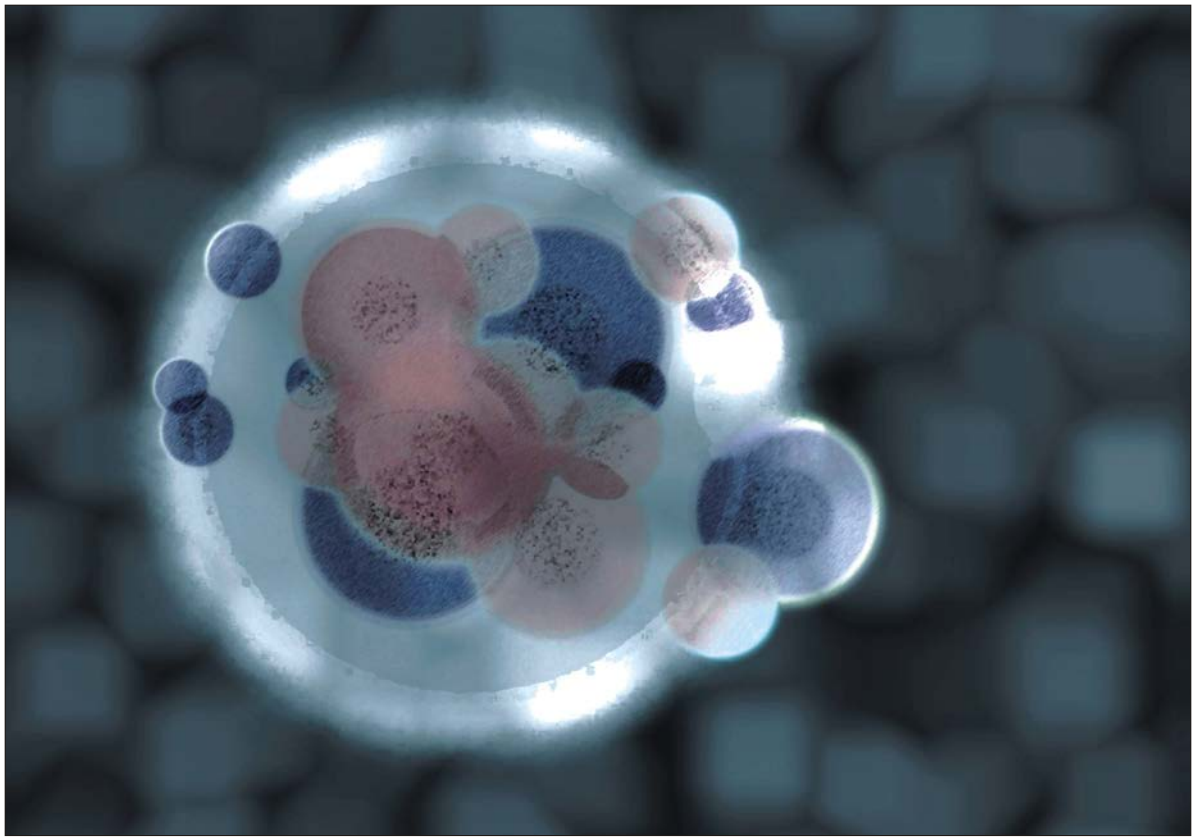




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Research Article

Profilin and Vicilin Genes of Allergens in Fingerprinting of Minor Legumes

Adam Kováčik, Jana Žiarovská, Aqsa Abbas and Dagmar Moravčíková

Faculty of Agrobiological and Food Resources, Institute of Plant and Environmental Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 94976, Nitra, Slovak Republic

Abstract

Background and Objective: Coding regions of plants are a good source for designing the DNA markers to describe genetic variability in specific genomic parts. Here, the genetic variability of five selected minor legume species was analyzed by DNA markers for profilins and vicilins. **Materials and Methods:** Varieties of *Phaseolus vulgaris* L., *Cicer arietinum* L., *Pisum sativum* L., *Lens culinaris* Med. and *Vicia faba* L. were analyzed by profilin-based amplified polymorphism (PBAP) and vicilin based amplified polymorphism (VBAP) methods. The heterozygosity index, polymorphism information content, effective multiplex ratio, marker index, diversity index and discrimination power were calculated by iMEC. Dendrograms were constructed based on the obtained binary matrices using the Jaccard coefficient of genetic similarity. Polymorphism and marker techniques effectiveness were compared. **Results:** Profilin-based polymorphism was found to be more effective in generating polymorphic fingerprints in all the analyzed legume species. Vicilin-based polymorphism provides only a species-limited tool for genomic variability analysis in common beans and chickpeas. **Conclusion:** Profilin-based polymorphism is more effective in generating polymorphic fingerprints. Vicilin-based polymorphism provides a species-limited tool for genomic variability analysis in legumes.

Key words: Allergen-based markers, coding region, length polymorphism, genomic, polymorphic fingerprints variability, vicilin, profilin

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Corresponding Author: Jana Žiarovská, Faculty of Agrobiological and Food Resources, Institute of Plant and Environmental Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 94976, Nitra, Slovak Republic

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Legumes are of great importance in human nutrition and in many parts of the world, they provide a main source of proteins along with this, they are of economic importance, too¹. Legumes are rich in proteins, complex carbohydrates and fiber and are a good source of minerals. Legume carbohydrates are absorbed slowly and are valued for their low glycemic index² and are rich in either monounsaturated or polyunsaturated fatty acids with favorable $\omega 3/\omega 6$ fatty acid ratio³. They also provide a sustainable variant in the production of high-quality proteins with many pros in agrotechnical procedures⁴. Demand for plant-based foods, such as minor legumes, has surged in recent years, with a particular focus on the growing popularity of the vegan diet, which is thought to be healthier and more ecologically friendly⁵. Connected to the nutrients and antinutrients of legumes, unfortunately, allergenic proteins have been identified in the majority of legumes. Allergenic responses to the legumes may range from mild skin reactions to life-threatening anaphylactic reaction⁶. In Europe, legumes are the fifth-leading cause of food allergies⁷. Food allergy is caused by a hypersensitivity response of the immune system to otherwise harmless proteins in food⁸. Groundnuts and soybeans are two of the top eight foods that cause the most food allergies in Europe and the USA⁹. In addition, other legumes such as garden beans, which are a common ingredient in wheat flour, are increasingly used in Europe¹⁰. The ability of lupin allergens was firstly reported as a result of cross-reaction with peanut allergens, but later it was also described as a primary sensitization¹¹. Cross-reactivity between different legume species is between 5 and 20%, with the highest between groundnut and garden bean (up to 20%). Cross-reactivity between legumes and nuts shows up to 33%. Allergenicity to peanuts increases the likelihood of allergy to oilseeds and sesame seeds by 25-40%¹². Foods, specifically legumes, can change their allergenicity by heat processing. Cooking reduces peanuts' allergenicity, but roasting them can increase it by up to 90 times. However, even after cooking, the allergens in lentils, chickpeas, green beans and peas remain highly stable¹³. Allergies to legumes such as edible lentils, green beans, peas and chickpeas may also be important in Mediterranean countries where these foods are sources of dietary fiber and valuable protein⁶.

Different studies of genetic diversity using the various morphological, chemical, biochemical and molecular markers are actual for legume germplasm characterization^{14,15}. Especially molecular and DNA marker-based characterization

of legume germplasm has great potential in determining genetic diversity and identification of unique variants that can be used in breeding programmes^{16,17}. The DNA markers are developed not only based on complete sequence information but an *in silico* prediction of potential DNA markers was reported to be efficient¹⁸. *In silico* approach is used to identify conserved parts in the amino acid sequences as well as in genomic sequences of allergens in plant species and based on the alignment, specific or degenerate primers can be designed¹⁹. Plant allergens share a high degree of sequence homology for proteins, as well as for genomic sequences^{20,21}, which allows us to predict DNA markers for them, too. Previously, Bet v 1-based amplified polymorphism was successfully applied as a fingerprint method for various plant species, as the sequences of Bet v 1-t main pollen allergen of birch is highly conserved in its epitopes in plants^{22,23}. Here, an abundant plant allergen profilin and legume-specific allergen vicilin were used as DNA markers. Both of them were reported to be applicable in the fingerprint of plants. In previous studies of soybean and groundnut, both of them provided polymorphic profiles. A total of 16 different amplicons were obtained for profilin and 17 different amplicons were obtained for vicilin in a set of 30 different soybean varieties and the PBAP (profilin-based amplified polymorphism) technique was able to distinguish all of the analyzed varieties²⁴. In the case of groundnut, both of the techniques distinguished 31 analyzed accessions and vicilin-based amplicon polymorphism (VBAP) provided polymorphism of 100%²⁵.

The study aimed to analyze the presence of conserved sequences of profilin and vicilin allergens in selected genotypes of five minor legume species and compare the intraspecies variability.

MATERIALS AND METHODS

Biological material: Seeds of five legume species (*Phaseolus vulgaris* L., *Cicer arietinum* L., *Pisum sativum* L., *Lens culinaris* Med. and *Vicia faba* L.) were obtained from GeneBank of Slovak Republic, Piešťany. All of them were planted in the laboratories of Research Centre AgroBioTech at Slovak University of Agriculture in Nitra during the season 2023-2024. Different numbers of individual varieties were randomly selected to analyze the natural intraspecies variability as old land, ancient as well as modern varieties were represented in Table 1. Young plants were obtained *in vitro* from sterilized seeds, basal Murashige and Skoog medium²⁶ were used with day-length 15 hrs and 20°C.

Table 1: Legume species accessions used in the study

<i>Phaseolus vulgaris</i> L.			
Albena	Meteorit	Avans	Mona
Alicante	Michael	Enso	Nordstern
Amanda	Olga	Favorit	Amulet
Atlanta	Pesak	Fruca Simpla	Katja
Belinda	SanCrop	Gangtok Bila	Kharkovskaya
Cabernet	Wawero	Golden Dream	Start
Canada	Zlaty Roh	Herold	
Fullcrop	Amethyst	Kaboon	
Marika	Augustynka	Goliat	
<i>Cicer arietinum</i> L.			
Kalika (a)	Farihame (g)	Sultano (m)	Sovhonznii (s)
Alfa (b)	Douyet (h)	Obraztsov chiflik n (n)	Gipsaphyla ttbelaia (t)
215055 (c)	Alcazaba (i)	Plovdiv 019 (o)	Bekescabai 1 (u)
Castuo (d)	Kompolti Bordo (j)	Lyons 512300 (p)	
Evros (e)	Sefiros (k)	Yialousa (q)	
Zahor (f)	Calia (l)	Rabat (r)	
<i>Pisum sativum</i> L.			
Ujmajori Sarga	Zidovicka Edelperle	Usatyj	Stretinskij
Iregi Sarga	Senator	Spiket	Skagit
Cicero	Libochovicky urodny	Frostar	Onsa
Parade	Juwel	Bulawa	Irkutskij
Weitor	Lancet	Recette	
Brunovsky	Verdorig	Rutts	
<i>Lens culinaris</i> Med.			
Peruanska (a)clumec (a)	Pelascgia (g)	Severnaia (m)	Mramor maskovskij (s)
Calisto (b)	Thessalia (h)	CDC Glamis (n)	Naslada (t)
Lesca (c)	Hungary 1987 (i)	CDC Milestone (o)	Zornitsa (u)
Krasnogradskaja 3 (d)	Didakte (j)	CDC Richlea (p)	Obrascov chiflik 7 (v)
Laird clasik (e)	Haliasko socivo (k)	Samos (q)	Colombia (w)
Pandora (f)	Limnos (l)	Pardina (r)	
<i>Vicia faba</i> L.			
Banner (a)	Kostlins (d)	Victor (g)	Outlook (j)
TIC Beans (b)	Stella spring (e)	Aurora (h)	Inovec (k)
Buldog (c)	Alfred (f)	Vega (i)	Koral (l)

Alphabets in the table are those used in the figures of constructed dendrograms

DNA extraction: Total genomic DNA was extracted by GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. The quantity and quality of extracted DNA were analyzed spectrophotometrically by NanoPhotometer™ (Implen) and functionality in PCR was checked by ITS primers²⁷.

Fingerprint analysis: For both of the allergens used in the study, profilin and vicilin type, primers were designed using the *in silico* analysis of their conserved sequences^{24,28}. A degenerated primer pair was used for profiling and nondegenerate for vicilin. DreamTaq™ DNA polymerase (Thermo Scientific, Waltham, Massachusetts, USA) was used in the analysis with 600 nmol of each primer. The PCR conditions were as follows: 95°C-5 min (95°C-45 sec; 55°C-45 sec and 72°C-35 sec) 40x plus 72°C-10 min. Obtained amplicons were separated in 2% agarose gel stained by GelRed® (Biotium, San Francisco, California, USA) and transformed into binary

matrices. Distance matrices were calculated by the Vegdist function in the Vegan package of Rstudio^{29,30}. The heterozygosity index, polymorphism information content, effective multiplex ratio, marker index, diversity index and discrimination power were calculated by iMEC (<https://irscope.shinyapps.io/iMEC/>). Dendrograms of genetic dissimilarity were created by the UPGMA using the Jaccard index³¹. Dendrograms of profilins were created by the hclust function in the stats package in RStudio³² and the dendrogram of vicilin in DendroUPGMA v.2.0 software³³. Heatmaps were created by the Pheatmap package in RStudio³⁴.

RESULTS

***Phaseolus vulgaris* L.:** A total of 142 amplicons were obtained in the set of analyzed common bean accessions when degenerated primers for profilin were used. The PBAP profile was mostly monomorphic with only one difference with the presence of the fragment with a length of 859 bp in

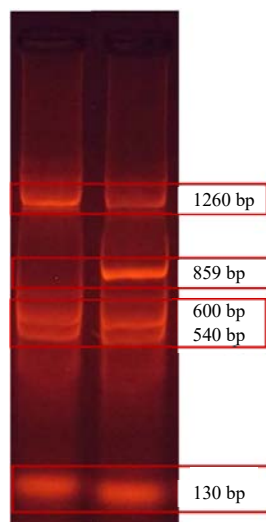


Fig. 1: PBAP profile types of *Phaseolus vulgaris* L. generated by degenerated profilin primers

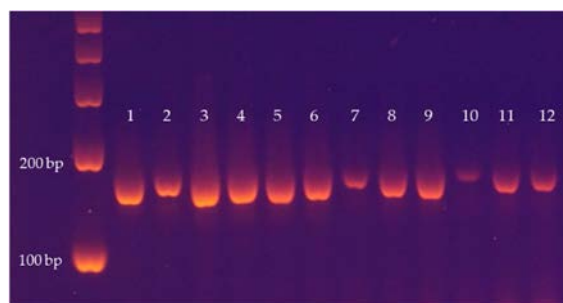


Fig. 2: Variants of vicilin homologs amplified by VBAP in selected common bean varieties

1: Albena, 2: Alicante, 3: Amanda, 4: Canada, 5: Fullcrop, 6: Goliat, 7: Marika, 8: Pesak, 9: SanCrop, 10: Wawero, 11: Zlaty Roh and 12: Avans

Fig. 1. A total of five different fragments were amplified-130, 540, 600, 859 and 1260 bp. Varieties where the amplicon of 859 bp was amplified are Cabernet, Canada, Goliat, Marika, Augustynka and Nordstern. All of the other analyzed common bean varieties' PBAP profiles were without this fragment.

For vicilin amplification, only one amplicon was generated in all of the analyzed common bean accession, with two length variants-160 and 176 bp in Fig. 2. Longer amplicon was obtained in varieties Alicante, Marika, Wawero, Favorit and Kaboon.

***Cicer arietinum* L.:** A total of 72 amplicons were obtained by degenerate profilin primer in the analyzed chickpea varieties. Amplified fragments were of length 126, 320, 400, 510, 580, 610, 730, 934, 986, 1050, 1300 and 1630 bp. The dendrogram generated from the obtained binary matrix has the value

of a cophenetic correlation coefficient of 0.92 in Fig. 3. Five different profile groups were generated and only two varieties, Alcazaba and Obratzov have the same PBAP profile. Based on Jaccard coefficients, varieties Evros, Castuo, Kalika and Sefiros were the most distinctive on their PBAP fingerprints.

The amplification pattern of vicilin in the set of analyzed chickpea varieties comprised three different fragments with lengths of 172, 344 and 916 bp. In the constructed dendrogram, three amplification patterns are visible plus the specific profile for the Sefiros variety in Fig. 4.

***Pisum sativum* L.:** For both of used marker techniques, amplification profiles in a set of analyzed pea varieties were monomorphic. For profiling, two fragments of 600 and 980 bp were obtained and for vicilin, an amplicon of the length of 100 bp was obtained.

***Lens culinaris* Med.:** In lentil varieties, only the PBAB technique provided amplification of PCR fragments. A total of nine different amplicon lengths were obtained-347, 622, 837, 880, 970, 1120, 1330, 1500 and 1705 bp. The dendrogram that was generated from the obtained binary matrix has the value of a cophenetic correlation coefficient of 0.87 in Fig. 5. Three main clusters were generated within two monomorphic groups. The first one comprises varieties Laird klasik, Didakte and Limnos and the second one is varieties Pandora, Severnaia and Pardina. Based on Jaccard coefficients, analyzed lentil varieties are very dissimilar in their PBAB fingerprints.

***Vicia faba* L.:** A total of 60 amplicons were obtained by a degenerate profiling primer in the analyzed faba bean varieties. Amplified fragments ranged in length from 133 up to 1500 bp. The dendrogram was generated from the obtained binary matrix and has the value of a cophenetic correlation coefficient of 0.78 in Fig. 6. Three main clusters were generated and the largest one comprised three subclusters. All the analyzed faba bean varieties were separated based on their PBAB profiles but shared a low degree of genetic dissimilarity.

For vicilin, only one amplicon of the length of 100 bp was obtained by the VBAP technique.

Comparing the PBAP and VBAP techniques used in the study, PBAP was more effective, because of amplification in all of the analyzed species and only in the case of *Pisum sativum*, L. was monomorphic. The ability of PBAP to detect polymorphism was comparable for chickpeas and faba beans shown in Table 2, but the effectiveness of polymorphism analysis was comparable. The highest discrimination power index was among the varieties of lentils.

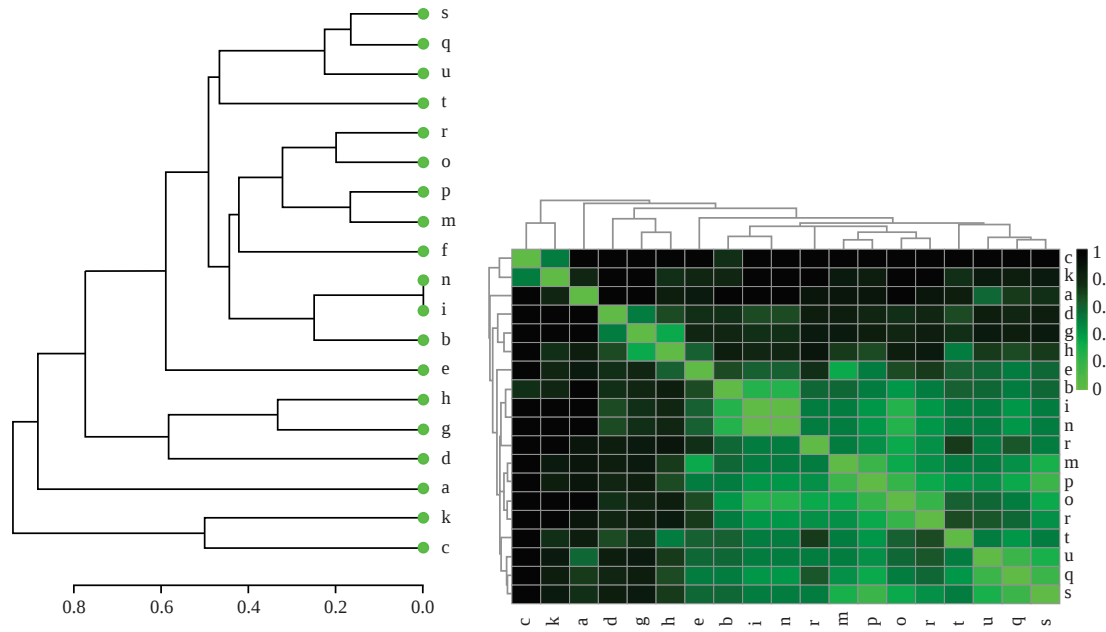


Fig. 3: Dendrogram of obtained PBAB profiles for analyzed chickpea varieties with a heatmap of distance matrix
Letters stand for varieties names as listed in Table 1

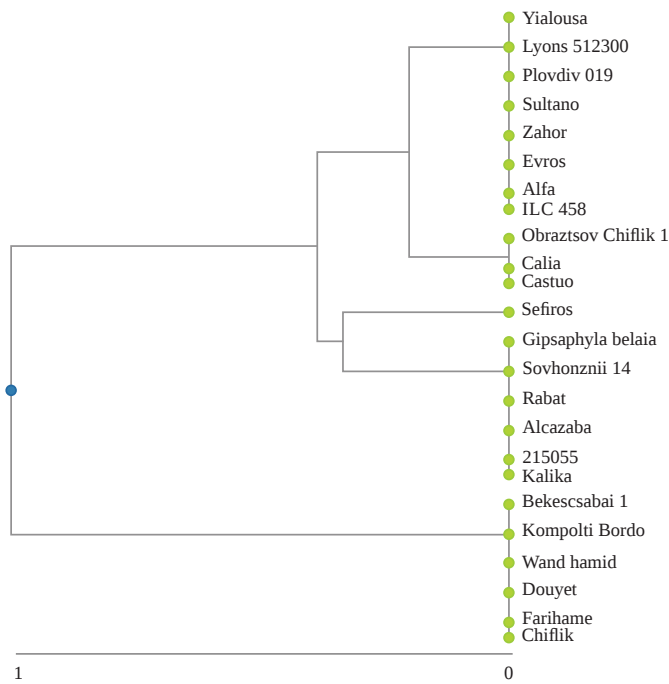


Fig. 4: Dendrogram of obtained VBAB profiles for analyzed chickpea varieties

Table 2: Characteristics of PBAP for chickpea, lentil and faba bean accessions

Species	H	PIC	E	MI	D	R
<i>Cicer arietinum</i> L.	0.43	0.34	3.83	0.01	0.89	4.77
<i>Lens culinaris</i> Med.	0.33	0.28	1.94	0.01	0.95	3.88
<i>Vicia faba</i> L.	0.49	0.37	5.00	0.02	0.79	5.45

H: Heterozygosity index, PIC: Polymorphism information content, E: Effective multiplex ratio, MI: Marker index, DI: Diversity index, D: Discrimination power and R: Resolution power

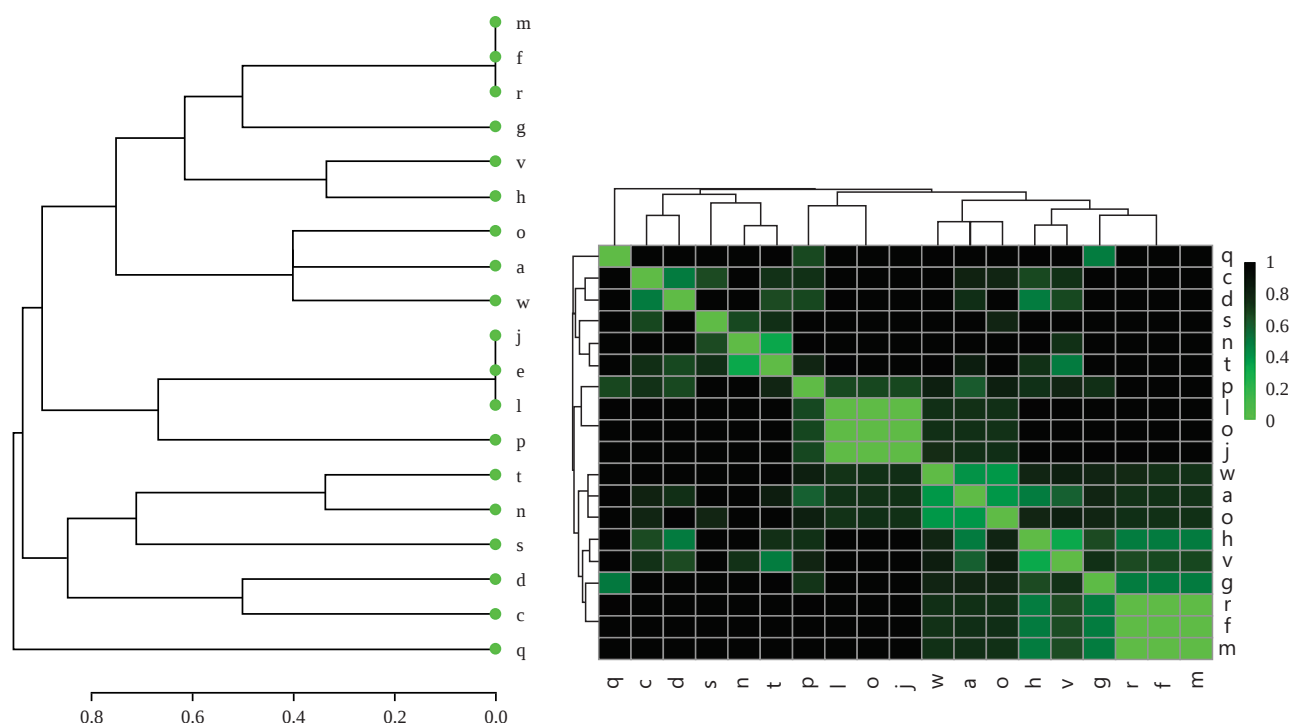


Fig. 5: Dendrogram of obtained PBAB profiles for analyzed lentil varieties with a heatmap of distance matrix
Letters stand for varieties names as listed in Table 1

DISCUSSION

Here, PBAP (profiling-based amplification polymorphism) and VBAP (vicilin-based amplification polymorphism) techniques were used to characterize selected varieties of five minor legume species. Profilins and actin-binding molecules belong to plant panallergens, which makes the PBAP universally in using³⁵. In legumes, soybean and groundnut genotypes were analyzed by PBAP previously^{24,25}. In both of these species, polymorphic profiles were generated. In the set of groundnut accessions, all were distinguishable but profiling-based marker technique, but in soybean, a couple of varieties (Sciaming and Krajina) produced the same fingerprint profiles. The PBAP fingerprints of peanuts were distributed in length ranging from 78 bp up to 1642 bp and for soybeans from 118 up to 1000 bp. This corresponded to the result of minor legumes analyzed in this study, where profiling-based markers generated polymorphic profiles in all the analyzed species except for peas. Such differences in the ability to generate polymorphism and distinguish analyzed genotypes are in concordance with the natural variability of profiling homologs in plants. Higher plants encode several profilin proteins, which are primarily classified into two classes according to the diverse ways in which they express

themselves in vegetative and reproductive tissues³⁶. Currently, more than 400 profilin proteins from plants are available in the National Center for Biotechnology Information (NCBI) gene database³⁷. According to earlier reports, even minor variations in the amino acid sequence can significantly impact the biochemical characteristics of profilin³⁸ and there is a tendency for profiling genes with evolutionary origins in the polyphyletic mode to have a higher degree of sequence variety³⁹.

Numerous plants contain vicilins, a family of allergenic seed storage proteins known as 7S globulins. They are termed vicilins based on the dominating presence of the Viciae group in the legumes⁴⁰. Cross-reactions among vicilin allergens are well-known among legumes based on protein similarity. Groundnuts are cross-reactive with allergens of lentils and peas, walnuts, cashews and hazelnut⁴¹, lupine⁴² and intraspecific with other groundnut allergens, too⁴³. The transferability of specific vicilin primers designed on the sequences of pea vicilin was reported previously by Klongová *et al.*²⁸. For the VBAP fingerprint, much higher variability exists in legumes when compared to PBAP. In the genotypes of groundnut and soybean, polymorphic profiles are generated^{24,25}, for chickpea, limited polymorphic distribution of fingerprint profiles was obtained and for

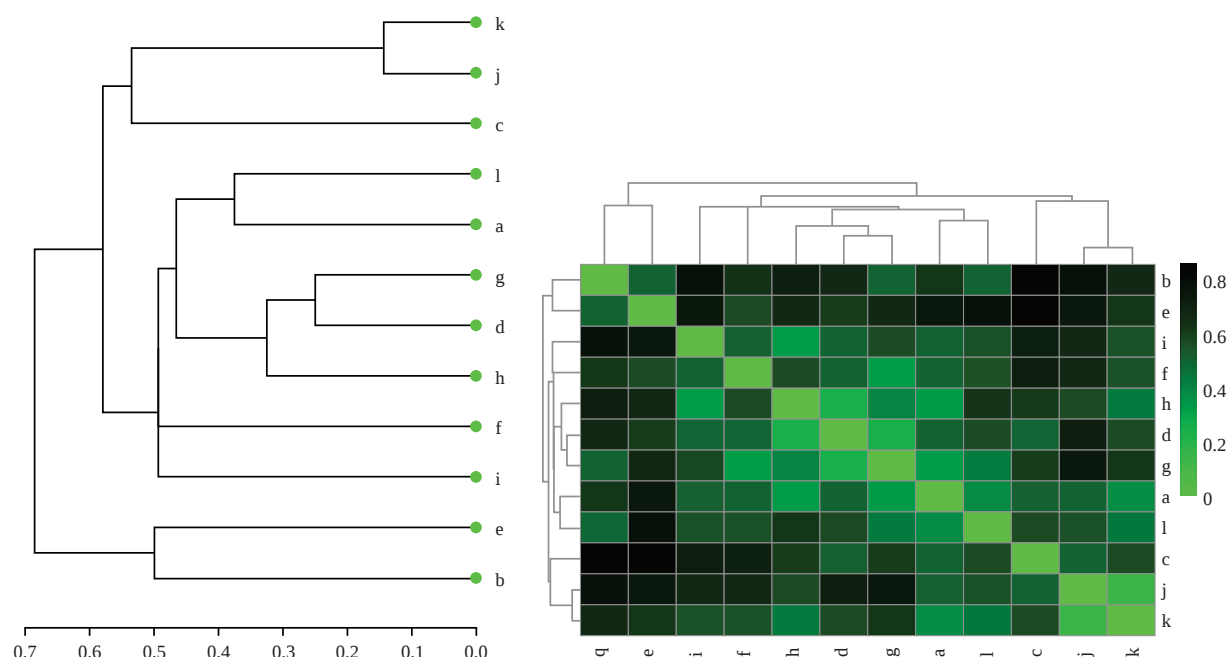


Fig. 6: Dendrogram of obtained PBAB profiles for analyzed faba bean varieties with a heatmap of distance matrix
Letters stand for varieties names as listed in Table 1

common bean two length variants exist, for pea and faba bean only one fragment is amplified and for lentil, this primer combination have no sequence to anneal with. Known sequential variability of vicilin allergen is high among the 61 members of this family in *Arachis hypogaea* L.⁴⁴, but for common beans, peas, chickpeas and faba beans, only limited information exists in public databases. For lentils, none of them exist.

The IgE-binding proteins have been identified in most legumes and the allergic response to these legumes can range from mild skin reactions to life-threatening anaphylactic reactions⁶. Food-induced anaphylaxis affects a significant proportion of the population and its prevalence is about 6-8% in children and 4% in adults. The number of people in whom allergens can trigger a strong and sometimes dangerous immune response is steadily increasing. This statement was supported by research⁴⁵⁻⁴⁷, the output of which shows a dramatic increase in food allergic reactions caused by legumes, especially in recent decades. High allergenicity was identified in legume species as follows: Edible lentil, chickpea, common pea, common bean and garden bean.

The DNA markers of coding space were applied previously in legume species for genetic diversity characterization. Start codon target (SCoT) markers are effective in common bean varieties, landraces and cultivars' genetic diversity characterization⁴⁸⁻⁵⁰ or in pea subspecies distinguishing⁵¹. Conserved DNA-derived

polymorphism (CDDP) was used in the description of variability of chickpea genotypes⁵². The study of genetic diversity is an inevitable part of the crop improvement process. Marker-based description of genetic relationships within plant germplasm based on DNA polymorphism generates knowledge for the simplification of the selection of germplasm for the breeding programs as well as for efficient conservation strategies of genetic resources^{53,54}. Allergen-based marker techniques are reported with different universality in plant studies. Along PBAP and VBAP, Bet v 1 based amplified polymorphism (BBAP) was applied in many flowering plants such as lime-tree⁵⁵ of fruits and vegetables^{22,23}. All of them provide unique marker techniques that should be useful for molecular breeding purposes.

CONCLUSION

The result obtained in this study provides data, that fulfill the PBAP and VBAP characteristics for their use in the analysis of allergen-based fingerprinting in legumes. Profilin-based polymorphism is more effective in generating polymorphic fingerprints. Vicilin-based polymorphism provides a species-limited tool for genomic variability analysis in legumes. Further specification for intraspecific and interspecific markers of allergens is needed, as different management strategies for the legume germplasm will be utilized toward the possibilities of allergenic management of food in the future.

SIGNIFICANCE STATEMENT

Food allergies have become one of the most relevant health difficulties that have arisen in the last decades. Despite that, only a limited number of protein families belong to plant allergens, their isoform variability in the combination of all of the plant individual varieties is important to be able to describe from the point of view of genetic variability. Here, *in silico* data were used to design DNA-based markers for the screening of the genomic length variability of homologs of profilins and vicilins in legumes. In the future, markers based on the coding regions of allergens will help to manage the plant germplasm more effectively toward the possible personalization of the nutritional needs of people suffering from food allergies.

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