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SHORT COMMUNICATION

PROPERTIES AND TAXONOMIC POSITION OF HOARY CRESS STRAIN OF *CUCUMBER MOSAIC VIRUS*

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SUMMARY

A strain of *Cucumber mosaic virus* naturally infecting hoary cress (*Lepidium draba*) in Shiraz, Iran (designated CMV-Ld), induces vein clearing and mosaic on the leaves of naturally infected plants. *Datura metel*, *D. stramonium*, *Lens culinaris*, *Lycopersicon esculentum*, *Nicotiana rustica* and *N. tabacum* "Turkish" showed line pattern after mechanical inoculation with the virus. These symptoms were different from those induced by a cucumber strain of CMV (CMV-Cu). CMV-Ld was serologically similar to the cucumber strain in gel immunodiffusion tests using homologous and heterologous antisera. SDS-polyacrylamide gel electrophoresis of purified virus preparation yielded a protein band with molecular weight of 25 kDa. Analysis of the nucleotide sequence of the coat protein (CP) gene showed that CMV-Ld, CMV-Cu and other CMV strains could be assigned to subgroups on the basis of *Eco*RI restriction site. The full length sequences of the CP gene of both strains showed that CMV-Ld and CMV-Cu belong to subgroups II and IA, respectively.

Key words: Lepidium draba, CMV subgroups, CMV strains, cloning, taxonomy.

Cucumber mosaic virus (CMV), the type member of the genus *Cucumovirus* (family *Bromoviridae*) has isometric particles with three linear single-stranded positive sense RNA molecules denoted RNA-1, RNA-2, and RNA-3. Subgenomic and satellite RNAs are also associated with some CMV strains (Suzuki *et al.*, 1991; Palukaitis, 1992; Gallitelli, 2000 García-Arenal *et al.*, 2000). The viral genome is encapsidated by a single species of coat protein (CP). Based on serology, nucleic acid hybridization and restriction fragment length polymorphism (RFLP), CMV isolates have been divided into two main subgroups denoted I and II. The members of subgroup I have been further divided into IA and IB based on phylogenetic analyses. They appear to be more prevalent than those of subgroup II (Gallitelli, 2000; Yu *et al.*, 2004).

CMV is one of the most devastating plant viruses with broad host range and worldwide distribution and can infect a large number of hosts among vegetables, field crops, ornamentals, fruits and weeds. Many weeds can serve as alternative hosts for CMV, support its population, and affect disease severity; therefore, they are important in virus epidemiology and disease control. CMV is transmitted in nature by many aphid species in a non-persistent manner (García-Arenal *et al.*, 2000; Sacristan *et al.*, 2004).

In Iran, CMV has been reported on many crops such as banana, cucurbits, lettuce, pepper, soybean, sugar beet, tomato, violet and zinnia (Rahimain and Izadpanah, 1978; Izadpanah, 1983; Ahoonmanesh *et al.*, 1997; Farhangi *et al.*, 2004; Golnaraghi *et al.*, 2004; Soleimani *et al.*, 2004; Ghotbi and Bananej, 2005). However, most of the CMV studies in Iran have been limited to virus detection by ELISA using commercial antisera and, in some cases, to mechanical inoculation of a host range. Little work has been carried out for complete characterization of CMV isolates at the molecular level and all molecularly characterized virus isolates from Iran are from cucurbits in the northwest of the country (Bashir *et al.*, 2006). Limited information is also available on the physicochemical properties of the virus. The wide host range, survival in many reservoir hosts and efficient natural transmission of CMV make its control difficult. Thus, information on genome sequence and genetic diversity of CMV strains is essential for planning modern control measures.

Hoary cress (*Lepidium draba*) is a common spring weed in many parts of Iran. Despite the relatively short life span, it may serve as a bridge for perpetuation of certain plant viruses (Izadpanah, 1983). In the present work a new strain of CMV (CMV-Ld) naturally infecting hoary cress in Shiraz is partially characterized biologically and molecularly. The use of certain molecular data in differentiation of CMV subgroups is also discussed.

Infected hoary cress plants were collected in Bajgah (15 km north of Shiraz) and used as the source of CMV-

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Table 1. Experimental host range of *Cucumber mosaic virus* from *Lepidium draba* following mechanical inoculation.

Test plants	Symptoms
Brassicaceae	
Brassica oleracea var. *gongyloides*	SSI
Raphanus sativus	Mo
Chenopodiaceae	
Chenopodium amaranticolar	CLL
C. quinoa	NLL
Cucurbitaceae	
Cucumis melo	CLL/ M, SCS
C. sativus	CLL/Mo
Cucurbita pepo	SCS
Fabaceae	
Lens culinaris	LP
Phaseolus auerus	NLL
P. vulgaris	NLL
Vicia faba	M
Vigna unguiculata	NLL
Solanaceae	
Capsicum annuum	M, Mo, LD, VC
Datura metel	CLL/ M, Mo, LP
D. stramonium	CLL/ M, Mo, LP, LD, SS
Lycopersicon esculentum	LP, M, OLP, SS
Nicotiana glutinosa	RS/ M, Mo, N, LD, D
N. rustica	M, Mo, LP
N. tabacum var. Turkish	CLL, CS/ M, Mo, LD, LP, N, VC, OLP, RS, D
Solanum melongena	M, Mo, VC

CLL: Chlorotic local lesions; CS: Chlorotic spots; D: Dwarfing; LF: Leaf deformation; LP: Line pattern; M: Mosaic; Mo: Mottling; N: Necrosis; NLL: Necrotic local lesions; OLP: Oak leaf pattern; RS: Ring spot; SCS: Systemic chlorotic spots; SS: Shoestring; SSI: Symptomless systemic infection; VC: Vein clearing.

Ld. The virus was isolated and maintained in *Datura stramonium* by sap inoculation. A cucumber strain of CMV (CMV-Cu) from *Cucumis sativus* was isolated from naturally infected cucumber tissue collected in a greenhouse in Isfahan province, and maintained in *Nicotiana rustica*. Mechanical inoculation was carried out by extracting infected tissue in cold 0.05 M potassium phosphate buffer pH 7, and rubbing the resulting extract onto test plants previously dusted with carborundum powder (Table 1). A number of test plants were also mechanically inoculated with CMV-Cu for comparison. The plants were kept in a greenhouse for symptom expression. Their infection was confirmed by back inoculation to *D. stramonium* or *Chenopodium quinoa* and by double diffusion test in gel using polyclonal antisera against CMV-Ld and CMV-Cu.

Naturally infected *L. draba* showed mosaic, vein clearing and slight leaf deformation (Fig. 1A). All 20 plant species from five families mechanically-inoculated with CMV-Ld were infected, with *C. amaranticolor*, *C. quinoa*, *Phaseolus aureus* and *Vigna unguiculata* showing local lesions on inoculated leaves (Table 1). The virus induced line pattern on the leaves of *D. metel, D. stramonium, Lens culinaris*, *Lycopersicon esculentum*, *Nicotiana rustica* and *N. tabacum* "Turkish" (Fig. 1B-D). The latter exhibited various symptoms, such as chlorotic local lesions, chlorotic spots, vein clearing, mosaic, oak leaf pattern, ringspots, dwarfing, leaf deformation and necrosis. Sato *et al.* (2000) found that line pattern induced in tobacco plants was linked to a novel satellite RNA of CMV-YW. However, no satellite RNAs were detected in CMV-Ld infected plants (data not shown). On *C. sativus*, chlorotic local lesions developed on inoculated cotyledons, and newly growing leaves occasionally showed mild mosaic which disappeared in a few days. *Brassica oleracea* var. *gongyloides* was infected symptomlessly. Back inoculation from such plants to *C. quinoa* resulted in the production of chlorotic local lesions.

CMV-Cu elicited systemic chlorotic spots in *Cucumis melo* and *Cucurbita pepo*, chlorotic spots on inoculated leaves of *D. metel*, mosaic and leaf deformation in *N. tabacum* "Turkish" and *Solanum melongena*, and mosaic, leaf deformation and shoestring in *N. glutinosa* and *N. rustica*. Symptoms of CMV-Cu on most test plants consisted of various types of mosaic and leaf distortion different from those induced by CMV-Ld. However, the symptoms induced in *C. sativus* and *V. unguiculata* by CMV-Cu were similar to those induced by CMV-Ld.

A simple procedure was adopted for purification of CMV-Ld. Virions were extracted from infected *D. stramonium* leaf tissue by grinding in 0.1M ammonium citrate buffer, containing 1% polyvinylpyrrolidone (PVP), 0.1% sodium diethyl-dithiocarbamate (DIECA) and 0.25% 2-mercaptoethanol, pH 6.0. After treatment with 20% chloroform and initial clarification, Triton X-100 was added at a final concentration of 7% and the extract was stirred at 4°C for 30 min. The virus was sedimented over a 20% sucrose cushion prepared in 0.01M ammonium citrate, pH 6.5, by high speed centrifugation and further purified by 10-40% sucrose density gradient centrifugation. Protein and RNA electrophoresis and electron microscopy were carried out as previously described (Laemmli, 1977; Sambrook *et al.*, 1989; Milne, 1993).

A yield of 5 mg virus per 100 g of infected tissue was estimated for the purified preparation. A protein band with expected molecular weight of 25 kDa was resolved after SDS-polyacrylamide gel electrophoresis of the purified preparation. Based on molecular weight, in addition to genomic RNAs, several extra bands were observed after RNA electrophoresis which may have been resulted from RNA degradation. Electron microscopy of the CMV-Ld purified preparation showed many isometric particles with an estimated diameter of *ca.* 30 nm.

A polyclonal antiserum was produced against CMV-

Ld in a white New Zealand rabbit by subcutaneous injections of purified virus (Ball *et al*., 1990). Serological relationships were determined by agar gel diffusion (AGD) as previously described (Ball, 1990). The antiserum prepared against CMV-Ld reacted positively with both CMV-Ld and CMV-Cu in AGD. Similarly, CMV-Cu antiserum detected both antigens. No precipitation lines were observed when these antisera were cross-absorbed with heterologus antigens. Positive results were obtained in AGD with naturally infected *L. draba* tissue extracts. Field collected symptomless *L. draba* tissue reacted negatively.

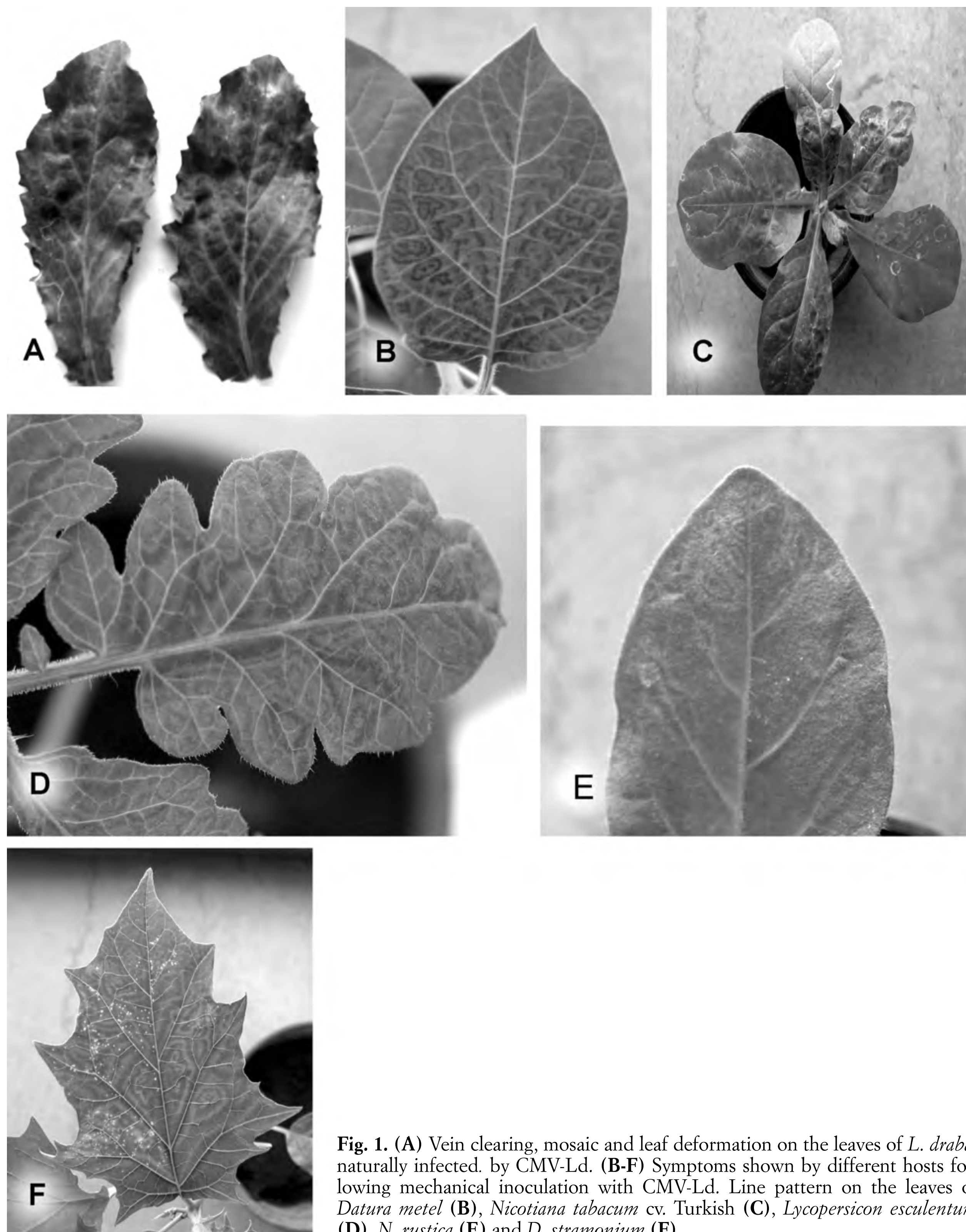

Fig. 1. (A) Vein clearing, mosaic and leaf deformation on the leaves of *L. draba*, naturally infected. by CMV-Ld. **(B-F)** Symptoms shown by different hosts following mechanical inoculation with CMV-Ld. Line pattern on the leaves of *Datura metel* **(B)**, *Nicotiana tabacum* cv. Turkish **(C)**, *Lycopersicon esculentum* **(D)**, *N. rustica* **(E)** and *D. stramonium* **(F)**.

Agar gel diffusion can differentiate certain serologically diverse isolates of CMV (Rahimian and Izadpanah, 1978; Ahoonmanesh *et al*., 1997). However, in the present study, CMV-Ld and CMV-Cu could not be differentiated by this method. Sensitive tests such as ELISA with monoclonal antibodies may be required for reliable distinction between serologically close strains.

Both CMV-Ld and CMV-Cu were subjected to RT-PCR, using the total RNA extraction protocol of Logemann *et al*. (1987) and *Cucumovirus* genus-specific primer pair CPTALL-3/CPTALL-5 (Choi *et al*., 1999). The primer pair CMVsat3H/sat5T7P was also used to investigate whether a satellite RNA was present (Sato *et al*., 2000). MMLV reverse transcriptase and Taq DNA polymerase were used as described by the manufacturers. Random amplification of cDNAs from CMV-Ld

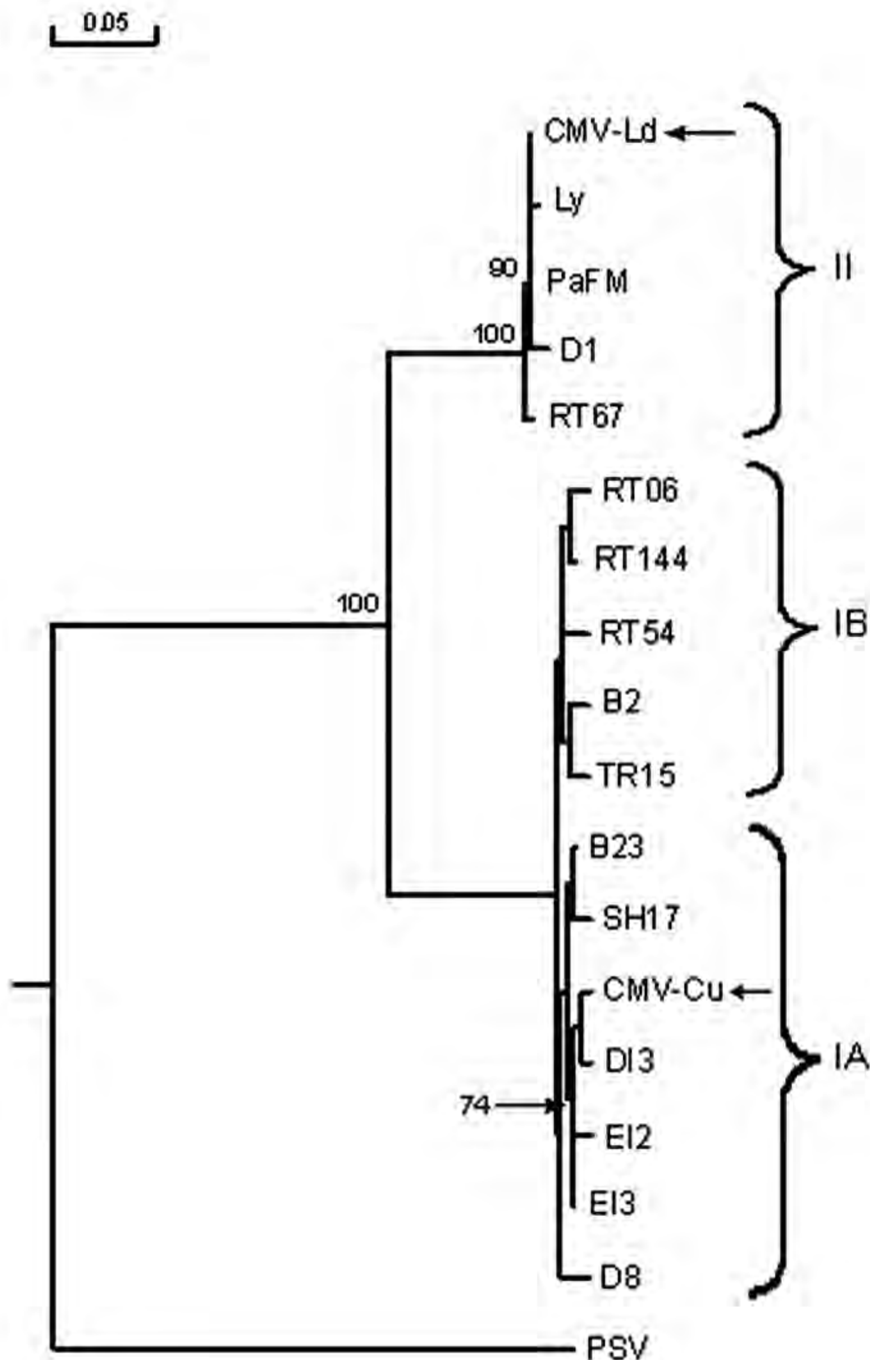

Fig. 2. Phylogenetic tree constructed after multiple sequence alignment of deduced coat protein gene amino acid sequences of selected CMV strains including CMV-Ld and CMV-Cu (arrows). Numbers above the nodes represent branches with more than 70% bootstrap scores. Virus isolates and GenBank accession numbers are: B23, AY871071; D8, AB004781; SH17, AY871068; EI3, DQ002882; EI2, DQ002881; DI3, DQ002879; B2, AB046951; TR15, AJ810264; RT06, AJ810265; RT54, AJ810263; RT144, AJ810262; D-1, DQ018292; PaFM, AB109908; LY, AF198103; RT67, AJ810253. Peanut stunt virus (PSV) was used as an outgroup.

genome was also done using random primers K1/K2 as described by Froussard (1992). PCR products were ligated into pTZ57R/T vector (Fermentas, Vilnius, Lithuania), cloned in *E. coli* strain DH5α and sequenced. Sequence data were compiled, analyzed and compared with those available in the GenBank database using NCBI/BLAST to search for related sequences. The deduced coat protein amino acid sequences of the selected CMV strains were extracted from the GenBank database for comparison with those of CMV-Ld and CMV-Cu. Sequence assembly, multiple sequence alignment and phylogenetic analysis were carried out using DNAMAN software (version 4.0.1.1). A neighbor-joining phylogenetic tree was constructed after 100 replicate bootstrapping the aligned sequences.

Expected fragments of approximately 940 nucleotides corresponding to the 3' half of RNA-3 from both CMV-Ld and CMV-Cu strains were amplified by RT-PCR using the primer pair CPTALL-3/CPTALL-5. Use of annealing temperature as low as 38°C was successful for PCR amplification of CMV strains by these primers, while Choi *et al.* (1999) obtained the best result with annealing temperature of 46°C.

Sequence data identified CMV-Ld as a member of CMV subgroup II. The cloned fragment from CMV-Ld contained 889 nt (accession No. EF050074) comprising 108 nt of the intergenic region of RNA-3 upstream of the CP gene, the full length CP gene (657 nt, 218 aa) and 134 nt of the 3´ end downstream of the CP gene. It had an *Eco*RI restriction site at nt 734 from its 5´ end (nt 626 of the CP gene). The cloned fragment from CMV-Cu contained 906 nt (accession No. EF620777) comprising 129 nt of the intergenic region of RNA-3 upstream of the CP gene, the whole CP gene (657 nt) and 120 nt of the 3´ end downstream of the CP gene. A search in the GenBank database showed CMV-Ld had highest similarity with members of subgroup II, while CMV-Cu was closer to members of subgroup IA. A randomly amplified 446 nt fragment of the movement protein gene of CMV-Ld (accession No. DQ683254) also showed highest similarity with members of subgroup II.

Digestion of recombinant plasmid or PCR product from CMV-Ld by *Eco*RI generated two fragments of *ca.*750 bp and 250 bp, while that from CMV-Cu (subgroup I) was not digested by this enzyme, providing evidence that the two strains are molecularly different. Presence of *Eco*RI restriction site in CMV-Ld and its absence in CMV-Cu, was confirmed by sequence data. A search was performed for the presence of *Eco*RI site in isolates M2, LS, Ly, Q, Xb, Trk7 and 241 (subgroup II), D8 and SNY (subgroup IA) and B2, Ix, SD and PBN (subgroup IB). The results showed that the *Eco*RI site is also present at the same position in members of subgroup II. In contrast, *Eco*RI site was either absent or positioned at nt 11-12 and 14-22 (numbered from the 5´end) in corresponding fragments of members of subgroup IA and IB, respectively. These results pose the possibility of identifying CMV subgroups by *Eco*RI digestion of PCR products obtained with primer pair CPTALL-3/CPTALL-5.

Multiple sequence alignment of the CP gene amino acid sequences revealed that CMV-Ld and CMV-Cu are 82.9% identical. CMV-Ld showed 82.5% amino acid sequence identity with isolates B13, SH17 and S337, and 82.9% with isolate B23, while the level of amino acid sequence identity between CMV-Cu and previously characterized Iranian isolates (Bashir *et al.*, 2006) was more than 97.7%. Therefore, the isolates B13, S337 and SH17 reported on cucumber in northwest of Iran (Bashir *et al.*, 2006) are close to CMV-Cu, a cucumber infecting isolate from Isfahan.

Because of the high similarity among Iranian isolates (Bashir *et al.*, 2006), the CP gene sequences from isolates B23, DI3, EI2, EI3 and SH17 were included as representatives in phylogenetic analysis and that from isolate SH12 (subgroup II) was excluded for unavailability of the complete CP sequence. The constructed neighbor-joining tree (Fig. 2) had two main well-separated clusters. One cluster containing CMV-Ld is related to CMV subgroup II. The other cluster related to CMV subgroup I is further divided into two sub-clusters, including members of IA and IB subgroups. CMV-Cu is located in the clade of subgroup IA.

In conclusion, CMV-Ld, a member of subgroup II, is reported on hoary cress for the first time. Line pattern, an unusual symptom of CMV strains (Sato *et al.*, 2000), was observed in CMV-Ld-infected plants but could not be associated with the presence of a satellite RNA. Many weeds serve as survival hosts for CMV, which is also transmitted through seed in some hosts including certain crucifers (Gallitelli, 2000). The population of hoary cress plants increases drastically around Shiraz during March-May. Therefore, it can serve as a primary source of the virus for subsequent infection of commercial hosts.

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