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Evaluation of the Genetic Diversity of several *Corylus avellana* Accessions from the Romanian National Hazelnut Collection

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Abstract

Romanian hazelnut (*Corylus avellana*) germplasm is held in a national collection at SCDP Valcea. A clear situation of the held accessions is necessary for an efficient management of the germplasm collection. In order to achieve this, the genetic variability of 43 accessions was assessed using 23 RAPD primers. The RAPD analysis was carried out as a screening test to confirm the genetic identity of some accessions. Based on the screening results, 12 accessions were selected for analysis using nine SSR primers. A high level of genetic diversity was observed (He=0.75, Ho=0.81, F=-0.061) among the analyzed samples. A genetic similarity matrix was constructed and the resulting UPGMA dendrogram revealed three major groups, corresponding to the geographical origin of the accessions. In order to increase the effectiveness of genebank management, the identification of duplicate and mislabeled accessions with the aid of molecular markers is of high interest, especially being the first one of this kind in a Romanian hazelnut germplasm collection.

Keywords: European hazelnut, germplasm collection, RAPD, SSR

Introduction

The European hazelnut (*Corylus avellana* L.) is a commercially important species among nut trees, being fifth in global importance after cashew (*Anacardium occidentale* L.), almond [*Prunus dulcis* (Miller) D. A. Webb], walnut (*Juglans regia* L.) and chestnut (*Castanea* spp.) (Petriccione *et al.*, 2009). Its fruits had been used as a food source since prehistoric times (Gokirmak *et al.*, 2009) and the kernels are consumed raw or processed. They contain unsaturated fatty acids that have been reported to decrease blood cholesterol levels and also control adverse effects of hypertension (Savage *et al.*, 1997; Amaral *et al.*, 2006).

The place and time of hazelnut domestication is not clear, although it was already cultivated by the Romans (Boccacci and Botta, 2009). Turkish and European hazelnut cultivars were obtained through selection, over many centuries, from local wild populations. In an effort to introduce new traits, wild species were crossed with *C. avellana* (Mehlenbacher, 1991).

The geographical distribution of *C. avellana* spans from the Atlantic coast of Europe to Norway. The northern boundary of its distribution includes the British Isles, Scandinavia and northern regions of the Russian Federation, the eastern boundary is represented by the Ural Mountains and its southern boundary includes Spain, Morocco and Algeria in the west through Italy, the former nation of Yugoslavia, Greece and Turkey to northwestern Iran and Transcaucasia (Kasapligil, 1942).

In the last decades, the scientific community became more interested in the rescue, collection and exploitation of hazelnut germplasm. Many Romanian populations of hazelnut are of natural origin, encompassing few trees separated by geographical natural obstacles. Genetic structures of such populations have been formed by natural selection and represent a valuable source of genetic diversity. The hazelnut accessions held at SCDP Valcea come from different populations. In this context, the scientific interest for the identification, evaluation and long time conservation in national collections is growing. The preservation of the hazelnut germplasm held at SCDP Valcea represents a real interest for Romania and in order to achieve this goal, genetic evaluation followed by vegetative regeneration has to be carried out. Traditional techniques of accession identification are based on morphological traits such as nut and husk. These traits can be influenced by environmental conditions, often being unreliable or imprecise indicators of plant genotype (Boccacci *et al.*, 2005).

The recent development of DNA markers, with high levels of polymorphism, has enabled the rapid identification of phenotypically extremely similar accessions. Furthermore, molecular identification techniques can be used at any stage of plant development and they are not affected by environmental factors. RAPD and SSR markers are the most common molecular markers used for identification

of the hazelnut accessions (Boccacci *et al.*, 2005; Boccacci and Batta, 2009; Miaja *et al.*, 2001; Gokirmak *et al.*, 2009). RAPD (random amplified polymorphic DNA) is an easy and cost-effective technique based on PCR (polymerase chain reaction) that can detect genetic polymorphisms randomly distributed throughout the genome by amplifying a set of DNA fragments using arbitrary primers (Botez *et al.*, 2009; Sestras *et al.*, 2009; Williams *et al.*, 1990). The use of RAPD as a first screening tool for accession identification has been suggested by Karp *et al.* (1996). Following the RAPD analysis, the identical accessions were excluded from the study and a molecular characterization of the remaining accessions was carried out with SSR markers. SSR markers have been used for accession fingerprinting and detection of mislabeling in germplasm collections (Botta *et al.*, 2005; Gokirmak *et al.*, 2009) and also for assessment of genetic relationships (Boccacci *et al.*, 2006; Boccacci *et al.*, 2008; Ghanbari *et al.*, 2005; Gokirmak *et al.*, 2009), being abundant and highly polymorphic tandemly repeated 1-6 bp sequence motifs that can be amplified by PCR.

In the present study, a total of 43 hazelnut accessions from SCDP Valcea have been assessed using RAPD markers. Following this analysis, 12 accessions have been fingerprinted using nine SSR markers published in previous studies (Boccacci *et al.*, 2006).

Materials and methods

Plant material

The 43 genotypes (Tab. 1) used in this study were obtained from the *Corylus* collection maintained at SCDP Valcea. Phenotypically identical accessions have the same name and differ by the code number following it.

DNA extraction

Young leaves from 43 accessions were collected in spring and immediately stored at -80°C. Total DNA was extracted using the protocol developed by Lodhi *et al.* (1994) and modified by Pop *et al.* (2003). Two pieces of 1 cm^2 of leaf tissue were ground to fine powder in liquid nitrogen in an Eppendorf tube. 700 µL of 65°C preheated extraction buffer [100 mM Tris-HCl, 20 mM sodium EDTA, pH=8.0, 1.4 M NaCl, 2% (w/v) CTAB, 2% PVP, 5 mM ascorbic acid and 4 mM DIECA, the last three components being added to the extraction buffer just before the heating at 65°C on the water bath] were then added to the tube and the mixture was incubated at 65°C for 25 min. The lysate was extracted with 700 µL of chloroform/isoamyl alcohol (24:1) and centrifuged for 15 min at 11000 rpm in a microcentrifuge. In order to precipitate the nucleic acids, the aqueous fraction was mixed with an equal volume of 5 M NaCl and then with 600 µL of ice cold 96% ethanol. The nucleic acid precipitate was washed two times in 76% ethanol and air dried before being resuspension in 50 µL TE buffer (10 mM Tris-HCl, pH=8.0, 1 mM disodium EDTA).

Tab. 1. The hazelnut genotypes used in the study
(In the dendrogram the accession is represented by the number in parenthesis)

Origin	Name
Croatia	'Okrogloplodna Leska' P1 (1), P2 (2)
France	'Corabel' P3
	'Red Lambert' (M) P7 (3), P5 (5)
	'Red Lambert MV1' P7* (4), MV3 P5* (6)
Greece	'Extra Giaghli'P1 (1), P2 (2)
Hungary	'Romai'
Italy	'Riccia de Talanico'
	'Tonda di Giffoni' P1 (1), P3 (3)
	'Tonda gentile Romana' P1 (1), P3 (3)
Netherlands	*C. avellana* T1 P7 (1), T1 P7 (2)
Romania	'Cozia' C1 (1), C2 (2)
	'Romavel' C1 P1 (1), C1 P2 (2)
	'Uriase de Valcea' P1 (1),
	'Valcea 22' C1 (1), C3 (3), C5 (5)
	'H 149-6-87' P1** (1), P2** (2)
Spain	'Closca Molla' P1 (1), P3 (3)
	'Fertile de Coutard' P1 (1), P3 (3)
	'Morell' P1 (1), P2 (2)
	'Pauetet' P1 (1), P2 (2)
	'Sant Pere' P3 (3)
	'Negret N9' P1(1), P2 (2)
USA	'Butler' C1 P1 (1), C1 P2 (2)
	'Clark' P2 (2)
	'Ennis' C1 P1 (1), C1 P2 (2)
	'Jamtegaard' 5 P1 (1), P6 (2)

* Mutation obtained at SCDP Valcea; ** Selection from SCDP Valcea

The concentration and purity of extracted DNA were determined using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific). DNA concentrations were between 300 ng/µL and 1870 ng/µL with A_{260}/A_{280} readings between 1.70-2.05. According to these measurements, DNA was diluted to 50 ng/µL and used for PCR amplification.

DNA amplification and electrophoresis conditions

PCR amplification reactions using RAPD markers were carried out as described by Williams *et al.* (1990). Reaction mixtures (total volume of 25 µL) consisted of 250 ng DNA, 9.3 µL distilled H_20 for PCR reactions, 2 µL PVP (poly vinyl pyrrolidone), 5 µL GoTaq Flexi green buffer (Promega), 2.5 µL $MgCl_2$ (Promega), 0.5 µL dNTP mix (Promega), 0.5 µL RAPD primer (Microsynth), 0.2 µL GoTaq polymerase (Promega). The RAPD primers used for differentiation of the analyzed *Corylus* accessions were decamer OPs and were synthesized by Microsynth.

The amplification was performed in a 96 Well Gradient Palm-Cycler CG1-96 (Corbett Research) programmed for 1 cycle of 3 min at 95°C, followed by 45 cycles of 1 min at 93°C, 1 min at 34°C and 1 min at 72°C. After a final incubation for 10 min at 72°C the samples were stored at 4°C prior to analysis.

The PCR amplicons were separated by electrophoresis on a 1.4% agarose (Sigma-Aldrich) gel in 1X TAE Buffer (242 g Tris Base, 57.1 mL Glacial Acetic Acid, 100 mL 0.5 M EDTA) at 0.29 V/cm^2, for approximately 2 hours. 100bp DNA Step Ladder (Promega) was used as molecular marker in order to evaluate the size of the amplified DNA fragments. Gels were visualized on a UV light Biospectrum AC Imaging System (UVP BioImaging Systems) after staining with 0.5 μg/μl ethidium bromide, for 25 min.

PCR amplification reactions using SSR markers were carried out by Microsynth AG (Balgach, Switzerland), according to their protocol and using specific primers selected from Boccacci *et al.* (2006).

Data analysis

Gel images were analyzed using TL120 software (Nonlinear Dynamics). Bands resulted after RAPD amplification were scored as present (1) or absent (0) and data entered into a binary matrix. Genetic distance between accessions was calculated using Nei and Li's coefficient of similarity (Nei and Li, 1979). Cluster analysis was conducted with FreeTree software using an UPGMA algorithm (Hampl *et al.,* 2001) and the dendrogram was visualized using TreeView software (Page, 1996). A synthetic outgroup was used for dendrogram rooting and bootstrap analysis was performed in 1000 repetitions.

Data obtained after SSR analysis was processed using CERVUS version 3.0.3 software (Kalinowski *et al.*, 2007) which calculated the number of alleles (n), allele frequencies, observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC) and non-exclusion probability of one parent (NEP1) (Weir, 1996). PIC was calculated using the formula from Botstein *et al.* (1980). For the UPGMA clustering of samples, the alleles were scored as present (1) or absent (0) and entered into a binary matrix which was used for calculating the genetic distance using Nei Li/Dice coefficient of similarity with FreeTree software and a bootstrap value of 1000. The generated image was visualised using TreeView software.

Results and discussions

RAPD analysis

A number of 23 decamer primers were used to amplify DNA extracted from the 43 hazelnut samples used in this study. The total number of polymorphic bands obtained was 216, with a mean of 9.4 polymorphic bands per primer. The most polymorphic bands (15) were obtained with OPO 14 primer (Tab. 2). The high polymorphism we obtained is characteristic for a germplasm collection that includes accessions from different countries of origin.

The smallest genetic distance obtained was 0 (between the genetic identical accessions), while the highest one was 0.43 (between 'Pauetet' and 'Cozia') and the mean genetic distance was 0.24, due to the presence of the identical accessions.

The dendrogram constructed using the FreeTree software is presented in Fig. 1, the numerical values showed near its nodes being the bootstrap values. The bootstrap values of 100 were characteristic to the identical accessions. The RAPD primers used did not cluster the accessions according to their origin. The genetically identical accessions were identified by RAPD analysis, thus, the screening purpose of these analyses was met. The following accessions were confirmed as genetically identical: 'Fertile de Coutard' 1 and 3, 'Okrogloplodna Leska' 1 and 2, 'Tonda gentile Romana' 1 and 3, 'Tonda di Giffoni' 1 and 3, 'Closca Molla' 1 and 2, 'H 149-6-87' 1 and 2, 'Extra Giaghli'1 and 2, 'Pauetet' 1 and 2, C. Avellana 1 and 2, 'Jamtegaard' 1 and 2 and 'Cozia' 1 and 2. The accessions 'Red Lambert' 3 and 5 were separated by the accessions 'Red Lambert' 4

Tab. 2. RAPD primers used for differentiation of the twenty four analyzed *Corylus* accessions

No.	Primer	No. of polymorphic bands
1	OPA 03	10
2	OPA 06	12
3	OPA 09	11
4	OPA 11	10
5	OPA 17	9
6	OPA 20	7
7	OPAB 11	14
8	OPAL 20	6
9	OPB 08	5
10	OPB 10	11
11	OPB 11	9
12	OPB 17	11
13	OPC 02	9
14	OPC 08	9
15	OPC 16	10
16	OPD 16	6
17	OPD 19	11
18	OPE 14	6
19	OPG 07	6
20	OPH 02	11
21	OPH 15	11
22	OPO 14	15
23	OPO 16	7

and 6 (the later being a mutation of the former), the genetic distance between the two groups being 0.087.

Our results are in agreement with earlier studies based on RAPD in *Corylus* species (Radicati *et al.*, 1997; Galderisi *et al.*, 1999; Miaja *et al.*, 2001). The markers have distinguished between accessions and have confirmed all the phenotipically identical accessions.

SSR analysis

The analyses were successful with nine primer pairs in the chosen accessions, a total number of 56 alleles being identified. The number of alleles per locus ranged from four (loci CaT-B509 and CaT-B512) to nine (locus CaT-B504) with an average of 6.22. The allele with the highest frequency (0.615) was 136 bp at locus CaT-B512 (Tab. 3). Twelve of the 56 alleles were unique, as they were detected in only one cultivar using the primers: CaT-B107 (111 bp - VL22, 115 bp - 'Extra Giaghli'and 141 bp -Tondagentile Romana), CaT-B504 (173 bp - 'Okrogloplodna Leska', 159 bp - Tondagentile Romana), CaT-B508 (161 bp - Extra Giaghli, 165 bp - 'Okrogloplodna Leska'), CaT-C001 (163 bp - VL22, 167 bp - 'Riccia de Talanico', 159 bp - Tondagentile Romana and 173 bp - 'Okrogloplodna Leska'), CaT-B501 (134 bp - Negret) and CaT-B511 (145 bp - VL22). This can be due to the great heterogeneity of the samples. The primer CaT-B508 identified a fixed allele of 155 bp, present in all accessions (Viruel *et al.*, 2005; Escribano *et al.*, 2004).

The alleles identified with the SSR primers had sizes between 106 bp and 183 bp (Tab. 3), comparable with the alleles obtained using the same primers by Boccacci *et al.* (2005, 2006). The differences of a few base pairs between our results and the previous published studies could be attributed to the different equipment we employed (Wünsch and Hormaza, 2002).

A statistical summary of the nine SSR loci based on the 12 accessions is presented in Tab. 4.

The PIC values ranged from 0.526 to 0.838 with an average of 0.68 (Tab. 4). The most polymorphic locus was CaT-B504 0 (PIC=0.838) and the least polymorphic locus was CaT-B512 (PIC=0.526). The observed heterozygosity (Ho) for individual loci ranged from 0.462 to 1 with an average of 0.80, while the expected heterozygosity (He) for individual loci ranged from 0.591 to 0.889 with an average of 0.74. The non-exclusion probability of one parent (NE-1P) for individual loci ranged from 0.454 to 0.825 with an average of 0.67. Only one primer (CaT-B501) had a positive estimated frequency of null alleles of +0.1707.

UPGMA clustering of the Corylus accessions

UPGMA clustering revealed three major groups (Fig. 2), each one containing accessions according to their geographic origin. We want to point out that the clustering is similar to that obtained by Gokirmak *et al.* (2009). The Black Sea Group includes Extra Giaghli, 'Romavel' and VL22 accessions. VL22 is a cultivar obtained at SCDP Valcea from a selection of Imperiale de Trebizonde, while 'Romavel' is obtained from a cross between Hall's giant x Imperiale de Trebizonde. According to Gokirmak *et al.* (2009), Imperiale de Trebizonde clustered together with 'Extra Giaghli'in the Black Sea Group 1. Another cluster is composed by 'Butler' and 'Uriase de Valcea', the later being obtained from a cross between 'Ennis' x 'Red Lambert'. 'Ennis' is a cultivar from USA and is grouped with 'Butler' in English Group 2 by Gokirmak *et al.* (2009). In our studies, 'Uriase de Valcea' and 'Butler' formed the English Group, next to the Black Sea Group. The third cluster

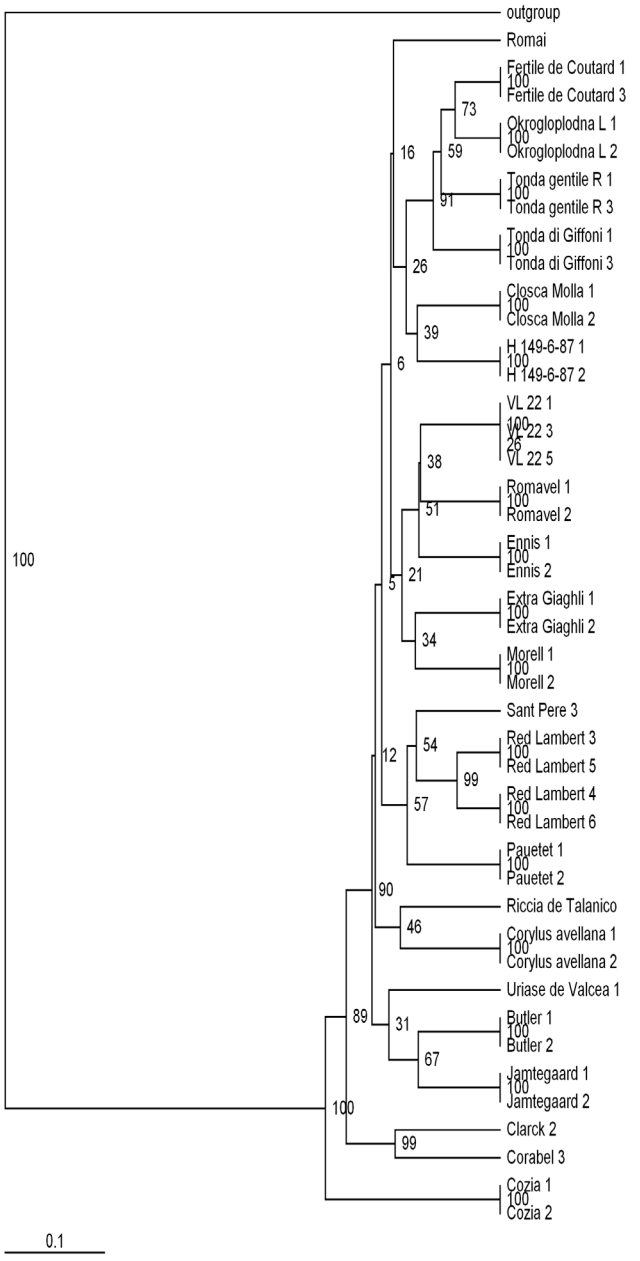

Fig. 1. UPGMA dendrogram based on the genetic relationships between the *Corylus* accessions

Tab. 3. Alleles obtained with SSR primers

Primer								
CaT-B107	CaT-B504	CaT-B508	CaT-B509	CaT-C001	CaT-B501	CaT-B503	CaT-B511	CaT-B512
Alele/Alleles								
111	157	151	106	153	116	117	139	136
115	159	153	108	159	122	125	141	138
117	167	155	110	163	124	127	143	141
119	169	157	114	165	130	129	145	148
121	171	159		167	134	131	149	
129	173	161		169			153	
133	175	165		171				
141	181			173				
	183							

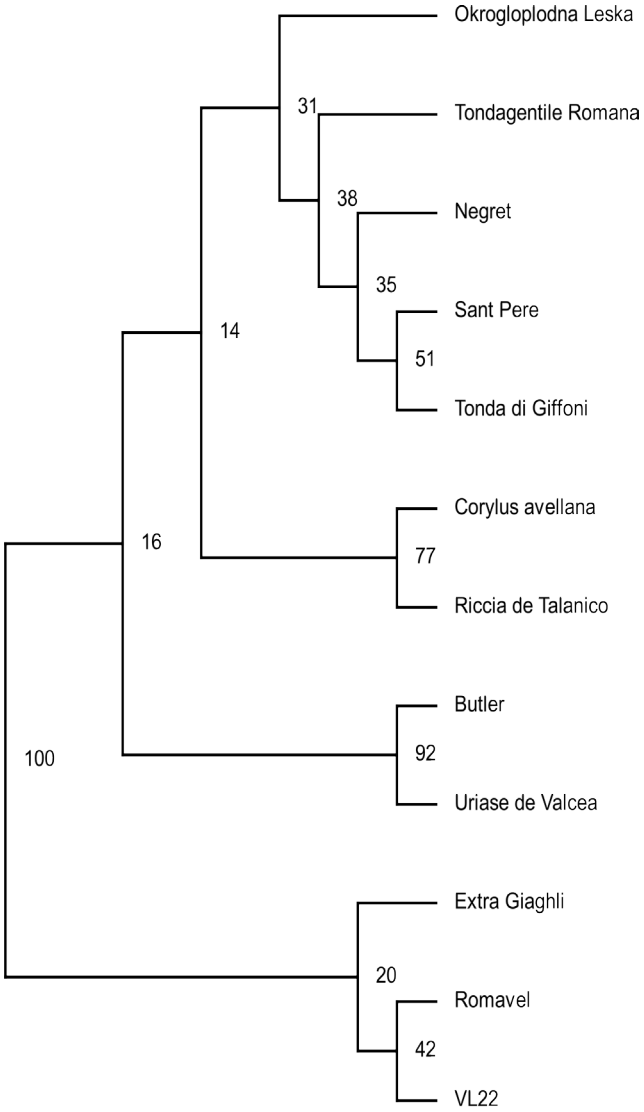

Fig. 2. Dendrogram generated based on the genetic relationships between some of the *Corylus* accessions, following SSR analysis

comprises the other accessions, most of Spanish-Italian origin. C*orylus avellana* and Okrogroplodna Leska accessions have origins in the Netherlands and Croatia, respectively. The present hazelnut genotypes have been obtained

Tab. 4. Description of 9 SSR loci in the 12 hazelnut accessions

SSR locus	n	Ho	He	PIC	NE-1P	F(Null)
CaT-B107	8	0.923	0.785	0.728	0.621	-0.1164
CaT-B504	9	1.000	0.889	0.838	0.454	-0.0806
CaT-B508	7	0.923	0.689	0.634	0.728	-0.2306
CaT-B509	4	0.846	0.748	0.666	0.708	-0.0809
CaT-C001	8	0.769	0.785	0.721	0.629	-0.0013
CaT-B501	5	0.462	0.683	0.607	0.755	+0.1707
CaT-B503	5	0.846	0.763	0.697	0.668	-0.0685
CaT-B511	6	0.769	0.763	0.698	0.663	-0.0111
CaT-B512	4	0.692	0.591	0.526	0.825	-0.1126
Total	56	7.23	6.696	6.115	6.051	-
Mean	6.22	0.80	0.74	0.68	0.67	-0.06

n=Number of alleles; Ho=observed heterozygosity; He=expected heterozygosity
PIC=polymorphic information content; NE-1P=non-exclusion probability of one parent

by a domestication process and the information regarding the origin of the cultivars is not very clear, but an exchange of germplasm took place during the human migration process (Boccacci *et al.,* 2006; Boccacci and Botta, 2009). Thus, the grouping of the Spanish-Italian cultivars is of no surprise.

Conclusions

In the present study, RAPD markers were used to confirm the genetic identity of 43 hazelnut accessions from the Romanian National Collection held at SCDP Valcea. Following this screening, 19 accessions were confirmed as genetically identical. A number of 12 accessions of interest were further characterized using SSR markers for the first time in Romania. The results obtained were similar to other studies (*e.g.* Boccacci *et al.*, 2006; Boccacci and Botta, 2009; Botta *et al.*, 2005; Gokirmak *et al.*, 2004; Ghanbari *et al.*, 2005; Gurkan *et al.*, 2009), indicating that molecular markers are of great use for the identification of identical accessions in a germplasm collection and for the assessment of the genetic diversity of the collection. In order to avoid the problem of synonyms in germplasm collections, morphological, phenological and molecular data

should be compared, our study representing a first step into this direction for the Romanian National Hazelnut Collection. In particular, we have genetically characterized the most important commercial hazelnut cultivars of our region, providing a valuable tool for plant breeders, the hazelnut industry and regulatory agencies.

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