# Analysis of the biological and molecular variability of Watermelon mosaic virus isolates from Iran

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**Abstract** Watermelon mosaic virus (WMV) is one of the most important viruses that causes different symptoms in *Cucurbitaceae*. WMV is a potyvirus with a worldwide distribution, but occurs most commonly in temperate and Mediterranean regions. Cucurbit species grown in Yazd, Esfahan, West Azerbaijan, Hormozgan, and Kerman provinces were surveyed for the relative incidence of WMV in 2004–2005. A total of 757 symptomatic cucurbit and 31 weed species were collected and assayed for infection with WMV. Of 788 leaf samples from cucurbit and weed plants, 190 samples were positive by double antibody sandwich ELISA (DAS-ELISA) using specific polyclonal antibody. Among the weed species tested only colocynth (*Citrullus colocynthis*) was found to be infected with WMV. The coat protein (CP) gene from 18 representative isolates was PCR amplified, cloned, sequenced, and compared with the sequences available in GeneBank. Phylogenetic analysis using 778 nucleotide long sequences of the coat protein gene showed that these isolates fell into two; groups I and II. Only one isolates (KER.JI.1) was classified in the group II. This isolate had a wider host range and infected *Nicotiana debneyii* and *Datura metel*. None of the other 17 isolates could infect these two species. Members of group I were divided into three subgroups; A, B, and C. The subgroup I$_B$ appears to be a new subgroup comprising only of the Iranian isolates. Phylogenetic analysis based on 200 nucleotides coding for the N-terminal segment of the CP showed that all Iranian isolates except KER.JI.1 clustered with the previously reported WMV strains. All Iranian isolates had a DAG amino acid triplet which is involved in aphid transmissibility. This is the first report on sequence analysis of the nearly full-length CP cDNA clones of WMV isolates from Iran.

**Keywords** Watermelon mosaic virus · Coat protein · Phylogeny · Iran

## Introduction

Watermelon mosaic virus (WMV) is a member of the genus *Potyvirus* and the family *Potyviridae* with approximately 750 nm long flexuous filamentous particles [20]. WMV appeared to share high sequence similarity with *Soybean mosaic virus* (SMV) [6]. The genome of WMV is a single, positive-sense RNA of around 10035-nt long. It contains a unique large open reading frame coding for a polyprotein of 3217 amino acids; it contains nine putative cleavage sites, yielding 10 putative functional proteins that have motifs conserved among homologous proteins of other potyviruses [6].

WMV causes economically important diseases on several horticultural crops, mostly cucurbits and some legumes, including pea [23] and orchids, vanilla [28], and *Habenaria radiata* [13]. It infects more than 170 plant species belonging to 27 families experimentally [26], including many weeds, that can host the virus between crops.

Sequence comparisons of coat protein (CP) genes can be used to identify and differentiate distinct potyviruses and their strains [24, 25]. Several studies have established phylogenetic relationships between strains of potyviruses

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based on CP gene sequences, e.g., potato virus Y [27], bean common mosaic virus [22], plum pox virus [4], papaya ring spot virus [29], lettuce mosaic virus [31], and zucchini yellow mosaic virus [7]. The sequences of other regions of the potyvirus genome, such as the 3′ nontranslated region (3′NTR), also can be used to discriminate strains within a virus species [11, 22].

Attempts have been made to analyze WMV variation at the nucleotide and amino acid levels. These studies have focused on analysis of the sequence of the P1, CP, and cylindrical inclusion (CI) genes. Moreno et al. [18] characterized 44 WMV isolates from Spain and demonstrated that variability existed in the CP and the cylindrical inclusion (CI) proteins.

Although WMV has long been known to occur in several regions of Iran [9, 16, 19, 21], a 218-nucleotide sequence of the CP N-terminal region of eight isolates from Iran has just recently been reported [8]. The goals of the present study were amplification of CP gene and assessment of the genetic variation among the CP sequences of the representative isolates collected from several regions of Iran. The availability of this geographically diverse range of WMV isolates and our observation that some of these isolates appeared to induce different symptoms in test plants prompted us to generate information on the molecular and biological variability of WMV.

## Materials and methods

### Collection and maintenance of virus isolates

In 2004, 178 and 127 samples in January, February, and April from Kerman and Hormozgan provinces, respectively, were monitored for virus identification. In 2005, 231, 136, and 116 samples during May to August from Yazd, West Azarbayjane, and Esfahan provinces, respectively, were conducted. Samples were collected from different cucurbit plants including cucumber, summer squash, winter squash, melon and weeds in the fields and greenhouses in major cucurbit growing regions of Iran. The cucurbit producing areas included were the southwest (West Azarbayjane province), center (provinces of Yazd and Esfahan), and southeast (provinces of Kerman and Hormozgan) (Table 1). The southwest areas of Iran are subject to severe winters, with average daily temperatures below freezing, and warm summers. The southeast of Iran has mild winters, with average January temperatures ranging from 7 to 18°C, summers are very humid and hot, with temperatures exceeding 40°C during July. The central part of Iran is marked by hot and dry summers and sporadic rainy winters, which is characterized by dry, warm winds in May to June. Cucurbit is grown as a summer crop in central and southwest of Iran; however, in the interior areas in the subtropical

**Table 1** Origins of WMV isolates from Iran, used in this study

| Isolates | Province–region | Host plant | Accession number |
|---|---|---|---|
| KER.JI.1[a] | Kerman–Jiroft | *Citrullus colocynthis* | EU667627 |
| KER.KE.1 | Kerman–Kerman | *Cucumis melo* L. | EU667644 |
| YAZ.SH.1 | Yazd–Sadogh | *C. melo* L. | EU667635 |
| YAZ.MO.1 | Yazd–Mohsenabad | *Cucumis sativus* L. (cucumber) | EU667638 |
| ESF.ES.1 | Esfahan–Esfahanak | *Cucurbita moschata*, Iranian cultivar | EU667637 |
| ESF.ES.2 | Esfahan–Esfahanak | *C. melo* L. | EU667640 |
| YAZ.MO.2 | Yazd–Mohsenabad | *Cucurbita pepo* (reported for the first time in this article) | EU667630 |
| ESF.GA.1 | Esfahan–Gaz | *C. sativus* L. (cucumber) | EU667641 |
| ESF.GA.2 | Esfahan–Gaz | *Cucurbita maxima* | EU667634 |
| ESF.ES.3 | Esfahan–Esfahanak | *C. sativus* L. (cucumber) | EU667633 |
| HOR.HA.1 | Hormozgan–Hajiabad | *C. melo* L. Shahabadi cultivar | EU667628 |
| ESF.GA.3 | Esfahan–Gaz | *C. sativus* L. (cucumber) | EU667643 |
| ESF.ZA.1 | Esfahan–Zarinshahr | *Cucurbita moschata* Duch (pumpkin), Iranian cultivar | EU667639 |
| ESF.ZA.2 | Esfahan–Zarinshahr | *C. melo* L. | EU667636 |
| URO.NA.1 | Uromiae–Naghadae | *C. maxima* Duch. E Lam. (winter squash) | EU667629 |
| URO.OS.1 | Uromiae–Oshnaviae | *C. melo* L., Kesavarz cultivar | EU667631 |
| ESF.ZA.3 | Esfahan–Zarinshahr | *C. sativus* L. (cucumber) | EU667642 |
| YAZ.TA.1 | Yazd–Taft | *Cucurbita pepo* (summer squash) | EU667632 |

[a] Each isolate was identified by location including province, regions, and series number

regions of the country in the southeast that is being frost-free in winter, cucumber is also grown as a winter crop.

A total of 788 samples comprised of 757 cucurbit and 31 weed plants (Table 2) were collected from 64 locations. In each field or greenhouse, cucurbit and plants showing mosaic and other typical virus symptoms were collected. Each sample consisted of the two youngest fully developed symptomatic leaves from cucurbit vines and similar ones from the weed plants. WMV, *Cucumber mosaic virus* (CMV), *Squash mosaic virus* (SqMV), *Papaya ring spot virus*-type W (PRSV-W), and *Zucchini yellow mosaic virus* (ZYMV) infection in all collected plants was verified by the double antibody sandwich enzyme-linked immunosorbent

assay (DAS-ELISA) [5] using the specific polyclonal antibodies (Bioreba, CH-4153 Reinach BL1, Switzerland). From WMV-positive sample set, 18 specimens were chosen according to hosts and agroecological considerations, and WMV isolates infecting these 18 specimens were biologically cloned by two local lesion passages in *Chenopodium amaranticolor*, followed by a single local lesion selection in *C. quinoa*. Such biologically cloned viruses are hereafter called isolates [18]. All WMV isolates for biological and molecular studies were maintained on *Cucurbita pepo* L.cv. Khoy in a temperature regulated insect-proof greenhouse. The isolates were named and numbered according to the abbreviated name of provinces and regions of collection (Table 1).

**Table 2** Reaction of selected weed plant species to different viruses that infect Cucurbitaceae[a] by ELISA

| Species | Family | Common name |
|---|---|---|
| *Solanum nigrum* L. | *Solanaceae* | Black nightshade |
| *Erigeron* sp. | *Asteraceae* | Fleabane |
| *Convolvolus arvensis* L. | *Convolvulaceae* | Field bindweed |
| *Conyza bonariensis* (L.) Cronq. | *Asteraceae* | Horseweed |
| *Salsola tragus* L. | *Chenopodiaceae* | Russian thistle |
| *Malva sylvestris* L. | *Malvaceae* | Common mallow |
| *Chenopodium album* L. | *Chenopodiaceae* | Lamb squarters |
| *Euphorbia* sp. | *Euphorbiaceae* | Wart weed |
| *Launaea acanthodes* (Boiss.) O.Kuntze | *Asteraceae* | Launaea |
| *Sonchus asper* (L.) Hill | *Asteraceae* | Prickly sow-thistle |
| *Cardaria* sp. | *Brassicaceae* | Hoary cress |
| *Citrullus colocynthis* (L.) Schrad. | *Cucurbitaceae* | Colocynth |
| *Orobanche* sp. | *Orobanchaceae* | Broomrape |
| *Tribulus terrestris* L. | *Zygophyllaceae* | Puncturevine |
| *Chrozophora* sp. | *Euphorbiaceae* | Turnsole |
| *Melilotus officinalis* (L.) Lam. | *Legominosae* | Yellow sweet clover |
| *Heliotropium europaeum* L. | *Boraginaceae* | European heliotrope |
| *Chenopodium murale* L. | *Chenopidiaceae* | Nettle-leaved goosefoot |
| *Cyperus* sp. | *Cyperaceae* | Nutgrass |
| *Polygonum* sp. | *Polygonaceae* | Knot weed |
| *Amaranthus* sp. | *Amaranthaceae* | Slim amaranth |
| *Hibiscus esculentus* L. | *Malvaceae* | Gombo |
| *Eragrostis* sp. | *Poaceae* | Love grass |
| *Echinochloa* spp. | *Poaceae* | Barnyard grass |
| *Datura stramonium* L. | *Solanaceae* | Jimson weed |
| *Carthamus tinctorius* L. | *Asteraceae* | Safflower |
| *Amaranthus* sp. | *Amaranthaceae* | Amaranth |
| *Chenopodium botrys* L. | *Chenopodiaceae* | Sticky goosefoot |
| *Solanum* sp. | *Solanaceae* | Nightshade |
| *Cressa cretica* Linn. (Rundati) | *Convolvulaceae* | Rudravanti |
| *Trigonella* sp. | Fabaceae | Fenugreek |
| *Rumex* sp. | *Polygonaceae* | Garden sorrel |
| *Alhagi persarum* Boiss & Buhse | Fabaceae | Camelthorne |

[a] Enzyme-linked immunosorbent assay (ELISA) was used for detection of viruses in weeds

## Biological characterization of the WMV isolates

Eighteen isolates were characterized biologically by reaction to inoculation of a variety of range of test plants. These samples were ground in 1% (w/v) solution of $K_2HPO_4$ at pH 7.5 containing 0.01% $Na_2SO_3$, 2% polyvinylpyrrolidone (PVP), and 0.05% ethylenediaminetetraacetic acid (EDTA). The extracts were then mechanically inoculated onto the following 13 plant species: *C. pepo* L.cv. Khoy., *Citrllus lantanus* Thunb.cv. Crimson Sweet, *Nicotiana clevelendii* Gray., *N. tabacum* L.cv. Samsun.N, *N. glutinosa* L., *N.debneyi* L, *N. bentamiana* L, *Datura metel* L, *Pisum sativum* L, *Phaseolus vulgaris* L.cv. Red Kidney, *C. quinoa* Willd, *C. amaranticolor* Coste & Reyn and *Cucumis melo* L.cv. Asgrown. Five plants of each species or cultivar were mechanically inoculated. After inoculation, plants were kept in an insect-proof greenhouse at 15–25°C for symptom development. ELISA was used as well as symptomology to check for presence of the respective viruses in inoculated plants.

## RNA isolation and RT-PCR

Total RNA from *C. pepo* L.cv. Khoy experimentally infected with each of the 18 WMV isolates was extracted using High Pure Viral Nucleic Acid Kit (Roche Biochemical, Germany) according to the manufacturer's instructions. One microgram of total RNA (200 ng/μl) plus 2.5 μl of diethyl pyrocarbonate (DEPC)-treated water were incubated at 65°C for 8 min and chilled on ice for 3 min to denature the RNA. To the denatured RNA extract, 15 μl of RT reaction mixture containing 1× M-MLV RT buffer, 1.5 Mm each dNTP, 20 units of RNasin (Fermentas, Vilnius, Lithuania), 0.1 μg (0.1 μM) of antisense primer WMV-R, 5′-ATT CAC GTC CCT TGC AGT GTG-3′ designed from WMV-Fr sequence, accession number AY437609 corresponding to nts 9744-9724 at the C-terminal region of CP, and 200 units of M-MLV reverse transcriptase were added to provide a final volume of 25 μl. Samples were incubated at 42°C for 1 h followed by 95°C for 3 min to terminate the RT reaction. PCR was carried out using 2.5 μl aliquots of the cDNA preparation in 50 μl reaction mixture containing 5 μl of 10× PCR buffer, 1.5 mM $MgCl_2$, 1 μl dNTP (10 mM each), 0.1 μM of antisense primer WMV-R, 170 ng (0.1 μM) of sense primer WMV-F, 5′-GAA TCA GTG TCT CTG CAA TCA GG-3′ designed from WMV-Fr sequence, accession number AF057533 corresponding to nts 8923-8945 at the N-terminal region of CP, *Taq* DNA polymerase (5 U/μl) (Fermentas) and deionized $H_2O$. The single-step RT-PCR reaction was carried out in a Techne TC 312 thermal cycler (Techne, Cambridge, UK) using the following program: initial denaturing for 3 min at

94°C, followed by 30 cycles of denaturation for 60 s at 94°C, annealing for 1 min at 60°C, extension for 1 min at 72°C, and a final extension step at 72°C for 10 min. In all tests positive and negative controls were used. The negative control was including distilled water instead of DNA template. Ten microliters of each PCR product was analyzed by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining.

## Cloning, sequencing, and phylogenetic analysis

The PCR products comprised of 804 bp of CP gene and 18 bp of the 3′ of the NIb gene were ligated into a pTZ57R/T vector using InsT/Aclone PCR Product Cloning Kit (Fermentas) according to the instruction of the manufacturer, and transformed into *Escherichia coli* strain DH5ἀ. Recombinant plasmids were extracted and nucleotide sequencing reactions were performed by Macrogen Company (South Korea) using dideoxy nucleotide chain termination method by universal primers for the plasmid.

The phylogenetic analysis of the 18 Iranian isolates were conducted by comparing the 778 bp of the CP gene and also the 200 nucleotides corresponding to 8922-9121 nts (accession number AY437609) coding for the N-terminal part of the CP gene with those of the other isolates of WMV obtained from GenBank (Tables 1, 3, and 4). The nucleotide and deduced amino acid sequences were compared with the counterpart sequences of WMV. Multiple sequence alignment was carried out using optimal alignment by DNAMAN software package (Lynnon, Biosoft, Quebec, Canada). Phylogenetic trees for grouping were constructed either by DNAMAN software package using similarity matrix and the neighbor-joining method and max parsimony (MEGA version 2.1) [15]. Tree branches were bootstrapped with 1000 replications. The validity of trees was evaluated by cophentic coefficient based on mantel test [17].

# Results

## WMV detection in the plant material

Of the 757 samples collected from cucumber, summer squash, winter squash and melon, tested by ELISA, 322, 190, 41, and 4 were infected with ZYMV, WMV, CMV, and SqMV, respectively. Double and triple infections involving different combinations of CMV, ZYMV, and WMV-2 were noted in 73 and 1 samples, respectively. WMV infection was detected only in one weed species (Colocynth; *Citrullus colocynthis*). Eighteen isolates of WMV were chosen as representative for further studies.

Biological characteristics of the WMV isolates

The isolates were compared with regard to symptoms produced on different test plants. All isolates infected *N. bentamiana* plants, systemically causing very mild mosaic. *C. amaranticolor* and *C. quinoa* reacted to all 18 isolates by production of local lesions in the inoculated leaves. Isolate KER.JI.1 caused a systemic mild mosaic on *N. debneyii* and vein banding and mosaic on *D. metel* (Fig. 1), whereas the remaining 17 isolates did not infect these two species. *C. pepo* and *C. melo* reacted differently to the isolates: ESF.ES.2, ESF.ES.3, ESF.ZA.2, ESF.ZA.3, and YAZ.MO.1 caused severe mosaic, leaf distortion, and vein clearing. Isolates ESF.ES.1, ESF.GA.1, ESF.GA.3, ESF.ZA.1, HOR.HA.1, KER.JI.1, KER.KE.1, URO.OS.1,

and YAZ.SH.1 produced mild mosaic symptoms on both plants. The remaining isolates induced varying degrees of mosaic and vein banding (YAZ.MO.2), vein clearing (ESF.GA.2), severe mosaic (URO.NA.1), and vein banding

**Table 3** GenBank accession number and origin of some of the previously reported WMV isolates used for phylogenetic comparison of a 804 nt long fragment of the genome including CP gene

| Isolate | Origin | Host plant | Accession number |
|---|---|---|---|
| USA | USA | – | D13913 |
| – | Tonga | *Vanilla tahitensis* | L22907 |
| – | Australia | – | D00535 |
| WMV-Fr | France | – | AY437609 |
| ZAR95.1[a] | Spain | *Cucumis melo* | AJ579482 |
| ZAR99.5 | Spain | *C. melo* | AJ579489 |
| MUR95.2 | Spain | *C. melo* | AJ579499 |
| BAD95.2 | Spain | *C. melo* | AJ579503 |
| BAD95.3 | Spain | *C. melo* | AJ579504 |
| MAD95.3 | Spain | *C. melo* | AJ579507 |
| MAD95.6 | Spain | *C. melo* | AJ579509 |
| MAD95.8 | Spain | *C. melo* | AJ579512 |
| MAD99.1 | Spain | *C. melo* | AJ579513 |
| SG99.1 | Spain | *C. melo* | AJ579517 |
| MAL99.2 | Spain | *C. melo* | AJ579521 |
| MAL99.5 | Spain | *C. melo* | AJ579524 |
| M116 | Spain | – | AF551334 |
| Habenaria | Japan | – | AB001994 |
| S96-6 | Japan | Pumpkin | AB353119 |
| WMV-HLJ | China | – | AY464948 |
| WMC-CHN | China | *Citrullus lanatus* | DQ399708 |
| Ch99/69 | China | *Cucurbita pepo* | EF127832 |
| WMV-PK | Pakistan | *Cucumis melo* var Flemenuosos | AB218280 |
| – | New Zealand | – | AY995215 |
| BZ[b] | China | *Pinellia ternata* | DQ360817 |

[a] The Spanish isolates were identified by location, year of sampling, and series number. ZAR, Zaragoza; MUR, Murcia; BAD, Badajoz; MAD, Madrid; SG, Segovia; MAL, Malaga (Moreno et al. [18])

[b] BZ is *Soybean mosaic virus* (SMV) and included as outgroup

**Table 4** GenBank accession number and origin of some of previously reported WMV isolates/strains used for phylogenetic comparison of the 200 nucleotides long coding sequence of the N-terminal part of the CP gene

| Isolate/strain | Origin | Accession number |
|---|---|---|
| ALG93 | Algeria | DQ845063 |
| ARG94 | Argentina | DQ845043 |
| AUST89 | Australia | DQ845040 |
| BAD95.2 | Spain | AJ579503 |
| BAD95.3 | Spain | AJ579504 |
| CH98-47 | China | DQ845034 |
| CHI87-620 | Chile | DQ845064 |
| CHI02-481 | Chile | DQ845056 |
| F-ALSACE | Herbsheim, France | DQ845052 |
| FBA02-06 | Balandran, France | DQ845067 |
| FMF00-LL1 | Montfavet, France | DQ845033 |
| F-RERP | Perpignan, France | DQ845062 |
| F-VAL | Valence, France | DQ845044 |
| IR00-43 | Iran | DQ845042 |
| IR00-133 | Iran | DQ845038 |
| IR00-170 | Iran | DQ845037 |
| IR02-01 | Iran | DQ845039 |
| IR02-10 | Iran | DQ845058 |
| IR02-54 | Iran | DQ845036 |
| IR02-57 | Iran | DQ845035 |
| IR02-79 | Iran | DQ845041 |
| MAD95.3 | Spain | AJ579507 |
| MAD95.6 | Spain | AJ579509 |
| MAD95.8 | Spain | AJ579512 |
| MAD99.1 | Spain | AJ579513 |
| MAL99.2 | Spain | AJ579521 |
| MAL99.5 | Spain | AJ579524 |
| MUR95.2 | Spain | AJ579499 |
| WMV-Fr | France | AY437609 |
| NZ | New Zealand | AY995215 |
| Pak | Pakistan | AB127934 |
| POLYNESIA | French Polynesia | DQ845065 |
| SG99.1 | Spain | AJ579517 |
| SL02-C2 | Slovakia | DQ845050 |
| TONGA | Tonga | L22907 |
| USA | Florida, USA | D13913 |
| USA92 | USA | DQ845032 |
| ZAR95.1 | Spain | AJ579482 |
| ZAR99.5 | Spain | AJ579489 |
| BZ[a] | China | DQ360817 |

[a] BZ is *Soybean mosaic virus* (SMV) and included as outgroup

**Fig. 1** Mild mosaic in *Nicotiana debneyii* leaves caused by KER.JI.1 isolate (**a**). Vein banding and mosaic in *Datura metel* leaves due to infection with isolate KER.JI.1 (**b**)
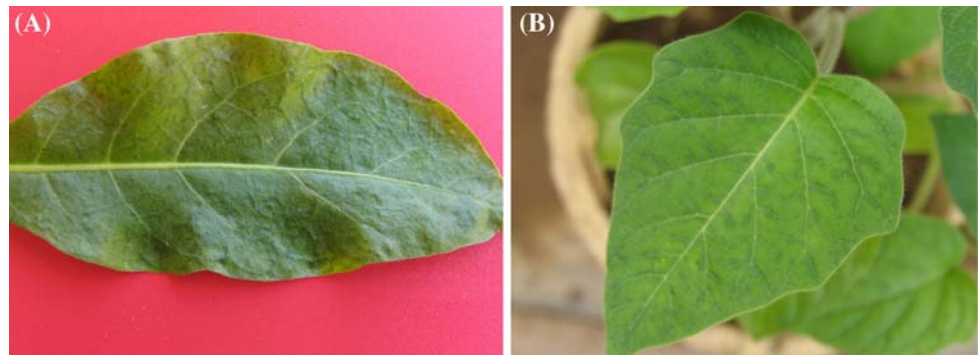
(YAZ.TA.1) only on the inoculated leaves of *C. pepo* and *C. melo*. None of the isolates infected *Citrllus lantanus*, *N. clevelendii*, *N. tabacum* cv. Samsun.NN., *N. glutinosa*, *Phaseolus vulgaris* and *Pisum sativum*.

Sequence data and identity matrix

The PCR-amplified fragment of approximately 822 bp was obtained by using primers WMV-F and WMV-R, when RNA isolated from 18 isolates was used as the template for the first-strand cDNA synthesis. The RT-PCR products were cloned and sequenced. Sequence information for these 18 WMV isolates has been submitted to NCBI-GenBank with the accession numbers from EU667627 to EU667644. The identity of WMV CP nucleotide sequences of the 18 Iranian isolates ranged from 92.7 to 99.9%. Sequence analysis demonstrated that 12 of the 18 isolates share 96.2–99.9% nucleotide homology with each other. The Iranian isolate KER.JI.1 in group II displayed the lowest (92.7%) and the highest (93.5%) nucleotide sequence identity with the isolates ESF.GA.1 and MAL99.2 in subgroup $I_A$, respectively. The nucleotide sequence of HOR.HA.1 and YAZ.TA.1 isolates in subgroup $I_B$ showed the highest (94.9%) and the lowest (93.3%) nucleotide sequence identity with KER.JI.1, respectively. The two isolates, Habenaria from Japan and WMV-HLJ from China in subgroup $I_C$, had the highest (95.5%) and the lowest (92.2%) nucleotide sequence identity with isolate KER.JI.1, respectively.

Identity levels in the amino acid of 14 Iranian isolates ranged between 97.4 and 100% (Table 5). The KER.JI.1 and YAZ.SH.1 isolates showed 91.6–93.1% and 92–93.4% identity at the amino acid sequence level with two other Iranian isolates YAS.TA.1 and YAZ.MO.I, respectively. The DAG box (Asp-Ala-Gly), which is believed to be crucial for potyvirus transmission by aphid, was found in the coat protein.

Phylogenetic analysis

To depict the diversity of the sequences, phylogenetic tree was generated using the 18 Iranian WMV CP sequences with representative sequences present in GenBank. WMV CP sequences formed two different groups with a strong boot-strap value (98%) estimated from 1000 resamplings (Fig. 2). Seventeen of the Iranian isolates fell into group II, which can be divided into two subgroups A and B with a bootstrap value of 100%. The subgroup $I_B$ is a new subgroup and only four Iranian isolates from two provinces (Yazd and Hormozgan) were classified in this subgroup. The majority of the Iranian isolates fell into the large subgroups $I_A$ with bootstrap values of 74–93%. The Iranian isolates YAZ.SH.1, KER.KE.1, and ESF.GA.3 were placed in a distinct but smaller sub-group, 7 out of 13 isolates in subgroup $I_A$ were collected from different regions of Esfahan province formed a distinct small group according to the geographical region. The remaining three Iranian isolates, URO.NA.1, URO.OS.1, and ESF.GA.1, were placed in a distinct but larger group that included the majority of Spanish isolates.

Group II contained only the KER.JI.1 isolate that was distant from Iranian isolates of group II (92.7–94.9% nucleotide and 92–93.4% amino acid identities). Comparison 778 nt segment comprising the CP gene of the KER.JI.1 isolate with those of two isolates from Spain (BAD95.2 and SG99.1), one from USA (D13913), and one from Tonga (L22907) clustered together in group II showed 96.6, 96.6, 96.2, and 95.5% nucleotide and 94.9, 94.2, 95.99, and 94.5% amino acid sequence identities, respectively. Thirty-six isolates clustered in group I: the sequences of 11 isolates from Spain and the 17 isolates from Iran were 93.9–97.2% identical at the nucleotide and 97.1–98.2% at the amino acid identity levels.

Phylogenetic analysis based on 200 nucleotides coding for the N-terminal part of the CP with other isolates obtained from GenBank showed three distinct groups. Seventeen Iranian isolates clustered in group 1. There was only one Iranian isolate (KER.JI.1) within group 2 (Fig. 3).

**Discussion**

WMV is the most prevalent potyvirus infecting cucurbits in Iran [16, 21] and other countries [10, 14]. WMV has a

**Table 5** Comparison of the percent nucleotide (top and right) and amino acid (bottom and left, in bold) identities among the 778 nt CP sequences of WMV isolated from Iran

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. ESF.ES.1 | * | 99.5% | 99.3% | 98.7% | 99.9% | 98.4% | 99.5% | 99.5% | 99.8% | 96.5% | 93.2% | 98.4% | 98.9% | 98.3% | 94.8% | 95.3% | 98.5% | 94.3% |
| 2. ESF.ES.2 | 100% | * | 99.5% | 98.4% | 99.6% | 98.2% | 99.5% | 99.3% | 99.8% | 96.2% | 92.9% | 98.2% | 98.7% | 98.1% | 94.5% | 95.0% | 98.3% | 94.0% |
| 3. ESF.ES.3 | 99.6% | 99.6% | * | 98.2% | 99.4% | 97.9% | 99.3% | 99.0% | 99.5% | 96.0% | 92.7% | 97.9% | 98.4% | 97.8% | 94.3% | 94.8% | 98.1% | 93.9% |
| 4. ESF.GA.1 | 98.5% | 98.5% | 98.2% | * | 98.8% | 97.8% | 98.4% | 98.4% | 98.7% | 96.6% | 93.3% | 97.8% | 99.0% | 98.4% | 95.1% | 95.6% | 97.9% | 94.6% |
| 5. ESF.GA.2 | 100.0% | 100.0% | 99.6% | 98.5% | * | 98.5% | 99.6% | 99.6% | 99.9% | 96.6% | 93.3% | 98.5% | 99.0% | 98.4% | 94.9% | 95.4% | 98.7% | 94.4% |
| 6. ESF.GA.3 | 99.3% | 99.3% | 98.9% | 98.5% | 99.3% | * | 98.2% | 98.5% | 98.4% | 96.1% | 92.8% | 98.8% | 98.3% | 97.4% | 94.6% | 95.1% | 98.3% | 94.2% |
| 7. ESF.ZA.1 | 100.0% | 100.0% | 99.6% | 98.5% | 100.0% | 99.3% | * | 99.3% | 99.8% | 96.2% | 92.9% | 98.2% | 98.7% | 98.1% | 94.5% | 95.0% | 98.7% | 94.0% |
| 8. ESF.ZA.2 | 99.3% | 99.3% | 98.9% | 97.8% | 99.3% | 98.5% | 99.3% | * | 99.5% | 96.2% | 92.9% | 98.2% | 98.7% | 98.3% | 94.8% | 95.0% | 98.3% | 94.0% |
| 9. ESF.ZA.3 | 100.0% | 100.0% | 99.6% | 98.5% | 100.0% | 99.3% | 100.0% | 99.3% | * | 96.5% | 93.2% | 98.4% | 98.9% | 98.3% | 94.8% | 95.3% | 98.5% | 94.3% |
| 10. HOR.HA.1 | 98.5% | 98.5% | 98.2% | 99.3% | 98.5% | 98.5% | 98.5% | 97.8% | 98.5% | * | 94.9% | 96.1% | 96.7% | 96.2% | 97.6% | 98.1% | 96.2% | 97.3% |
| 11. KER.JI.1 | 92.7% | 92.7% | 92.3% | 92.7% | 92.7% | 92.7% | 92.7% | 92.0% | 92.7% | 92.7% | * | 93.4% | 93.4% | 92.7% | 93.9% | 94.4% | 93.4% | 93.3% |
| 12. KER.KE.1 | 99.3% | 99.3% | 98.9% | 98.5% | 99.3% | 99.3% | 99.3% | 98.5% | 99.3% | 98.5% | 92.7% | * | 98.1% | 97.4% | 94.6% | 95.1% | 98.7% | 94.3% |
| 13. URO.OS.1 | 98.2% | 98.2% | 97.8% | 98.9% | 98.2% | 98.9% | 98.2% | 97.4% | 98.2% | 98.9% | 93.1% | 98.2% | * | 98.7% | 95.0% | 95.5% | 98.2% | 94.5% |
| 14. URO.NA.1 | 98.9% | 98.9% | 98.5% | 99.6% | 98.9% | 98.9% | 98.9% | 98.2% | 98.9% | 99.6% | 93.1% | 98.9% | 99.3% | * | 94.5% | 95.0% | 97.6% | 94.0% |
| 15. YAZ.MO.1 | 97.4% | 97.4% | 97.1% | 98.2% | 97.4% | 97.4% | 97.4% | 97.4% | 97.4% | 98.2% | 92.0% | 97.4 | 97.8% | 98.5% | * | 99.3% | 95.0% | 97.8% |
| 16. YAZ.MO.2 | 98.2% | 98.2% | 97.8% | 98.9% | 98.2% | 98.2% | 98.2% | 97.4% | 98.2% | 98.9% | 92.7% | 98.2% | 98.5% | 99.3% | 98.5% | * | 95.5% | 98.3% |
| 17. YAZ.SH.1 | 95.3% | 95.3% | 94.9% | 94.5% | 95.3% | 95.3% | 95.3% | 94.5% | 95.3% | 94.5% | 92.7% | 95.3% | 94.2% | 94.9% | 93.4% | 94.2% | * | 94.5% |
| 18. YAZ.TA.1 | 97.1% | 97.1% | 96.7% | 97.8% | 97.1% | 97.1% | 97.1% | 96.4% | 97.1% | 97.8% | 91.6% | 97.1% | 97.4% | 98.2% | 96.7% | 97.4% | 93.1% | * |
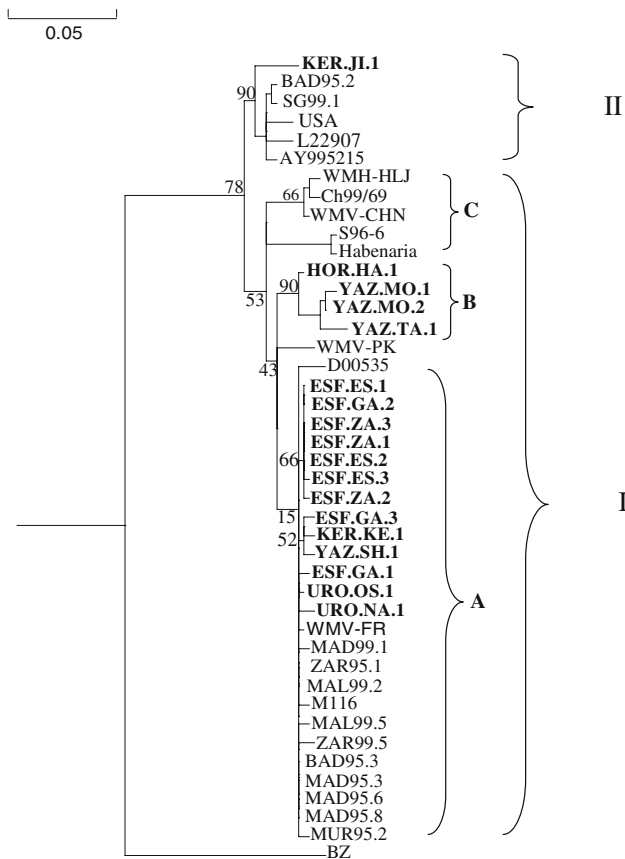
**Fig. 2** Neighbor-joining tree illustrating the Phylogenetic relationships between the Iranian WMV isolates (Table 1) and other published WMV isolates (Table 3). The tree was drawn using the 778 bp CP sequences. Multiple sequence alignments of the nucleotide sequences were generated with DNAMAN program (Version 5.2.2; Lynnon Biosoft, Quebec, Canada). Numbers associated with nodes represent the percentage of 1000 bootstrap interactions supporting the nodes. The Iranian isolates are shown in bold. BZ is SMV and included as outgroup

**Fig. 3** Neighbor-joining tree illustrating the phylogenetic relationships between the Iranian WMV isolates (Table 1) and other published WMV isolates (Table 4). The tree was drawn using 200 nucleotides coding for the N-terminal part of the CP. Multiple sequence alignments of the nucleotide sequences were generated with DNAMAN program (Version 5.2.2; Lynnon Biosoft, Quebec, Canada). Numbers associated with nodes represent the percentage of 1000 bootstrap interactions supporting the nodes. The name of Iranian isolates is shown in bold letters. BZ is SMV and included as outgroup

relatively wide host range infecting plant in 23 families of dicotyledonous plants, and occurs naturally in cucurbit and legume crops [20]. In Iran, WMV has not been detected in non-cucurbit plants, except for a few weeds [16], and cucumber, watermelon, squash, and melon are by far the most important crop affected. Hence, the WMV population could be well represented by isolates recovered from cucumber, squash, and melon. Cucurbits in fields and greenhouses are grown in southwest, central, and southeast of Iran. In this work, care was also taken that most of the geographic areas and agroecological conditions where these cucurbits are grown be represented.

Phylogenetic trees were created using neighbor-joining (DNAMAN) and max parsimony methods for either 200 nt including NIb-CP gene fragment of WMV and 778 nucleotide long sequences of the coat protein gene. The cophentic coefficient of Fig. 2 which belong to group of
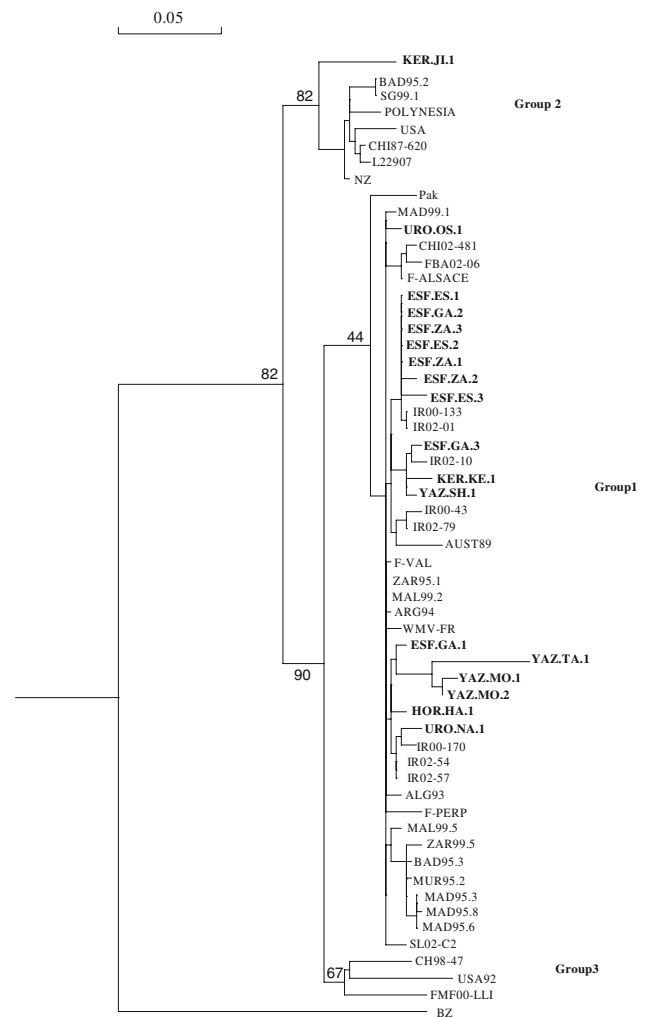
isolates based on 778 bp was 0.97, which means the high validity of this dendenograms.

In agreement with the published data [18], existence of at least two groups of WMV isolates can be demonstrated in some districts of Iran. Group I included three subgroups A, B, and C. Isolates in subgroup $I_A$ were the most frequently detected isolates worldwide and included most of the Spanish melon isolates and Iranian cucurbit isolates. Most Iranian isolates of this subgroup were from melon (*C. melo*), cucumber (*C. sativus*), pumpkin (*Cucurbita moschata*), winter squash (*C. maxima*), and summer squash (*C. pepo*) (Table 1).

There were several distinct sub-clusters that could be related to geographical areas rather than to the host plants. For example, isolates collected from Esfahan (ESF.ZA.1, ESF.ZA.3, ESF.ES.3, ESF.ES.2, ESF.GA.2, ESF.ES.1, and ESF.ZA.2) clustered in subgroups $I_A$, whereas isolates from Yazd province (YAZ.MO.1, YAZ.MO.2, and YAZ.TA.1) gathered in subgroups $I_B$. Nonetheless, the subgroup $I_A$ was comprised of some sub-clusters, which did not strictly follow the geographical origin of the isolates. For instance, isolates ESF.GA.3 from Esfahan fell into a sub-cluster separate from the other isolates from this province. These data would imply movement of WMV isolates between or into provinces.

Subgroup $I_B$ also contains three out of four isolates collected from Yazd province. This subgroup, being reported for the first time herein, is comprised of only four Iranian isolates collected from summer squash, cucumber, and melon. According to the results of this study and those of Moreno et al. [18], the isolates of group I are the most widespread isolates of WMV.

Adams et al. [1] propose that 76–77% nt sequence identity at the CP is the optimal species demarcation criterion for potyviruses. Since the CP nt sequence identities between the WMV isolates were in the range of 92.7–99.5% (Table 5), all isolates studied here can confidently be assigned to one virus species, WMV.

Host range and symptoms have been used for the identification and differentiation of strains and pathotypes of viruses [26, 30]. Sap inoculation of 18 isolates of WMV onto a range of plants revealed differences in symptoms and host range. Some isolates showed similar biological properties, suggesting that they are closely related; others appeared to be exceptional strains, and these observations seemed to be consistent with their phylogenetic grouping. Isolates ESF.ZA3 and ESF.ES.2 were not only similar in symptoms and host range but also shared a CP amino acid sequence identity of 100%. YAZ.SH.1, KER.KE.1, and ESF.GA.3 isolates also caused mild symptoms on *C. pepo* and *C. melo*, and all are in the same sub-cluster of $II_B$. Based on reactions to the two test plants *C. pepo* and *C. melo*, the Iranian WMV isolates were clustered in three groups. The first group caused severe mosaic, leaf distortion, and vein clearing. The second group produced mild mosaic symptoms on both test plants and the third group depending on WMV isolates induced varying degrees of mosaic and vein banding and vein clearing. But the reaction of WMV isolated that was reported by Moreno et al. [18] on these two test plants was different. All mentioned isolates caused systemic mosaic and leaf lamina deformation in *C. melo* and systemic mosaic, leaf lamina deformation, and vein clearing in *C. pepo*. Therefore, the differences on symptoms may be related to cultivars of these two test plants.

Of particular interest is the case of KER.JI.1: although it was collected from the weed Colocynth, it had a varied host range and infected both *N. debneyii* and *D. metel* systemically. However, none of the other 17 Iranian isolates could infect these two test plants. In spite of different host range of this isolate, the CP amino acid sequence identity (>92%) of KER.JI.1 with other WMV isolates indicated that is an isolate of WMV, because the CP amino acid sequence identity among potyvirus isolates and between different potyviruses is >90% and <70%, respectively [2, 25]. Therefore, nucleotide sequence identity showed that KER.JI.1 is a unique isolate of WMV. We have been unable to ascertain the exact origin of this isolate but our results clearly show that it is more closely related to the Tonga, two Spanish, and American (US) isolates than to the other Iranian isolates of WMV (Fig. 2). The comparison made in the present work only reflects about one eleventh of the total genome of WMV; presumably, there are other differences in the remaining part of the genome of this isolate. Comparisons involving a full-length genome of this isolate are needed to clarify this. The other point is that no information about the reaction of other isolates placed them in group I is on *N. debneyii* and *D. metel*.

Phylogenetic analysis based on previous analysis of the 273 nt including NIb-CP gene fragment of WMV isolates [8] has shown three distinct molecular groups. In this article, phylogenetic analysis based on 200 nucleotides coding for the N-terminal part of the CP showed that all Iranian isolates clustered with previously reported WMV isolates in group I, whereas the KER.JI.1 isolate clustered in group 2 (Fig. 3). As mentioned by Desbiez et al. [8] also these three groups correlated with one motif at the N-terminal extremity of the CP. All Iranian isolates except KER.JI.1 and URO.OS.1 had a "KEA" motif at positions 3–5 in the CP. These two Iranian isolates (KER.JI.1 and URO.OS.1) had the "KET" motif instead of "KEA".

The presented data provide useful information concerning WMV isolates found in Iran. These findings may be particularly relevant in light of recent reports of the presence of WMV in Spain. A more exhaustive analysis of pathogenicity determinants and vectors present in the same surveyed area should be carried out, considering that all of the Iranian isolates had the motif responsible for aphid transmission, which is similar to other aphid-transmissible potyviruses [3, 12].

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