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Original Research

DNA-Based Variability of Length Polymorphism of Plant Allergens Coding Genes Homologs in Selected *Lamiaceae* Herbs

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Abstract

Medicinal plants have been a part of human life from a very early age. In the field of plant genetics, they are still widely investigated for their genomic variability. This study used two DNA marker techniques to obtain polymorphic profiles in selected species from Lamiaceae. Both are based on the variability of plant genes that code for allergens - BBAP (Bet v 1-Based Amplicon Polymorphism) and PBAP (Profilin-Based Amplicon Polymorphism). Variability of Bet v 1 homologues within individual genomes showed similarity of basil and oregano as well

as basil with common sage and rosemary with creeping thyme. PBAP profiles were the most similar profiles for basil and rosemary.

Keywords
Allergen coding genes; polymorphism; DNA markers

1. Introduction

Humans inherited the great part of their knowledge about the positive or negative effects of herbs as medicines from ancestors. Until the time of the first synthetic drugs in the 19th century [1], herbs were the only means for treating diseases. Recently, significant emphasis has again been placed on their beneficial effects on health. Herbal preparations are increasingly popular over synthetic drugs in common diseases with weaker symptoms, such as flu, colds, abdominal infections, etc. [2].

Even though we know many positive (as well as negative) effects of herbs and spices, often this knowledge is only empirically verified or referred to as "can help" statement. This is due to the unknown impacts on the human body at the molecular level, the correct dosages, their overall biochemical composition, and possible toxicity, which causes distrust of their broader use in conservative medicine [3]. Moreover, regular use of herbs can also be dangerous for specific groups of people (diabetics, pregnant women, babies, etc.). Herbs can cause allergies, interfere with classical treatment, or even stimulate hypermanifestations of some symptoms [4].

Herbs represent clinically not common allergens with some exceptions such as elecampane (*Inula helenium*) or arnica (*Arnica montana*), dandelion (*Taraxacum officinale*), plantain (*Plantago lanceolata*) or purple cornflower (*Echinacea purpurea*) [5, 6]. Members of the *Astraceae* family are usually associated with the potency to evoke allergic reaction. To this family belong some widely used herbs such as the mentioned arnica, calendula, and chamomilla [7]. Some spices can invoke allergic reactions, but they are scarce. Cross allergies are typical, particularly with birch and mugwort. However, diagnosis is difficult (especially for spice-herb mixtures), and allergen studies focus on more regular allergens [8]. Individual cases reported in the literature, such as a patient with IgE-mediated allergic reaction to oregano and thyme, with a previous diagnosis of sensitivity to grasses [9, 10].

Up to now, different DNA-based markers were used to analyze medicinal plants and herbs' genomic variability. In the case of classical DNA markers, restriction fragment length of polymorphisms (RFLPs) was one of the first markers used to detect variation in a DNA sequence. RFLP analysis was performed in the *Hypericum perforatum* L. [11], and similar profiles were found in somaclonal plants. Randomly amplified polymorphic DNA (RAPD) was used in the differentiation of *Codonopsis pilosula* [12] or *Glycyrrhiza glabra* L. [13]. Amplified fragment length polymorphism (AFLP) was used to find a correlation with agronomic traits in the genome of *Ocimum* genus [14]. Single sequence repeats (SSR) was influential in the differentiation of *Rhodiola rosea* L. [15]. New generations of DNA markers based on the coding regions of genes were reported as efficient and informative in the analysis of medicinal plants and herbs [16]. Codon-targeted polymorphism (SCoT)

was used to explore the genetic variability of *Andrographis paniculata* (Burm. f.) [17] and *Senna obtusifolia* L. [18].

The very specific group of genes that are very often abundant and homologous in plants are those for allergens, such as genes for PR-10 proteins (Bet v 1 homologs) or profilins. Bet v 1 sequences have a high homology in the regions that are matched by primers used in the BBAP method [19]. Bet v 1 is a major birch pollen allergen and is a member of plant pathogenesis-related protein family PR-10 that is abundant throughout the plant kingdom [20]. Multigene families encode PR-10 proteins [21], and the phylogenetic analysis of PR-10 isoforms showed strong coordinated evolution of this multigene family [22]. Pathogen-related proteins (PR) are assumed to be ubiquitous among plants. They have been described as proteins induced by biotic or abiotic stress. Their expression is evoked mainly by salicylic acid, jasmonic acid, or ethylene as signal molecules and possesses antimicrobial activity, contact toxicity, or another defense signaling. PR proteins were divided into 17 families based on primary structure and biological and serological activities [23, 24]. Profilins were characterized as actin-binding molecules that are one of the plant panallergens group [25]. This group is present in almost all eukaryotic cells and is highly cross-reactive [26], making them a good marker to detect plant genomic-based variability [27]. We know no work has been devoted to allergen-based DNA polymorphic techniques in medicinal plants and herbs.

The Present study focused on the methodology's applicability. The Bet v 1 homologs of selected species have not yet been sequenced; knowledge about the genes is limited in the literature, and thus, the obtained results offer valuable information about the presence and sequence variability of these genes in chosen genomes.

2. Materials and Methods

2.1 Biological Material

The plant material of basil (*Ocimum basilicum* L.), oregano (*Origanum vulgare* L.), clary sage (*Salvia sclarea* L.), rosemary (*Salvia rosmarinus* S.), common sage (*Salvia officinalis* L.), common thyme (*Thymus vulgaris* L.), peppermint (*Mentha* × *piperita* L.) and creeping thyme (*Thymus serpyllum* L.), were purchased at store chain as single-species teas/spices. Biological material was stored at 4°C in cooling devices.

2.2 gDNA Extraction, PCR Functionality Checking, and Amplicons' Visualisation

gDNA was extracted using the EliGene® kit (Elisabeth Pharmacon) in accordance with the protocol. gDNA quality and quantity were determined by NanoPhotometer™ (Implen®). EliZyme HS Robust MIX (Elisabeth Pharmacon) and commercial ITS primers (ITS1 and ITS4) were used to check the functionality of DNAs (White et al., 1990). Amplicons were separated by electrophoresis in 1.5% agarose gels stained by GelRed™ (Biotium) and visualised in UV lamp BioDocAnalyze Box 2 (Biometra).

2.3 BBAP and PBAP Marker Techniques

BBAP (Bet v 1-Based Amplicon Polymorphism) and PBAP (Profilin-Based Amplicon Polymorphism) marker techniques were used to characterise polymorphisms of allergen homologues - Bet v 1 and profilins – in chosen genomes [19, 28, 29]. BBAP technique uses non-degenerate forward primer

and a combination of degenerate reverse primer and its non-degenerate variants [19, 25]. EliZyme HS Robust MIX (Elisabeth Pharmacon), 400 nM primers, ten times diluted DNAs (final concentration of 50 ng) and following PCR protocol were used for the analysis: primary denaturation at 95°C (5 min); 40 cycles of denaturation at 95°C (45 s), primers annealing at 54°C (45 s), polymerisation at 72°C (35 s); and final polymerisation at 72°C for 10 minutes. The same chemical concentrations were used to the PBAP technique. A PCR protocol was as follows: primary denaturation at 95°C (5 min); 40 cycles of denaturation at 95°C (45 s), primers annealing at 55°C (45 s), polymerization at 72°C (35 s); and final polymerization at 72°C (10 min). PBAP technique uses only one primer pair (Klongová et al., 2021). Amplicons were separated by electrophoresis in 3% AGE, stained by GelRed™ (Biotium) and visualized the same way as ITS amplicons mentioned above.

2.4 BBAP and PBAP Output Analysis

PCR profiles of obtained amplicons were analysed by the software GelAnalyzer 23.1 and transcribed into binary matrices. Matrices and dendrograms were calculated in RStudio using method “jaccard” [30]. Statistical properties such as polymorphism information content (PIC) or percentage of polymorphism and others were calculated for each primer pair. PIC is an expression of a degree to which two genotypes are identical [31]. For dominant markers such as BBAP and PBAP, the PIC value indicates the probability of finding this marker in two states (present/absent) with two randomly selected individuals in the population [32].

2.5 In Silico Analysis

In silico analyses were performed to evaluate the genome/nucleotide sequence information available in the NCBI database about the studied species and their genera. Subsequently, BLAST analysis was performed with a discontinuous mega blast algorithm to evaluate the similarity of the available sequences of the studied species with the genes on which the methodologies BBAP and PBAP were based. The accession number in the NCBI database, AJ289771, was used for the YPR 10 gene, and FM 887030.1 was used for the profilin gene.

3. Results

3.1 BBAP Polymorphism Analyse

Analysed plant species amplified 291 alleles by a combination of all degenerate and non-degenerate primer pairs divided into 28 to 36 loci (Table 1). Obtained BBAP amplicons had lengths from 59 bp to 1710 bp.

Table 1 Comparison of characteristics of BBAP primer pairs.

Primer pair	Number of amplicons	Number of different lengths of amplicons	Number of unique amplicons	% of polymorphism	PIC	Amplicon length range
F plus R	73	36	18	100	0.349	59-1413 bp
F plus R1	64	32	11	100	0.346	67-1545 bp
F plus R2	67	35	17	100	0.335	79-1710 bp
F plus R3	49	28	12	100	0.313	76-1516 bp
F plus R4	59	30	14	100	0.342	72-1691 bp

The variability of Bet v 1 homologs (Figure 1) within individual genomes is shown in the dendrograms created in RStudio (Figure 2). FR2 and FR3 primer pair combinations showed the similarity of basil and oregano species (0.4 and 0.2). FR1 showed the following similarities: basil with common sage (0.385) and rosemary with creeping thyme (0.25) (Figure 1). The most similar profiles by FR4 were basil and peppermint (0.385), oregano, and common thyme (0.444).

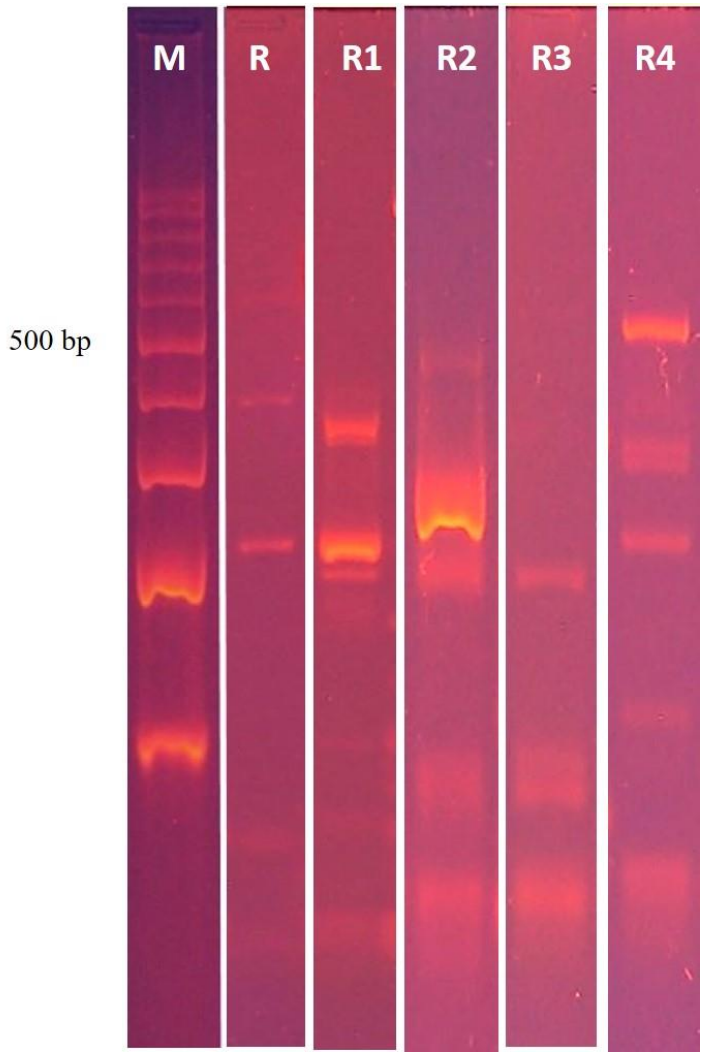

Figure 1 BBAP profiles of *Salvia sclarea* for individual BBAP primer pairs.

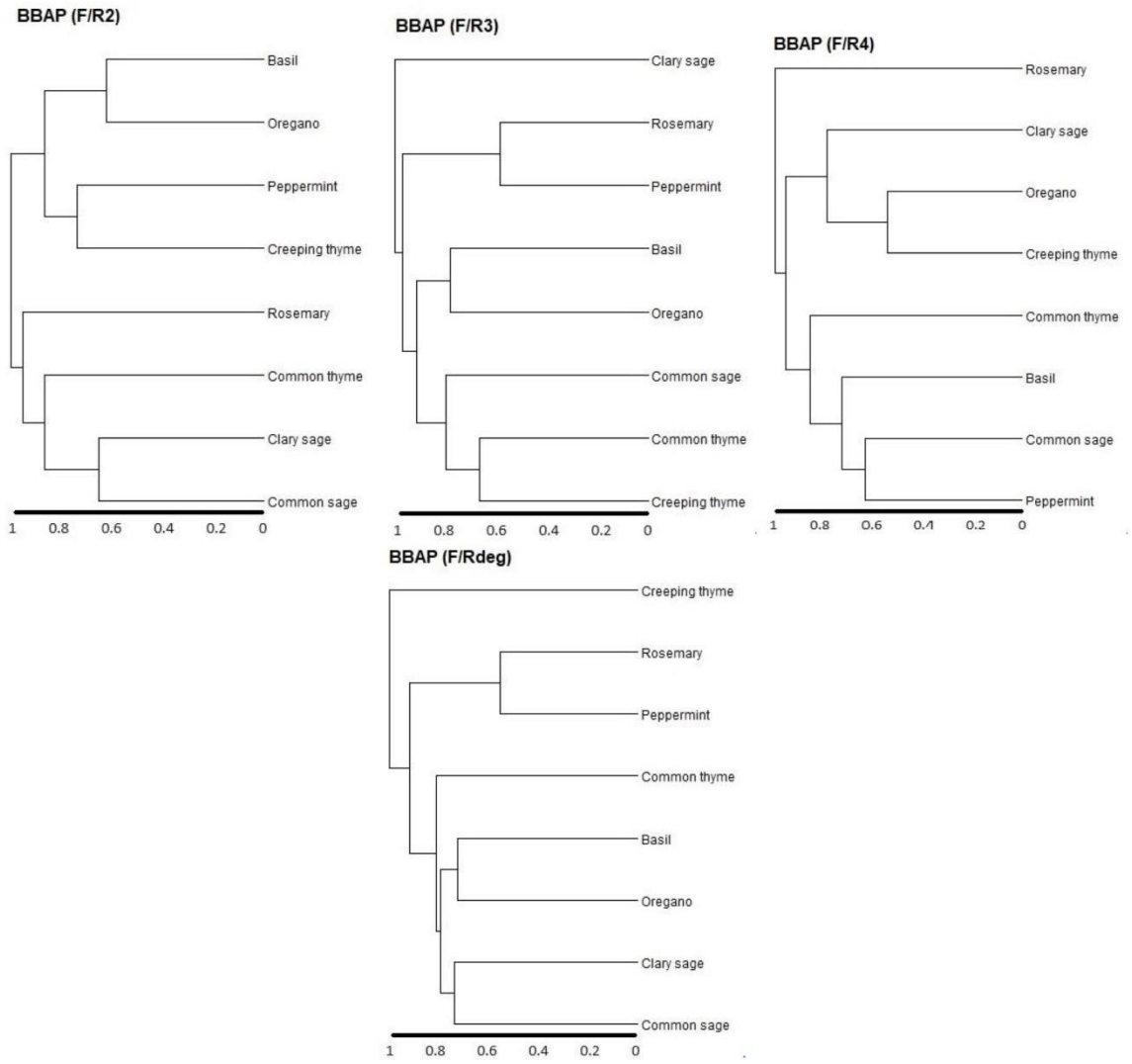

Figure 2 Dendrograms of BBAP profiles for degenerate reverse primer (A) and its non-degenerate variants (B-R1; C-R2; D-R3; E-R4).

The variety of associations among analysed plant species indicates the binding site variability of Bet v 1 allergen homologs. Dendrograms BBAP technique points out clary sage with the most variable BBAP profile (Figure 3), followed by common sage, while basil, oregano, and thyme are very variable in BBAP profiles.

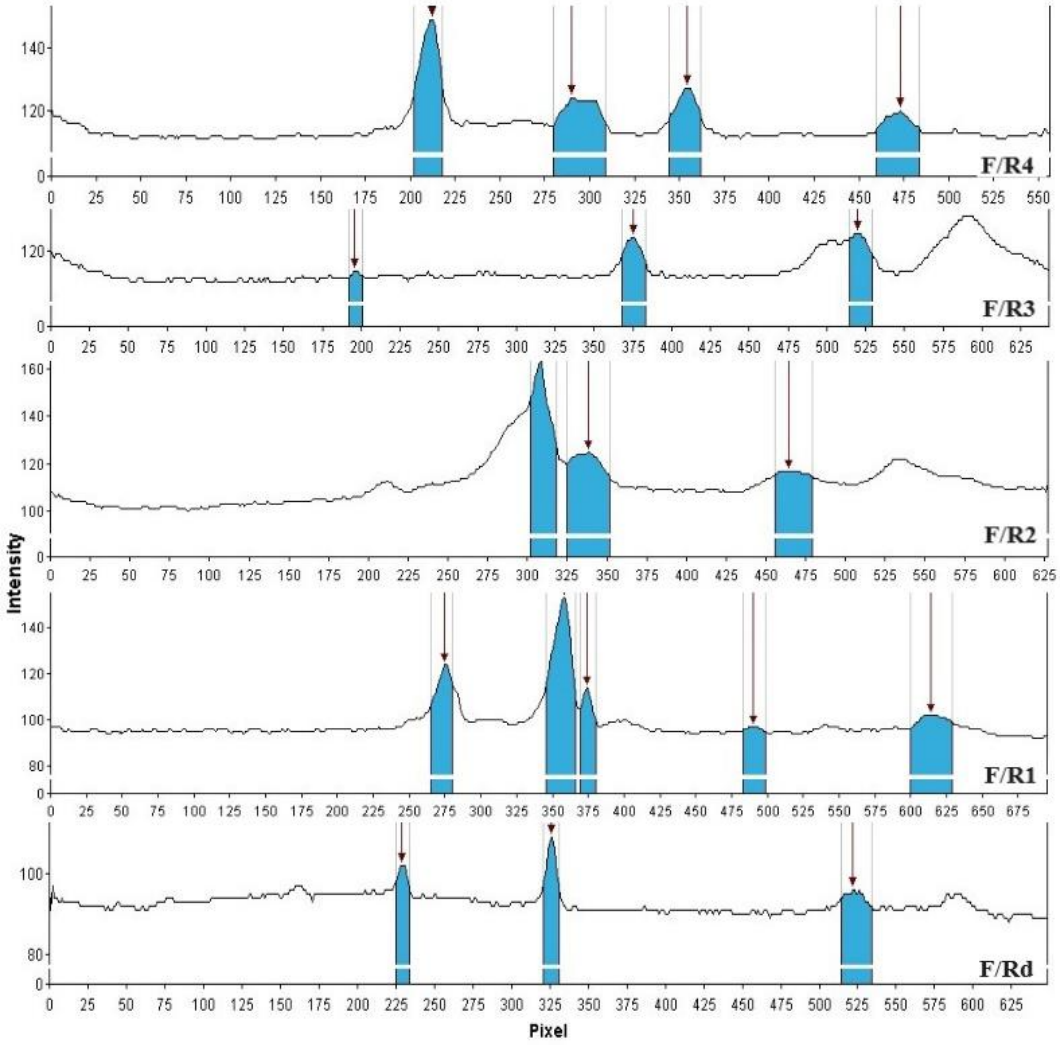

Figure 3 Demonstration of BBAP profiles of individual primer pairs (F/Rd + R1 - R4) for clary sage. Graphs represent the presence and location of amplicons amplified with the primer pairs used in the AGE gel in digital form in GelAnalyzer. The sizes of amplicons in bp is converted based on the molecular ladder.

3.2 PBAP Polymorphism Analyse

One primer pair was applied to generate PBAP profiles (Figure 4), which amplified 69 alleles divided into 31 loci using biological material (Table 2). Amplicons of lengths 72 bp to 1187 bp were obtained.

Table 2 Comparison of characteristics of PBAP primer pairs.

Primer pair	Number of amplicons	Number of different length of amplicon	Number of unique amplicons	% of polymorphism	PIC	Amplicon length range
PBA	69	31	14	100	0.323	72-1187 bp

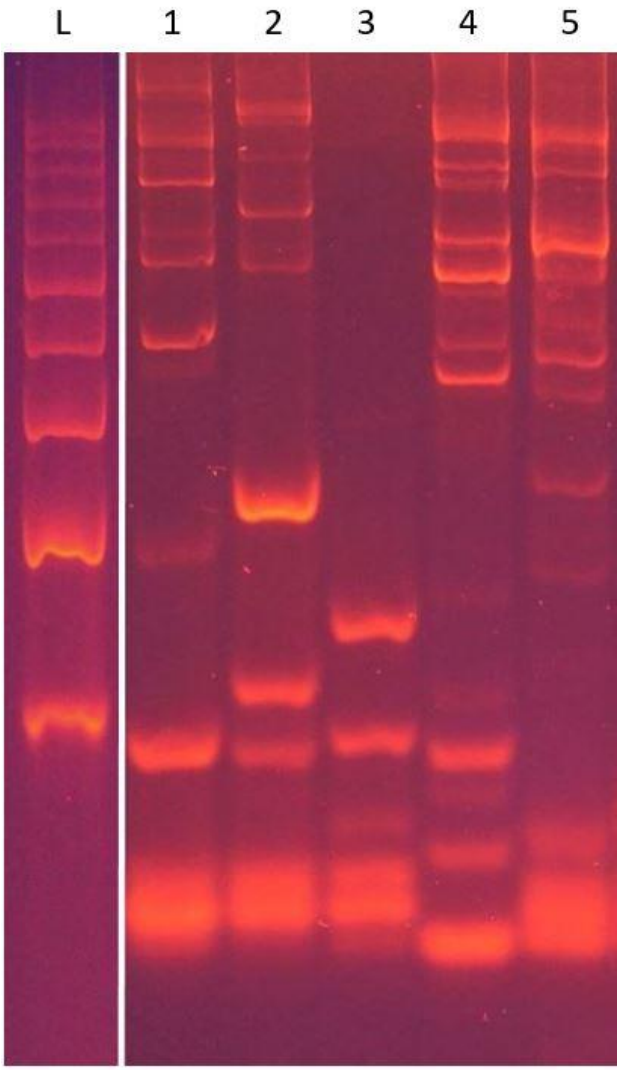

Figure 4 PBAP profiles of basil (*1*), oregano (*2*), clary sage (*3*), rosemary (*4*), and common sage (*5*).

PBAP profiles of plant species were grouped into three clusters (Figure 5). The most similar profiles shared basil and rosemary (0.417). The first cluster included basil, rosemary, common sage, peppermint, and thyme. The other cluster consisted of creeping thyme oregano and clary sage. The two species of sage are not very similar in PBAP profiling profiles.

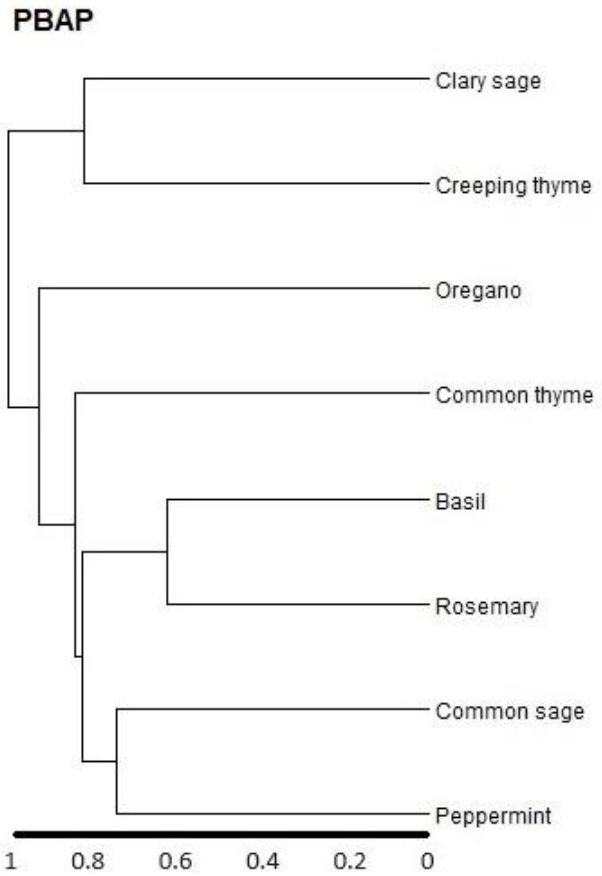

Figure 5 Dendrogram of PBAP profile of analysed medicinal plant species.

3.3 In Silico Analyse

The results of the in silico analysis indicated that there is still not enough genomic information on the studied species. A BLAST comparison of available sequences of the studied species with the genes on which the methods used in the work were based showed no match, which may be because the species have not been sequenced so far. Figure 4 and Figure 5 show the BLAST matches of sequences of other species within the studied genera, mostly within the Salvia L. genus (Table 3).

Table 3 Results from blast comparison of available nucleotide sequence accessions of the studied species and their genera in the NCBI database with genes FM887030.1 and AJ289771.

	BLAST result (discontignous megablast)	
Analysed species	Homology with profilin gene FM 887030.1	Homology with YPR 10 gene AJ289771
Ocimum basilicum L.	no homology	no homology
Origanum vulgare L.	no homology	no homology
Salvia sclarea Crantz	no homology	no homology
Salvia officinalis L.	no homology	no homology
Salvia rosmarinus Spenn.	no homology	no homology
Thymus vulgaris L.	no homology	no homology

Thymus serpyllum L.	no homology	no homology
Mentha × *piperita* L.	no homology	no homology
Analysed genus	Homology with profilin gene FM 887030.1	Homology with YPR 10 gene AJ289771
Ocimum spp.	no homology	no homology
Origanum spp.	no homology	no homology
Salvia spp.	homology with 28 sequence accessions (Figure 6)	homology with 7 sequence accessions (Figure 7)
Thymus spp.	no homology	no homology
Mentha spp.	no homology	no homology

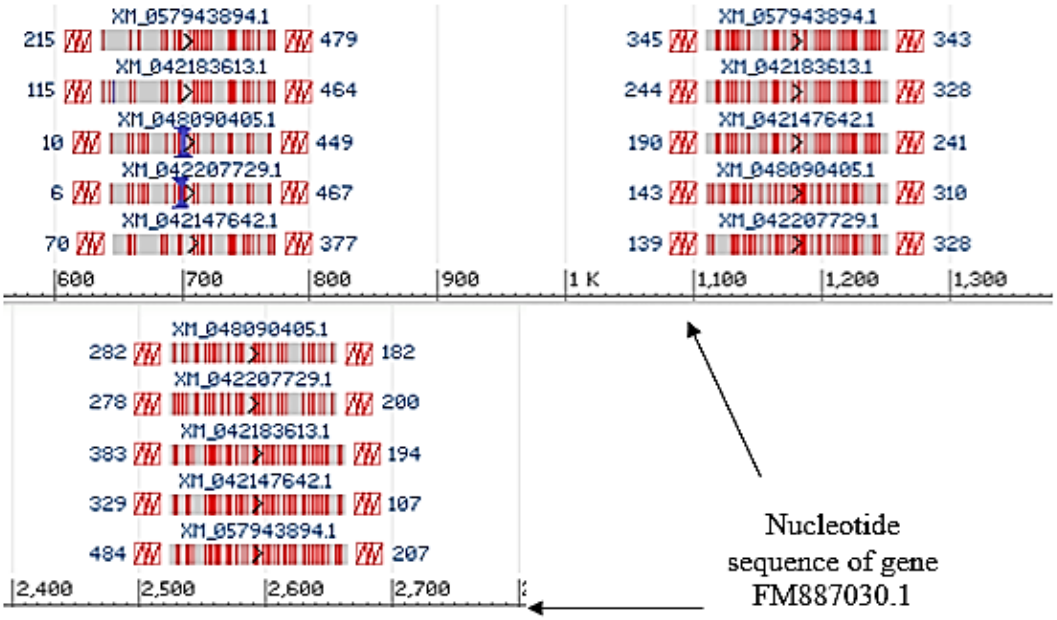

Figure 6 BLAST result of the comparison of FM887030.1 gene with nucleotide accessions of the studied genera (modified image).

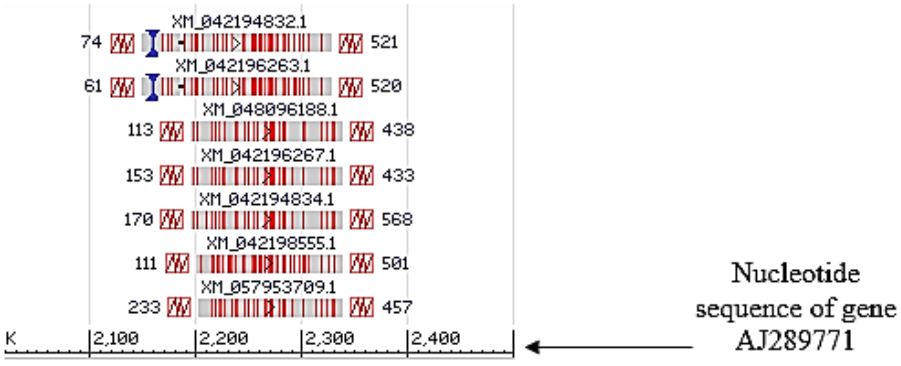

Figure 7 BLAST result of the comparison of AJ289771 gene with nucleotide accessions of the studied genera (modified image).

Of the herbs studied, only one species was sequenced, namely *Salvia sclarea* L. as for *Origanum vulgare* L., not a single gene record is available, and for *Thymus vulgaris* L. there are only 2 records (Table 4).

Table 4 Result of NCBI assembly search of the studied species.

Analysed species	Genome assembly	Number of nucleotide accessions	Genes
Ocimum basilicum L.	0	25524	134
Origanum vulgare L.	0	964	0
Salvia sclarea Crantz	1	302	132
Salvia officinalis L.	0	1053	134
Thymus vulgaris L.	0	390	2
Thymus serpyllum L.	0	158	134
Mentha × *piperita* L.	0	1815	134

The NCBI database records showed that the genus *Origanum* sp. does not have any of its species sequenced. As for the other genera, *Salvia* sp. has five whole-genome sequenced species, and *Ocinum* sp. and *Thymus* sp. have two genome assemblies. *Mentha* sp. has one genome assembly (individual species shown in Table 5.

Table 5 Results of NCBI assembly search of the studied genera.

Analysed genus	Genome assembly	Accession number	Species
Ocimum L.	2	GCA_001278415.1	*Ocimum tenuiflorum* L.
		GCA_001748785.1	*Ocimum tenuiflorum* L.
Origanum L.	0	-	
Salvia L.	9	GCA_028751815.1	*Salvia miltiorrhiza* Bunge
		GCA_023119035.1	*Salvia hispanica* L.
		GCA_004379255.2	*Salvia splendens* Sellow ex Nees
		GCA_036873715.1	*Salvia sclarea* Crantz
		GCA_028566235.1	*Salvia elegans* Vahl
		GCA_029641045.1	*Salvia hispanica* L.
		GCA_030788125.1	*Salvia hispanica* L.
		GCA_016432925.1	*Salvia miltiorrhiza* Bunge
		GCA_948473435.1	*Salvia hispanica* L.
Thymus L.	2	GCA_024222315.1	*Thymus quinqnecostatus* Benth
		GCA_029379335.1	*Thymus mandschuricus* Ronniger
Mentha L.	1	GCA_001642375.2	*Mentha longifolia* L.

4. Discussion

High chemical polymorphism of medicinal plants and herbs implies a high genetic diversity within the individual species, however studies on the genetic variability for them are rather scarce. Several molecular techniques have already been used successfully to describe some herb and spice genomes. DNA based methods are often used, for example RAPD (Random amplified polymorphic DNA), SSR (simple sequence repeats), RFLP (restriction fragment length polymorphism), AFLP (Amplified fragment length polymorphism) etc. or more superior methods such as hybridization or sequencing [33, 34], however, most of the studies have been interested in genomic variability associated with aromatic traits. Allergen studies are naturally focused mainly on very common allergies occurring in a large part of the human population. However, monitoring non-allergenic forms is necessary to understand the relationship between the non-allergenic gene and a gene encoding a protein provoking the immune system.

Ocinum basilicum L., known as sweet basil, is a popular culinary herb [35] that grows in tropical and subtropical climates. More than 30 species of herbs and shrubs of *Ocinum* L. genus exist. Basil contains more than 100 bioactive compounds [36]. The exact biochemical composition can depend on parameters such as genotype, cropping seasons and geographical properties [37]. Genetic diversity of Ocimum spp. was previously reported [38] and the results indicated that domesticated species, *O. minimum* L., *O. basilicum* L. and *O.* ×*citriodorum* Vis. share the highest similarity indices within species, while the nondomesticated *O. americanum* L., *O. gratissimum* L. and *O. kilimandscharicum* Guerke showed the lowest similarity. RAPD results also indicated that *O. minimum* should not be considered a distinct species but rather a variety of *O. basilicum*. Another study [39] analyzed the genetic diversity and population structure of *Ocimum basilicum* L. by DArTseq markers and identified two genetic groups.

Origanum vulgare L. is native to the Mediterranean region and western Eurasia. The plant contains various medicinally active components, such as flavonoids and phenolic glycosides [40]. Studies on systematic genetic diversity and identification in Origanum spp. were most often aimed at detecting DNA polymorphism between or within germplasm and identifying phylogenetic relationships using different DNA markers such as AFLP, ISSR, SAMPL, SSR, RAPD, and CAPS [41]. Classical DNA markers such as RAPD were utilized in the studies of the genetic variability of this species previously [42], as well as modern ones, such as sequence-related amplified polymorphism (SRAP), to reveal the diversity in natural populations [43].

Salvia sclarea L., *Salvia rosmarinus* S., and *Salvia officinalis* L. belong to the genus *Salvia* L., which is the largest group in the *Lamiaceae* L. family, consisting of 900 species and contains an extensive range of bioactive substances [44-46]. Genetic relationships among salvia species were identified by RAPD and ISSR markers [47]. Expressed sequence tags (ESTs) were used to study the natural polymorphism of Salvia species germplasm naturally occurring in the Mediterranean region of Turkey [48]. In Salvia studies, CAAT box-derived polymorphism (CBDP markers) provided high genetic diversity among studied species [49].

Thymus vulgaris L. is an evergreen herb native to the Mediterranean and southern Europe. Traditionally, it has been used to treat skin inflammation and various diseases of the respiratory and gastrointestinal system [50]. The plant essential oil has potent bactericidal activity [51], antioxidant, and cytotoxic activity against cancer cell lines [52]. RAPD markers allowed a perfect distinction between Thymus species, including *Thymus vulgaris* [53]. DNA markers based on the inter simple

sequence repeats (ISSR) were used in the characterization of thymus species [54] as well as in the differentiation of *Thymus vulgaris* L. chemotypes [55].

Like the previous herbs, Peppermint *Mentha* × *piperita* L. belongs to the *Lamiaceae* family. Peppermint is a perennial herb native to Europe and cultivated in many parts of the world [56]. Genetic diversity among peppermint cultivars was analysed previously, and all genotypes were differentiated by RAPD markers [57]. Using the EST sequences in the peppermint genetic polymorphism study, the dinucleotide repeat of SSRs was most frequent with the AG/CT repeat motif followed by AT/AT [58].

gDNA extraction from biological material was successful, samples were diluted ten times, and the functionality was checked by ITS primers in PCR. ITS amplicons were about 450 bp in length (data not shown). Both BBAP and PBAP techniques were able to generate different sample fingerprints. PIC values ranged from 0.313 to 0.349 for BBAP and 0.376 for PBAP. This confirmed that both methods can detect genetic distances and describe dominant markers as highly informative [32]. As expected, the *ypr5* variability (PBAP) was lower than the ypr10 (BBAP) variability. At the species level, the genomic similarity of *ypr10* genes can be 50 to more than 90%, and the general protein structure of PR-10 is more conserved, so they can easily enter allergen cross-reactions [21]. On the other hand, profilins are highly conserved, especially in sites essential for the actin-binding mechanism [59].

Plant allergen genes are generally well described [22, 60, 61], which predetermines the use of them as DNA-based markers. As mentioned, BBAP amplified 291 alleles that is under previous studies, and homologic allergen genes encode several isoforms [59, 62, 63]. *In silico* analysis, high variability in amino acid sequences of Bet v 1 homologues among plant species [64] enables primer annealing to various sequence sites and the observation of polymorphisms. On the other hand, the amino acid sequence homology of Bet v 1 is conservative enough to use BBAP as a marker technique, especially as it includes a confirmed IgE binding site (Uehara *et al*., 2001). Reverse primers were designed to amplify a relatively variable region of *ypr-10* gene. Therefore, they can capture the variability in position 19 of the amino acid sequence (NCBI: P15494) (Breiteneder et al., 2000). The BBAP technique was used to analyze intraspecies variability of apple (*Malus domestica* Borkh.) where obtained BBAP profiles have been more monomorphic, indicating more excellent stability of *ypr10* genes in one species [65], but two of four primers still allow intraspecific analysis. This corresponds to the high structural homology of Bet v 1 proteins in the apple proteome [66] despite the high variability of ypr10 genes (50–90% similarity) in the apple genome [29]. Polymorphism of BBAP profiles of chosen herbs was 100%.

Profilins are actin-binding proteins belonging to plant pan-allergens [67]. Firstly, it was described as a birch-pollen Bet v 2 allergen [25]. It is a confirmed allergen in pear, peach, apple, watermelon, tomatoes, celery, pumpkin, peanut, and many other species [64]. This technique was also used in legumes, where lengths of amplicons were 46 bp to 669 bp [68]. There are possible functional and regulatory consequences of sequence polymorphism in pollen profilins, and this polymorphism may represent a mechanism for generating multiple profilin covariants between species [69]. Profilin isoforms show low sequence divergence in plants. Allergenic profilins are found exclusively in flowering plants. Due to their high degree of sequence conservation, they form a family of cross-reactive allergens in monocot and dicot pollens and plant foods [22]. Pairwise alignments of more than 100 profilin sequences of plant species showed a range of identity of 68.7–99.2% among plant reproductive profilin proteins and 66.0–90.0% of identity among plant vegetative profilins [70]. Our

results point to an immense sequence variability in the studied herbs; the detected polymorphism is 100%, and the technique did not create clusters according to the plant genus, which is an exciting result since the method was able to distinguish cereal species according to our unpublished results.

5. Conclusions

Two techniques that analyze the length polymorphism of plant allergen homologs were applied to medicinal plants. The study's result showed that both techniques could be used as polymorphic DNA marker systems to study plant diversity. The marker system is also highly informative according to the PIC value, which was 0.376.

Author Contributions

Conceptualization - L.U., J.Ž.; methodology - J.Ž.; software - S.F., I.S. and M.K.; validation - S.F., J.Ž., L.U. and M.K.; formal analysis - S.F., I.S., V.Š.; investigation - J.Ž. and L.U.; resources - J.Ž.; writing original draft preparation - L.U. and S.F.; writing review and editing - J.Ž. and M.K.; visualization - S.F., L.U. and V.Š.; supervision - J.Ž. and M.K.; project administration - J.Ž. and M.K.

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Competing Interests

The authors have declared that no competing interests exist.

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