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ASSESSMENT OF THE INFORMATIVENESS OF IPBS MARKERS FOR IDENTIFICATION AND DIFFERENTIATION OF UKRAINIAN HAZELNUT VARIETIES

Aim. *To assess the informativeness of iPBS markers for the identification and differentiation of Ukrainian hazelnut varieties.* ***Methods.*** *Thirty Corylus spp. specimens (25 varieties and 5 wild plants) were analyzed using PCR amplification with 24 iPBS primers. The informativeness indices were calculated based on PCR banding profiles.* ***Results.*** *Six primers with the highest polymorphism were selected. They produced 95 bands (15.8 per primer on average), only one of which was monomorphic. The mean resolving power (Rp) of these primers was 6.760 and discriminating power (D_L) 0.948, while the number of non-differentiated pairs (ND) averaged 3.2 per primer. Primer 2402 distinguished all varieties, and two other primers were unable to differentiate only between one pair. In comparison with SSR markers, iPBS demonstrated higher Rp and D_L and significantly lower ND values.* ***Conclusions.*** *iPBS markers are capable to provide an efficient and reliable tool for the differentiation of varieties and can be successfully applied in hazelnut breeding programs.*

Keywords: *Corylus spp., hazelnut, variety, breeding, iPBS markers.*

Introduction

The genus *Corylus* comprises a diverse group of deciduous shrubs and trees found in temperate forests of the Northern Hemisphere. Among *Corylus* species, hazelnut is the most economically significant, occupying third position in the ranking of nut-bearing woody crops globally in terms of harvested area, which encompassed approximately 1,078,192 ha in 2023 (FAO, 2025), following cashew and almond. The average annual global production of hazelnut in 2023 was about 975,259 t,

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with Turkey (650,000 t) and Italy (102,740 t) as the leading producers. Other significant contributors include the USA (85,460 t), Azerbaijan (75,409 t), Georgia (36,900 t), and China (36,900 t), while smaller production areas are found in Chile, Spain, France, Romania, Serbia, and Russia. The global hazelnut market was valued around 8.52 billion USD in 2023 [1]. However, global prices fluctuate considerably due to the strong dependence of Turkish yields on climatic factors, particularly spring frosts in the Black Sea region [2].

The global demand for hazelnuts is predominantly driven by their utilization in the production of confectionery products, with less than 10% of the total production being sold in raw form. Currently, industry standards for nut quality, shape, and size are based on nuts of Italian cultivars *Tonda Gentile de Langhe*, *Tonda di Giffoni*, and *Tonda Romana* [3].

In recent decades, rapid progress has been achieved in the genetics, biology, and breeding of hazelnut, including both the improvement of traditional approaches and the development of new tools, particularly molecular methods. Substantial advances have been made in genetic studies, breeding efficiency, and in understanding the genetic basis of traits such as resistance to Eastern Filbert Blight (EFB), red leaf pigmentation, chlorophyll deficiency, pollen coloration, and self-compatibility [2, 4].

A series of marker-assisted selection (MAS) studies have been conducted, the results of which have revealed that the varieties *Gasaway*, *Zimmerman*, and *Ratoli* exhibit resistance against EFB, a fungal disease that limits hazelnut cultivation in eastern North America [5]. Research has been conducted on quantitative characteristics such as resistance to bud mites, ease of husk removal, and nut quality, with a view to providing valuable information for crop improvement. In addition, high-resolution simple sequence repeat (SSR) markers have also been developed, facilitating research into genetic diversity and intragenus relationships [3, 6].

The development of approaches for varietal identification using DNA markers and pedigree analysis has the potential to enhance the efficiency of breeding, particularly by facilitating the selection of genetic material for further crosses and enhancing genetic analysis of resulting hybrids. To obtain progeny with desirable traits, pedigree information is of paramount importance, especially for specimens included in crossing programs [7].

Despite the widespread use and high efficiency of SSR markers in breeding and genetic studies, their application is not always feasible across laboratories due to the need for fluorescently labeled primers and specialized equipment for PCR fragment separation and detection. A more straightforward and economical option would be to employ PCR with dominant markers, among which iPBS markers have been shown to be especially promising [8]. The iPBS method relies on the presence of the reverse transcriptase primer binding site (PBS) in LTR retrotransposons, which is used for PCR primer annealing, followed by amplification of DNA fragments located between the adjacent PBS. Since LTR retrotransposons constitute 50—90% of a plant genome and are distributed evenly across it, iPBS is regarded as a universal and sensitive approach, albeit with reduced reproducibility than SSR analysis.

The aim of this study was to assess the informativeness of iPBS markers for identification and differentiation of Ukrainian hazelnut varieties.

Materials and Methods

Plant material and DNA extraction

To assess the informativeness of iPBS markers, a total of 30 specimens were used, including 25 hazelnut varieties and 5 plants of three *Corylus* species (*C. avellana*, *C. colurna*, and *C. chinensis*), obtained from the pomological collection of the National Dendrological Park "Sofiyivka" of the National Academy of Sciences of Ukraine (Table 1). The specimens were kindly provided by Corresponding Member of NAS of Ukraine I. S. Kosenko.

DNA isolation

Young leaves were collected in May and subsequently dried using silica gel, prior to being subjected to

DNA extraction. The material was stored in sealed plastic bags with silica gel at ambient temperature for several months prior to extraction. DNA was isolated using a cetyltrimethylammonium bromide (CTAB)-based protocol, which included preliminary removal of water-soluble secondary metabolites [9]. The quality and concentration of extracted DNA were assessed by agarose gel electrophoresis using λ phage DNA of known concentration as a standard, and with a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific).

Primers and PCR conditions

For PCR analysis, 24 iPBS primers that had been described earlier [8] were used; characteristics of these primers are provided in Table 2. PCR reactions (20 μL total volume) contained 1× ammonium sulfate PCR buffer (Thermo Fisher Scientific) with 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of Taq DNA polymerase (Thermo Fisher Scientific), 30 ng of template DNA, and 1 μM of primer. Amplifications were carried out in a 5PRIMEG (Techne) thermocycler under the following program: 94 °C for 3 min; 28—35 cycles of 94 °C for 15 s, T_{ann} (see Table 2) for 60 s, 72 °C for 60 s; 72 °C for 5 min.

PCR products were fractionated by electrophoresis in a 1.4% agarose gel containing 0.5 μg/mL ethidium bromide and 1× SB buffer (5 mM $Na_2B_4O_7$, pH 8.5). Electrophoresis was carried out at 30 V for 20 minutes, then at 80—100 V until the dye front completely migrated out of the gel. The gel was imaged under UV light.

Table 1. **Cultivated hazelnut varieties and Corylus spp. from the collection of the National Dendrological Park "Sofiyivka" of the NAS of Ukraine used to assess the informativeness of iPBS markers**

No.	Species / variety	No.	Species / variety
1	*C. avellana* f. *fuscorubra*	16	Shedevr [2]
		17	Hrandioznyi [2]
2	*C. chinensis*	18	Badius [2]
3	*C. colurna*	19	Dokhidnyi [2]
4	Sofiyivskyi 15 [1]	20	Funduk 85 [2]
5	Garibaldi [4]	21	Peremoha 74 [2]
6	Sofiyivskyi 1 [1]	22	Lozivskyi bulavovydnyi [2]
7	Sofiyivskyi 2 [1]		
8	Dar Pavlenka [2]	23	Pyrizhok [2]
9	Ukraina 50 [2]	24	Zhovtnevyi [2]
10	Cherkeskyi 2 [3]	25	Funduk 45 [2]
11	Lozivskyi sharovydnyi [2]	26	Darunok yunnatam [2]
12	Koronchastyi [2]	27	Svichkovyi [2]
13	Morozivskyi [3]	28	Trapezund [5]
14	Hordiienko [3]	29	*C. avellana*
15	Karamanivskyi [3]	30	*C. avellana*

Notes: The breeding centers, where the respective varieties were developed: [1] — National Dendrological Park "Sofiyivka" (Uman); [2] — Ukrainian Research Institute of Forestry and Forest Melioration named after G. M. Vysotsky (Kharkiv); [3] — Scientific Research Institute of Floriculture and Subtropical Crops (Sochi); [4] — Italy; [5] — Turkey.

Data analysis

The digital images of electrophoretic gels with PCR products were processed using Totallab TL120 v2008 (Nonlinear Dynamics Ltd.) software. The amplification products of each primer were scored as present (1) or absent (0) bands to construct a binary matrix.

The following parameters were calculated to evaluate the informativeness of the markers: total number of amplified bands per primer (B), resolving power (Rp) [10], discriminating power (D_L), number of non-differentiated pairs (ND) [11], and polymorphic information content (PIC) [12, 13]. Expected heterozygosity (He) and unbiased expected heterozygosity (uHe) were calculated using GenAlEx v.6.502 software [14, 15]. Pairwise genetic distance matrix was also generated in GenAlEx, and then used to construct a UPGMA dendrogram of genetic similarity in MEGA6 software [16].

D_L reflects the probability that two closely related genotypes can be distinguished and ranges from 0 to 1. Rp characterizes the level of differentiation between individuals detectable by a given primer and is considered a qualitative index without fixed limits. ND is indicative of the number of

genotype pairs cannot be distinguished by a primer and thus provides a quantitative measure. PIC represents the ability of a marker to detect polymorphism within a population and range from 0 to 0.5 for dominant markers.

Results and Discussion

At the initial stage of the study, the iPBS primers, described by Kalendar *et al.* (2010), were screened to identify those that yielded the most polymorphic amplification products with DNA from *Corylus* varieties and species. In total, 24 iPBS primers (see Table 2) were tested in PCR with DNA from five *Corylus* specimens. This approach enabled the identification of primers that generated the most polymorphic amplification profiles for subsequent analyses (Fig. 1).

First, the primers were assessed based on the clarity and polymorphism of amplification profiles obtained with DNA from the test specimen set. The primers that yielded predominantly monomorphic or poorly resolved bands were eliminated from further consideration. Electrophoretic profile analysis allowed us to select six primers that exhibited the most clearly distinguishable and polymorphic amplification products, namely 2229, 2232, 2252, 2277, 2395, and 2402 (Table 2). Then, their informativeness was evaluated using PCR with DNA from 30 specimens of hazelnut varieties and *Corylus* species. The calculated informativeness indices of the selected primers are presented in Table 3.

The total number of scored bands that were obtained using six primers for 25 hazelnut varieties was 95, ranging from 11 for primer 2229 to 25 for primer 2402, with an average of 15.8 bands per primer. The majority of the selected primers yielded polymorphic bands, with the exception of primer 2395, which exhibited a single monomorphic fragment among 14 amplified products. The number of amplified bands generated by the iPBS primers in our study often exceeds the comparable data reported by other authors. For instance, in the study of 18 parsley (*Petroselinum crispum*) genotypes from Turkey, the average number of fragments per primer was 11.3 [17]. In the case of iPBS analysis of 35 and 13 grapevine varieties from China and Slovakia, respectively, the values obtained were 6.6 and 11.6 [18, 19]. Similarly, in the sample of 80 specimens representing three *Zanthoxylum* species, the average number of bands per primer was 12.7 [20], while for cabbage it reached 14 [21]. It is evident that the primers selected in the present study exhibited amplification profiles that were

Table 2. **List of initially screened iPBS primers**

Name	Sequence	T_{ann} (°C)	B	PB	PPB (%)
2231	ACTTGGATGCTGATACCA	52	10	6	60
2249	AACCGACCTCTGATACCA	54.7	12	9	75
2085	ATGCCGATACCA	53	9	7	77.8
2378	GCTCATGATACCA	51	10	8	80
2076	GCTCCGATGCCA	59.2	8	6	75
2380	CAACCTGATCCA	50.5	12	12	100
2382	TGTTGGCTTCCA	50.5	11	10	90.9
2232	AGAGAGGCTCGGATACCA	55.4	13	12	92.3
2277	GGCGATGATACCA	52	15	14	93.3
2272	GGCTCAGATGCCA	55	11	9	81.8
2252	TCATGGCTCATGATACCA	51.6	12	12	100
2251	GAACAGGCGATGATACCA	53.2	25	16	64
2253	TCGAGGCTCTAGATACCA	51	20	16	80
2376	TAGATGGCACCA	52	8	5	62.5
2402	TCTAAGCTCTTGATACCA	50	22	21	95.4
2415	CATCGTAGGTGGGCGCCA	61	14	11	78.6
2273	GCTCATCATGCCA	56.5	8	7	87.5
2378	GGTCCTCATCCA	53	6	4	66.7
2229	CGACCTGTTCTGATACCA	53.5	17	16	94.1
2219	GAACTTATGCCGATACCA	53	9	9	100
2377	ACGAAGGGACCA	53	11	9	81.8
2395	TCCCCAGCGGAGTCGCCA	52.8	12	12	100
2373	GAACTTGCTCCGATGCCA	51	11	7	63.6
2374	CCCAGCAAACCA	47.1	13	9	69.2

Notes: primers highlighted in bold were selected for further analysis with the complete specimen set. T_{ann} — annealing temperature of the primer; B — the total number of bands; PB — the number of polymorphic bands; PPB — the percentage of polymorphic bands. The B, PB, and PPB indicators were calculated based on the analysis of 5 test specimens.

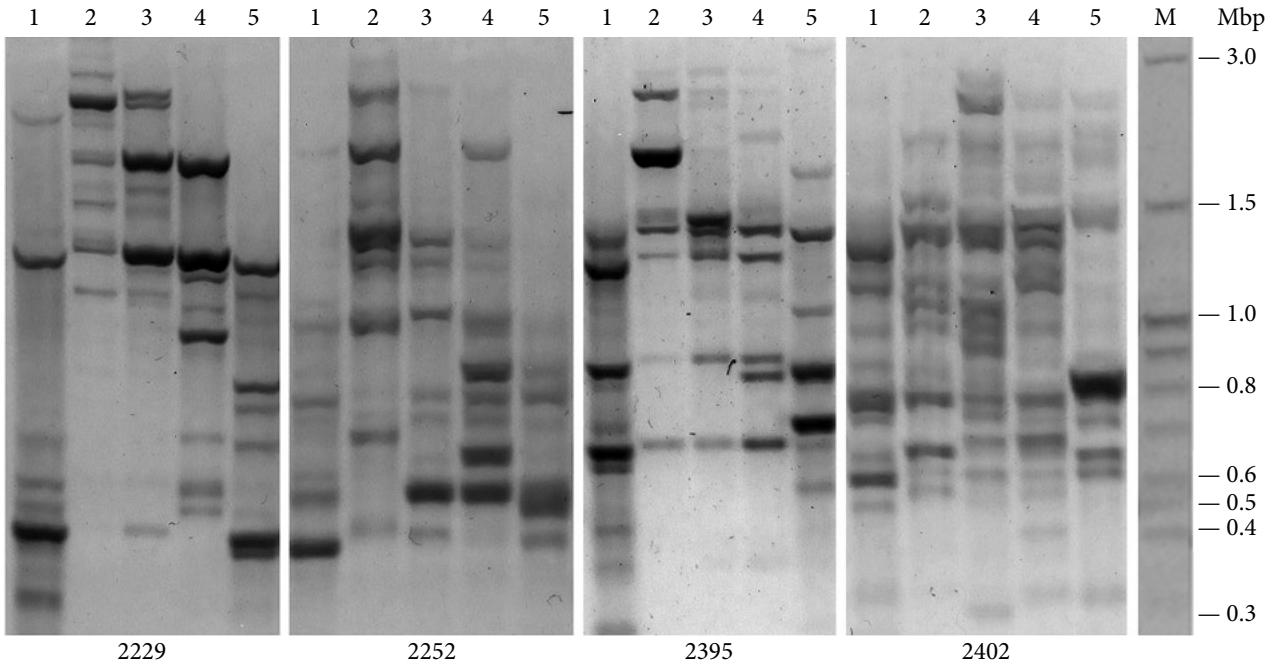

Fig. 1. Electrophoretic banding patterns of PCR products obtained with the test specimen set using selected iPBS primers (indicated under the panels): 1 — *C. avellana* f. *fuscorubra*; 2 — *C. chinensis*; 3 — Garibaldi; 4 — Dar Pavlenka; 5 — Ukraina 50. M — DNA size marker (1kb ladder)

generally comparable to or exceeded by the informativeness those documented in the existing literature.

The polymorphism information content for the selected primers ranged from 0.258 for primer 2252 to 0.325 for primers 2229 and 2232, with an average of 0.299. These values indicate the ability of the primers to detect inter-varietal differences and are satisfactory for dominant iPBS markers, for which the maximum possible PIC is 0.5. Similar results were reported in other studies using iPBS markers. For instance, an average PIC value of 0.330 was observed in the study of common bean [22]. In the analysis of genetic diversity in

Table 3. **Informativeness indices of the selected iPBS primers**

Parameter	2229	2232	2252	2277	2395	2402	Average value
B	11.000	14.000	12.000	19.000	14.000	25.000	15.800
Rp	5.690	6.960	3.540	8.800	5.040	10.560	6.760
D_L	0.942	0.946	0.931	0.955	0.957	0.960	0.948
ND	4.000	4.000	9.000	1.000	1.000	0	3.200
He	0.285	0.224	0.199	0.253	0.216	0.270	0.240
uHe	0.289	0.226	0.201	0.256	0.218	0.273	0.240
PIC	0.325	0.325	0.258	0.316	0.279	0.291	0.299

Notes: B — total number of amplified fragments; Rp — resolving power; D_L — discriminating power; ND — number of non-differentiated pairs; He — expected heterozygosity; uHe — unbiased expected heterozygosity; PIC — polymorphism information content. The B, PB, and PPB indicators were calculated based on the analysis of 25 hazelnut varieties.

Leonurus cardiaca L., the mean PIC value was 0.290 [23], while in the study of potato it was somewhat lower — 0.230 [24].

The average resolving power (Rp) of the six iPBS primers used was 6.76. The values ranged from 3.54 for primer 2252, which showed the lowest resolving power, to 10.56 for primer 2402.

The discriminating power (D_L) of the selected iPBS markers was also high, averaging 0.948 and ranging from 0.931 to 0.960. This indicates that the probability of correctly distinguishing two randomly selected genotypes in the dataset exceeds 93%, which is an exceptionally high indicator for dominant markers.

The number of non-differentiated pairs (ND) for the selected iPBS markers ranged from 0 to 9, with an average of 3.2. Primer 2402 differentiated all 25 genotypes of the studied hazelnut varieties, while primers 2277 and 2395 failed to distinguish only one pair each.

To determine the optimal marker combinations, we evaluated the efficiency with which the iPBS primers differentiate hazelnut varieties by calculating the number of non-differentiated pairs for samples of 200, 500, and 1,000 specimens. For the ND calculation, we assumed independence of the product sets generated by the primers (Table 4). In theory, a single primer 2402 should be sufficient for analyzing small samples of up to 30 specimens. However, to increase identification reliability, it is advisable to use combinations of at least two primers, particularly 2402 with either 2277 or 2395. This is especially important when analyzing samples of more than 200 specimens. Other primers from the selected set may also be used for genetic analysis of hazelnut varieties, if needed.

We assessed the efficiency of the selected iPBS primers by comparison them with the SSR primers. The informativeness of SSR primers for identifying Ukrainian hazelnut varieties was demonstrated in previous study [25]. We compared the resolving power (Rp), discriminating power (D_L), and the number of non-differentiated pairs (ND) calculated from the data obtained on the same dataset of 25 varieties for two types of markers. Using the same set of genotypes in both studies ensured correct comparison of the results.

For the SSR primers, Rp values were lower, ranging from 1.210 to 2.730, about three times lower than the corresponding values for the iPBS primers. The substantially higher Rp of the iPBS primers is likely due to the greater number of polymorphic fragments produced per primer. In the SSR marker analysis, a single primer pair is used to amplify one locus, which, in diploid organisms, is represented by a maximum of two alleles. In contrast, the iPBS primers can generate a significantly larger number of polymorphic fragments in a single PCR-reaction, explaining the higher Rp values.

For the SSR primers, D_L values ranged from 0.490 to 0.901 with an average of 0.805, which is lower than the average of 0.948 observed for the iPBS primers.

For the SSR primers, ND values were substantially higher, ranging from 31.92 to 213.2, which is several orders of magnitude above the values for the iPBS primers. Moreover, among the SSR markers used, none was capable of differentiating all studied specimens. The comparison results demonstrate the higher sensitivity of the iPBS primers and their ability to efficiently differentiate Ukrainian hazelnut varieties using only one or two primers.

Based on the matrix of pairwise genetic distances, a dendrogram of genetic similarity among the studied hazelnut varieties and *Corylus* species was

Table 4. **Expected efficiency of different marker combinations for genetic differentiation of Ukrainian hazelnut varieties in samples of various sizes**

Combination of primers	ND (200)	ND (500)	ND (1,000)
2402*	0	0	0
2277 or 2395	14.58	39.58	81.25
2277 and 2395	0.05	0.13	0.27

Notes: ND — number of non-differentiated genotype pairs, calculated under the assumption of independence of the product sets generated by each primer, for sample sizes of 200, 500, or 1,000; * — these estimates are based on the data obtained and may change if more genotypes are analyzed.

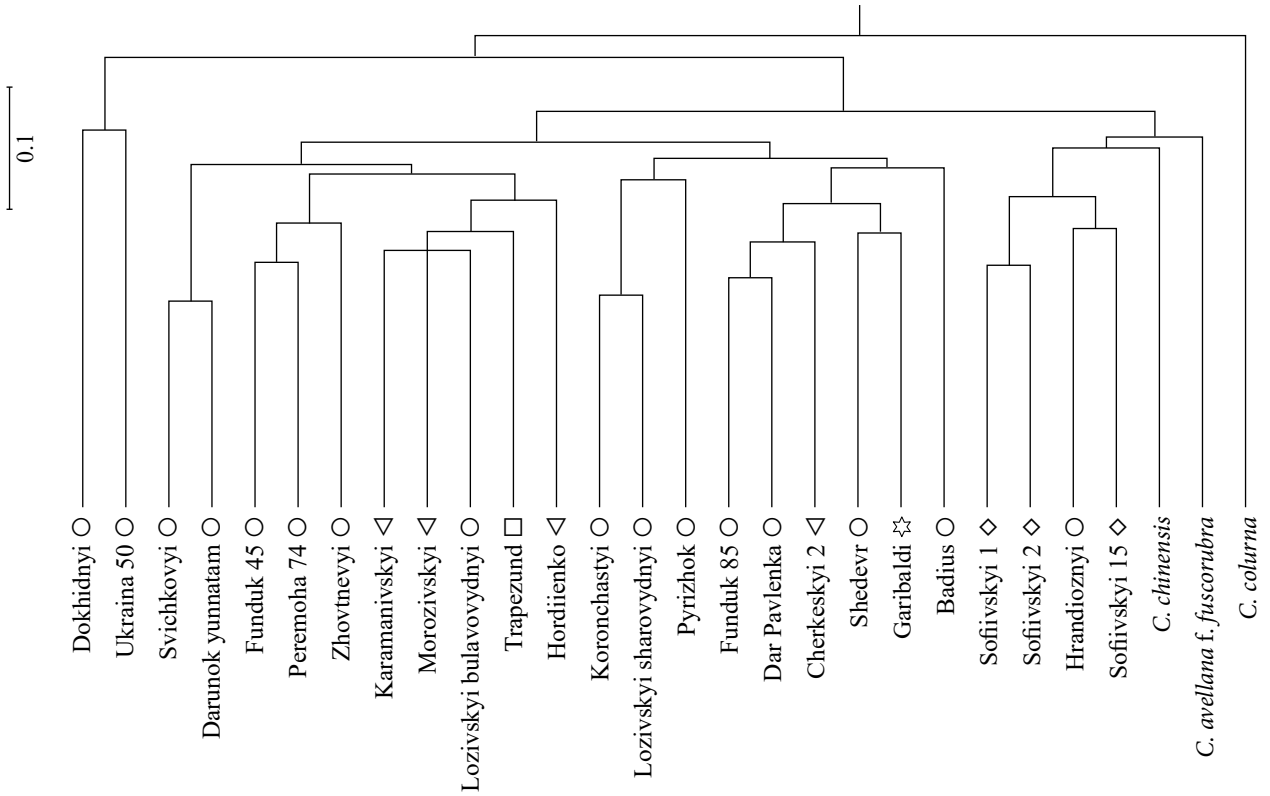

Fig. 2. UPGMA dendrogram of genetic similarity among the studied Ukrainian hazelnut varieties. The breeding centers, where the respective varieties were developed, are indicated as follows: ◇ — National Dendrological Park "Sofiyivka" (Uman); ○ — Ukrainian Research Institute of Forestry and Forest Melioration named after G. M. Vysotsky (Kharkiv); ◁ — Scientific Research Institute of Floriculture and Subtropical Crops (Sochi); ☆ — Italy; □ — Turkey

constructed (Fig. 2). The *C. colurna* specimen is positioned separately from all formed groups on the obtained dendrogram. This is because this species was not used to breed the analyzed hazelnut varieties. The varieties created at the National Dendrological Park "Sofiyivka" cluster with *C. chinensis* and *C. avellana* f. *fuscorubra*, which corresponds to their breeding origin, as these plants were indeed used in crossings to produce the aforementioned varieties. Another distinct cluster consists of varieties developed at the Scientific Research Institute of Floriculture and Subtropical Crops. These varieties are grouped together with the Turkish variety Trapezund. In contrast, the varieties bred at the Ukrainian Research Institute of Forestry and Forest Melioration named after G.M. Vysotsky are evenly distributed across the dendrogram and do not form a clear cluster, which may reflect the high diversity of the initial breeding material used in their development.

Thus, in terms of their ability to differentiate genotypes, iPBS markers are not inferior to SSR markers, and in certain parameters they even surpass them. The SSR marker analysis remains an important tool in genetic studies of cultivated plants due to their codominant nature and the ability to estimate allele frequencies. From a practical perspective, however, iPBS markers provide comparable, and in some cases higher, efficiency. Given the simplicity, universality, and lower cost of the iPBS marker analysis, it can be considered a full complement or even an alternative to the SSR analysis for solving applied tasks in hazelnut breeding and genetic studies.

Conclusion

This study demonstrated that the iPBS marker analysis is an effective tool for genetic identification of Ukrainian hazelnut varieties. Following the initial screening of 24 iPBS primers, six were selected based on the highest levels of polymorphism. Two of the selected primers were sufficient to generate informative banding profiles and distinguish all analyzed genotypes. Dendrogram analysis largely confirmed the relationship between cluster structure and the breeding origin of hazelnut varieties. A comparison with SSR markers revealed that iPBS markers possess comparable or higher informativeness while requiring less labor and incurring lower costs. These results underscore the practical value of iPBS markers for identifying hazelnut varieties and for further use in hazelnut breeding programs.

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ОЦІНКА ІНФОРМАТИВНОСТІ iPBS-МАРКЕРІВ ДЛЯ ІДЕНТИФІКАЦІЇ ТА ДИФЕРЕНЦІАЦІЇ СОРТІВ ФУНДУКА УКРАЇНСЬКОЇ СЕЛЕКЦІЇ

Мета. Оцінити інформативність iPBS-маркерів для ідентифікації та диференціації українських сортів фундука. ***Методи.*** Тридцять зразків *Corylus* spp. (25 сортів та 5 видів) було проаналізовано за допомогою ПЛР-ампліфікації з 24 iPBS-праймерами. Показники інформативності були розраховані на основі ПЛР-профілів отриманих смуг. ***Результати.*** Було відібрано шість найполіморфніших праймерів. Всього відібрані праймери забезпечили 95 смуг (в середньому 15,8 на праймер), лише одна з яких була мономорфною. Середня роздільна здатність (Rp) цих праймерів становила 6,760, а дискримінаційна здатність (D_L) 0,948, тоді як кількість недиференційованих пар (ND) становила в середньому 3,2 на праймер. Праймер 2402 диференціював усі сорти, а два інші праймери не могли розрізняти лише одну пару. Порівняно з SSR-маркерами, iPBS-маркери продемонстрували вищі Rp та D_L та значно нижчі значення ND. ***Висновки.*** iPBS-маркери є ефективним та надійним інструментом для диференціації сортів і можуть бути успішно застосовані в селекційних програмах фундука.

Ключові слова: *Corylus* spp., фундук, сорт, селекція, iPBS-маркери.