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Original Article

An Investigation on characterization of cucumber mosaic virus isolated from lily green house in Damavand County, Iran

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Abstract

Background and Aims: Virus infections represent some of the most important diseases of lily, plants because of the devastating effects caused to the crops and the absence of effective treatments. A survey for virus diseases of lilies, revealed the occurrence of *Cucumber mosaic virus* (CMV) in plants growing in Tehran province, Iran.

Materials and Methods: During 2013, 50 lily samples with virus-like symptoms were collected and tested by ELISA. The presence of the CMV was confirmed by biological assay and RT-PCR tests. Phylogenetic structure, statistical tests of neutrality and genetic differentiation and the gene flow level between populations were evaluated using 2b gene.

Results: Thirty seven (74%) out of 50 lily samples were infected with CMV. The host rang of lily isolates was limited to *Nicotiana benthamiana*, which confirmed by ELISA and RT-PCR. The complete 2b gene nucleotide sequence of lily isolates were 243 nt long. The highest nucleotide identity (99%) was indicated with South Korean LiCB isolate (Ac. no. AB506799). Phylogenetic analysis using 2b gene showed two main groups which, lily isolates were classified into a separate branch in group I. Using the maximum likelihood method, amino acid 55 S in the 2b protein of the CMV isolates in group I was found to be under positive selection.

Conclusion: CMV has been previously reported from Iran but, the association of CMV in lily plants represents the first record from Iran. Our information will help to better understand epidemiology and to develop a successful management program for reducing the impact of this disease.

Keywords: Lily, CMV, RT-PCR, 2b gene

Introduction

Lily (Lilium spp.) belong to Liliaceae family, is ranked within the top ten flowers in the export market, and one of the most productive and commercially valuable ornamental plants with high economic value (1). There are 80 to 100 species of lilies, with more than 4500 cultivated varieties, and most are native to the Northern Hemisphere in Asia, Europe and North America. The three commercially important divisions of lily include Easter lily (Lilum longiflorum), Asiatic hybrids and Oriental hybrids (2). Virus infections represent some of the most important diseases of lily, because of the devastating effects caused to the crops and the absence of effective treatments (3). Cucumber mosaic virus (CMV), the type member of the genus Cucumovirus in the family

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Bromoviridae, is one of the most and commonly occurring viruses of lily. It has been reported in many Asia, Europe countries, and United States (3). The CMV genome contains five genes, expressed from the three genomic RNAs and two subgenomic RNAs. The 1a and 2a are involved in virus replication, whereas the 2b protein is an RNA silencing suppressor, an antagonist of other host defense mechanisms and a viral recombination effectors protein. The 3a and coat protein (3b) are essential for both cell-to-cell and long-distance movement, processes affected by all of the CMV-encoded proteins (4, 5). The RNA3 of most CMV lily isolates have been sequenced and had extremely high sequence similarity to one another (6, 7, 8, 9). Based on their nucleic acid sequence similarity, CMV strains can be divided broadly into two major subgroups, designated I and II, with subgroup I strains divided into two (A and B). Generally, there are no clear differences in the host range of isolates belonging to those groups, and most CMV strains can easily be transmitted into numerous herbaceous plants. The exception was a number of CMV isolates found in lily plants (3, 10). The isolates from lily can infect only a small number of host plant species because of a specific adaptation to lily plants. Most of them were not able to infect many species of tobacco and cucurbits and they induced mild systemic symptoms on other herbaceous test plants (6, 8, 11). During 2013, viral-like symptoms were observed in lily plants, in Damavand County in Tehran province, Iran. Infected plants showed mottling, mosaic, color breaking and leaf deformation (Fig. 1). The possible infection of lily samples with CMV was checked using biological and molecular analysis.

Methods

Collection of lily samples

Damavand County is one of the main lily cut flower production areas in Tehran province, Iran. A survey was conducted in lily greenhouses in Damavand and totally 50 leaf samples with virus-like symptoms including mosaic, mottling, leaf deformation, chlorosis, color breaking, and stunting were collected from their own cultivated bulbs (Fig. 1) .

Serological virus identification

Leaf samples tested for CMV infection using double antibody sandwich (DAS)-ELISA (12). ELISA plates (Nunc Maxisorb, denmark) were coated with 100 µl of the recommended dilution of IgG in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 5 mM NaN_3, pH 9.6) and incubated overnight at 4°C. Samples were extracted (1:5 wt/vol) in phosphate buffered saline (PBS) (2.7 mM KCl, 5 mM NaN_3, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, and 0.13 M NaCl) containing 2% polyvinylpyrrolidone (PVP)-24,000 and 0.05% Tween 20, pH 7.4. Plates were washed four times at 5-min intervals with washing buffer (PBS containing 0.05% Tween 20), and 100 µl of plant extract was added to each well and incubated overnight at 4°C. After washing the plates, 100 µl of alkaline phosphatase-conjugated IgG diluted in conjugate buffer (PBS, pH 7.4, containing 2% PVP-24,000, 0.05% Tween 20, 0.2% bovine serum albumin, and 1 mM $MgCl_2$) was added and incubated 3 h at 37°C. Wells were washed and incubated with 100 µl of substrate (9.7% diethanolamine, pH 9.8, containing 1 mg/ml of p-nitrophenyl phosphate and 5 mM NaN_3) for 1 h at room temperature, and the absorbance was determined at 405 nm using an ELISAreader (Multiscan-334, Labsystems, Finland). A sample was considered positive (infected) if the absorbance at 405 nm was greater than or equal to three times the average value of negative (healthy) samples .

Virus isolates and host tests

Leaf tissue extracts from the ELISA positive samples were used to mechanically inoculate appropriate herbaceous indicator plants. Plant species used were Gomphrena globosa (Asteraceae), *Chenopodium amaranticolor* and *Ch. quinoa* (Chenopodiaceae), *Phaseolus vulgaris* cv. Bountiful and *Vigna unguiculata* (Fabaceae), *Datura metel*, *D. stramonium*, *Nicotiana benthamiana*, *N. glutinosa*, *N. rustica*, *N. tabacum* cv. Samsun, *N. tabacum* cv. White Burley and *Petunia hybrida* (Solanaceae). For mechanical inoculations, the selected leaf samples were ground in 0.1 M K-

phosphate buffer (pH 7.0) containing 0.2% sodium sulfite .

A total of five plants of each experimental species were inoculated and bioassay was repeated three times .

Viral RNA extraction and sequencing

Four weeks post inoculation all symptomatic and asymptomatic plants were assayed by ELISA. Total RNA was extracted from ELISA positive lily samples using Tri-reagent (Sigma, USA) and first-strand cDNA synthesis was performed using M-MuLV reverse transcriptase (Fermentas, Lithuania), according to the manufacturer's instructions. First, CMV isolates were tested for the identification of the corresponding subgroups using specific primers as described previously (13). Subsequent PCR reactions were performed using specific primers for CMV 2b gene (CMV 2bF: 5'-ACATGGAAGCTAAGGTGATGGA -3' and CMV 2bR:5'-TAGCCGT(A/T)AGCTGGATGGACAACCC G-3'), that were designed based on complete RNA2 sequences in GenBank. Single-strand cDNA was synthesized using 5 µl of template RNA (1.5 µg), one µl of the primer CMV 2bR (20 pmol/µl) and one µl of RevertAidTM M-MuLV reverse transcriptase (200 unit/µl) (Fermentas, Lithuania) in 20 µl reaction volume at 42°C for 60 min and then at 72°C for 10 min to inactivate the enzyme, according to manufacturer's instructions. For the amplification reaction cDNA was used as a template in PCR using Pfu DNA polymerase (Cinnaclone, Tehran, Iran) and 2b gene specific primers. PCR program included 95°C for 5 minutes, 35 cycles of 95°C for 1 minute, 47°C for 1 minute, 72°C for 1 minute, with the final extension step at 72°C for 10 minutes. The RT-PCR product was isolated by excising the band from agarose gel and purified using Wizard PCR DNA purification system (Promega, Madison, WA, USA) according to the manufacturer's recommendations. CMV 2bR and CMV 2bF primers were used for sequencing DNA amplicons in both directions using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit and an Applied Biosystems Genetic Analyzer DNA model 310 (Applied Biosystems, Foster City, CA, USA). Complete or partial 2b gene nucleotide sequences of nine lily isolates of CMV in GenBank including IRN-LiD21, with 31 Iranian CMV isolates belong to subgroups IA and IB; and three representative isolates TrK7, Ly and Ls from group II were aligned using CLUSTAL X2 program (14). The phylogenetic tree was inferred using the Neighbor-Joining (NJ) method implemented in MEGA 4 (15). The average nucleotide distance between the 2b sequences was estimated using Kimura's two parameters implemented in PHYLIP version 3.5 (16).

Population analysis

The Kimura 2-parameter (17) was used to estimate the nucleotide diversity among the 2b gene of Iranian isolates characterized in this study and of other isolates using MEGA v. 4.1 program (15). DNASP version 5.10.01 (18) was used to estimate haplotype diversity. Haplotype diversity was calculated based on the frequency and number of haplotypes in the population. Seeking for determining the selective forces that might be operating during the evolutionary diversification of CMV 2b gene, we used Tajima's D test (19) which implemented in the DNASP version 5.10.01 (18). Genetic differentiation and the gene flow level between populations were evaluated by the statistical test F_{ST} (pairwise measures of population differentiation) (20). F_{ST} values >0.33 suggest infrequent gene flow.

Selection pressure

The selective forces that likely operate during the evolutionary diversification of 2b gene were determined. The pattern of selection was inferred through the non-synonymous to synonymous nucleotide substitution rate ratio (ω) with $\omega < 1$ for negative selection, $\omega = 1$ for neutral evolution and $\omega > 1$ for positive selection perssure (21). The ω ratio was assessed using an ML codon substitution model implemented in the CODEML program of the PAML4 package (22). Six site models, including M0 (one ratio), M1a (nearly neutral), M2a (positive selection), M3 (discrete), M7 (b) and M8 (β plus ω), were exploited as described previously (21, 23, 24). Three likelihood ratio tests (LRT) (M3 vs M0, M2a vs M1a and M8 vs M7) were used to assess the models' fit to

the data (24). If LRTs suggested positive selection, the Bayes Empirical Bayes (BEB) approach (23) was used to identify amino acids subjected to positive selection (posterior probabilities 95%).

Results

According to the serological ELISA assay, 37 lily samples (74%) were infected with CMV. Part of symptomatic samples (26%) was not infected by CMV and this may indicate their infection with other viruses. Among the indictor plants just mild systemic mottle symptoms was appeared on *Nicotiana benthamiana*, in which CMV infection was confirmed with ELISA. Using subgroup I specific primers, a DNA fragment of the expected size (about 600 bp) was amplified in lily isolates, whereas CMV subgroup II could not be detected in any of the tested isolates. The complete 2b nucleotide sequence of IRN-LiD21 isolate was 243 nt long. On the basis of 2b sequences analysis, CMV isolates fell into two main groups. All lily isolates including Iranian isolate IRN-LiD21 were classified into a separate branch in group I (Fig. 2a). Overall 2b nucleotide identity between CMV isolates in group I, ranged from 91.5 % to 100 %. Pairwise nucleotide distances also confirmed two main groups with the highest and lowest similarities for groups I and II, respectively. Nucleotide distances from 0.00 to 25.7% were found in group I. The low nucleotide distance was found for group I (0.00-12.9%), whereas, nucleotide distance for lily isolates subgroup was 12.9 to 25.7% (Fig.2b). Comparative sequence analysis revealed that IRN-LiD21 isolate shares the highest nucleotide sequence identity (99 %) with LiCB isolate from South Korea (AB506799) .

The mean evolutionary diversity for the entire population was 0.136±0.016. In addition, the within-population diversities (0.010 ± 0.003, 0.016±0.006 and 0.020± 0.006) were identified for group I, subgroup lily and group II, respectively (Table 1). According to the results for F_{ST} estimations (Table 2), there was a frequent gene flow between groups (I and II) and (I and lily subgroup) populations. F_{ST} ranges from 0 to 1 for undifferentiated to fully differentiated populations, respectively. An absolute value of F_{ST} 0.33 suggests infrequent gene flow.

Regarding the diversity of function and interaction of different proteins, it is not surprising that evolutionary constraints vary between proteins. The various proteins encoded by the CMV genome play different roles in the infection cycle of the virus. They are all involved in various phases of infection within the plant (replication, movement, or seed infection). The mean ω value for 2b protein was estimated (0.40-0.65) indicating purifying selection. In this study, using the BEB inference (23) an amino acid site (55 S) of 2b protein was identified under positive selection pressure (P>95%).

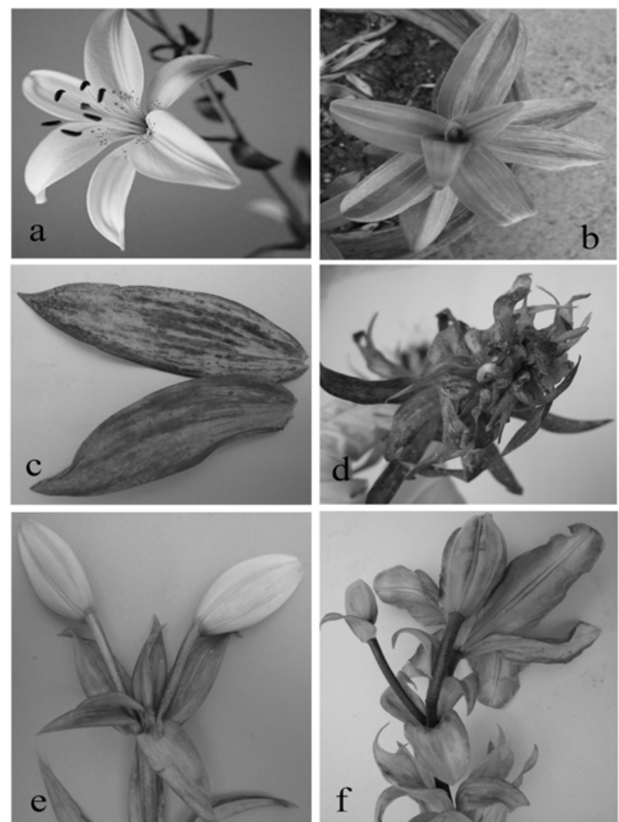

Fig. 1. Virus symptom observed in lily. (a) Healthy lilium; (b) Vein chlorosis; (c) leaf mosaic; (d) leaf deformation and stunting; (e) twisted and malformation in buds; (f) Flower deformation and color breaking.

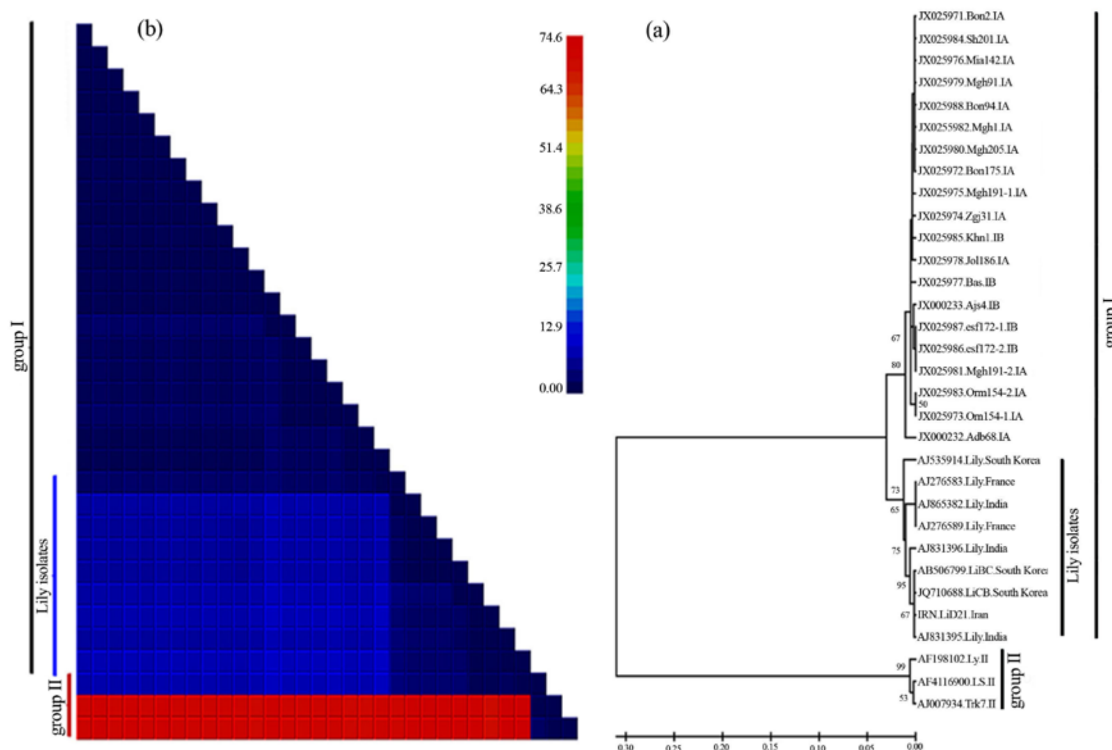

Fig. 2. (a) Phylogenetic tree based on nucleotide sequences of 2b protein gene of *Cucumber mosaic virus* isolates inferred using the Neighbor-Joining method implemented in MEGA 4 (Tamura et al. 2007). (b) Two dimensional pairwise nucleotide distances plot of 2b sequences of *Cucumber mosaic virus* isolates.

Table 1: Tajima's D test, haplotype and nucleotide diversity of each *Cucumber mosaic virus* population using 2b gene.

Phylogenetic groups	Haplotype diversity	Nucleotide diversity	Tajima's D test
Group I	0.926 (0.000)	0.010 (0.003)	-1.78222
Subgroup Lily	0.888 (0.001)	0.016 (0.006)	-0.33238
Group II	1.000 (0.000)	0.020 (0.006)	*

Numbers in parentheses indicate standard deviations. *) Number of sequences was not enough.

Table 2: Nucleotide diversity and gene flow between phylogenetic groups using 2b gene of *Cucumber mosaic virus* populations.

Phylogenetic groups	(Between two population)		
	GI & GII	GI & Lily G	GII & Lily G
Nucleotide diversity	0.643 (0.079)	0.060 (0.016)	0.619 (0.076)
F_{ST}	0.0703	0.1400	0.6990

Discussion

CMV is one of the commons plant viruses in many plants species, including pepper, tobacco, cucurbits, ornamentals, vegetables, and legumes in Iran. In this paper, we characterized biological and molecular properties of lily isolates of CMV.According to the ELISA assay, 37 lily samples (74%) were infected with CMV which indicated the high incidence of this virus among lily samples. Part of symptomatic samples (26%) was not infected by CMV and this may indicate their infection with other viruses. In general, most of CMV lily isolates, which they induced mild systemic symptoms in some indicator plants or failed infection many species of tobacco or cucurbit plants. In addition, CMV lily isolates had a relatively narrow host range, compared to the other CMV strains (11). However, CMV isolates from various plants infected well many species of tobacco and cucurbit plants (5). Similarly, biological reactions of CMV lily isolates were limited to *N. benthamiana* which, confirmed by RT-PCR .

CMV isolates of subgroups IA (25), subgroup IB (26) and II (27) had previously been reported from Iran. Our findings show for the first time the occurrence CMV lily isolates in Iran. Understanding the evolutionary history of viruses and evolutionary mechanisms driving their selection and diversification is an important aspect of evolutionary biology that could help manage viral diseases (28). Despite of some described lily isolates in group II (29), all of the lily isolates were classified to group I, and their sequences were highly conserved, regardless of lily species and/or variety as well as geographic origin (9). Therefore it was proposed that lily isolates constitute a unique pathological population, evolutionary adapted to lily plants regardless of their geographic origins (11). In addition, it has been shown that 2b induce symptoms in CMV-infected plants in both host-specific and virus strain-specific manner; therefore, it is thinkable that 2b is essential for the viral adaptation to lily plants .

The within-population diversities were identified for these populations that were different to the between-population diversity, indicating that there was genetic differentiation between these populations (Table 1 and 2). Phylogenetic analyses using 2b gene revealed that CMV populations in group I are closely related to each other, as supported by the lowest nucleotide diversity and high haplotype diversity (Table 1). A combination for high haplotype diversity and low genetic diversity, assessed by mitochondrial DNA markers, is taken as evidence of a recent population expansion after a genetic bottleneck. Unlike, relatively small amount of genetic differentiation among CMV populations, the significantly negative values of Tajima's D test (Table 1) suggest the demographic expansion of CMV populations and suggest that the CMV isolates are in a state of increasing population size. Furthermore, the Tajima's D test gave negative demonstrate either a decrease of the genetic variation by elimination of deleterious mutations by purifying selection or a rapid population size increase following a bottleneck or founder event. The direction of Tajima's D test is potentially informative about the evolutionary and demographic forces that a population has experienced. For example, negative values reflect an excess of rare polymorphisms in a population, which is consistent with either positive selection or an increase in population size. In contrast positive values indicate an excess of intermediate-frequency alleles in a population and can result from either balancing selection or population bottlenecks. The values of F_{ST} between the CMV phylogenetic group II and lily subgroup, was < 0.33 suggesting a relative level of differentiation. This value was > 0.33 between two populations (I and II) and (I and lily), which is an indication of infrequent gene flow (Table 2). Furthermore, it was indicated that gene flow is responsible for hinder populations from diverging, and therefore they become integrated (30).

The mean ω value for 2b protein indicated purifying selection which, agrees with Moury (31). This amino acid site was conserved among the 2b protein of different Iranian CMV isolates in group I, but not in lily subgroup (including Iranian isolate) or group II. Meanwhile, Moury (2004) indicated that the

evolutionary constraints exerted on proteins 1a, 2a, and 3a are larger than those exerted on proteins 2b and 3b (31). Positive selection pressure acting directly on functionally significant amino acids is rarely demonstrated because the fitness-related phenotypic consequences of individual amino acids are usually unknown. However, if any sites are positively selected along gene, it is likely that the sites involved increase fitness. It was shown that the substitutions at three amino acid positions (25, 76, and 214) in CP gene of CMV affect transmission efficiency by Myzus persicae but not by Aphis gossypii (32). Also rapid evolution of amino acid at position 25 in the CP was detected in independent analyses by Moury (31). Therefore, adaptation to the plant or to the vector may explain why diversifying selection affects several sites in the genome.

Because CMV in lily is normally transmitted through the bulb, once it infects a certain lily, it can exist for generations in the infected lily and its vegetative offspring (3). In Iran, despite of expanding of lily cultivation, healthy bulb production is scarce and the flower bulb industry is supplied mainly from the Netherlands. Furthermore, cut flower producers in Damavand County (Tehran province), save and multiply their own bulbs for next cultivation, which increase the source of infection. Lily plants grown from virus-infected bulbs can greatly affects the quality of flowers, which may be a hindrance to trade. Our information on the high infection of lily with CMV in various commercial lily greenhouses in Tehran province will help to better understand epidemiology and to develop a successful management program for reducing the impact of this disease.

Conclusion

CMV has been previously reported from Iran but, the association of CMV in lily plants represents the first record from Iran. Experimental evolution approaches using plant viruses as biological models have opened promising fields in evolutionary and experimental biology studies to address questions regarding the dynamics of adaptation, genetic diversification, and deleterious effects of mutations in populations. The accurate picture of phylogenetic relationships and comparisons among distantly related virus genomes provides an important insight into basic evolutionary mechanisms. Additionally, analysis of these variations is necessary for making advances in control strategies for viral diseases in order to hinder their spread.

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