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Polymorphism of resistance genes to biotic stresses in some edible vegetable plants revealed by NBS profiling

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Submission Date: July 8th, 2025, **Acceptance Date:** July 24th, 2025, **Publication Date:** August 7th, 2025

Please cite this article as: Harutyunyan R., Badalyan M., Aloyan T., Kharatyan S., Dilanyan V., Harutyunyan M., Hovhannisyan M., Sargsyan L., Melikya A. Polymorphism of resistance genes to biotic stresses in some edible vegetable plants revealed by NBS profiling. *Functional Food Science* 2025; 5(8): 349 - 363. DOI: https://doi.org/10.31989/ffs.v5i8.1712

ABSTRACT

Background: The flora of Armenia is extremely rich and diverse. It is a source of vast reserves of many different useful plants. Wild plants have played an important role in human life since ancient times: they have been used for food, medicine, fiber, and other purposes. Wild edible plants (WEPs) serve as a source of carbohydrates, proteins, and fibers. They are also particularly rich in vitamins and minerals such as vitamin A, vitamin C, zinc, iron, calcium, iodine, thiamine, riboflavin, niacin, and folate. At all stages of ontogenesis, plants are constantly exposed to the risk of infection by various pathogens. Due to their lack of mobility, plants have evolved several molecular and chemical mechanisms that allow them to withstand biotic stresses.

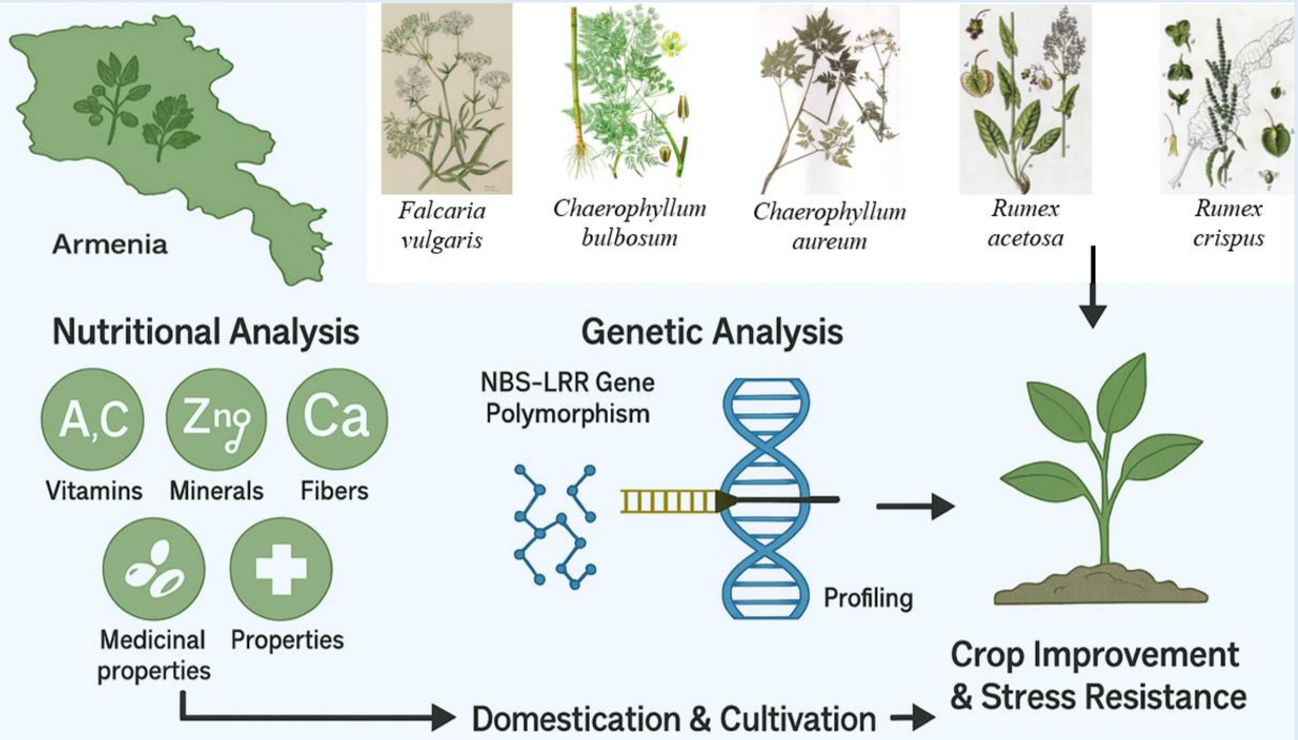
Objective: The aim of the research is two-fold. First, to study the nutritional value of some wild, edible vegetable plants distributed in Armenia. Second, to study the polymorphism of NBS-LRR genes, which are associated with resistance to biotic stresses, and conduct population genetic analysis using the NBS-profiling method.

Methods: Experimental studies were performed at the Biological Research Laboratory of the “Agrobiotechnology Scientific Center” branch of ANAU. The objects of study were some wild edible vegetable plants common in Armenia *Falcaria vulgaris Bernh., Chaerophyllum aureum L., Chaerophyllum bulbosum L., Rumex acetosa L., Rumex crispus L.*).

Results: The studied wild edible vegetable plants are rich in nutrients, mineral salts, vitamins, and have medicinal properties. The population-genetic indicators of all observed species are relatively high, therefore, according to the scale for assessing the state of the gene pool of herbaceous plant species, they are in a satisfactory state.

Conclusion: Wild edible vegetable plants are a significant potential food source, and their domestication and introduction into cultivation is an urgent priority. The results presented in the work clearly demonstrate the advantage of NBS profiling in R and RGA gene marker and population genetic studies, therefore NBS profiling can serve as a important new tool in the process of obtaining varieties resistant to biotic stresses of agricultural crops and improving existing ones.

Keywords: Wild edible plants, NBS profiling, nutritional value, biotic stresses, polymorphism.



Graphical Abstract: Polymorphism of resistance genes to biotic stresses in some edible vegetable plants revealed by NBS profiling

INTRODUCTION

The flora of Armenia is extremely rich and diverse, serving as a major reservoir of numerous valuable plant species - medicinal, industrial, edible, fodder, and more [1-2].

Wild plants have played a vital role in human life since ancient times, being used for food, medicine, fiber, and various other purposes. Wild edible plants (WEPs) are important sources of essential nutrients, including vitamins, carbohydrates, proteins, fiber, and minerals. In particular the WEPs are rich in vitamins A and C, as well as zinc, iron, calcium, iodine, thiamine, riboflavin, niacin, and folate [1, 3-4].

The role of fresh plants as a source of vitamins is particularly important, as most of these vitamins are not synthesized in the human species. Vitamin deficiency disrupts key biochemical and physiological processes in the human body. This can lead to decreased work capacity, reduced resistance to adverse environmental factors, impaired tissue regeneration, delayed blood clotting, weakened adaptation mechanisms, and the development of various serious diseases, even in cases where the diet is rich in high-calorie foods [5-6].

Vitamin A is found in plants in the form of provitamins (carotenoids), which are then converted into the active form of the vitamin A in the human body. Consuming 50–70 grams of wild vegetables per day can provide an adult with the recommended daily intake of vitamins [4].

Beyond their basic nutritional value, many wild edible plants are classified as functional foods, as defined by the Functional Food Center (FFC) - that is, natural or processed foods that contain bioactive compounds providing clinically proven health benefits beyond essential nutrition [7–10]. These bioactive compounds include antioxidants, flavonoids, polyphenols, alkaloids, saponins, and various essential oils that assist in the prevention and management of chronic diseases.

The bioactive components of wild vegetables have been shown to exhibit antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, and even anticancer activities. The antioxidant capacity plays a crucial role in neutralizing harmful free radicals in the body, thereby reducing oxidative stress and lowering the risk of chronic diseases like cardiovascular disorders, diabetes, and cancer. Anti-inflammatory properties contribute in mitigating inflammation, which is a common underlying factor in many metabolic and degenerative diseases. Moreover, certain wild vegetables contain antimicrobial compounds that can inhibit the growth of pathogenic bacteria and fungi, supporting the body's innate defense mechanisms. The regular consumption of wild vegetables thus provides a natural means of enhancing overall health and resilience [11-12].

As such, the regular consumption of wild edible plants can contribute not only to nutritional adequacy but also to health promotion and reduce disease risk, placing them firmly within the framework of functional foods as recognized by the FFC [8, 13, 14].

Humans have long relied on nature to provide resources that have not yet been domesticated, such as edible wild plants. The biological characteristics and potential for cultivation of these plants are insufficiently studied and require further research.

Wild food resources serve as additional sources of nutrition and help meet the dietary needs of rural communities, particularly during periods of food scarcity or crisis [14].

The nutritional properties of wild edible plants have been examined in only a limited number of studies [1, 15, 16].

Unfortunately, due to intensive land use, industrial development, global climate change, atmospheric pollution, the spread of pathogens and pests, and other factors, the natural habitats of wild edible plants have been significantly reduced [1].

Taking the above into account, efforts are currently underway to domesticate several valuable wild edible vegetable species. In this process, the prevention and control of diseases of various origins is critically important - particularly in reducing the risk of pathogen and pest transmission from wild populations to cultivated fields.

It is well established that plants are continuously exposed to infection by various pathogens throughout all stages of their ontogeny, making disease resistance a key consideration in domestication and breeding programs.

No comprehensive research has yet addressed the pests affecting wild edible plants in Armenia. Several studies have been conducted in Morocco to assess the antimicrobial activity of wild food plants traditionally used by local communities against specific bacterial species [17-18].

Due to their immobility, plants have evolved diverse molecular and chemical defense mechanisms to cope with biotic stresses. In this context, a key current priority is the identification and study of genes responsible for resistance to various plant pathogens, their conservation, and the development of seed collections that can be utilized both in breeding programs and directly in human diets.

The plant immune system relies on receptors that recognize molecules linked to a broad range of pathogens. It has been demonstrated that plant resistance to biotic stresses is managed by a family of resistance genes, commonly referred to as R genes (Resistance genes) [19].

R gene products (R proteins) are believed to recognize specific pathogen-derived signaling molecules, rapidly modulating cellular metabolism and physiological processes. This response establishes a defense mechanism that directly inhibits the growth and development of the pathogen. To date, more than one hundred R genes have been cloned, providing valuable insights into the molecular basis of plant immune responses and offering promising targets for genetic improvement of disease resistance in crops [20].

Five unique protein structures are known to play a role in plant biotic resistance: TIR-NBS-LRR (TNL) [21], CC-NBS-LRR (cNLS) [22], receptor-like kinases (RLKs) [23], receptor-like proteins (RLPs) [24], and the Pto protein kinase [25]. Among these, the most diverse group is the NLR family (nucleotide-binding leucine-rich repeat proteins) [25-26], which is characterized by the presence of a leucine-rich repeat (LRR) domain [27]. The last one contains nucleotide-binding sites (NBS) and a leucine-rich repeat (LRR) domain [28].

The presence of conserved domains in the proteins encoded by R genes enables the analysis of the plant genome through the amplification of resistance gene analogs (RGAs) [25].

NBS-LRR receptors consist of two main domains: a central NBS (Nucleotide Binding Site) domain and a C-terminal LRRs (Leucine-Rich Repeats) domain [29]. At the same time, unlike the relatively conserved LRR domain, the NBS domain is polymorphic and consists of conserved motifs such as the P-loop, kinase-2, and Gly-Leu-Pro-Leu (GLPL) regions, mutations in which can lead to impaired receptor function, potentially compromising the plant's ability to recognize and respond to pathogenic threats [26].

Molecular markers are effective tools for investigating the genetic diversity of resistance to pathogens. NBS (nucleotide-binding site) profiling is a PCR-based technique used to study genetic variation by specifically targeting chromosomal regions that contain R genes and their analogs. NBS profiling is based on the analysis of DNA fragments amplified using specific primers. One primer is complementary to the conserved sequence within the NBS domain of R genes, while the other primer targets a restriction fragment of the genomic DNA. NBS profiling enables the assessment of polymorphism in R genes and the identification of specific markers for resistance genes and their analogs.

Currently, multilocus NBS marker profiling is widely used to study the diversity of RGA loci in both cultivated crops [30, 31] and wild species [25, 32]. Additionally, NBS profiling is employed in evolutionary studies of R genes, genotyping gene bank collections, and developing" (assuming the sentence continues) [33].

This study aimed to investigate the polymorphism and conduct population genetic analysis of NBS-LRR genes associated with resistance to biotic stresses in selected wild edible vegetable species distributed in Armenia, using the NBS-profiling method. The overarching goals were to support the prevention and control of diseases and pests' transmission during the domestication process, to map R genes, to apply the findings in marker-assisted and genomic selection, and to contribute to the supply of healthy food while minimizing the use of pesticides - thus protecting both the environment and the food chain.

MATERIAL AND METHODS

The experimental studies were conducted at the Biological Research Laboratory of the "Agrobiotechnology Scientific Center", a branch of the Armenian National Agrarian University. The study focused on several wild edible vegetable species commonly found in Armenia, including *Falcaria vulgaris* Bernh., *Chaerophyllum aureum* L., *Chaerophyllum bulbosum* L., *Rumex acetosa* L., and *Rumex crispus* L.

Dry matter content was determined using the refractometric method, carbohydrate levels were quantified by the Bertrand method, and vitamins A and C were analyzed using the Moore method.

Genomic DNA was extracted from seeds using an automated nucleic acid isolation system from BIOBASE, with reagents supplied by the same manufacturer. DNA concentrations in the samples were measured using a NanoDrop One spectrophotometer, and the purity of the DNA was assessed based on A260/A280 absorbance ratios.

Genomic DNA restriction was performed by preparing a restriction mixture containing 2 µL of the appropriate 10× reaction buffer, 5 µL of nuclease-free deionized water, 2 µL of DNA sample, and 1 µL of Mse I restriction enzyme (5 U/µL). The reaction mixture was incubated at 37 °C for 5 hours. When necessary, the restriction reaction was stopped by adding 1 µL of 0.5 M Toluol B buffer (pH=7.5).

An appropriate adapter was ligated to the ends of the DNA restriction fragments. The 3′ end of the short strand of the adapter was blocked to prevent extension by *Taq* polymerase, while the 5′ end was phosphorylated to enable ligation to the blunt-ended DNA restriction fragments (Table 1).

Table 1. Nucleotide sequences of primers and adapters used in NBS profiling.

Adapter, primer	Nucleotide sequences
Adapter long arm	5′-ACTCGATTCTCAACCCGAAAGTATAGATCCCA-3′
Adapter short arm	5′-TGGGATCTATACTT-3′ (with 3′ amino group)
Adapter primer	5′-ACTCGATTCTCAACCCGAAAG-3′
NBS2	5′-GTWGTYTTICCYRAICCISSCAT-3′
NBS3	5′-GTWGTYTTICCYRAICCIGGCATITGG-3′
NBS5	5′-YYTKRTHGTMITKGATGATGTITGG-3′
NBS7	5′-ATTGTTGGRATGGGMGGIMTIGG-3′
NBS9	5′-TGTGGAGGRTTACCTCTAGC-3′

For Mse I ligation, the short adapter strand was extended to be complementary to the Mse I restriction site. Adapter ligation was performed using high-concentration ligase (5 U/µl) at 20°C for 16 hours. The reaction was terminated by heat inactivation.

Amplification of NBS fragments was carried out in two PCR stages using TPersonal thermocycler (Biometra, Germany). The first step was linear (asymmetric) PCR using a limited amount of NBS-specific primer (1.5 µM), 10 µl of dNTPs, 0.4 U of HotStarTaq (Qiagen, Germany), and 2.5 µl of HotStarTaq PCR buffer in a total reaction volume of 25 µl. The program consisted of 30 cycles of 30 seconds at 95°C, 1 minute 40 seconds at 55–60°C, and 2 minutes at 72°C. The annealing temperature was 55°C for primers NBS5 and NBS7, and 60°C for NBS2.

Asymmetric PCR was followed by exponential PCR with NBS and adapter primers, adding 15 mmol of each primer, 200 µl of dNTPs, 0.4 U HotStarTaq, and 2.5 µl of HotStarTaq PCR buffer to the linear PCR product, in a final volume of 50 µl. Finally, the PCR products were labeled by primer extension using NBS-specific primers labeled at the 5' end with [γ-33P] ATP [33].

The separation of PCR amplicons was performed using 6% polyacrylamide gel electrophoresis on a Multigel-long electrophoresis apparatus (Biometra, Germany). A 100 bp DNA ladder was used to determine the sizes of the DNA fragments. Prior to electrophoresis, an equal volume of denaturing buffer was added to the amplification products.. The sample mixture was incubated at 99°C for 10 minutes, mixed with an equal volume of Ladder, and rapidly cooled. The denaturing buffer consisted of the following components: 98% formamide, 0.01 M EDTA, 3.61 mM bromophenol blue, and 4.64 mM xylene cyanol.

After electrophoresis, gel imaging and result analysis were conducted using the GelDoc Go system (Bio-Rad, USA).

The genomic structure of the studied samples was analyzed using Structure v. 2.3.4 software, which enables identification of common genetic clusters and determination of their proportions within each sample [34].

The dendrogram was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method [35].

Population genetic analysis was performed using POPGENE 1.32 and the GenAlEx Macros specialized for Microsoft Excel softwares, calculating the proportion of polymorphic loci (P_{95}), expected heterozygosity (H_e), absolute (n_a) and effective (n_e) numbers of alleles per locus, Shannon’s information index (I), number of rare alleles (R), population differentiation index (G_{ST}), and population fixation index (F_{IT}), following methodologies previously applied in our studies of other crop species [36, 37].

Statistical data processing was performed using SPSS and MS Excel programs, using standard methods of population-genetic research.

RESULTS

A significant share of the global biomass produced globally comes from plants, which plays a crucial role in the survival of all other living organisms. Plants serve as the primary source of carbohydrates, which should account for more than 50% of the diet during intense physical activity and under extreme conditions. Thanks to rapidly digestible plant sugars (glucose, fructose, sucrose), the body's energy expenditure can be replenished in a very short time. Wild vegetables are rich in high-molecular-weight carbohydrates, such as dietary fiber, which stimulate intestinal peristalsis and promote the activity of beneficial gut microbiota. Wild edible vegetables are rich in minerals essential for the formation of body tissues - particularly the skeletal system, as well as for the proper functioning of endocrine glands, metabolic processes, and energy production, especially water-electrolyte balance. Wild vegetables contain significant amounts of potassium, magnesium, copper, and other essential micronutrients. Considering these factors, along with the ongoing challenges posed by changing climatic conditions, our research aimed to assess several key qualitative indicators in wild vegetable plants, including the content of dry matter, carbohydrates, and vitamins A and C. The species *Chaerophyllum aureum* L. and *Chaerophyllum bulbosum* L. are of particular interest due to their high dry matter content, amounting to 19.8% and 20.2%, respectively. In the other plant species studied, the dry matter content ranges from 10.4% to 12.1% (Table 2).

Table 2. Qualitative indicators of the studied species.

№	**Plant species**	**Chemical composition**			
		dry matter, %	**carbohydrates, %**	**vitamin C, mg%**	**vitamin A, mg%**
1	*Falcaria vulgaris* Bernh.	10.4	7.6	61.8	4.7
2	*Chaerophyllum aureum* L.	19.8	10.6	78.5	4.4
3	*Chaerophyllum bulbosum* L.	20.2	11.8	80.3	4.6
4	*Rumex acetosa* L.	12.1	3.4	68.4	5.2
5	*Rumex crispus* L.	11.5	3.2	61.3	4.9

The highest carbohydrate content, similar to that of dry matter, was recorded in *Ch. bulbosum* L. (11.8%) and *Ch. aureum* L. (10.6%). In contrast, the carbohydrate content in species of the *Rumex* genus was on average 3.5 times lower. The species *F. vulgaris* Bernh. occupies an intermediate position in terms of carbohydrate content, with a value of 7.6% (Table 2).

The ascorbic acid content in the studied plant species is relatively high, ranging from 61.3 mg% (*R. crispus* L.) to 80.3 mg% (*Ch. bulbosum* L.). As for vitamin A, the values across the studied species are relatively similar, ranging from 0.1 to 0.3 mg% (Table 2).

In general, excluding vitamin A, the highest values for the studied indicators were recorded in species of the genus *Chaerophyllum*.

During evolution, plants have developed various mechanisms to cope with interspecific interactions and defend themselves against biological threats. NBS profiling, as a novel molecular marker technology, is currently widely employed to analyze both interspecific and intraspecific polymorphism of R genes. In this study, the NBS profiling method was employed to analyze the polymorphism and phylogenetic relationships of NBS-LRR gene family members in selected wild edible vegetable plants.

In molecular genetic studies, including NBS profiling, the primers we used were evaluated for their informativeness and efficiency to ensure their suitability as molecular markers. Highly efficient markers are characterized by a high level of polymorphism. Quantitatively, polymorphism is measured by two parameters, one of which is the polymorphism information content (PIC). For this purpose, all primers selected for our study were individually tested on the genomic DNA of the target species using the PCR method.

Based on the evaluation of the efficiency of the NBS primers we used, it was decided to profile *Falcaria vulgaris* Bernh. and *Chaerophyllum bulbosum* L. using the NBS7/Mse I and NBS9/Mse I primer pairs, while *Chaerophyllum aureum* L., *Rumex acetosa* L., and *Rumex crispus* L. were profiled using the NBS2/Mse I, NBS5/Mse I, and NBS7/Mse I primer pairs.

The latter primers are complementary to the sequences of two NBS domains, the P-loop and GLPL motifs, enabling the amplification of both the 5´ and 3´ regions of NBS-LRR family genes and their homologs (Figures 1 and 2).

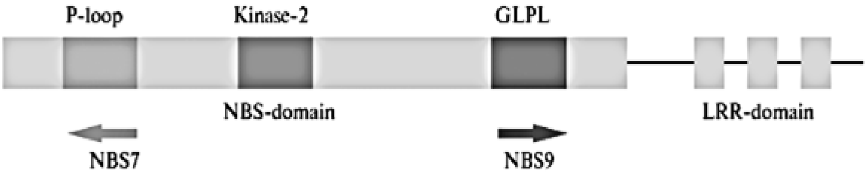


Figure 1. Schematic diagram of the arrangement of NBS7 and NBS9 primers for NBS profiling: NBS – Nucleotide Binding Site, LRR – Leucine-Rich Repeats, GLPL – Gly-Leu-Pro-Leu.

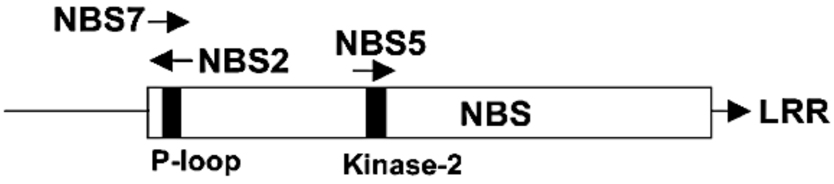


Figure 2. Schematic diagram of the nucleotide binding site (NBS domain) of disease resistance (R) genes, indicating the positions of the modified primers (NBS2, NBS5, and NBS7) used in this study.

Of the five primers tested on *F. vulgaris* Bernh., two – NBS7 and NBS9 – demonstrated very high efficiency, with an average of 14.5 amplified DNA fragments. The maximum number of fragments amplified was 18 (NBS7), and the minimum was 11 (NBS9). For the NBS7 primer, 14 polymorphic fragments were detected, corresponding to 78%, while for the NBS9 primer, 9 polymorphic fragments were detected, corresponding to 82%. In the genomic DNA of the *F. vulgaris* Bernh. species, two rare alleles were identified (Table 3).

Table 3. DNA polymorphism of the studied species based on NBS primers.

Plant species	Primer/ Enzyme	Length of amplified fragments (bp)	Number of DNA fragments	Number of polymorphic fragments	DNA polymorphism (%)	Number of unique fragments
***Falcaria vulgaris* Bernh.**	NBS2/Mse I	265-480	11	4	36	0
	NBS3/Mse I	250-620	9	3	36	0
	NBS5/Mse I	280-550	14	4	29	0
	NBS7/Mse I	280-680	18	14	78	1
	NBS9/Mse I	300-630	11	9	82	1
***Chaerophyllum aureum* L.**	**NBS2/Mse I**	280-450	14	12	86	0
	NBS3/Mse I	340-500	8	2	25	0
	NBS5/Mse I	200-680	16	13	81	1
	NBS7/Mse I	300-750	14	11	79	1
	NBS9/Mse I	260-520	7	2	29	0
	NBS2/Mse I	290-480	12	5	42	0

Plant species	Primer/ Enzyme	Length of amplified fragments (bp)	Number of DNA fragments	Number of polymorphic fragments	DNA polymorphism (%)	Number of unique fragments
***Chaerophyllum bulbosum* L.**	NBS3/Mse I	310-530	8	3	38	0
	NBS5/Mse I	265-630	11	3	27	0
	NBS7/Mse I	240-560	12	9	75	1
	NBS9/Mse I	350-640	15	11	73	1
***Rumex acetosa* L.**	**NBS2/Mse I**	310-560	11	9	82	0
	NBS3/Mse I	240-660	6	2	33	0
	NBS5/Mse I	430-700	16	13	81	1
	NBS7/Mse I	350-680	12	8	67	0
	NBS9/Mse I	280-550	14	5	36	0
***Rumex crispus* L.**	**NBS2/Mse I**	350-630	12	10	83	1
	NBS3/Mse I	280-655	8	3	37	0
	NBS5/Mse I	230-650	13	9	69	0
	NBS7/Mse I	380-680	15	11	73	1
	NBS9/Mse I	255-720	11	4	36	0

For *Ch. aureum* L., three of the five primers tested (NBS2, NBS5 and NBS7) demonstrated high efficiency. The average number of DNA fragments amplified by these primers was 14.7. The maximum number of fragments was 16 (NBS5), while the minimum was 14 (NBS2 and NBS5). Overall, out of the 44 fragments amplified by these primers, 36 (86%) were polymorphic, with fragment sizes ranging from 200 to 750 bp (Table 3). The selected group exhibited a maximum of 13 polymorphic loci (NBS5) and a minimum of 11 polymorphic loci (NBS7). Fragments with a frequency of occurrence below 5% or unique fragments number two in each of the species.

Marker analysis of *Ch. bulbosum* L. revealed that the NBS7 and NBS9 primers exhibited very high efficiency. Seventy-four percent of the DNA fragments amplified by these primers were polymorphic, with the highest polymorphism observed for NBS7 (75%) and the lowest for NBS9 (73%). Notably, as in *F. vulgaris*, there are two unique fragments with a frequency of occurrence below 5% in this case as well (Table 3).

During the NBS marker assay of *R. acetosa* L., like *Ch. aureum*, the primers NBS2, NBS5, and NBS7 exhibited very high activity, amplifying a total of 39 fragments, with a maximum of 16 (NBS5) and a minimum of 11 (NBS2). The average number of polymorphic fragments amplified by these primers was 10, with polymorphism rates of 82%, 81%, and 67% for NBS2, NBS5, and NBS9, respectively. One rare allele was identified in this species, detected during amplification with the NBS/Mse primers (Table 3).

In the NBS marker assay of *R. crispus* L., the primers NBS2, NBS5, and NBS7 demonstrated high efficiency, amplifying an average of 13.3 DNA fragments, of which 10 (75%) were polymorphic. The sizes of these fragments ranged from 230 to 680 bp. Two rare fragments were identified in the experimental group for this species (Table 3).

Population genetic analysis was performed to evaluate the intraspecific and interspecific genetic diversity of R genes and their homologs conferring nematode resistance in the studied species. For this purpose, the proportion of polymorphic loci (P_{95}), expected heterozygosity (H_E), absolute (n_a) and effective (n_e) allele numbers per locus, Shannon's information index (I), number of rare alleles (R), population differentiation coefficient (G_{ST}), and population fixation index (F_{IT}) were estimated (Table 4).

Table 4. Genetic diversity indices in populations of the studied species based on NBS profiling.

Plant species	Genetic-mathematical indices							
	H_e	n_a	n_e	P_{95}	R	G_{ST}	I	F_{IT}
Falcaria vulgaris Bernh.	0.131 (0.011)*	14.500 (0.913)	11.500 (0.513)	0.800	2	0.141	0.204 (0.018)	0.038
Chaerophyllum aureum L.	0.156 (0.014)	14.666 (1.101)	12.000 (0.684)	0.820	2	0.138	0.217 (0.022)	0.054
Chaerophyllum bulbosum L.	0.163 (0.018)	13.000 (1.104)	10.000 (0.764)	0.748	2	0.218	0.206 (0.011)	0.063
Rumex acetosa L.	0.136 (0.016)	13.000 (1.163)	10.000 (0.714)	0.766	1	0.174	0.198 (0.024)	0.076
Rumex crispus L.	0.145 (0.028)	13.330 (1.088)	10.000 (0.515)	0.754	2	0.201	0.184 (0.018)	0.088

* Numbers in brackets correspond to Standard Deviations (SD).

Expected heterozygosity, also known as gene diversity in a multilocus system, refers to the probability that an individual will be heterozygous at a given locus. The expected heterozygosity in the populations of the species we studied ranges from 0.131 to 0.163. Such a low heterozygosity index indicates limited genetic diversity within the populations.

The effective number of alleles, as a measure of population genetic diversity, represents the number of alleles for which the observed and expected frequencies of homozygotes are equal. In the studied species, this value is relatively similar, ranging from 10 to 12.

The Shannon information index, which reflects the level of genetic diversity within a population, was 0.217, 0.198, 0.204, 0.184, and 0.206 for *F. vulgaris* Bernh., *Ch. aureum* L., *Ch. bulbosum* L., *R. acetosa* L., and *R. crispus* L., respectively (Table 4).

In all studied species, except *R. acetosa*, two rare alleles were identified. This serves as an indicator of the uniqueness of genes responsible for biotic stress resistance within the observed populations.

Similar to various bioeconomic traits, natural disease resistance is determined by the polymorphism of specific genes. Among the studied species, the highest number of polymorphic loci was recorded in *Ch. aureum*, with a value of 82% (P_{95} = 0.820). In *Ch. bulbosum, R. acetosa*, and *R. crispus*, the proportion of polymorphic loci was similarly high, ranging from 74% to 76%. *F. vulgaris* occupied an intermediate position among the studied species, with P_{95} = 0.800 (Table 4).

The genetic similarity index between pairs of homologous individuals within the populations was also analyzed. The widest range of similarity was observed in *R. crispus* and *Ch. bulbosum*, while the lowest values were recorded in *F. vulgaris*.

The population fixation index (F_{IT}) in the species studied is greater than zero, ranging from 0.038 to 0.088. This indicates a deficit of heterozygous genotypes in the overall population, likely resulting from deviations in genotype frequencies (Table 4).

The population differentiation index (G_{ST}) of the studied species is relatively low, ranging from 0.138 to 0.218. This indicates that the majority of genetic diversity, approximately 79–87%, is concentrated within populations, while intrapopulation variability accounts for 13–21% (Table 4).

Based on the results of NBS profiling, the genetic distance (D_N) among the studied species was estimated using polymorphic alleles of the NBS-LRR genes and their frequencies. Overall, the genetic distance ranged from 0.026 to 0.057. The species pairs *Ch. aureum* and *R. acetosa*, *Ch. aureum* and *R. crispus*, *Ch. aureum* and *Ch. bulbosum*, *R. acetosa* and *R. crispus*, and *F. vulgaris* and *R. crispus* showed relatively close genetic relationships. The highest genetic distances were observed between *R. crispus* and *Ch. bulbosum* (D_N = 0.044), *F. vulgaris* and *Ch. bulbosum* (D_N = 0.057), *R. acetosa* and *F. vulgaris* (D_N = 0.051), and *Ch. aureum* and *F. vulgaris* (D_N = 0.048) (Table 5).

Table 5. Genetic distances among the studied species based on NBS profiling results.

Plant species	*Falcaria vulgaris* Bernh.	*Chaerophyllum aureum* L.	*Chaerophyllum bulbosum* L.	*Rumex acetosa* L.	*Rumex crispus* L.
F. vulgaris Bernh.	0.000	0.042	0.057	0.051	0.038
Ch. aureum L.		0.000	0.036	0.034	0.038
Ch. bulbosum L.			0.000	0.048	0.044
R. acetosa L.				0.000	0.026
R. crispus L.					0.000

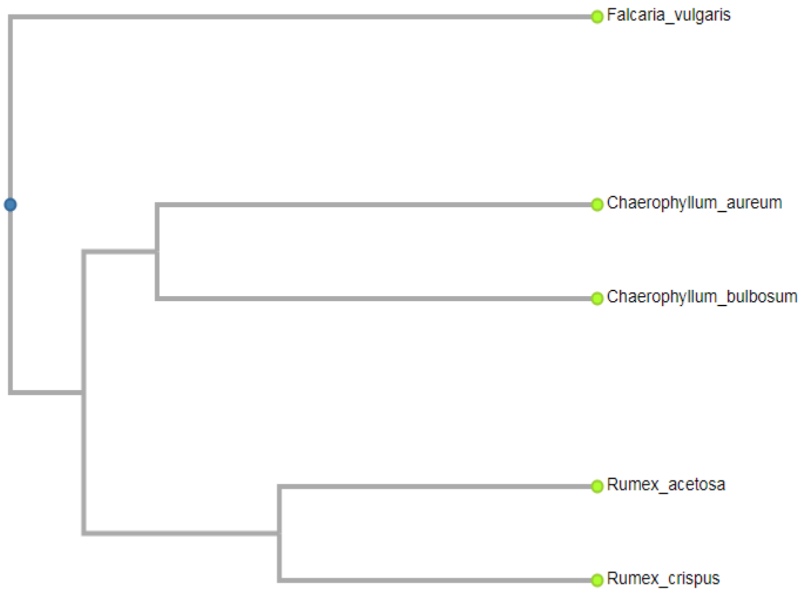


Figure 3. Genetic clustering of studied samples using NBS profiling.

Based on the obtained NBS profiles (D_N), a dendrogram was constructed, where the included samples formed highly polymerized clusters (Figure 3).

Clustering analysis of the studied species resulted in the formation of two distinct clusters. The first cluster comprises *Ch. aureum* and *Ch. bulbosum*, while the second includes *R. acetosa* and *R. crispus*. Such separation indicates significant differences in the groups of genes conferring resistance among different species, which is likely associated with the evolution and genetic diversity of NBS-LRR genes.

DISCUSSION

Plants frequently employ a defense mechanism based on the "gene-for-gene" principle, whereby the product of a resistance (R) gene recognizes the product of a pathogen's avirulence gene. Most of the R genes identified to date belong to the NBS-LRR family, characterized by cytoplasmic nucleotide-binding sites and leucine-rich repeat domains.

In this study, we describe the NBS-profiling approach, which generates a large collection of fragments from R genes and their analogs (RGAs), uncovers genetic diversity, and enables the identification of relevant molecular markers. It should be noted that research on NBS marker profiling of R genes and their analogs conferring stress tolerance in wild edible vegetable plants is scarce or nonexistent. So, in order to comprehensively profile the NBS-LRR gene family, we used four high-efficiency NBS primer/enzyme combinations simultaneously.

Of the 176 NBS fragments obtained from the five species studied, 139 (79%) were polymorphic, with nine rare fragments identified. These rare alleles may represent unique evolutionary adaptations and could serve as valuable sources of novel resistance traits. The species exhibiting the most unique R gene spectra may be considered promising donors for resistance genes in future breeding efforts. In contrast, 21% of observed monomorphic fragments likely represent conserved genes or allelic variants conferring resistance to specific, perhaps widespread, phytopathogens.

The polymorphism of the R genes in the species we studied is extremely high, indicating the successful selection of the primer/enzyme system for NBS marker profiling. Numerous cases have been reported where incorrect estimation of primer informativeness leads to the detection of only a minimal number of polymorphic fragments during genotyping of the studied cultivars or their wild relatives [38].

The analysis of the data suggests that the level of gene polymorphism depends both on the number and variability of R genes in the species' genome. Additionally, the potential clustering of samples based on resistance to various phytopathogens was analyzed, with the cophenetic correlation coefficient (CP) found to be relatively high (CP = 0.7325).

In terms of population genetic analysis, only *R. acetosa* was characterized as basal or typical, exhibiting the minimum number (R = 1) and frequency of rare alleles. All other species possess a more unique gene pool, indicating that they harbor a greater number of rare alleles that are not characteristic of the studied region.

The genetic diversity index in the populations of the studied species is relatively high, whereas the low expected heterozygosity observed in *R. acetosa* and *F. vulgaris* indicates a certain degree of gene pool depletion. Nevertheless, the population genetic indices for all examined species remain quite high, suggesting that, according to the assessment scale for the state of the herbaceous gene pool, these populations are in a satisfactory condition.

The high genetic polymorphism observed in the NBS-LRR resistance genes of wild edible vegetable species not only reflects their resilience to potential biotic stresses but also suggests a stable genetic basis for maintaining their nutritional qualities under environmental pressures. Since the nutritional value of wild edible plants, rich in essential minerals, vitamins, and bioactive compounds, is closely linked to their physiological state and stress resistance mechanisms, preserving and characterizing these resistance genes is crucial [39, 40].

Understanding and preserving the adaptive variation in R genes contributes to more than just plant protection. It informs the strategic conservation of wild genetic resources, supports marker-assisted selection for disease-resistant varieties, and provides opportunities for the domestication of more plants rich in bioactive compounds. These efforts can accelerate the development of functional foods, which combine agronomic performance with clinically relevant health benefits.

Therefore, integrating molecular marker data with biochemical and agronomic traits may facilitate the selection of elite genotypes that are both nutritionally superior and resilient to pathogens, contributing to sustainable agriculture, climate-smart breeding programs, and the functional food industry. Such interdisciplinary applications position wild edible plants not only as reservoirs of genetic resistance but also as potential drivers of innovation in health-oriented food systems.

CONCLUSION

Thus, through multi-locus analysis using the NBS profiling method, five different species of wild edible vegetable plants were characterized for the first time in the Republic of Armenia. Among these species, a high level of polymorphism in NBS-LRR genes was detected. Groups of samples exhibiting both highly similar and distinctly different NBS-LRR gene patterns were identified.

The results presented in this study demonstrate the advantages of NBS profiling for marker analysis of R and RGA genes as well as population genetic research. Therefore, NBS profiling has the potential to become an important tool in developing biotic stress-resistant crop varieties and improving existing ones.

This approach is particularly valuable for wild edible plants. By maintaining their resistance, wild edible plants can serve as natural, nutrient-dense food sources, thereby supporting the development of a healthy diet and the conservation of plant biodiversity simultaneously.

Abbreviations: RA: Republic of Armenia, NBS: nucleotide-binding site, LRR: leucine-rich repeat, WEPs: Wild edible plants, RLK: receptor-like kinase, RGAs: resistance gene analogs, GLPL: Gly-Leu-Pro-Leu, DNA: deoxyribonucleic acid, PCR: polymerase chain reaction, PIC: polymorphism information content, bp: base pair, EDTA: Ethylenediaminetetraacetic acid, UPGMA: Unweighted Pair Group Method with Arithmetic Mean, CP: cophenetic correlation coefficient.

Competing interests: The authors declare that there are no competing interests.

Authors' contributions: RH: conceptualization, methodology, validation, resources, data curation, writing-original draft preparation, writing-review and editing; MB: conceptualization, methodology, resources, data curation, writing-original draft preparation, writing-review and editing; TA: conceptualization, methodology, data curation, writing-original draft preparation, writing-review and editing, SKh: methodology, data curation, writing-review and editing, VD: methodology, data curation, writing-review and editing, MH: methodology, resources, writing-review and editing; MH: resources, writing-review and editing, LS: resources, writing-review and editing; AM: conceptualization, methodology, validation, resources, data curation, supervision, writing-original draft preparation, writing-review and editing. All authors read and approved the final version of the manuscript.

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