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Identification of viruses infecting cucurbits and determination of genetic diversity of *Cucumber mosaic virus* in Lorestan province, Iran

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Abstract

Various viral pathogens infect Cucurbitaceae and cause economic losses. The aim of the present study was to detect plant viral pathogens including *Cucumber mosaic virus* (CMV), *Cucumber green mottle mosaic virus* (CGMMV), *Zucchini yellow mosaic virus* (ZYMV), *Cucurbit yellow stunting disorder virus* (CYSDV) and *Cucurbit chlorotic yellows virus* (CCYV) in Lorestan province, in western Iran, and also to determine CMV genetic diversity in Iranian populations. A total of 569 symptomatic leaf samples were collected in 2013 and 2014 from cucurbits growing regions in Lorestan province. The collected samples were assessed for viral diseases by ELISA. The results showed virus incidences in most regions. Then, the infection of 40 samples to CMV was confirmed by RT-PCR. Moreover, to distinguish between the two groups (I and II) of CMV, PCR products were digested by two restriction enzymes *Xho*I and *Eco*RI. Results of the digestion showed that the isolates of Lorestan belonged to group I. The CMV-coat protein gene of eight isolates from different regions and hosts was sequenced and phylogenetic analysis was performed. Subsequent analyses showed even more genetic variation among Lorestan isolates. The phylogenetic tree revealed that Lorestan province isolates belonged to two IA and IB subgroups and could be classified together with East Azerbaijan province isolates. The results of the present study indicate a wide distribution of CMV, ZYMV, CGMMV, CYSDV and CCYV viruses in cucurbits fields of Lorestan province and for the first time subgroup IB of CMV was reported on melon from Iran.

Key words: CMV subgroup, genetic diversity, RFLP

Introduction

The members of the Cucurbitaceae family are among the most important agricultural crops grown in tropical and subtropical regions. The main species of cultivated cucurbits are cucumber (*Cucumis sativus*), melon (*C. melo*), watermelon (*Citrullus lanatus*), and different squash species (*Cucurbita pepo*, *C. moschata*, and *C. maxima*) (Robinson and Decker-Walters 1997). Cucurbits are grown throughout Lorestan province of Iran. Around 35 viruses have been reported on the Cucurbitaceae worldwide (Provvidenti 1996). Some of these viruses which include: *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV), *Cucumber green mottle mosaic virus* (CGMMV), *Cucumber*

yellow stunting disorder virus (CYSDV) and *Cucurbit chlorotic yellows virus* (CCYV) have been reported from field-grown cucurbit crops in Iran (Rahimian and Izadpanah 1977; Rahimian and Izadpanah 1978; Ghorbani 1988; Keshavarz and Izadpanah 2005; Bananej *et al*. 2013; Keshavarz *et al*. 2013a).

ZYMV is a dangerous virus and it is widely reported in more than 50 countries (Desbiez and Lecoq 1997). CGMMV causes the appearance of mosaic and green or white spots on the leaves and fruit as well as damage to the quality and quantity of fruit. The watermelon strain of CGMMV causes severe disease symptoms in plants, particularly the decline of fruit which results in

economic losses to watermelon growers (Shim *et al.* 2005). CYSDV is a destructive cucurbit virus in large areas of the world that reduces the yield from 30 to 50 percent (Louro *et al.* 2000). This virus has spread in recent years, and has been found in many regions of Iran (Keshavarz *et al.* 2013b). CCYV is a new white-fly-transmitted crinivirus which causes serious harm to melon and cucumber crops. It causes yield losses in cucumber and a considerable decrease in the sugar content of melons (Gyoutoku *et al.* 2009).

CMV is one of the members of the genus *Cucumovirus* in the family Bromoviridae. CMV has three single-stranded plus-sense RNAs, that code for five functional proteins [1a, 2a, 2b, 3a and coat protein (CP)]. Proteins 1a and 2a, encoded by RNA1 and RNA2, respectively, are essential for replication. Protein 2b is expressed from subgenomic RNA 4A, that is derived from RNA2 (Soards *et al.* 2002). The RNA3 cods for two proteins, movement protein (MP) and coat protein. The CP is translated from the subgenomic RNA4 and is required for encapsidation, systemic infection or long distance movement, aphid transmission and host range (Boccard and Baulcombe 1993). According to serology, nucleic acid hybridization and restriction fragment length polymorphism (RFLP), CMV isolates have been divided into two main groups I and II (Palukaitis and García-Arenal 2003). The members of group I based on phylogenetic analysis have been divided into two subgroups IA and IB. CMV has a broad host range in 85 separate families of dicotyledons and monocotyledons. The virus is transmitted by 80 species of aphids in a non-persistent and non-circulative manner (Palukaitis and García-Arenal 2003).

Although the five mentioned cucurbit viruses have been reported in various regions of Iran no comprehensive study on the existence of these viruses in the western region of Iran has been carried out. Despite extensive cultivation of cucurbits in Lorestan province of Iran, surveys for viral infections and differentiation of viral isolates have not been done. This report summarizes the findings of a survey made during two years (2013 and 2014), in order to identify the viruses infecting cucurbit crops (squash, cucumber and melon) in Lorestan province. Since CMV has been reported in many areas of the country, another objective of this study was to determine CMV subgroups and genetic diversity. The results of this study would be useful for the management of cucurbit virus diseases.

Materials and Methods

Surveys and sample collection

Different cucurbit fields in six regions were visited on several occasions in the springs and summers of 2013 and 2014. During these surveys 569 symptomatic leaf samples of cucumber, squash and pumpkin were collected from several locations in Khorramabad, Poledokhtar, Doureye-e-chegini, Alashtar, Koohdasht and Broujerd.

ELISA

The infectivity of all collected samples were examined by indirect-ELISA (Converse and Martin 1999) using specific polyclonal antisera against CMV, ZYMV, CGMMV, CYSDV and CCYV. The CMV, CYSDV and CCYV (1 : 300) antisera were obtained from the Plant Virology Research Center (PVRC), Shiraz, Iran. ZYMV (1 : 5,000) and CGMMV (1 : 3,000) antisera were obtained from DSMZ-Germany. Goat anti-rabbit-IgG alkaline phosphatase conjugate (Promega, USA) was used as a secondary antibody at a 1 : 7,500 dilution. Absorbance was measured using an ELISA reader (Anthos 2.020) at 405 nm. Samples were considered positive if their absorbance was equal to or greater than twice that of healthy control mean values. Each sample was tested in duplicate.

RNA extraction, RT-PCR and sequencing

Total RNA extraction of the 40 ELISA-positive samples to CMV antibody was performed using the RNX-Plus kit (CinnaGen, Tehran, Iran) according to the manufacturer's instructions.

The RT-PCR was performed using CMVCPf (5'--GCTTCTCCGCGAG-3') and CMVCPr (5'-GCCGTA-AGCTGGATGGAC-3') primer pair corresponding to the CP region of CMV (Davies and Symons 1988). The reverse transcription was performed using the hyper-Script RT premix kit as described by the manufacturer (GeneAll, South Korea). Approximately 300 ng of total RNA was subjected to hyperScript RT premix for 1 h at 42°C in a 20 µl reaction mixture with 20 pmol of the reverse primer. PCR was performed in a 25 µl reaction mixture containing 10 µl of the reverse transcribed mixture, 2.5 µl of 10X PCR buffer, 2 mM $MgCl_2$, 2.5 pmol CMVCPr and 1.25 Units *Taq* DNA polymerase (Fermentas, Lithuania). The RT-PCR reaction was carried out in a SensQuest thermocycler (Germany). After an initial denaturation step at 94°C for 4 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 45 s and extension at 72°C for 1 min, followed by a final polymerization at 72°C for 5 min were performed. The PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide (0.5 µg · ml⁻¹) to determine the size of the amplicons. Gel electrophoresis of RT-PCR products revealed an expected size amplicon of approximately 866 bp. PCR products were gel excised, purified using (GeneAll, South Korea) and sequenced in both directions (Macrogen Inc, Seoul,

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Table 1. Selected isolates abbreviations, accession numbers and origins of *Cucumber mosaic virus* from Iran and isolates from other countries were obtained from the GenBank database

Isolates	Accession numbers	Abbreviation	Host	Isolates	Accession numbers	Abbreviation	Host
TEH_A	JX865605	ToV29	*Lycopercicon esculentum*	**LOR_H**	KT279572	LOR_H	*C. sativus*
TEH_B	JX865604	ToV11	*L. esculentum*	**IND_A**	EU573928	Lucknow-SKR	*Gladiolus* sp.
TEH_C	JX86560	ToV2	*L. esculentum*	**IND_B**	AY754359	Kerala	*Vanilla planifolia*
TEH_D	JX865598	CuV	*Cucumis sativus*	**IND_C**	GU253913	–	*Ricinus communis*
TEH_E	JX865597	CuV4	*C. sativus*	**IND_D**	AJ890465	–	*Lilium tigrinum*
TEH_F	JX865594	ChV35	*Chenopodium* sp.	**IND_E**	AJ890464	–	*Oriental lily*
AZB-A	AY871069	S337	*C. sativus*	**IND_F**	LN649264	Palampur	–
AZB-B	JX025994	Jol186	muskmelon	**JAP_A**	AF103991	Pepo	–
AZB-C	JX025997	Mgh91	muskmelon	**JAP_B**	AB004781	D8	*Japanese radish*
AZB-D	JX025999	Ajs4	*L. esculentum*	**JAP_C**	D28487	FT	*L. esculentum esculentum*
AZB-E	JX025991	Bon94	*Cucurbita* sp.	**JAP_D**	D28489	cs	*Limonium sinuatum*
AZB-F	JX025990	Zdj31	*C. sativus*	**JAP_E**	D28486	N	*L. sinuatum*
Kho_A	JX865596	CuKh54	*C. sativus*	**JAP_F**	AB049568	HL	–
Kho_B	JX865595	CuKh16	*C. melo*	**KOR_A**	L36251	KOR	–
Kho_C	KC122254	ToKR1	*L. esculentum*	**KOR_B**	AJ276481	MF	–
Kho_D	KC122255	ToKR2	*L. esculentum*	**KOR_C**	KC527770	RP46	*Capsicum annuum*
Kho_E	KC122256	ToKR3	*L. esculentum*	**KOR_D**	KC527769	RP45	*C. annuum*
Kho_F	KC122257	ToKR4	*L. esculentum*	**KOR_E**	L36525	ABI	Gladiolus
ESF_A	KF873620	Cuk	*C. sativus*	**KOR_F**	AF013291	AS	–
ESF_B	KF873618	Cud	*C. sativus*	**SPA_A**	AJ829769	MAD99/2	melon
ESF_C	KF873617	Csu	*Cucurbita pepo*	**SPA_B**	AJ829770	MAD99/4	*C. pepo*
ESF_D	KF873615	Cues	*C. sativus*	**SPA_C**	AJ829771	MAD96/3	melon
ESF_E	EF620777	Ld	*C. sativus*	**SPA_D**	AJ829772	MAD93/1	melon
ESF_ E	KF873616	Kryz	*Vigna radiata*	**SPA_E**	AJ829773	MAD01/3	*Diplotaxis erucoides*
LOR_A	KT279565	LOR_A	*C. sativus*	**SPA_F**	AJ829774	MAD96/2	melon
LOR_B	KT279566	LOR_B	*C. sativus*	**USA_A**	JF918967	N1-04	*Vinca minor*
LOR_C	KT279567	LOR_C	*C. sativus*	**USA_B**	JF918964	N1-05	*V. minor*
LOR_D	KT279568	LOR D	*C. sativus*	**USA_C**	JF918966	N1-03	*V. minor*
LOR_E	KT279569	LOR_E	*C. sativus*	**USA_D**	GU362669	–	*Mertensia virginica*
LOR_F	KT279570	LOR_F	*C. melo*	**USA_E**	KJ486271	CM3	*Mazus reptans*
LOR_G	KT279571	LOR_G	*Cucurbita* sp.	**USA_F**	AF523351	CK54	–

South Korea) using an ABI3700 DN sequencer. The accession numbers of the CP sequence of CMV deposited in GenBank database and the other CP sequences obtained from GenBank are shown in table 1.

In silico restriction enzyme analysis

In silico digestions were performed with the pDRAW32 software, which is useful for generation of virtual agarose gels, predicting a restriction pattern of a sequence (Kield 2006). The sequence of nine isolates chosen for restriction enzyme digestion pattern comparisons and *in silico* restriction fragment length polymorphism (RFLP) were exported to the pDRAW32 program.

For validation and confirmation of the *in silico* predicted pattern of restriction enzyme analysis, the RT-PCR amplified fragments were digested by two enzymes: *Xho*I and *EcoR*I (Fermentas Inc., Lithuania). The digestions of 38 samples were performed according to the manufacturer's instructions. Approximately 100 ng of each PCR product was digested in 5 units of enzyme and 20 μl of 1X restriction buffer containing

100 ng · ml⁻¹ bovine serum albumin (BSA). The reactions were allowed to take place at 37°C for 2–3 h, and then analyzed on a 1.5% agarose gel.

Sequence alignment, estimation of population genetic parameters

Multiple nucleotide sequence alignments were performed using CLUSTALW implemented in MEGA version 5 software (Tamura *et al*. 2011). Aligned CMV sequences were assessed using DnaSP version 5.1 (Rozas *et al*. 2003) and GenAlEx software (Peakall and Smouse 2006). The number of segregation sites (S), Watterson's estimator of θ_w (Watterson 1975), and Pi (the average number of nucleotide differences between two random sequences in a population) (Tajima 1983) which is also called genetic diversity were estimated. Haplotype diversity (Hd) was also used to assess selection pressure imposed upon the CP region of the 62 sequence of CMV isolates (Table 1), non-synonymous [Pi(a), amino-acid altering] and synonymous [Pi(s), silent] substitution rates and their associated ratios Pi(s)/Pi(a) were estimated.

Recombination analysis

To detect putative recombinants between the 32 sequences of the Iranian CMV isolates (Table 1), they were aligned and analyzed using RDP3 (Martin *et al*. 2010). A total of 7 recombination detection methods including RDP, Bootscan, GENECONV, MaxChi, Chimaera, Siscan and 3SEQ were performed.

Phylogenetic analysis

For reconstructing molecular phylogenetic relationships, the CP gene sequence of each isolate was compared with those of the other isolates available in GenBank using the BLAST homology search program. The selected DNA sequences were aligned by Clustal X2 (http://www.clustal.org//) using the default parameters. The aligned data sets were manually edited in MEGA 5. The model of base substitution was selected using MrModeltest 2 (Posada and Crandall 1998). The Akaike-supported model, a general time reversible model, including among-site rate heterogeneity (GTR+G) was used in phylogenetic analyses. Bayesian analysis was performed using MrBayes v3.1.2 running the chains for one million generations (Huelsenbeck and Ronquist 2001). After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The Markov Chain Monte Carlo (MCMC) method within a Bayesian framework was used to estimate the posterior probabilities of the phylogenetic trees using the 50% majority rule (Larget and Simon 1999). For phylogenetic analysis of the CP gene, the isolate CMV-Q (accession number M21464) was used as the out-group.

Results

Incidence and detection of viruses in cucurbit samples

An analysis of the 352 collected samples in 2013 showed that 30.11% (106 out of 352), 8% (24 out of 300), and 11% (33 out of 300) of the samples were infected with CMV, ZYMV and CGMMV, respectively. Additionally, 17.92% of the samples (19 out of 106) reacted to the CYSDV-CCYV antisera. However, monitoring of samples in 2014 showed that 22.11% (48 out of 217), 16.4% (17 out of 104), and 9.2% (20 out of 217) of the samples were infected with CMV, ZYMV and CG-MMV, respectively. Additionally, 7.6% of the samples (8 out of 104) reacted to the CYSDV-CCYV antisera. Also 43.07%, 27.5%, 26.13%, 12.5%, 24.44%, 15.94% of the total samples from Khorramabad, Poledokhtar, Doureye-e-chegini, Koohdasht, Borujerd and Alashtar locations in Lorestan province were infected with CMV, respectively.

Mixed infections

Part of the samples were infected with more than one virus. The following percentages, 3.58% (7 out of 195), 2.89% (6 out of 207), 2.2% (4 out of 181), 2.12% (2 out of 94), 1.47% (1 out of 68) and 2.5% (2 out of 80) were simultaneously infected with CMV and ZYMV, CMV and CGMMV, CMV and SYSDV or CCYV, ZYMV and CGMMV, ZYMV and CYSDV or CCYV and CGMMV and SYSDV or CCYV, respectively. Also 1.2% (3 out of 248) of the samples were infected with CMV, ZYMV and CGMMV.

RT-PCR and RFLP analysis

The extracted RNA of the ELISA-positive samples to CMV antibody resulted in a clear amplicon of approximately 866 bp using reverse transcription-polymerase chain reaction with the CMVCPf/CMVCPr primer pair. The obtained RFLP pattern of virtual digestion with *Xho*I and *Eco*RI of the CP sequences of CMV clearly differentiated the isolates into two distinct groups. Virtual digestion showed that *Xho*I can potentially separate the CMV samples into two groups, I and II. The *Xho*I restriction enzyme divided the subgroups IA and IB into two fragments of 664 and 202 bp whereas none of the members of group II were digested. Therefore, *Xho*I was considered an efficient and suitable enzyme for differentiation of CMV samples into groups I and II. Furthermore, the *Eco*RI enzyme
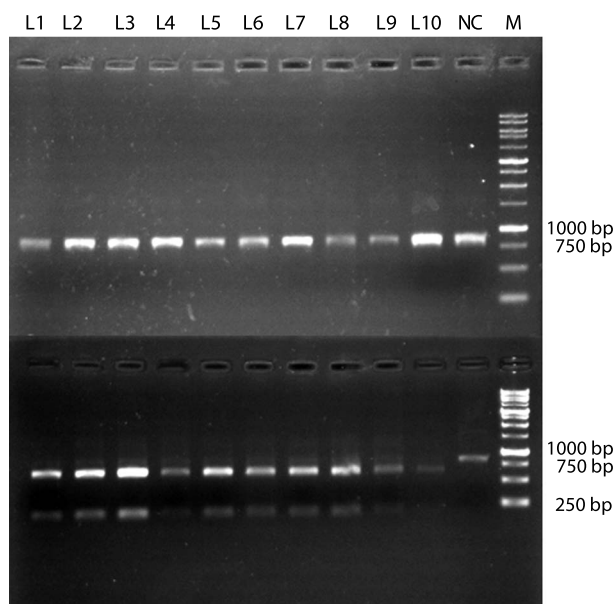
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Fig. 1. Electrophoretic pattern of the virus coat protein gene of *Cucumber mosaic virus* – treated cutting restriction enzymes *Eco*RI (top) and *Xho*I (bottom); L1 to L10 – samples; NC – samples that have not been digested by enzyme; M – GeneRuler™ 1 kb DNA ladder, Fermentas

did not digest subgroups IA and IB whereas it digested members of group II into two parts 704 and 162 bp. Therefore, enzymes *Eco*RI and *Xho*I confirmed each other's results.

The digestion pattern of our amplified 38 samples showed that they were digested only by the *Xho*I enzyme, but none were cut by enzyme *Eco*RI (Fig. 1), so according to the results mentioned it was demonstrated that our samples belonged to group I of CMV. For the first time in the present study, we used the *Xho*I enzyme for differentiation of CMV samples and we introduced this enzyme as a potential separator of CMV member groups I and II.

Recombination analysis

The results obtained in the present study indicated that some of the examined strains showed evidence of recombination. However, since the recombination in some strains was supported by less than four RDP3 programs and their p-value was less than 1.0×10^{-6}, they therefore were considered as 'tentative' recombinants.

Phylogenetic analysis

The isolates of LORA, LORB, LORD, LORE and LORH were placed in a separate group adjacent to the isolates of subgroup IA. LORA, LORB, LORD, and LORH isolates were placed near Iranian isolates S337 and Jol186 that were respectively reported in Shabestar and Julfa regions, East Azerbaijan province. The LORE isolate

was placed adjacent to Mgh91 isolate from Maragheh. It is therefore concluded that Lorestan province isolates were phylogenetically close to East Azerbaijan province isolates. In previous studies, it has been demonstrated that Jol186, S337, E5 and Mgh91 isolates were members of subgroup IA (Bashir *et al.* 2006; Nematollahi *et al.* 2012). It is evident that LORA, LORB, LORD, LORE and LORH isolates of Lorestan province can be placed in subgroup IA, while other isolates, including LARC, LORF and LORG belong to a separate group adjacent to subgroup IB isolates. These isolates were more closely related to Iranian Esf172 and Khn1, East Azerbaijan province isolates (Nematollahi *et al.* 2012). As a result, LORA, LORB, LORD, LORE and LORH isolates of Lorestan province could be placed in subgroup IA (Fig. 2). The sequences reported in this paper were submitted to GenBank and are presented in Table 1.

Genetic diversity of selected CMV isolates

The population genetic parameters shown in Table 2, were estimated and analyzed. This analysis includes π, the average pairwise nucleotide difference per site, and θw, the mutation rate from segregating number. These estimators were used as two indicators of genetic diversity for the CP region. Accordingly, Lorestan and Tehran provinces showed the highest and lowest genetic diversity, respectively. Based on the two parameters Wθ (0.035) and π (0.043), the genetic diversity of CMV in Lorestan province was the highest. Also according to the same parameters (Wθ and π), Tehran province isolates showed the lowest variation.

Genetic parameters between populations of CMV

Calculation of genetic variation among CMV populations determined with GenAlEx software revealed that Lorestan and Tehran provinces had the highest and the lowest values of the parameters of population genetics, respectively. Shannon's (Brown and Weir 1983) information index and diversity of Nei (Nei 1978) were 0.198 and 0.135, respectively for Lorestan, Shannon and Nei parameters were 0.014 and 0.010 for Tehran province. Polymorphism (32.8) amount in Lorestan province was more than other regions of Iran. Generally, the India CMV population (50.54) had the highest rank in polymorphism among the assessed populations.

Variations between populations of CMV

Based on the analysis of molecular and population variations calculated with GenAlEx software, 186 characters were changed in CMV isolates. Seventy per cent of the variations occurred within a population, and 30%
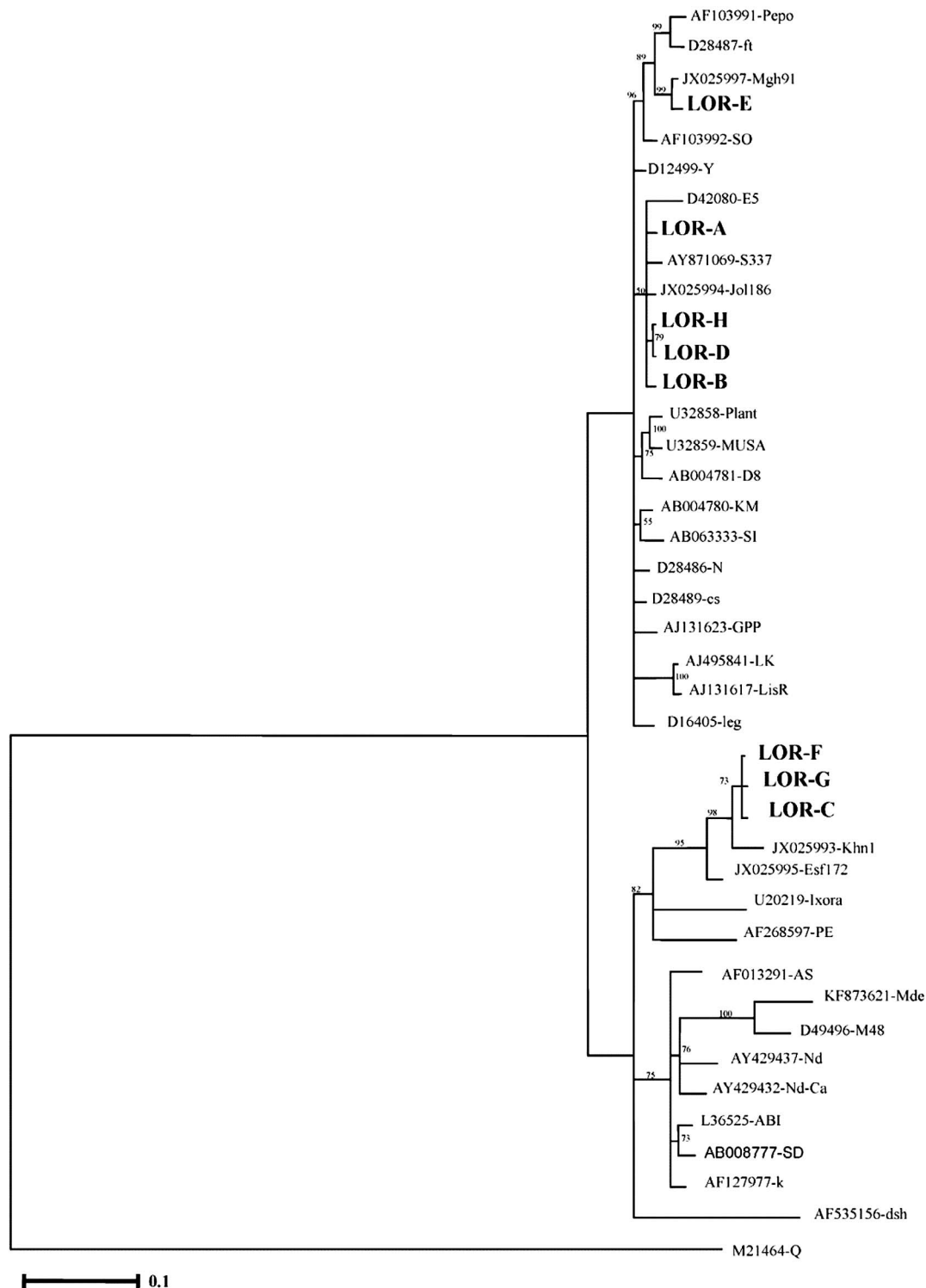
Fig. 2. Bayesian phylogenetic trees of *Cucumber mosaic virus* isolates based on CP gene including field collected (n = 8) and from other regions of the world. The sequence of CMV-Q isolate (Acc. no. M21464) was used as out group. Isolates LORA, LORB, LORD, LORE and LORH in subgroup IA and Isolates LARC, LORF and LORG were placed in IB subgroup

occurred between populations. These values indicate that within a population variation is greater than between variations.

Then, using NTSYSpc software (Rohlf 1998) and by algorithm UPGMA, dendrogram dissimilarity was drawn. The dendrogram revealed that the population of Lorestan province was close to the population from East Azerbaijan province. Also isolates of Isfahan, Tehran and Khorasan provinces were located near each other according to the dendrogram (Fig. 2).

Selection pressure on the CP region

The obtained results showed that the ratio of Pi(a)/Pi(s) for the CP region of CMV isolates was less than one (Table 2). In regions of the genome where the number

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Table 2. Population parameters estimated based on the coat protein gene of CMV

Population	No. of sequences	No. of sites	S[a]	Θ_w[b]	Pi[c]	Hd[d]	Pi(a)[e]	Pi(s)[f]	Pi(a)/Pi(s)
Lorestan	8	657	61	0.03581±(0.01589)	0.04376±(0.00672)	0.964±(0.077)	0.01160	0.14899	0.078
Khorasan	6	657	11	0.00737±(0.00397)	0.00866±(0.00158)	0.933±(0.122)	0.00794	0.01108	0.717
Tehran	6	657	4	0.00267±(0.00172)	0.00345±(0.00055)	0.933±(0.122)	0.00236	0.00670	0.352
Isfahan	6	657	38	0.02533±(0.01240)	0.02192±(0.00506)	1.000±(0.096)	0.01726	0.03734	0.462
East Azerbaijan	6	657	57	0.03800±(0.00503)	0.03907±(0.00927)	1.000±(0.096)	0.01033	0.12895	0.080
Japan	6	657	50	0.03333±(0.01615)	0.03115±(0.00450)	1.000±(0.096)	0.01068	0.09637	0.111
Korea	6	657	69	0.04600±(0.00554)	0.04820±(0.00850)	1.000±(0.096)	0.01720	0.15071	0.114
India	6	657	93	0.06209±(0.02962)	0.06362±(0.01314)	1.000±(0.096)	0.01550	0.13812	0.112
USA	6	657	86	0.05733±(0.02739)	0.05530±(0.01546)	1.000±(0.096)	0.02179	0.17110	0.127
Spain	6	657	10	0.00667±(0.00211)	0.00578±(0.00118)	1.000±(0.096)	0.00069	0.01705	0.041

[a] number of segregating sites
[b] the mutation rate from segregating number
[c] the average number of nucleotide differences between two random sequences in a population
[d] haplotype diversity
[e] the average number of non-synonymous substitutions per non-synonymous sites
[f] the average number of synonymous substitutions per synonymous sites

of synonymous substitutions was more than non-synonymous substitutions, the type of mutations were neutral and amino acid did not change (purifying selection).

Discussion

Investigation of incidence of CMV, ZYMV, CGMMV, CYSDV and CCYV in cucurbit growing regions of Lorestan province by ELISA showed that cucurbit plants were infected with all five tested viruses. The obtained results of the present study agree with other reports from Iran which showed that these viruses were present in most growing cucurbit areas of Iran (Bananej and Vahdat 2008). The symptoms of the virus are hidden in hot weather. Therefore spring and autumn, when it is cool, are suitable seasons for sampling. In the spring, the symptoms are easily observable (Valkonen *et al.* 1992).

Determination of the CMV subgroup is important for epidemiological studies and the determination of genetic diversity is an effective step in virus management (Lin *et al.* 2003). In this study, CMV subgroups were determined by RFLF and nucleotide sequencing. Therefore, *Xho*I and *Eco*RI were applied to diagnose subgroups. In *in silico* analysis, we found that group I (IA and IB) was digested by *Xho*I restriction enzyme and resulted in two fragments of 664 and 202 bp, while it was not digested by *Eco*RI. In contrast, members of group II were digested into two fragments, 704 and 162 bp, by *Eco*RI. Therefore, these two enzymes were able to distinguish the two groups I (IA and IB) and II

of CMV. According to virtual digestion, all isolates of Lorestan province were digested into two fragments of 664 and 202 bp by the enzyme *Xho*I, but were not cut by the enzyme *Eco*RI and revealed that the isolates of Lorestan province belonged to subgroup I. We recommend the two enzymes, *Eco*RI and *Xho*I for diagnosis of subgroups and confirmed it by our experimental results. Use of these enzymes is a fast procedure for distinguishing CMV subgroups.

Considering the overall results of the experimental and enzymatic digestion, one can conclude that all investigated CMV isolates belonged to group I, and that group II was not detected in cucurbit growing regions in Lorestan province. In general, the group I isolates of CMV are more dispersed than isolates of group II (Crescenzi *et al.* 1993). It has been shown that 80% of the known isolates belong to subgroup I and the frequency of subgroup IA is more than that of IB (Gallitelli 2000). Group II is more common in cold climates (Roossinck 2002) and since Iran is a subtropical country, it was expected that more Iranian isolates would belong to group I, although group II has been reported from Iran (Bashir *et al.* 2006; Sokhandan *et al.* 2007; Rasoulpour and Izadpanah 2008; Nematollahi *et al.* 2012). In our research, we detected IB subgroup strains from melon in Iran for the first time.

Phylogenetic study showed that five isolates (LORA, LORB, LORD, LORE and LORH) were more closely related to isolates Mgh91, S337, E5 and Jol186. Mgh91 and Jol186 isolates were placed in subgroup IA by Nematollahi *et al.* (2012) in their study to assess CP and MP genes, and draw a phylogenetic tree. LORA, LORB, LORD, LORE and LORH were placed in

subgroup IA close to S337 and E5 isolates (Bashir *et al.* 2006). Khn1 and Esf172 isolates from East Azerbaijan province of Iran, by assessing CP and MP genes, were placed in subgroup IB and LARC, LORF and LORG isolates in our research were grouped with Khn1 and Esf172 isolates (Nematollahi *et al.* 2012). According to the phylogenetic analysis it could be concluded that isolates from Lorestan province (in western Iran) and East Azerbaijan province (in northwestern Iran) have a high phylogenetic similarity and may have the same origin.

Also, the results of the present study showed that there were more IA subgroups than IB. Isolates of subgroup IB are more common in East Asian countries such as India, China, Philippines and Indonesia (Pratap *et al.* 2012). This indicates that these isolates are more likely to have originated from East Asia.

The observed variation in Lorestan isolates is not unexpected since the sequenced samples were collected from different regions of Lorestan province where there are very different climates due to its vast geographical expanse. Poldokhtar, in the south of Lorestan, has a tropical climate, with a mean annual temperature of 29.8°C. Boroujerd, located in the northern part of the province, has cooler weather with a mean annual temperature of 21.1°C (Anonymous 2011). Tehran samples were collected from areas with similar climate and most likely the Tehran isolates were derived from one source. Calculation of genetic variation among populations by GenAlEx software, revealed that climatic variation and distance between sampling regions could explain the results. Therefore, it would be expected that the genetic variation in Lorestan would be significantly higher than in other provinces.

CMV has a wide host range and different strains of CMV have been reported from over 1,300 host plants belonging to 500 genera (Palukaitis and García-Arenal 2003). The virus diversity cannot be attributed to the effects of hosting as has been demonstrated in previous research from USA and China (Liu *et al.* 2009; Nouri *et al.* 2014). Our results also showed that host differences cannot be attributed to the genetic diversity.

Since observed variation among Iranian CMV population was 30%, it could be concluded that genetic differences of populations reflect their geographic origin. The differentiated population group in this study belonged to a particular geographic area. According to molecular variance and population variations in CMV, it is probable that the observed variation among populations is due to a combination of some processes similar to mutation, recombination and assortment of genome segments.

The ratio values determined for the CP region by DnaSP software showed that the CP region has purifying selection. In other words, the below one Pi(a)/Pi(s) ratio indicates negative selection to preserve the genome of this region and the nature of the proteins encoded by CP gene is preserved and cannot be changed. Negative selection can reduce genetic variation in a population of viruses and increase the maintenance of coat protein stability. Negative selection of CMV coat protein abundance has been reported in previous studies (Liu *et al.* 2009; Nouri *et al.* 2014).

According to the dendrogram of dissimilarity matrix, isolates of Isfahan, Tehran and Khorasan were located in the same cluster. Based on the dendrogram, these isolates have genetic similarity and belong to subgroup IA. Therefore high genetic correlation is reflected among these isolates. Also according to the position of isolates on the dendrogram, it is implied that the population of East Azerbaijan and Lorestan provinces were correlated to East Asian countries such as Korea, Japan and India. Since the subgroup IB isolates are more frequent in East Asia and subgroup IB was detected in two provinces (East Azerbaijan and Lorestan), these results are expected and most likely represent the common origin of the isolates.

In conclusion, the present study showed a wide distribution of CMV, ZYMV, CGMMV, CYSDV and CCYV in cucurbits fields of Lorestan province and genetic diversity of CMV in Iran for the first time. In addition, this is the first report of subgroup IB on melon from Iran. Finally, considering the spread of CMV in Iran, in order to better understand the population genetic structure, the genome of CMV isolates from other regions of Iran should be sequenced.

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