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Genetic Diversity and Evolutionary Relationships of *Faba Bean Necrotic Yellows Virus* in Lentil and Chickpea from Western Iran

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ABSTRACT

Faba bean necrotic yellows virus (FBNYV, genus *Nanovirus*, family *Nanoviridae*) is an economically important nanovirus associated with severe yellowing disease in chickpea (*Cicer arietinum* L.) and lentil (*Lens culinaris* Medik). In this study, we determined the complete genome sequences of six Iranian FBNYV isolates collected from chickpea and lentil fields in western Iran. The genetic distance, genetic differentiation, gene flow and selection pressure of the Iranian populations, which studied, and global populations of this virus were also analyzed, using population genetic and statistical approaches. Pairwise sequence comparisons revealed that the Iranian isolates shared the highest nucleotide identity with the isolates from Azerbaijan. Phylogenetic analyses, which were based on full-length genome sequences, confirmed a close relationship between the Iranian isolates and Azerbaijani FBNYV isolate [AZ; Lahic12]. The isolates clustered according to geographical origin rather than host species. To investigate evolutionary forces shaping FBNYV diversity, isolates were divided into three geographical populations. The Caspian Sea population (Iran and Azerbaijan) exhibited higher genetic diversity compared to Mediterranean populations (Syria/Egypt and Tunisia/Morocco/Spain). Selection pressure analysis indicated that, except for DNA-C (encoding the C-link protein), all genomic components were under purifying (negative) selection, suggesting functional constraints and coordinated genome evolution. The DNA-C component showed an evidence of positive selection. Gene flow analysis (Fst) revealed limited genetic exchange among the three populations, which indicated significant population differentiation. Neutrality tests suggested possible population expansion events, although results were not statistically significant. Overall, our findings suggest that FBNYV populations may share a common geographical origin but have evolved independently in distinct regions, with founder effects contributing to their current genetic structure.

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Introduction

Lentil (*Lens culinaris* Medik.) and chickpea (*Cicer arietinum* L.) are important food legumes widely cultivated in West Asia and North Africa (WANA). These crops represent important sources of plant protein for human consumption and animal feed, contributing significantly to soil fertility through nitrogen fixation. According to FAOSTAT (2022), during the 2021-2022 growing seasons, chickpea cultivation in Iran

covered approximately 535920 ha with a production of 356746 tons, whereas lentil cultivation occupied 116132 ha with a production of 72104 tons (FAOSTAT, 2022). Yellowing symptoms are among the most common manifestations of viral disease in legume fields worldwide and may be caused by various plant viruses. One of the most economically significant agents is *Faba bean necrotic yellows virus* (FBNYV), a member of the genus Nanovirus within the family Nanoviridae. Members of this family possess multipartite circular single-stranded DNA (ssDNA) genomes (Kraberger *et al*., 2018; Lal *et al*., 2020).

Nanoviruses consist of eight circular ssDNA components (~1 kb each), each encoding a single protein and encapsidating individually within small icosahedral particles (18-20 nm in diameter) (Vetten *et al*., 2012). Five genomic components (DNA-R, -S, -C, -M, and -N) are conserved among nanoviruses, encoding the master replication initiator protein (M-Rep), capsid protein (CP), cell cycle link protein (Clink), movement protein (MP), and nuclear shuttle protein (NSP), respectively (Vetten, 2008). Three additional components (DNA-U1, -U2, and -U4) encode proteins of currently unclear or partially characterized functions (Vetten, 2008; Grigoras *et al*., 2009). The role of DNA-U1 and DNA-U2 in symptom severity and aphid transmission efficiency has been demonstrated (Grigoras *et al*., 2018). Nanoviruses are frequently associated with Alpha-satellites, which are additional circular DNA molecules dependent on helper viruses for replication (Briddon *et al*., 2011). FBNYV was first reported in Syria in 1988 and subsequently identified in numerous countries across the WANA region, including Ethiopia, Egypt, Tunisia, Libya, Sudan, Morocco, Algeria, Iran, Iraq, Lebanon, Jordan, Pakistan, Yemen, and Turkey (Katul *et al*., 1997:1998; Kumari and Makkouk, 2007; Grigoras *et al*., 2014). It was later detected in Spain (Babin *et al*., 2000) and Azerbaijan (Kumari *et al*., 2009), indicating its spread into Europe. In Iran, FBNYV was first reported from some chickpea and lentil fields in Kermanshah, Lorestan and Kurdistan provinces (in the west of Iran), Kerman and Khorasan provinces (in the east), West Azerbaijan province (in the north), and also some fababean fields in one district of Kermanshah province (located in the west of Iran) (Makkouk *et al*., 2003; Lotfipour *et al*., 2017; Avish-Koohshahi *et al*., 2021).

Despite its wide distribution and economic importance, the molecular evolution and population genetic structure of FBNYV remain to be understood poorly. Our previous study (Sokhansanj *et al*., 2018) reported the molecular characterization and phylogenetic analysis of three Iranian chickpea isolates of FBNYV. In the present study, we substantially extend that work by incorporating additional isolates, conducting comprehensive evolutionary, and population genetic analyses that were beyond the scope of the previous report. The knowledge generated in this study advances our understanding of the genetic diversity and evolutionary dynamics of FBNYV, providing a valuable foundation for developing sustainable disease management strategies aimed at limiting the spread of genetically diverse virus populations in legume crops.

Materials and Methods

Virus isolation

A total number of 177 (27 lentil and 150 chickpea) samples showing leaf yellowing and plant stunting were collected from 45 fields (12 lentil and 33 chickpea field) in 12 regions of Lorestan and Kermanshah provinces in the west of Iran during the 2017-2018 growing seasons. Lentil samples (15 of 27) were collected from seven lentil fields in four regions (Noorabad, Alashtar, Zagheh, and Boroujerd) of Lorestan province and 12 other samples were collected from five lentil fields in four regions (Ravansar, Kangavar, Sararood, and Eslamabad) of Kermanshah province. Number of samples and geographic regions are given in Table 1. Chickpea samples (70 of 150) were collected from 15 fields in six regions (Noorabad, Alashtar, Khoramabad, Firouzabad, Zagheh, and Boroujerd) of Lorestan province and 80 other samples were collected from 18 fields in six regions (Ravansar, Kangavar, Harsin, Sararood, Kerand, and Eslamabad) of Kermanshah province. The numbers of samples and sampling locations are summarized in Table 1.

DNA extraction and PCR analysis

Total DNA was extracted from samples by Vivantis DNA Extraction Kit (Vivantis

Technologies Sdn. Bhd., Malaysia), according to the manufacturer's instructions. PCR amplification was performed using the nanovirus-specific degenerate primer pair NanoF103/NanoR101 derived from consensus sequences in the DNA-R of nanoviruses (Kumari *et al*., 2010) and yielded the expected amplicon size of ~770 bp, whereas such amplification was not obtained from healthy plants. Samples with positive reaction with the degenerate primers were then tested by PCR and specific primers denoted FBNYV-C5F/R for specific FBNYV detection (Kumari *et al*., 2010). PCR performed in a thermocycler (BioRad, iCycler, Germany) with a temperature profile of a minute at 95 °C for initial denaturation followed by 30 cycles of 45 s at 95 °C for denaturation, 30 s at 44 °C for annealing, and 30 s at 72 °C for extension, followed by a final extension of 10 min at 72 °C.

Table1. Incidence of FBNYV obtained by PCR test using specific primer (DNA-S genomic section) for chickpea and lentil hosts collected from different districts of Kermanshah and Lorestan provinces of Iran.

Provinces	Districts	Infected samples[a]	Chickpea		Lentil	
		No	No[b]	%[c]	No	%
Kermanshah	Ravansar	2/21	2/18	11.1	0/3	0
	Kangavar	2/14	1/12	8.33	1/2	50
	Harsin	1/12	1/12	8.33	ND	ND
	Sararoud	2/17	1/13	7.69	1/4	25
	Kerand	0/10	0/10	0	ND	ND
	Eslam-abad	1/18	1/15	6.66	0/3	0
Percentage		(8.69%)[d]	(7.5%)[e]		(16.66%)	
Lorestan	Nourabad	2/13	1/11	9.09	1/2	50
	Alashtar	2/15	2/11	18.18	0/4	0
	Khoramabad	1/10	1/10	10	ND	ND
	Firouzabad	1/12	1/12	8.33	ND	ND
	Zagheh	2/19	1/13	7.69	1/6	16.6
	Boroujerd	0/16	0/13	0	0/3	0
		(9.41%)	(8.57%)		(13.33%)	
Total No.		16/177 [f]	12/150		4/27	
Percentage		(9.03%)[g]	(8%)		(14.81%)	

[a] No. of infected samples over number of samples collected for different districts of each province; [b] No. of infected samples over number of samples collected from each host; [c] Percentage of FBNYV infection in each host for different districts of each province; [d] Percentage of FBNYV infection in each province; [e] Percentage of FBNYV infection in each host; [f] Total No. of infected samples over number of samples collected from all provinces, [g] Average percentage of FBNYV infection for all provinces.

Rolling circle amplification and whole-genome detection

Circular DNAs present in one µl of total DNA preparations of FBNYV-positive samples were amplified by rolling circle amplification (RCA) (Inoue-Nagata *et al*., 2004) using an Illustra TempliPhi Amplification Kit (GE Healthcare, USA) according to the manufacturer's instructions and incubated at 30 °C for 18 h, followed by 10 min at 65 °C. The RCA products of ~1 kb in length that were consistent with nanovirus DNA components (Grigoras *et al*., 2009) were digested with AatII restriction endonuclease. As described previously, the AatII site (GACGTC) was conserved in many nanoviruses (Grigoras *et al*., 2009). The RCA derived DNA was subsequently used as a template in PCR amplification reaction with FBNYV component-specific primer pairs (Grigoras *et al*., 2014) to amplify eight genomic fragments (DNA-R, -S, -C, -M, -N, -U1, -U2 and -U4). PCR was performed in a thermocycler (BioRad, iCycler, Germany) with a temperature profile of 5 min at 95 °C for initial denaturation, followed by 35 cycles of 30 s at 95 °C for denaturation, 1 min at 55 °C for annealing, and 2 min at 72 °C for extension, followed by a final extension of 5 min at 72°C.

Sequencing and phylogenetic analysis

The PCR amplicons were purified from Agarose gels using the Vivantis Gel DNA Recovery Kit (Vivantis Technologies Sdn. Bhd., Malaysia). Purified amplicons (~1 kb) were ligated into the pGEM-T Easy Vector (Promega, Mannheim, Germany) according to the manufacturer's instructions and transformed into competent

Escherichia coli DH5α cells. Recombinant plasmids were purified using the Vivantis Plasmid DNA Extraction Kit (Vivantis Technologies Sdn. Bhd., Malaysia) and sequenced bidirectionally by Macrogen Inc. (Seoul, South Korea) using the Sanger dideoxy chain-termination method. For each genomic component, at least three independent recombinant clones were sequenced. Minor nucleotide differences occasionally observed among clones, which were considered likely to be resulted from PCR amplification or cloning artifacts. Consensus sequences were generated based on agreement, among independent clone sequences, and ambiguous nucleotide positions were confirmed by resequencing.

Six FBNYV isolates were selected from the sixteen PCR-positive samples to represent the geographical distribution, host range, and genetic diversity of the virus population in the western Iran, while avoiding redundant sequencing of highly similar isolates. Selection was based on geographical origin (Lorestan and Kermanshah provinces), host species (chickpea and lentil), symptom characteristics, and the quality of rolling-circle amplification (RCA) products suitable for complete genome sequencing. The selected isolates included IR-Lor-1, IR-Lor-28 (chickpea), and IR-Lor-51 (lentil) from Lorestan province, together with IR-Ker-15, IR-Ker-21 (chickpea), and IR-Ker-55 (lentil) from Kermanshah province.

Complete genome sequences were assembled by reference-guided assembly using CLC Genomics Workbench and the MegAlign Module of the Lasergene package (version 15.2.0; DNASTAR, Madison, WI, USA). Sequence quality was assessed by examining bidirectional sequences, and only high-quality consensus sequences derived from independent clone agreement were retained for subsequent analyses. Sequence identity analysis was performed using BLASTn by comparing the sequences available in the GenBank database (Altschul *et al*., 1997). To investigate the evolutionary relationships of the Iranian FBNYV isolates, phylogenetic analyses were performed using complete genome sequences together with all publicly available full-length FBNYV genomes retrieved from GenBank. Homologous nucleotide sequences were aligned using CLUSTALX version 1.8 (Pearson and Lipman, 1988). Phylogenetic trees were reconstructed using the Neighbor-joining algorithm (Saitou and Nei, 1987) with the p-distance substitution model (Nei and Kumar, 2000) implemented in MEGA version 5.05 (Tamura *et al*., 2011). The robustness of tree topology was evaluated using 1,000 bootstrap replicates, and branches with bootstrap support below 75% were collapsed. Pairwise nucleotide identity matrices were calculated using SDT version 1.2 (Muhire *et al*., 2013).

Genetic statistical analysis

Calculation of genetic distance within and between FBNYV geographical groups and mean diversity in the entire population was done using the MEGA 5.05 program (Tamura *et al*., 2011) and based on the Kimura two-parameter model (Kimura, 1980). For the population genetic analyses, the FBNYV isolates were classified into three geographical populations based on their country or region of origin using the metadata associated with complete genome sequences deposited in GenBank. These geographical groups correspond to the principal epidemiological regions currently represented by available genomic data from the West Asia and North Africa (WANA) region. This classification was adopted to facilitate comparisons of genetic differentiation, gene flow, and population structure among regional virus populations. Alternative grouping strategies, such as host-based classification, were not applied because the available dataset contained an unequal representation of host species, whereas geographical origin provided a more robust and biologically relevant basis for population genetic inference.

Selection pressure was estimated for genomic region of each component using the ratio between non-synonymous and synonymous substitution (Pi(a)/Pi(s)) by the Pamilo-Bianchi-Li method (Pamilo and Bianchi, 1993) in DnaSP 5.10 software (Librado and Rozas, 2009). Pi(a)/Pi(s) ≈ 0 indicates neutral evolution, Pi(a)/Pi(s)< 1 suggests negative or purifying selection, while Pi(a)/Pi(s)> 1 suggests positive or adaptive selection. Genetic differentiation between FBNYV populations were calculated by three permutation-based statistical tests, Ks*, Z* and Snn (Hudson *et al*., 2000; Hudson, 1992), using

DnaSP 5.10 program (Librado and Rozas, 2009). Only geographical areas with more than two isolates of the virus were considered as a population. The level of gene flow between populations was estimated by Fst statistics using DnaSP 5.10 (Librado and Rozas, 2009). Generally, gene flow between populations is frequent if Fst-value< 0.33 and gene flow is infrequent if Fst-value> 0.33 (Weir and Cockerham, 1984). The absolute values of Fst from 0 to 1 represent undifferentiated to fully differentiated populations.

Three population genetic statistics *(Tajima's D, Fu and Li's D, and F*) (Fu and Li, 1993) were calculated using DnaSP 5.10 to evaluate the demographic forces acting on the FBNYV populations. Negative values reflect an excess of rare polymorphisms caused by population expansions. The total number of segregating sites (S), the mean nucleotide differences between sequences (K), the nucleotide diversity (π), the number of haplotypes (H), the total number of mutations (Eta), the total number of singleton mutations Eta(s), and the haplotype diversity (Hd) were calculated using DnaSP5.10 software to measure the molecular variability.

Results

PCR analysis and incidence of FBNYV infection in symptomatic samples

PCR analysis revealed that 16.4% of the samples (29 of 177) generated the expected amplicon (~770 bp) using the nanovirus DNA-R primers (F103 and R101) (Kumari *et al*., 2010). Among these positive samples, 55.1% (16 of 29) yielded the expected PCR product with the FBNYV coat protein-specific primer pair (FBNYV-C5F/R) (Supplementary table S1). FBNYV was detected across several districts of Lorestan and Kermanshah provinces, with 14 of 45 surveyed fields exhibiting infection. Overall, 16 of 177 symptomatic chickpea and lentil samples tested positive, corresponding to a total incidence of 9.03% (Table 1). The incidence in Lorestan and Kermanshah provinces was 9.41% and 8.69%, respectively (Table 1).

Sequence and phylogenetic analyses

RCA-derived DNA products were digested with AatII endonuclease, which yielded fragments of approximately 1 kb, as visualized on 1% agarose gel. Eight distinct circular ssDNAs were amplified, using RCA-derived DNA and component-specific primers, cloned, and completely sequenced.

Complete nucleotide sequences of six Iranian isolates were determined and deposited in GenBank under accession numbers MG827045-MG827060, MG950192-MG950199, MG950208-MG950215, MG827061-MG827068, and MG950200-MG950207 for isolates IR-Lor-1, IR-Lor-28, L-Lor-51, IR-Ker-15, IR-Ker-21, and IR-Ker-55, respectively. The eight circular ssDNA components of Iranian FBNYV isolates ranged from 987 to 1005 bp. BLAST analysis showed >96% nucleotide and >92% amino acid sequence identity among the Iranian isolates. All eight FBNYV DNA components shared >81% nucleotide identity with previously published full genomes, confirmed by SDT analysis. DNA-M exhibited the lowest identity, while DNA-R was the most conserved among isolates.

Phylogenetic analyses, which were based on full-genome sequences, indicated that the six Iranian isolates clustered together and showed the highest similarity with isolates from Azerbaijan. Comparisons with previously reported Iranian isolates from Kerman and Northern Khorasan provinces revealed 93–95% identity with IR:Raz:88 and IR:Raz:77 isolates (Fig. 1). Previously reported Iranian isolate Sy36 clustered with Egyptian and Syrian isolates and showed 87-90% nucleotide identity with other Iranian isolates based on full-genome analysis (Figs. 1 and 2). Tunisian isolates were highly similar to Spanish isolates, whereas Moroccan isolate Mor23 grouped differently depending on the DNA component (Figs 1 and 2). Full-genome phylogeny classified FBNYV isolates into two major groups: Group 1 included isolates from Iran, Azerbaijan, Morocco, Spain, and Tunisia; Group 2 comprised isolates from Syria, Egypt, and one Iranian isolate, with high bootstrap support (Fig. 1). Subgrouping within Group 1 showed Iranian and Azerbaijani isolates forming one subgroup, and Moroccan, Spanish, and Tunisian isolates forming another.

Genetic analysis

Twenty-three FBNYV isolates from Tunisia, Spain, Morocco, Iran, Azerbaijan, Egypt, and

Syria were grouped geographically: G1 (Iran and Azerbaijan), G2 (Syria and Egypt), and G3 (Spain, Tunisia, and Morocco). Mean inter-group genetic distances for U4 were highest (G1 vs G2: 0.151, G1 vs G3: 0.141, and G2 vs G3: 0.144), whereas DNA-R and DNA-N exhibited the lowest distances (Table 2). Total genome nucleotide distances were greater between groups (0.081-0.064) than within groups (0.017–0.42) (Table 2).

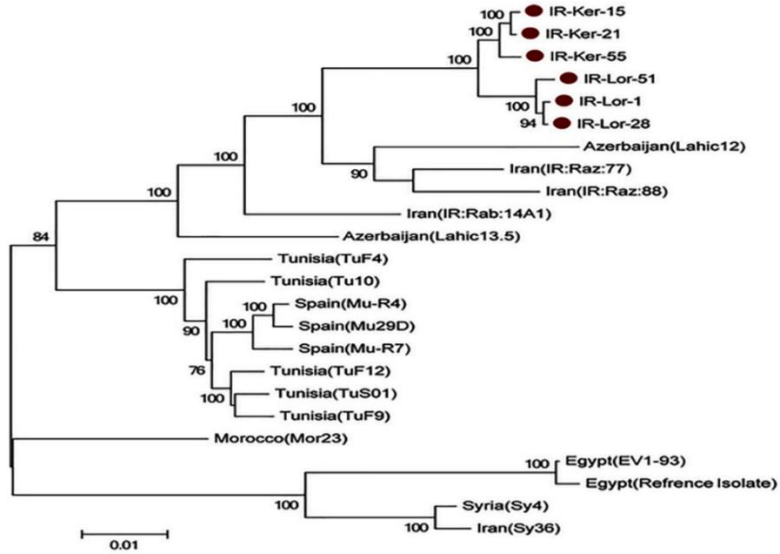

Fig 1. Phylogenetic trees constructed on the full-length genome sequence of different genomic components as well as the full length of the genome of Iranian FNYV isolates, together with homologue sequences of global FBNYV listed in Table 3. The sequences determined in this study are indicated by the red circle symbol. The phylogenetic tree is inferred from the "Neighbor-joining method" and numbers on branches derived from bootstrap resembled data sets, indicated as percentage of support from 1000 bootstrap replications. Branch lengths represent bootstrap values. Nodes with less than 70% bootstrap support were collapsed. The bar represents 0.05 changes per site.

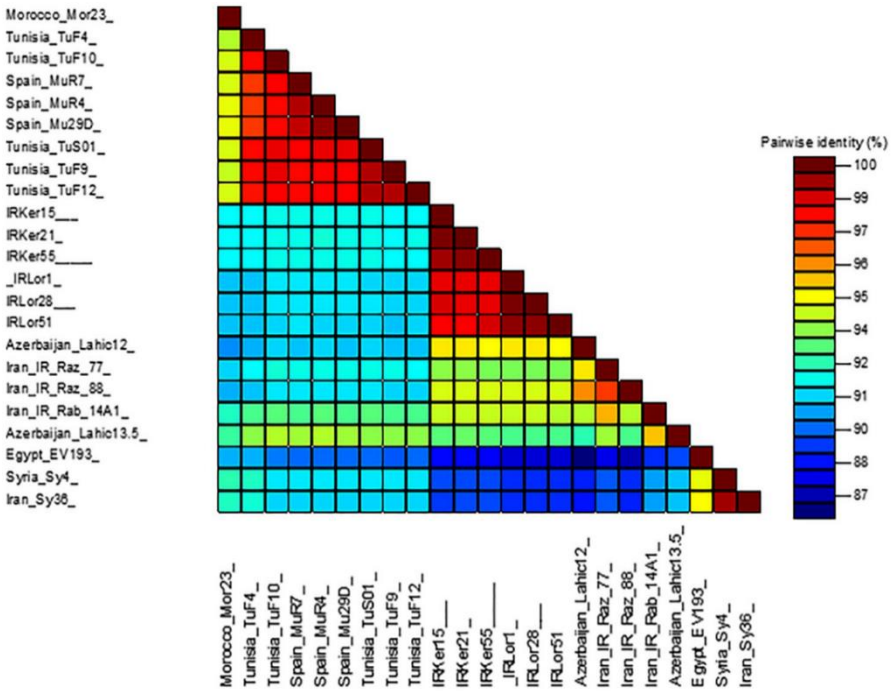

Fig 2. Graphical illustration of pairwise nucleotide identities (%) between Iranian FBNYV and other full genome FBNYV isolates available in GenBank database, using SDT software.

Table 2. Genetic distance within and between geographical groups and mean diversity in entire population calculated for different components and full genome of three FBNYV populations using the MEGA 5.05 program.

Component	Mean distance within group			Mean distance between groups			Mean Diversity in Entire Population
	G3	G2	G1	G2 and G3	G1 and G3	G1 and G2	
R	0.016	0.009	0.019	0.039	0.030	0.041	0.027
S	0.062	0.004	0.008	0.095	0.086	0.069	0.069
C	0.049	0.033	0.013	0.072	0.059	0.045	0.051
M	0.052	0.026	0.012	0.123	0.066	0.127	0.068
N	0.028	0.010	0.009	0.047	0.036	0.030	0.031
U1	0.058	0.025	0.019	0.97	0.083	0.044	0.066
U2	0.045	0.084	0.040	0.098	0.078	0.090	0.069
U4	0.060	0.029	0.033	0.151	0.141	0.144	0.108
Full genome	0.042	0.024	0.017	0.081	0.065	0.064	0.055

Group 1 includes the countries around Caspian Sea (Iran and Azerbaijan), group 2 includes the countries of south-eastern part of Mediterranean basin (Syria and Egypt) and group 3 includes the countries of western and south-western part of Mediterranean basin (Spain, Tunisia and Morocco).

Nucleotide diversity (π) was highest in DNA-S (groups 1 and 2) and DNA-U2/U4 (group 3), but overall below 0.041. Group 1 exhibited the highest total-genome π (0.041), while group 3 had the lowest (0.017). Haplotype diversity (Hd) was 1.000 across all groups. Selection pressure analysis Pi(a)/Pi(s) indicated strong purifying selection in DNA-R and DNA-N, while DNA-C showed positive selection, suggesting adaptive evolution of the C-link protein (Table 3). Population differentiation, assessed using Ks*, Z*, and Snn statistics, indicated significant genetic separation between G1 and G2, G1 and G3, and G2 and G3 for most genome components (Table 4). Fst analysis revealed limited gene flow between populations, indicating strong differentiation. Neutrality tests (Tajima's D, Fu & Li's F* and D*) for total-genome sequences of populations G1 and G3 were negative, although not statistically significant (P> 0.05), which were consistent with potential population expansion (Table 3). Due to limited sequence numbers, neutrality tests for the second population were inconclusive.

Discussion

This study provides a comprehensive genome-wide characterization of six Iranian isolates of Faba bean necrotic yellows virus (FBNYV), which infect chickpea and lentil in western Iran. Although three of the Iranian chickpea isolates included in this study were previously reported with respect to their molecular characterization and phylogenetic relationships (Sokhansanj *et al*., 2018), the objectives and scope of the present study are fundamentally different. The earlier work was primarily limited to the characterization of the genomic components of individual isolates and their phylogenetic placement. In contrast, the present study integrates these previously characterized isolates with three additional Iranian isolates to investigate the evolutionary dynamics and population genetic structure of FBNYV at the whole-genome level. By applying comprehensive population genetic and statistical analyses, the present work provides new insights into the genetic diversity, geographical differentiation, and evolutionary relationships of Iranian FBNYV populations within the broader West Asia and North Africa (WANA) region. These analyses extend substantially beyond the descriptive molecular characterization presented previously and provide a more comprehensive understanding of the evolutionary biology of FBNYV, thereby establishing a foundation for future epidemiological investigations and disease management strategies. The evolutionary patterns observed in the present study are also consistent with the broader characteristics of members of the family *Nanoviridae*. Multipartite nanoviruses possess independently encapsidated circular ssDNA genome components that evolve under a combination of mutation, reassortment, selection, and geographical isolation. Recent comparative studies have emphasized that these evolutionary processes contribute substantially to the diversification of nanovirus populations and the emergence of regionally distinct viral lineages infecting economically important crops throughout the Asia and Africa regions and beyond (Lal *et al*., 2020). Therefore, the population genetic framework presented here extends previous molecular characterization studies by placing Iranian FBNYV isolates within the broader evolutionary context of the *Nanoviridae* family.

Table 3. Genetic structure parameters estimated for different components and full genome of three FBNYV populations. Group 1 includes the countries around Caspian Sea (Iran and Azerbaijan), group 2 includes the countries of south-eastern part of Mediterranean basin (Syria and Egypt) and group 3 includes the countries of western and south-western part of Mediterranean basin (Spain, Tunisia and Morocco).

GR	GG	No. of sequences	S	H	Hd	π	k	Eta	Eta(s)	Pi(s)	Pi(a)	Ratio Pi(a)/Pi(s)	Tajima's D†	Fu and Li's D†	Fu and Li's F†
R	1	12	46	12	1	0.01582	13.33	48	25	0.05497	0.00460	0.081	-0.73862	-0.72219	-0.82825
R	2	2	11	2	1	0.00870	7.33	12	1	0.02557	0.00406	0.156	ND	ND	ND
R	3	9	51	9	1	0.01905	16.05	51	28	0.06711	0.00522	0.074	-0.73643	-0.53301	-0.65397
S	1	12	82	9	0.93	0.06244	31.03	93	33	0.11545	0.04648	0.383	0.08124	0.02955	0.04926
S	2	2	3	2	0.66	0.00402	2	3	2	0	0.00524	-	ND	ND	ND
S	3	9	11	7	0.94	0.00814	4.05	11	3	0.03061	0.00159	0.051	0.00958	0.50817	0.43389
C	1	12	70	11	0.98	0.04909	24.15	77	18	0.01936	0.05632	2.985	-0.15948	0.58567	0.44408
C	2	2	24	2	1	0.03252	16	24	3	0.02462	0.03327	1.360	ND	ND	ND
C	3	9	18	9	1	0.01287	6.33	18	10	0.00698	0.01348	1.940	-0.21361	-0.53236	-0.51008
M	1	12	53	12	1	0.05180	15.95	68	32	0.16226	0.03198	0.179	-0.16860	-0.50069	-0.47128
M	2	2	12	2	1	0.02597	8	13	4	0.04619	0.01996	0.424	ND	ND	ND
M	3	9	12	8	0.97	0.01227	3.77	15	10	0.04437	0.00232	0.051	-1.13382	-0.93673	-1.10174
N	1	12	36	11	0.98	0.02690	12.10	38	13	0.11689	0.00202	0.016	-0.17240	0.08571	0.02047
N	2	2	7	2	0.66	0.01037	4.66	7	0	0.03392	0.00379	0.109	ND	ND	ND
N	3	9	10	7	0.91	0.00790	3.55	11	6	0.03628	0	0	-0.57103	-0.47335	-0.55538
U1	1	12	64	7	0.89	0.05606	25.22	68	17	0.18122	0.02358	0.115	0.55682	0.50938	0.59518
U1	2	2	16	2	0.66	0.02370	10.66	14	6	0.04225	0.01876	0.437	ND	ND	ND
U1	3	9	27	9	1	0.01852	8.33	29	22	0.04594	0.01122	0.238	-1.09692	-1.33224	-1.43051
U2	1	12	50	8	0.90	0.04554	16.39	52	22	0.14042	0.01802	0.117	-0.22023	-0.28067	-0.30188
U2	2	2	43	2	0.66	0.07963	28.66	46	37	0.15511	0.05773	0.346	ND	ND	ND
U2	3	9	42	6	0.91	0.03519	12.66	42	30	0.08626	0.02042	0.226	-0.91502	-1.17809	-1.25097
U4	1	12	67	10	0.96	0.06056	18.16	69	34	0.13090	0.03955	0.282	-0.94844	-0.60243	-0.79099
U4	2	2	13	2	0.66	0.02889	8.66	15	7	0.08088	0.01424	0.168	ND	ND	ND
U4	3	9	38	6	0.88	0.03528	10.58	40	33	0.08656	0.02015	0.222	-1.42325	-1.60967	-1.75617
Full genome	1	12	460	12	1	0.04192	157.54	499	179	0.04895	0.03587	0.726	-0.11331	-0.01186	-0.02423
Full genome	2	2	134	2	1	0.02377	89.33	149	72	0.03565	0.02038	0.566	ND	ND	ND
Full genome	3	9	201	9	1	0.01729	64.97	210	130	0.03577	0.01220	0.336	-0.79718	-0.82688	-0.92159

GR: Genomic region; GG: Geographical group; S: number of segregating (polymorphic) sites; H: number of haplotypes; π: nucleotide diversity; K: average number of genetic differences; Eta: total number of mutations; Eta(s): total number of singleton mutations; Pi(s): synonymous nucleotide diversity; Pi (a): non synonymous nucleotide diversity. † Tajima's D and Fu & Li's D* and F* tests measure the departure from neutrality for all mutations in a genomic region (Tajima, 1989; Fu and Li, 1993); ND: not determined

FBNYV was initially believed to be restricted to the West Asia and North Africa regions, until it was first reported in Spain (Babin *et al.*, 2000) indicating a potential path of FBNYV introduction into southern Europe (Grigoras *et al.*, 2014). Genome sequence comparison showed that all studied Iranian isolates are closely similar to the isolates from Azerbaijan on the basis of their full-length genome sequence. Iran and Azerbaijan are the neighboring countries and are bordered by the Caspian Sea to the north and east parts of Iran respectively; therefore, these two countries may facilitate virus movements via trafficking of infected plant materials or natural aphid dispersal, as described previously for Spain and Tunisia (Kraberger *et al*., 2018). A previous study suggested that, FBNYV- [AZ; Lahic13.5] may represent a FBNYV type common to the Caspian Sea region and Iran (Grigoras *et al*., 2014); but the data obtained in the present study showed 94% and 92% nucleotide identity between six Iranian isolates in full genome with AZ; Lahic12 and AZ; Lahic13.5 isolates from Azerbaijan, respectively. Moreover, isolate IR: Rab: 14A1 from Kerman in southern part of Iran was genetically closer to the isolate AZ; Lahic13.5 (sharing approximately 96% nucleotide sequence identities). Iranian isolate Sy36 that was recovered in Kraberger *et al.*, study in 2018 (Sy36) (Kraberger *et al*., 2018) was most similar to the Syrian isolate (Sy4) and sharing approximately 99% nucleotide sequence identities in all eight components.

Similar geographical clustering has been reported for several members of the family *Nanoviridae*, where virus populations frequently exhibit strong regional structuring because long-distance dispersal is limited despite efficient aphid transmission. Although aphid vectors can spread nanoviruses locally, continental-scale dissemination appears to occur relatively infrequently, resulting in geographically differentiated populations that reflect both historical dispersal events and regional evolutionary processes (Lal *et al*., 2020).

Phylogenetic analyses can contribute to better epidemic control strategies by extending our knowledge on the important molecular features of the viral genome such as mutation, recombination, selection and genetic diversity of plant virus populations (Pagan, 2018). Phylogenetic analyses, which were based on full-length genomes revealed a clear geographical structuring of FBNYV populations. Iranian isolates clustered consistently with Azerbaijani isolates, forming a distinct Caspian Sea lineage. This clustering is consistent with regional virus movement and/or shared ancestry, potentially facilitated by aphid-mediated transmission and regional agricultural exchange. In contrast, isolates from Syria and Egypt formed a separate clade, while western Mediterranean isolates (Tunisia, Spain, and Morocco) grouped independently, indicating limited interregional mixing.

The absence of a clear correlation between host species (lentil versus chickpea) and phylogenetic grouping in the present dataset suggests that geographical origin may exert a stronger influence on the genetic structure of FBNYV populations than host association. However, this interpretation should be made with caution because the number of lentil-derived isolates included in the analysis was limited. Additional genome sequences obtained from lentil and other legume, which hosts across broader geographical regions, will be necessary to determine more conclusively whether host adaptation contributes to the evolutionary diversification of FBNYV. The relatively high genetic variability observed among Iranian FBNYV isolates may reflect the country's diverse agro-ecological conditions together with long-term local virus circulation, which could facilitate the emergence of geographically differentiated viral populations. This observation is also consistent with recent reports, showing that geographical distribution rather than host specialization may represent a stronger determinant of population structure for several nanovirus species, although broader sampling across alternative hosts is still required to confirm this hypothesis for FBNYV.

The geographical populations analyzed in this study were defined according to the distribution of complete genome sequences currently available in GenBank and previously recognized epidemiological regions within the West Asia and North Africa (WANA) region, providing a practical framework for comparative population genetic analyses.

Table 4. Neutrality tests, gene flow, and genetic differentiation analyses based on variation in different components and full genome between three FBNYV populations. Group 1 includes the countries around Caspian Sea (Iran and Azerbaijan), group 2 includes the countries of south-eastern part of Mediterranean basin (Syria and Egypt), and group 3 includes the countries of western and south-western part of Mediterranean basin (Spain, Tunisia and Morocco).

GR (Genomic region)	Between populations	F_{st}‡	K_s*	Z*	S_{nn}
R	G1 and G2	0.68475	2.39**	3.31**	0.86
R	G1 and G3	0.42852	2.45***	3.83***	0.85**
R	G2 and G3	0.65975	2.37**	2.77**	0.87*
S	G1 and G2	0.65554	2.93**	3.36***	0.93*
S	G1 andG 3	0.60021	2.45***	3.78***	0.90***
S	G2 and G3	0.91484	1.40**	2.70**	1**
C	G1 and G2	0.43537	2.84*	3.44*	0.86
C	G1 and G3	0.47237	2.45 ***	3.83 ***	0.95***
C	G2 and G3	0.49157	1.92**	2.80**	0.91**
M	G1 and G2	0.68458	2.57**	3.34**	0.86
M	G1 and G3	0.52280	2.35***	3.77***	0.95**
M	G2 and G3	0.84900	1.54**	2.76***	1*
N	G1 and G2	0.60012	2.25**	3.37**	0.82
N	G1 and G3	0.50222	1.94**	3.86**	0.81**
N	G2 and G3	0.70241	1.36**	2.75**	1**
U1	G1 and G2	0.57184	2.61**	3.39**	0.86
U1	G1 and G3	0.53839	2.40***	3.85***	0.90**
U1	G2 and G3	0.49208	1.99*	2.78*	0.83
U2	G1 and G2	0.34642	2.47***	3.38**	0.86*
U2	G1 and G3	0.44783	2.33***	3.86***	0.85**
U2	G2 and G3	0.32771	2.19	3.02	0.83
U4	G1 and G2	0.69294	2.45**	3.35**	0.86
U4	G1 and G3	0.66028	2.23***	3.72***	0.85**
U4	G2 and G3	0.78235	1.78**	2.76**	0.91**
TOTAL	G1 andG 2	0.58975	4.70**	3.32**	0.86
TOTAL	G1 and G3	0.54585	4.47***	3.73***	0.90***
TOTAL	G2 and G3	0.67848	3.98**	2.71**	1**

K_s*, Z* and S_{nn} are test statistics of genetic differentiation; ‡F_{st} examines the extent of genetic differentiation between geographical isolates.ns: non-significant values, *Significant values, 0.01<P<0.05, *Significant values, 0.001<P<0.01, *Significant values, P<0.001. P < 0.05 was considered as the criterion for rejecting the null hypothesis that there is no genetic differentiation between two populations.

Population structure of some DNA viruses has shown a clear evidence of population differentiation according to geographical origin (Lal *et al*., 2020; Alishiri *et al*., 2017; García-Arenal *et al*., 2001). The population genetic analyses further supported this conclusion. The Caspian Sea population (Iran-Azerbaijan) exhibited the highest nucleotide diversity (π), indicating either long-term local circulation or multiple introduction events (Table 3). In contrast, Mediterranean populations displayed lower genetic variability, possibly reflecting founder effects or historical bottlenecks. The observation that between-population genetic distances exceeded within-population distances reinforces the existence of well-structured, geographically differentiated subpopulations (Table 2).

The highest and lowest genetic diversity were observed in DNA-U4 and DNA-R, respectively (Table 3). High genetic diversity has often been shown to be positively correlated with indicator values of individual fitness and adaptability to changing environmental conditions (Booy *et al*., 2000). For both individual genome components and complete genome sequences, between-group genetic distances exceeded within-group genetic distances, supporting the existence of geographically differentiated viral populations (Table 2). Genetic difference of Iranian Sy36 isolate with other Iranian isolates was also evident in phylogenetic trees (Fig. 1). Consistent with this observation, it was shown that genetic distance between two different isolates in Azerbaijan, with only ~500 m, is greater than that between the FBNYV isolates from Spain and Morocco countries (Grigoras *et al.*, 2014).

Selection pressure analysis revealed strong purifying selection acting on most genomic components, particularly DNA-R encoding the master replication initiator protein. Such constraint is expected, given the central role of M-Rep in coordinating replication of all genome components (Table 4). In contrast, DNA-C (C-link protein) showed evidence of positive selection across all populations, suggesting adaptive evolution. Given that C-link interacts with host cell cycle regulators and the ubiquitin pathway (Aronson *et al*., 2000), adaptive changes in this protein may reflect host-driven selective pressures or optimization of replication efficiency. Positive selection reflects adaptive genetic changes that enhance the fitness of viral variants under specific environmental conditions (García-Arenal *et al*., 2001). Similar evolutionary constraints have been described for other nanoviruses, where essential replication-associated proteins are generally subject to strong purifying selection because of their indispensable roles in genome replication, whereas proteins involved in virus–host interactions may experience relatively relaxed or positive selection. Such contrasting selective pressures are considered characteristic features of multipartite nanovirus evolution (Grigoras *et al*., 2014; Grigoras *et al*., 2018).

The relatively weaker purifying selection observed in Group 1 at the whole-genome level is consistent with the higher genetic variability detected within this population. Neutrality test results (negative Tajima's D and Fu & Li statistics in groups 1 and 3) may indicate recent population expansion following bottleneck or founder events; however, statistical non-significance warrants cautious interpretation. Larger datasets will be necessary to clarify demographic histories more conclusively. Gene flow analysis (Fst> 0.33 for most comparisons) indicated infrequent genetic exchange between geographic populations. Together with significant Ks*, Z*, and Snn statistics (Table 4), these results demonstrate strong population differentiation. Limited gene flow combined with purifying selection suggests that local evolutionary processes dominate over frequent long-distance viral dispersal. Conversely, in most of plant viruses, gene flow among distinct geographical areas is responsible for shaping the genetic structure of viral populations by reducing the genetic diversity and forming genetic uniformity between viral populations (Moya *et al*., 2004; Murad *et al*., 2004). Overall, our findings support the hypothesis that FBNYV populations in the WANA region have diverged primarily through geographic isolation, restricted gene flow, and localized evolutionary pressures, following divergence from a common ancestral population.

Although recombination has been recognized as an important evolutionary mechanism contributing to the diversification of members of the family *Nanoviridae*, reassortment among independently encapsidated genome components has likewise been identified as an important source of genetic variation in multipartite nanoviruses. Recent reviews have emphasized that both recombination and reassortment contribute substantially to nanovirus evolution and host adaptation (Lal *et al*., 2020). Because the primary objective of the present study was to investigate phylogenetic relationships, population genetic diversity, and evolutionary differentiation among complete FBNYV genomes, dedicated recombination analyses were beyond the scope of this work. Future studies integrating larger collections of complete genomes with recombination and reassortment analyses will provide a more comprehensive understanding of the evolutionary mechanisms shaping FBNYV populations throughout the WANA region.

Conclusion

This study provides a comprehensive genome-wide comparative and population genetic analysis of six complete Iranian *Faba bean necrotic yellows virus* (FBNYV) isolates collected from chickpea and lentil in western Iran. While the complete genome sequences of three chickpea isolates had been reported previously, the present study substantially extends current knowledge by integrating these previously characterized isolates with three newly sequenced isolates and performing comprehensive phylogenetic, evolutionary, and population genetic analyses across all eight genomic components. This integrated approach enabled a broader assessment of the genetic diversity, evolutionary relationships, and population structure of FBNYV in Iran and within the West Asia and North Africa (WANA) region. The analyses demonstrated a generally high level of nucleotide conservation among Iranian isolates while revealing component-specific evolutionary patterns, clear geographical population structure, and evolutionary differentiation that are consistent with the multipartite organization of the nanovirus genome. Population genetic analyses revealed significant geographical differentiation among regional populations, together with evidence of restricted gene flow and a shared evolutionary history among neighboring countries. Furthermore, selection pressure analyses indicated that most genomic components are evolving under strong purifying selection, whereas adaptive evolution may have contributed to the diversification of specific genome components, highlighting that the distinct evolutionary constraints acted on the multipartite FBNYV genome. Overall, this study expands the available genomic resources for FBNYV, providing new insights into the evolutionary dynamics, population structure, and geographical diversification of this economically important virus. These findings establish a robust foundation for future investigations of FBNYV evolution using larger collections of complete genome sequences, including studies of recombination, genome component reassortment, host adaptation, and regional epidemiology, thereby contributing to improved understanding and long-term management of FBNYV-associated diseases.

Conflict of interests

The authors declare that they have no conflict of interest.

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