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Host range and molecular characterization of an Iranian isolate of alfalfa mosaic virus

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Abstract

Alfalfa mosaic virus (AMV, *Alfamovirus AMV*) is the type species of the genus in the family *Bromoviridae* and is transmitted in non-persistent manner by aphids. Based on the previous studies, four Iranian AMV isolates collected in Khuzestan Province phylogenetically were clustered into a distinctive group. One out of the four isolates (Kh.Be.A) was selected for further studies. This isolate was biologically characterized by inoculating a range of test plants from *Solanaceae*, *Fabaceae* and *Chenopodiaceae* families. Among inoculated plants, AMV induced symptoms on *Nicotian glutinosa* L., *N. debneyii* L., *Datura maxima* L. and *D. stramonium* L.. In molecular studies, the total length of the P2 gene of AMV was amplified using three specific primer pairs in the RT-PCR test and sequenced to be 2418 nucleotides. Sequence comparison of the P2 gene with counterpart sequences available in the GenBank showed that the AMV isolates are classified into three groups. Members of each group were divided into two subgroups, A and B. Group IIA comprise Iranian isolates together with two Australian isolates (LC485016.1 and MK913781.1). Sequence comparison of the P2 gene of the Kh.Be.A isolate with those of the two isolates- shared 96.8 and 95% nucleotide and 96.6 and 93.7% amino acid sequence identities, respectively. According to the result of this study, phylogenetic analysis based on the P2 gene indicates three main groups of AMV isolates occurring in different countries and plant hosts.

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Introduction

Alfalfa mosaic virus (AMV) is one of the most important plant viruses that was first reported by Weimer in 1931 from alfalfa (*Medicago sativa*) in the United States of America as the causal agent of mosaic disease in this plant (Hull, 1969). This virus has a wide host range and infects more than 600 plant species belonging to 245 genera of 70 families (Wang et al., 2023), most of which belong to the legume family (Jaspars & Bos, 1980). The majority of plant species infected by this virus belong to the *Fabaceae*, *Solanaceae*, *Compositae*, and *Umbelliferae* families (Crill et al., 1970; Smith, 1972; Miczynski & Hiruki, 1987, Wang et al., 2023). AMV causes symptoms such as mottle, chlorosis between the lateral veins of the leaves, vein clearing, leaf deformation, necrosis and death of the plant. In sensitive varieties, symptoms include severe dwarfism, malformation and chlorotic and mosaic stripes especially in newly grown leaves. The virus also affects the nodulation of plants, causing a decrease in plant vigor and a significant decrease in yield (Tu & Holmes, 1980).

AMV, the single member of the genus *Alfamovirus* (Bujarski et al., 2012) has single-stranded, positive-sense RNA, consisting of three genomic (RNA1, RNA2 and RNA3) and a fourth subgenomic RNA (sgRNA) (Jung et al., 2000). RNA1 and RNA2 encode the viral replicase proteins P1 and P2, respectively, and RNA3 encode the movement protein (MP) as well as coat protein (CP) and serves as a template for the synthesis of subgenomic RNA4, which encodes the CP. All three genomic RNAs of AMV are flanked by 5′ and 3′ UTRs, which are highly structured and presumed to

contain important cis-acting regulatory elements involved in translation and replication (Vlot et al., 2001). The 3′ terminal 145 nts of the three genomic RNAs preceded by 18 to 34 nts unique for each RNA can form an almost identical secondary structure (Vlot & Bol, 2003). Between the two ORFs in RNA3, a non-coding intergenic region contains approximately 250 nucleotides. In this intergenic region, there is an internal Poly A sequence, which ends at a distance of 20 bases from the 5′ region of the CP gene. It has also been determined that the first 9 bases of RNA4 include the last nine bases of the intergenic region (Langereis et al., 1986).

So far, several strains of AMV, which are biologically different and molecularly divided into at least two groups, have been reported in the world (Hull, 1969; Parrella et al., 2011; Xu & Nie, 2006). According to the previous studies based on the nucleotide sequence of the MP gene of AMV, one isolate of the virus (Kh.Be.A) is different from the other Iranian isolates (Alipour et al., 2021). This study further investigates the host range and molecular analysis of the isolate.

Materials and Methods

The source of the virus isolate

According to previous molecular studies (Alipour et al., 2021), the Kh.Be.A isolate, which was stored in the virus source of the Department of Plant Pathology, Faculty of Agriculture, Shahid Bahonar University, Kerman, was selected. An indirect ELISA test was performed based on the Clark and Adams (1977) method, using AMV polyclonal antibody, and the Kh.Be.A isolate showed a positive reaction to AMV infection. As a result, for further molecular and biological studies, the mentioned isolate was propagated in greenhouse conditions on host plants of AMV. N. *tabacum* cv. Samsun and *N. glutinosa* were used as propagation hosts for the Kh.Be.A isolate in this study.

RNA extraction and RT-PCR

In order to perform the polymerase chain reaction test, total RNA was extracted from tobacco plants (*N. glutinosa*) that were infected with Kh.Be.A isolate using a High Pure Viral Nucleic Acid Kit (Roche Biochemical, Germany). First-strand cDNA synthesis was performed using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase by revers primers as described previously by Sharifi et al., (2008) (Table 1). The resulting viral cDNAs were used for consecutive PCR reactions with appropriate primers designed by FAST-PCR software based on HZ strain (HQ316636.1) (https://primerdigital.com/fastpcr.html) (Table 1). The following thermal cycling conditions were used: initial denaturation at 94°C for 60 s, followed by 30 cycles of denaturation at 94°C for 60 s, annealing at appropriate temperatures of each primer for 60 s, and extension at 72°C for 60 s, and a final 10 min extension at 72°C. The PCR products were subsequently analyzed using 1% agarose gel electrophoresis.

Table 1. PCR primers used for the detection and amplification of RNA2 of the isolate Kh.Be.A

Primer	Sequence (5´-3´)	Genomic location	Annealing temperature (°C)
AMV1 F	TCGCGATTGAAAAGATAAGT	12-32	56
AMV1 R	AGGTATCATCCTCCCCAACGT	841-862	
AMV30 F	TGGATGACGTTGGGGAGGATG	845-856	56-58
AMV30 R	CTTGCATCAAAGGCTTCAGCA	1612-1623	
AMV52 F	GCTGAAGCCTTTGATGCAAGT	1613-1624	56
AMV52 R	CATCAAGCTCGGCGTGCCCTTG	2408-2430	

Sequencing, phylogenetic analysis and genetic diversity

PCR products were sequenced directly at Macrogen Inc in two directions (South Korea) and aligned using the Blast tool in the NCBI database (http://www.ncbi.nlm.nih). Alignments with AMV isolate sequences available in GenBank and those determined in this study were performed using the Neighbor-Joining algorithm in kimura 2-parameter (k2p) model at MEGA 7 with 1000 bootstrap (Kumar et al., 2016). The pairwise identities of nucleotide (nt) and amino acid (aa) were analyzed using the SDTv 1.0 program.

Host range assay

Thirteen plant species and cultivars from three families were used to determine the host range and identify the symptoms. The seeds of different plants were planted in small pots containing one-part sand and one part of leaf

soil and were kept in a temperature regulated insect-proof greenhouse. Then 0.5 grams of test plants leaves that showed positive AMV infection in the RT-PCR were extracted using 0.1 M potassium phosphate buffer and carborundum powder, and inoculated on the surface of the leaves of the test plants. After inoculation, the examined plants were kept in an insect-proof greenhouse at 15–25°C for symptom development. ELISA and symptomology were used to check for the presence of the virus in inoculated plants.

Results

RT-PCR assay

RT-PCR-amplified fragments of approximately 822, 785 and 827 bps were obtained using primer pairs AMV-F1/AMV-R1, AMV-F30/AMV-R30, AMV-F52/AMV-R52, respectively, and then sequenced. After determining the synonymy, different fragments are inserted, and the overlapping regions of the complete synonymy of the P2 gene are removed. The length of this gene for Kh.Be.A isolate was determined to be 2373 nts and deposited in GenBank with accession number OM864517.

Host range and phylogenetic analysis

In order to investigate the host range of the Iranian isolate of Kh.Be.A, test plants belonging to the three families *Solanaceae*, *Fabaceae* and *Chenopodiaceae* were used. Based on the results of RT-PCR and ELISA tests, the Kh.Be.A isolate infect all tested plants from the *Solanaceae* family (Table 2). The *N. glutinosa* and *N. debnyii* were systemically infected by the Kh.Be.A, and they showed yellowing, blistering and malformation symptoms, however, the inoculated *N. clevelandii*, *N. tabacum* cv. Turkish and *N. tabacum* cv. Samsun did not show any symptoms. *D. stramonium* and *D. maxima* reacted to the Kh.Be.A isolate, showing yellowing and local lesions in the inoculated leaves, respectively (Fig. 1).

This research assessed the evolutionary status of the AMV isolate (OM864517, Kh.Be.A) by analyzing it alongside 73 other isolate sequences available in GenBank. Based on the phylogenetic tree of P2 gene, AMV isolates were clustered into three groups (I, II and III), each further divided into subgroups A and B (Fig. 2). Four isolates from America (all from soybeans), one isolate from Canada (potato) and a several isolates reported from China clustered in subgroup IA. Two Iranian isolates IR-VM (MW014930.1, Lesser periwinkle) and IR-WS (MW014933.1, Wisteria) (Moradi & Mehrvar, 2021) along with five other isolates reported from different countries (England, Germany, Italy and China) were classified in subgroup IB. Based on the phylogenetic tree, the Iranian isolate Kh.Be.A along with four isolates LC485016.1 (Australia, chickpea), MK913781.1 (Australia, chickpea), KC881009.1 (Argentina, alfalfa) and MT951267.1 (China, alfalfa) were clustered in group IIA. The Kh.Be.A isolate also shared the highest nucleotide (98.3%) and amino acid (98.5%) identities with isolate 295 (Australia, MK913781.1).

Table 2. Symptoms induced by the Iranian isolate Kh.Be.A on selected plant species

Plant family	Test plant species	Symptoms
Solanaceae		
	Datura stramonium L	PG, LI
	Datura maxima L.	PG, NLL, LI
	Nicotiana glutinosa L.	PG, B, LD, LI
	Nicotiana debneyii L.	PG, B, LD, LI
	Nicotiana tabacum cv. Turkish	LI
	Nicotiana tabacum var Samsun NN.	LI
	Nicotiana Clevelendii Gray.	LI
Fabaceae		
	Phaseolus vulgaris	-
	Vigna unguiculata cv Mashhad.	-
	Vigna radiata	-
	Cicer arietinum	-
	Lens culinaris	-
Chenopodiaceae		
	Chenopodium amaranticolor Coste et Reyn	-

LD, leaf deformation; **NLL,** necrotic local lesions; **B,** blistering; **PG,** pale green; **LI,** latent infection; **-,** no infection

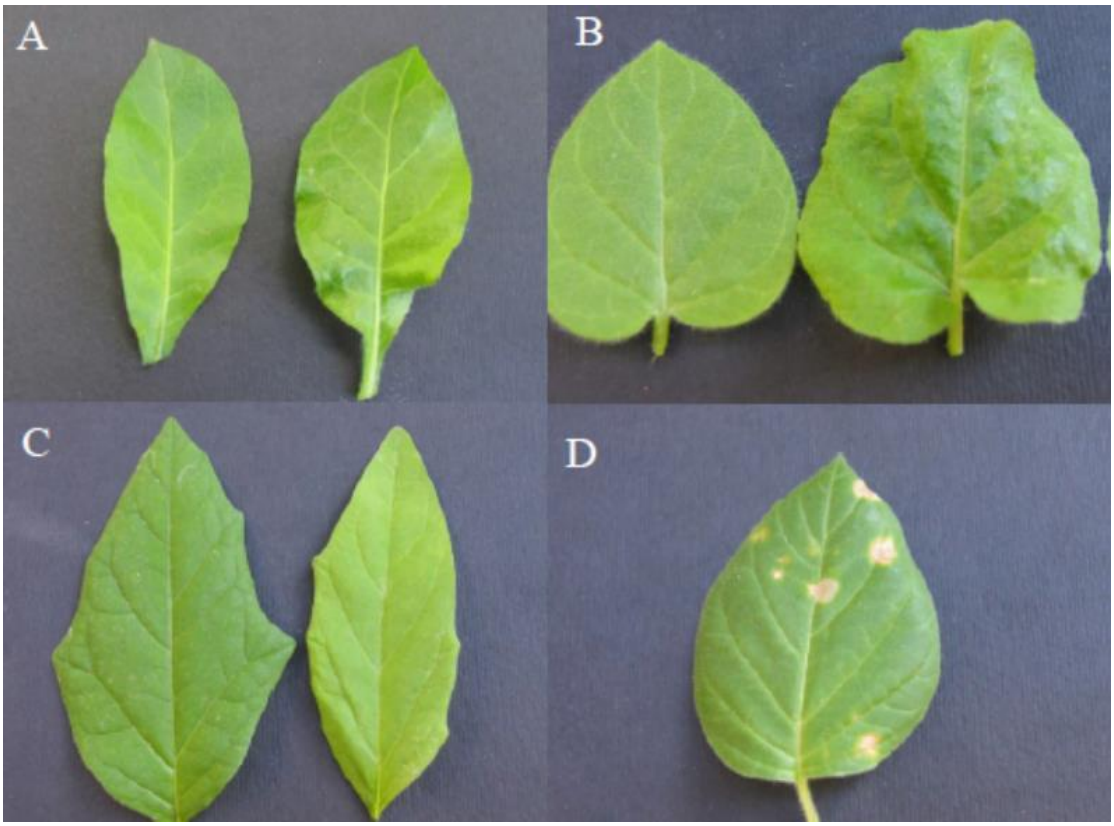

Fig. 1. Symptoms induced by Kh.Be.A isolate of AMV in inoculated test plants under greenhouse conditions. (A) Malformation and blistering in *Nicotiana debneyii* (right) and healthy plant (left). (B) Blistering and mosaic in *Nicotiana glutinosa* (right) and healthy plant (left). (C) Mild mosaic and clearing in *Datura stramonium* (left) and healthy plant (right). (D) Necrotic local lesion on *Datura maxima*.

On the other hand, isolates Tec1 (Spain, FR715041.1) and Yichuan_H7 (China, OL773505.1) shared the lowest nucleotide (95.3%) and amino acid (94.9%) identities with the Kh.Be.A isolate, respectively. In comparing three Iranian isolates, who's the P2 gene nucleotide identity was determined, Kh.Be.A isolate shared 96.3% nucleotide and amino acid identities with IR-VM and IR-WS isolates (Fig. 3). Subgroup IIB also includes eight isolates reported from China (alfalfa) and three isolates from Spain (FR715041.1, Cape honeysuckle), Italy (MT093210.1, Chayote) and Germany (MZ405630.1, *Nicotiana tabacum*). Furthermore, group IIIA includes 15 isolates, all reported from China and the alfalfa plant, except MT874920.1 isolated from *Impatiens balsamina* plant. Fifteen other isolates derived from different plants such as alfalfa, tobacco, potato, soybean and countries of China, Argentina, Netherlands, America and Canada were also included in IIIB subgroup.

Discussion

AMV has a relatively wide distribution, especially in the perennial alfalfa fields in Iran. The symptoms caused by this virus on the alfalfa plant depend on the growth stage of the plant, weather conditions, host variety and virus strain (Latham & Jones, 2001; Li et al., 2022; Amin et al., 2023; Wang et al., 2023). Host range and symptoms have been used to identify and differentiate strains and pathotypes of viruses (Shukla et al., 1994; Xiao et al., 1993). Based on biological studies, it was resolved that the isolate investigated in this research differed in host range compared to other Iranian isolates. Thus, the Kh.Be.A isolate induced symptoms such as mosaic and vein clearing only in *D. stramonium* L., *D. maxima* L., *N. glutinosa* L., and *N. debneyii* L., while remaining asymptomatic in most other test plants. According to the report of Mangeli et al. (2019), most Iranian isolates cause symptoms such as yellowing, formation of necrotic spots and severe mosaic in leaves, etc., on a wide range of test plants such as N. tabacum L. cv. Turkish, *N. tabacum* var Samsun NN., *N. tabacum* L. cv. White burly, *Lycopersicon esculentum* L. cv. Global, *Phaseolus aureus* (*Vigna radiata*) and

Lens culinaris Medic. According to the phylogenetic analysis of the complete CP sequence of AMV isolates (657 nucleotides), they were classified into two main groups, I and II (Parrella et al., 2000). Group II is also divided into two subgroups, IIA and IIB (Parrella et al., 2011).

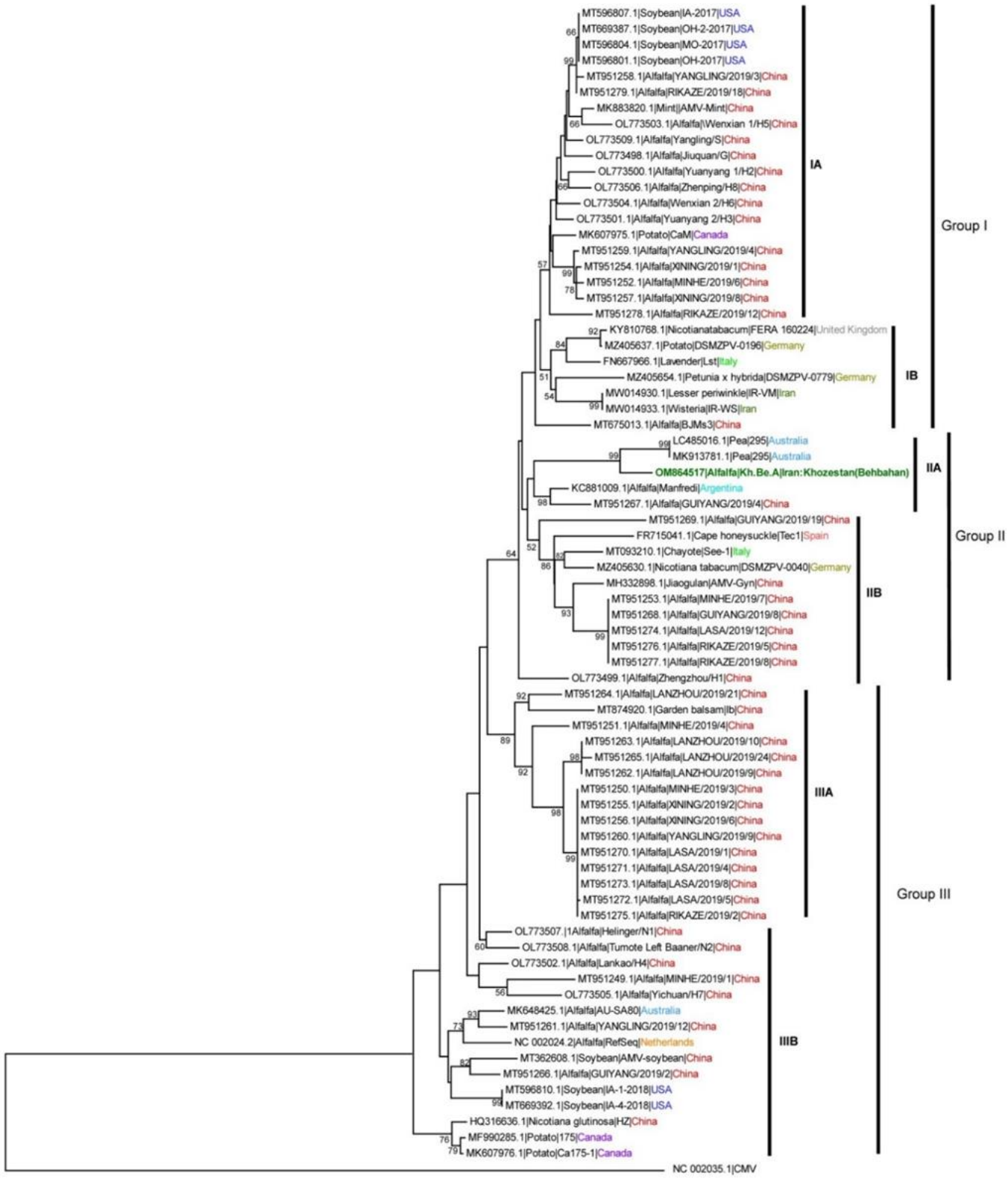

Fig. 2. Neighbor-Joining phylogenetic tree of the sequences P2 gene of the Kh.Be.A isolate of AMV and 73 GenBank isolates of the virus by MEGA 7. Numbers of branches represent percent bootstrap values (1,000 replicates) over 50. The Iranian isolate (Kh.Be.A) is shown in green color and bold. Cucumber mosaic virus (NC_002035) was chosen as outgroup.

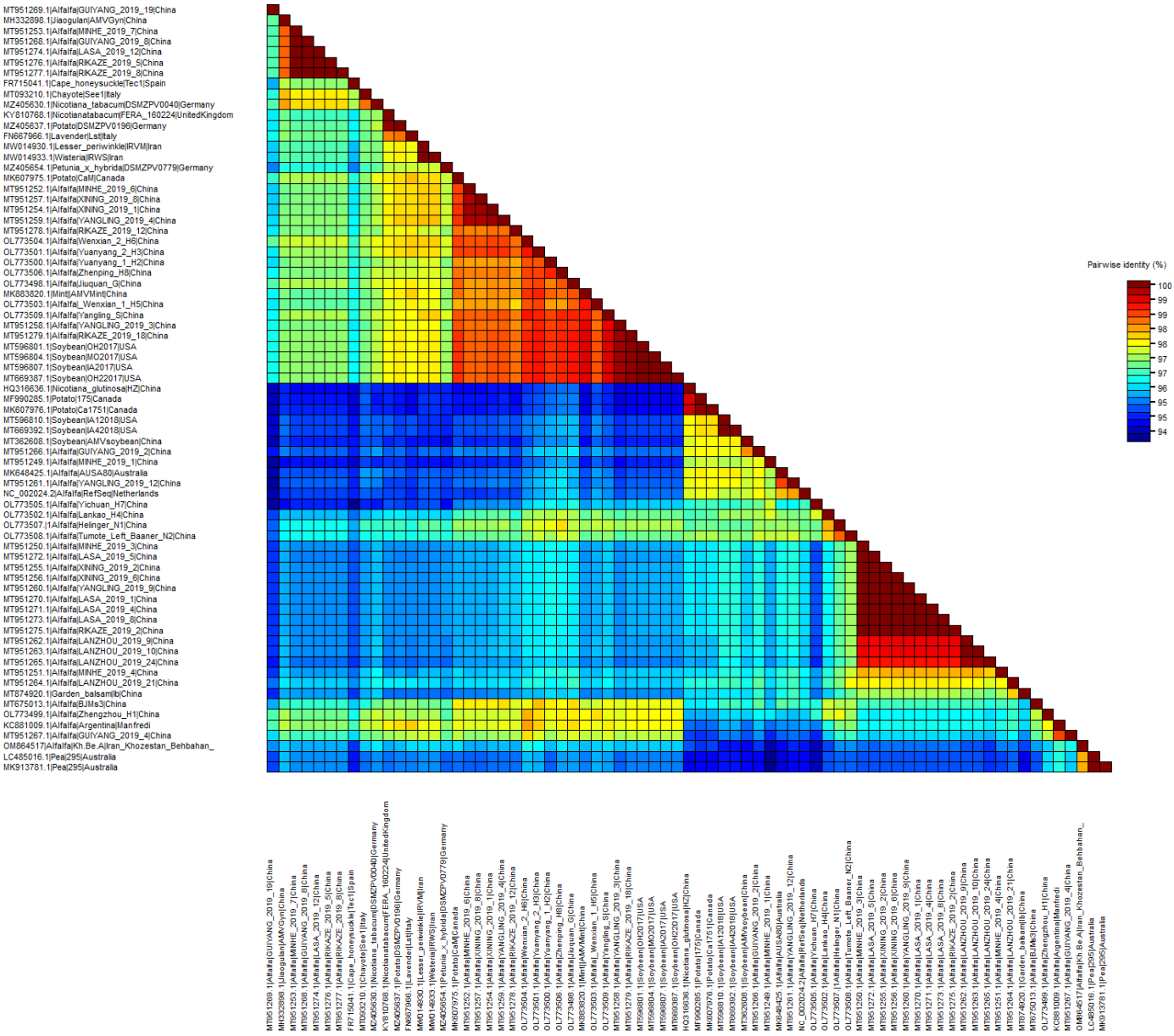

Fig. 3. Sequence-based pairwise identity of the AMV isolate (Kh.Be.A) identified in this study and some available isolates in GenBank using the SDT v1.2 program.

Before this study, two researches had been conducted in the field to investigate 59 Iranian isolates of alfalfa mosaic virus based on the CP gene in Iran (Mangeli et al., 2019; Alipour et al., 2021). The phylogenetic analysis of these Iranian isolates was conducted, and a comparison with the sequences available in GenBank, revealed that most of the isolates including worldwide and Iranian isolates (145 number) belong to group I, while only 28 other isolates are classified in the second group. According to these results, the isolates definitive in group IIB (Parrella et al., 2011) are considered group III. However in this grouping, four Iranian isolates (Ke.Ma.A, Kh.Be.A, Kh.Ah.A and Si.Ni.A) were clustered in a distinct subgroup from the other isolates. This study aimed to further investigate the biological and molecular properties of the Kh.Be.A strain, which

diverges from other Iranian strains in its CP and MP genes (Mangeli et al., 2019; Alipour et al., 2021). According to Codoner et al. (2015), regarding the use of nucleotide sequence of whole genome, to identify the phylogenetic relationships between members of the family Bromoviridae, the present study attempted to determine the sequence of the RNA2 genome of strain Kh.Be.A, which contains the P2 gene. The position of the mentioned strain in the phylogenetic tree was further investigated. Our phylogenetic analysis based on the P2 gene indicates three main groups of AMV isolates occurring in different countries and plant hosts. AMV isolates have been reported in China (Fig. 2). No correlation was observed between AMV isolates, the geographical origin of host plants, and variability in the P2 gene sequence. Sequence data of the Kh.Be.A isolate suggests this isolate is most closely related to two Australian isolates from chickpea (Maina et al., 2019). Considering that the alfalfa plant is native to the Near East and Central Asia and the AMV was identified in alfalfa as the main hosts (Sun et al., 2019), the virus may have spread through the global trade of alfalfa material to Australia.

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Conflict of interest

The authors declare that there are no conflicts of interest present

CRediT author statement

N. Pourseyyedi: Field and laboratory works. **H. Massumi:** Supervision, methodology, writing, reviewing & editing. **J. Heydarnejad** and **A. Hosseinipour:** Counseling, reviewing & editing. **M. Maddahian:** Data analyzing and editing.

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