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Natural Variability of Genomic Sequences of Mal d 1 Allergen in Apples as Revealed by Restriction Profiles and Homolog Polymorphism

Lucia Urbanová [1], Jana Bilčíková [1], Dagmar Moravčíková [2] and Jana Žiarovská [2,*]

[1] Research Centre AgroBioTech, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia; lucia.urbanova@uniag.sk (L.U.); jana.bilcikova@uniag.sk (J.B.)
[2] Institute of Plant and Environmental Sciences, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia; xmoravcikova@uniag.sk
* Correspondence: jana.ziarovska@uniag.sk; Tel.: +421-37-6414244

Abstract: Apples are a popular fruit worldwide, with many health and nutritional benefits. However, this fruit is also among those that, particularly in Central and Northern Europe, are allergenic due to the Mal d 1 allergen. Mal d 1 is a homologous allergen to Bet v 1—the main pollen allergen of birch. In this study, two different approaches were used to identify the natural length polymorphism of Bet v 1 homologs in apple varieties, with the aim of characterizing their effectiveness. BBAP (Bet v 1 based amplified polymorphism) and RFLP (restriction fragments length polymorphism) profiles were characterized and compared. RFLP analysis recognizes the genetic diversity of *M. domestica* Mal d 1 sequences at a relatively low level. In BBAP profiles, the genetic dissimilarity was up to 50%, which appears suitable for intraspecific fingerprinting and serves as an additional method for RFLP analysis. RFLP analysis was able to distinguish some varieties that BBAP could not, such as Sonet.

Keywords: Bet v 1; Mal d 1; polymorphism; *Malus domestica* Borkh

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1. Introduction

Different DNA-marker-based techniques have been developed to effectively analyze the genomic variability of plants. In principle, these techniques amplify coding or non-coding regions in a polymerase chain reaction (PCR) to generate variable length polymorphism, enabling the distinction or characterization of plant germplasm. For these techniques, where coding regions serve as markers, methods based on the variability of homolog genes for plant allergens have been developed [1–3]. One of them, Bet v 1-based amplified polymorphism (BBAP), amplifies the different lengths of the homologs of Bet v 1, a main birch allergen, and is anchored in the linear epitope sequence through degenerate primers [4]. Birch pollen (*Betulaceae*) is considered the most allergenic in Northern, Central, and Eastern Europe [5] and it is the main tree pollen allergen in Northern Europe [6]. Its major allergen, Bet v 1, is thought to trigger cross-reactions with some food allergens, which form the PR-10 protein family [7]. The percentage of people suffering from birch pollen allergy and who also respond to at least one of the PR-10 group is 50–93%, what is known as PFS (pollen-food syndrome).

In 2015, the EAACI reported that 7 million patients suffer from food allergies (URL4), with the pediatric population being the most vulnerable. Current studies suggest that food allergy is becoming a common population disease and affects 5–10% of the population [8]. One of the most significant Bet v 1 cross-reactions is connected to apple consumption, which affects >70% of patients allergic to birch pollen [9].

M. domestica Borkh. belongs to the *Rosaceae* family, genus *Malus*. Cultivation is widespread, especially in temperate areas, with more than 10,000 varieties being grown [10]. Apple are valued for their sweet taste and juice with a high content of minerals, vitamins, or

various bioactive substances, such as polyphenols, polysaccharides, flavonoids, or various health-promoting acids [11–13]. The content of all substances is influenced by the type of cultivar or breeding purpose. The main apple allergen is Mal d 1, a 17.5 kDA protein that belongs to the PR-10 protein family.

Mal d 1 consists of a complex gene family composed of 31 different loci, each encoding a different isoallergen [14]. Moreover, the variability of the major apple allergen is amplified by the existence of isoallergen alleles with small differences, that encode isoallergenic variants/isoforms [14–16]. Differences in the amino acid sequence of isoforms can alter ligand binding sites, influencing the allergenic potential of the allergen. Reduced or increased affinity to human IgE has been confirmed between Mal d 1 isoforms from different apple varieties [17] or by amino acid substitutions in the epitopes [18,19]. Based on different isoforms, a restriction fragment length polymorphism (RFLP) can be applied to analyze genomic variability, as was previously proven for part of the *Ypr 10* gene promotor in the apple varieties, Santana, Cripps Pink, Jonagold, and Gala [20].

Despite the relatively variable residual nucleotide sequence of *ypr10* genes (genomic similarity 50–90%) [21], proteins of the PR-10 family share a very similar to identical protein structure due to highly conserved sequences [22]. The conserved sequences are probably specific IgE-binding sites, known as epitopes. A probable IgE epitope could be located between the 42nd and 52nd amino acids [23], with Glu45 considered essential. Its essentiality has been confirmed by several mutations, demonstrating the variability with which the mutants bind specifically [24–26].

In this study, two different approaches were used to identify the natural length polymorphism of Bet v 1 homologs in apple varieties, with the aim to characterizing their effectiveness. BBAP- and RFLP-generated polymorphic profiles were characterized and compared.

2. Materials and Methods

2.1. Biological Material and DNA Extraction

The biological material consisted of a total of 70 apple varieties, as listed in Table 1. All samples were collected from a private orchard (Brodno, Žilina, Slovakia) at the stage of physiological maturity and were frozen at −20 °C. Genomic DNA was isolated using GeneJet™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific®, Shanghai, China). The quality and quantity of DNAs were verified three times: using a Nanophotometer™ (IMPLEN, Munich, Germany), 1% agarose gel and PCR with ITS primers [27].

Table 1. List of the apple varieties with relevant sample numbers used in all figures.

Sample Number	Apple Variety	Sample Number	Apple Variety	Sample Number	Apple Variety	Sample Number	Apple Variety
1	Santana	19	Tabor	37	Rozela	55	Hael 616
2	Spencer	20	Sonet	38	Melrose	56	Orion
3	Primadela	21	Parkerovo	39	Fiesta	57	Maj Gold
4	Ecolette	22	Jantár	40	HL 782	58	Sirius
5	Akame	23	Rubigold	41	Delor	59	Paula Red
6	Karneval	24	Topaz	42	Spigold	60	Dulcit
7	Waltz	25	Ligol	43	Rucla	61	Harmony
8	Winesap	26	Pocomoke	44	Heliodor	62	Pinova
9	Čistecké lahôdkové	27	Shalimar	45	Stela	63	Sentima
10	Melrose 24628	28	Florina	46	RubinStep	64	Mutsu
11	Selena	29	Rezistent	47	Aneta	65	Viktory
12	Lotos	30	Bolero	48	Jonagold Decosta	66	Lipno
13	Produkta	31	Ligol	49	Delbarestivale	67	Pikant
14	Jonalord	32	Alkmene	50	Goldstar	68	Fanny
15	Angold	33	Bohemia	51	Blanik	69	Admiral
16	Rezistent Opal	34	Dalila	52	HL 189	70	Freyberg

Table 1. *Cont.*

Sample Number	Apple Variety	Sample Number	Apple Variety	Sample Number	Apple Variety	Sample Number	Apple Variety
17	Kamzi	35	Linda	53	Rajka	71	Kristian
18	Meteor	36	Alkmene	54	Biogolden		

2.2. BBAP

Non-degenerated forward and degenerated reverse primers for the BBAP technique were designed for Bet v 1 sequences with NCBI accession numbers AJ289770.1 and AJ28977.1 [4]. The sequence of the forward primer without degeneration, matching the conservative region, was F: 5′ CCT GGA ACC ATC AAG AAG 3′. The sequence of the reverse degenerated primer (at the 12th and 14th positions), matching the variable region, was R: 5′ TTG GTG TGG TAS TKG CTG 3′, while S = G/C and K = T/G. Individual variants of the reverse primer combinations were used in the analysis separately with the codes R1, R2, R3 and R4.

For the amplification reactions, EliZyme HS Robust MIX was used with 10 ng of DNA and 400 nM of each primer. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C, 45 s; annealing at 54 °C, 45 s; polymerization 72 °C, 35 s; and a final elongation at 72 °C for 10 min.

2.3. RFLP

The Mal d 1 sequence of the apple Bet v 1 homolog (NCBI AF020542.1) was divided into three parts for amplification, with primer sequences listed in Table 2.

Table 2. Primers used to amplify Mal d 1 sequence for RFLP.

Amplicon	Primers	Position	Length
Mal d 1–part 1	F: 5′GCTCGATCACGATAAACTAAGG 3′ R: 5′ATGAGGATGGGGTGTTGAAG 3′	nt 3–522	520 bp
Mal d 1–part 2	F: 5′ACATCCAGTACCGGGGATGA 3′ R: 5′GGGTGCAATCTTGGGGATGA 3′	nt 833–1470	638 bp
Mal d 1–part 3	F: 5′GATGCTTTGACAGACACCATTG 3′ R: 5′TTTCAAACAAATACATAAAGGGCAAC3′	nt 1801–2190	390 bp

In silico DNA restriction analysis was used to find eligible restriction enzymes by the NEBcutter V2.0 website (www.nc2.neb.com/NEBcutter2; accessed on 20 March 2024). Two restriction enzymes were selected to each sequence part: a specific enzyme for the amplified part and a common enzyme for all of the amplified parts (Table 3). Restriction cleavage conditions followed the manufacturer's instructions.

Table 3. Restriction enzymes chosen for RFLP analysis.

Restriction Enzyme	Cutting Site	Part of Mal d 1 Sequence
AseI	AT↓TA↑AT	part 1, 2, 3
NcoI	C↓CATG↑G	part 3
NlaIII	↑CATG↓	part 2
SpeI	A↓CTAG↑T	part 1

The cleaved products were separated in 10% PAGE and stained by GelRedTM (Biotium). Arrows stand for restriction site.

2.4. Data Processing

BBAP as well as RFLP profiles were analyzed using the freely available GelAnalyzer software (www.gelanalyzer.com; accessed on 15 January 2024) and converted into binary

matrices. The matrices from both analyses were processed using the UPGMA method, employing the Nei-Li coefficient of genetic distances, to create dendrograms. The Mantel test, cophenetic coefficients, *phi* coefficient, effective multiplex ratio, marker index, resolving power, as well as heatmap design and dendrogram comparison were all carried out in the R studio environment [28].

3. Results

3.1. BBAP Variability

The analysis of a length-based polymorphism for one of the nucleotide sequences of the Bet v 1 epitope and its homologs is a universal technique, with different results obtained based on the individual reverse primers used in their non-degenerated forms.

Primers F + R1 (Bet v 1 isoforms with His-119) amplified a total of 13 amplicons with the following lengths: 940 bp, 910 bp, 850 bp, 690 bp, 665 bp, 600 bp, 560 bp, 385 bp, 355 bp, 340 bp, 285 bp, 200 bp, and 140 bp. A dendrogram could not be constructed due to very similar or identical profiles among the individual apple varieties analyzed. However, all the analyzed apple varieties could be grouped into four groups (Table 4) based on their F + R1 amplicon profiles. Notably, no PCR product was amplified in the Alkmene variety using this primer combination, even after repetition. Amplicons of 385 bp and 200 bp were found in all samples with a stronger signal, indicating a higher number of these loci in the analyzed genotypes and a preference for their amplification. The control amplicon of 385 bp, which matched the in silico prediction of PCR results, was amplified in all other apple varieties.

Table 4. Groups of analyzed apple varieties based on the differences in BBAP F + R1 primer combination fingerprints profiles.

Group	Variety
I.	Santana, Spencer, Primadela, Ecolette, Produkta, Rezistent Opal, Meteor, Sonet, Jantar, Rezistent, Bolero, Fiesta, Rucla, Heliodor, Rajka, Dulcit, Harmony, Sentima, Admiral
II.	Akame, Karneval, Waltz, Winesap, Melrose 24628, Selena, Lotos, Tabor, Parkerovo, Rubigold, Topaz, Pocomoke, Shalimar, Florina, Ligol, Alkmene, Bohemia, Dalila, Linda, Rozela, Melrose, HL 782, Delor, Spigold, Stela, Delbarestivale, Orion, Maigold, Sirius, Paula Red, Lipno, Pikant, Fanny, Freyberg, Kristian
III.	Čistecké lahôdkové, Kamzi, Rubinstep, Aneta, Jonagold Decosta, Goldstar, Blanik, HL 189, Biogolden, Hael 616, Pinova
IV.	Jonalord, Angold, Mutsu, Viktory

Primers F + R2 (Bet v 1 isoforms with Asp-119) amplified two lengths of 210 bp and 385 bp in all analyzed apple varieties. Only in the case of ten apple varieties—Winesap, Melrose 24628, Rubinstep, Jonagold Decosta, Goldstar, Paula Red, Dulcit, Harmony, Pinova and Sentima—were additional amplicons of 180 bp and 150 bp obtained.

Primers F + R3 (Bet v 1 isoforms with Glu-119) generated fingerprint profiles that were grouped the varieties into six different clusters, with five samples showing unique profiles (Figure 1).

The most distinct profile was observed in the Primadela variety. The greatest interspecific genetic distance was up to 50%, while the most similar groups, the fifth and sixth, shared approx. 80% genetic similarity. A total of eight PCR products were amplified: 385 bp, 220 bp, 210 bp, 150 bp, 140 bp, 125 bp, and two shorter than 50 bp, with the 385 bp and 210 bp amplicons being present in all varieties except for Primadela and Admiral.

The most polymorphic results were generated by the F + R4 primer pair annealing to the *Ypr10* gene, which has an isoform with 119-Lys in its amino acid sequences. A total of 20 PCR products were amplified, with following lengths: 1300 bp, 1200 bp, 1100 bp, 970 bp, 690 bp, 585 bp, 385 bp, 340 bp, 290 bp, 260 bp, 235 bp, 210 bp, 185 bp, 170 bp, 150 bp, 140 bp, 120 bp, 110 bp, 95 bp, and 75 bp. Despite the polymorphic BBAP profiles, identical profiles were obtained among several pairs of varieties: Maigold–Sirius; HL 782–Orion;

Winesap–Delor; Waltz–Fiesta; Spencer–Bohemia; Resistent Opal–Florina; Meteor–Bolero–Ligol. All varieties were grouped into seven clusters in the dendrogram (Figure 2), with the Fanny variety showing a unique profile. The first three groups in the dendrogram shared more than 45% similarity and were the most represented. The Jantar, Heliodor, and Aneta varieties were distinguished from all other varieties by almost 95%, with their genetic distance between 50 and 70%.

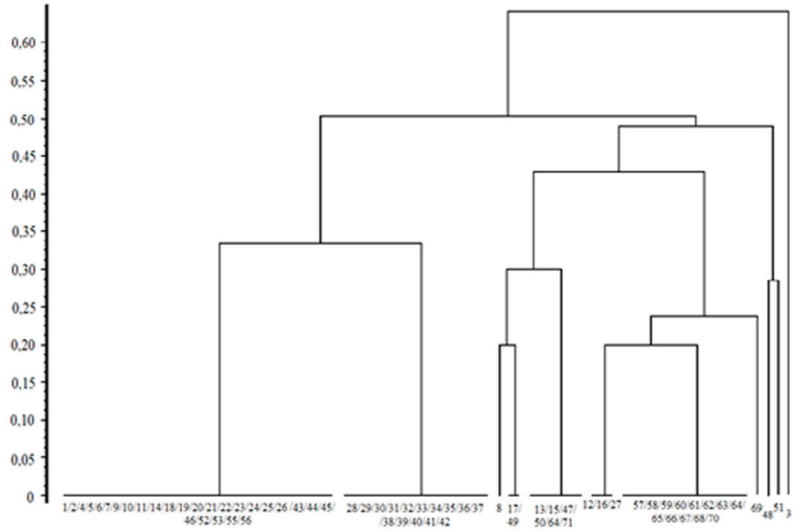


Figure 1. UPGMA dendrogram of Nei-Li coefficient of genetic similarity values among analyzed apple varieties for BBAP F + R3 fingerprints. Number codes of varieties are listed in Table 1.

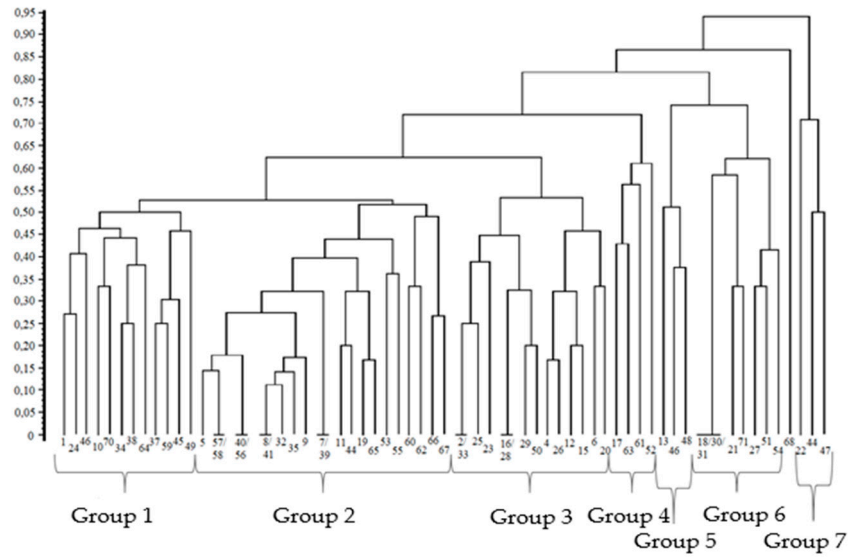


Figure 2. UPGMA dendrogram of Nei-Li coefficient of genetic similarity values among analyzed apple varieties for BBAP F + R4 fingerprints. Number codes of varieties are as listed in Table 1.

Combining the results from the individual reverse primers into one binary matrix, groups A–F were identified based on the similarity of some varieties (Table 5). The highest genetic similarities were found in group F, varying from 70 to 90%, in contrast to group K, which had the lowest similarity of all groups (only 30% between varieties Jantar and Heliodor. Two of the analyzed varieties, Maigold and Sirius, generated identical profiles for all four BBAP primers. The second-highest similarity in all BBAP profiles (almost 90%) was between Alkmene and Linda.

Table 5. Groups of varieties that share the highest similarities of BBAP profiles.

Group	Variety	Similarity in %
Group A	Topaz, Stela	~50
Group B	Dalila, Rozela, Melrose	60–70
Group C	Akame, Orion, Waltz, Selena, Tabor	60–80
Group D	Čistecké lahôdkové, Hael 616	~60
Group E	Maigold, Sirius, Lipno, Pikant	50–65
Group F	HL 782, Linda	~75
Group G	Bohemia, Florina	~55
Group H	Spencer, Ecolette, Sonet	50–55
Group I	Rubigold, Pocomoke, Karneval	50–55
Group J	Harmony, Sentima	~45
Group K	Jantar, Heliodor	~30

The results indicate a good ability of the BBAP technique to generate an intraspecific polymorphism that reflects the sequence variability of *M. domestica* Borkh. *ypr10* genes. Primers F + R2 point to intraspecific stability, while primers F + R4 demonstrate high variability of *ypr10* genes within the *M. domestica* Borkh. genome. The most stable amplicon lengths amplified across all primer combinations were 210 bp and 388 bp, what corresponding with predicted in silico data [4]. Only two completely identical profiles were obtained for varieties Maigold and Sirius; all other varieties differed by at least 10% in their BBAP profiles.

3.2. RFPL Variability

In RFLP, a total of 20 to 36 fragments were obtained, with a high degree of polymorphism. The lowest number of fragments (20) was observed in the Sonet variety, while the most abundant profile (38 fragments) was found in the Kamzi variety. Identical cleavage profiles were identified for the varieties, Blanik-HL 189-Dulcit, Jonagold-Maigold, and Alkemene-Florina.

In the first amplified part of the Mal d 1 gene, the Sonet variety profile exhibited the lowest number of cleavage fragments (n = 3), while the Rozela variety showed the highest (n = 12) when combining restriction fragments from both restriction enzymes used. The highest number of restriction fragments for Ase 1 was 11 and for Spe I it was 4 (Figure 3). The cleavage of the part 1 region generated 39 different cleavage profiles, 24 of which were unique. Unique cleavage profiles were found in some low-allergenic varieties, such as Santana and Pink Lady, as well as the highly allergenic variety, Golden Delicious.

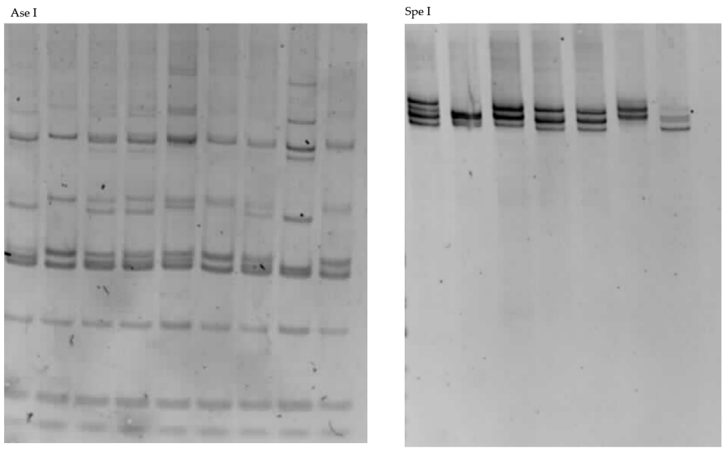


Figure 3. Selection of restriction pattern generated by Ase I and Spe I in the first amplified part of Mal d 1 gene.

In the second amplified part of the Mal d 1 gene, the AseI enzyme cut amplicon to 4–8 fragments, forming 16 restriction profiles, of which three were unique (Gala, HL 782 and Sonet). Again, the Sonet variety had the lowest number of fragments (n = 4). In the Golden Delicious variety, eight restriction fragments were obtained. The most numerous restriction profile was found in 22 varieties and consisted of five cleavage fragments. A restriction fragment of 250 bp was present in all varieties except for Sonet. Using the NlaIII enzyme, 6 to 11 fragments were obtained, resulting in 22 different restriction profiles. Unique profiles were observed in the varieties Dalila, Golden Delicious, Kamzi, Pink Lady, Produkta, and Spencer. Up to five restriction fragments were present in the restriction profiles of all 71 varieties, with lengths of 110 bp, 400 bp, 510 bp, 580 bp, and 630 bp.

In the third amplified part of the Mal d 1 gene, the cleavage profiles showed the highest degree of monomorphism with the selected enzymes AseI and NcoI. The sequence was cleaved into 2 to 5 fragments by AseI, forming five cleavage profiles, and 2 to 5 fragments by NcoI forming three restriction profiles. No variety showed a unique profile. The NcoI fragments were 83 bp and 37 bp in size, corresponding to the in silico cleavage profile fragments.

The enzymes used in the RFLP analysis cleaved all three amplified parts of the Mal d 1 sequence along the entire gene length. SpeI and NlaIII cleaved the Mal d 1/*ypr10* gene sequence upstream of the region amplified in the BBAP, NcoI cleaved downstream of this region, while AseI cleaved both upstream and downstream. The constructed dendrogram, based on the binary matrix of restriction profiles, showed that the RFLP analysis was able to differentiate between most varieties with a dissimilarity coefficient of 0.18 (Figure 4). The most unique varieties according to RFLP was Sonet, the most dissimilar to all of the other analyzed varieties was Victory. The most similar restriction profiles were obtained for the varieties Čistecké lahôdkové, Lotos, Florina, Linda, and Lipno.

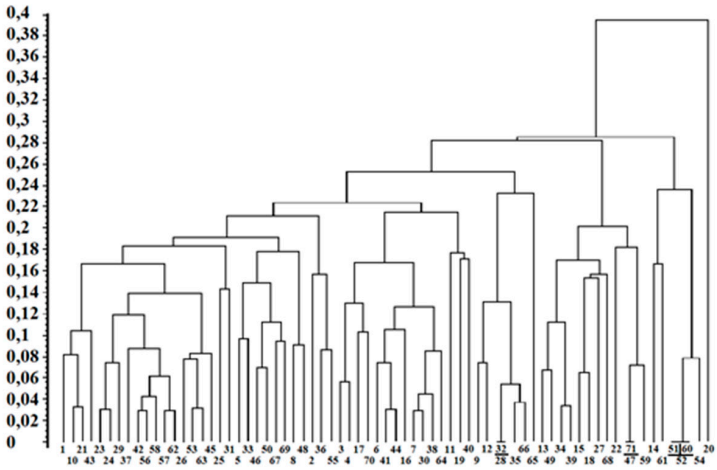


Figure 4. UPGMA dendrogram of Nei-Li coefficient of genetic similarity values among analyzed apple varieties for RFLP restriction profiles. Number codes of varieties are as listed in Table 1.

3.3. Comparative Potential of BBAP and RFLP in Mal d 1 Length Variability Analysis in the Apple Germplasm

Comparing the techniques used in the study, BBAP was able to generate fully different fingerprints, and all the analyzed apple varieties were grouped in a dendrogram without any similar pattern (Figure 5). In contrast, the RFLP restriction pattern failed to distinguish seven of the analyzed varieties in the final dendrogram. In the case of Jantár, Jonagold and Rubin Step, all of these varieties were grouped in a very different pattern, as was in differences in BBAP F + R1 primer combination fingerprint profiles.

The individual binary matrices with the Nei-Li coefficient showed no significant correlation in the Mantel test based on Pearson's product–moment correlation as well as the phi coefficient for binary matrices. Cophenetic coefficients for individual dendrograms were 0.672 for the RFLP data and 0.669 for the BBAP data.

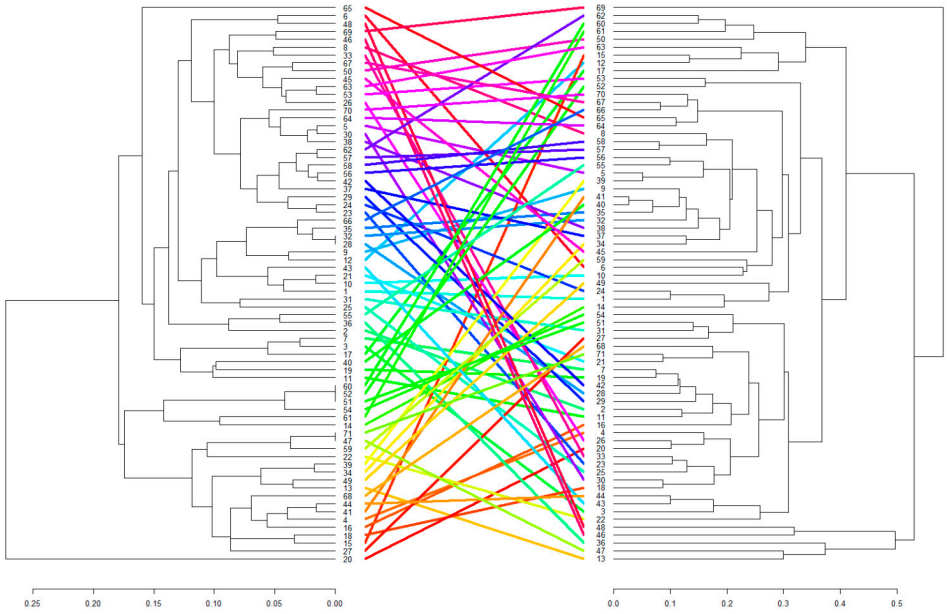


Figure 5. The relationship of the location of individual apple varieties in summary dendrograms for both of the methods used in the study. Number codes of varieties are listed in Table 1. Individual colours of lines represent individual varieties and their positions in dendrograms.

The BBAP is comparable in effectiveness and the ability to analyze genetic dissimilarities between individual varieties with the RFLP (Figures 6 and 7). The coefficient values were closely aligned when marking the universal length polymorphism of Bet v 1 homologs compared to those generated by restriction fragment length polymorphism.

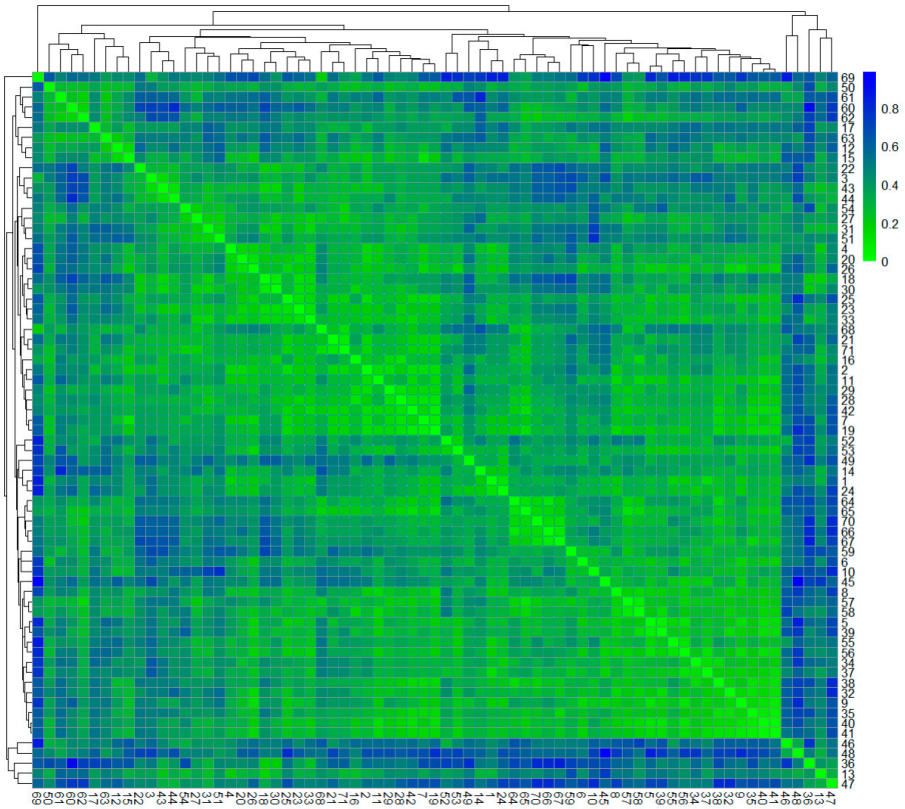


Figure 6. Heatmap of dissimilarity coefficient distribution among apple varieties analyzed by BBAP. Number codes of varieties are listed in Table 1.

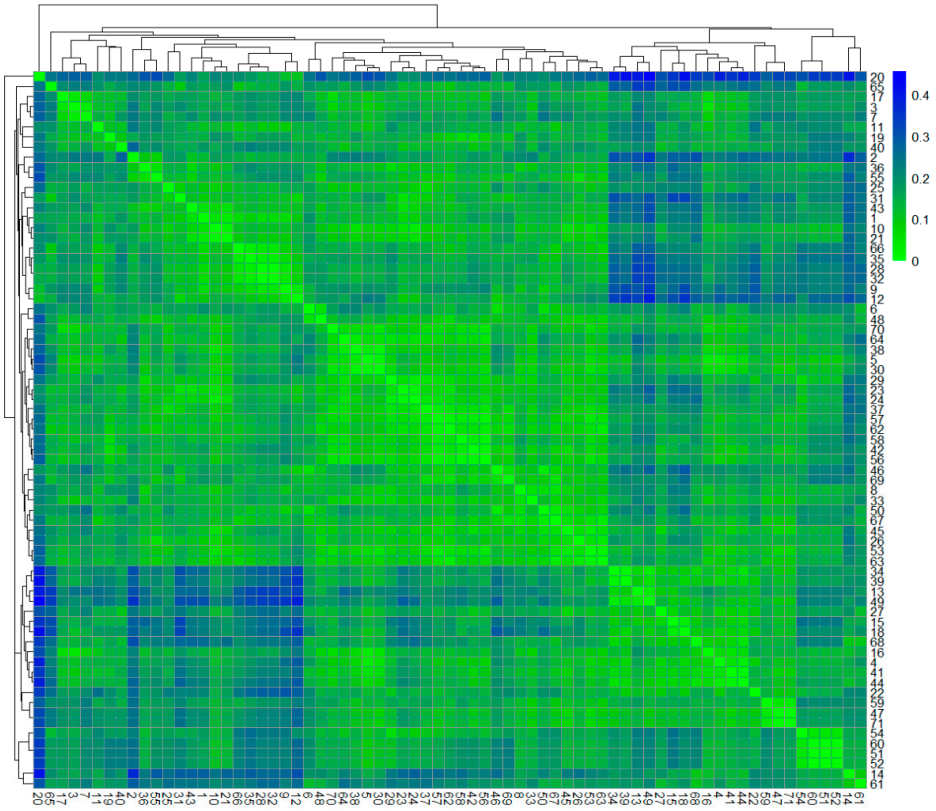


Figure 7. Heatmap of dissimilarity coefficient distribution among apple varieties analyzed by RFLP. The number codes of varieties are listed in Table 1.

The probability of detecting polymorphism with BBAP in the analysis of apple germplasm was 0.67, compared to 0.59 for RFLP. The effectiveness of both marker techniques in multiplex testing, as calculated by the effective multiplex ratio, was 32.67 for BBAP and 40.91 for RFLP. The Marker index, which estimates the total utility of the marker technique, was 18.96 for BBAP and 24.17 for RFLP. The resolving power of differences detection was 0.59 for BBAP and 0.66 for RFLP.

4. Discussion

Thus far, various DNA-based markers have been utilized in apple germplasm studies, each offering different characteristics. According to the available literature, the simple sequence repeat (SSR) technique is suitable for studying a polymorphism of *M. domestica* Borkh. due to its high level of polymorphism, co-dominant inheritance, and reproducibility [29,30]. A high level of genetic diversity was demonstrated using SSR across the species (He > 0.7) and no significant reduction in the diversity over the last eight centuries. Although, less diversity has been confirmed for commercially used varieties [31]. The conserved DNA-derived polymorphism technique (CDDP) has also been used to produce polymorphic amplification patterns in a set of 15 apple varieties from this study. In some primer combinations, identical amplification profiles were observed in a few varieties, similar to the RFPL results in this study. Identical CDDP profiles were found in the pairs of varieties: Red Delicious–Granny Smith; Maigold–Paula Red; Selena–Melodie and Maigold–Paula [32]. Gene-specific markers are reported to determine fruit storage life (ethylene production), fruit skin color, *Alternaria* resistance, and scab resistance in apples [33].

An in silico comparison of amino acid sequences of known Bet v 1 allergen isoforms typically shows significant variability among individual plant species [34]. Such variability is a good background for using these regions as DNA-based marker techniques to reveal the variability of Bet v 1 homologs in flowering plants and to be universal in their application. A high homology exists in the amino acid sequences in the region of the forward primer for

the BBAP strategy, which includes a confirmed epitope for IgE [2,35]. The reverse primers amplify a variable region of the *ypr10* gene, matching the amino acid variability at position 119 of the Bet v 1 protein (P15494) [34]. This study analyzed different apple varieties to prove the efficiency of the *in silico*-generated markers for Bet v 1 homologs compared to the restriction cleavage of species-specific Bet v 1 homologs in the apple genome–Mal d 1 gene, to provide polymorphic fingerprints among them. The universality of this DNA-based technique has been reported for different groups of flowering plants relevant to food allergies, such as fruits, vegetables, nuts, or legumes [2,36,37]. For vegetables, genetic distance analysis using the Nei-Li coefficient showed a narrow range (the difference was only 0.36), but the genetic diversity was relatively high, corresponding to the findings of variability among plant Bet v 1 homolog proteins [35,38].

Several studies have shown a high degree of natural genetic diversity manifested in Mal d 1/*ypr10* genes in *M. domestica* Borkh. At the genetic level, Mal d 1 is a complex gene family with 31 loci, each encoding a different isoallergen [14], while each isoallergen can be divided into isoallergenic variants due to single-allele substitutions [14–16]. The similarity level among individual coding sequences of Mal d 1/*ypr10* genes ranges from 53.1% to 97.7% and among isovariants from 95% to 99.8%. At the protein level, the sequences of isoallergens are identical between 37% (e.g., Mal d 1.08 and Mal d 1.11 A with 102 amino acid substitutions) and 96% (Mal d 1.06 A and Mal d 1.06 D with 7 substitutions [12]. Considering the limited number of analyzed varieties [16] and the existence of more than 7000 varieties, the variability in *ypr10* genes across the entire apple genome is likely several times greater. This study focused on monitoring the natural variability in the Mal d 1 allergen across 71 varieties, including old varieties, such as Akane, Alkmene, Spencer, Spigold or Winesap, as well as newer and commercially important varieties, such as Blanik, Kamzi or Rozela. For primary screening, fingerprinting via BBAP and RFLP techniques was chosen due to its specificity and reported effectiveness [36,39].

The results of BBAP profiles confirm the ability of the primers to create intraspecific polymorphisms, reflecting the variability of the *ypr10* genes in M. *domestica* Borkh. and complementing the data obtained *in silico*. Primer pair F + R2 amplifies isoforms that are stable across varieties, while primer pair F + R4 captures the high variability of *ypr10* genes within the *M. domestica* Borkh. genome. Previous research used degenerated reverse BBAP primers that anneal to variable and conserved parts of Bet v 1 homologues genes in the BBAP technique to analyze the intraspecific variability in *M. domestica* Borkh. varieties [40]. The generated amplicons formed relatively monomorphic profiles, indicating the stability of the Bet v 1 isoforms within the selected apple varieties, corresponding to the results of F + R1 and F + R2 combinations in this study. Bet v 1 homologs in the apple genome possess a high-protein structure homology due to short amino acid sequences, which are highly conserved among plant species, resulting in a very similar or identical protein structure [22], despite the relatively variable nucleotide sequences of the *ypr10* genes (genomic similarity from 50 to more than 90%) [22].

Data mining and experimental verification were previously applied for Mal d 1 RFLP analysis [20]. Virtual cleavage maps were prepared for available sequences of the apple variety McIntosh, which has been referred to as hypoallergenic, as well as the variety Santana [41–43]. The RFLP technique has been reported to be useful for *M. domestica* when differentiating between closely related apple varieties [39] or when pedigree information is missing [44]. Phylogenetic relationships are well illustrated for apple germplasms when using RFLP [45]. The inter-apple and intra-apple variability concerning the Mal d 1 gene and allergenicity is well documented [46]. The amount of Mal d 1 in apples classified as containing low concentrations of allergen may be sufficient to induce both clinical symptoms and skin reactivity in birch-pollen-allergic patients. Differences in the concentration of Mal d 1 proteins year-on-year or even between two growing sites have also been confirmed [47]. Different aspects in the assessment of allergen identification in plant food sources, variability of allergenic molecules, and genetic relationships is important

not only for the scientific community but also for the consumers as the number of allergy sufferers increases every year, which results in an impact on the quality of their life.

5. Conclusions

The results of the BBAP profiles confirm the ability of the primers to create an intraspecific polymorphism that reflects the variability of the Mal d 1/*ypr10* genes in *M. domestica* Borkh. Primer pair F + R2 captures isoforms stably occurring across the entire species, while primer pair F + R4 captures the high variability of *ypr10* genes within the *M. domestica* Borkh. genome. RFLP recognizes the genetic diversity of *M. domestica ypr10* genes at a relatively low level (with a dissimilarity coefficient up to 0,18). Compared to BBAP analysis, genetic dissimilarity was up to 50% (for F/R4), making the gene suitable for intraspecific fingerprinting and an additional method for the Bet v 1 protein family. However, RFLP analysis was able to distinguish some varieties which BBAP was not, such as Sonet.

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