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Determination of genetic relationship among some walnut (*Juglans regia* L.) genotypes and their early-bearing progenies using RAPD markers

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Abstract

Random amplified polymorphic DNA (RAPD) method was used to study the genetic relationships among eight walnut genotypes grown in Turkey. A total of 45 10-mer primers were tested with the RAPD technique. Thirty-seven primers showed reproducible polymorphic pattern. These primers produced 513 bands, out of which 340 were polymorphic. The molecular size varied between 200 – 5000 bp. The dendrogram realized from the RAPD markers grouped the eight genotypes into three major clusters. The highest similarity was found between NO-2 and NO-8 genotypes (0.779). The cophenetic correlation coefficient between the similarity matrix and the cophenetic matrix of the dendrogram was very high (r = 0.9131), supporting the validity of the dendrogram. Based on these results, RAPD analysis can be used for the characterization and grouping of walnut genotypes.

Keywords: *Juglans regia* L., Genetic diversity, RAPD

Introduction

The genus *Juglans* is characterized by monoecious and heterodichogamous habit, including about 20 species. Among those, *Juglans regia*, the English or Persian walnut, is the most economically important species. Its cultivars are grown primarily for nut production, as varietal clones [1]

Walnut grows well in areas with a temperate climate. Production is concentrated in China (29% of world production) and USA (17%). Turkey, with 172 000 (10%) Mt of nuts is the third largest walnut producing country [2]. Anatolia with various eco-geographical regions is one of the major centers of origin for Persian walnut diversity. There are 4.5 million natural seedlings of intra-specific hybrid walnut trees on their own roots in Turkey. These trees are an important source of genetic diversity for *J. regia* in Turkey. Because of high heterozygosity caused by continual sexual propagation, a very rich genetic material has formed among these local walnut populations [3]. Walnut germplasm in Anatolia has been used extensively in selection studies for production of superior walnut clones. The first selection study from natural populations of the Marmara region was performed by OLEZ [4]. Afterwards, SEN [5] and CELEBIOGLU [6] selected a few genotypes according to their yield and nut characteristics.

The knowledge of the genetic relationships among walnut genotypes and their pomological characteristics will be very useful in walnut cross-breeding programs. Several

techniques have been used to examine genetic diversity and relationships among cultivars of Persian (English) walnut, including isozymes [7, 8, 9, 10, 11] restriction fragment-length polymorphism (RFLP) [12], random amplified polymorphic DNA (RAPD) markers [13, 14, 15] and inter-simple sequence repeat (ISSR) markers [16]. RAPD markers have been used to evaluate genotype characterization among walnuts [14, 15, 17, 18, 19] and in other nut crops as almond [20, 21], pistachio [22], chestnut [23] and hazelnut [24, 25]. RAPD markers are not only important for the characterization of the germplasm but can also be used to evaluate the effects of selection over time and to aid in the development of crossing schemes in walnut improvement programs, since this method allows the study of the genetic diversity of the available germplasm [14].

The aim of this study was to determine the genetic relationships among some native and foreign genotypes and their early-bearing natural hybrids, with RAPD markers.

Materials and Methods

Plant Material

Analyses were carried out on eight walnut genotypes (six native and two foreign) (Table 1). Among these genotypes, NO-1, NO-2 and NO-8 are chance seedlings *(J.regia x J.regia*), obtained from open pollination. NO-1 is a seedling of Franquette. NO-2 and NO-8 are seedlings of Kaman 1. These chance seedlings gave fruit in the second year. In the first observation, NO-1 is leafing out about in the same time with Pedro. NO-2 and NO-8 are coming into leaf about in the same time with Franquette. Pedro and Bursa 95 are other cultivars in the orchard gathering seeds of Kaman 1.

Genomic DNA extraction

Young leaf samples were collected from individual trees, stored in an ice box and transported to the laboratory where DNA was extracted in the same day. Genomic DNA was extracted by a modified CTAB method of [26, 27]. The samples of 0.2g of fresh whole leaves were ground in liquid nitrogen with mortar and pestle. To each sample, 600 µl 1% CTAB was added. After vortexing, the samples were incubated at 65°C for 10 min. Then an equal volume (600 µl) of 24:1 chloroform:iso-amyl alcohol was added. After vortexing, centrifugation was performed at 11,200 *g* at 4°C for 5 min. After the supernatant was transferred and washed with choloroform:iso-amyl alcohol twice, 750 µl cold 2-propanol was added and inverted gently. The samples were incubated at 4°C for 10 min in the refrigerator. The final centrifugation was performed at 11,200 *g* at 4°C for 15 min, producing a pellet. The aqueous phase was decanted. The samples were rinsed with 500 µl of 70% ethanol. The samples were dried at 26°C in the sterile hood with continuous air flow. The pellet was dissolved in 100 µl TE. The resuspended DNA was treated with RNase and the concentration of extracted DNA was determined using a spectrophotometer at 260 nm. DNA was diluted to 10 ng/µl and used for PCR amplification.

RAPD reaction and electrophoresis conditions

Forty-five primers (Bio Basic Inc., Ontario, Canada) were used for PCR amplifications (Table 2). The PCR amplification reactions were carried out: 12.5µl volumes containing 1.25µl 10x reaction buffer, 1.25µl 25mM $MgCl_2$, 1.00µl 2.5mM of each dNTP (Takara), 0.125µl 5.0 unit of Taq DNA polymerase (Takara Taq) 0.5µl primer and 10 ng of genomic DNA. The total 45 RAPD primers were tested in this study and polymorphism obtained primers were shown in Table 2.

The thermalcycler (Creacon T-Cy) was programmed as one cycle of 3 min at 94^{o}C, followed by 40 cycles of 30 sec at 94^{o}C, 1 min at 38^{o}C, and 2 min at 72^{o}C, followed by a final incubation for 10 min at 72^{o}C then brought down to 4^{o}C.

Amplified DNA fragments were analyzed by gel electrophoresis in 1.2% agarose (BioBasic) (to which ethidium bromide (BioBasic) was added) in 1 x Tris Borate EDTA (TBE) buffer. A 100bp-10kb ladder (BioBasic) was used to estimate the approximate molecular weight of the amplified products.

Data analysis

The resulting band patterns from the PCR were scored as 0 and 1, absent and present, respectively. The dissimilarity matrix was calculated with the Pearson correlation using Dice's coefficient with the un-weighted pair group method using arithmetic average (UPGMA) as a clustering algorithm. The dendrogram was drawn using SAHN module in NTSYSpc v.2.2 software [28]. For the determination of the correlation between the similarity matrix and the dendrogram drawn, the Mantel test was performed to calculate a cophenetic value using MXCOMP module. The polymorphic information content (PIC) was computed as: $PIC_i=2f_i(1-f_i)$, where PIC_i is the polymorphic information content 'i', f_i is the frequency of the amplified allele (band present), and $(1-f_i)$ is the frequency of the null allele (band absent) [29,30]. The Principle Component Analysis (PCA) was performed to determine eigenvalues, percent and cumulative variance. PCA is often applied to standardized data because the results are sensitive to the choices of units of measurement and the choice of units is often arbitrary [28].

Table 1. Walnut genotypes used in this study.

Code	Genotype	Original source	Description
1	Franquette	France	Old French cultivar
2	NO-1	Turkey (Marmara Region)	Chance seedling
3	Kaman 1	Turkey (Central Anatolia)	Local cultivar
4	NO-2	Turkey (Marmara Region)	Chance seedling
5	NO-8	Turkey (Marmara Region)	Chance seedling
6	Bursa 95	Turkey (Marmara Region)	Local cultivar
7	Pedro	Univ.of California	Conway-Mayette X Payne
8	Kaman 5	Turkey (Central Anatolia)	Local cultivar

Results and Discussion

A total of 45 custom-designed 10-mer primers were used with all genotypes in the first screening of the experiment. In total, 37 primers out of 45 primers gave good and reproducible polymorphic band. Table 2 shows codes and sequences of the used primers, total number of amplification fragments (bands) resultant from eight walnuts genotypes and the number of polymorphic fragments for each primer. Three hundred and forty polymorphic fragments were obtained from 513 amplified fragments derived from the use of 37 primers on the studied walnut genotypes (Table 2). These primers ranged in their amplification fragments between two (S 56 and S101) and twenty (S-139) with an average number of 9.18 polymorphic fragments per primer. Figure 1 shows the amplification patterns resultant from primer S 22 that produced thirteen polymorphic bands. The relative number of polymorphic fragments to the total number of amplified fragments ranged between 25.00% (S133) and 95.23% (S139) with an average of 66.10% for all primers. PIC values ranged from 0.37 (S 165 and S 271) to 0.50 (S35, S38, S124, S129, S161, S177, S188, S 248, S 418 primers) for the polymorphic data set (Table 2).

Table 2. The used random primers along with their sequences, band size, total number of bands, the number of polymorphic bands and PIC value resultant from all tested walnut genotypes.

Primer	Sequence (5'→3')	Band size (bp) min-max	Total bands	Polymorphic bands	Polymorphism ratio (%)	PIC
S 21	CAGGCCCTTC	500-3000	14	10	71.42	0.46
S 22	TGCCGAGCTG	300-2700	20	13	65.00	0.41
S 32	TCGGCGATAG	300-5000	9	8	88.88	0.43
S 34	TCTGTGCTGG	670-2750	11	7	63.63	0.42
S 35	TTCCGAACCC	900-2500	12	10	83.33	0.50
S 38	AGGTGACCGT	970- 4500	11	9	81.81	0.50
S 56	AGGGCGTAAG	850-3000	7	2	28.57	0.49
S 98	GGCTCATGTG	630-2700	11	8	72.72	0.49
S 101	GGTCGGAGAA	1050-3000	4	2	50.00	0.49
S 120	GGGAGACATC	700-2100	9	8	88.88	0.47
S 122	GAGGATCCCT	350-2500	15	8	53.33	0.47
S 124	GGTGATCAGG	600-2950	14	10	71.42	0.50
S 125	CCGAATTCCC	450-2750	11	8	72.72	0.49
S 126	GGGAATTCGG	1500-2950	11	8	72.72	0.49
S 127	CCGATATCCC	500-2250	8	6	75.00	0.44
S 128	GGGATATCGG	1000-4000	8	4	50.00	0.42
S 129	CCAAGCTTCC	450-3500	16	6	37.50	0.50
S 130	GGAAGCTTGG	650-3000	16	9	56.25	0.49
S 132	ACGGTACCAG	400-3000	13	7	53.84	0.48
S 133	GGCTGCAGAA	600-3000	12	4	25.00	0.46
S 134	TGCTGGAGGT	700-2500	15	6	40.00	0.48
S 135	CCAGTACTCC	750-4000	16	13	81.25	0.47
S 139	CCTCTAGACC	200-3000	21	20	95.23	0.48
S 151	GAGTCTCAGG	600-5000	13	10	76.92	0.49
S 156	GGTGACTGTG	450-4500	24	16	66.66	0.49
S 161	ACCTGGACAC	3750-1000	13	9	69.23	0.50
S 165	TGTTCCACGG	1250-2850	6	5	83.33	0.37
S 169	TGGAGAGCAG	600-2800	21	13	61.90	0.49
S 177	GGTGGTGATG	450-3500	23	18	78.26	0.50
S 188	TTCAGGGTGG	1000-4000	14	11	78.57	0.50
S 248	GGCGAAGGTT	1000-4500	19	14	73.68	0.50
S 271	CTGATGCGTG	750-3250	16	10	62.50	0.37
S 280	TGTGGCAGCA	600-3800	23	14	60.86	0.49
S 418	CACCATCCGT	770-2600	9	7	77.77	0.50
S 443	CTGTTGCTAC	200-3000	17	10	58.83	0.49
S 444	AAGTCCGCTC	550-3500	13	7	61.53	0.44
S 461	GTAGCACTCC	650-3000	18	10	55.55	0.49
Total			513	340		
Mean			13.87	9.18	66.10	

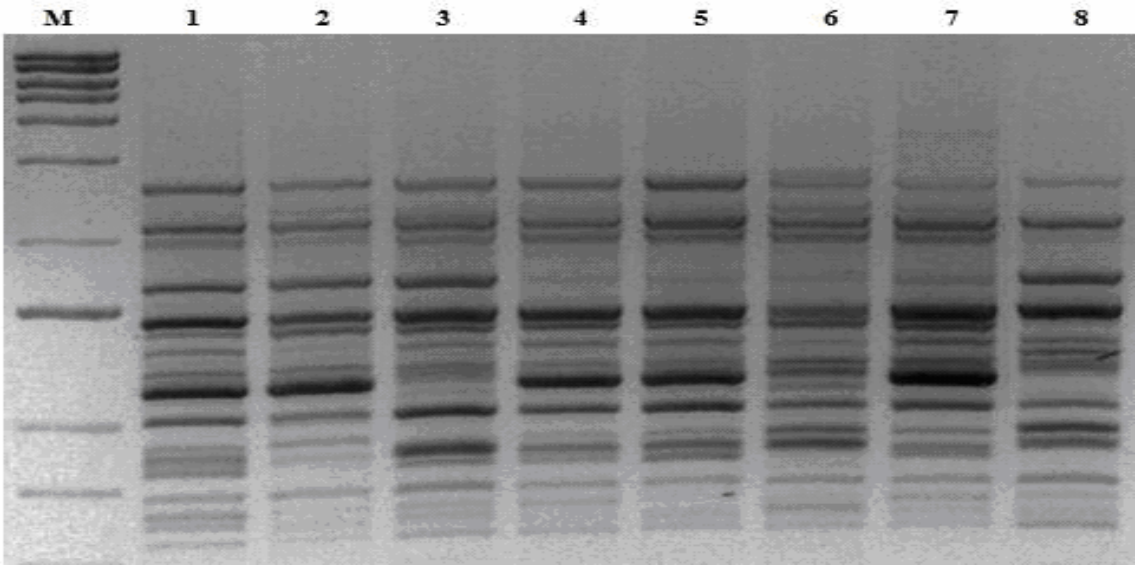

Figure 1. Identification of 8 walnut genotypes with primer S 22. M: 100bp-10 kb ladder lanes 1 through 8 correspond to the genotypes listed in Table 1.

According to the similarity matrix (Table 3), the cultivar Kaman 1 appears to be more closely related to its progenies (NO-2 and NO-8), with a similarity value of 0.751 and 0.716, respectively. NO-2 had a similarity value of 0.779 with NO-8. Pedro showed high similarity values with NO-2 and NO-8 (0.754 and 0.725 respectively) than with Bursa 95 (0.554 and 0.580, respectively). The similarity index of NO-1 and its maternal parent (Franquette) was 0.654. Kaman 5 is relatively distant to Kaman 1 (similarity value=0.587) which was selected from the same location placed in the central region of Turkey (Kırşehir).

Table 3. The similarity matrix of genotypes in walnuts

	Franquette	NO-1	Kaman 1	NO-2	NO-8	Bursa95	Pedro	Kaman 5
Franquette	1.000							
NO-1	0.654	1.000						
Kaman 1	0.624	0.539	1.000					
NO-2	0.673	0.654	0.751	1.000				
NO-8	0.656	0.641	0.716	**0.779**	1.000			
Bursa 95	0.595	0.533	0.529	0.554	0.580	1.000		
Pedro	0.675	0.645	0.630	0.754	0.725	0.559	1.000	
Kaman 5	0.489	0.522	0.587	0.535	0.544	**0.457**	0.479	1.000

UPGMA cluster analysis (Figure 2) separated the walnut genotypes included in this study into three clusters. The first group included Kaman 1, Franquette, NO-2, NO-8, NO-1 and Pedro. The second cluster contained Bursa 95. The third group included Kaman 5. The markers S 38- 990, S 280 -1200 and S 151-1050 were only present in Kaman 1 and its progeny (NO-8). The marker S 151-1100 was only present in Kaman 1 and its progeny (NO-2); S120-700 and S120-950 were only present in NO-2 and Bursa 95; and S188-1150 and S169-2800 were only present in NO-2 and Pedro. The marker S132-300 was present only in NO-8 and Pedro (Figure 3). Either Pedro or Bursa 95 could be the paternal parent for NO-2

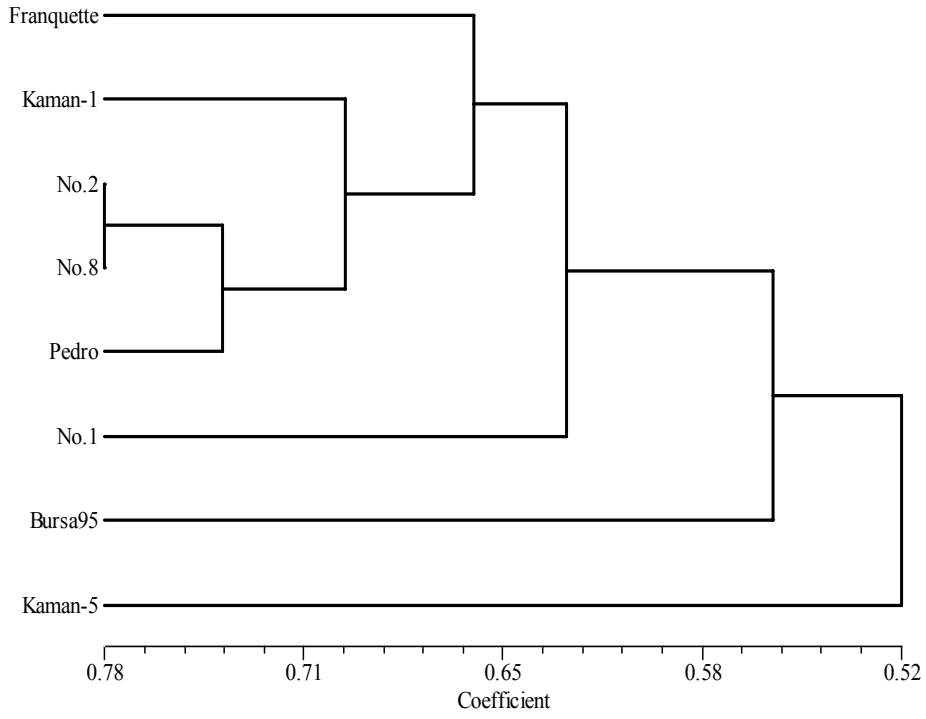

Figure 2. UPGMA dendrogram showing the relationship of walnut genotypes,

and NO-8. Besides, NO-2, NO-8 and Pedro are present within the group I. This result indicates that genotypes within the group I are genetically closer to each other. Pedro showed high similarity values with NO-2 and NO-8 (0.754 and 0.725 respectively) than Bursa 95 (0.554 and 0.580 respectively). Therefore, Pedro is more likely to be the paternal parent of the progenies NO-2 and NO-8 than Bursa-95. NO-1 is Franquette's progeny. Five markers (S177-1450, S177-825, S177-750, S177-730, and S177-600) were only present in Franquette and NO-1 (Figure 3). As expected, most of the fragments amplified from DNA obtained from the progenies were also present in the parents. The occurrence of parental bands has been reported in walnut with RAPD [14].

Among all the genotypes tested, Bursa 95 and Kaman 5 appear to be the most distantly related to others. They are replaced in separate groups. The fingerprint of each genotype is defined by multiple RAPD bands presumably at multiple genetic loci. But some markers have found specific to certain genotypes. For example, markers S35-1000, S120-1600, S248-1150, S127-1650, S161-1900, S139-2400, S134-1270, S126-2000, S 248-1500 did only exist in Bursa 95; these markers can distinguish Bursa 95 from the other genotypes. Bursa 95 was originally selected from location placed in the west region of Turkey (Bursa). Kaman 1 and Kaman 5 tend to have the same tree habit of growth. However, their fruit characteristics such as fruit weight (Kaman 1: 11.7 g, Kaman 5: 16.0 g) and kernel weight (Kaman 1: 5.87 g, Kaman 5: 7.7 g) are different [31, 32]

Code	Franquette	No-1	Kaman 1	No-2	No -8	Bursa 95	Pedro	Kaman 5
S177-1450	■	■						
S177-825	■	■						
S177-750	■	■						
S177-730	■	■						
S177-600	■	■						
S38- 990			■		■			
S151-1050			■		■			
S280 -1200			■		■			
S151-1100			■	■				
S120-700				■		■		
S120-950				■		■		
S188-1150				■			■	
S169-2800				■			■	
S132-300					■		■	

Figure 3. Polymorphic bands identified by RAPD with 8 selected primers on 8 genotypes. A dark gray box represents the presence of the marker; the white box represents the absence of the marker

A dendrogram was constructed according to calculated genetic similarities with the highest cophenetic correlation coefficient (r=0.9131) (Figure 2). Therefore, there is a very high cophenetic correlation between the similarity matrix and the dendrogram. This result indicated that 37 random primers gave a better understanding of genetic relationships between walnut genotypes. NICESE & al. [14] found the correlation coefficient 0.65 for 19 walnut genotypes with 23 random primers. They suggested that a higher number of markers will be needed to obtain a dendrogram that accurately reflects the similarity matrix. MIRALI and

NABULSKI [21] studied the genetic diversity of 19 almond cultivars and found that 30 random primers were sufficient of the genetic relationships between almonds cultivars.

The relationships among genotypes were defined by the first three principle components (PC) (eigenvectors), accounting for 57.23% of the total variation at the molecular level. The genotypes were placed -0.12 to 0.38 and -0.24 to 0.23 in dimension-1 and in dimension-2, respectively. Kaman 1, NO-2, NO-8 and Pedro quite closely located from others in the three dimensional graph (Figure 4). This also confirms the results in the UPGMA analysis (Figure 2). PCA can be used in yield and quality components, taxonomic similarities, and association between genetic and environmental attributes in horticultural crops [33]. By comparison, the first three PC of 43 male fig (DALKILIC & et al., unpublished) and that of five PC of 10 accessions of European chestnuts [34] explained together 40.91% and 51.00% of the total variation at the molecular level, respectively.

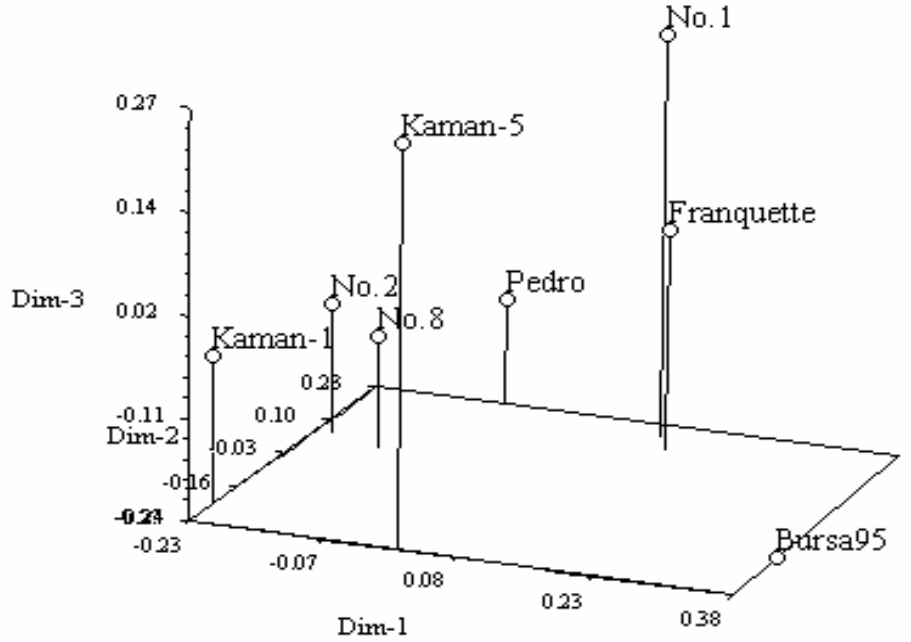

Figure 4. Principle component analysis (PCA) of RAPDs generated by 37 primers for 8 walnut genotypes

Conclusion

In this study, the described markers were able to distinguish some native and foreign genotypes and their early-bearing progenies. The differences present among these walnut genotypes suggest that they originated from genetically divergent parents or have adaptation to their respective micro-climatic regions. On the other hand, early-bearing trait of walnuts is important for cross breeding. Previous research showed that early-bearing trait, controlled by polygene (gene clusters), was a heritable quantitative character with segregation phenomenon [35]. Screening markers linked to walnut early-bearing character by RAPD technique have been reported by YANG & al. [36], WANG & al. [37] and ZHANG & al. [38]. So the work for the next step is to recover, clone and sequence the remaining markers and convert these RAPDs into sequence characterized amplified region (SCAR) markers, which will establish the foundation for constructing genetic linkage map linked to walnut early-bearing character. These works are significant for further walnut breeding program and development of the walnut crop in Turkey.

Acknowledgements

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