*, 2015). ZYMV has an experimental host range that includes species of 11 families of dicotyledons although it infects mostly cucurbits in natural conditions (Romay *et al.*, 2014).

Several serological procedures have been used to detect ZYMV, the enzyme linked immunosorbent assay (ELISA) and direct tissue blot immunoassay (DTBIA) tests are the most widely serological methods developed for virus detection, including ZYMV (Mostafa and Abou-Ela, 2011).

RT-PCR has successfully been utilized for the detection of ZYMV (Simmons *et al.*, 2011) and sequence data for the 3' terminus of the ZYMV genome, including region coding for the coat protein (CP), have been obtained for many isolates worldwide (Desbiez *et al.*, 2002). Amino acid sequence identity in the CP of distinct ZYMV isolates is over 90%, except for the Singapore and Reunion isolates, which are particularly variable in the N-terminus of the CP-coding region (Zhao *et al.*, 2003).

Sequences of the ZYMV coat protein gene available from public molecular databases revealed three major groups of this virus isolates worldwide (Coutts *et al.*, 2011) with differing geographical distributions (Zhao *et al.*, 2003; Simmons *et al.*, 2008). Group I contained the majority of European isolates, as well as some from China and Japan, and a single Californian isolate. ZYMV isolates from Asia were formed Group II, while group III included several Chinese isolates. Notably, while group I and II isolates resulted in mosaic symptoms on leaves and fruit distortion, group III viruses did not cause symptoms on the fruit, but induced severe mosaic symptoms on the leaves (Zhao *et al.*, 2003).

Differences in biological, serological and molecular properties of ZYMV isolates have been reported from various locations in the world (Desbiez *et al.*, 2002; Zhao *et al.*, 2003; Yakoubi *et al.*, 2008). The coat protein is considered the best marker for investigating closely related ZYMV isolates, because it is the only gene product which shares little sequence identity with the corresponding protein of other virus groups (Berger *et al.*, 1997), and sequence variability may have important implications in the use of the CP gene for transgenic resistance (Lin *et al.*, 2000).

The aim of this study was to identify, know the molecular variability of the Egyptian ZYMV isolate and its phylogenetic relationship with other isolates in different geographical locations to understand the complexity and epidemiology of this pathogen and hence management of the disease.

2. Material and Methods

Samples collection and virus isolation

Squash leaves showing typical symptoms of ZYMV were collected from the open field, Faculty of Agriculture; Ain Shams University, Cairo, Egypt during growing season 2013/2014. ZYMV isolate in this study was singled out by successive local lesion transfers to *Chenopodium amaranticolor* (Yeh *et al.*, 1984) and a single local lesion was used to inoculate of *C. pepo*. cv. Eskandarani as propagative host plant. Young symptomatic squash leaves were ground in 0.02 M potassium phosphate buffer pH 7, containing 0.02 M sodium sulfite and carborundum as an

abrasive. The extract was rubbed onto squash cotyledons and true leaves and the inoculated plants were maintained under insect proof greenhouse conditions at 25-28°C for 21 days. Test plants were observed for symptoms development.

Host range

Plant hosts listed in Table 1 were tested by mechanical sap inoculation under insect proof greenhouse conditions. The infection of test-plants was determined by examining the occurrence of local and/or systemic symptoms at 7, 14, and 21 days after inoculation. Virus infection was confirmed by direct ELISA, DTBIA and RT-PCR analyses as described below.

Direct enzyme linked immunosorbent assay

The inoculated plants were tested for the presence of ZYMV using direct ELISA as described by (Voller *et al.*, 1978; Mowat and Dawson, 1987) using polyclonal antiserum conjugated with alkaline phosphatase. Appropriate negative and positive controls were included. The reaction was considered positive when the O.D. 405 nm reading was at least three times that of the healthy sample.

Direct tissue blot immunosorbent assay (DTBIA)

Healthy, infected squash and host plant leaf samples were pressed directly on the nitrocellulose membrane (BioRad Laboratories, CA). The blotted membranes were left to dry for 30 min and processed in plastic trays using direct immunological test modified from Lin *et al.* (1990). The membrane was placed in a solution of 1% BSA in PBS and incubated for 30 min at room temperature, with gentle shaking in a plastic dish on a shaker. Membranes were incubated for 1 to 2 h at room temperature for the ZYMV alkaline phosphates polyclonal antibodies conjugate. Substrate was prepared from Sigma Fast BCIP (5-bromo-4-chloro-3-indoly phosphate)/NBT (nitroblu tetrazolium) tablets (Sigma Chemical Co., St. Louis, MO). Membranes were incubated in the substrate solution for 5 to 20 min. The reaction was stopped by washing the membrane in distilled water for 5 min. The results were documented by photography.

Electron microscopic examination (EM)

Leaf dip assay was carried out according to Ahlawat and Varma (1997). The grids were negatively stained with aqueous 2% uranyl acetate and examined under a JOEL-JM-100-C transmission electron microscope (TEM) (Electron Microscope Unit, Al-Azhar University, Cairo, Egypt).

RNA isolation, RT-PCR and sequencing

Total RNA was isolated from *C. pepo* cv. Eskandarani experimentally infected with ZYMV isolate using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's protocol. RT-PCR was done as described by (Sharifi *et al.*, 2008). Oligonucleotide primers were designed in the CP gene

conserved region according to the published nucleotide sequences of ZYMV (accession No. AF343979). The sense primer, ZYMV-F (5' ATC AGG CAC TCA GCC AAC TGC 3') is located in a region from nt 8541 to nt 8562 of the complete gene of ZYMV isolate; the antisense primer ZYMV-R (5' ACA CTA AAG CTT CCG ACA GGA C 3') corresponds to a region spanning from nt 9524 to nt 9546. The amplified fragment was expected to be 1005 bp containing the complete coat protein gene and most of the 3' untranslated region. Amplification of the CP gene was done using the first-strand cDNAs procedure (Sharifi *et al.*, 2008; Massumi *et al.*, 2011) with the antisense primer ZYMV-R. PCR consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 30 sec at 94°C for denaturation, 30 sec at 56°C for primer annealing and 45 sec at 72°C for elongation. The reaction was terminated by a final elongation step of 5 min at 72°C. PCR product was analyzed on 1 % agarose at 60 V in TAE (40 mM Tris/acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide and visualized by UV transilluminator. The amplified DNA fragment was purified using the Wizard® SV Gel and PCR Clean-

Up System (Promega, Madison, WI, USA). Specific PCR product of the ZYMV Egyptian isolate amplified from infected squash samples was used directly for sequencing. Partial nucleotide sequence of the coat protein gene (~362 bp) was performed using 3500 Genetic Analyzer (Applied Biosystems) at Colors For Research, Cairo, Egypt.

Sequence analysis

The resulting nucleotide sequence was assembled and analyzed using MEGA6 program. Deduced amino acid sequences were obtained by using an online translation tools (www.expasy.ch/tool/translate). Nucleotide and protein sequence data was subjected to sequence similarity searches against GenBank database using BLAST program.

Phylogenetic trees were constructed after multiple sequence alignments using Clustal W embedded in MEGA6 program and neighbor-joining method with 500 bootstrap replicates (Tamura *et al.*, 2013).

3. Results

Virus isolate and symptomatology

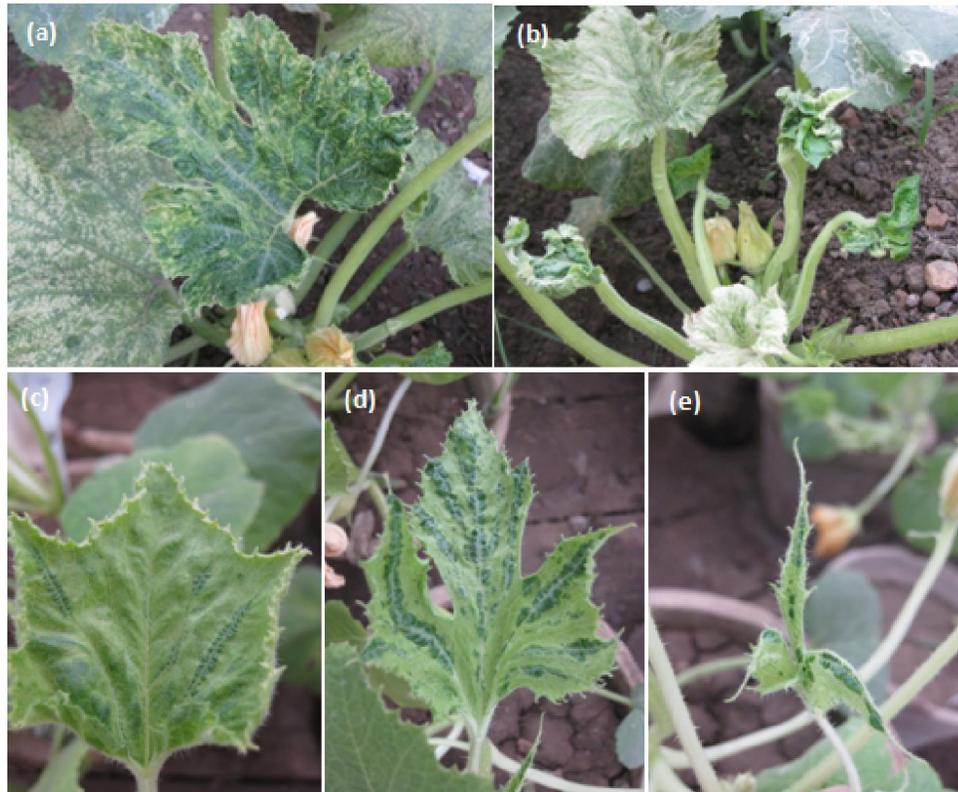


Fig. 1 Disease symptoms of ZYMV-EG isolate on *C. pepo*. cv. Eskandarani. (a, b) represent field symptoms (severe mosaic and leaf deformation), (c, d and e) symptoms on *C. pepo*. cv. Eskandarani following sap transmission (vein-banding, severe mosaic and leaf deformation).

ZYMV was isolated from naturally infected squash plants in the open field which showed vein-banding, yellowing, severe mosaic, leaf malformation and stunting symptoms using single local lesion assay and maintained on healthy seedlings of *C. pepo* cv. Eskandarani by mechanical sap inoculation under insect proof greenhouse conditions and squash plants inoculated with the present isolate showed leaf deformation, severe mosaic and vein-banding (Fig. 1).

Host range

ZYMV isolate was detected and identified using host range and indicator plants which gave distinct symptoms on *Cucurbitaceae* family, including *Cucumis sativus* cv. Beit Alpha, *Luffa acutangula* L., and *Citrulus lanatus*. ZYMV isolate gave severe

mosaic, vein-banding and leaf deformation on *Cucurbita pepo* cv. Eskandarani (Fig. 1). *Cucumis sativus* cv. Beit Alpha showed mosaic symptoms at 2 weeks of post inoculation. *Luffa acutangula* L., and *Citrulus lanatus* revealed similar symptoms severe mosaic and leaf deformation. In *Chenopodiaceae* family, including *Chenopodium amaranticolor* and *Chenopodium quinoa*, ZYMV induced chlorotic local lesions surrounded with red hollow (Fig. 2), while no symptoms were appeared on the plant species of *Solanaceae* family as shown in Table 1. In *Fabaceae* family, ZYMV isolate did not induce any symptoms in *Vicia faba* while *Phaseolus vulgaris* L. showed mosaic after 21 days of inoculation.

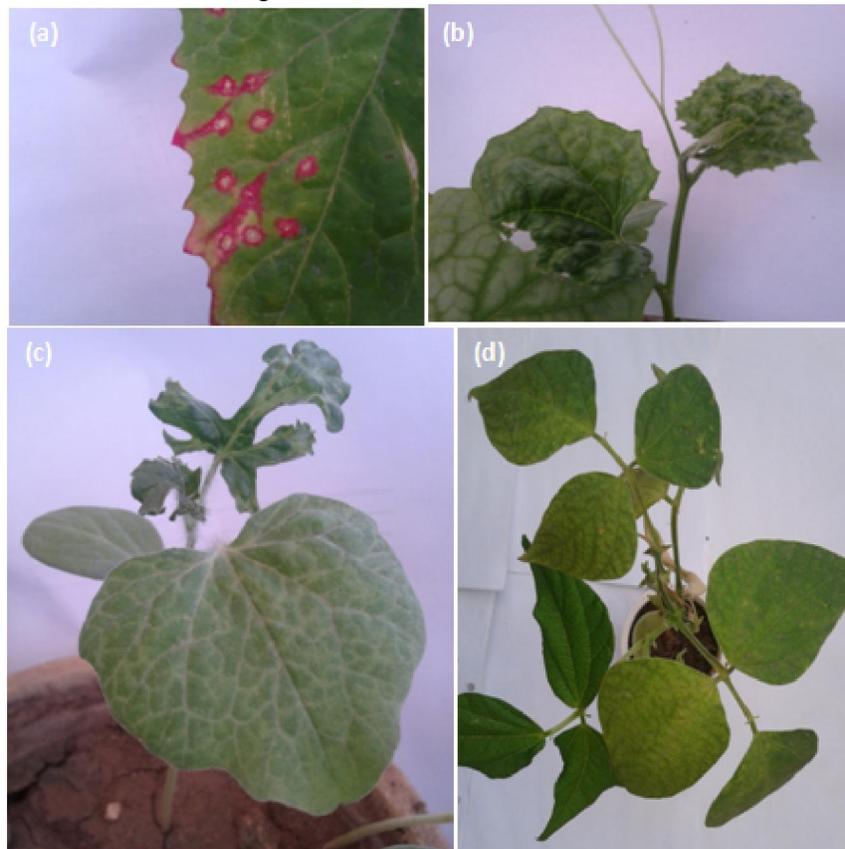


Fig. 2 ZYMV-EG symptoms on indicator and host plants (a) *C. amaranticolor* showing chlorotic local lesions surrounded by red hollows, (b) *L. acutangula* L, showing severe mosaic and leaf deformation, (c) *C. lanatus* revealed severe mosaic and leaf deformation and (d) *Phaseolus vulgaris* showing mosaic.

Direct-ELISA

After the virus under study has been identified by biological assays as ZYMV, direct ELISA was performed to confirm its identity. ZYMV polyclonal antiserum was used to detect the virus in all samples as shown in Table 1.

Direct tissue blot immunosorbent assay (DTBIA)

ZYMV polyclonal antibodies gave positive signals with all infected samples as shown in (Fig. 3);

while healthy leaves did not show any purple color in the whole blot.

ZYMV Morphology

Electron microscopy of leaf dip preparation of infected squash leaves showed long flexuous filamentous virus particles measuring (750X13 nm) (Fig. 4).

Table 1 Reaction of diagnostic species to ZYMV-EG infecting cucurbits

Family, species	Symptoms	Direct-ELISA*
Cucurbitaceae		
<i>Cucurbitapepocv.</i> Eskandarani	SM, Vb and LD	+
<i>Luffaacutangula</i> L.	SM, LD	+
<i>Citruslanatus</i>	SM, LD	+
<i>Cucumissativusc.</i> Beit Alpha	M	+
Chenopodiaceae		
<i>Chenopodiumamaranticolor</i>	Chll	+
<i>Chenopodium quinoa</i>	Chll	+
Solanaceae		
<i>Nicotianatabacumcv.</i> Samsun	--	-
<i>Nicotianarustica</i>	--	-
<i>Nicotianaglutinosa</i>	--	-
<i>Physalisfloridana</i>	--	-
<i>Gomphrenaglobosa</i>	--	-
<i>Daturastramonium</i>	--	-
Fabaceae		
<i>Phaseolus vulgaris</i> L.	M	+
<i>Viciafaba</i>	--	-

SM Severe Mosaic, Vb Vein-banding, LD Leaf Deformation, M Mosaic, Chll Chlorotic local lesion, -- no symptoms
 *Direct-ELISA readings (absorbance at 405 nm) of extract of inoculated plants

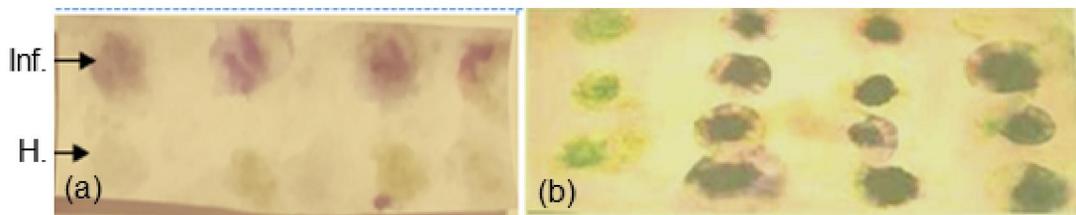


Fig. 3 Direct tissue blot immunosorbent assay (DTBIA) of (a) mechanically inoculated host plants and (b) ZYMV-EG infected *C. pepo* cv. Eskandarani using polyclonal antibodies (ZYMV/PAb). (H.) indicates healthy tissues showed no reaction and (Inf.) indicates ZYMV-infected plants.

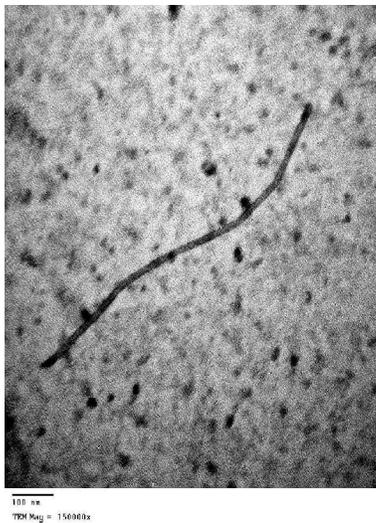


Fig. 4 Electron micrograph of ZYMV-EG negatively stained with 2% uranyl acetate (X-150000). Bar represents 100 nm.

PCR detection

RT-PCR was used successfully to amplify the viral DNA fragment of the CP gene and part of 3'UTR (1005 bp) by using specific primer for ZYMV, as indicated in (Fig. 5).

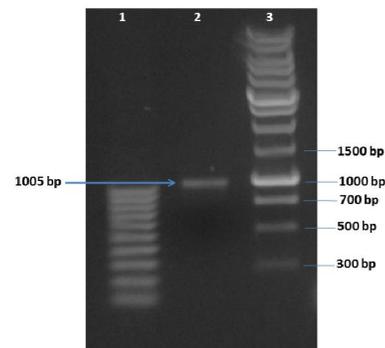


Fig. 5 Gel electrophoresis showing PCR product of ZYMV-CP and most of 3' UTR (1005 bp). Lane (1) 100 bp step ladder, lane (2) Arrow indicates PCR amplified product of the whole Cp gene, part of 3'UTR for ZYMV-EG using specific primers and lane (3) 300-10,000bp DNA Ladder.

Sequence analysis

The identity of the virus was confirmed by partially characterizing its CP gene and the relationship with other 30 selected ZYMV isolates registered in GenBank was done as illustrated in Table 2. The ZYMV-F and ZYMV-R primers amplified a fragment of approximately 1005 bp containing the complete coat protein gene of the virus; the partial

coat protein gene sequence comprised 362 nucleotides, encoding 120 amino acids which identified as conserved domains of potyvirus coat protein in GenBank database (pfam00767). BLAST analysis of the partial CP gene showed high sequence homology to different ZYMV sequences from different geographic origins ranged from 95 % to 97 %.

Table 2 The evolutionary distances and similarity percentage of amino acid sequence of Cp gene for ZYMV-EG isolate and 30 other ZYMV isolates retrieved from GenBank database.

Accession No.	ZYMV-EG	Distance*	Isolate	Country
KU127244	100	0.000	ZYMV-EG	Egypt
NC_003224	95.15	0.030	TW-TN3	Taiwan
AF435425	95.15	0.030	Hangzhou	China
AF127933	95.15	0.030	TW-NT1	Taiwan
KP872577	95.15	0.030	K17	Turkey
KP872571	95.15	0.030	D14	Turkey
KP872570	95.15	0.030	H1M	Turkey
KP872566	95.15	0.030	C5	Turkey
KJ866938	95.15	0.030	TN TNV SG1	India
KJ866937	95.15	0.030	TN UDU PUM1	India
KJ848769	92.23	0.059	Shanshan-1	China
JX502677	95.15	0.030	ZYMV-Fe	Brazil
JX502668	96.12	0.020	ZYMV-RN	Brazil
JX502667	95.15	0.030	ZYMV-DF	Brazil
JX310117	96.08	0.030	VE10-117	Venezuela
JX310111	95.15	0.030	VE05-026	Venezuela
JX310105	96.08	0.030	VE04-017	Venezuela
JX262127	92.23	0.059	390-10	Serbia
JX262118	92.23	0.059	115-08	Serbia
JX028593	95.15	0.030	RSHS	France
JN861005	96.12	0.020	E15	France
JN315860	92.23	0.059	151-08	Serbia
JF795799	96.08	0.030	Cvn-15	Australia
JF795793	96.08	0.030	Cvn-9	Australia
JF795792	96.08	0.030	Cvn-8	Australia
HQ543133	95.15	0.030	S112	USA
HM768193	95.15	0.030	GC84	USA
FJ705253	96.12	0.020	Azr.Sha.C	Iran
EU371649	95.15	0.030	leaves	UAS
AB063251	96.12	0.020	Melon	Japan
AB004641	95.15	0.030	M	Japan

*The evolutionary distances were computed using the p-distance method (Tamura et al. 2013) and are in the units of the number of amino acid differences per site.

Phylogenetic analysis

Phylogenetic analyses were carried out using amino acids sequences of the CP gene from the Egyptian ZYMV isolate and 30 ZYMV isolates registered in Genbank.

The amino acids sequence identity ranged from 92.23 to 96.12% from different countries. The CP gene sequence from the ZYMV-EG isolate and 30

isolates from different parts of the world was aligned (Fig. 6), and a phylogenetic tree was constructed (Fig. 8). ZYMV isolates formed two distinct clusters, named A and B; cluster A was divided into two subclusters, I and II as proposed by Romay *et al.* (2014). The Egyptian isolate was clustered within subclusters II of cluster A.

The genetic diversity between Egyptian isolate and other ZYMV from different continents was appeared in (Fig. 7) as variable sites in amino acid sequences. The Egyptian ZYMV isolate has glutamic acid (E) instead of lysine (K) and alanine (A) substitutes for threonine (T). Glutamine (Q) changes to histidine (H) compared to other ZYMV isolates. These sequences are specific only to the Egyptian isolate and form a distinct branch in the phylogenetic tree. The evolutionary distances were computed using

the p-distance method (Tamura *et al.*, 2013) between the Egyptian isolate and ZYMV isolates worldwide as shown in Table 2. The Egyptian isolate was more related to isolates from Taiwan, China, Brazil, USA, Venezuela, France, Turkey, Iran, Japan and Australia (95.15-96.12% identity) and more distant from Serbian isolates (390-10, 115-08 and 151-08) and Chinese isolate (Shanshan-1) (92.23% identity).

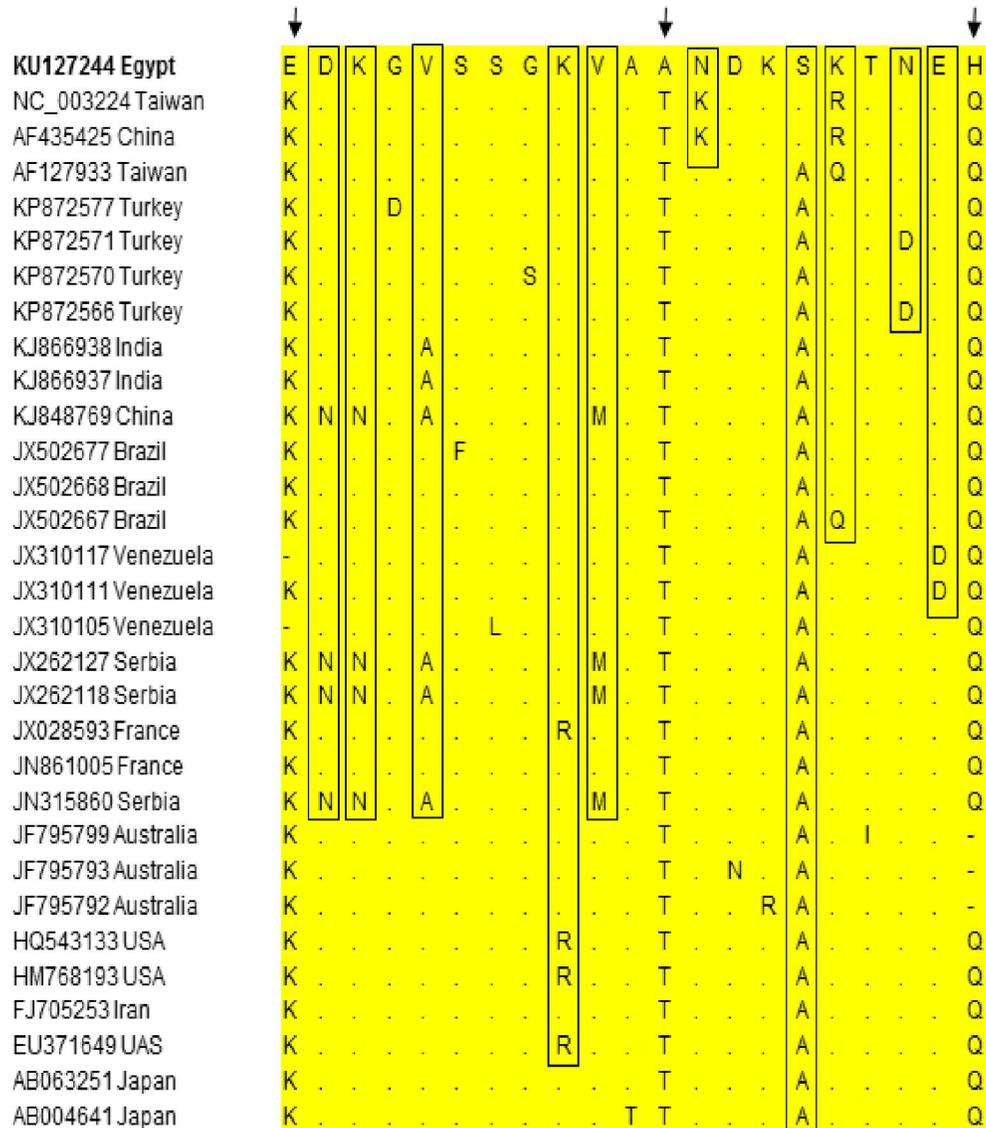


Fig. 7 The variable sites of aligned partial amino acid sequence of ZYMV Egyptian isolate (KU127244) to those of 30 ZYMV isolates registered in Genbank.; boxes indicate amino-acid parsimony-informative site; black arrows indicate specific amino acid differences sites between Egyptian and other ZYMV isolates; «-» indicates lacking amino acid; «.» indicates identical amino acid residues in the alignment.

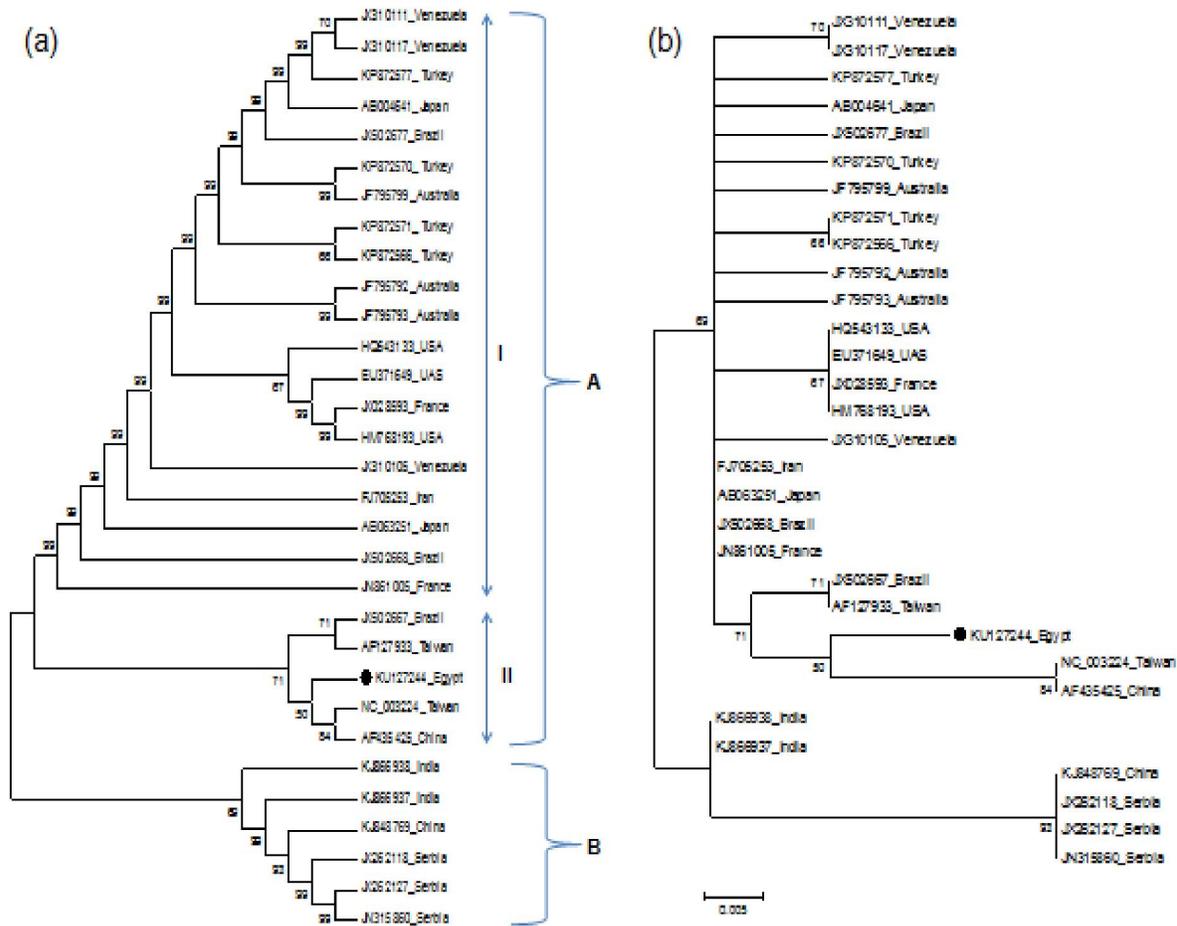


Fig. 8 (a) Phylogenetic tree showing the relationship of Egyptian ZYMV isolate (is marked with dot) with 30 ZYMV sequences from worldwide isolates available in GenBank using partial amino acid sequence of the coat protein gene. Neighbour-joining tree was built with the program MEGA6, phylogenetic relations based on p-distance method. Bootstrap values of 500 resembling as percents are indicated at key nodes. Only bootstrap values above or equal 50% are shown. (b) Original tree (uncondensed).

4. Discussion

In this paper, we report results of identification and phylogenetic analysis of ZYMV in Egypt, aiming at determining the genetic diversity of ZYMV, one of the main dangerous viruses infecting the major cucurbit crops. Biological, serological and molecular diagnostic tests were used on symptomatic and asymptomatic samples.

Our results have shown that ZYMV-EG isolate causes severe mosaic, vein-banding and leaf deformation when the virus isolate was inoculated on the main host in greenhouse similar to those observed in the open field. In the later stages of infection leaves develop a yellow mosaic and often show dark green blisters, these results in accordance to (Desbiez and Lecoq, 1997; Gal-On, 2007; Lecoq and Desbiez, 2012; Al-Saleh *et al.*, 2014).

Use of local lesion host plant species has been very useful to prove the pathogenicity of the viruses. ZYMV-EG on *Chenopodium amaranticolor* inoculated leaves causes local chlorotic lesions which become surrounded with red hollow in a few days, the virus isolate also caused systemic infections, mosaic symptoms and leaf deformation on *L. acutangula* L., and *C. lanatus*, (Hasiów-Jaroszewska *et al.*, 2013; Spadotti *et al.*, 2015). Strains of ZYMV can be distinguished by differences in symptomatology and incubation periods in both squash and melon (Wang *et al.*, 1992). Other indicator host, Black Turtle 2 and red kidney beans, along with *Nicotiana benthamiana*, are useful for differentiation of these strains. However the infection of *P. vulgaris* cv. Pinto is strain specific and remains symptomless with some French and North American strains (Provvidenti *et al.*, 1984a; Singh *et*

al., 2003). An experimental indicator host study of ZYMV-EG isolate revealed some variation in their biological properties. During the present studies ZYMV-EG infected *Phaseolus vulgaris* L. systemically and gave mosaic although it is not susceptible to many isolates of ZYMV but gave local infection on inoculated leaves only (Provvidenti *et al.*, 1984a). The Florida strain (ZYMV-FL) induced local infection on *Phaseolus vulgaris* Black Turtle2 (Provvidenti *et al.*, 1984a) but the California strain (ZYMV-Ca) did not induce any local or systemic reaction on *Phaseolus vulgaris* Black Turtle2 (Glasa *et al.*, 2007). This suggests a difference in the infectivity and virulence of ZYMV-EG with other ZYMV isolates. Differences among ZYMV isolates in their host range, pathogenicity, and serological characteristics have been defined worldwide (Glasa *et al.*, 2007; Pfosser and Baumann, 2002).

ELISA and DTBIA are considered routine methods for plant viruses detection and diagnosis (Hsu and Lawson, 1991). Our results showed that both ELISA and DTBIA gave positive results with ZYMV infection.

Flexuous, rod shape structures (750X13 nm) of ZYMV particles were observed in the infected leaf sap under electron microscope after negative staining. Similar results have been reported by (Gal-On, 2007; Zechmann and Zellnig, 2009; Johnson *et al.*, 2013).

RT-PCR was used to identify ZYMV in leaves of infected squash. Oligonucleotide primers which annealed to regions in the whole coat protein (CP) gene and part of 3'UTR generated a 1005-bp product from ZYMV; this result was in agreement with (Thomson *et al.*, 1995; Mostafa and Abou-Ela 2011; Simmons *et al.*, 2011).

The Egyptian ZYMV isolate was partially sequenced and the sequence was deposited in the GenBank database (accession number KU127244) compared to the corresponding nucleotide and amino acid sequences of ZYMV isolates from different geographic origins, nucleotide identity of 95–97%, whereas that of the deduced amino acid sequences ranged from 92.23-96.12%, suggesting a low genetic diversity between ZYMV-EG isolate and other ZYMV isolates in the world.

The results of phylogenetic analyses indicate that the Egyptian ZYMV isolate is located in a distinct clade in cluster A (Coutts *et al.*, 2011; Lecoq and Desbiez, 2012; Romay *et al.*, 2014) contained two subcluster I and II. Subcluster I included ZYMV isolates from USA, Venezuela, Brazil, France, Turkey, Iran, Japan and Australia and subcluster II was exclusively composed of viruses from Taiwan, China, and Brazil. While cluster B was phylogenetically distinct that they are assigned as

separate group, included one Chinese isolate, two Indian isolates, and three Serbian ZYMV isolates.

The Egyptian ZYMV isolate found in this study clustered into subcluster II of the cluster A (Fig. 8) which is apparently the most widespread throughout the world (Coutts *et al.*, 2011; Lecoq and Desbiez, 2012; Romay *et al.*, 2014; Spadotti *et al.*, 2015).

But there was a noticeable level of divergence between Egyptian ZYMV isolate, Chinese (KJ848769) and Serbian ZYMV isolates (92.23% identity), although one ZYMV isolate from China (AF435425) was in the same phylogenetic clade with Egyptian isolate, this means that Chinese isolates have a high genetic variability and they already clustered into two distinct groups I/II and III or A and C as were recorded in previous studies (Simmons *et al.*, 2008; Coutts *et al.*, 2011; Romay *et al.*, 2014). Indian isolates (TN TNV SG1 and TN UDU PUM1), Serbian isolates (390-10, 115-08 and 151-08) and Chinese isolate (Shanshan-1) were grouped into genetically distinct cluster B can be explained by variable sites with in amino acids sequence compared with an the Egyptian ZYMV isolate (Tobias and Palkovics, 2003).

The branch in which the Egyptian, two Taiwan ZYMV isolates (TW-TN3 and TW-NT1) and Brazilian isolate ZYMV-DF clustered in the phylogenetic tree was statistically significant (71% bootstrap), based on the degrees of coat protein (CP) homology, Lin *et al.* (2000) proved the TW-TN3 isolate and most of Taiwan ZYMV isolates were placed in genotype I. Thus, the Egyptian ZYMV isolate may be belonging to this genotype. In addition to that molecular variability between the Egyptian and other ZYMV isolates was determined by certain amino acid positions in the CP, there were amino acid residues specific for the Egyptian ZYMV isolate that differ from amino acid sequences of other ZYMV, and these results were in accordance to (Lin *et al.*, 2000; Tobias and Palkovics, 2003).

The Brazilian isolate ZYMV-DF was in group A, subgroup II, with isolates from North America and Europe and our results proved that ZYMV-DF isolate was located in the same clade with an Egyptian ZYMV isolate in cluster A. However, ZYMV-Fe and RN isolates clustered in group A, subgroup I, along with a Brazilian isolate previously collected in the state of São Paulo State (GU586790) and with isolates from Asia and Europe (Spadotti *et al.*, 2015), and our results showed that the Brazilian isolates (ZYMV-Fe and RN) were also located in subcluster I of cluster A.

High similarities were also observed between ZYMV-EG and isolates from far distance countries (Brazil and Japan) (96.12% identity). Our results revealed that there is little evidence clustering of the isolates according to their geographical origin. The Egyptian ZYMV isolate grouped together with

Taiwan, Chinese, and Brazilian isolates. However, isolates sampled from adjoining locations are not always related (Pfosser and Baumann 2002), suggesting that biogeographical structure may, to some extent, be determined by the international trading of infected seeds between different countries (Desbiez *et al.*, 2002; Tobias and Palkovics, 2003; Hasiów-Jaroszewska *et al.*, 2013).

The objective of this study was to identify ZYMV infecting *Cucurbita pepo* in Egypt, and to evaluate the genetic variability between the Egyptian isolate and other ZYMV isolates worldwide. The Egyptian isolate has specific amino acid sequences in coat protein and form a distinct branch in the phylogenetic tree including Taiwan, China and Brazil isolates, more distant from Serbian isolates and Chinese isolate (Shanshan-1).

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