Research Article

Differentiation and phylogeny of *Cucumber mosaic virus* isolates originating from ornamentals in Iran; concerning genetic structure of virus

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Abstract: *Cucumber mosaic virus* (CMV; genus *Cucumovirus*, family: *Bromoviridae*) has the widest host range of any known plant viruses. Seven virus isolates, originated from different ornamental plant species and greenhouses, were biologically purified, mechanically inoculated onto test plants and their serological differences were assayed based on reactivity with 11 CMV-specific monoclonal antibodies. Following total RNA extraction, coat protein (CP) coding region of CMV isolates was amplified. Based on biological, serological and phylogenetic analysis, only one isolate belonged to CMV subgroup II and other six isolates were equally distributed among the two IA and IB subgroups. Aphid transmission assay showed that no significant difference was observed between transmission efficiency of CMV subgroups IA, IB and II members by *Aphis gossypii*. The genetic variation and evolution of CMV in Iran was studied by sequence analysis of the CP gene and comparison with equivalent sequences of isolates from other continents that exhibited low genetic diversity and close evolutionary relationships among isolates in subpopulations. Analysis of various population genetics parameters and distribution of synonymous and nonsynonymous mutations revealed that most of the amino acid sites were under negative selection and only one site was under positive selection.

Keywords: *Cucumber mosaic virus*, genetic structure, ornamental plants, population genetics, transmission efficiency

Introduction

Cucumber mosaic virus (CMV, *Cucumovirus*, *Bromoviridae*) has the widest host range of any known viruses (Palukaitis and Garcia- Arenal, 2003b; Garcia- Arenal and Palukaitis, 2008). It infects more than 1200 dicotyledonous and monocotyledonous plant species (Edwardson and Christie, 1991; Roossinck, 2002). CMV is easily transmitted by mechanical inoculation of plant sap and naturally transmitted by 80 species of aphids in non-persistent manner (Perry *et al.*, 1994; Gallitelli, 2000), with *Myzus persicae* and *Aphis gossypii* as the most efficient vectors for the virus (Perry *et al.,* 1998; Tian *et al.,* 2012). The rate of transmission can be affected by the virus strain, aphid species and the quality of the recipient and donor plants (Perry *et al.,* 1994; Gallitelli, 2000). Some CMV strains are not transmissible or are poorly transmitted by aphids. These differences in transmissibility are probably not only attributed to differences in the amino acid composition of the virus capsid, but also to

specific segments of the genome whose structure may affect protein/protein or RNA/protein interactions with a dramatic impact on particle stability. Complete loss or reduced rate of aphid transmissibility may represent a genetic 'bottleneck' for many natural reassortants, a trait that correlates positively with their rare occurrence in nature (Zitter and Gonsalves, 1991).

The existence of many CMV strains that have a very broad host range suggests that the multicomponent nature of the virus does not constrain its ecological success or epidemiological competence (Gallitelli, 2000). Strains of CMV can be divided into subgroups I and II on the basis of serological relationships, peptide mapping of the coat protein, nucleic acid hybridization and nucleotide sequence identity (Palukaitis *et al.,* 1992; Rizos *et al.,* 1992). Surveys of naturally infected crops suggest that sub-group I strains are more frequent than those of subgroup II and sometimes they represent more than 80% of all isolates (Fraile *et al.,* 1997). Sequence data show that a number of CMV strains within sub-group I and originating from Asia differ by 7–12% in sequence arrangement from other subgroup I strains (Gallitelli, 2000). Accordingly, Palukaitis and Zaitlin, (1997) proposed that subgroup I should be split by placing the 'Asian strains' in subgroup IB and the others in subgroup IA. Strains in the same sub-group differ by only 2-3% of their sequence homology. Phylogenetic analyses of CMV using the whole genome or the ORFs of single genes strongly support the sub-division of sub-group I, considering CMV strains so far sequenced (Roossinck, 1999). CMV subgroup I strain generally induce more severe disease than those of subgroup II and have historically been predominant throughout the world (Hord *et al.,* 2001; Tian *et al.,* 2009).

CMV plays an important role in the deterioration of ornamental quality because it not only causes direct damage to the host but also predisposes the plant to secondary invaders. Also, infected plants are considered as the main source of the virus movement and spreading (Samuitiene and Navalinskiene, 2008). In the current study, we provided useful information of biological, serological and molecular characteristics of ornamental CMV isolates in Iran, concerning analysis of genetic variation and population genetics parameters.

Materials and Methods

Virus Isolates
In July 2012, during a survey to determine the presence of *Cucumber mosaic virus* (CMV) on ornamentals, total of 294 samples belonging to 28 families of ornamental plants, showing yellowing, mosaic and distortion were collected from green houses in Alborz and Tehran provinces of Iran.

DAS-ELISA
Collected symptomatic leaf samples were tested by double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977) using CMV specific polyclonal antibody (AS-0929, DSMZ, Germany) The reaction was considered positive when absorbance values at 405 nm reached more than threefold the mean value of the healthy controls.

Biological assays
Among the serologically CMV-positive samples, seven isolates originating from different hosts, except two later ones with different symptoms on test plants (designated as CMV-Bo1, -Co1, -Go5, -Ka16 and -Os7 isolated from *Bougainvillea spectabilis* Willd., *Coleus blumei* Benth., *Gomphrena globose* L., *Kalanchoe blossfeldiana* Poelln., *Osteospermum caulescens* Harv., respectively; and CMV-Zin1 and -Zin2 both from *Zinnia elegans* L.) were selected. Isolates were mechanically inoculated on *Chenopodium quinoa* Willd. and *Chenopodium amaranticolor* Coste & Reyn. local lesion hosts for biological purification. Single local lesions were then inoculated on *Nicotiana benthamiana* L. plants and then systemically infected plants were used as the source of virus isolate inoculum for all the subsequent studies. Twelve test plant species representing four families were

mechanically inoculated in early stages of growth, using carborundum as an abrasive. The inocula were prepared by homogenized tobacco infected plant leaf tissues in 0.1 M phosphate buffer pH 7 (1: 10, w/v), containing 0.2% sodium diethyldithiocarbamate as virus stabilizer.

Aphid transmission experiments of four selected CMV isolates (CMV-Bo1, -Ka16, -Os7 and -Zin2, belonging to different subgroups) by *Aphis gossypii* was carried out according to the methods described by Noordam, (1973). The inoculated *N. benthamiana* plants were kept in an insect proof cage (20 ± 1 °C and 16 h light photoperiod) and observed for six weeks for symptom development, if any. The presence of virus in all inoculated test plants (mechanically or by vector) was also confirmed by DAS-ELISA.

The relative concentration of CMV in aphid inoculated plants was estimated by semi-quantitative DAS-ELISA method as described previously (Mazier *et al.,* 2004), using 1: 10 dilution of inoculated plants leaf tissue extract in extraction buffer at 28 dpi, so that the relationship between OD_{405} and the viral concentration was linear. CMV accumulation level data were statistically analyzed by ANOVA (Proc. GLM) followed by Student-Newman-Keuls (SNK) test at α = 5% to estimate the differences among the variants using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA).

Virus transmission rates (i.e., the ratio of infected plants to the total number of inoculated ones at 28 dpi) was analyzed by logistic regression using the GENMOD procedure of SAS, with CMV isolate as class variable; means were separated using the least-squares statement of SAS (Neter *et al.,* 1990).

The transmission rate of a single aphid (P*) was calculated by using the formula suggested by Gibbs and Grower (1960): $P^* = 1 - (1-T)^{1/I}$

Where T = transmission rate as T = R/N; R = number of infected plants; N = number of inoculated plants: I = number of aphids per inoculated plant.

TAS-ELISA

Infected *N. benthamiana* plants were subjected to the indirect triple antibody sandwich ELISA (TAS-ELISA) as described previously by Thomas *et al.* (1986) using 11 monoclonal antibodies (MAb) kindly provided by DSMZ, Germany (Table 1) to determine the subgroup of each isolate. CMV polyclonal antibody (DSMZ AS-0929) was used for coating the plates, followed by loading of systemically infected *N. benthamiana* plants leaf extract in ELISA extraction buffer. The reaction was considered positive when absorbance values reached more than threefold the mean value of the healthy controls.

Table 1 Reactivity of CMV isolates with CMV specific monoclonal antibodies in TAS ELISA.

Isolates	CMV monoclonal antibody										
	657.1	656.1	486.1	490.1	656.3	489.1	487.1	488.1	491.8	655.5	656.7
Bo1	++++	-	-	+++++	-	-	-	++++	++++	++++	-
Co1	++++	-	-	+++++	-	-	-	++++	++++	++++	-
Go5	+++	-	-	+++++	-	-	-	++++	++++	++++	-
Ka16	+++	-	-	+++++	-	-	-	++++	+++	++++	-
Os7	++++	-	-	+++++	-	-	-	+++++	++++	+++++	-
Zin1	+	-	-	+++++	-	-	-	++++	++++	++++	-
Zin2	-	++	+++	-	++++	+++	++	-	-	-	+++

TAS ELISA reactions were scored based on mean actual absorbance value of three wells of sample at 405nm. (-)= not different from values obtained for mock inoculated controls with OD < 0.1; (+) = 0.1 < OD > 0.3; (++) = 0.3 < OD > 0.6; (+++) = 0.6 < OD >1; (++++) = 1 < OD > 2; (+++++) = OD > 2.

RNA extraction, RT-PCR, cloning and sequencing

Total RNA was extracted from *N. benthamiana* infected plants leaf tissues using phenol chloroform method (Sambrook *et al.,* 1989). The quality of extracted RNA was analyzed by electrophoresis on 1% agarose gel (as below).

Reverse transcription polymerase chain reaction (RT-PCR) was performed to amplify complete *coat protein* (CP) gene of the isolates using extracted total RNA and subgroup-specific primer pairs (Lin *et al.,* 2004). cDNA was synthesized in a 20µl reaction with M-MuLV reverse transcriptase (Fermentas GmbH, St. Leon- Rot, Germany) at 42 °C for 60 min in a thermocycler (Palm cycler, CG1-96, Corbett Research, Mortlake, Australia). Touchdown PCR was performed for subgroup I- specific primers containing reactions and the normal PCR was performed for subgroup II- specific primers containing reactions. Amplified fragment of CMV isolates were extracted from agarose gel, subsequently TA cloned into the pTG19-T cloning vector (Vivantis, Malaysia) and sent for sequencing in both directions to Bioneer Inc. (Daejeon, South Korea).

Sequence analysis

Nucleotide sequence of amplified fragment was analyzed by online Blastn software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and CP ORF was predicted using online ORF finder software (www.ncbi.nlm.nih.gov/projects/gorf/) to find out start and stop codons. The nucleotide and translated amino acid sequence of our seven CMV isolates ORF CP were multiple aligned with 54 different CMV isolates sequences (belonging to CMV S-IA, S-IB and S-II, including Iranian isolates), already are available in GenBank (Table 2), by using Muscle within the MEGA 6 sequence analysis package, using the default parameters (Edgar, 2004; Tamura *et al.,* 2013).

Model evaluation was carried out for the nucleotide and translated amino acid sequence dataset within MEGA. This analysis showed that Kimura two parameter (K2P) (Kimura, 1980) and Jones-Taylor-Thornton (JTT) (Jones *et al.,* 1992) with gamma distributed rates amongst sites were the best models, respectively. Phylogenetic trees for the nucleotide and translated amino acid sequences were then constructed using maximum likelihood within MEGA6 using the K2P + G and JTT + G substitution models, respectively. Each model was tested with 1000 bootstraps replicates and branches with < 60% bootstrap supports were collapsed. *Tomato aspermy virus* (TAV) *CP* gene (accession no. AJ550020) was used as outgroup.

Population genetics for the CMV *coat protein* gene was estimated within and between different continent or geographical regions (Iranian, Asian, European and American subpopulations) by parameters with respect to: statistic Watterson's estimator of Θ (Θ_w) (Watterson, 1975), number of segregating sites (S), nucleotide diversity π (Pi), the average number of nucleotide differences per site between two sequences (Nei, 1987); and synonymous codon usage biases were calculated by the DnaSP version 5.0 program (Rozas *et al.,* 2003). Synonymous codon usage bias was measured by quantifying the "effective" number of codons (ENC) (Wright, 1990) that are used in a gene. For the nuclear universal genetic code, the value of ENC ranges from 20 (if only one codon is used for each amino acid, i.e., the codon bias is maximum) to 61 (if all synonymous codons for each amino acid are equally used, i.e., no codon bias).

To assess the genetic differentiation and the gene flow level between subpopulations, the statistic F_{ST} (Weir and Cockerham, 1984) was used that was implemented in the DnaSP 5.0 program (Rozas *et al.,* 2003).

To study the role of natural selection at the molecular level, the rate of synonymous substitutions per synonymous site (Pi_s) and the rate of nonsynonymous substitutions per nonsynonymous site (Pi_a) were estimated by DnaSP 5.0 program by the method described by Nei and Gojobori, (1986).

Table 2 *Coat protein* gene sequences of various *Cucumber mosaic virus* strains and isolates used for comparison.

CMV isolate/strain	Accession NO	Original host	County of origin	Subgroup
473-12	KC878465	*Citrullus lanatus*	Serbia	-
Ab	KJ173756	*Abutilon theophrasti*	Iran	IB
Ajs4	JX025999	*Lycopersicum esculentum*	Iran	IB
ALS-IPO	AJ304399	*Alstroemeria sp.*	The Netherlands	II
B13	AY871070	*Cucumis sativus*	Iran	IA
B2	AB069971	*Musa sapientum*	Indonesia	IB
Bas3	JX025989	*Cucurbita pepo*	Iran	IB
BKD257	AJ131621	Ornamental crops	The Netherlands	II
Bn57	HF572916	*Phaseolus vulgaris*	USA	IA
Bo1	KP662629	*Bougainvillea spectabilis*	Iran	this study
Ce27	KJ173754	*Apium graveolens*	Iran	IB
CMV-G10	AY541691	*Solanum lycopersicum*	Greece	IB
CMV-G2	AY450854	*Solanum lycopersicum*	Greece	IA
Co1	KP662630	*Coleus blumei*	Iran	this study
Cu49	KJ173755	*Cucumis sativus*	Iran	IA
Cues	KF873615	*Cucumis sativus*	Iran	IA
D	AJ131624	*Phaseolus vulgaris*	USA	-
Datura	EF593024	*Datura innoxia*	India	IB
Esf172	JX025995	*Lycopersicum esculentum*	Iran	IB
EUS	AJ131627	*Lisianthus* sp.	Taiwan	IB
Fny	D10538	*Cucumis melo*	USA	IA
Ft	D28487	*Lycopersicum esculentum*	Japan	IA
GDG2	FJ403474	*Capsicum annuum*	China	-
Go5	KP455738	*Gomphrena globosa*	Iran	this study
GPP	AJ131623	*Gladiolus sp.*	The Netherlands	IA
Ka16	KP455737	*Kalanchoe blossfeldiana*	Iran	this study
Ker.Ker.mel2	JX112020	*Citrullus lanatus*	Iran	IB
Ker.Ker.pepo	JX112021	*Capsicum annuum*	Iran	IB
KKG	HQ343232	*Solanum melongena*	India	IB
Kor	L36251	-	South Korea	IA
Kzn-Bm1	KJ789892	*Cucurbita pepo*	South Africa	IA
LBO	AJ131615	*Lilium sp.*	The Netherlands	IA
Legume	D16405	*Vigna unguiculata*	Japan	IA
LiSR	AJ131617	*Lilium sp.*	The Netherlands	IA
LS	AF127976	*Lactuca sativa*	USA	II
Mf	AJ276481	*Melandryum firmum*	South Korea	IA
Musa	U32859	*Musa sapientum*	Columbia	IA
N1-04	JF918967	*Vinca minor*	USA	IA
N11	AJ276587	*Alstroemeria sp.*	The Netherlands	II
Nd-S	AJ131620	Ornamental crops	The Netherlands	II
Ns	AJ511990	*Nicotiana glutinosa*	USA	IA
NT9	D28780	*Lycopersicum esculentum*	Taiwan	IB
Ny	U22821	-	Australia	IA
Oahu	U31220	*Musa sapientum*	USA	IB
Os7	KU695261	*Osteospermum caulescens*	Iran	this study
PE	AF268597	*Passiflora incarnata*	China	IB
PoCMV9	AB448696	*Solanum tuberosum*	Syria	IA
Q	M21464	*Capsicum annuum*	Australia	II
Ri-8	AM183119	*Lycopersicum esculentum*	Spain	IA
Rs	AJ517802	*Raphanus sativus*	Hungary	-
S	AF063610	-	South Africa	II
Sn	U22822	*Trifolium subterraneum*	Australia	II
Sny	U66094	*Cucumis melo*	USA	IA
SP103	U10923	*Spinacia oleracea*	USA	II
Tfn	Y16926	*Lycopersicum esculentum*	Italy	IB
To33	KJ173753	*Lycopersicum esculentum*	Iran	IB
Tob	KJ173757	*Nicotiana tabacum*	Iran	IA
Trk7	L15336	*Trifolium repens*	Hungary	II
Xb	AF268598	*Musa sapientum*	China	II
Zin1	KP455736	*Zinnia elegans*	Iran	this study
Zin2	KP662628	*Zinnia elegans*	Iran	this study

Selection at individual codons was statistically tested by the fixed effects likelihood (FEL) and single likelihood ancestor counting (SLAC) methods available from the DATAMONKEY (Kosakovsky-Pond and Frost, 2005) server (http://www.datamonkey.org). To classify a site as positively or negatively selected, the cut-off P-value was selected to be 0.1 and only selections determined to be significant by both methods were considered as positive selections.

Results

Symptomology and serological assays

DAS-ELISA results revealed that 64 out of 294 symptomatic ornamental plants collected from green houses in Alborz and Tehran provinces of Iran were infected with CMV. All infected plants showed mosaic, yellowing and distortion symptoms in comparison with healthy plants. Among biologically purified isolates only CMV-Zin2 isolate reacted positively with a set of six (out of 11 ones) CMV specific MAbs in TAS-ELISA, while the other six isolates (CMV-Bo1, -Co1, -Go5, -Ka16, -Os7 and -Zin1), reacted positively with another set of five CMV-MAbs, as determined by OD_{405} values (Table 1), while no positive reaction were observed with the healthy plant tissues used as the negative control.

Biological assays

Following mechanical inoculation, all seven isolates induced similar symptoms on most of infected test plants including chlorotic local lesions on *C. quinoa* and *C. amaranticolor* at 6-8 days post-inoculation (dpi) (Fig. 1A-B), systemic mosaic on *Cucurbita pepo* L. and *Capsicum annuum* L. (Fig. 1C-D), mosaic and/or distortion symptoms on *Nicotiana tabacum* L. cv. Samsun, *N. rustica* (L.) Opiz., *N. tabacum* L. and *N. benthamiana* (Fig. 1E-H), *N. tabacum* L. cv. White Burley, *N. debneyi* L. and also *Vicia faba* L. at 25-30 dpi. Six CMV isolates induced large necrotic local

lesions on *Vigna unguiculata* (L.) Walp. while only isolate Zin2 induced pin point necrotic local lesions (Fig. 1I-J).

A. gossypii transmitted successfully all four CMV-Bo1, -Ka16, -Os7 and -Zin2 isolates in non-persistent manner from systemically infected *N. benthamiana* to *C. pepo* plants. Infection of *C. pepo* plants, exhibiting severe mosaic symptoms at 25- 30 dpi, was confirmed by DAS-ELISA. Aphid transmission rate of CMV showed no significant difference among four different isolates; however, CMV-Os7 and CMV-Bo1 were transmitted maximally (80%) and minimally (60%) by *A. gossypii*, respectively. In addition, calculated transmission rate of a single aphid (P*) for four CMV isolates showed that -Os7 and -Bo1 isolates were transmitted with highest and lowest efficiency, respectively (Table 3). Statistical comparison of the relative concentrations of the virus between inoculated plants with CMV-Bo1, -Co1, -Ka16, -Zin2 isolates by aphids showed no significant difference between isolates (P< 0.05), while mock inoculated plants were free of virus with significantly lower OD_{405} values.

RT-PCR and sequences analysis

Gel electrophoresis analysis of RT-PCR products revealed that a cDNA fragment was amplified by using Q1/Q3 primer pair for CMV-Zin2 isolate in predicted size (642 bp), while for the other isolates (except for CMV-Bo1, with amplified cDNA of 606 bp in length) RT-PCR resulted in amplification of an approximately 678 bp DNA fragment using F1/F3 primer pair covered the CP gene (657 nts) plus 12 and nine nts of 5' and 3' flanking regions, respectively. In contrast, no amplicon was obtained in healthy sample using either F1/F3 or Q1/Q3 primers. The nucleotide sequence of ORF CP of seven CMV isolates in this study, were deposited in the NCBI GenBank under accession numbers KP662629, KP662630, KP455737, KP455738, KP455736, KP662628 and KU695261 (Table 2).
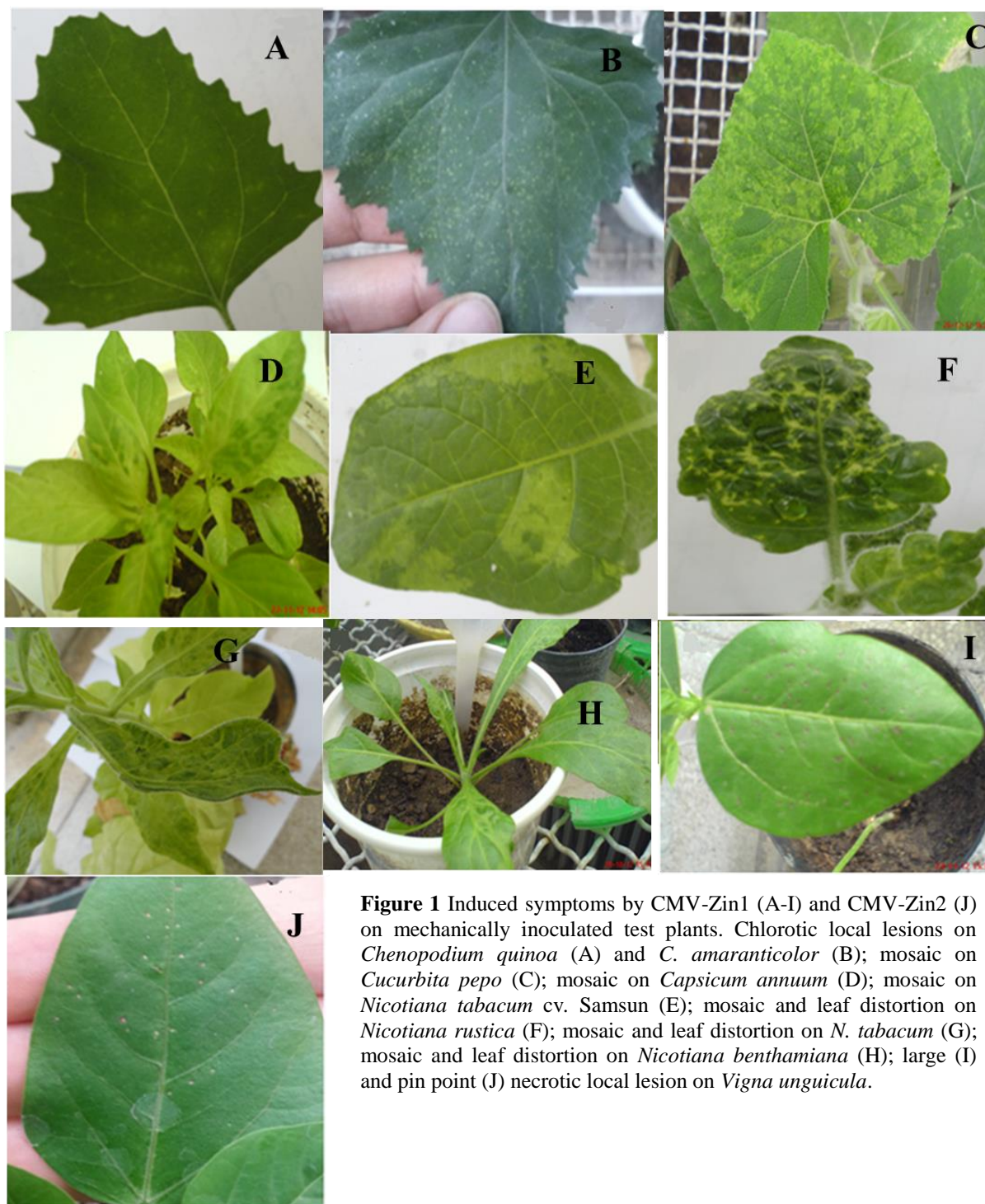
Figure 1 Induced symptoms by CMV-Zin1 (A-I) and CMV-Zin2 (J) on mechanically inoculated test plants. Chlorotic local lesions on *Chenopodium quinoa* (A) and *C. amaranticolor* (B); mosaic on *Cucurbita pepo* (C); mosaic on *Capsicum annuum* (D); mosaic on *Nicotiana tabacum* cv. Samsun (E); mosaic and leaf distortion on *Nicotiana rustica* (F); mosaic and leaf distortion on *N. tabacum* (G); mosaic and leaf distortion on *Nicotiana benthamiana* (H); large (I) and pin point (J) necrotic local lesion on *Vigna unguicula*.

Table 3 Comparison of CMV isolates transmission by *A. gossypii* and their relative concentration (OD_{405}) in inoculated plants.

CMV isolates	Number of inoculated (infected) plants	Transmission rate (%)	I	P	OD_{405}
Os7	15 (12)	80.0 (a)	6	0.24	0.76 ± 0.14 (a)
Bo1	15 (9)	60.0 (a)	6	0.15	0.60 ± 0.10 (a)
Ka16	15 (11)	73.3 (a)	6	0.20	0.68 ± 0.08 (a)
Zin2	15 (10)	66.6 (a)	6	0.17	0.78 ± 0.09 (a)
Mock inoculation	10 (0)	0	6	0	0.14 ± 0.01 (b)

I = Number of aphids per inoculated plant.
P = Transmission rates for single aphids according to the formula of Gibbs and Grower (1960).

Nucleotide sequence comparison of ORF CP of ornamental CMV isolates in this study and other isolates revealed identities of 94.2-100% among CMV-Ka16, -Os7 and -Zin1 with CMV S-IA, 84- 93.6% between CMV-Bo1, -Co1 and -Go5 with CMV S-IB and 95.3- 96.5% among CMV- Zin2 with CMV S-II members. Phylogenetic analysis of 61 CMV isolates CP gene nucleotide and translated amino acid sequences (54 reference CMV sequences obtained from GenBank and seven newly identified isolates in this study), using maximum likelihood by the K2P + G and JTT + G substitution models, respectively, resulted in re-construction of a phylogenetic tree (Fig. 2). Divergence of subgroups I-A, I-B and II was observed with > 98% supporting values. In this phylogenetic tree CMV-Ka16, -Os7 and -Zin1 isolates fall into S-IA, whereas CMV-Bo1, -Co1 and -Go5 isolates fall into CMV S-IB and CMV- Zin2 isolate grouped into S-II. Based on these analyses, subgroup I was the predominant subgroup among the Iranian CMV isolates originating from ornamentals included in this investigation (Fig. 2).

To evaluate the genetic variation of four CMV subpopulations (Iranian, Asian, European and American) associated population genetic parameters were estimated (Table 4). Based on the results Asian isolates showed the highest (π: 0.08; S: 146 and Θ_w: 0.078 ± 0.006) and American isolates showed the lowest (π: 0.024; S: 42 and Θ_w: $0.028 \pm$ 0.004) genetic variation among four subpopulations. Overall, nucleotide diversity for all CMV isolates was estimated with a mean value of 0.05. To understand the evolutionary constraints imposed on different populations the ratio of Pi_a/Pi_s (G꞉) for all four populations were calculated from pairwise comparisons between sequences. The G꞉ values were all < 1 (Table 4), indicating that the CP gene was subjected to negative selection and is strongly conserved. Determining of site specific selection pressure in coat protein coding region showed one positive selection on codon 65 (Table 5). Calculation of the F_{ST}, the coefficient used to evaluate the extent of genetic differentiation or the gene flow between CMV subpopulations, showed that all the values of F_{ST} among populations were < 0.33, suggesting frequent gene flow (Table 6).

Synonymous codon usage bias was calculated by using the ENC. The value of ENC for these coding regions ranged from 58.14 to 60.66, suggesting that all these populations had only a slight bias in codon choice (Table 4).

Figure 2 Nucleotide (A) and amino acid (B) phylogenetic tree of CMV isolates and reference CMV based on their coat protein sequences reconstructed by maximum likelihood analysis with 1000 bootstrap replication. The CP sequence of of *Tomato aspermy virus* (TAV) another *Cucumovirus* was used as outgroup. Bootstrap values greater than 60% are shown at the nodes. Iranian isolates of CMV in current study are boxed (Figure 2 continued in the next page).

Figure 2 Continued.

Table 4 Population genetic parameters estimated for CMV CP gene of 59 isolates.

Isolates	N[1]	π (P$_i$)[2]	S[3]	Θw[4]	dS (Pi$_s$)[5]	dN (Pi$_a$)[6]	Pi$_a$/Pi$_s$[7]	ENC (%)[8]
Iran	23	0.052	118	0.052 ± 0.004	0.14	0.018	0.12	59.34
Europe	15	0.035	89	0.041 ± 0.004	0.12	0.005	0.05	60.14
US America	11	0.024	42	0.028 ± 0.004	0.07	0.007	0.08	60.66
Asia	10	0.081	146	0.078 ± 0.006	0.24	0.023	0.1	58.14

1. number of isolates.
2. Nucleotide diversity, π (Pi), the average number of nucleotide differences in total sites analyzed.
3. number of segregating sites.
4. Mutation rate estimated from segregating sites.
5. The average number of pairwise differences per synonymous site.
6. The average number of pairwise differences per non-synonymous site.
7. dS and dN were estimated by the method described by Nei and Gojobori (1986).
8. ENC, effective number of codons.

Table 5 Positive selection of CMV CP coding region in the subpopulations of 59 isolates.

Site	SLAC dN/dS	SLAC P- value	FEL dN/dS	FEL P-value
25[1]	-	-	6.10	0.2609
65[2]	4.47	0.0755	Infinite	**0.0286**

1. Sites detected as statistically significant by one method.
2. Sites detected as statistically significant by two methods.
Significant values have been illustrated as bold.

Table 6 F_{ST} values for pairs of temporal subpopulations of 59 isolates of CMV *CP* gene.

Subpopulation	F_{ST}		
	Asia	US america	Europe
Iran	0.0980	0.2453	0.1520
Europe	0.1386	0.0356	
US America	0.2351		

Discussion

CMV has the broadest host range among the plant viruses and has been reported infecting ornamental plants from various parts of the world. Our attempt for this study was to characterize and classify isolates of this virus, from ornamentals, up to genomic level and to find out any similarity with the other strains and also genetic variation and evolution was assessed.

In this study DAS-ELISA analysis using anti-CMV polyclonal antibody revealed the presence of virus infections in 21.7% (64 out of 294) symptomatic ornamental leaf tissue samples, collected from the nurseries of two provinces in Iran. Although the remaining samples showed typical virus infection symptoms such as mosaic, leaf distortion, plant yellowing and stunting, the ELISA did no detect CMV in them. This may indicate that other viruses were present in the plants (data not shown).

TAS- ELISA results on seven samples revealed meaningful serological difference between CMV- Zin2 isolate and the other six isolates (Table 1), indicating these antibodies have high efficiency for subgroup differentiation.

In contrast to previous reports concerning more severe symptoms induced by S-I than those of S-II (Zhang *et al.,* 1994) and also S-IB than those of S-IA (Hellwald *et al.,* 2000), our seven isolates caused symptoms of similar severity on inoculated *Nicotiana* species and capsicum, regardless of their categorization to subgroups IB, IA or S-II, which was in agreement with pervious report (Eyvazi *et al.,* 2015).

Mechanical inoculation of isolates on *V. unguiculata,* CMV-Zin2 (S-II isolate) induced pin point necrotic local lesions in contrast to large necrotic local lesions induced by other isolates (S-I strains), which was in accordance with a previous report (Palukaitis and Garcia-Arenal, 2003a).

The transmission of CMV in nature is dependent upon aphid vectors. *Myzus persicae* and *A. gossypii* are the most important and essential aphid species in the transmission, epidemiology and regional distributions of

different CMV subgroup isolates (Tian *et al.,* 2012). Based on Tian *et al.* (2012), subgroup II (AG) was transmitted more efficiently (73%) than subgroup I (32%) by *A. gossypii*. In our study, however, there were no significant differences in transmission of subgroups IA (Ka16 and Os7), IB (Bo1) and II (Zin2) by this aphid species, regardless of their categorization to subgroups. The CMV transmission efficiency depends on the variability of virus strain but also aphid vector clones. Considering the variability of CMV transmission rates for both aphid and virus sides, the prediction and modeling of virus spreading seems to be difficult to organize and are closely dependent on the variability of each protagonist-aphid and virus (Bosquee *et al.,* 2016). Studies of strains showing very poor transmission efficiency by aphids demonstrated that the CP was the sole viral determinant for vector transmission (Jacquemond, 2012). Amino acids that affect virus transmission by *A. gossypii* have been mapped to two capsid protein positions, 129 and 162 (Perry *et al.,* 1994). Pierrugues *et al.* (2007) showed that position 161 affects both virion instability and loss of aphid transmission. Coat protein amino acid alignment in our study showed that these three positions (proline, alanine and glutamine, respectively) are conserved among subgroups, including newly studied isolate in current work. On the other hand, the variability between clones of the same aphid species in the transmission ability of a particular virus has been previously reported for CMV (Simons 1959).

Sequence analysis revealed that the isolate Ka16 showed 100% identity with the Fny and Ny strains (subgroup IA), while, Os7 and Zin1 showed 99.8% and 96.4% in nucleotide identity with the Fny, respectively. Go5 and Co1 showed highest identity (93%) in nucleotide with Tfn and Nt9 strains (subgroup IB). CMV-Zin2, the only subgroup II isolate in current study showed highest identity (96.5%) in nucleotide with Q strain. Phylogenetic trees based on *CP* gene sequence revealed the presence of both subgroups I and II in ornamentals of Iran, supported by 100% bootstrap value. Strains belonging to subgroup

I, which is called heat-resistant, are more frequent than those of subgroup II in plants worldwide (Jacquemond, 2012) and subgroup IB is mainly restricted to Asia (Roossinck, 2002). In Iran, it has been reported that S-I is more prevalent than S-II in field and horticultural crops and most of the S-I strains belong to S-IA (Sokhandan Bashir *et al.,* 2006). The results of this investigation showed that six out of seven CMV isolates, collected from ornamentals of Alborz and Tehran provinces, belong to subgroup S-I, while among newly collected six S-I isolates both IA and IB isolates were equally detected. The higher incidence of S-I isolates in Iran (based on the results of this study and previous ones (Sokhandan Bashir *et al.,* 2006; Nematollahi *et al.,* 2012; Eyvazi *et al.,* 2015)) could not be attributed to their host advantage (Hayakawa *et al.,* 1989), as both S-I and S-II strains were isolated from cucumber (Sokhandan Bashir *et al.,* 2006) and *Zinnia elegans* (this study). In our study no correlation was found between phylogenetic groups, geographic location or host plant species, which is consistent with previously findings (Lin *et al.,* 2004; Nouri *et al.,* 2014).

Analysis of genetic variation among CMV subpopulations revealed that American subpopulation has the lowest, while Asian subpopulation has the highest genetic variation. Higher genetic variation in Asian CMV isolates, including Iranian ones, might be related to restriction of subgroup IB to Asia. However, higher variation among Iranian isolates in contrast with those from California (θ: 0.02494; π: 0.01489) (Lin et al. 2004) and U.S (θ: 0.028; π: 0.030) (Nouri *et al.,* 2014), which might be related to host variation of Iranian isolates, as described before by Lin *et al.* (2004). Overall, this low genetic variation in CMV subpopulations is true for most plant virus populations (Garcia-Arenal *et al.,* 2001). A founder effect has been suggested as a partial explanation potentially shaping observed genetic structure of CMV (Fraile *et al.,* 1997; Lin *et al.,* 2004). Genetic bottleneck(s) may also have contributed to this observed low genetic diversity and might have functioned to minimize the

extent of genetic variation. Genetic bottlenecks during CMV systemic movement in host plants and CMV transmission by the aphid vector(s) have previously been reported (Li and Roossinck, 2004; Ali *et al.,* 2006). However, the low F_{ST} values in all comparisons suggested a certain gene flow between geographically distant populations, but this was limited, enabling some genetic differentiation among subpopulations. An analysis of natural selection for all of the populations showed that CMV coat protein coding region in all investigated geographical regions is under the negative selection (purifying selection). This type of selection imposed on CMV encoded proteins, including CP, has been shown previously (Lin *et al.,* 2004; Moury, 2004; Liu *et al.,* 2009). Selection can be associated with various factors such as structural features of the virus, host plant and an arthropod vector. Garcia-Arenal *et al.* (2001) illustrated that negative selection predominates during evolution of plant viruses when the entire genome is assayed and that this purifying selection is principally due to the internal and external constraints.

Acknowledgments

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تمایز و تبارزائی جدایه‌های ویروس موزاییک خیار جداشده از گیاهان زینتی ایران، با تأکید بر ساختار ژنتیکی ویروس

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چکیده: ویروس موزاییک خیار (*Cucumber mosaic virus, Cucumovirus, Bromoviridae*) دارای وسیع‌ترین دامنه میزبانی در بین ویروس‌های گیاهی است. هفت جدایه ویروس از میزبان‌های مختلف گیاهان زینتی انتخاب و پس از خالص‌سازی بیولوژیکی، به روش مکانیکی بر روی گیاهان محک مایه‌زنی شدند. تفاوت سرولوژیکی جدایه‌ها براساس واکنش به ۱۱ آنتی‌بادی تک‌همسانه‌ای اختصاصی ویروس موزاییک خیار ارزیابی و پس از استخراج آر ان ا کل، ژن پروتئین پوششی جدایه‌ها تکثیر شد. براساس تحلیل‌های بیولوژیکی، سرولوژیکی و تبارزائی، فقط یک جدایه متعلق به زیرگروه II و شش جدایه‌ی دیگر به‌طور مساوی در دو زیر گروه IA و IB قرار گرفتند. ارزیابی انتقال جدایه‌ها با شته *Aphis gossypii* نشان داد که اختلاف معنی‌داری بین کارآیی انتقال جدایه‌های متعلق به زیرگروه‌های IA، IB و II وجود نداشت. مطالعه تنوع ژنتیکی و تکاملی CMV در ایران براساس ژن پروتئین پوششی و مقایسه آن با توالی‌های متناظر جدایه‌های سایر قاره‌ها نشان از تنوع ژنتیکی کم و ارتباط تکاملی نزدیک بین جدایه‌ها در جمعیت‌ها داشت. تحلیل شاخص‌های تنوع ژنتیکی جمعیت و توزیع جهش‌های هم‌نام و غیرهم‌نام نشان داد که اغلب جایگاه‌های آمینواسیدی تحت انتخاب منفی قرار داشته و تنها یک جایگاه دارای انتخاب مثبت بود.

واژگان کلیدی: ژنتیک جمعیت، ساختار ژنتیکی، کارآیی انتقال، گیاهان زینتی، ویروس موزاییک خیار