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RESEARCH ARTICLE

Study on Genetic Diversity of Resistance to the Rust in Iranian Garlic Clones (*Allium sativum* L.) Using NBS profiling Technique

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ABSTRACT: Garlic rust is one of the most important diseases of garlic worldwide, which hardly can be controlled by applying fungicides while the weather condition goes on the favor of the disease progress. The NBS-profiling approach is one of the effective methods for separating the replicated parts of resistance gene analogues (RGA). In this study, 12 primers (NBS-LRR) were used on 16 Iranian garlic clones. Out of 499 scored marker sites in the range of 100 to 800 bp for NBS, from which 477 sites were multi-faceted (95.59 percent). The highest number of marker sites was for the primer combination NBS1-AluI and the lowest was for the primer combination NBS7-RsaI. The highest polymorphism occurred with combination NBS2-AluI and NBS1-AluI with 70 alleles and the lowest polymorphic composition occurred in NBS7-RsaI combination. The results of cluster analysis using UPGMA divided the clones into eight separate groups. This study showed that there is a significant diversity in the homologues of resistance genes in the Iranian garlic clones, which can be exploited in plant breeding programs. In addition, the results indicated that the NBS profiling technique is an efficient method for investigation on diversity of resistance genes in various plant species, including garlic. Using of NBS-profiling technique to study the diversity of resistance genes in garlic clones was addressed for the first time in the world in this study.

KEYWORDS: Garlic rust, NBS-LRR Markers, Resistance genes, UPGMA method.

INTRODUCTION

Garlic (*Allium sativum* L.) is one of the most important vegetables grown in the world and is the second most important in terms of nutritional value and cultivation after onions [1]. This vegetable has a worldwide production of more than 26 million tons per year, which has grown significantly in production and consumption during the last 20 years (1996-2016), its production has been tripled [1]. Asia is leading the world with over 92% of the garlic production [1].

Plant diseases are one of the major constraints in the production of agricultural crops. Of the various plant pathogens, the fungi are particular importance [2]. The causal agent of the garlic rust is a fungus called *Puccinia allii* Rud., which is usually developed by the wind and spreads the disease. The disease is characterized by sores or pustules in the surface of the leaves [2].

Development and usage of resistant cultivars is one of the important ways of disease management in plants which reducing the damage caused by them, however, if genetic control with high heritability is maintained [3]. Among these, plant resistant genes (R-genes) are valuable genetic resources that can be used to improve plants resistance to stressors. Genetic resistance reduces or eliminates the amount of used chemical pesticides and as a result, it is

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the most economic, environment-friendly, and the healthiest way to fight plant diseases [4].

NBS-profiling approach is one of the effective methods for separating replicated RGA fragments. This method can be used in different species with little or no change in primers and protocols [5]. Most of the proteins encoded by the genes of resistance have a nucleotide binding site (Nucleotide Binding Site: NBS) which is associated with a repetitive Leucien-rich (Leucien-Rich Repeats: LRR) variable length at the end of carboxyl. These domains participate in protein-protein interactions and signal transduction of molecular messages [6]. Resistance genes of the NBS-LRR group are the most common group of resistance genes [7]. The studies on the resistance gene (R-genes) in monocotyledonous and dicotyledonous plants have shown that most of these genes contain protected domains (NBS-LRR). Several homologous resistance genes of RGHs from the genome family of rice, corn, soybeans, potatoes, Arabidopsis, wheat and tomatoes, and even perennial herbs such as apples and recently dates, have been isolated. Resistance genes extracted from different species are the source of resistance to viruses, bacteria, fungi and even insects and nematodes. These highly protected motifs are an appropriate option for the identification of R-gene in different species of plants and investigate the type of resistance. Comparison of amino acid sequences of proteins derived from matched resistance genes in different plants has shown that there are a number of protected motifs [8]. Until now, the only proven role for encoding genes of proteins with NBS-LRR in plants has been resistance to diseases or pests [9]. NBS amplification of resistant genes has several advantages for identifying homologous sequences through motifs within the NBS region in new species. Protected motifs of an unchangeable region can be used to determine the phylogeny relationships and the classification of NBS-LRR genes [10, 11, and 12].

A significant collection of protected NBS-LRR sequences is now available in various plant species. Many of these sequences have affected by chromosomal regions carrying large resistance genes and associated with quantitative resistance loci (QRL) effectively located in resistance to disease [13].

In spite of the economic importance of garlic in the world as well as the amount of damage to garlic rust in different regions, the main control measures are limited to chemical control methods, which hardly can be effective when the weather condition goes on the favor of the disease progress. In order to find resistance genes for development of new resistant cultivars, screening of local clones of garlic may address valuable source of R-genes. To achieve this goal, NBS-profiling technique was used to evaluate the genetic diversity of the 16 local garlic clones.

MATERIALS AND METHODS

Plant material

Thirty-six Iranian garlic clones were collected based on genetic diversity and geographic origin from main garlic production areas in Iran. The cloves were planted after disinfection with fungicide carbendazim and confidor insecticide at 2 ppm concentration in a field trial in Chavarzagh Tarom Zanjan with geographical location (48 46' 43.34" E - 36 59' 51.33" N) and altitude of 484 meter from sea level and semi-arid climate in a completely randomized block design with three replications during two consecutive years. By examining the resistance to the disease in the first year, 16 resistant clones to rust disease with the highest performance and morphological diversity were selected and re-cultivated in the 2nd year (Table 1).

Evaluation of resistance to the rust fungus:

In order to evaluate the frequency of leaf infection by rust fungus among the clones, the numbers of pustules were recorded at 4 stages (20 April, 5 May, 20 May and 5 June). The evaluation method of infection frequency of the disease was as follows: 10 plants per plot were randomly selected [14]. The average of number of pustules on leaves of 10 plants was determined as the percentage of rust infection in each clone [14, 15, 16].

DNA extraction

The harvested bulbs were collected and stored in optimum storage condition for the second year. In early spring were grown in the pot in greenhouse conditions. Extraction of genomic DNA from young leaves of each clone was done by CTAB method [17]. Assessment of the quantity and the quality of DNA was carried out by using Nano drop machine and 2% agarose gel electrophoresis.

NBS Profiling method

This step was carried out according to the Van der Linden method [5] with a few changes as follows on genomic DNA.

Table 1. Origin specifications of Iranian garlic clones

Clone	Province	City/Region	Longitude	Latitude	Altitude (m)	Resistance rank
Dezfol	Khozestan	Dezfol	48° 25' 52.57" E	32° 22' 59. 08" N	143	S
Khorva biabanak	Esfehan	Khor va biabanak	54^0 36' 34.18"E	33^0 53'20.56" N	937	SS
Sahneh	Kermanshah	Sahneh	47° 41' 41.08" E	34° 28' 26. 53" N	1354	SR
Lalejin	Hamedan	Lalejin	48° 28' 34.42" E	34° 58' 25. 22" N	1700	R
Azar shahr1	Azarbayejan sharghy	Azar shahr	45° 58' 59.78" E	37° 44' 1. 64" N	1415	SR
Lahijan	Gilan	Lahijan	50° 0' 12.07" E	37° 12' 25. 46" N	4	SS
Khaf	Khorasan razavi	Khaf	60° 8' 50.48" E	34° 34' 18. 53" N	975	SR
Sojas	Zanjan	Sojas	48° 33' 04.45" E	36° 14' 24. 14" N	1774	SR
Hesar	Zanjan	Hesar	47° 43' 7.24" E	36° 57' 26. 62" N	1158	SR
Gilvan 1	Zanjan	Gilvan 1	49° 4' 48.88" E	36° 48' 10. 53" N	340	R
Gilvan 2	Zanjan	Gilvan 2	49° 7' 51.54" E	36° 47' 10. 94" N	340	SS
Chavarzagh	Zanjan	Chavarzagh	48° 46' 43.34" E	36° 59' 51. 31" N	484	S
Harsin	kermanshah	Harsin	47°36' 16.62" E	34° 16' 18. 88" N	1570	SS
kermanshah	kermanshah	kermanshah	47° 4' 40.01" E	34° 19' 39. 76" N	1468	SS
Hamedan	Hamedan	Hamedan	48° 30' 54.08" E	34° 47' 55. 89" N	1829	SR
Azar shahr2	Azarbayejan sharghy	Azar shahr	45° 59' 13.93" E	37° 44' 39. 52" N	1522	SS

R: Resistant, SM: Semi-resistant, SS: Semi-susceptible, S: Susceptible

Table 2. Components of restriction endonucleases digestion reaction and adapter ligation for one sample

Reaction components	per reaction(ul)
10x R/L buffer	6
ATP(10mM)	6
Restriction Enzyme	1
Adapter reverse	3
Adapter Forward.blunt	3
T4 DNA Ligase	0.2
PEG	2
DNA (400ng)	5
D.D.Water	33.8
Total	60

Restriction digestion and adapter ligation

At this stage, about 400 ng of genomic DNA was treated by the shear enzyme with the flat residue (*Alu*I and *Rsa*I). At the same time, T4 and ATP were used (Table 2) and (Table 5). After preparing the main mixture, the samples were incubated at 37 ℃ for 4 hours. Then for inactivation of the restriction enzymes, the samples were heated at 65 ℃ for 15 minutes.

Table 5. Sequence of primers and adapters used in multiplication of PCR & digestion and ligation stage

Primer and Adapter	Primer and Adapter sequences	Length (mer)	TM (℃)
NBS1	5'- GCI ARW GTW GTY TTI CCY RAI CC -3'	23	59.4
NBS2	5'- GTW GTY TTI CCY RAI CCI SSC AT -3'	23	62.7
NBS3	5'- GTW GTY TTI CCY RAI CCI SSC ATI CC -3'	26	67.4
NBS5A	5- 'YYT KRT HGT MIT KGA TGA YGT ITG G -3'	25	61.9
NBS6A	5'- YYT KRT HGT MIT KGA TGAYAT ITG G -3'	25	59.7
NBS7	5'- ATT GTT GGR ATG GGM GGI MTI GG -3'	23	64.4
Adapter primer	5'-ACT CGA TTC TCA ACC CGA AAG -3'	21	54.6
Adapter Reverse. Blunt	5'-TGG GAT CTA TAC TT-C 7-Aminolink -3'	14	38.0
Adapter Forward.Blunt	5'-ACT CGA TTC TCA ACC CGA AAG TAY AGA TCC CA -3'	32	66.7

PCR methods

The polymerase chain reaction was performed in two steps. In the pre-selection stage, only a fraction of the components in the digestion and ligation step was multiplied. In the first step, the final product obtained from the digestion and annealing of the adapters was diluted to 1:6 ratio with water and used as a template for the primary multiplication reaction (Table 3).

The PCR program was conducted using a C1000 thermal cycler (BIO-RAD, USA). The amplification conditions were: 15 min at 95°C (one cycle), followed by 30-35 cycles of 30 s at 95°C, 1.40 min at 55-60°C (depending on motif-specific primer) (Table 5), 2 min at 72°C (30-35 cycles), 20 min at 72°C (one cycles) and hold at 4°C.

Finally, 6 µl of the PCR reaction mixture was loaded onto 2% agarose metaphor gel with a TBE 2X buffer at a constant voltage of 85 in an electrophoresis apparatus.

In the second step, the selective replication chain reaction, the product of the initial multiplication reaction was diluted 1:9, and was used as a template in the second step reaction (Table 4).

The amplification conditions were: 4 min at 95°C (one cycle), followed by 30-35 cycles of 30 s at 95°C, 1.40 min at 55-60°C (depending on motif-specific primer) (Table 5), 2 min at 72°C (30-35 cycles), 20 min at 72°C (one cycles) and hold at 4°C. Finally, 10µl of the reaction mixture of selective amplification was loaded on a 2% agarose metaphor gel with a TBE 2X buffer at a constant voltage of 85 volts in an electrophoresis apparatus.

Data analysis

Each of the replicated DNA fragments in the samples was graded based on the presence and absence of the band and displayed respectively with numbers one and zero. Due to the difficulty of scoring NBS bands, as well as errors caused by the quality of the gel, the blurred and rare bands were neglected [18]. The data were transferred to Excel 2013 software with the aim of calculating the similarity matrix and cluster analysis using Nei's similarity coefficient and UPGMA method to NTSYS software. The PIC multicast content index was calculated using PIC=1-ΣPi2 formula. In this formula, Pi is determined the frequency of the i - th allele in a given location [19].

Genetic variability and polymorphism were also evaluated using Popgene32 software [20]. Also, this software was used to analyze the molecular variance (AMOVA).

Table 3. Reaction components of first step PCR for one sample

Reaction components	per reaction(ul)
PCR buffer (with 15mM $MgCl_2$)	2.5
Hot start *Taq* polymerase (10 (u/ul))	0.08
Domain specific primer	2
dNTP mix (5 mM)	1
Adapter primer	2
Template (1: 6 ratio with water)	5
D.D.Water	12.42
Total volume	25

Table 4. Selective replication of second step PCR for one sample

Reaction components	per reaction(ul)
PCR buffer	1
dNTP mix (5 mM)	0.4
Motif-specific primer	0.3
Adapter primer	0.6
$MgCl_2$	0.3
Taq polymerase (10 (u/ul))	0.05
Diluted mixture first PCR(1:9 ratio with water)	5
D.D.Water	2.35
Total volume	10

RESULTS AND DISCUSSION

In this study, 12 primers (NBS-LRR) were investigated on 16 Iranian garlic clones. Out of 499 scored markers sites in the range of 100 to 800 bp for NBS, 477 sites were multi-faceted (95.59 percent). The average score for each combination was 41.58 (Table 6). The highest number of marker sites was for the primer combination NBS1-AluI and the lowest was for the primer combination NBS7-RsaI.The highest polymorphism was for primer combination NBS2-AluI and NBS1-AluI with 70 alleles and the lowest was for the primer combination NBS7-RsaI with 77.77% alleles. The primers NBS1-AluI and NBS1-RsaI had the highest index of Shannon (Table 6). The high amount of this index in this primers compared to other primers imply that these primers can better justify genetic variation within the population. Therefore, these primers can be used as effective primers to study the genetic diversity of garlic clones in next studies.

Table 6. Results of Genetic Diversity Analysis of 16 Iranian Garlic clones using NBS Markers

Primer	NTB	NPB	PPB	N	Na	Ne	I	He	PIC
NBS1-*Alu*I	72	70	97/22	3.750	1.464	1.499	0.372	0.263	0.529
NBS2-*Alu*I	71	70	98/59	4.000	1.208	1.290	0.218	0.154	0.414
NBS3-*Alu*I	59	55	93/22	4.000	0.885	1.216	0.199	0.131	0.907
NBS5A-*Alu*I	38	37	97/36	3.000	1.250	1.350	0.300	0.202	0.678
NBS6A-*Alu*I	51	49	96/07	4.000	1.375	1.367	0.277	0.195	0.203
NBS7-*Alu*I	25	25	100	3.750	1.188	1.304	0.275	0.184	0.829
NBS1- *Rsa* I	48	47	97/91	3.750	1.531	1.424	0.387	0.257	0.825
NBS2- *Rsa* I	35	33	94/28	4.000	1.075	1.248	0.243	0.155	0.941
NBS3- *Rsa* I	20	16	80	2.250	0.571	1.119	0.112	0.072	0.897
NBS5A- *Rsa* I	51	48	94/11	3.750	1.071	1.258	0.220	0.149	0.749
NBS6A- *Rsa* I	20	20	100	3.500	1.250	1.308	0.272	0.182	0.753
NBS7- *Rsa* I	9	7	77/77	1.750	0.688	1.082	0.087	0.055	0.892
Mean	41.58	29.81	70.40	3.458	1.130	1.289	0.247	0.167	0.718

NTB= number of total band; NPB= number of polymorphic bund; PPB= percentage of polymorphic bund; N= total number of alleles; Na= number of different alleles; Ne= number of effective alleles; He= expected heterozygosity; I= Shannon's information index; PIC= polymorphic information content

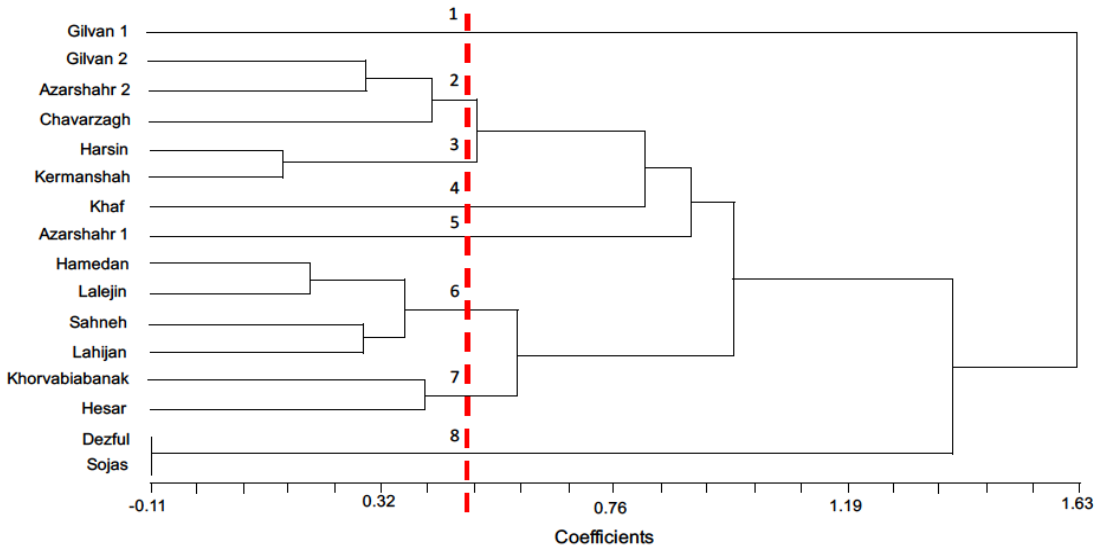

Figure 1. Grouping of Iranian Garlic clones based on NBS-LRR by UPGMA method

The PIC, which represents the value of a primer for the diagnosis of polymorphism in a population, was calculated based on the number of identified alleles and their distribution patterns. The highest level of (PIC) polymorphism in the total population was 0.941 and was related to the location NBS2-RsaI, and a minimum of 0.203, in which related to location NBS6A-AluI (Table 6). The average PIC for the total population was calculated as 0.718. With respect to the calculated polymorphic.

Content, the PIC greater than 0.5 has the most useful polymorphism, and the closer we get to the polymorphism, the more favorable the polymorphisms are data between 0.5<PIC>0.25 have reasonable information [21].

UPGMA method showed that the reference line at the similarity level was about 0.5 based on dendrogram (Fig 1) the population was divided into eight clusters. In the first cluster, Gilvan1 garlic clone was located in the Tarom area of Zanjan, which showed the highest resistance in terms of resistance to disease under farm conditions (Table 1).

In the second cluster there were three clones Gilvan2, Azar shahr, Chavarzagh all three of which were purple

clones. In the third cluster, two clones Harsin and Kermanshah were taken from the Kermanshah province. The fourth cluster consisted of a clone Khaf from northeastern Iran and the fifth cluster containing a distinct clone Azarshahr1 of the northwest of Iran. The sixth cluster, which was also the largest cluster, was the fourth clone Hamedan, Lalejin, Kermanshah and Lahijan which contained white Iranian garlic. In the seventh cluster there were two clones Khor va biabanak and Hesar and the eighth cluster contained two clones Dezfol and Sojas. These results are not consistent with the geographic distribution of the groups as well as the result of morphological analyzes and also their different phenotypic characteristics had a high match (in terms of outer bulb color, shape, and structure of the Bulb - Data not shown). Based on dendrogram can be seen that due to the non-sexual reproduction of garlic, the clones collected from adjacent ecological regions were also located in less distant.

Also, the clones collected from different geographic regions in the same clusters can be indicative of material exchanges among regions or genetic similarity between the clones. It might be also due to identical genetics and geographical origin of some clones that received different names. Grouping based on cluster analysis showed that the clones Gilvan1, Khaf and Azar shahr were completely separated from other clones. This result was fully consistent with the results of evaluation the resistance of the clones to rust disease in farm conditions.

Using NBS-LRR markers, the clone Gilvan1 had distinct characteristics compared to other clones (Fig 1). Study on genetic diversity of 58 durum wheat genotypes by AFLP and SSR markers and the NBS indexing method, and by comparing the correlation of these three methods, which was equal to r = 0.73 and r = 0.76 respectively, it was shown that NBS profiling could be a valid classification tool for genetic variation [22].

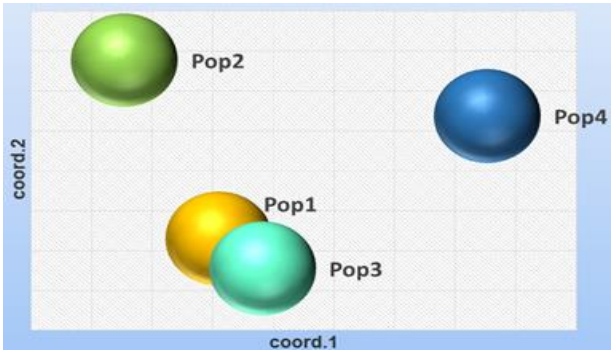

Figure 2. The position of Iranian garlic groups on the coordinate axis based on the two primary output axes and molecular data

Table 7. Analysis of variance of group Iranian garlic clones based on molecular data

Source	Df	SS	MS	Est.var.	%
Among groups	3	66.646	22/215	1/835	11
Within groups	12	182.167	15/181	15/181	89
Total	15	248.813		17/016	100
PhiPT Value=0.090* Value=0.108					

*: significant at %5 probability levels, respectively

According to the descriptor recommended by the International Plant Genetic Resources Institute [23, 24], this clone has violet color with code 5, number of bulb skin 6-8, side view shape of the compound bulb is broadly ovate, basal plate event, shape of matured bulb is globe and type of bulb structure is regular two-fan group of cloves. The distinction of this clone with other violet clones is in the number of skins.

In order to find the genetic distances of the groups in multidimensional form and to have a better view of the analysis to the main vectors, Principle coordinate analysis (PCO) were performed on four groups using molecular data (Fig 2). The first axis alone explained 42.53% of the variations and total of 83.05% of the total justification was explained by first two axes. PCO results showed good agreement with the results of cluster analysis of groups with the help of POPGEN software.

The molecular variance analysis (AMOVA) for distributed genotypes was performed among the groups and the molecular results indicated that the major part of the genetic diversity was related to intra-group diversity (89%) and the partial part of genetic diversity was interdisciplinary (11%) in which these results were significant at 5% level (Table 7).

DISCUSSION

NBS profiling can produce polymorphic markers of genes associated with resistance to disease.

Combination NBS2-AluI and NBS1-AluI were the best primer in NBS-Profiling technique to study the diversity of resistance genes in garlic clones.

The results of cluster analysis using UPGMA divided the clones into eight separate groups, which were consistent with the resistance of the clones in field conditions.

In addition, our results showed that the NBS profiling could be very suitable for genetic variation assays and more importantly, for studies of genetic mapping or gene detection or QTLs responsible for resistance to garlic diseases.

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بررسی تنوع ژنتیکی مقاومت به زنگ در توده های بومی سیر ایرانی(*Allium sativum* L.) با استفاده از تکنیک NBS-profiling

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چکیده

زنگ سیر یکی از مهمترین بیماری‌های سیر در جهان و ایران محسوب می‌شود که در سال‌های مساعد از نظر آب و هوایی استفاده از قارچکش‌ها تاثیر چندانی در کنترل آن ندارد. رهیافت NBS- profiling یکی از روش‌های جدید برای جداسازی قطعات RGA تکثیرشده می باشد. در این مطالعه، ۱۲ ترکیب آغازگری NBS-LRR روی ۱۶ توده سیر ایرانی بررسی شد. از مجموع ۴۹۹ مکان نشانگری امتیازدهی شده در محدوده ۱۰۰تا ۸۰۰ جفت باز برای NBS ، ۴۷۷ مکان چند شکل بودند (۹۵/۵۹ درصد). بیشترین تعداد مکان نشانگری مربوط به ترکیب آغازگر NBS1-*Alu*I و کمترین مربوط به ترکیب آغازگر NBS7- *Rsa*I بود. بیشترین میزان چند شکلی متعلق به ترکیب آغازگری NBS2-*Alu*I و NBS1-*Alu*I با ۷۰ آلل و کمترین درصد چند شکلی مربوط به ترکیب آغازگری NBS7- *Rsa*I بود. نتایج تجزیه خوشه‌ای به روش UPGMA نشان داد که توده ها در ۸ گروه مجزا قرارگرفتند. نتایج این تحقیق نشان داد که در توده‌های بومی سیر ایران تنوع قابل توجهی برای همولوگ‌های ژنهای مقاومت وجود دارد که از آنها می توان در برنامه های اصلاحی بهره‌برداری کرد. علاوه بر این نتایج نشان داد که تکنیک NBS- profiling می تواند به عنوان یک ابزار معتبر در بررسی تنوع ژن‌های مقاومت در گونه های مختلف گیاهی از جمله سیر مورد استفاده قرار گیرد. استفاده از تکنیک NBS-Profiling برای بررسی تنوع ژنهای مقاومت در توده های بومی سیر برای اولین بار در دنیا در این تحقیق مورد ارزیابی قرار گرفت.

کلمات کلیدی: بیماری زنگ سیر، نشانگرهای NBS-LRR، ژن‌های مقاومت، تکنیک UPGMA