Complete Genome Sequence of *Zucchini Yellow Mosaic Virus* Strain Kurdistan, Iran

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Abstract

The complete genome sequence of *Zucchini yellow mosaic virus* strain Kurdistan (ZYMV-Kurdistan) infecting squash from Iran was determined from 13 overlapping fragments. Excluding the poly (A) tail, ZYMV-Kurdistan genome consisted of 9593 nucleotides (nt), with 138 and 211 nt at the 5' and 3' non-translated regions, respectively. It contained two open-reading frames (ORFs), the large ORF encoding a polyprotein of 3080 amino acids (aa) and the small overlapping ORF encoding a P3N-PIPO protein of 74 aa. This isolate had six unique aa differences compared to other ZYMV isolates and shared 79.6–98.8% identities with other ZYMV genome sequences at the nt level and 90.1–99% identities at the aa level. A phylogenetic tree of ZYMV complete genomic sequences showed that Iranian and Central European isolates are closely related and form a phylogenetically homogenous group. All values in the ratio of substitution rates at non-synonymous and synonymous sites (d_N/d_S) were below 1, suggestive of strong negative selection forces during ZYMV protein history. This is the first report of complete genome sequence information of the most prevalent virus in the west of Iran. This study helps our understanding of the genetic diversity of ZYMV isolates infecting cucurbit plants in Iran, virus evolution and epidemiology and can assist in designing better diagnostic tools.

Keywords Genetic diversity · Negative selection · Overlapping fragments · Phylogenetic analysis · ZYMV

Introduction

Zucchini yellow mosaic virus (ZYMV) is a member of the genus *Potyvirus* in the family *Potyviridae* which cause mosaic disease on cucurbits (Adams et al. 2005). ZYMV genome contains a single positive-sense ssRNA molecule with 5'VPg and 3'polyA tail which codes for a polyprotein that is proteolytically processed into ten smaller mature proteins; P1 (protease), HC (helper component/protease), P3, 6K1, CI (cylindrical inclusion), 6K2, NIa (nuclear inclusion a), VPg (viral protein linked genome), NIb (nuclear inclusion b) and CP (coat protein) (Riechmann et al. 1992; Lin et al. 2001). In addition, a short ORF embedded within the

P3 cistron (*pipo*) was found which is translated in the +2 reading frame (Chung et al. 2008). This virus is efficiently transmitted in a stylet-borne by aphids such as *Myzus persicae*, and via seeds (Lisa et al. 1981).

Cucurbit plants are economical crops grown in all parts of Iran because the climate is favorable for their growth. ZYMV is the most common virus infecting cucurbitaceous hosts in Iran and it has been reported from several cucurbits-growing regions (Bananej et al. 2008; Massumi et al. 2011; Mohammadi et al. 2016; Sokhandan-Bashir et al. 2013). So far, the complete genome of three isolates of squash (*Cucurbita pepo*) from south (Fars) (Acc. No. JN183062), center (IKA) (Acc. No. KU528623), and north (SANRU) (Acc. No. KU198853) of Iran have been sequenced.

The present study aims to determine complete genome sequence of an Iranian ZYMV isolate from a different geographic region of Iran where cucurbit fields are mainly affected by ZYMV (Mohammadi et al. 2016). Accordingly, we determined the complete genome sequence of an Iranian ZYMV isolate (ZYMV-Kurdistan) in the west of Iran from a squash host with mild symptoms. We amplified overlapping fragments and compared with the available

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full-length genome of the other ZYMV strains to determine their genetic diversity, evolutionary relationship and recombination events. More information about the whole genome sequence of a ZYMV isolate will further reveal molecular variation of this virus, thus facilitating its management and prevention of the disease.

Materials and methods

The source of the ZYMV isolate used in this research was a field-grown squash (*Cucurbita pepo*) with typical symptoms including leaf deformation and mosaic. The host was collected in August 2016 from the west part of Iran (Nashour, Sanandaj, Kurdistan province). To obtain complete genome of ZYMV-Kurdistan, total nucleic acid was extracted by silica-capture method (Foissac et al. 2000) and then used as a template for first-strand cDNA synthesis with random hexamer primers, and a HyperScriptTM Reverse Transcriptase kit (GeneAll, Seoul, Korea). PCR reactions were done with 13 primer pairs (Table 1) and amplicons of the expected size were eluted from agarose gel by Squeeze DNA Gel Extraction Spin Columns (Bio-Rad Laboratories,

Hercules, CA, USA), directly ligated into pTG-19 (Sina-Clon, Tehran, Iran) and transformed into *Escherichia coli* DH5 α . Recombinant plasmids were sequenced in both directions and the obtained sequences were assembled and the complete genome of ZYMV-Kurdistan was obtained from 13 overlapping fragments. The 5' and 3' proximal ends of the ZYMV-Kurdistan genome were directly amplified by specific designed primers.

Genetic distances between the newly sequenced isolate and other ZYMV isolates were estimated using Kimura two-parameter method (Kimura 1980) implemented in MEGA6 software (Tamura et al. 2013). A phylogenetic unrooted tree was constructed by the maximum likelihood (ML) method implemented in MEGA6 software and the branch support was computed using the bootstrap method based on 1000 replicates. The direction and degree of selective pressure operating in coding regions were estimated by calculating d_N/d_S values (the ratio between nucleotide diversity values in non-synonymous and synonymous positions) (Boulila 2011) using DnaSP v.5.10.01 (Rozas 2009).

To detect recombination event(s) in the Kurdistan isolate, 23 aligned complete genome sequences of ZYMV using several methods (Chimaera, Geneconv, MaxChi, RDP, Siscan,

Table 1 Primers designed in this study for determining complete sequence of ZYMV-Kurdistan

Primer name	Sequence (5–3)	Genome positions ^a	Expected DNA (bp) ^b
ZY5UTF	AAAATTGAAACAAATCACAAAGACTAC	5'-UTR + pP1	253
ZY253R	ATGTTGCCATGTGGCCAG		
ZY140F	ATGGCCTCCATTATGATTGGTTC	P1	944
ZY1084R	TCCGGTTGCGACGAATAGTG		
ZY1019F	GATTGTGAACGCGCTGGAAC	pHC-Pro	882
ZY1899R	GCTGATGTGAGTGGCTTCTTCTC		
ZY1811F	CATAGGCTCATTGATTGTACCAC	pHC-Pro + P3	953
ZY2760R	GACAACCCCTTCATGATTTCAG		
ZY2715F	TGATAAGCGAAGCTTCACCAC	pP3 + PIPO	719
ZY3432R	TCCTCAAGATCTGGTCTCAC		
ZY3389F	CGCCATATACAATGACTTTC	pP3 + 6K1 + pCI	818
ZY4206R	ATTGAAAGCTATGGTCGCAC		
ZY4162F	ATAGACGAATGCCATGTCAC	pCI	863
ZY5024R	ACCTTGTTGAGCACCATTTC		
ZY4986F	GTTCAAACCTCAGGGATTTCAG	pCI + p6K2	707
ZY5692R	GTTTCATTGATCTTTTTCGTG		
ZY5617F	TGATATTGGCGATTATGACAC	P6K2 + NIa-VPg + pNIa-Pro	794
ZY6411R	TCCATTAGTGATGATTATCGG		
ZY6341F	GATTCTGATGGCCTCAAGG	pNIa-Pro + pNIb	916
ZY7260R	CCAATTGATTAACAGTGACGG		
ZY7134F	AACACACGCAGAAGCGAG	pNIb	871
ZY8004R	TTTCATGCACGCATAGTAAATAG		
ZY7945F	ACACACTAATGGTTGTGATCTC	pNIb + pCP	899
ZY8844R	GAATTGCTGATGAGACGCTCG		
ZY8830F	TCTCATCAGCAATTCGCCTC	pCP + 3'-UTR	762
ZY-UTR3R	T(14)AGGCTTGCAAACGGAG		

^ap partial

^bCorresponded to TW-TN3 isolate (NC-003224)

Fig. 1 Organization of the genome, the lengths of amino acids consisting of mature proteins and predicted cleavage sites of the polyprotein of ZYMV-Kurdistan. Each cleavage site is indicated by a slash symbol (a). Unrooted phylogenetic tree of whole genome sequences of 20 ZYMV strains obtained from GenBank database and isolate

ZYMV-Kurdistan sequenced in this study. Each strain is represented by strain name followed by country of isolation and its accession number in bracket. The phylogenetic analysis was constructed in MEGA6 software using Maximum likelihood algorithm. Bootstrap values are shown next to the branches (b)

LARD and 3Seq) implemented in the Recombination Detection Program (RDP4) (Martin et al. 2015) were assessed. These analyses were done using default settings for the different methods. Only those recombination event detected by at least three out of the seven methods were accepted.

Results and discussion

The complete genome of ZYMV-Kurdistan was deposited in GenBank under accession number MF684760. The genome of this isolate was 9593 nt in length excluding the poly(A) tail, which is two and four nucleotides longer than Fars and IKA isolates, respectively, and the same as that in SANRU. These insertions and deletions were found in 3' and 5'-UTR

Table 2 Sequence identity comparisons between the ZYMV-Kurdistan isolate and the corresponding genome regions of other ZYMV isolates at the nucleotides and amino acid (in parentheses) level

Isolates	Accession numbers	Genome regions													
		5'UTR	P1	HC-Pro	P3	P3N-PIPO	6K1	CI	6k2	VPg	N1a	N1b	CP	3'UTR	Poly protein
10itSDE	KT59822	87.5	87.7 (85.3)	91.7 (97)	92.3 (96.2)	93.2 (96.4)	90.8 (100)	93.6 (98.5)	91 (95.9)	94.8 (98.8)	92 (98.2)	94.8 (97.4)	93.8 (98.5)	95.4 (96.6)	92 (96.6)
Kuchyna	DG124239	97.1	97.2 (96.5)	98.2 (97.9)	98.7 (98.5)	98.4 (98.7)	99.3 (100)	98.8 (98.6)	98.7 (100)	98.5 (98.8)	98.7 (99.1)	99.3 (99.2)	98.5 (97.8)	98.5 (98.4)	98.5 (98.4)
SE04T	KF976713	97.1	97.5 (97.2)	98 (97.7)	98.9 (98.5)	98.7 (98.7)	99.3 (100)	99 (99.2)	98.7 (100)	98.7 (99.4)	99.2 (100)	99.4 (99.2)	98.7 (98.2)	99 (98.7)	98.6 (98.7)
H	KF976712	96.3	97.3 (97.2)	98.2 (98.1)	98.8 (98.5)	98.4 (98.2)	99.3 (100)	98.9 (99.2)	98.7 (100)	98.7 (99.4)	99 (100)	99.4 (99.4)	98.7 (98.2)	98.5 (98.8)	98.6 (98.8)
B*	AY188994	98.5	98 (97.9)	98.9 (98.8)	98.8 (98.5)	98.5 (98.2)	97.1 (97.5)	99 (99.2)	98.1 (100)	98.7 (99.4)	99.3 (100)	99.4 (99.4)	98.8 (98.5)	98.5 (99)	98.8 (99)
IKA	KU528623	93.4	98.7 (97.2)	97.6 (97)	97.8 (96.6)	97.3 (94.5)	98.6 (100)	95.7 (98.3)	98.7 (98)	95.8 (94)	98.4 (97.8)	99 (98.6)	91.3 (87.7)	99 (96.6)	96.9 (96.6)
SANRU	KU198853	94.9	96.4 (95.1)	98.5 (98.6)	97.6 (97)	98.5 (99.1)	97.8 (95)	98.5 (97.6)	99.4 (100)	97 (97.6)	98.4 (99.1)	98.9 (98.8)	99 (99.3)	96.4 (98)	98.1 (98)
AP Gherkin	KT778297	93.4	96 (94.7)	97.9 (98.4)	98.4 (98.1)	98.7 (98.7)	97.8 (100)	98.4 (98.6)	98.7 (100)	97.4 (99.4)	98.6 (99.6)	98.9 (99)	98.1 (98.9)	97.5 (98.4)	97.9 (98.4)
Fars	JN183062	96.3	95.7 (92.9)	98.2 (99.1)	98.3 (98.5)	98.7 (99.1)	97.1 (97.5)	98.8 (99)	99.4 (100)	98.5 (98.8)	98.2 (100)	98.9 (98.8)	99.3 (99.6)	98 (98.5)	98.2 (98.5)
PA_2006	JQ716413	ND	97 (95.5)	97.9 (98.6)	97.6 (97.4)	97.2 (96.8)	96.4 (92.4)	98.1 (98.8)	98.7 (100)	97 (99.4)	94.7 (99.1)	96.2 (97.8)	94.5 (98.9)	ND (98.1)	96.4 (98.1)
TW-TN3	AF127929	83.1	94.2 (94.7)	93.3 (97.7)	91.7 (97)	93.2 (95.5)	94.1 (100)	92.6 (98.1)	94 (98)	95.8 (98.2)	96.1 (100)	96.2 (98)	93.2 (97.8)	95.4 (97.7)	93.5 (97.7)
KR-PS	AY279000	85.3	90.9 (91.1)	93.5 (98.1)	92 (95)	91.9 (93.1)	91.6 (100)	91.7 (97.3)	93.2 (95.9)	92.7 (99.4)	93 (100)	95 (98)	93 (97.1)	95.5 (97.1)	92.2 (97.1)
KR-PE	AY278999	86	91.3 (90.7)	93.6 (98.1)	91.9 (95)	91.6 (92.6)	92.4 (100)	91.4 (96.9)	93.2 (95.9)	92.7 (99.4)	93 (100)	95.1 (98.2)	93 (97.1)	95.5 (97)	92.2 (97)
KR-PA	AY278998	83.1	91.8 (90.3)	93.4 (98.6)	91.9 (97)	92.8 (95.5)	91.6 (100)	90.5 (97.1)	92.5 (95.9)	90.7 (98.8)	93.5 (99.6)	95.3 (97.4)	92.5 (96.7)	97.5 (97)	91.9 (97)
AG	EF062583	98.5	97.9 (97.6)	98.7 (98.6)	98.9 (98.5)	98.7 (98.7)	97.1 (97.5)	99 (99.2)	98.1 (100)	98.7 (99.4)	99.4 (100)	99.2 (99.8)	99 (98.5)	98.5 (98.9)	98.7 (98.9)
NAT	EF062582	98.5	97.9 (97.6)	98.8 (98.8)	98.9 (98.5)	98.7 (98.7)	97.1 (97.5)	99 (99.2)	98.1 (100)	98.7 (99.4)	99.4 (100)	99.3 (99.8)	99 (98.5)	98.5 (98.9)	98.8 (98.9)
2002	AB188116	87.5	89.4 (87.3)	91.5 (97.2)	92.4 (96.6)	93.5 (95.9)	93.3 (100)	97.1 (98.8)	97.4 (98)	98.5 (98.8)	97.2 (99.6)	96.2 (98.6)	95.3 (98.9)	97 (97.3)	94.6 (97.3)
Z5-1	AB188115	87.5	89.4 (87.3)	92.1 (98.1)	92.7 (96.6)	93.5 (95.9)	93.3 (100)	97.1 (98.6)	97.4 (98)	98.5 (98.8)	97.2 (99.6)	96.9 (98.8)	95.1 (98.9)	97 (97.4)	94.6 (97.4)

Table 2 (continued)

Isolates	Accession numbers	Genome regions													
		5'UTR	P1	HC-Pro	P3	P3N-PIPO	6K1	CI	6k2	VPg	NIa	NIb	CP	3'UTR	Poly protein
RD	AB369279	85.3	92 (91.1)	93.6 (98.4)	90.6 (95)	92 (93.1)	90 (97.5)	91.2 (97.4)	93.2 (95.9)	90.5 (97.6)	93.1 (99.1)	95.1 (97.8)	89.9 (95.6)	95.9	91.7 (96.7)
Singapore	AF014811	76.9	44.3 (48.5)	76 (88.5)	88.4 (92.6)	92.2 (92.7)	86.3 (97.5)	84 (95.9)	80.6 (93.8)	82.4 (96.4)	84.8 (96.9)	89.2 (95.3)	85 (91.7)	97	79.6 (90.1)

These comparisons were obtained in MEGA6 program using p-distance method

Nd not determined

regions. It contained two open-reading frames (ORFs), the large ORF encoding a polyprotein of 3080 amino acids (aa) and the small ORF encoding a PIPO protein of 74 aa, which overlapped the large ORF. As predicted by Pirone and Blonc (1996) and Choi et al. (2007) for other ZYMV variants, nine putative cleavage sites at amino acid positions 310, 766, 1112, 1164, 1798, 1851, 2041, 2284 and 2801 were also found in the polyprotein of ZYMV-Kurdistan that give rise to ten mature proteins: P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and CP (Fig. 1a). All conserved potyvirus motifs were identified in amino acid sequences of ZYMV-Kurdistan. In HC-Pro protein, PTK (Phe-Thr-Lys) (Glasa and Pittnerová 2006) and KLSC (Lys-Leu-Ser-Cys) (Desbiez et al. 2003) motifs were identified that could play an important role in aphid-transmission. In comparison to other Iranian isolates of ZYMV, KITC (Lys-Ile-Thr-Cys) motif is generally found in potyviruses (Desbiez et al. 2003) including majority of ZYMV isolates but in the newly studied isolate, the motif KLSC was present instead. Glasa and Pittnerová (2006) have reported that KLSC instead of KITC in ZYMV-Kuchyna was permissive for transmission and efficient aphid transmissibility to zucchini and cucumber. Collectively, ZYMV-Kurdistan had six unique aa differences in comparison to other ZYMV isolates (Supplementary Table 1). In P3 of the new isolates, we found that it had an Arginine at position 917 of the polyprotein (corresponded to position 151 of P3), encoded by an AGG, while all aggressive variants of ZYMV had different amino acids at this position (Glasa et al. 2007).

The complete genome of isolate Kurdistan was compared with the available 20 whole genome sequences of ZYMV worldwide (Table 2). This isolate shared 79.6–98.8% identities with the other reported ZYMV isolates at the nt level and 90.1–99% at the aa level. Furthermore, the identity percentage between isolate Kurdistan with other isolates for each of the ORFs (P1, HC-Pro, P3, PIPO, 6K1, CI, 6K2, NIa, VPg, NIb, CP) at the nt and aa levels were determined. In addition, the highest genetic distance was obtained for 5'UTR (0.136 ± 0.016), followed by P1 (0.105 ± 0.006), HC-Pro (0.067 ± 0.004), NIb (0.067 ± 0.004), CP (0.066 ± 0.005), 6K1 (0.065 ± 0.012), CI (0.060 ± 0.003), VPg (0.059 ± 0.005), 6K2 (0.057 ± 0.010), NIa (0.056 ± 0.005), 3'UTR (0.036 ± 0.006) and PIPO (0.035 ± 0.008). The greatest variability between potyvirus genomes has been reported in the P1 gene (Adams et al. 2005; Lin et al. 2001) and likewise, we also found that P1 gene to be the most variable region in the ZYMV strains genome. Therefore, it is useful for investigation on the evolutionary relationship and differentiation between strains of potyviruses.

The phylogenetic tree was constructed by the maximum likelihood (ML) method implemented in MEGA6 (Tamura et al. 2013) with 1000 replicates (bootstrap). ZYMV isolates clustered into three groups (Fig. 1b). A single isolate

from Singapore (Singapore) was in Group III, whereas collectively 15 isolates from Israel, India, Slovakia, Czech Republic, Iran, Japan and the USA were in Group I, and 6 isolates from Taiwan, Argentina and South Korea in Group II. A correlation appeared to exist between the phylogeny and geographic origin of the isolates being in agreement with the report by Glasa et al. (2007), where a close genetic relationship linking the ZYMV isolates from the central European region (Slovakia and Czech Republic) and here was found. We suggest that the Iranian isolates of ZYMV have a common ancestor with ZYMV isolates from central Europe or have been spread from unique infection focus.

d_N/d_S ratio differed greatly among different ORFs as follows: 0.255 (P1), 0.061 (HC-Pro), 0.064 (P3), 0.114 (P3N-PIPO), 0.067 (6K1), 0.039 (CI), 0.057 (6K2), 0.036 (VPg), 0.024 (NIa), 0.033 (NIb) and 0.089 (CP). All the ZYMV-encoded proteins were under negative selection ($d_N/d_S > 1$), while this value for NIa was the lowest (0.024) suggesting that this protein is under strong negative (purifying) selection pressure. NIa protein plays many roles during infection (Martínez et al. 2016), therefore, the rate of synonymous substitution is much larger than the non-synonymous rate in this protein, reflecting that the majority of non-synonymous mutations were removed by strong purifying selection over the course of evolution. Vice versa, in consistent with previous studies of other potyviruses such as *Soybean mosaic virus* (Ahangaran et al. 2013), *Watermelon mosaic virus* (Moreno et al. 2004), d_N/d_S ratio was the highest (0.255) for P1 protein due to high sequence variability among ZYMV isolates (0.105 ± 0.006), reflecting different evolutionary dynamics acting on the various potyviral genes.

No recombination event was found in ZYMV-Kurdistan, but this isolate has been identified as the major parent of another Iranian isolate, IKA (Acc. No. KU528623) (data not shown). Recombination in IKA isolate was confirmed by the phylogenetic tree wherein this isolate was separate from the remaining Iranian isolates including those from Fars, SANRU and Kurdistan (Fig. 1b). Each recombination event was usually parented by the isolates from the same geographical region as the recombinant.

In conclusion, this is the first report of complete genome sequence information of the most prevalent virus in west of Iran. This study helps us to further understand the genetic diversity, evolution and epidemiology of ZYMV isolates infecting cucurbit plants in Iran and assists in implementing better diagnostic tools and virus control strategies.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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