Eur J Plant Pathol
https://doi.org/10.1007/s10658-021-02253-4

Molecular phylogenetic analysis shows that causal agent of maize rough dwarf disease in Iran is closer to rice black-streaked dwarf virus

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Accepted: 4 March 2021

Abstract Maize rough dwarf disease (MRDD) is one of the most important viral diseases around the world. Rice black-streaked dwarf virus (RBSDV) and maize rough dwarf virus (MRDV) cause MRDD in East Asia and Mediterranean regions, respectively. RBSDV and MRDV are similar in many features such as host range, disease symptoms, vector, and serological relationships, which make it hard to differentiate them based on serological and biological properties. However, molecular analyses as powerful tools could be used for differentiating species of fijiviruses. In this study, the complete genome sequence, genetic variation of 13 isolates using the nucleotide sequence of segment 10 ORF, and host range of MRDD causal agent in Iran were determined. Whole-genome comparisons and phylogenetic analysis showed that Iranian isolate shared high nucleotide sequence identities ranging from 86.5–91.3% and closest evolutionary relationship to the RBSDV Chinese isolates. The Iranian isolates showed over 99% homology based on amino acid sequence of ORF10 and were grouped in one clade through phylogenetic analysis. The results revealed that ORF10 of MRDD causal agent was under negative selection. Iranian isolate of MRDD was experimentally transmitted to maize, rice, barley, wheat, millet, oat, rye, and some grasses by *Loadelphax striatellus* with about 32% efficiency. The results of this study revealed that the MRDD causal agent in Iran had the closest genetic similarity to RBSDV demonstrating it is closer to RBSDV than MRDV.

Keywords Maize rough dwarf disease · Sequence analysis · Rice black-streaked dwarf virus · Transmission

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Introduction

Maize rough dwarf disease (MRDD) is one of the most important diseases caused by fijiviruses and leads to severe yield losses in maize (Lenardon et al. 1998; Zhang et al. 2001; Dovas et al. 2004; Achon et al. 2015). The infected plants show dwarfing, leaf darkening, vein enations on the undersurface of leaves, no flower and ear formation (Milne and Lovisolo 1977). So far, four viruses have been reported as causal agent of MRDD around the world including maize rough dwarf virus (MRDV) in Europe (Dovas et al. 2004; Mingfang et al. 2016; Svanella-Dumas et al. 2016), mal de Rio Cuarto virus (MRCV) in South America (Distefano et al. 2003), rice black-streaked dwarf virus (RBSDV)

Published online: 08 March 2021

(Isogai et al. 2001; Bai et al. 2002) and southern rice black-streaked dwarf virus (SRBSDV) in East Asia (Zhou et al. 2008).

The plant-infecting members of the *Reoviridae* family were grouped into three genera *Phytoreovirus*, *Fijivirus*, and *Oryzavirus* based on virion morphology, the number of genome segments, serological relationship and insect vector specificity. Fijiviruses have icosahedral particles approximately 70—80 nm in diameter containing ten genome segments of linear double-stranded RNAs named S1 to S10 based on their size and mobility in polyacrylamide gel electrophoresis (Nuss and Dall 1990). The current eight species of fijiviruses are classified into five groups based on the host range, insect vector, serological relationship, and sequence homology. MRDV, RBSDV, SRBSDV, and MRCV are included in group 2 (Attoui et al. 2012).

MRDV was first reported in Italy in the late 1940s (Boccardo and Milne 1984). It is responsible for MRDD in Italy, Spain, France, and Greece (Dovas et al. 2004; Mingfang et al. 2016; Svanella-Dumas et al. 2016; Achon et al. 2015). MRDV naturally infects maize but not rice and causes severe and economically significant disease in Europe (Milne and Lovisolo 1977; Boccardo and Milne 1984; Dovas et al. 2004). It is transmitted by the planthopper *Laodelphax striatellus* Fallen (Hemiptera: *Delphacidae*) in a persistent and propagative manner and transmitted through eggs (Milne and Lovisolo 1977). RBSDV causes black-streaked dwarf symptoms in rice, and rough dwarf symptoms in maize (Isogai et al. 2001; Bai et al. 2002). It is transmitted by *Laodelphax striatellus* in a persistent and propagative manner, but not transmitted through eggs (Milne and Lovisolo 1977). RBSDV was first observed in Japan in the 1950s (Kuribayashi and Shinkai 1952). To date, RBSDV has been reported in Japan, China, South Korea, and other Asian countries (Isogai et al. 2001; Bai et al. 2002). It infects gramineous host species and some plants in Asteraceae and Amaranthaceae families (Yang and Ma 1983). SRBSDV or rice black-streaked dwarf virus 2 (RBSDV-2) was reported from rice in 2001 from China. It naturally infects rice, maize, sorghum, and other gramineous weeds. SRBSDV was first considered as an isolate of RBSDV due to its symptoms and serological similarity. However, differences between the viruses in their genomes and insect vectors, identified the virus as a novel fijivirus in group 2. It is transmitted by the white-backed planthopper *Sogatella furcifera* Horváth (Hemiptera: *Delphacidae*) in a persistent and propagative manner (Zhou et al. 2008). MRCV was first recorded in the 1976 in maize fields of Rı'o Cuarto (C'ordoba, Argentina). It causes the most critical maize disease in Argentina (Lenardon et al. 1998). It is not able to infect rice in nature. MRCV is transmitted by *Delphacodes kuscheli* Fennah (Hemiptera: *Delphacidae*) in a persistent and propagative manner (Lenardon et al. 1998; Distefano et al. 2003).

MRDV, RBSDV, SRBSDV, and MRCV belonging to *Fijivirus* group 2 in the family *Reoviridae* (Attoui et al. 2012) and share common characteristics including particle morphology, genomic profile, serological relationships, and symptoms on maize (Milne and Luisoni 1977). It has been suggested that these four viruses considered as geographical races of same species (Azuhata et al. 1993).

In Iran, MRDD is an important and economic maize disease. The symptoms were first observed in 1981. The causal agent of MRDD based on serological relationships and biological properties considered as MRDV (Izadpanah et al. 1983). However, to date, no comprehensive studies have been done on the molecular properties of the MRDD causal agent. In the present research, we obtained the complete genome of MRDD causal agent by Illumina sequencing method and investigated its experimental host range and the genetic variation of virus isolates based on ORF10 sequence. These analyses help to comprehend the etiology of MRDD in Iran and the relationship among members of fijiviruses group 2.

Materials and methods

Plant material

A total of 68 maize plants with symptoms of dwarfing and enation on the lower side of leaves were collected from three maize growing provinces Isfahan with 50 samples, Chaharmahal-o-Bakhtiari with 10 samples and Fars with eight samples during 2016–2017.

RNA extraction and cDNA synthesis

Total RNA was extracted from infected leaves, as described by Uyeda et al. (1998). The RNA was electrophoresed on 1.5% agarose gels in TBE (Tris-borate EDTA buffer) running buffer to assess the quality and quantity of the RNA. Total RNA was used as a template

for cDNA synthesis. To identify causal agent of MRDD in maize, three primer pairs including RBSDVS10F/RBSDVS10R (Bong-Choon et al. 2005), MRS6–1/MRS6–2 (Hengmu et al. 2001) and MRDV-F1/MRDV-R2 (Dovas et al. 2004) were used in reverse transcription-polymerase chain reaction (RT-PCR) (Table 1). cDNA synthesis was primarily performed in 10 μl reaction mixtures containing 5 μl total RNA, 1 μl reverse primer (10 pmol/μl) and 4 μl sterile distilled water. The mixture was incubated at 70 °C for 10 min and immediately chilled on ice. Then 4 μl RT-buffer (5X), 0.5 μl dNTPs (10 mM), 0.25 μl M-MuLV reverse transcriptase (200 units/μl) and 5.25 μl sterile distilled water were added to each reaction followed by incubation at 42 °C for 1 h.

PCR and sequencing

For the amplification of the target segments, synthesized cDNA was used as a template in a 20 μl PCR reaction containing 4 μl cDNA, 7 μl 2X Master mix, 1 μl of each forward and reverse primers (10 pmol/μl) and 7 μl sterile distilled water. PCR products were run on 1% agarose gel in TBE running buffer. A total of 11 samples representing different regions were sent to the Macrogen Company (Seoul, South Korea) for Sanger sequencing. The sequences were analyzed by the BLASTn program available at www.ncbi.nlm.nih.gov with an e-value threshold of 10e-20 (Altschul et al. 1997).

Genome data

RNA preparation for transcriptome sequencing

Based on the results of virus detection explained in section 1, causal agent of MRDD from Isfahan province (RBSDV-Ir) was selected for RNA sequencing. Hence, total RNA was extracted by the P-biozol reagent and RNeasy column (Qiagen kit). Briefly, 50–100 mg of the leaf was homogenized in 1 ml P-biozol and stored at room temperature for 5 min. 0.2 ml chloroform per ml P-biozol was added to the homogenate and shaken vigorously for 20 s, then the sample was placed at room temperature for 2–3 min and centrifuged at 10,000 rpm for 18 min at 4 °C. The aqueous phase was transferred to a new sterile RNase-free tube, and an equal volume of 100% RNA-free EtOH was added. The mixture was Loaded (up to 700 ml) into a RNeasy column seated in a collection tube and centrifuged for 30 s at 8000 rpm. The Flow-through liquid was discarded. The column was washed by RW1 (RNA Wash) and RPE (RNA Precipitating Elution) buffers to remove any residual impurities according to the manufacturer's instructions. The column was placed in a new clean 1.5 μl tube and 30–50 μl of RNase-free water was directly added onto the column membrane. The sample was left at room temperature for 1–2 min and then spun 1 min at 8000 rpm to elute RNA. RNA was stored at −80 °C until used.

Library preparation, sequencing, and virus genome assembly

The RNA was sent to Macrogen (Seoul, South Korea) for transcriptome sequencing using the Illumina HiSeq 2000 platform to generate paired end reads with 150 bp length. The total RNA quality was assessed using the Agilent 2100 Bioanalyzer and the cDNA rapid library was prepared according to the TruSeq Stranded Total RNA Sample Prep Guide.

The raw reads were processed through Trimmomatic v 0.36 (Bolger et al. 2014) with ILLUMINACLIP: 2:30:10, LEADING: 3, TRAILING: 3, SLIDINGWINDOW: 4:20 and MINLEN: 50 parameters to remove adaptor

Table 1 Primers used for detection of MRDD causal agent (RBSDV-Ir) in the present study

Primers	Direction	Sequence (5′ to 3′)	Size of PCR product (bp)
RBSDV S10F	forward	TGGCTGTACCTTGTTTTGAT	501
RBSDV S10R	reverse	GACAATAGCTGAATTTCCCCC	
MRS6–1	forward	GCCGTTCTCATCACTATA	562
MRS6–2	reverse	GTCTGCAAAATTGTGAATC	
MRDV-F1	forward	AGCGGAGAACGTTTGGATC	568
MRDV-R2	reverse	TTAACAACAGCAGCTTCACC	

sequences and short, low quality reads. The cleaned-up sequencing reads were mapped against the maize reference genome v4 (Andorf et al. 2016). Then Samtools view (Li et al. 2009) was used to extract unmapped reads and convert .sam file into a compressed version (.bam). High-quality unmapped reads were used for de novo assembly by using Trinity v 2.0.6 (Grabherr et al. 2011). The final transcriptome assembly was blasted against the previously published rice black-streaked dwarf virus genome (Wang et al. 2003).

Sequence analysis and phylogenetic relationships

Genome organization, ORFs, and deduced amino acid sequences were obtained with DNAMAN v. 7 (Lynnon Biosoft, Quebec, Canada). To construct a phylogenetic tree, the sequences belonging to different Fijiviruses including: MRDV Italian isolate, French isolate; RBSDV-ZJ, -AH, -HeB, -Hub, -JS isolates; SRBSDV-GD, -HN, -YN isolates; MRCV, FDV, OSDV; NLRV and rice ragged stunt virus (RRSV) were obtained through NCBI (Table 2).

The nucleotide sequences for each segment were collected into a fasta file and were multiply aligned using MUSCLE v3.8.31 (Edgar 2004). The resulting alignment outputs were subsequently utilized in filtering out the poorly aligned regions via the trimAl v1.4 (Capella-Gutiérrez et al. 2009) on the basis of the parameter "-gt 0.95 -st 0.001". Alignments of all single-copy genes were concatenated by SequenceMatrix (Vaidya et al. 2011). For the concatenated sequences, RAxML version 8.2.11 (Stamatakis et al. 2005) was used to reconstruct the maximum likelihood (ML) tree under the GTRGAMMA model with 100 bootstrap replicates. As the second scenario, for each alignment sequence, RAxML was executed with similar parameters. Then all gene trees were fed to ASTRAL-II (Mirarab and Warnow 2015) to make the consensus tree. All the phylogenetic trees were plotted using the FigTree v1.4.0 software (Rambaut and Drummond 2012).

The planthopper *Loadelphax striatellus* and virus transmission experiments

Preparation of a vector population

A colony of *Loadelphax striatellus* was obtained based on Hortamani et al. (2018) method. Briefly, the planthoppers were collected from the field and a fertile female was put on barley plants in a cage for oviposition. After several generation of rearing, the nymphs were used for transmission tests. Insect rearing was done in a growth chamber with a photoperiod 16 h light/8 h dark, at 25 ± 1 °C and 60 ± 5% relative humidity (Hortamani et al. 2018).

Host range of virus and transmission efficiency

Transmission tests were carried out as described by Hortamani et al. (2018). The seeds of gramineous species, including *Zea mays, Oryza sativa, Triticum aestivum, Hordeum vulgare, Panicum miliaceum, Avena sativa, Secale cereale, Lolium perenne, Agropyron desertorum, Festuca arundinacea* were sown into plastic pots. Five pots containing 5 seedlings per pot were used in transmission test for each plant species. For determination of the host range of RBSDV-Ir, a large number of the second and third instar nymphs of *Loadelphax striatellus* were released on infected maize plant for 72 h acquisition access feeding (AAF). After AAF, the nymphs were transferred to healthy barley seedlings for the latent period of 3–4 weeks. Then the insects were transferred to gramineous plants in the coleoptile stage for 72 h transmission feeding. The plants were kept in a growth chamber with a 16 h light/8 h dark cycle, at 25 ± 1 °C and 60 ± 5% relative humidity for 30 days. After 30 days post-inoculation (dpi) plants were checked for the appearance of symptoms and tested by RT-PCR (described in section 1.3). The healthy plants (non-inoculated) were used as controls.

For transmission efficiency test, 70 viruliferous planthoppers were individually put on maize seedling in coleoptile stage to die. After 30 dpi plants were checked for the appearance of symptoms and tested by RT-PCR.

Genetic diversity of Iranian isolates of MRDD causal agent

A total of 13 maize plants with MRDD symptoms were collected from three maize growing provinces, Isfahan with six samples, Fars with four samples and Chaharmahal-o-Bakhtiari with three samples, from 2016 to 2017.

Table 2 List of GenBank accession numbers used for phylogenetic tree construction with 10 segments and RdRp gene of fijiviruses

Isolate or Virus	Accession number									
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
MRDV Italian isolate	HQ637550	HQ637551	HQ637552	HQ637553	HQ637554	HQ637555	HQ637556	HQ637557	HQ637558	HQ637559
MRDV French isolate	KU984966	KU984967	KU984968	KU984969	KU984970	KU984971	KU984972	KU984973	KU984974	KU984975
RBSDV-ZJ	AJ294757	AJ409145	AJ293984	AJ409146	AJ409147	AJ409148	AJ297427	AJ297431	AJ297430	AJ297433
RBSDV-AH	HF954985	HF954986	HF954987	HF954988	HF954989	HF954990	HF954991	HF954992	HF954993	HF954994
RBSDV-HeB	KC134289	KC134290	KC134291	KC134292	KC134293	KC134294	KC134295	KC134296	KC134297	KC134298
RBSDV-Hub	AY144568	AF521806	AF432355	AY160687	AY144569	AY144570	AF397894	AF399826	AF536564	AF227206
RBSDV-JS	KM921673	KM921674	KM921675	KM921676	KM921677	KM921678	KM921679	KM921680	KM921681	KM921682
SRBSDV-GD	FN563983	FN563984	FN563985	FN563986	FN563987	FN563988	EU784841	EU784842	EU784843	EU784840
SRBSDV-HN	FN563989	FN563990	FN563991	FN563992	FN563993	FN563994	FN563995	FN563996	EU523359	EU523360
SRBSDV-YN	JQ773420	JQ773421	JQ773422	JQ773423	JQ773424	JQ773425	JQ773426	JQ773427	JQ773428	JQ773429
MRCV	AF499925	AF499926	AF499928	AF395873	AY607587	AF499927	AY923115	AF395872	DQ023312	AY607586
FDV	AY029520	AF049704	AF359556	AF049705	AY029521	AF356083	AY789927	AY297693	AF050086	AY297694
OSDV	–	–	–	–	–	–	AJ583250	AJ583251	AB011026	AB011027
NLRV	D49693	D49694	D49695	D49696	D49697	D49698	D49699	D49700	D26127	D14691
RRSV	AF020334	AF020335	AF020336	AF020337	U66712	U66713	U66714	U33633	U46682	L38899

'—' denotes not available sequences

RT-PCR and sequencing

RNA extraction from infected maize plants and quality controls were performed as explained in section 1.2. Two Specific primer pairs were designed for amplifying ORF10 of the virus isolates according to the segment 10 of RBSDV-Ir and sequences obtained from GenBank (accession numbers: HF954994.1, KM921682.1, HF955014.1, HM209078.1, HM209077.1, HM209075.1, HM209074.1, HM209073.1, HM209072.1, HM209071.1) (Table 3). RT-PCR was done as described in section 1.2, and PCR products were sent to the Macrogen Company (Seoul, South Korea) for Sanger sequencing.

Data analysis

ORF10 nucleotide sequences of 13 Iranian isolates of MRDD causal agent were assembled using the Vector NTI v.11.0 (Invitrogen) and subsequently multiple alignment was performed by adding other RBSDV sequences available at NCBI for phylogenetic analysis as described in section 2.3. Selection pressure on P10 encoded was estimated with the dN-dS method using the MEGA 6.06 software (Tamura et al. 2013). MEGA 6.06 software was also used for evaluation of mean evolutionary rate with the Jones-Taylor-Thornton model (+G) and nucleotide diversity (πt).

Results

Virus identification

Three primer pairs of RBSDVS10F/RBSDVS10R, MRS6–1/MRS6–2, and MRDV-F1/MRDV-R2 were used at RT-PCR to detect the causal agent of MRDD in Iran. In RT-PCR with the RBSDVS10F/RBSDVS10R primer pair which specifically detects RBSDV (Bong-Choon et al. 2005), a fragment of expected size of 501 bp was amplified in all samples with MRDD symptoms (data not shown). However, no amplification was obtained using the MRS6–1/MRS6–2 primer pair for specific detection of MRDV. RT-PCR product using MRDV-F1/MRDV-R2 was the expected size of 568 bp (Dovas et al. 2004) (data not shown). This primer pair detects both MRDV and RBSDV. PCR products of 501 bp and 568 bp were sequenced. Comparisons of the resulting sequences with the GenBank database demonstrated that the amplicons have high homology with RBSDV Chinese isolates. The nucleotide sequence of 501 bp segment amplified using RBSDVS10F/RBSDVS10R showed the highest identity (96%) to Chinese isolate (accession number HQ394210.1) and 568 bp segment amplified using MRDV-F1/MRDV-R2 primer pair showed the highest identity (92.17%) to Chinese isolate (accession number HQ394209.1).

Summary of NGS results of RBSDV-Ir genome

To obtain the full sequence of RBSDV-Ir genome, NGS was performed with the Illumina Hiseq platform generating a total of 38,545,730 paired-end reads with 150 bp length from one sample (a maize sample collected from Isfahan province, RBSDV-Ir). To remove reads with similarities to maize genome sequence, sequence reads were aligned to the reference sequence of maize genomic DNA database. The alignment revealed 99% reads were identified as the maize genome source. About 1,061,081 clean reads were used to assemble via Trinity, and further analyzed using BLASTn (http://www.ncbi.nlm.nih.gov/BLASTn), to identify fijivirus sequences. According to the Trinity results, only 12 contigs were obtained with valuable length which were associated to the RBSDV genome. Most contigs had less than 500 bp in length. These were not further analyzed for homology to database entries.

Table 3 Primers designed for amplification of ORF10 of RBSDV-Ir

Primers	Direction	Sequence (5′ to 3′)	Band size (bp)	Annealing temperature (°C)
RBSDVisf1	forward	CCTCACCCATAATGGCTGAC	885	60
RBSDisfr1	reverse	CTACTGCGCTCCAAGTYTGT		
RBSDVisf2	forward	TTCKCGTCAAATGTTCGAACG	1010	60
RBSDisfr2	reverse	CTGAAGAYCGCACAGCACTG		

Genome data

The complete genome sequence of RBSDV Iranian isolate (RBSDV-Ir) was identified and submitted into GenBank under the accession numbers: (MH699993-MH700002). An overview of the genome features is presented in Table 4. Based on NGS data, the genome of the RBSDV-Ir is 29,141 nt in length with a low G + C content ranging from 31.4% to 38% and 10 genomic segments encoding 13 viral proteins. ORF predictions by DNAMAN software indicated that each genome segment contained one ORF, except for S5, S7, and S9, which had two ORFs (overlapping or non-overlapping) as in fijiviruses of group 2. The 5′ and 3′ non-coding regions (NCRs) of all genomic segments respectively, ranged from 14 to 81 bp and 71 to 185 bp in length, including the same genus-specific terminal conserved sequences (5′-AAGTTTTTT…CAGCTNNNGTC-3′) as reported for RBSDV, MRDV (McMahon et al. 1999), MRCV (Distefano et al. 2003) and SRBSDV (Wang et al. 2010), previously. Also, there was a segment-specific, perfect, or imperfect inverted repeat with 7–11 bp in length, adjacent to the terminal conserved sequences (Table 5). These sequences are characteristic of different reoviruses and possess significant role in replication, sorting, and assembly of viral RNAs (Anzola et al. 1987).

Sequence comparison

Nucleotide (nt) sequences of 10 segments and amino acid (aa) sequences of 13 ORFs of RBSDV-Ir were compared to those of other fijiviruses. Multiple alignment of complete genome of RBSDV-Ir comprising all segments showed high identity ranging from 56 to 91% in nucleotide sequence and 44–97% in amino acid sequence between RBSDV-Ir and other fijiviruses of GenBank. RBSDV-Ir had the highest identity (nt: 86.5–91.3%), (aa: 87.9–97.8%) with the RBSDV isolates from China and Japan, respectively and lowest identity (nt: 56.3–73.6%), (aa: 44–84%) with the MRCV. The identities between RBSDV-Ir and MRDV and RBSDV-Ir and SRBSV were (nt: 84.8–88.5%), (aa: 85.2–97.1%) and (nt: 68.7–79.4%), (aa: 62–88.4%), respectively (Table 6). RBSDV-Ir segments shared the least identity (43–65% nt, 14.81–63.6% aa) with members of other groups of fijiviruses, containing FDV (group 1), OSDV (group 3) and NLRV (group 5) (Table 6).

The genome of RBSDV-Ir isolate contained 13 putative ORFs and encoded proteins, including P1, P2, P3, P4, P5–1, P5–2, P6, P7–1, P7–2, P8, P9–1, P9–2, and P10. Some properties of these ORFs and their encoded proteins are summarized in Table 4.

Protein analysis showed that RBSDV-Ir S1 ORF encoded a putative protein (P1 protein) of 1464 aa with a predicted molecular mass of 168.5 kDa. The P1 of RBSDV-Ir shared the highest identity (94.3–95.9%) with other RBSDV isolates. The central region of P1 protein possessed amino acid motifs of a reoviral RNA-dependent RNA polymerase (RdRp) including GDD and other adjacent conserved sequences (Nakashima et al. 1996) which is approximately identical with members of group 2 of fijiviruses (Fig. 1).

P8 protein encoded by S8 had 591 amino acids and a predicted molecular mass of 68 kDa. This protein comprised two previously defined motifs (McQualter et al. 2004). Motif I is an ATP-GTP binding motif and its role probably is RNA replication. The function of motif II is unknown. These two conserved motifs are present in fijiviruses such as RBSDV-Ir and some other reoviruses (Fig. 2).

Phylogenetic relationships

To evaluate the phylogenetic relationship between RBSDV-Ir and other fijiviruses, two scenarios were applied to construct phylogenetic trees. Because of the existence of bias in the concatenated method (Soorni et al. 2019), the ASTRAL-II method was also used. ASTRAL tree (Fig. 3) showed that the RBSDV-Ir is clustered with RBSDV isolates and separated from MRDV and other group 2 members with strongly supported bootstrap (100%). According to this analysis, there was a close relationship between RBSDV Iranian isolate (RBSDV-Ir) and other RBSDV isolates than those of MRDV isolates. However, the RBSDV-Ir formed a unique sister clade and separated from other RBSDVs. The phylogenetic tree constructed with deduced amino acid sequences of RdRp (Fig. 4) and other genes confirmed the above results (data not shown).

Experimental host range of RBSDV-Ir and its transmission efficiency by *L. striatellus*

The seedlings of the test plants in each pot were inoculated by 10 insects that fed on infected maize for 72 h AAF and kept on healthy barley for 3–4 weeks for

Table 4 Genome characteristics of RBSDV-Ir

Genome segment	Accession number	length (bp)	G+C (%)	ORFs (nts)	Protein (kDa)	Predicted function
S1	MH699993	4501	32.6	36–4430	168.458	RdRp
S2	MH699994	3812	33.5	46–3726	141.188	Major core
S3	MH699995	3572	33.5	15–3455	131.962	Capping enzyme
S4	MH699996	3617	31.4	34–3543	134.946	Outer shell B spike protein
S5	MH699997	3164	37.2	16–2829	107.158	Viroplasm
				2462–3073	23.512	Nonstructural
S6	MH699998	2645	38	82–2460	89.614	Viroplasm
S7	MH699999	2193	34	42–1130	40.964	Tubular
				1183–2112	35.958	Nonstructural
S8	MH700000	1936	34.5	25–1800	68.007	Minor core
S9	MH700001	1900	33.8	52–1095	39.946	Viroplasm
				1160–1789	24.097	Nonstructural
S10	MH700002	1801	36.2	22–1698	63	Major outer capsid

incubation period. Inoculated and non-inoculated gramineous plants were checked for the appearance of symptoms and tested by RT-PCR at 30 dpi. Inoculated plants of *Agropyron desertorum* and *Festuca arundinacea* showed no symptoms and tested negative, while *Zea mays, Oryza sativa, Triticum aestivum, Hordeum vulgare, Panicum miliaceum, Avena sativa, Secale cereal* and *Lolium perenne,* tested positive by RT-PCR and showed typical symptoms of MRDD including dwarfing, deformation and darkening of leaves. At 60 dpi, all infected plants died because of the disease severity except maize that showed symptoms of stunting and rough enation along the veins and abaxial surface of the leaf. Non-inoculated plants (controls) were symptom-free and did not amplify product of expected size in RT-PCR.

In transmission efficiency test, 22 out of 70 inoculated maize plant were positive tested and the transmission efficiency of RBSDV-Ir by *L. striatellus* was calculates as 32%.

Genetic diversity of RBSDV Iranian isolates

To estimate the genetic variation of RBSDV Iranian isolates, ORF10 of 13 isolates were identified and after

Table 5 The terminal sequences of 10 segments of RBSDV-Ir

		5′ NCR	3′ NCR	
Segments	Length (bp)	Terminal sequences	Terminal sequences	Length (bp)
S1	35	AAGTTTTTTTGCCGACCTA	CATGGCGGGAACAGCTGATGTC	71
S2	45	AAGTTTTTTCCCGG	CCGGTAAATTCAGCTATTGTC	86
S3	14	AAGTTTTTTTCAGG	CCTGAAAGCAGCTATTGTC	117
S4	33	AAGTTTTTTTCCATGT	ACATGGGAACAGCTATCGTC	74
S5	15	AAGTTTTTTTCACTC	GAGTGAATACAGCTGATGTC	91
S6	81	AAGTTTTTTGAGTCT	AGACTCAAATCAGCTGATGTC	185
S7	41	AAGTTTTTTTTCGACCT	AGGTCGAAATGCAGCTGATGTC	81
S8	24	AAGTTTTTTTCGCAC	GTGCGAGAATTCAGCTACTGTC	136
S9	51	AAGTTTTTTTAGCCTGG	CCGGCTTACAGCTATCGTC	110
S10	21	AAGTTTTTTTCCTC	GGGGAAATTCAGCTATTGTC	102

*Inverted repeats are underlined

Table 6 Comparison of amino acid sequences of the ORFs of RBSDV-Ir and other fijiviruses

RBSDV-Ir		**Fijiviruses***						
		Group 2				**Group 5**	**Group 3**	**Group 1**
Genome segment	Viral protein	RBSDV	MRDV	SRBSDV	MRCV	FDV	OSDV	NLRV
S1	P1	94.3–95.9	94	84.1	78.2	63.6	–	32
S2	P2	97.6–97.8	97.1	88.4	84	56.1	–	23.1
S3	P3	94.5–95.1	93.7	73.4	74	56	–	22.1
S4	P4	91.4–92	91.2	84.5	57	37.8	–	22.2
S5	P5–1	91.7–92.4	91.1	69.2	61.6	31.1	–	19.9
	P5–2	90.6–91.6	85.2	62	51	–	–	–
S6	P6	88.5–89.5	86.2	62.6	44.8	25.2	–	18.8
S7	P7–1	96.7–97.5	93.4	80.7	60.2	52.6	31.1	19.5
	P7–2	88–89.6	87.4	62.5	44	25.8	14.81	–
S8	P8	87.9–92.7	91.5	71.6	59	38.7	26.1	20.1
S9	P9–1	91.4–93.4	89.6	76.9	62.6	35.7	25.3	15.7
	P9–2	96.2–96.7	94.7	72.2	52.2	38.8	23.2	29.8
S10	P10	95–96.6	95.5	84.4	71.7	49	32.2	21.6

*For sequence accession numbers see supplementary file

'—' denotes not available sequences

processing submitted to the GenBank under the accession numbers: MN442989-MN443001.

Multiple alignment of sequences showed 99.66% nucleotide and 99.92% amino acid identity. Further-

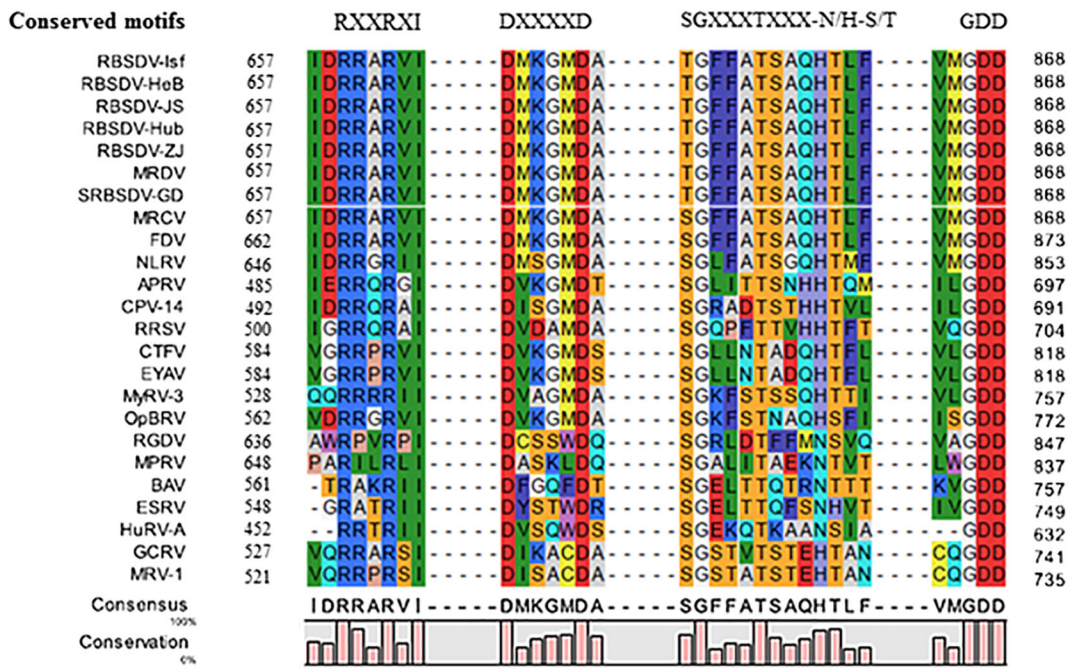

Fig. 1 Conserved amino acids in the central region of RNA-dependent RNA polymerase (RdRp) of RBSDV-Ir and other fijiviruses (For sequence accession numbers see supplementary file)

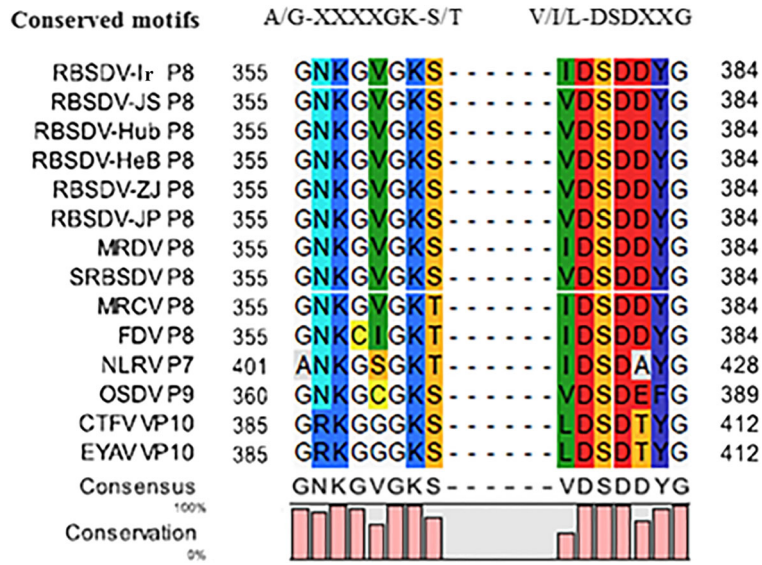

Fig. 2 Multiple amino acid sequence alignment of RBSDV-Ir P8 and other fijiviruses (for sequences accession numbers see Table 2) and two members of the genus *Coltivirus* [Colorado tick fever virus (CTFV) (NP-690899.1) and Eyach virus (EYAV) (AAM18352.1)]

more, isolates shared 90.5–91.3% nucleotide and 95–96.6% amino acid identity with other RBSDV isolates. (Fig. 5). The phylogenetic tree showed that Iranian isolates were grouped in a separate clade from Chinese and Korean Isolates.

Alignment of P10 sequences from Iranian isolates represented the mean of dN-dS ranging from −3.88 to 0.99. Five positions were found under positive selection and position 368 showed the highest dN-dS value. Therefore, most of the codons were found to be under neutral evolution or negative selection. Result from Tajima's Neutrality Test indicated that nucleotide diversity (πT) in P10 of RBSDV Iranian isolates was 0.006.

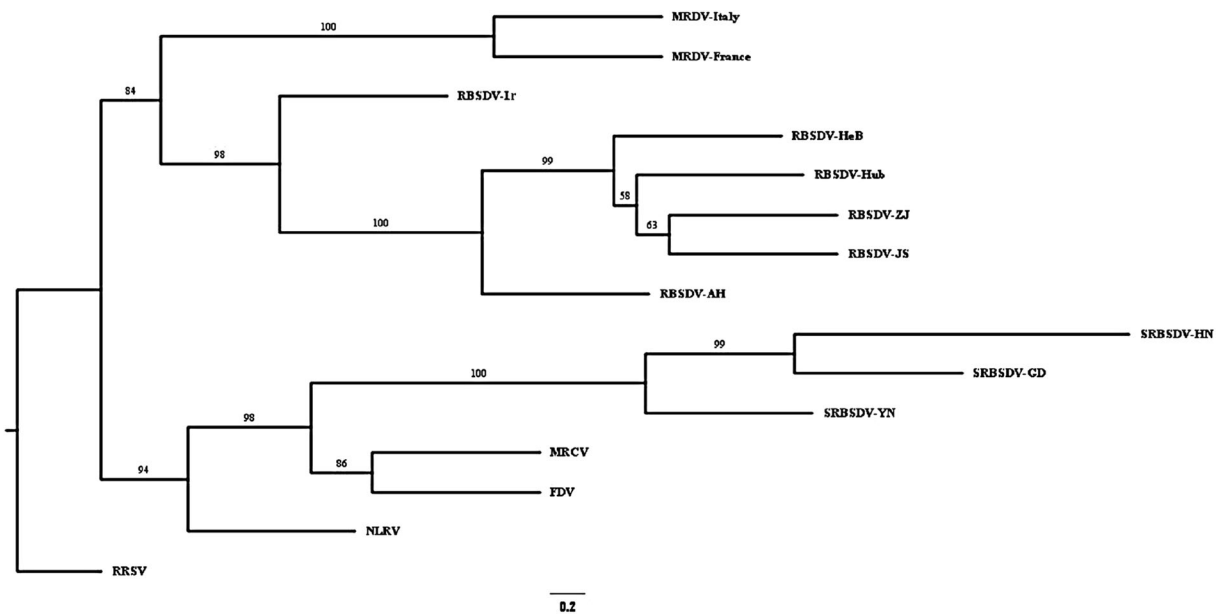

Fig. 3 Phylogenetic tree (consensus tree) constructed with nucleotide sequences of 10 segments of RBSDV-Ir and other fijiviruses using the ASTRAL approach. RRSV used as outgroup (For sequence accession number see Table 2)

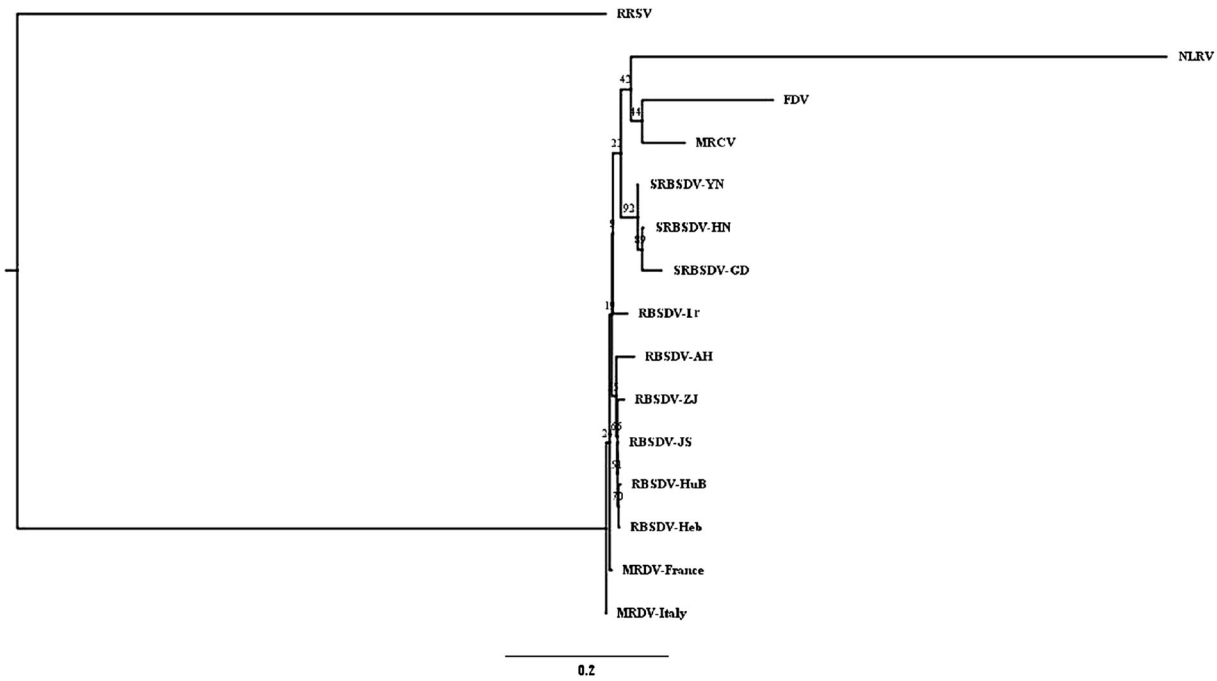

Fig. 4 Phylogenetic tree constructed with amino acid sequences of RdRp of RBSDV-Ir and other fijiviruses. RRSV used as outgroup (For sequence accession number see Table 2)

Discussion

Rice and maize are the second and third most important crops after wheat, respectively (Giraldo et al. 2019). Fijiviruses, causing dwarfing diseases, have been isolated from maize and rice in Iran (Izadpanah et al. 1983; Kamran et al. 2000). Iranian rice isolate shows a close relationship to RBSDV (Kamran et al. 2000) and Iranian maize isolate was considered as MRDV based on biological and serological analysis (Izadpanah et al. 1983). In transmission tests, small brown planthopper *Laodelphax striatellus* transmitted the rice isolate to rice, maize, wheat, barley, oat and some grasses (Kamran et al., 2000). Biological studies showed that Iranian maize and rice isolates have similar host range and symptoms and were transmitted efficiently by *L. striatellus*. RBSDV and MRDV have the same experimental host range and symptoms (Yang and Ma 1983, Boccardo and Milne 1984). Gramineous plants such as maize, wheat, millet, barley, oat, rye, rice, and some other grasses are considered as main hosts of RBSDV or MRDV. Experimental host range studies showed that the symptoms of infected plants are stunting, enation and leaf darkening. Since the RBSDV and MRDV have similar or identical biological properties and due to some cross serological reactions between RBSDV and MRDV, molecular properties could be used as accurate criteria to differentiate between RBSDV and MRDV. Species demarcation criteria in the genus *Fijivirus* are based upon the biological, serological and molecular properties. However, molecular analyses are the most critical criterion for species distinction between closely related viruses (King et al. 2011).

In this study, the complete nucleotide sequence of 10 segments (S1-S10) of the RBSDV-Ir was determined. Full length genome of RBSDV-Ir consisted of 29,141 nucleotides, 3–4 nt shorter than genome of MRDV isolates. (Mingfang et al. 2016; Svanella-Dumas et al. 2016). Genome organization and features of RBSDV-Ir were similar to other RBSDV isolates that were previously characterized. (Wang et al. 2003; Zhang et al. 2001). Comparisons of genome segments of RBSDV-Ir at the nucleotide and amino acid levels with corresponding segments of other fijiviruses revealed a higher similarity with other RBSDV isolates than with MRDV. RBSDV-Ir shared a strong homology with the Chinese isolates of RBSDV and a relatively lower homology with MRDV isolates. Similar results have been reported for other RBSDV and

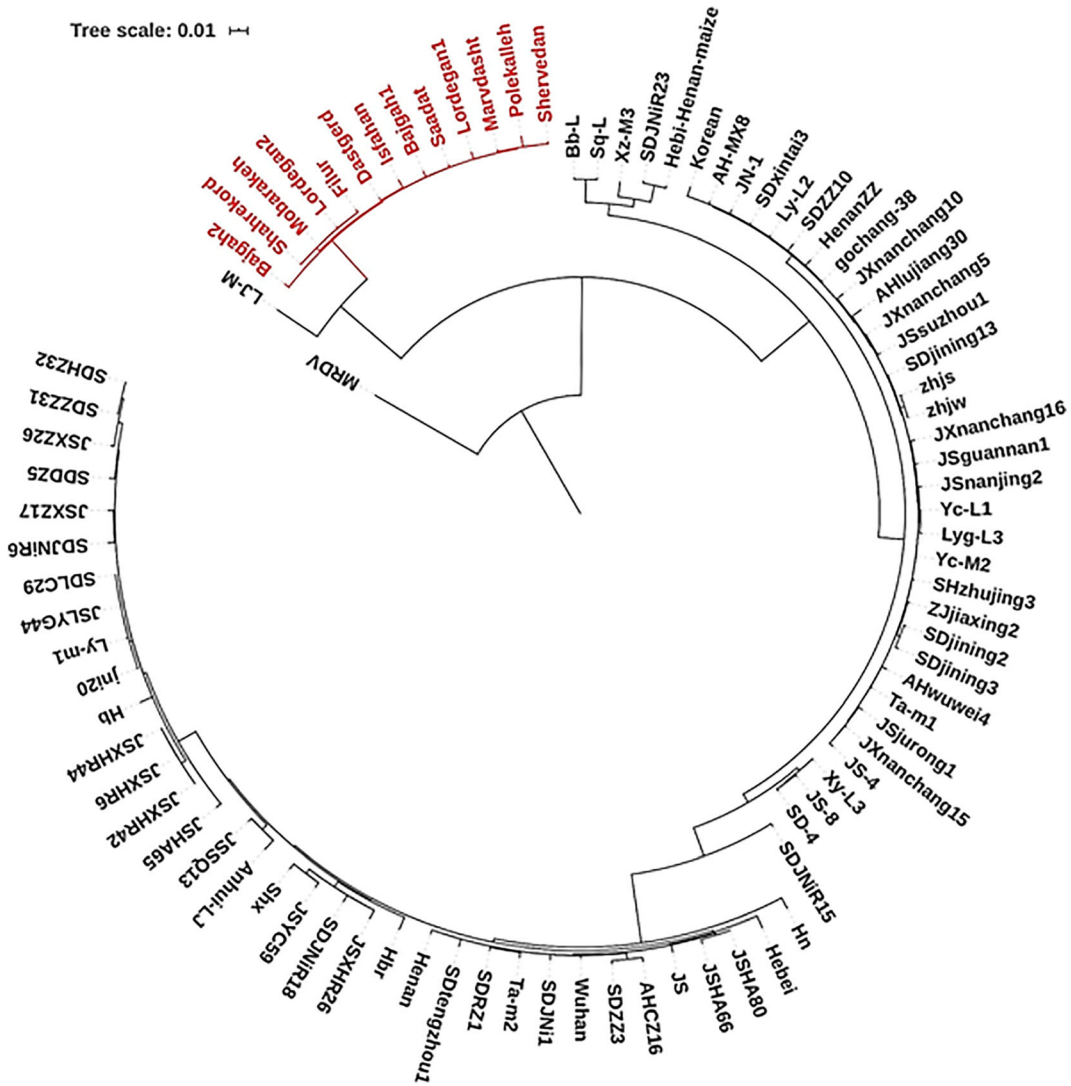

Fig. 5 Phylogenetic tree constructed with ORF10 nucleotide sequence of RBSDV-Ir and other RBSDV isolates. MRDV used as outgroup. Bajgah 1, Bajgah 2, Shervedan, Polekalleh, Marvdasht, Lordegan 1, Lordegan 2, Saadatshahr, Isfahan, Dastgerd, Filur, Mobarake and Shahrekord are Iranian isolates. Gochang-38 (JX994207.1) and Korean (HQ670674.1) are South Korean isolates and other isolates are from China (For sequence accession numbers see supplementary file)

MRDV isolates throughout the world (Wang et al. 2003). Amino acid sequences identities (Table 6) show P1 (encoded by S1), P2 (encoded by S2), and P10 (encoded by S10) were relatively more conserved than other proteins which probably indicating the protein's function. P1, P2, and P10 are involved in RNA-dependent RNA polymerase (RdRp), major core capsid proteins, and major outer capsid proteins, respectively. The conservation is possibly leading to similar morphology and replication mechanism among fijiviruses of group 2 (Wang et al. 2010).

In phylogenetic trees, RBSDV-Ir was separated from MRDV isolates and clustered with other RBSDV isolates. Previous studies indicate RBSDV Chinese isolates are closer to RBSDV Japanese isolate than to MRDV (Zhang et al. 2001; Bai et al. 2002; Wang et al. 2003). Our data revealed that although the causal agent of maize rough dwarf disease in Iran had a close relationship with RBSDV isolates, it is not entirely similar to them and relatively placed between the two species (RBSDV and MRDV). These results reinforced the previous hypothesis that RBSDV and MRDV may have the same origin

and because of geographical differentiation may have evolved separately (Marzachi et al. 1995).

Our insights into phylogenetic relationships, epidemiology, and dispersal of RBSDV could be more comprehensive with increasing information on the genetic diversity of the virus. In this study, Iranian isolates showed more than 99% homology and formed a single clade that indicating low genetic variability among them. Similarly, Chinese RBSDV isolates showed identities of 94.7–100% (nt) and 97.3–100% (aa) and classified into two groups based on the S10 sequences, regardless of host or geographic region (Li et al. 2012). In earlier study in Fars province, phylogenetic analysis based on partial sequences of S6, S8 and S10 of fijivirus rice isolate placed it in a clade between MRDV and RBSDV, distinct from both but somewhat closer to RBSDV (Nouri, S. personal communication). In this study, the rice fields and wild gramineous plants in Isfahan and Fars provinces were surveyed for virus presence, but no fijivirus symptoms were observed. As partial sequences of the rice isolate were not submitted to GenBank (Nouri, S. personal communication), no comparison was made between isolates of this study and the rice isolate. It seems maize and rice isolates are very similar, but additional works such as determination of complete genome of the rice isolate is needed for reliable conclusion.

Our data revealed that the P10 was under neutral or negative selection like the results that have been reported for Chinese RBSDV isolates (Li et al. 2012). P10 is the major capsid protein and responsible for the assembly and stability of the virus particle, hence the neutral evolution and low genetic diversity of this protein may be associated with the conservation of the P10 functions. In the vector-borne plant viruses, low genetic diversity may be due to role of vector as strong genetic bottlenecks. In a study, cucumber mosaic virus (CMV) populations transmitted by aphids showed a significantly lower mutation rate than CMV populations when inoculated mechanically (Ali et al. 2006).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-021-02253-4.

Declarations

Conflict of interest They have no conflicts of interest.

We used the insects as vector in this work. All applicable international guidelines for the care and use of animals were followed. This article contains no studies involving human participants performed by any of the authors.

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