

Communication

Genetic Identification of Hybrid Walnuts (*Juglans* × *intermedia* Carr.) in Hungary, the Hidden Potential for Future Breeding

Klára Cseke ^{1,*}, Géza Bujdosó ², Mátyás Báder ³, Tamás Mertl ¹, Attila Benke ¹ and József Dezső Kámpel ⁴

¹ Forest Research Institute, University of Sopron, 9600 Sarvar, Hungary; mertl.tamas@uni-sopron.hu (T.M.); benke.attila@uni-sopron.hu (A.B.)

² Research Centre for Fruit Growing, Institute for Horticultural Sciences, Hungarian University of Agriculture and Life Sciences, 1223 Budapest, Hungary; bujdoso.geza@uni-mate.hu

³ Faculty of Wood Engineering and Creative Industries, University of Sopron, 9400 Sopron, Hungary; bader.matyas@uni-sopron.hu

⁴ Kiszalföldi ASzC Herman Mg Tech, 9700 Szombathely, Hungary; kempel.jozsef.dezso@hermanszombathely.hu

* Correspondence: cseke.klara@uni-sopron.hu

Abstract: The question of the hybrid walnut (*Juglans* × *intermedia* Carr.) is still under debate in the Central European region. There is not simply just an underutilization, rather, even the existence of these hybrid forms is not broadly accepted. On the contrary, there is an intensive cross-breeding activity in the western part of Europe resulting in commercially available hybrid clones. In Hungary, several individuals have been reported with intermediate morphology from different old black walnut plantations. Due to the lacking information, a preliminary study was conducted in order to prove the difference of these selected trees and to test the hybrid state. For this purpose, DNA fingerprinting was used by applying 13 simple sequence repeat (SSR) markers for the identification of 22 hybrid genotypes selected from one study plot. A comparative analysis with a reference sample set of the ‘parental’ species and other known hybrids was performed as well. The genetic analysis resulted distinct, unique genotypes for all of the samples. Based on the genetic pattern, the analyzed hybrid group was clearly distinguishable from the other two walnut groups. The result of this study also highlights the hidden potential in walnut breeding in the Central European region. Future concepts concerning hybrid walnut utilization in plantation forestry, agroforestry or as breeding material are also discussed.

Keywords: hybrid walnut; *Juglans* × *intermedia*; SSR fingerprint; walnut breeding



Citation: Cseke, K.; Bujdosó, G.; Báder, M.; Mertl, T.; Benke, A.; Kámpel, J.D. Genetic Identification of Hybrid Walnuts (*Juglans* × *intermedia* Carr.) in Hungary, the Hidden Potential for Future Breeding. *Sustainability* **2022**, *14*, 4782. <https://doi.org/10.3390/su14084782>

Academic Editor: Svein Øivind Solberg

Received: 31 March 2022

Accepted: 14 April 2022

Published: 16 April 2022

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The genus *Juglans* is composed of four sections with 21 species worldwide, and comprises one of the most important nut species, *Juglans regia* L. (English or Persian or common walnut) [1]. Besides their definitely highest importance as nut fruit genetic resources, *Juglans* species also provide valuable timber, with the North-American Eastern black walnut (*Juglans nigra* L.) having the highest rank from this aspect. *Juglans nigra* has a central importance, especially in Europe, as an exotic tree species for highly valuable timber production, due to the superior wood quality and its remarkable light to dark brown colour with lilac traces [2]. Interestingly, black walnut also exhibits several varieties for nut production; however, it is considered only locally important in some parts of the US [3,4].

Hybridization is a relatively common phenomenon among *Juglans* species in the same section, and a rather rare one among species from different sections [5]. The question of hybridization is a key topic not only in the conventional walnut breeding [6], but also from the point of view of conservation biology strategies, as it is the case for the North-American butternut, *Juglans cinerea* L., for instance [7]. Furthermore, there is a well-defined group of hybrid walnuts that hold great potential for future walnut management in a broader context, namely the hybrids between the different North-American black walnut species

and common walnut. There is a long tradition of interspecific cross-breeding of walnuts in the US that originates from the aim of improving the nut quality of the local edible black walnut selections with soft-shelled nuts. The most famous American hybrids are probably from the Burbank's 'Paradox' group, a series of combination of *J. hindsii* × *J. regia* crosses [8]. Nowadays, this name refers to any black walnut–common walnut combination in a broader sense with outstanding performance, which are used as rootstocks for common walnut scions [9]. Even clonally propagated clones are available for this purpose, like the *J. hindsii* × *J. regia* 'Vlach' and 'VX211', or the *J. microcarpa* × *J. regia* 'RX1' clone, with moderate to high resistance against *Phytophthora*, crown gall disease (*Agrobacterium*) and root lesion nematodes [10], combined with remarkable drought tolerance in the case of 'RX1' [11].

In Europe, the so-called hybrid walnut (*Juglans* × *intermedia* Carr.), derived from a *J. nigra* and *J. regia* cross, has reached the best reputation due to the higher growth rate (better timber production), better resistance against biological diseases, and its good cold-resistance compared to *J. regia* [4]. Hybrids can arise spontaneously where individuals of the two species occur together; however, it is not a common phenomenon due to time-shift differences of the species' phenology. The exploration of appropriate hybridogenic partners for a high ratio hybridization was reported to be essential in the Mediterranean region, with predominantly the combination of a female *J. nigra* and a pollinator *J. regia* [12]. Some others are spontaneous hybrids, such as IRTA X-80 described in Spain [13], and the several hybrid saplings discovered in a park in Bressanvido, Italy [14]. On the other hand, directional crosses were conducted to obtain new hybrids with the appropriate 'partners' in the frame of several breeding projects, not only in the US, but also in France and Spain [15]. The well-known French hybrids, NG38 × RA, NG23 × RA and MJ209 × RA (*J. major* × *J. regia*) are the most frequently used hybrid walnuts in Europe, propagated vegetatively with in vitro micropropagation or produced in seed orchards as full-sib progenies [16]. Interestingly, while in the US the main aim of hybrids is the superior rootstock development, in Europe, walnut hybrids are used predominantly in plantations for high quality timber production. All the aforementioned hybrids have been reported to show higher vigor and remarkably fast growth combined with higher hardiness and resistance in general [15]. These unique features are probably due to a 'hybrid vigor' that could be obtained with interspecific crosses, similarly as in the case of hybrid poplars [17].

Nevertheless, in Central and Eastern Europe, the interspecific walnut hybrids are almost unknown. There are stochastic observations in black walnut stands about interesting individuals with an intermediate morphology towards the common walnut [18], but most of the information is built around personal communication. On the other hand, only in Hungary, several 'hybrid-like' trees have been reported recently after a quick enquiry from different parts of the country. A draft conclusion has been made that the spontaneous occurrence of these hybrids appears to be more frequent in this part of Europe than it was previously reported from the Western Mediterranean countries. As no detailed inventories are available about the putative hybridization in the region, and compared to black walnut, the use of the registered French hybrids is also untypical in plantations in this part of Europe, a first attempt was made to discover these hidden but rather apparent potentials in the Hungarian forests.

For this purpose, a preliminary study was conducted on the most interesting plot in Hungary, where 27 trees were observed with intermediate morphology within one hectare. After the first field assessment, the observed 27 putative hybrid trees with intermediate morphology gave almost 50% of the whole walnut stand on the study plot. The transitional characteristics between the typical black walnut and common walnut features were apparent in the case of the bark, twigs and the compound leaves. A preliminary hypothesis has been suggested that putative hybrids are originated from one (or a few) *Juglans nigra* mother tree(s), and were mingled with other *J. nigra* half-sibs. The main aim of the current analysis is to provide satisfactory evidence for the hybrid stage of the recognized trees. For this purpose, DNA fingerprinting was used applying simple sequence repeat (SSR)

markers, as this method proved to be a reliable tool for the identification of hybrids and the ‘parental’ species [14,19]. Moreover, a recommendation regarding the future application of the selected superior hybrid genotypes will be discussed.

2. Materials and Methods

The sample plot is approx. 1 hectare and located in north-west Hungary in a privately owned forest (47.36141267 N, 16.75932137 E, 182 m a.s.l.), near the Ablánc brook in a riparian mixed forest with partly planted origin. Regarding the tree composition, the forest compartment consists of a considerably high ratio of common ash (*Fraxinus excelsior* L., 38%) and pedunculate oak (*Quercus robur* L., 21%), mixed with hornbeam (*Carpinus betulus* L., 15%), sycamore (*Acer pseudoplatanus* L., 9%), Turkey oak (*Quercus cerris* L., 9%) and black walnut (*Juglans nigra* L., 2%), the latter standing within one block. The average age of the forest compartment is 67 years, the closure is 92%. The average height of black walnuts is 23 m, and the average diameter at breast height (DBH) is 29 cm based on the National Forestry Database. The black walnut block is close to its planned rotation age of 80 years.

Twenty-seven putative hybrids (trees with intermediate morphology) were signed individually and sampled for a leaf morphology description and a DNA-test. Plant material was collected in July 2021. The composed leaves were scanned and photo-documented, and the leaves for the DNA analysis were put in a deep freezer, at a temperature of $-20\text{ }^{\circ}\text{C}$. Bark characteristics were also checked (Figure A1 in Appendix A). Diameters of the putative hybrids were individually measured, as well as eight black walnut trees selected randomly from the plot as part of a broader stand assessment.

For the DNA analysis, the same method was used as previously for fingerprinting the common walnut assortment in Hungary [20], based on the original protocols [21–23]. In this study, 13 SSR markers were arranged in three marker sets as follows: set1: WGA89, WGA276, WGA202, WGA9, WGA69; set2: WGA72, JR 6160, WGA27, JR 1817, WGA 118; and set3: WGA01, WGA04, WGA321, WGA331, WGA349. For the comparative analysis, besides 28 hybrid genotypes (22 selected from the study plot, two other putative hybrids from Hungary and four known hybrid cultivars), the reference sample set of the two original species was included with 22 *Juglans regia* and 10 *Juglans nigra* samples (Table 1). Data analysis was conducted by GenAlEx 6.5, calculating the main marker indices, P_{ID} , the genetic distance matrix, PCoA and AMOVA [24,25]. Moreover, an UPGMA dendrogram was constructed based on the genetic distance matrix by PAST [26], and STRUCTURE analysis was conducted with the following settings: 200,000 burn-in period, 500,000 MCMC replicates, estimating K from 1 to 6 with 10 repetitions using the admixture model and correlated allele frequencies [27]. STRUCTURE Harvester was used to decide the most likely K with the plateau criterion by Prichard et al. [28], and the deltaK method described by Evanno et al. [29].

Table 1. List of the analyzed *Juglans regia*, *Juglans nigra* and *Juglans* × *intermedia* samples.

Nb.	Sample ID	Taxon	Clonal Name/Information	Region	Origin
<i>Juglans regia</i> sample set					
1	JR[A117]	<i>Juglans regia</i>	Alsószentiváni 117	Hungary	local selection
2	JR[M10]	<i>Juglans regia</i>	Milotai 10	Hungary	local selection
3	JR[T83]	<i>Juglans regia</i>	Tiszacsécsi 83	Hungary	local selection
4	JR[M_intenziv]	<i>Juglans regia</i>	Milotai intenzív (Milotai 10 × Pedro)	Hungary	from cross breeding
5	JR[Bonifac]	<i>Juglans regia</i>	Bonifác (Alsószentiváni 117 × Pedro)	Hungary	from cross breeding
6	JR[A_kesei]	<i>Juglans regia</i>	Alsószentiváni kései (A 117 × Pedro)	Hungary	from cross breeding
7	JR[BD6]	<i>Juglans regia</i>	Érdiő1 (syn.: BD6)	Hungary	from cross breeding
8	JR[EII_Fertod]	<i>Juglans regia</i>	Esterhazy II	Hungary	local selection
9	JR[Szentbalázsi]	<i>Juglans regia</i>	Szentbalázsi	Hungary	local selection
10	JR[Pladany10]	<i>Juglans regia</i>	Püspökladány10	Hungary	local selection
11	JR[Franquette]	<i>Juglans regia</i>	Franquette	France	local selection
12	JR[Corne]	<i>Juglans regia</i>	Corne	France	local selection

Table 1. Cont.

Nb.	Sample ID	Taxon	Clonal Name/Information	Region	Origin
13	JR[Parisienne]	<i>Juglans regia</i>	Parisienne	France	local selection
14	JR[Mayette]	<i>Juglans regia</i>	Mayette	France	local selection
15	JR[Hartley]	<i>Juglans regia</i>	Hartley	US	local selection
16	JR[Tehama]	<i>Juglans regia</i>	Tehama (Waterloo × Payne)	US	from cross breeding
17	JR[Waterloo]	<i>Juglans regia</i>	Waterloo	US	local selection
18	JR[Adams]	<i>Juglans regia</i>	Adams	US	local selection
19	JR[Serr]	<i>Juglans regia</i>	Serr (Payne × PI 159568)	US	from cross breeding
20	JR[Chandler]	<i>Juglans regia</i>	Chandler (Pedro × UC 56-224)	US	from cross breeding
21	JR[Pedro]	<i>Juglans regia</i>	Pedro (Conway-Mayette × Payne)	US	from cross breeding
22	JR[Caucasus3]	<i>Juglans regia</i>	seed origin	Caucasus	local selection
<i>Juglans nigra</i> sample set					
23	JN[Tata]	<i>Juglans nigra</i>	Tata—forest	Hungary	introduced, planted forest
24	JN[Csepreg1]	<i>Juglans nigra</i>	Csepreg—study site	Hungary	introduced, planted forest
25	JN[Csepreg2]	<i>Juglans nigra</i>	Csepreg—study site	Hungary	introduced, planted forest
26	JN[Csepreg3]	<i>Juglans nigra</i>	Csepreg—study site	Hungary	introduced, planted forest
27	JN[Csepreg4]	<i>Juglans nigra</i>	Csepreg—study site	Hungary	introduced, planted forest
28	JN[Csepreg5]	<i>Juglans nigra</i>	Csepreg—study site	Hungary	introduced, planted forest
29	JN[Csepreg6]	<i>Juglans nigra</i>	Csepreg—study site	Hungary	introduced, planted forest
30	JN[Csepreg7]	<i>Juglans nigra</i>	Csepreg—study site	Hungary	introduced, planted forest
31	JN[Csepreg8]	<i>Juglans nigra</i>	Csepreg—study site	Hungary	introduced, planted forest
32	JN[Érd]	<i>Juglans nigra</i>	Érd (MATE Fruit Research Institute)	Hungary	introduced, gene collection
<i>Juglans × intermedia</i> sample set					
33	HYB[Csepreg1]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
34	HYB[Csepreg3]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
35	HYB[Csepreg4]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
36	HYB[Csepreg6]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
37	HYB[Csepreg7]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
38	HYB[Csepreg9]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
39	HYB[Csepreg10]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
40	HYB[Csepreg11]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
41	HYB[Csepreg12]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
42	HYB[Csepreg13]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
43	HYB[Csepreg14]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
44	HYB[Csepreg15]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
45	HYB[Csepreg16]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
46	HYB[Csepreg18]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
47	HYB[Csepreg19]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
48	HYB[Csepreg20]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
49	HYB[Csepreg21]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
50	HYB[Csepreg22]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
51	HYB[Csepreg23]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
52	HYB[Csepreg24]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
53	HYB[Csepreg25]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
54	HYB[Csepreg27]	<i>Juglans × intermedia</i>	Csepreg—study site	Hungary	planted forest
55	HYB[Kámon]	<i>Juglans × intermedia</i>	Szombathely (Kámon Botanical Garden)	Hungary	introduced, origin not known
56	HYB[Röjtök2]	<i>Juglans × intermedia</i>	Röjtökmuzsaj—forest	Hungary	planted forest
57	HYB[NG38×RA]	<i>Juglans × intermedia</i>	NG38 × RA	France	commercially available clone, forestry
58	HYB[MJ209×RA]	<i>J. major</i> × <i>J. regia</i>	MJ209 × RA	France	commercially available clone, forestry
59	HYB[NG23×RA]	<i>Juglans × intermedia</i>	NG23 × RA	France	commercially available clone, forestry
60	HYB[VLACH]	<i>J. hindsii</i> × <i>J. regia</i>	Vlach	US	commercially available clone, rootstock

3. Results

The applied 13 markers were all polymorphic, and it can be concluded that the method was appropriate for genetic fingerprinting as the P_{ID} value (in other words, the probability of random matching) was very low (1.1×10^{-9} for the JR (*J. regia*) group, 7.5×10^{-15} for the JN (*J. nigra*) group, and 4.4×10^{-14} for the HYB (*J. × intermedia*) group). The main genetic indices are presented in Table 2. The genetic analysis resulted in distinct, unique genotypes for all samples. Based on the allelic parameters, the highest genetic diversity can be seen in the HYB and the JN groups, while the analyzed JR sample set shows lower

variability. Private alleles occur in all the three groups, including the hybrid group, with a moderately high number.

Table 2. Main genetic parameters of the applied simple sequence repeat (SSR) markers in the three analyzed sample sets (where N: sample number, N_a : allele number, N_e : effective allele number, N_p : private allele number, H_o : observed heterozygosity, H_e : expected heterozygosity, F: fixation index).

<i>Juglans regia</i> samples (JR)							
Locus	N	N_a	N_e	N_p	H_o	H_e	F
WGA89	22	4	2.367	0	0.682	0.577	−0.181
WGA276	21	8	5.040	5	0.524	0.802	0.347
WGA118	22	5	2.839	1	0.500	0.648	0.228
WGA202	22	12	3.967	5	0.545	0.748	0.271
WGA72	22	3	1.501	0	0.318	0.334	0.046
JR 6160	22	4	1.686	0	0.227	0.407	0.442
WGA27	22	4	2.185	2	0.500	0.542	0.078
JR 1817	22	2	1.198	0	0.182	0.165	−0.100
WGA09	21	5	3.486	2	0.714	0.713	−0.002
WGA01	22	7	3.113	1	0.500	0.679	0.263
WGA04	22	2	1.996	0	0.500	0.499	−0.002
WGA321	22	6	4.632	0	0.818	0.784	−0.043
WGA331	22	4	3.073	1	0.636	0.675	0.057
Mean	21.846	5.077	2.852	1.308	0.511	0.583	0.108
SE	0.104	0.755	0.331	0.479	0.051	0.053	0.052
<i>Juglans nigra</i> samples (JN)							
Locus	N	N_a	N_e	N_p	H_o	H_e	F
WGA89	10	10	6.667	4	0.900	0.850	−0.059
WGA276	10	10	7.143	5	0.700	0.860	0.186
WGA118	10	5	2.985	3	1.000	0.665	−0.504
WGA202	10	8	3.922	6	0.500	0.745	0.329
WGA72	10	5	2.703	2	0.800	0.630	−0.270
JR 6160	10	6	4.444	2	0.900	0.775	−0.161
WGA27	10	6	4.444	3	0.600	0.775	0.226
JR 1817	10	6	4.348	3	0.800	0.770	−0.039
WGA09	10	7	4.545	2	0.700	0.780	0.103
WGA01	10	7	4.762	1	0.700	0.790	0.114
WGA04	10	6	3.846	4	0.700	0.740	0.054
WGA321	10	7	4.545	0	0.600	0.780	0.231
WGA331	10	6	5.128	4	0.700	0.805	0.130
Mean	10.000	6.846	4.576	3.000	0.738	0.767	0.026
SE	0.000	0.451	0.343	0.435	0.038	0.018	0.064
<i>Juglans × intermedia</i> samples (HYB)							
Locus	N	N_a	N_e	N_p	H_o	H_e	F
WGA89	28	10	6.297	1	0.893	0.841	−0.061
WGA276	27	11	5.098	3	0.852	0.804	−0.060
WGA118	28	11	3.751	5	0.964	0.733	−0.315
WGA202	28	10	3.910	2	0.929	0.744	−0.248
WGA72	27	6	2.899	1	0.963	0.655	−0.470
JR 6160	28	6	4.532	1	0.964	0.779	−0.237
WGA27	28	6	3.033	1	0.250	0.670	0.627
JR 1817	28	8	3.233	3	1.000	0.691	−0.448
WGA09	28	8	5.352	2	0.964	0.813	−0.186
WGA01	28	8	5.765	0	0.929	0.827	−0.123
WGA04	28	7	4.238	3	1.000	0.764	−0.309
WGA321	27	9	5.461	2	0.741	0.817	0.093
WGA331	28	9	1.997	4	0.357	0.499	0.285
Mean	27.769	8.385	4.274	2.154	0.831	0.741	−0.112
SE	0.122	0.500	0.356	0.374	0.068	0.026	0.084

To prove the distinctiveness of the two species groups and the hybrids, the molecular variance was checked by AMOVA, as well. AMOVA proved that 19% of the total variance came from the variance among groups (with $p = 0.0001$ confidence interval and based on standard permutation after 9999 repetitions). The pairwise F_{st} values, representing the ratio of molecular variance among groups, are the following: 0.292 between the *J. regia* and *J. nigra* groups; 0.184 between the hybrids and the *J. regia* group; and 0.113 between the hybrids and the *J. nigra* group.

For the visualization of the genetic relatedness of the analyzed samples, a principal coordinate analysis (PCoA) was conducted based on the genetic distance matrix. While the two parental species' groups are completely separated, putative hybrids are situated between them, but closer to the black walnut group (Figure 1). The UPGMA dendrogram supported this grouping, common walnut samples separated within one distinct cluster, while black walnut samples in another cluster together with a subgroup of the putative hybrids selected from the study plot (Figure A2 in Appendix A). On the other hand, the four known hybrid clones, as well as one included hybrid genotype, found also in Hungary, are grouped with the common walnut samples in this analysis, indicating a higher genetic relatedness between these groups by the clustering approach.

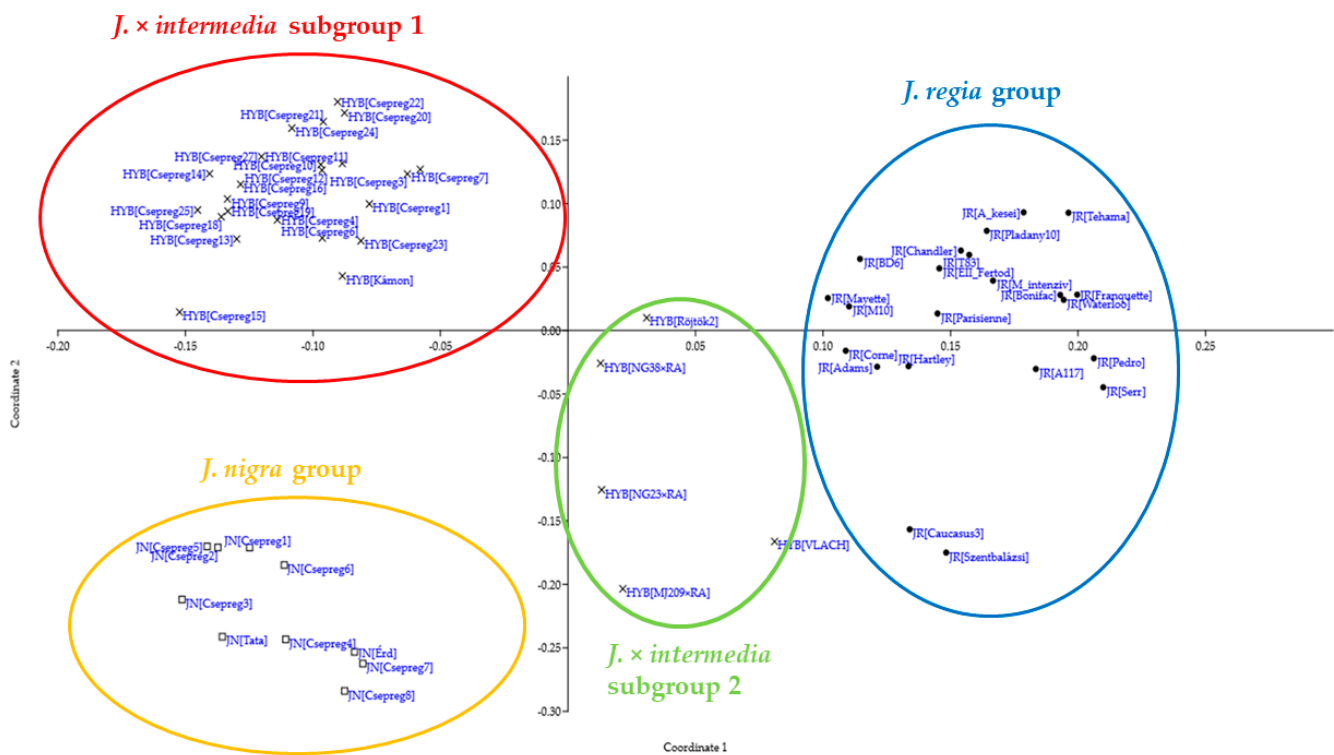


Figure 1. Result of the principal coordinate analysis (PoCA). The different groups of samples are signed with the following colors: blue—*J. regia*, yellow—*J. nigra*, red—*J. × intermedia* subgroup 1 (from the study plot), green—other hybrids, subgroup 2.

Based on the STRUCTURE analysis, the existence of four clusters has the highest probability based on the mean of the estimated Ln probabilities, while the Evanno method also supports the existence of $K = 4$ clusters (Figure 2, for the supported deltaK see Figure A3 in Appendix A). Interestingly, the separation of the analyzed groups is apparent, where the hybrids also form two distinct, unique clusters. The $K = 4$ pattern refers partly to a sub-clustering within the *Juglans nigra* group as well.

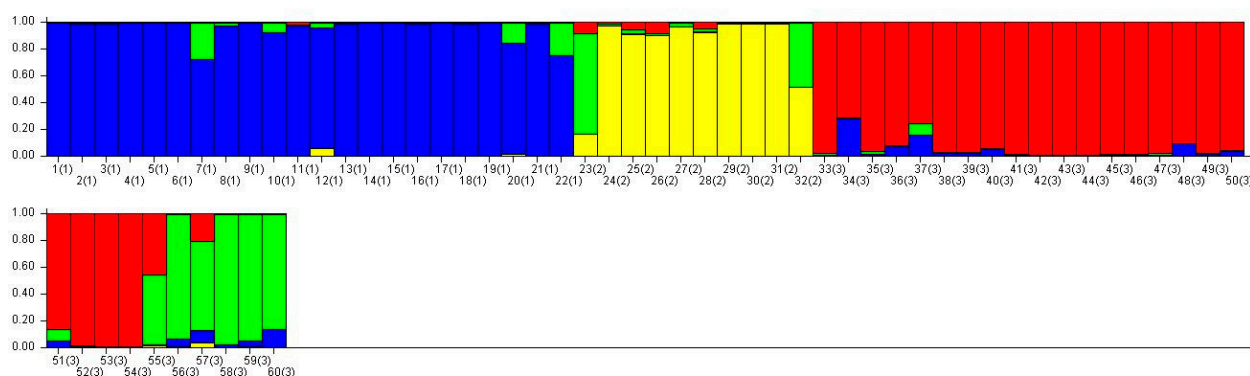


Figure 2. Result of the STRUCTURE analysis, with the most probable $K = 4$ subgroups (where numbers in brackets (1) refer to the samples of the *Juglans regia* group, (2) to *Juglans nigra*, and (3) to *J. × intermedia*).

4. Discussion

The hint of a former hybridization event was detected and studied in an old black walnut stand in Hungary. Twenty-two putative hybrid trees with intermediate morphology were selected for deeper investigation. The selected hybrids represent half of the walnut stand, and 18 trees show even superior diameter growth and height compared with the pure black walnut individuals. Out of these, four outstanding trees with DBH above 45 cm were designated for tree breeding purposes as selected plus trees. A detailed forestry assessment is in progress, with a complex survey of tree height, crown structure and trunk quality. Observations of phenological traits (leafing period, flowering features, fruitification, etc.) have been started as well, to provide a full description of the selected plus trees.

As the spontaneous occurrence of hybrids in such a high number is not a common phenomenon, in this preliminary study, a genetic analysis was conducted to confirm the hybrid state of the observed trees. Based on the genetic analysis, it is clearly visible that the two parental species' groups are completely separated, while putative hybrids sampled from the study site form a distinct group. The applied two ordination methods (PCoA, UPGMA clustering) divide the analyzed hybrids into two subgroups. Both approaches set the study plot's hybrid subgroup (with one additional hybrid from Kámon, Hungary) closer to the black walnut group, while the subgroup of the other hybrids (including the known hybrid cultivars) is located between the parental species (in the case of PCoA), or even closer to the *J. regia* group (based on the UPGMA).

On the other hand, the Bayesian analysis by STRUCTURE shows a different genetic pattern along the analyzed data set, where the hybrids of the study site form a completely distinct group as if the samples of a third species had been evaluated. On the contrary, the genotypes of the reference hybrids follow a different pattern. From the latter result, the question would arise whether the putative hybrids are real hybrids at all, or if they are rather the progenies of a third *Juglans* species. As the origin of the propagation material is unknown, this scenario should also be considered. The use of full- or half-sib progenies for forestation was not uncommon in Hungary, especially for exotic tree species—mainly conifers, but also in some rare indigenous broadleaved species—where the propagation material was not available. In these cases, seed collection from solitary trees in parks or botanical gardens used to be a general practice in forestry. In Hungary, two other walnut species can be found besides *J. regia* and *J. nigra*. One is the North American butternut (*J. cinerea* L.), which belongs to the *Cardiocaryon* section. Another possible species would be the Manchurian walnut (*Juglans mandshurica* Maxim.) also from the *Cardiocaryon* section, but from Asia. This walnut species occupies the most northern area and is a very frost resistant type [4,6]. Both mentioned *Juglans* species are quite rare in Hungary, they can be found mainly individually in botanical gardens or dendrological collections. It is not unlikely, however, that *J. mandshurica* individuals were used in forestry test experiments between the 1950s and 1980s in Hungary, originated from the territory of the former Soviet Union.

Nevertheless, no descriptions or notes are available proving this hypothesis. Morphological characteristics of the selected trees are also not congruent with the ones of butternut and Manchurian walnut. The typical large leaves with pubescent hairiness and the brownish and hairy twigs are missing, as well as the very emblematic ‘moustache’ above the leaf scar in the case of butternut. Being a hybrid of one of these mentioned species is again not very likely. Butternut forms hybrids only within its own section, and not with the black walnut or common walnut. Manchurian walnut hybridization may occur with common walnut, but the morphological features are still lacking. For these reasons, it can be concluded that the origin of the analyzed hybrids is rather the one that was proposed previously. However, this first STRUCTURE result can be verified in a future analysis with a broader sample set from various resources of different *Juglans* species, or with different approaches, such as nuclear barcoding markers or even biochemical profiling [30–32].

Nevertheless, the applied fingerprinting method was suitable to prove the uniqueness of untypical trees in the black walnut stand. One certain result of the study is the unrelatedness of the observed hybrid trees and the black walnuts on the study site. The sampled hybrids and black walnut trees formed two distinct groups with different allele compositions, and even private alleles occurred in both groups in a moderately high number. The preliminary hypothesis, that putative hybrids are originated from one (or a few) *Juglans nigra* mother tree(s) and were mingled with other *J. nigra* half-sibs, can certainly be excluded. The applied fingerprinting method proved to be a reliable tool for identifying half- or full-sibs based on the shared allele content. On the other hand, field observations with morphology descriptions can provide a reliable and trustable result as well. The random sampling of a few selected individuals could be a timesaving and less expensive approach for the future.

The main goal of the present study was to highlight the existence of spontaneous hybrids in older black walnut stands in the Central European forests, even if the origin of the seed lot is not evident. These hybrids may represent a considerably high economic value due to the increased growth and timber production, combined with better hardiness and resistance, as it has already been recognized for the NG38 × RA, NG23 × RA and MJ209 × RA hybrid walnut clones in the Mediterranean region. However, these well-known hybrid varieties have not yet been tested in the eastern and rather continental part of Europe, and the chance of a lower tolerance towards continental environmental conditions can be predicted. The fact that the locally adapted genetic resources are given a high priority in various national common walnut breeding programmes would also point towards this concept [33–35]. From this aspect, the selection of local hybrids, originated from local resources, can be a key question, as the *J. regia* trees acting as pollinator in the hybridization process represent a quite different race (Carpathian race) compared to the members of the Atlantic–Mediterranean French race [36]. Nevertheless, this hypothesis should still be proved, and for this purpose, the establishment of common garden trials is needed besides the genetic analysis.

We can specify at least three aims for hybrid walnut selection: timber production, multi-purpose usage in agroforestry, or as future breeding material. The most straightforward option is the use of high-quality timber, similarly to the other hybrid cultivars. The most expensive, but also the most profitable option would be the intensive clonal plantation for timber production purposes applying vegetatively propagated hybrid genotypes. A more affordable solution for forestry application could be the use of hybrids with generative origin, or mixing the expensive vegetative plating material with seedlings of walnut or other noble hardwood species. From the aspect of timber production, the low fertility or even sterility of the hybrids would be preferred, as trees could allocate all their resources for vegetative development. An additional benefit of sterility would be the blocked invasiveness, promoting the acceptance and use of such non-native, clonal material in vulnerable riparian forests.

Another potential option is agroforestry and the multi-purpose usage, provided hybrids are fertile and produce edible nuts. Even if the European consumption is focusing

mainly on the common walnut, hybrid nuts could fill in a niche and help the diversification of the nut market. The recent invasion of the Walnut husk fly (*Rhagoletis completa* Cresson) [37] is again an emerging and serious problem that would characterize future breeding concepts with the application of less sensitive genotypes of other *Juglans* species through directional crosses.

Finally, considering that the locally selected hybrids as potential genetic resources for future breeding is a less defined field of usage, manifold aspects can be included within it. We have already referred to the walnut husk fly damage and a possible solution using new genotypes with less sensitivity. Another important field of improvement is rootstock breeding. In fact, this is one of the main pillars of plantation management and is not highlighted enough in our region. Furthermore, from the ongoing climate change point of view, the test of various genetic resources with higher resistance and hardiness, and the selection of locally adapted genotypes, would have more and more importance not only in fruit breeding, but also in forestry [38,39]. All this already existing knowledge should be built into a future breeding plan focusing on the changes we are facing locally or worldwide.

Author Contributions: Conceptualization, K.C. and J.D.K.; methodology, K.C. and A.B.; investigation, K.C.; resources, K.C., J.D.K., G.B., M.B., T.M. and A.B.; writing—original draft preparation, K.C.; writing—review and editing, K.C., M.B., T.M., A.B., G.B. and J.D.K.; visualization, K.C., M.B., T.M., A.B., G.B. and J.D.K.; funding acquisition, A.B. All authors have read and agreed to the published version of the manuscript.

Funding: Project no. TKP2021-NKTA-43 has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the TKP2021-NKTA funding scheme.

Acknowledgments: We are grateful, first of all, to the owner of the private forest for the opportunity of conducting the field study and sample analysis. We also thank Denis Vauthier (INRAE Centre de Recherche, Unité expérimentale Entomologie et Forêt Méditerranéenne, Avignon, France) for his valuable comments and the experiences he shared with us during our visit at the *Juglans* field trial in Avignon. Special thanks for Norbert Somogyi, counselor of the Hungarian Embassy in France, for promoting the Hungarian walnut breeding and research abroad. Finally, special thanks also for Paola Pollegioni (Research Institute on Terrestrial Ecosystems, National Research Council, Porano, Italy), who kindly gave an insight into the topic several years ago.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

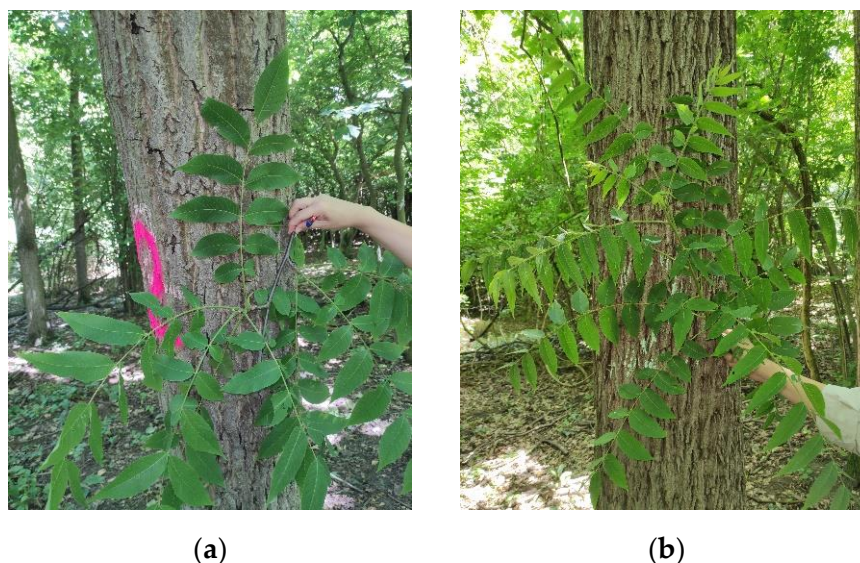


Figure A1. Cont.

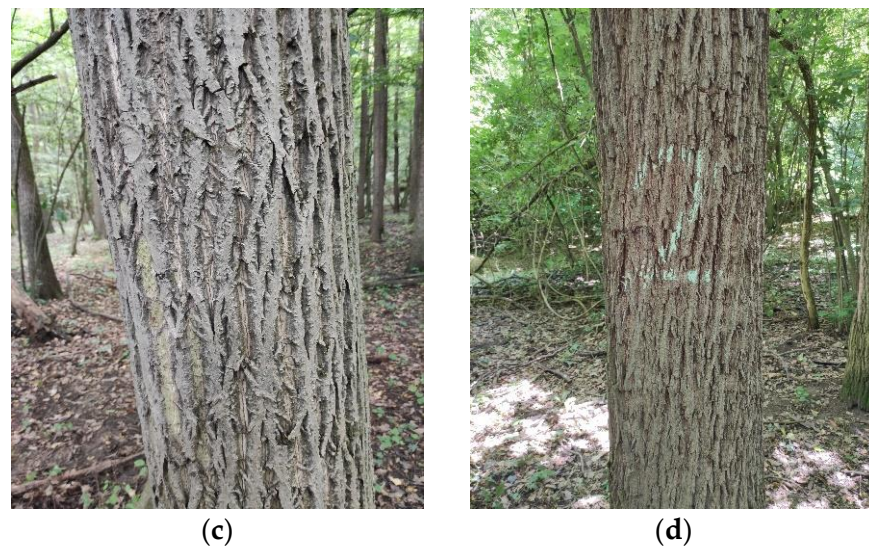


Figure A1. Intermediate morphology of the hybrid walnut (*Juglans × intermedia*) compared with the black walnut (*Juglans nigra*). (a) compound leaf of the hybrid walnut; (b) compound leaf of the black walnut; (c) bark of the hybrid walnut; (d) bark of the black walnut.

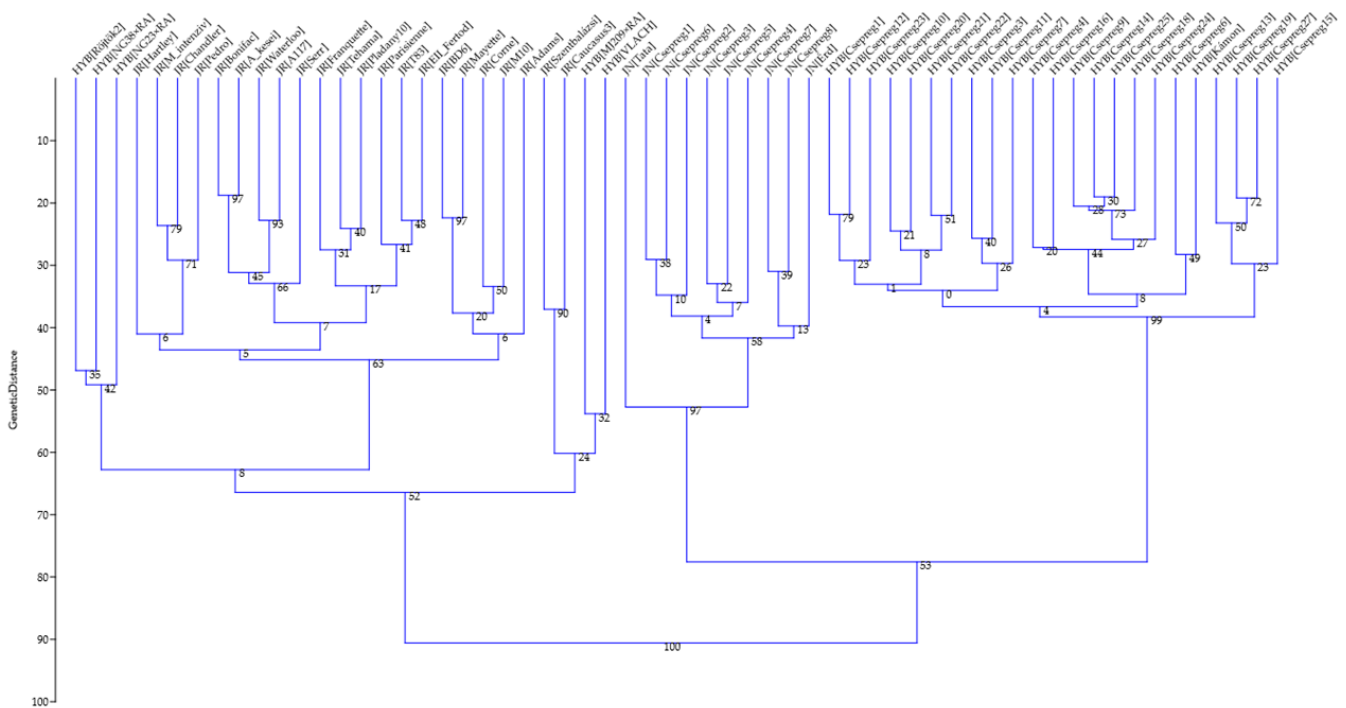


Figure A2. UPGMA (unweighted pair group method with arithmetic mean) dendrogram of the analyzed walnut samples (where JR refers to *Juglans regia*, JN—*Juglans nigra*, HYB—*J. × intermedia*).

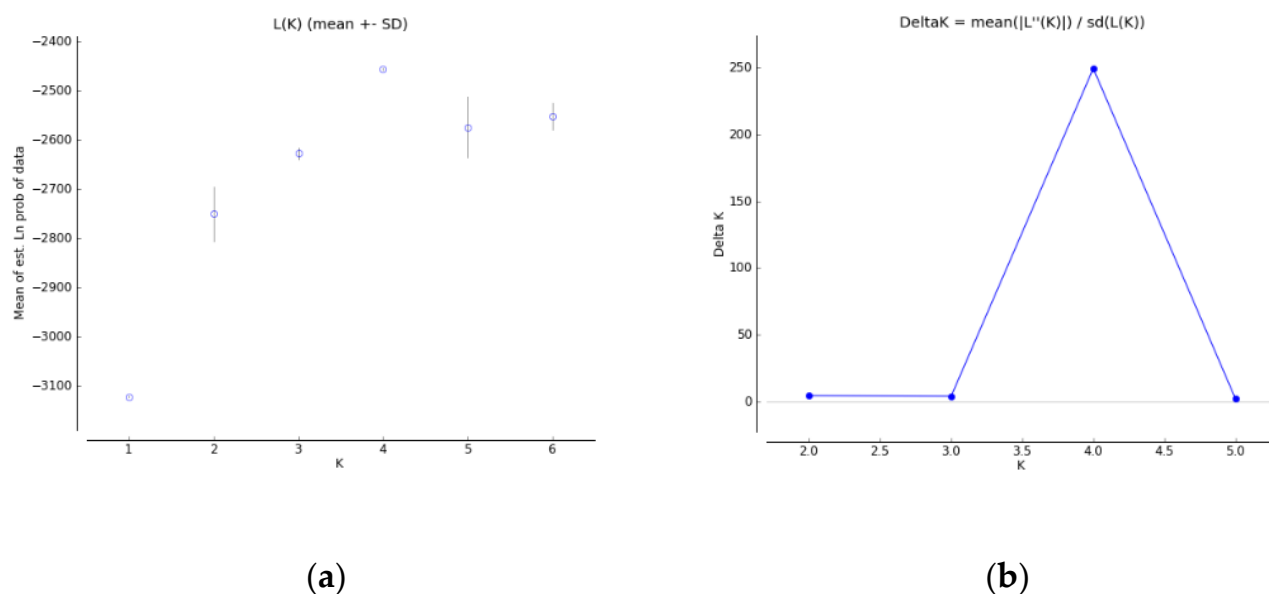


Figure A3. The supported K = 4 cluster scheme from the STRUCTURE–Harvester analysis, based on (a) the mean of the estimated Ln probabilities; and (b) the Evanno method calculating the deltaK.

References

- Manning, W.E. The Classification Within the Juglandaceae. *Ann. Mo. Bot. Gard.* **1978**, *65*, 1058–1087. [\[CrossRef\]](#)
- Nicolescu, V.-N.; Rédei, K.; Vor, T.; Bastien, J.-C.; Brus, R.; Benčať, T.; Đodan, M.; Cvjetkovic, B.; Andrašev, S.; La Porta, N.; et al. A review of black walnut (*Juglans nigra* L.) ecology and management in Europe. *Trees* **2020**, *34*, 1087–1112. [\[CrossRef\]](#)
- Zhao, P.; Zhou, H.; Coggeshall, M.V.; Reid, B.; Woeste, K.E. Discrimination and assessment of black walnut (*Juglans nigra* L.) cultivars using phenology and microsatellite markers (SSRs). *Can. J. Plant Sci.* **2018**, *98*, 616–627. [\[CrossRef\]](#)
- Woeste, K.; Michler, C. *Juglans*. In *Wild Crop Relatives: Genomic and Breeding Resources*; Kole, C., Ed.; Springer: Berlin/Heidelberg, Germany, 2011; pp. 77–88. [\[CrossRef\]](#)
- Williams, R.D. *Juglans nigra* L. black walnut. In *Silvics of North America, Hardwoods*; Burns, R.M., Honkala, B.H., Eds.; Technical Coordinators; Agric Handbook 654; U.S. Department of Agriculture, Forest Service: Washington, DC, USA, 1990; Volume 2, pp. 391–399. Available online: https://www.srs.fs.usda.gov/pubs/misc/ag_654_vol2.pdf (accessed on 25 March 2022).
- Bernard, A.; Lheureux, F.; Dirlwanger, E. Walnut: Past and future of genetic improvement. *Tree Genet. Genomes* **2018**, *14*, 1. [\[CrossRef\]](#)
- Farlee, L.; Mckenna, J.; Ostry, M.; Woeste, K.; Weeks, S. *Identification of Butternut and Butternut Hybrids FNR-420-W*; Purdue University Cooperative Extension Service: West Lafayette, IN, USA, 2010.
- Preece, J.; Mcgranahan, G. Luther Burbanks Contributions to Walnuts. *HortScience* **2015**, *50*, 201–204. [\[CrossRef\]](#)
- Potter, D.; Gao, F.; Baggett, S.; McKenna, J.R.; McGranahan, G.H. Defining the sources of Paradox: DNA sequence markers for North American walnut (*Juglans* L.) species and hybrids. *Sci. Hortic.* **2002**, *94*, 157–170. [\[CrossRef\]](#)
- Hasey, J. Selecting the Right Clonal Rootstock for Managing Soil and Pest Problems. University of California Agriculture and Natural Resources. 2016. Available online: <http://www.sacvalleyorchards.com/blog/walnuts-blog/selecting-the-right-clonal-rootstock-for-managing-soil-and-pest-problems/> (accessed on 25 March 2022).
- Knipfer, T.; Reyes, C.; Momayyezi, M.; Brown, P.J.; Kluepfel, D.; McElrone, A.J. A comparative study on physiological responses to drought in walnut genotypes (RX1, Vlach, VX211) commercially available as rootstocks. *Trees* **2020**, *34*, 665–678. [\[CrossRef\]](#)
- Pollegioni, P.; Woeste, K.; Mugnozza, G.S.; Malvolti, M.E. Retrospective identification of hybridogenic walnut plants by SSR fingerprinting and parentage analysis. *Mol. Breed.* **2009**, *24*, 321–335. [\[CrossRef\]](#)
- Aletà, N. Current research in Spain on walnut for wood production. In *Black Walnut in a New Century, Proceedings of the 6th Walnut Council Research Symposium, Lafayette, IN, USA, 25–28 July 2004*; Michler, C.H., Pijut, P.M., Van Sambeek, J.W., Coggeshall, M.V., Seifert, J., Woeste, K., Overton, R., Ponder, F., Jr., Eds.; Gen. Tech. Rep. NC-243; U.S. Department of Agriculture, Forest Service, North Central Research Station: St. Paul, MN, USA, 2004; 188p.
- Pollegioni, P.; Woeste, K.; Major, A.; Mugnozza, G.S.; Malvolti, M.E. Characterization of *Juglans nigra* (L.), *Juglans regia* (L.) and *Juglans x intermedia* (Carr.) by SSR markers: A case study in Italy. *Silvae Genet.* **2009**, *58*, 68–78. [\[CrossRef\]](#)
- Fady, B.; Ducci, F.; Aletà, N.; Becquey, J.; Vazquez, R.D.; Lopez, F.F.; Jay-Allemand, C.; Lefèvre, F.; Ninot, A.; Panetsos, K.; et al. Walnut demonstrates strong genetic variability for adaptive and wood quality traits in a network of juvenile field tests across Europe. *New For.* **2003**, *25*, 211–225. [\[CrossRef\]](#)

16. Fernández-Moya, J.; Urbán-Martínez, I.; Pelleri, F.; Castro, G.; Bergante, S.; Giorcelli, A.; Gennaro, M.; Licea-Moreno, R.J.; Santacruz Pérez, D.; Gutiérrez-Tejón, E.; et al. *Silvicultural Guide to Managing Walnut Plantations for Timber Production*; 2019; p. 76. ISBN 978-84-09-12163-2. Available online: <http://woodnat.seistaglabs.com/research/index.html> (accessed on 24 March 2022).
17. McKown, A.D.; Guy, R.D. Hybrid vigour—Poplars play it cool. *Tree Physiol.* **2018**, *38*, 785–788. [[CrossRef](#)] [[PubMed](#)]
18. Hřib, M.; Koblížek, J.; Maděra, P. *Juglans × intermedia* Carr.—An interesting finding in the Židlochovice Forest Enterprise. *J. For. Sci.* **2002**, *48*, 475–481. [[CrossRef](#)]
19. Weising, K.; Nybom, H.; Wolff, K.; Kahl, G. *DNA Fingerprinting in Plants: Principles, Methods, and Applications*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2005; p. 472.
20. Bujdosó, G.; Cseke, K. The Persian (English) walnut (*Juglans regia* L.) assortment of Hungary: Nut characteristics and origin. *Sci. Hortic.* **2021**, *283*, 110035. [[CrossRef](#)]
21. Woeste, K.; Burns, R.; Rhodes, O.; Michler, C. Thirty polymorphic nuclear microsatellite loci from black walnut. *J. Hered.* **2002**, *93*, 58–60. [[CrossRef](#)]
22. Dangl, G.S.; Woeste, K.; Aradhya, M.K.; Koehmstedt, A.; Simon, C.; Potter, D.; Leslie, C.A.; McGranahan, G. Characterization of 14 Microsatellite Markers for Genetic Analysis and Cultivar Identification of Walnut. *J. Am. Soc. Hortic. Sci.* **2005**, *130*, 348–354. [[CrossRef](#)]
23. Dang, M.; Zhang, T.; Hu, Y.; Zhou, H.; Woeste, K.; Zhao, P. De Novo Assembly and Characterization of Bud, Leaf and Flowers Transcriptome from *Juglans regia* L. for the Identification and Characterization of New EST-SSRs. *Forests* **2016**, *7*, 247. [[CrossRef](#)]
24. Peakall, R.; Smouse, P.E. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **2006**, *6*, 288–295. [[CrossRef](#)]
25. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update. *Bioinformatics* **2012**, *28*, 2537–2539. [[CrossRef](#)]
26. Hammer, Ø.; Harper, D.A.T.; Ryan, P.D. PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontol. Electron.* **2001**, *1*, 9.
27. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959. [[CrossRef](#)]
28. Earl, D.A.; vonHoldt, B.M. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **2012**, *4*, 359–361. [[CrossRef](#)]
29. Evanno, G.; Regnaut, S.; Goudet, J. Detecting the number of clusters of individuals using the software structure: A simulation study. *Mol. Ecol.* **2005**, *14*, 2611–2620. [[CrossRef](#)] [[PubMed](#)]
30. Suo, Z.; Pei, D.; Ma, Q.; Jin, X. Genetic Formation of Paradox Hybrids (*Juglans* L.) Revealed by nrDNA IGS8-ETS1 Region. *AASRI Procedia* **2012**, *1*, 156–165. [[CrossRef](#)]
31. Dong, W.; Xu, C.; Li, W.; Xie, X.; Lu, Y.; Liu, Y.; Jin, X.; Suo, Z. Phylogenetic Resolution in *Juglans* Based on Complete Chloroplast Genomes and Nuclear DNA Sequences. *Front. Plant Sci.* **2017**, *8*, 1148. [[CrossRef](#)] [[PubMed](#)]
32. Likhanov, A.F.; Burda, R.I.; Koniakin, S.N.; Kozyr, M.S. Identifying species and hybrids in the genus *Juglans* by biochemical profiling of bark. *Mod. Phytomorphol.* **2020**, *14*, 27–34. [[CrossRef](#)]
33. Bernard, A.; Dirlewanger, E.; Lheureux, F. The French walnut improvement program: Preliminary investigations. *Acta Hortic.* **2020**, *1280*, 77–84. [[CrossRef](#)]
34. Akça, Y.; Yuldaşulu, Y.B.; Murad, E.; Vahdati, K. Exploring of Walnut Genetic Resources in Kazakhstan and Evaluation of Promising Selections. *IJHST* **2020**, *2*, 93–102. [[CrossRef](#)]
35. Bujdosó, G.; Izsépi, F.; Bartha, K.S.; Varjas, V.; Szentiványi, P. Persian walnut breeding program at NARIC Fruticulture Research Institute in Hungary. *Acta Hortic.* **2020**, *1280*, 89–94. [[CrossRef](#)]
36. Ebrahimi, A.; Zarei, A.; McKenna, J.R.; Bujdosó, G.; Woeste, K.E. Genetic diversity of Persian walnut (*Juglans regia*) in the cold-temperate zone of the United States and Europe. *Sci. Hortic.* **2017**, *220*, 36–41. [[CrossRef](#)]
37. Augustinos, A.; Moraiti, C.; Drosopoulou, E.; Kounatidis, I.; Mavragani-Tsipidou, P.; Bourtzis, K.; Papadopoulos, N. Old residents and new arrivals of *Rhagoletis* species in Europe. *Bull. Entomol. Res.* **2019**, *109*, 701–712. [[CrossRef](#)]
38. Vahdati, K.; Arab, M.M.; Sarikhani, S.; Sadat-Hosseini, M.; Leslie, C.A.; Brown, P.J. Advances in Persian Walnut (*Juglans regia* L.) Breeding Strategies. In *Advances in Plant Breeding Strategies: Nut and Beverage Crops*; Al-Khayri, J., Jain, S., Johnson, D., Eds.; Springer: Cham, Switzerland, 2019; pp. 401–472. [[CrossRef](#)]
39. Hemery, G.E. *Forest Management and Silvicultural Responses to Predicted Climate Change Impacts on Valuable Broadleaved Species*; Short-Term Scientific Mission Report for Working Group 1, COST Action E42; 2007; p. 73. Available online: https://sylva.org.uk/forestryhorizons/documents/STSM_COST-E42_GabrielHemery_May07_final.pdf (accessed on 24 March 2022).