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Evolutionary Characterization and Genetic Structure of Iranian Isolates of *Beet Necrotic Yellow Vein Virus* Population Based on p25 Protein

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ABSTRACT

To study molecular evolutionary characteristics and genetics of *beet necrotic yellow vein virus* (BNYVV) isolates population from Iran, nucleotide sequences of p25 and coat protein (CP) were determined and the amino acids sequences thus deduced were analyzed using phylogenetic and population genetics methods. A survey of BNYVV in Iran indicated the infection of 288 collected samples out of 392 samples in most beet growing areas and that most of the isolates (92%) were of the *A*-type and the rest of isolates (8%) were *P*-type. Our molecular evolutionary analysis showed that CP was highly conserved but allowed to assign all isolates to three distinct groups. Different parts of p25 coding regions were under different evolutionary constraints. The most positive selection was detected at the position 68, the second amino acid of the tetrad motif. Iranian isolates were found to cluster with European isolates into three distinct clusters based on p25 sequences. Population genetics analysis revealed that BNYVV populations have low differentiation (Kt= 3.97145) and low diversity (πT= 0.006, Hd= 0.860) with frequent gene flow indicating lack of phylogeographic structure between populations.

Keywords: Evolution, Phylogenetic analysis, Plant virus, Positive selection.

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV) causes an economically important disease of sugar beets called rhizomania, which is distributed worldwide (Tamada and Baba, 1973). It was initially reported from Italy (Canova, 1959) and is transmitted by the soil-borne plasmodiophorid *Polymixa betae* (Beemster and de Heij, 1987). Rhizomania typical symptoms include massive lateral root proliferation resulting in a beard-like appearance, constriction of the main taproot and stunting of the infected plant. Leaves may become flabby and wilt without discoloration. Sometimes, there is a proliferation of smaller leaves generated at the crown. Because infected roots are inefficient in water and nutrient uptake, general foliar symptoms are similar to water stress or nitrogen deficiency. The disease causes economic loss of sugar beet by reducing yield. It can cause a decrease in root yield of 30-90% and reduce sugar yield up to 70% (Yardimci and Çulal Kiliç, 2011).

BNYVV is the type species of a rod-shaped particle genus of *Benyvirus*. It contains four or five plus-sense single-stranded genomic RNAs with 10 to 11 open reading frames (ORFs) and 5′-cap and a 3′-poly (A) tail (Tamada and Abe, 1989; King *et al.*, 2012). RNA 1 encodes the RNA-dependent RNA polymerase; RNA 2

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encodes coat protein (CP), the triple block involved in cell-to-cell movement and a 14 KDa protein, which is a silencing suppressor; RNA 3 has three ORFs encoding p25 and two other small proteins (King *et al.*, 2012). P25 is responsible for symptom development and previous studies have shown that recently occurring resistance-breaking isolates possess increased p25 variability (Thiel and Varrelmann, 2009). Also, previous studies suggested that p25 is involved in this inhibition of virus translocation in the taproots of the resistant cultivar Rizor (Tamada *et al.,* 1999). Other studies demonstrated the role of RNA3-encoded p25 in the exacerbation of virus-induced symptoms on inoculated leaves (Jupin *et al.,* 1992; Tamada *et al.,* 1999; Klein *et al.,* 2007) and the role of its subcellular localization (Vetter *et al.,* 2004). RNAs 4 and 5 each contain one ORF; RNA 4 encodes p31 that is involved in efficient vector transmission, symptom severity and RNA silencing suppression; RNA 5 encodes p26 and is a pathogenicity factor (Link *et al.*, 2005; King *et al.*, 2012). RNA-5-containing isolates have been reported from France (Koenig *et al.*, 1997), Kazakhstan (Koenig, 2000), China (Li *et al.*, 1999), the UK (Harju *et al.*, 2002) and Japan (Tamada *et al.*, 1996).

Based on molecular characteristics, including RNA composition and nucleotide sequence divergence (Koenig *et al.*, 1995), BNYVV has been classified in three major pathotypes designated as *A, B* and *P*. Type *A* is widespread in most European countries, USA, Iran, China and Japan whereas the *B* type is more restricted and mainly found in Germany, France and in some cases in Sweden, China and Japan (Schirmer *et al.*, 2005; Pavli *et al.*, 2011). *P*-type isolates have been reported from France, UK, Kazakhstan (Schirmer *et al.*, 2005) and recently from Iran (Mehrvar *et al.*, 2009).

Molecular diversity of BNYVV was studied for the first time by single-strand conformation polymorphism (SSCP) and restriction fragment polymorphism (RFLP) analyses that confirmed the existence of three major groups of BNYVV isolates (Kruse *et al.*, 1994; Koenig *et al.*, 1995). Subsequent studies were based on amino acid sequences so that aligned amino acid sequences of p25 showed some key residue substitutions in different types of BNYVV isolates (Miyanishi *et al.*, 1999). Phylogenetic analysis of CP, p25 and p26 amino acid sequences collected from worldwide specimens have shown high variability in p25, especially within amino acid residues 67-70 (Schirmer *et al.*, 2005). A survey detected BNYVV infection in six different regions of China and revealed the presence of *A*- and *B*-type isolates similar to European isolates (Li *et al.*, 2008).

Rhizomania disease symptoms were first reported in sugar beet fields in Fars province of Iran in 1996 (Izadpanah *et al.*, 1996), where subsequent soil bioassays indicated the widespread occurrence of *P. betae* (Kamran *et al.*, 2000). In other researches, the main Iranian sugar beet cultivation areas were surveyed for the presence of BNYVV and two other soil born viruses (*Beet virus Q* and *Beet black scorch virus*) and their soil borne vector, *P. betae* using serological methods (Farzadfar *et al.*, 2007) and RT-PCR (Shahnejat-Bushehri *et al.*, 2006). In addition, some researchers tried to find and tag rhizomania-resistant genes (Nouhi *et al.*, 2008; Salari *et al.*, 2008). In another study, sequence analyses were conducted on the coat protein gene (CP), and parts of RNA3 and RNA4 of an Iranian strain of BNYVV from the Fars province. Sequence alignments of CP with other isolates showed closed similarities at nucleotide and amino acid levels with BNYVV pathotype *A* isolates; S from Japan, and YU2 from Yugoslavia (Sohi and Maleki, 2004). Recently studies revealed that RNA-5 was not found in Iranian isolates (Mehrvar *et al.*, 2009).

Knowledge of evolutionary history and the genetic structure of virus populations provides different levels of information about ecology and epidemiology that are important in the selection of control strategies. Virus-resistant plants offer the most cost effective strategies for rhizomania disease control. Therefore, the effective use of BNYVV-resistant cultivars and management of rhizomania requires a better understanding of the genetic structure and evolutionary trajectories of the virus

population (Richards *et al.*, 2005). Population diversity suggests that this virus is capable of rapid evolution and adaptation in response to changing agricultural practices. DNA sequence data can be used to infer the process of population diversity and to identify evolutionary forces that have shaped the genetic structure of virus population (Canova, 1959). Also, evolutionary characterization of the viruses is essential to develop better strategies for controlling all infections. Since the first report of BNYVV in 1952, different studies were performed on various aspects of rhizomania disease and BNYVV. However, there is little information about evolutionary characteristics, population genetic structure and parameters of BNYVV populations. This study hopes to fill some of these gaps.

MATERIALS AND METHODS

Virus Isolation and Sequences Sources

Sugar beet roots with rhizomania-like symptoms of 392 samples of sugar beet were collected from most regions of Iran, which are listed as follows: Ardabil, North and South Azarbeijan, Khorasan Razavi and Shomali, Semnan, Ghazvin, Zanjan, Ilam, Kermanshah, Hamadan, Fars and Kerman Provinces (Figure 1). Detection of BNYVV and amplification of the p25 coding region of all isolates were carried out by means of RT-PCR as described by Meunier *et al.* (2003). Also, the CP coding region of an isolate was amplified by the same method. The nucleotide sequences of the PCR products were obtained by using an ABI377 sequencer (Applied Biosystems, USA). The nucleotide sequences of p25 coding region were submitted to the GenBank under Accession Nos. AM745439-AM745638, FJ230957-FJ230958 and FM210563-FM210625; and the nucleotide sequence of the CP coding region of IR-GR1 were catalogued in the GenBank under Accession No. FM210683 and were compared with other 45 sequences obtained from GenBank including two Iranain Isolates (AF522462 and AY277887; Jahromi *et al.*, 2005; Sohi and Maleki, 2004) and CP sequences of other

Figure 1. Geographical distribution of BNYVV where sample populations used in this study were collected.

countries. The sequences of other isolates used in this study were obtained from GenBank and their accession numbers are presented in Figure 2.

Multiple Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignments were performed using Muscle (Edgar, 2004) with the default parameters. The most appropriate model was determined using the Bayesian Information Criterion (BIC) implement in MEGA version 5.0 program (Tamura *et al.,* 2011). The alignments were used to calculate genetic distance by T92+G method (Tamura, 1992), which was selected by the lowest BIC. Phylogenetic analyses were done by the neighbor-joining methods using MEGA 5.0 under the assumption of the substitution models proposed. The robustness of inferred evolutionary relationships was assessed by 1,000 bootstrap replicates.

Molecular evolution of p25 neutrality test, namely Tajima's D test was run using DnaSP 4.0 (Rozas *et al.*, 2003). Tajima's D was used to test if the sequence variant distribution followed the model of neutral evolution. Also, using the MEGA 5.0 program (Tamura *et al.*, 2011), positive selection was inferred by the counting method described by Nei and Gojobori and, later on, by Suzuki and Gojobori (Nei and Gojobori, 1986; Suzuki and Gojobori,

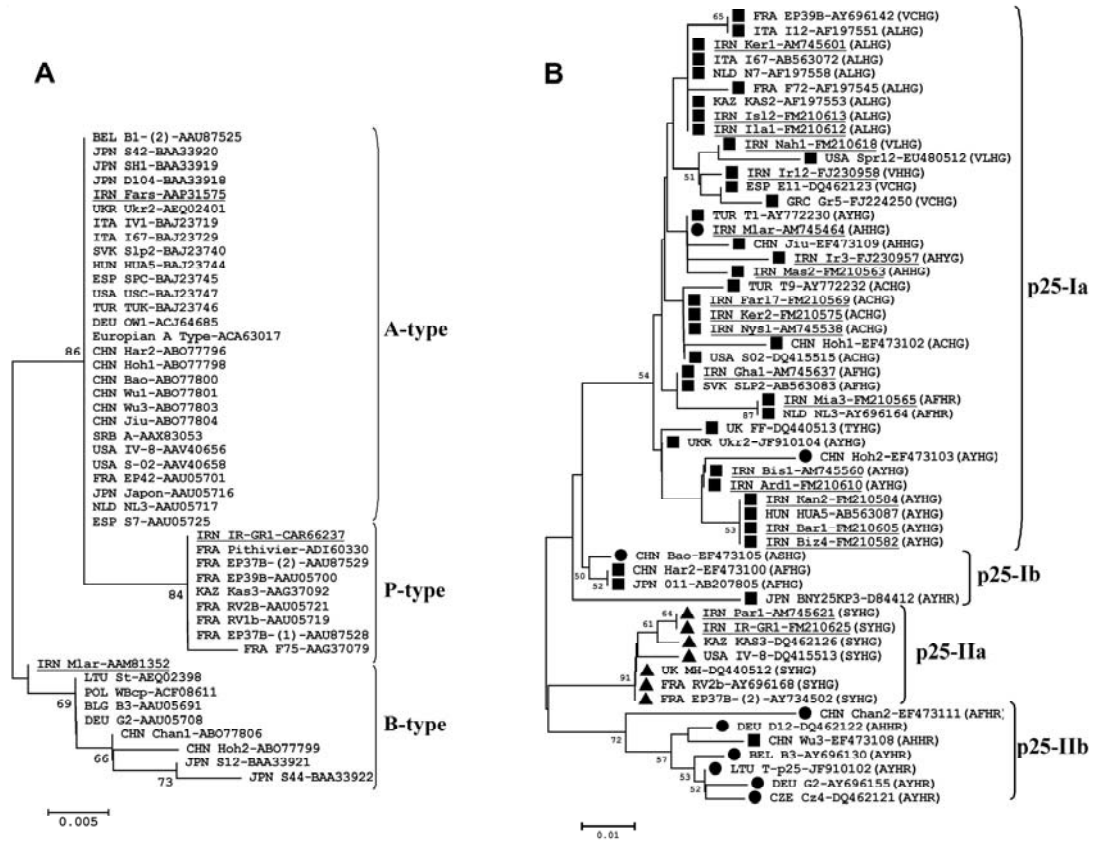

Figure 2. Phylogenetic analysis of BNYVV based on CP (A) and p25 coding region (B) using neighbor-joining method and T92+G model with 1,000 bootstraps (using MEGA 5.0 software). Black square, circles and triangles correspond to *A-, B-* and *P*-type isolates, respectively. All Iranian isolates were shown as underlined. The tetrad motifs of p25 are indicated in parentheses.

1999). According to these methods, the phylogenetic tree of sequences analyzed was used. Selective pressures on each codon were evaluated using the difference between nonsynonymous (d_N) and synonymous (d_S) substitution rates per codon. Values of d_N-$d_S < 0, = 0$ or > 0 indicate negative selection, neutral evolution and positive selection, respectively. Estimates of the difference in substitution rates were made from a phylogenetic tree inferred using the Neighbor-Joining algorithm with distances corrected under the T92+G model.

Population Parameter Estimation

The total population of Iranian BNYVV isolates was divided into 10 subpopulations based on different sampling regions. Within and between populations, diversities were estimated according to Nei (Nei, 1987) based on the T92+G model. Evolutionary analyses were conducted in MEGA version 5.0 (Tamura *et al*., 2011). The numbers of base substitution per site from averaging over all sequence pairs within each population and between populations were calculated and standard errors were obtained by a bootstrap procedure (500 replicates). The rate variation among sites was modeled with a gamma distribution (Shape parameter= 0.2). The analysis involved 262 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$ and all positions containing gaps and missing data were eliminated so that there were a total of 660 positions in the final dataset. Phylogenetic relationships among different BNYVV populations from Iran were constructed using Neighbor-Joining (NJ) method based on between population distances. Genetic differentiation between populations was examined by *Z* test which represents a sequence-based statistical test for genetic differentiation (Hudson *et al.,* 1992). The extent of genetic differentiation or the level of gene flow between populations was measured by estimating F_{st} (the interpopulational component of genetic variation or the standardized variance in allele frequencies across populations). F_{st} ranges from 0 to 1 for undifferentiated to fully differentiated populations, respectively. Normally, an absolute value of F_{st}= 0.33 suggests infrequent gene flow. The statistical tests for genetic differentiation, estimation of F_{st}, number of haplotypes (h) and haplotype diversity (H_d) were performed by DnaSP 4.0 (Rozas *et al.*, 2003). Potential recombination events between nucleotide sequences of p25 coding regions were assayed with SimPlot 3.5.1 (Lole *et al.*, 1999).

RESULTS

Phylogenetic Analysis

The BNYVV was detected in 288 out of 392 sugar beet root tested samples. Most of these isolates were identified as *A*-type (92%) and the rest of the isolates were *P*-type. Although an Iranian isolate, M1ar (Jahromi *et al.*, 2005) was clustered in *B*-type group, the *B*-type isolate was not observed among our 288 samples. The phylogenetic tree based on CP sequences displayed three main lineages associated with the three types. M1ar, Fars and IR-GR1 isolates were clustered to *B, A* and *P*-type groups, respectively. Although the M1ar isolate was grouped in the *B*-type cluster, it differed by an $S_{62}T$ substitution ($T_{62}N_{103}F_{172}$). The *B*-type group was more diverse and included all *B*-type isolates from Europe and Asia (Iran, China and Japan). East Asian *B*-type isolates differed by an $N_{126}H$ substitution that was not seen in other *B*-type isolates. The Fars Isolate was grouped in the *A*-type group, which was more conserved. All CP (28 sequences) sequences within the *A*-type group in this study were identical. In addition, the *P*-type isolates were conservative and the IR-GR1 isolate was identical in sequences to the European *P*-type isolates. These results indicated the occurrence of three types of BNYVV isolates in Iran.

Phylogenetic analysis of p25 revealed high variability within a four-amino acid sequence that is referred to as the tetrad in position 67-70, downstream of the nuclear localization signal motif (Vetter *et al.*, 2004) and upstream of the zinc finger motif (Jupin *et al.*, 1992). The phylogenetic tree of the p25 showed two main groups with four subgroups (Figure 2-B). Iranian isolates mostly belong to subgroup p25-Ia together with common European isolates. Some isolates from west of Iran (Kermanshah and Ilam) were clustered with those of Kazakhstan, the Netherlands and Italy, which all had ALHG motif (Figure 2-B). The Nahavand1 isolate was similar to the Spanish isolate with VCHG tetrad but had the tetrad VLHG that has not been seen in other regions of Iran. An isolate from Khorasan Razavi (Mas2) with the tetrad AHHG stayed together with isolates M1ar from Hamadan and the Chinese isolate Jiu. Totally, the widespread tetrad AXHG was found to occur most commonly including the isolates of Fars, Kermanshah2, Nyshabour1 (ACHG), Ghazvin1 (AFHG), Ardabil1, Bisotoon1, Barsir1, Biz2, Kangavr4 (AYHG) which were grouped with European and American isolates. Isolate Miandoab3 (AFHR) was similar to NL3 isolate from the Netherlands. The p25-Ib cluster was associated with East Asian isolates and both of the subgroups, p25-Ia and p25-Ib, were related to the *A*-type isolates except the isolate M1ar which was grouped in B-type and belongs to the p25-Ia cluster. The distinct cluster p25-IIa matched completely with the *P*-type isolates with the conserved motif SYHG including two isolates from the East (IR-GR1) and the West (Pars1) of Iran. Finally, the p25-IIb cluster was found to be related to *B*-type.

Molecular Evolution Analysis of p25

The nucleotide substitution pattern for p25 coding region showed that the rates of different transitional substitutions varied from 11.46 to 23.95, and those of transversional substitutions varied from 3.93 to 6.75. The nucleotide frequencies were: 0.229 (A), 0.35 (T/U), 0.203 (C), and 0.217 (G). The transition/transversion rate ratios were k_1= 2.734 (purines) and k_2= 3.547 (pyrimidines). The overall transition/transversion bias was R= 1.497, where $R= [AGk_1 +TCk_2]/[(A+G)(T+C)]$.

Mean (relative) evolutionary rates are shown for each site next to the site number (Figure 3). These rates are scaled such that the average evolutionary rate across all sites is 1. This means that sites showing a rate< 1 are evolving more slowly than average whereas those with a rate> 1 are evolving faster than average. These relative rates were estimated using the Jones-Taylor-Thornton (1992) model (+G) (Jones *et al.*, 1992). A discrete Gamma (+G) distribution was used to model evolutionary rate differences

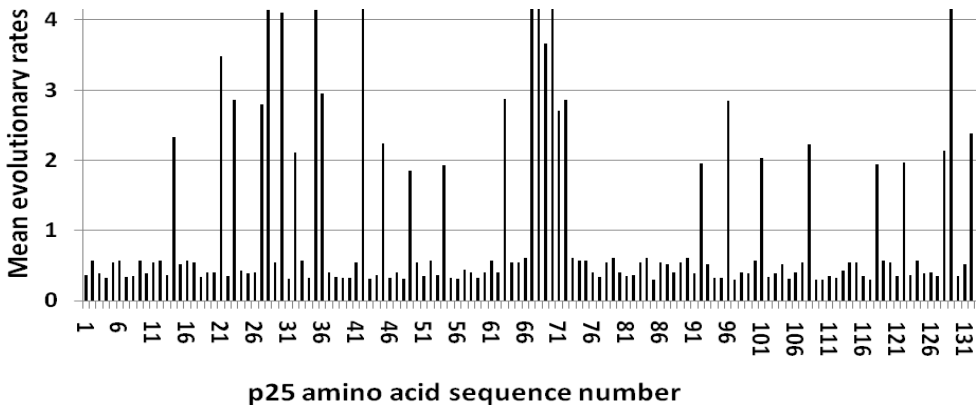

Figure 3. Mean (relative) evolutionary rates over p25 amino acid sequence.

among sites (5 categories). The ML estimate of the gamma shape parameter was 0.2561. The maximum Log likelihood for this computation was -792.779. The analysis involved 262 amino acid sequences. No recombination events were detected in the p25 coding region of Iranian BNYVV isolates.

Positive Selection

The high genetic stability of viruses can be attributed to negative or purifying selection to maintain the functional integrity of the viral genome. The degree of negative selection in genes, or the degree of functional constraint for the maintenance of the encoded protein sequence, can be estimated by two standard approaches. The first approach, which is based on Tajima's D method, tests if the number of segregating sites in the sample significantly departs from the neutral expectation. The test suggests that p25 is evolving neutrally in Iran but this test showed a strong positive selection for worldwide samples. These results may be due to sampling error. Therefore, it is convenient to use an alternative test. To overcome this drawback of Tajima's D, the second approach was to evaluate the neutrality by the index of the nucleotide diversities by differences between nonsynonymous and synonymous positions (d_N-d_S). The d_N-d_S index below zero is consistent with negative selection against protein change. In contrast, a d_N-d_S index above zero may be an indication that adaptive or positive selection is driving gene divergence. In this study, comparisons of all Iranian p25 coding regions showed heterogeneous mean d_N-d_S values (from -2.94 to 3.63). The positions 68 and 4 showed the highest and the lowest d_N-d_S values, and were under highly positive and negative selection, respectively (Figure 4).

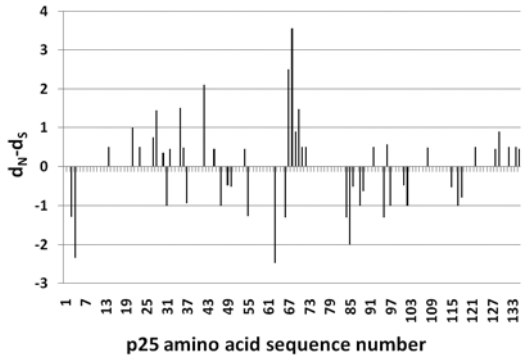

Figure 4. The d_N-d_S along p25 amino acid sequence.

Population Genetic Structure

All sequenced p25 collected from Iran were grouped into 10 subpopulations based on geographical regions (Table 1). Totally 27 haplotypes out of 262 samples were detected with haplotype diversity ranging from 0.466 to 1.0 and nucleotide diversity from 0.001 to 0.009. Estimation of within population distance (D_w) and π showed that the population of Ardabil and the populations of Fars and Kerman represented the most and the lowest population diversities in Iran, respectively. However, the lowest value of haplotype diversity was for Ardabil population indicating the presence of a few haplotypes with high nucleotide differences. Estimation of evolutionary divergence over sequence pairs between groups showed the highest distance between the populations from Azarbaeijan Gharbi and Hamadan and that on the whole Azarbaeijan Gharbi was more divergent compared to other populations (data not shown).

The number of base substitutions per site obtained by averaging overall sequence pairs was 0.006±0.002. The mean evolutionary diversity within subpopulations was 0.005±0.001. Mean interpopulational evolutionary diversity was 0.005. The coefficient of evolutionary differentiation was 0.246±.041. Result of Z test strongly supported the hypothesis that all subpopulations were genetically undifferentiated (P= 0). Measurement of the coefficient F_{st} also showed the existence of gene flow as follows: the values of F_{st} between the subpopulation from Ardabil and

Table 1. Origin of BNYVV populations and estimates of average evolutionary divergence over sequence pairs within populations, population parameters and Tajima's D index based on the ORF encoded p25.

L[a]	N[b]	h[c]	H_d[d]	D_w[e] (SE)[f]	K[g]	π[h]	D[i]
Ardabil	13	4	0.46667	0.010(0.003)	6.15238	0.00932	1.00558
Azarbaeijan Gharbi	18	6	0.81699	0.008(0.002)	5.34641	0.00810	-0.31406
Azarbaeijan Sharghi	6	4	0.80000	0.003(0.002)	2.06667	0.00313	-0.31466
Fars, Kerman	25	5	0.68308	0.001(0.001)	0.96308	0.00146	-0.75093
Ghazvin, Zanjan	10	7	0.90909	0.003(0.001)	2.10909	0.00320	0.11709
Hamadan	16	9	0.90441	0.005(0.002)	2.88235	0.00437	-0.42353
Ilam	5	5	1.0	0.004(0.002)	2.60000	0.00394	0.56199
Khorasan Shomali, Semnan	15	5	0.61254	0.003(0.001)	1.93162	0.00293	-1.9647*
Kermanshah	37	13	0.87838	0.006(0.002)	3.55556	0.00539	-1.30373
Khorasan Razavi	117	12	0.64395	0.004(0.001)	2.58455	0.00392	-1.44412
Total	262	27	0.86076	0.0047(0.0017)	K_t= 3.97145	π_T= 0.00602	-0.96553

[a] Location (Province); [b] Number of isolates; [c] Number of haplotypes; [d] Haplotype diversity; [e] Within population distance; [f] Standard error; [g] Average number of differences; [h] Nucleotide diversity; [i] Tajima's D neutrality test, * Significant (P= 0.05).

other subpopulations, between Fars, Kerman and Khorasan Razavi, and between Khorasan shomali, Semnan and Azarbaeijan Sharghi were all > 0.33 (data not shown), which is an indication of infrequent gene flow. The absolute values of F_{st} for all other subpopulation pairs were < 0.33, suggesting frequent gene flow.

DISCUSSION

This study provides an overview of the relationships between BNYVV isolates collected from Iran and isolates from other countries. We compared molecular evolutionary characteristics of BNYVV based on CP and p25 sequences. Our analyses support the following conclusions:

First, BNYVV isolates were widely distributed in all beet growing regions in Iran. Second, our data showed that the isolates collected from Iran were very diverse including *A* and *P*-types in addition to at least one previously reported isolate (M1ar), which was similar to *B*-type. Third, phylogenetic analysis of CP amino acid sequences collected worldwide revealed that this ORF was highly conserved but allowed to assign isolates to three distinct groups (Figure 2) consisting of *A*-type ($K_{17}T_{62}V_{102}S_{103}L_{172}$), *B*-type ($K_{17}S_{62}V_{102}N_{103}F_{172}$) and *P*-type ($R_{17}T_{62}I_{102}S_{103}$ L_{172}) (Kruse *et al.*, 1994, Lennefors *et al.*, 2005). The high conservation in CP sequences collected worldwide showed that coat protein was required for basic function(s); stability or reproduction in BNYVV as shown for other conserved proteins (Boyko *et al.*, 1992, Morozov and Solovyev, 2003, Ghazala *et al.*, 2008) while most other plant viruses have variable CPs. Such evolutionary conservation occurs because mutations of these amino acids were harmful to protein function, and were lost over time. Also, low variability of CP could separate three distinctive lineages with different pathogenicities. Amino acid comparison of *A*- and *B*-type revealed that the three conserved amino acids in *A*-type isolates ($K_{17}T_{62}V_{102}S_{103}L_{172}$) were replaced by functionally similar amino acids in *B*-type isolates ($K_{17}S_{62}V_{102}N_{103}F_{172}$). Both S and T in position 62 were carbohydrate linking amino acid, N and S in position 103 were carbohydrate N-link and O-link, respectively. Although, L and F in position 172 were both hydrophobic, the residue F was more so (Wimley and White, 1996).

Furthermore, *P*-type isolates had two conserved amino acids in comparison with the *A*-type; Furthermore, P-type isolates had two conserved amino acid substitutions in comparison with A-type: the R17K and I102V so that each pairs were functionally similar to each other (basic hydrophilic and hydrophobic, respectively). Therefore, we propose that these amino acid substitutions cannot be important for differences in pathogenicity among *A-, B-* and *P*-types. Although some substitutions had occurred along coat protein, they did not affect their function. The isolate M1ar which was clustered in *B*-type group and differed in position 62, had an $S_{62}T$ substitution, which was named pseudo-*B*-type group due to function similarity between S and T.

According to the sequence comparison and phylogenetic analysis of p25, the diversity of BNYVV p25 in Iran is reported to be the highest in the world. Two distinct main groups were determined based on p25 sequence analysis. The p25-I included *A*-type isolates which was divided into two subgroups; p25-Ia that can be divided into some other subgroups based on their tetrad motif. *A*-type isolates were widespread and have been reported from Asia, Europe and America (Schirmer *et al.*, 2005). Ten of the thirteen different known tetrads have been found in the Iranian isolates (Mehrvar *et al.*, 2009), no VCHG, TYHG and ASHG tetrads were found in Iran, while they are found only in Europe, UK and China, respectively (Li *et al.*, 2008). The tetrads AHYG (for Ir3 isolate) and VHHG (for Ir12 isolate) have not been reported previously in other countries (Schirmer *et al.*, 2005, Li *et al.*, 2008). The tetrad profile in this group was [AV][LCHFY]H[GR] for which the residue H_{69} was very conserved in all isolates, therefore, it seems to be involved in the active site of p25. The hydrophobic residues A and V were aliphatic neutral amino acids, therefore their replacement with each other could not change protein functionally but the previously reported $V_{67}E_{135}$ was a signature for resistance-breaking variants of BNYVV from the US as opposed to the WT motif $A_{67}D_{135}$ (Acosta-Leal and Rush, 2007). Also, it has been shown that $V_{67}A$ substitution results in higher virus accumulation (Koenig *et al.*, 2009). The position 68 was the most divergent position; the residues L, C, F and Y were hydrophobic but H was hydrophilic and could change protein structure and function. Existence of H residue at position 68 is rare and is only observed in Iranian, Chinese and Dutch isolates. The residue G in position 70 was conserved and rarely replaced with R in the Miandoab3 isolate. The residue G was neutral but R was a polar hydrophilic positively charged residue whose replacement may affect protein function significantly. The subgroup p25-Ib included East Asian *A*-type isolates with the profile A [SHY]HG. The group p25-II was divided into two subgroups; p25-IIa included *P*-type isolates which were more conserved than other subgroups with a unique tetrad SYHG. These findings were in agreement with those that showed the high degree sequence identity among the P-type isolates from Iran and those of Kazakhstan (Mehrvar et al., 2009)." Existence of S at the position 67 was important evidence. The residue S_{67} in *P*-type isolates was replaced by $[AV]_{67}$ in *A*-type isolates since A was a hydrophobic amino acid and S was a polar hydrophilic amino acid which can be covalently linked with carbohydrates. This property may be the reason for *P*-type high pathogenicity. These results were in agreement with those of Chiba *et al.* (2008) who found that the p25 was an avirulence factor. Another group, p25-IIb, was completely matched with *B*-type isolates having the tetrad profile A[FHY]HR and did not include Iranian isolates even the isolate M1ar which grouped in B-type based on CP. Also, some Chinese isolates which were grouped into *A*- (Wu3) and *B*-type isolates (Bao and Hoh2) based on CP, were grouped into p25-IIb, p25-Ib and p25-Ia, respectively. These results showed reassortment events in mixed infections by both *A*- and *B*-type isolates. Finally, based on p25 phylogenetic analysis it was supposed that the origin of the Iranian

BNYVV was more likely from European isolates and was different from South Eastern Asian.

Estimating transitional and transversional substitutions showed that high transitional substitution events along p25 coding region caused a slow evolution rate. Molecular population genetics analysis based on Tajima's test suggested that demographic change had occurred. In other words, negative Tajima's D suggested that demographic forces were acting on the population and there had been a demographic expansion of BNYVV subpopulations resulting in low frequency polymorphism. A total of 31 segregating sites were identified along 405 positions representing more than 7.6% of the total number of sequence bases, indicating low frequency of segregating sites.

Estimation of mean evolutionary rate for each site showed that the positions 68, 67 and 70 (three positions of tetrad) had the highest evolutionary rates, respectively, which was confirmed by strongly positive selection. Therefore, these amino acids evolve more than those in the other positions. The lowest rates were related to the positions 89 (in the zinc finger motif), 109, 110 and 121. These rates for the nuclear localization signal motif (amino acids 57-62) and the zinc finger motif (amino acids 73-90) were very low (0.3-0.6) which indicate the important role of these motifs for virus evolution. The evolutionary rates for the first 13 N-terminal amino acids were very low that might be having major unknown functions. These results were confirmed by the index d_N-d_S; because a strongly positive selection was observed on tetrad and negative selection was detected on the third and fourth nucleotide, the zinc finger motif means any changes in this region might result in virus extinction. There was no positive or negative selection on the NLS site and it had neutral evolution with low evolutionary rate. We assume that the zinc finger site and conserved region in its N-terminal were more important than NLS. Furthermore, the most positive pressure on position 68 indicated the role of this position for virus survival and flexibility against evolutionary forces (Figure 4).

A good understanding of population genetic structure can support the design of an effective disease management program. Lack of phylogeographic structure (Figure 5) between all populations, except for Ardabil and Azarbaeijan Gharbi, indicated high gene flow among these populations that was supported by F_{st} estimation. There was no gene flow between Ardabil population and other populations, between Fars, Kerman and Khorasan Razavi and between Khorasan Shomali and Azarbaeijan Sharghi. The last two pairs of population were geographically far away from the others but Ardabil population had no gene flow with its neighbor populations. Low differentiation ($K<4$) and high gene flow ($F_{st}> 0.33$) among all populations except Ardabil and Azarbaeijan Gharbi, were usually interpreted as a signature of recent range expansion from a single source population.

The population also exhibited an overall low genetic diversity, averaging

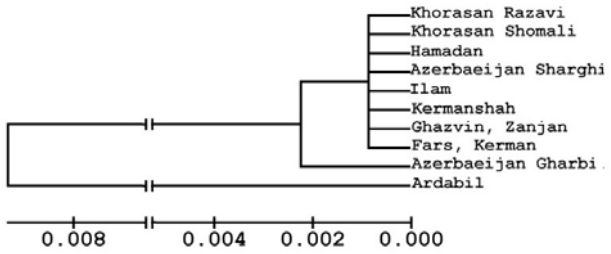

Figure 5. Phylogenetic relationships among different BNYVV populations from Iran, constructed using NJ method based on between population distances.

0.0047±0.0017 (Table 1). Similar genetic structure and diversity have been found in populations of most plant viruses studied so far (Li *et al.*, 2008). Among populations of Iranian BNYVV isolates analyzed here, the population of Ardabil and Azarbaeijan Gharbi showed significantly higher genetic diversity (π= 0.00932 and π= 0.00810, respectively) whereas other populations exhibited low diversity (Table 1). These results in combination with those of population distances indicated that all populations except Ardabil and Azarbeijan Gharbi were closely related and could be considered as one population. These data suggest that these populations most likely were derived via a founder event from a common ancestor and they only recently colonized and spread within the area of Iran. However, we cannot rule out the possibility that the low diversity (π_T= 0.00602) and close evolutionary relationship among isolates could be due to selection by the host plant, because all the populations were collected from beet, or due to need of preserve the function of viral protein (García-Arenal *et al.*, 2003).

Gene flow in the region suggests a significant potential risk for the spread of novel strains that might contribute to breakdown of resistance genes. Approaches such as using RNA silencing for breeding should be applied to those parts of genes with low variability and negative selection. Since p25 is a pathogenicity factor and is responsible for the production of rhizomania symptoms (Chiba *et al.*, 2008), based on our findings on evolutionary rates and selective constraints along p25, it is proposed that breeding efforts could examine the possibility of the use of the zinc finger site and the p25 N-terminal for RNA silencing targeting.

REFERENCES

1. Acosta-Leal, R. and Rush, C. M. 2007. Mutations Associated with Resistance-Breaking Isolates of *Beet Necrotic Yellow Vein Virus* and Their Allelic Discrimination Using Taqman Technology. *Phytopathol.,* **97**: 325-330.
2. Beemster, A. B. R. and De Heij, A. 1987. A Method for Detecting *Polymyxa betae* and *Beet Necrotic Yellow Vein Virus* in Soil Using Sugar-beet as a Bait Plant. *Neth. J. Plant Pathol.,* **93**: 91-93.
3. Boyko, V. P., Karasev, A. V., Agranovsky, A. A., Koonin, E. V. and Dolja, V. V. 1992. Coat Protein Gene Duplication in a Filamentous RNA Virus of Plants. *Proc. Natl. Acad. Sci. USA,* **89**: 9156-60.
4. Canova, A. 1959. On the Pathology of Sugarbeet. *Informatore Fitopatologico,* **9**: 390-396.
5. Chiba, S., Miyanishi, M., Andika, I. B., Kondo, H. and Tamada, T**.** 2008. Identification of Amino Acids of the *Beet Necrotic Yellow Vein Virus* p25 Protein Required for Induction of the Resistance Response in Leaves of *Beta vulgaris* Plants. *J. Gen. Virol.,* **89**: 1314-1323.
6. Edgar, R. C. 2004. Muscle: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res.,* **32**: 1792-1797.
7. Farzadfar, S., Pourrahim, R., Golnaraghi, A. R. and Ahoonmanesh, A. 2007. Surveys of *Beet Necrotic Yellow Vein Virus*, Beet Soil-borne Virus, *Beet Virus Q* and *Polymyxa betae* in Sugar Beet Fields in Iran. *J. Plant Pathol.,* **89**: 277-281.
8. García-Arenal, F., Fraile, A. and Malpica, J. M. 2003. Variation and Evolution of Plant Virus Populations. *Int. Microbiol.,* **6**: 225-232.
9. Ghazala, W., Waltermann, A., Pilot, R., Winter, S. and Varrelmann, M. 2008. Functional Characterization and Subcellular Llocalization of the 16k Cysteine-rich Suppressor of Gene Silencing Protein of Tobacco Rattle Virus. *J. Gen. Virol.,* **89**: 1748-58.
10. Harju, V. A., Mumford, R. A., Blockley, A., Boonham, N., Clovert, G. R. G., Weekes, R. and Henry, C. M. 2002. Occurrence in the United Kingdom of *Beet Necrotic Yellow Vein Virus* Isolates which Contain RNA 5. *Plant Pathol.,* **51**: 811.
11. Hudson, R. R., Boos, D. D. and Kaplan, N. L. 1992. A Statistical Test for Detecting Geographic Subdivision. *Mol. Biol. Evol.,* **9**: 138-51.

12. Izadpanah, K., Hashemi, P., Kamran, R., Pakniat, M., Sahandpour, A. and Masumi, M. 1996. Widespread Occurrence of Rhizomania-like Disease of Sugar Beet in Fars. *Iran. J. Plant Pathol.,* **32**: 200-206.
13. Jahromi, Z. M., Azimipour, M., Rashidian, J., Rastgoo, N. and Arbabi, M. 2005. Cloning and Expression Recombinant Coat Protein of an Iranian Isolate of *Beet Necrotic Yellow Vein Virus* (BNYVV) in *E.coli*. *The 4th National Biotechnology Congress, Kerman Iran,* 35 PP.
14. Jones, D. T., Taylor, W. R. and Thornton, J. M. 1992. The Rapid Generation of Mutation Data Matrices from Protein Sequences. *Comput. Appl. Biosci.,* **8**: 275-282.
15. Jupin, I., Guilley, H., Richards, K. E. and Jonard, G. 1992. Two Proteins Encoded by *Beet Necrotic Yellow Vein Virus* RNA 3 Influence Symptom Phenotype on Leaves. *EMBO J.,* **11**: 479-488.
16. Kamran, R., Izadpanah, K. and Shivani, A. 2000. Distribution of *Polymyxa betae*, the Vector of Sugar-beet Rhizomania, in Fars Province. *Proceedings of the 14th Iranian Plant Protection Congress,* Isfahan, Iran, 74 pp.
17. King, A. M. Q., Adams, M. J., Carstens, E. B. and Lefkowitz, E. J. 2012. *Virus Taxonomi: Ninth Report of the International Committee on Taxonomy of Viruses.* Elsevier Inc., 1163 PP.
18. Klein, E., Link, D., Schirmer, A., Erhardt, M. and Gilmer, D. 2007. Sequence Variation within *Beet Necrotic Yellow Vein Virus* p25 Protein Influences Its Oligomerization and Isolate Pathogenicity on Tetragonia Expansa. *Virus Res.,* **126**: 53-61.
19. Koenig, R. 2000. Deletions in the Kter-encoding Domain, which Is Needed for Polymyxa Transmission, in Manually Transmitted Isolates of *Beet Necrotic Yellow Vein Benyvirus*. *Arch. Virol.,* **145**: 165-170.
20. Koenig, R., Haeberlé, A. M. and Commandeur, U. 1997. Detection and Characterization of a Distinct Type of *Beet Necrotic Yellow Vein Virus* RNA 5 in a Sugarbeet Growing Area in Europe. *Arch. Virol.,* **142**: 1499-1504.
21. Koenig, R., Loss, S., Specht, J., Varrelmann, M., Lüddecke, P. and Demi, G. 2009. A Single U/C Nucleotide Substitution Changing Alanine to Valine in the *Beet Necrotic Yellow Vein Virus* p25 Protein Promotes Increased Virus Accumulation in Roots of Mechanically Inoculated, Partially Resistant Sugar Beet Seedlings. *J. Gen. Virol.,* **90**: 759-763.
22. Koenig, R., Luddecke, P. and Haeberle, A. M. 1995. Detection of *Beet Necrotic Yellow Vein Virus* Strains, Variants and Mixed Infections by Examining Single-strand Conformation Polymorphisms of Iimmunocapture RT-PCR Products. *J. Gen. Virol.,* **76**: 2051-2055.
23. Kruse, M., Koenig, R., Hoffmann, A., Kaufmann, A., Commandeur, U., Solovyev, A. G., Savenkov, I. and Burgermeister, W. 1994. Restriction Fragment Length Polymorphism Analysis of Reverse Transcription-PCR Products Reveals the Existence of Two Major Strain Groups of *Beet Necrotic Yellow Vein Virus. J. Gen. Virol.,* **75**: 1835-1842.
24. Lennefors, B. L., Savenkov, E. I., Mukasa, S. B. and Valkonen, J. P. T. 2005. Sequence Divergence of Four Soilborne Sugarbeet-infecting Viruses. *Virus Genes,* **31**: 57-64.
25. Li, D. W., Yu, J. L., Han, C. G., Lui, T., Quin, S. C., Lui, Y. and Koenig, R. 1999. Detection and Nucleotide Sequence Analysis of *Beet Necrotic Yellow Vein Virus* RNA 5 Isolated from China. *Chin. J. Biotechnol.,* **15**: 461-465.
26. Li, M., Liu, T., Wang, B., Han, C., Li, D. and Yu, J. 2008. Phylogenetic Analysis of *Beet Necrotic Yellow Vein Virus* Isolates from China. *Virus Genes,* **36**: 429-432.
27. Link, D., Schmidlin, L., Schirmer, A., Klein, E., Erhardt, M., Geldreich, A., Lemaire, O. and Gilmer, D. 2005. Functional Characterization of the *Beet Necrotic Yellow Vein Virus* RNA-5-encoded p26 Protein: Evidence for Structural Pathgenicity Determinants. *J. Gen. Virol.,* **86**: 2115-2125.
28. Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadkari, D., Kulkarni, S. S., Novak, N. G., Ingersoll, R., Sheppard, H. W. and Ray, S. C. 1999. Full-length Human Immunodeficiency Virus Type 1 Genomes from Subtype C-infected Seroconverters in India, with Evidence of Intersubtype Recombination. *J. Virol.,* **73**: 152-160.
29. Mehrvar, M., Valizadeh, J., Koenig, R. and Bragard, C. G. 2009. Iranian *Beet Necrotic Yellow Vein Virus* (BNYVV): Pronounced Diversity of the p25 Coding Region in *A*-type BNYVV and Identification of *P*-type BNYVV Lacking a Fifth RNA Species. *Arch. Virol.,* **154**: 501-506.

30. Meunier, A., Schmit, J. F., Stas, A., Kutluk, N. and Bragard, C. 2003. Multiplex Reverse Transcription-PCR for Simultaneous Detection of *Beet Necrotic Yellow Vein Virus*, Beet Soilborne Virus, and *Beet Virus Q* and Their Vector Polymyxa Betae Keskin on Sugar Beet. *Appl. Environ. Microbiol.,* **69**: 2356-2360.
31. Miyanishi, M., Kusume, T., Saito, M. and Tamada, T. 1999. Evidence for Three Groups of Sequence Variants of *Beet Necrotic Yellow Vein Virus* RNA 5. *Arch. Virol.,* **144**: 879-892.
32. Morozov, S. Y. and Solovyev, A. G. 2003. Triple Gene Block: Modular Design of a Multifunctional Machine for Plant Virus Movement. *J. Gen. Virol.,* **84**: 1351-66.
33. Nei, M. 1987. *Molecular Evolutionary Genetics.* Columbia Univ. Press, New York, 512 pp.
34. Nei, M. and Gojobori, T. 1986. Simple Methods for Estimating the Numbers of Synonymous and Nonsynonymous Nucleotide Substitutions. *Mol. Biol. Evol.,* **3**: 418-426.
35. Nouhi, A., Amiri, R., Haghnazari, A., Saba, J. and Mesbah, M**.** 2008. Tagging of Resistance Gene(s) to Rhizomania Disease in Sugar Beet (*Beta vulgaris* L.). *Afr. J. Biotechnol.,* **7**: 430-433.
36. Pavli, O., Prins, M., Goldbach, R. and Skaracis, G. N. 2011. Efficiency of rz1-based Rhizomania Resistance and Molecular Studies on BNYVV Isolates from Sugar Beet Cultivation in Greece. *Eur. J. Plant Pathol.,* **130**: 133-142.
37. Richards, K., Jonard, G., Guilley, H. and Van Dun, C. M. P. 2005. Method of Conveying BNYVV Resistance to Sugar Beet Plants. *N.V./S.A. (BE),* United States, Patent US **6956149.**
38. Rozas, J., Sánchez-Delbarrio, J. C., Messeguer, X. and Rozas, R. 2003. DnaSP, DNA Polymorphism Analyses by the Coalescent and Other Methods. *Bioinformatics,* **19**: 2496-2497.
39. Salari, V., Norouzi, P., Omidi, M., Amiri, R. and Zandieh, I. 2008. Screening of Sugar Beet Tissue Culture Clones for Resistance to Rhizomania Disease. *Pak. J. Biol. Sci.,* **11**: 1610-1614.
40. Schirmer, A., Link, D., Cognat, V., Moury, B., Beuve, M., Meunier, A., Bragard, C., Gilmer, D. and Lemaire, O. 2005. Phylogenetic Analysis of Isolates of *Beet Necrotic Yellow Vein Virus* collected worldwide. *J. Gen. Virol.,* **86**: 2897-2911.
41. Shahnejat-Bushehri, A. A., Adel, J. and Koohi Habibi Dehkordi, M. 2006. Detection of *Beet Necrotic Yellow Vein Virus* with Reverse Transcription-polymerase Chain Reaction. *IJAB,* **8**: 280-285.
42. Sohi, H. H. and Maleki, M. 2004. Evidence for Presence of Types *A* and *B* of *Beet Necrotic Yellow Vein Virus* (BNYVV) in Iran. *Virus Genes,* **29**: 353-8.
43. Suzuki, Y. and Gojobori, T. 1999. A Method for Detecting Positive Selection at Single Amino Acid Sites. *Mol. Biol. Evol.,* **16**: 1315-1328.
44. Tamada, T. and Abe, H. 1989. Evidence that *Beet Necrotic Yellow Vein Virus* RNA-4 Is Essential for Efficient Transmission by the Fungus *Polymyxa betae*. *J. Gen. Virol.,* **70**: 3391-3398.
45. Tamada, T. and Baba, T. 1973. *Beet Necrotic Yellow Vein Virus* from Rhizomania-Affected Sugar Beet in Japan. *Ann. Phytopathol. Soc. Japan,* **39**: 325-332.
46. Tamada, T., Kusume, T., Uchino, H., Kiguchi, T. and Saito, M. 1996. Evidence that *Beet Necrotic Yellow Vein Virus* RNA-5 Is Involved in Symptom Development of Sugarbeet Roots. *Proceedings of the 3rd Symposium of the International Working Group on Plant Viruses with Fungal Vectors, American Society of Sugar Beet Technologists,* Denver, 49 PP.
47. Tamada, T., Uchino, H., Kusume, T. and Saito, M. 1999. RNA 3 Deletion Mutants of *Beet Necrotic Yellow Vein Virus* Do Not Cause Rhizomania Disease in Sugar Beets. *Phytopathol.,* **89**: 1000-1006.
48. Tamura, K. 1992. Estimation of the Number of Nucleotide Substitutions When There Are Strong Transition-transversion and G+C-content Biases. *Mol. Biol. Evol.,* **9**: 678-687.
49. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. Mega5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.,* **28**: 2731-2739.
50. Thiel, H. and Varrelmann, M. 2009. Identification of *Beet Necrotic Yellow Vein Virus* p25 Pathogenicity Factor-interacting Sugar Beet Proteins that Represent Putative Virus Targets or Components of Plant

Resistance. *Mol. Plant-Microbe Interact.,* **22**: 999-1010.
51. Vetter, G., Hily, J. M., Klein, E., Schmidlin, L., Haas, M., Merkle, T. and Gilmer, D. 2004. Nucleo-cytoplasmic Shuttling of the *Beet Necrotic Yellow Vein Virus* RNA-3-Enclosed p25 Protein. *J. Gen. Virol.,* **85**: 2459-2469.
52. Wimley, W. C. and White, S. H. 1996. Experimentally Determined Hydrophobicity Scale for Proteins at Membrane Interfaces. *Nat. Struct. Biol.,* **3**: 842-8.
53. Yardimci, N. and Çulal Kiliç, H. 2011. Identification of *Beet Necrotic Yellow Vein Virus* in Lakes District: A Major Beet Growing Area in Turkey. *Indian J. Virol.*, **22**:127-130.

تعیین خصوصیات تکاملی و ساختار ژنتیک جمعیت جدایه‌های ایرانی ویروس زردی نکروتیک رگبرگ چغندر بر اساس پروتئین p25

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چکیده

به منظور مطالعه خصوصیات تکاملی و ژنتیک جمعیت جدایه‌های ایرانی ویروس زردی نکروتیک رگبرگ چغندر (BNYVV) توالی نوکلئوتیدی p25 و پروتئین پوششی (CP) آن تعیین شد و توالی آمینواسیدی حاصل از آن با استفاده از روش‌های فیلوژنتیکی و ژنتیک جمعیت تجزیه و تحلیل شد. غربالگری BNYVV در ایران آلودگی ۲۸۸ جدایه از ۳۹۲ نمونه را نشان داد که حاکی از آلودگی بیشتر مناطق رشد چغندر است. بیشتر جدایه‌ها (۹۲ درصد) از تیپ A و مابقی (هشت درصد) از تیپ P بودند. تجزیه و تحلیل تکاملی نشان داد که به رغم اینکه پروتئین پوششی بسیار حفاظت شده است، امکان تقسیم بندی جدایه‌ها به سه گروه مشخص را فراهم می‌سازد. نتایج نشان دادند که قسمت‌های مختلف رمزکننده p25 زیر فشارهای تکاملی متفاوتی بوده اند. بیشترین فشار انتخاب مثبت در موقعیت ۶۸ مشاهده گردید که دومین اسید آمینه از یک موتیف چهارتایی است. بر اساس توالی p25 جدایه‌های ایرانی با جدایه‌های اروپایی در سه گروه مشخص دسته بندی شدند. بررسی ژنتیک جمعیت نشان داد که جمعیت‌های BNYVV تفرق کم ($Kt= 3.97145$)، تنوع پایین ($H_d= 0.860$, $\pi_T= 0.006$) و جریان ژنی بالایی دارند که نشان می‌دهد میان جمعیت‌ها ساختار فیلوژئوگرافیک وجود ندارد.