

Genetic Variability in Accessions of 'Amasya' Apple Cultivar Using RAPD Markers

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Abstract

'Amasya' is a native and standard cultivar for commercial apple (*Malus domestica* Borkh.) production in Turkey. The aim of this study was to determine the genetic variability among some accessions of 'Amasya' apple cultivar. Ten 'Amasya' accessions, all selected from agronomic evaluations, and 'Granny Smith' (as control) were analyzed by RAPD marker technique. Of the total 441 RAPD loci that were amplified by 38 random primers, 180 were polymorphic with 39.98% diversity. Cluster analysis divided the apples into two major clusters; the first cluster involved 10 accessions of 'Amasya', and the second cluster comprised 'Granny Smith'.

Keywords: *Malus domestica* Borkh, 'Amasya', genetic similarity, RAPD

Introduction

Turkey is one of the most important producers of apples (*Malus domestica* Borkh.) with a production of nearly 2504490 T per year (FAO, 2008). There are over 460 local apple cultivars in Turkey, all with different qualities (Ozbek, 1978). In terms of fruit characteristics, 'Amasya' is one of the best cultivars among these native apple cultivars. This cultivar has been cultivated in Anatolia since ancient times. It has a red skin color, white flesh color and high flesh firmness, and long storage life without significant loss of quality. However, two main drawbacks of this cultivar involve different fruit types and definite alternate bearing habits. Many 'Amasya' accessions from Anatolia were evaluated according to their agronomic performances, and some of them were selected because of their characteristics such as alternate bearing, skin color, fruit size, taste, and skin toughness (Eltez and Kaşka, 1985; Kaplan *et al.*, 2002). Apart from phenotypic characteristics, the genetic analysis through molecular marker is very important for cultivar and clone characterization. In addition, molecular markers allow genetic relatedness among cultivars and clones to be assessed and determined more accurately (Masi *et al.*, 2005; Lattoo *et al.*, 2008). RAPD markers have been used to identify apple cultivars (Mulcahy *et al.*, 1993; Koller *et al.*, 1993; Zhu *et al.*, 1997; Royo and Itoiz, 2004), to create genetic maps (Hemmat *et al.*, 1994; Liebhard *et al.*, 2003), as markers of resistance genes (Gygax *et al.*, 2004), and to determine paternity (Harada *et al.*, 1993; Gardiner *et al.*, 1996). This study was carried out to find possible genotypic differences, if any, within selected 'Amasya' apple accessions with a wide range of phenotypic variation.

Materials and methods

Plant material

The plants were maintained at the Atatürk Central Horticultural Research Institute (ACHRI) Yalova, Turkey. Leaf samples of 10 'Amasya' accessions ('Amasya 20', 'Amasya 21', 'Amasya Uludag', 'Amasya 22', 'Amasya 41', 'Amasya 9', 'Amasya 38', 'Amasya', 'Amasya 40' and 'Amasya 50') and 'Granny Smith' cultivar, which was used as control, were collected in May for DNA extraction.

DNA isolation

DNA was isolated from fresh leaves using 'Bio Basic EZ-10 Spin Column Genomic DNA Isolation Kit' according to the manufacturer's instructions after mechanical disruption of 100 mg of tissue in liquid nitrogen. Pure DNA was eluted with 50 µl of PCR Elution Buffer. The concentration and purity of extracted DNA were assessed by a spectrophotometer then diluted to 10 ng/ml for PCR amplification.

RAPD-PCR procedure

Forty-five RAPD primers (Bio Basic Inc., Ontario, Canada) were used in this study. The PCR amplification reactions were carried out in 12.5 µl volumes containing 1.25 µl 10x reaction buffer, 1.25 µl 25 mM MgCl₂, 1.00 µl 2.5 mM of each dNTP (Takara), 0.125 µl 5.0 units of Taq DNA polymerase (Takara Taq), 0.5 µl primer and 10 ng of genomic DNA. The thermal cycler (Creacon T-Cy) was programmed as follows: one cycle of 3 min at 94°C,

40 cycles of 30 sec at 94°C, 1 min at 38°C, 2 min at 72°C and a final incubation for 10 min at 72°C then brought down to 4°C. Amplified DNA fragments were analyzed by gel electrophoresis in 1.2% agarose (Bio Basic) (to which ethidium bromide (Bio Basic) was added) in 1 x Tris Borate EDTA (TBE) buffer. A 100 bp-10 kb ladder (Bio Basic) 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 for absent and 1 for present. The similarity matrix was calculated with the Pearson correlation using Dice's coefficient with the unweighted pair group method using arithmetic average (UPGMA) as a clustering algorithm. The dendrogram was drawn using the SAHN module in NT-SYSPc v.2.2 software (Rohlf, 2008). For the determination of a Mantel test, a cophenetic value was calculated 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) (Roldán-Ruiz *et al.*, 2000; Soengas *et al.*, 2006). PIC provides an estimate of the discriminatory power of a locus by taking into account the number of alleles that are expressed and the relative frequencies of those alleles (Smith *et al.*, 1997). PIC values range from 0 (monomorphic) to 1 (very highly discriminative with many alleles in equal frequencies).

Results and discussion

A total of 45 custom-designed 10-mer primers were screened in initial experiments. In total, 38 primers out of 45 primers produced clear and reproducible bands. Table 1 shows codes and sequences of the used primers, total number of the amplification fragments (bands) resultant from apple samples and the number of polymorphic fragments for each primer. A total of 441 RAPD fragments were detected for the ten 'Amasya' accessions and 'Granny Smith' cultivar. Thirty-eight primers generated 180 polymorphic fragments, which distinguished 'Granny Smith' from all the 'Amasya' accessions. The amplified fragments per primer ranged from one (S 156 and S186) to twelve (S 34) with an average of 4.73 (Tab. 1). The relative number of polymorphic fragments to the total number of amplified fragments ranged between 11.76% (S169) and 70.58% (S34) with an average of 39.98 % for all primers. The PIC values for the 38 primers ranged from 0.00 to 0.49 in this study.

UPGMA cluster analysis of the similarity matrix (Fig. 1) divided the apple samples into two main groups. Based on the secondary branching, all 'Amasya' acces-

sions formed the first group, whereas 'Granny Smith' was placed into the second group; its similarity value was 0.04 with group 1. A similarity matrix was calculated using 441 RAPD fragments according to the simple matching method. The cophenetic correlation between an ultrametric similarities tree and the similarity matrix was found to be high ($r = 0.997$, $P < 0.01$), suggesting that the cluster analysis strongly represents the similarity matrix. From the similarity matrix constructed for the ten accessions (Tab. 2), similarity values were close to 1.000 for all pair combinations. Analysis of the 'Amasya' accessions revealed a very high degree of similarity, or even genetic identity.

When the analysis was confined only to the 'Amasya' accessions, 17 primers out of 38 primers gave polymorphic bands. The remaining 21 primers exhibited monomorphic patterns among ten apple samples and are useless for their discrimination. (Tab.1, Fig. 2). The 38 primers produced 402 discrete DNA fragments, 26 of which showed polymorphism in one or more accessions, indicating a low degree of polymorphism (Tab. 1, Fig. 3). A total of 402 bands were scored, of which only eight extra bands in six of the ten accessions studied were observed; therefore, the frequency of variation was found to be very low. While 'Amasya 38' and 'Amasya 41' had two extra bands, 'Amasya 40', 'Amasya Uludag', 'Amasya 9' and 'Amasya' had only one extra band amplified by different primers. The 900 bp fragment by primer S101 and 1100 bp fragment amplified by primer S 125 was observed in 'Amasya 41'. Only 'Amasya 40' and 'Amasya Uludag' had the 1750-bp fragment and the 1250 bp fragment amplified by primer S35, respectively. 'Amasya 41' lacked the 3750 bp fragment amplified by primer S 98 and 'Amasya 40' lacked the 850 bp fragment amplified by primer S 443, which were shared by all other 'Amasya' accessions. In contrast, the 1250 bp fragment of primer S 443 was observed in the 'Amasya' and 'Amasya 50' clones (Fig. 4). It has been suggested (Williams, 1993) that altered RAPD patterns, *i.e.*, the loss or gain of a band, may be due to the variety of genomic alterations, such as a mutation in the priming site or a deletion/insertion between the priming sites. Altered RAPD patterns could also be the result of altered competition for priming sites based on rearrangement/amplification in certain regions of the genome (DeVerno *et al.*, 1994). In our study, analysis of RAPD confirmed few genetic differences among these Amasya accessions. Further, the PIC values confirmed the low polymorphism (Tab. 1). Genetic similarity values among the 10 apple accessions of group I were 0.94 or higher. Two accessions ('Amasya 20' and 'Amasya 21') had a similarity index of 100%, and this observation suggests that there are clones among the analyzed accessions. The phenotypic variations are probably due to differential gene expression in response to varying environmental conditions and growing practices.

Tab. 1. List of RAPD primers, their numbers of total (T) and polymorphic fragments (P) and percentage of polymorphism and polymorphism information contents (PIC) used in this study

Primer	Sequence (5'→3')	'Amasya' and 'Granny Smith'			'Amasya' Accessions			
		T	P	Polymorphic DNA fragments (%)	T	P	Polymorphic DNA fragments (%)	PIC
S 21	CAGGCCCTTC	13	3	23.07	12	1	8.33	0.32
S 32	TCGGCGATAG	9	4	44.44	9	3	33.33	0.48
S 34	TCTGTGCTGG	17	12	70.58	16	0	0.00	0.00
S 35	TTCCGAACCC	17	9	52.94	14	2	14.28	0.18
S 38	AGGTGACCGT	14	4	28.57	12	1	8.33	0.50
S 56	AGGGCGTAAG	14	5	35.71	11	0	0.00	0.00
S 98	GGCTCATGTG	13	6	46.15	10	1	10.00	0.18
S 101	GGTCGGAGAA	15	10	66.66	14	3	21.42	0.42
S 120	GGGAGACATC	13	6	46.15	11	1	9.09	0.32
S 122	GAGGATCCCT	15	5	33.33	11	0	0.00	0.00
S 124	GGTGATCAGG	10	5	50.00	9	0	0.00	0.00
S 125	CCGAATTCCC	18	7	38.88	15	2	13.33	0.18
S 126	GGGAATTCCG	7	3	42.85	6	1	16.66	0.18
S 128	GGGATATCGG	7	3	42.85	7	1	14.28	0.32
S 129	CCAAGCTTCC	16	4	25.00	15	0	0.00	0.00
S 130	GGAAGCTTGG	13	3	23.00	13	0	0.00	0.00
S 132	ACGGTACCAG	11	4	36.36	10	0	0.00	0.00
S 133	GGCTGCAGAA	18	7	38.88	17	0	0.00	0.00
S 134	TGCTGGAGGT	13	3	23.07	11	0	0.00	0.00
S 135	CCAGTACTCC	9	6	66.66	6	0	0.00	0.00
S 139	CCTCTAGACC	14	6	42.85	13	2	15.38	0.18
S 151	GAGTCTCAGG	9	4	44.44	8	0	0.00	0.00
S 156	GGTGACTGTG	7	1	14.28	6	0	0.00	0.00
S 161	ACCTGGACAC	9	3	33.33	9	1	11.11	0.18
S 165	TGTTCCACGG	6	3	50.00	6	1	16.66	0.32
S 169	TGGAGAGCAG	17	2	11.76	17	0	0.00	0.00
S 177	GGTGGTGATG	12	4	33.33	9	0	0.00	0.00
S 186	GAT ACC TCGG	5	1	20.00	5	0	0.00	0.00
S 188	TTCAGGGTGG	15	9	60.00	14	2	14.28	0.49
S 218	GATGCCAGAC	6	4	66.66	5	0	0.00	0.00
S 222	AGTCACTCCC	14	5	35.71	11	0	0.00	0.00
S 248	GGCGAAGGTT	12	3	25.00	11	0	0.00	0.00
S 271	CTGATGCGTG	6	3	50.00	5	0	0.00	0.00
S 280	TGTGGCAGCA	19	7	36.00	17	1	5.88	0.18
S 418	CACCATCCGT	6	3	50.00	6	0	0.00	0.00
S 443	CTGTTGCTAC	11	4	36.36	10	2	20.00	0.49
S 444	AAGTCCGCTC	11	3	27.27	10	0	0.00	0.00
S 461	GTAGCACTCC	13	6	46.15	11	1	9.09	0.32
Total	-	441	180	-	402	26	-	0.13
Mean	-	11.94	4.73	39.98	10.57	0.36	4.17	-

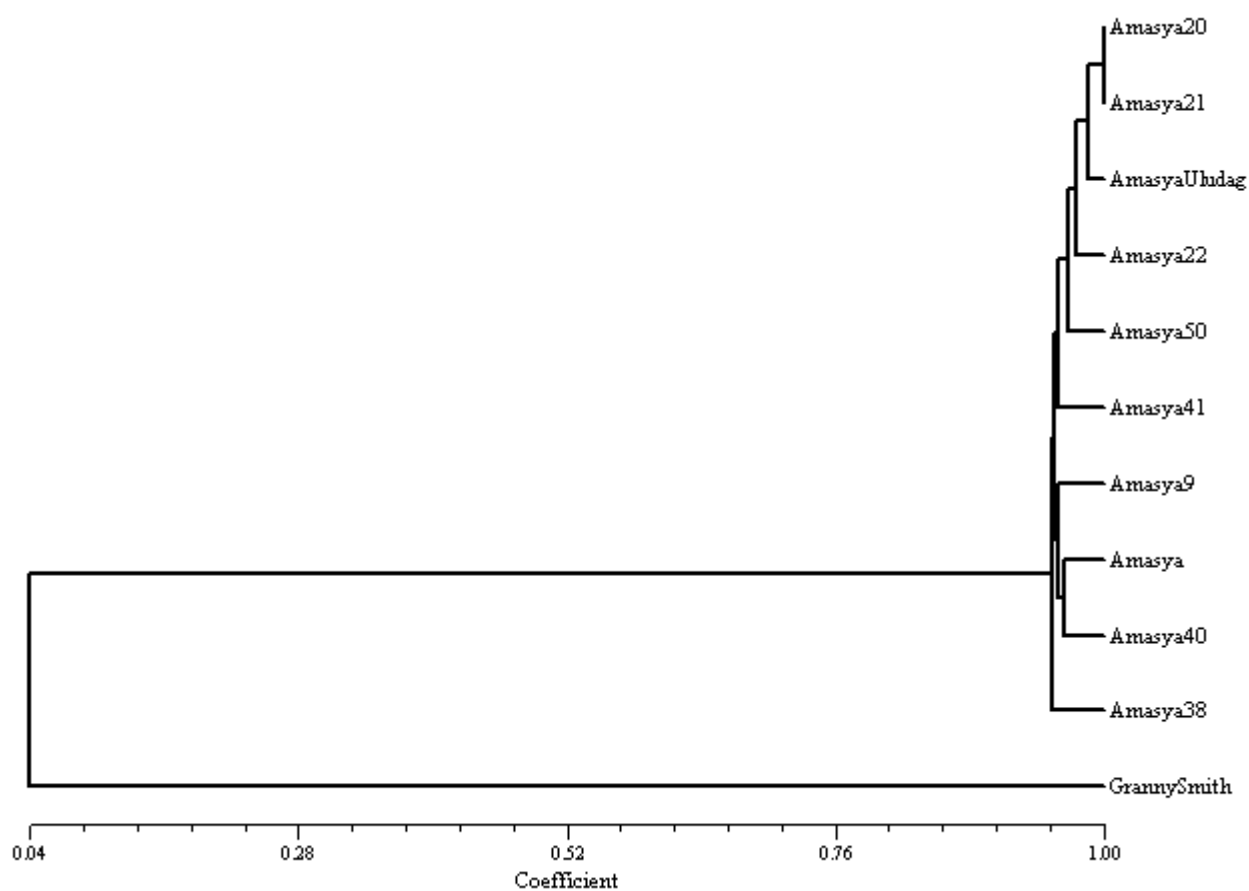
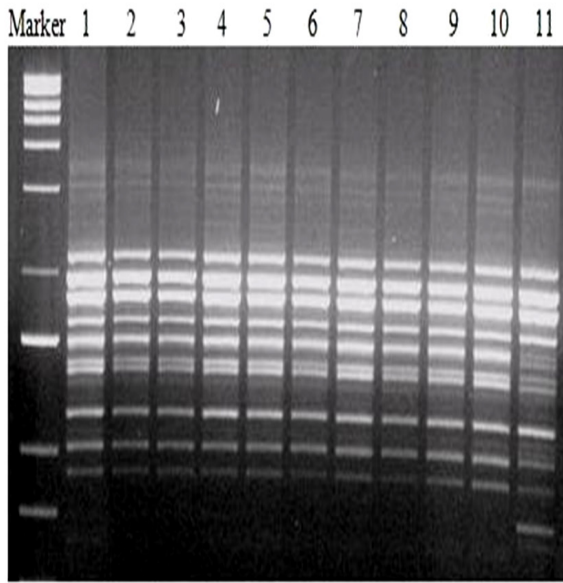


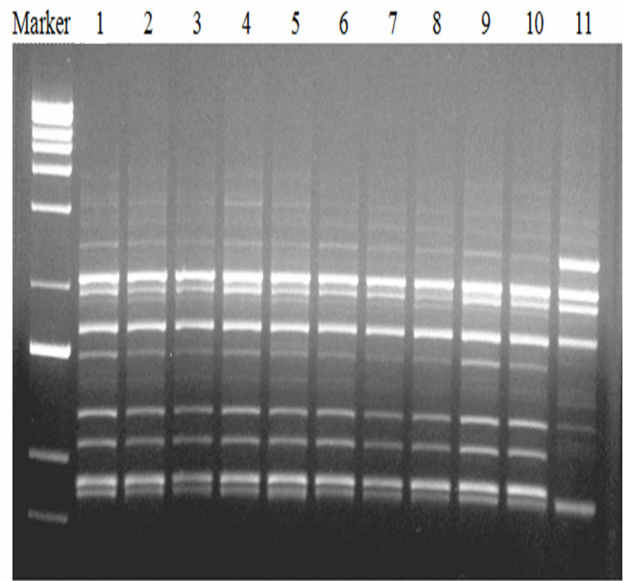
Fig. 1. Dendrogram of relationships among 'Amasya' accessions and 'Granny Smith' apple cultivar analyzed using RAPD marker

Tab. 2. Genetic similarity matrix based on RAPD data among 'Amasya' apple accessions estimated according to Dice's method

	'Amasya 20'	'Amasya 21'	'Amasya Uludag'	'Amasya 22'	'Amasya 41'	'Amasya 9'	'Amasya 38'	'Amasya'	'Amasya 40'	'Amasya 50'	'Granny Smith'
'Amasya 20'	1.000										
'Amasya 21'	1.000	1.000									
'Amasya Uludag'	0.986	0.986	1.000								
'Amasya 22'	0.977	0.977	0.973	1.000							
'Amasya 41'	0.959	0.959	0.955	0.964	1.000						
'Amasya 9'	0.959	0.959	0.955	0.955	0.955	1.000					
'Amasya 38'	0.954	0.954	0.959	0.959	0.950	0.959	1.000				
'Amasya'	0.959	0.959	0.964	0.964	0.946	0.956	0.960	1.000			
'Amasya 40'	0.969	0.950	0.946	0.964	0.946	0.965	0.942	0.965	1.000		
'Amasya 50'	0.968	0.968	0.964	0.973	0.964	0.964	0.950	0.965	0.952	1.000	
'Granny Smith'	0.036	0.036	0.035	0.058	0.047	0.034	0.047	0.045	0.034	0.057	1.000

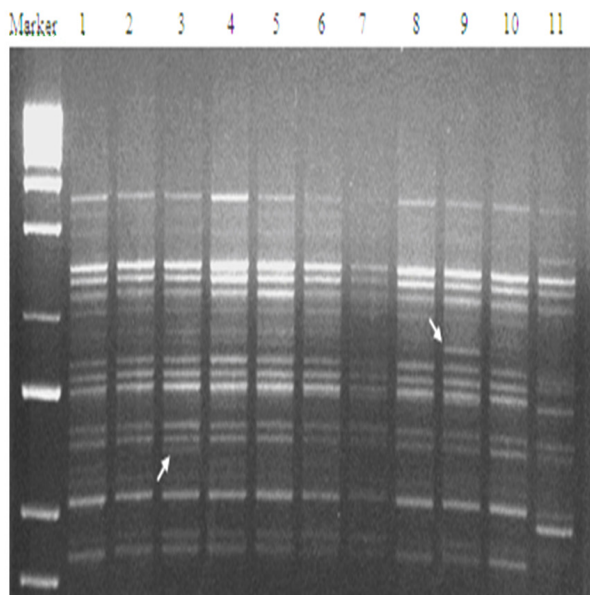


S 248

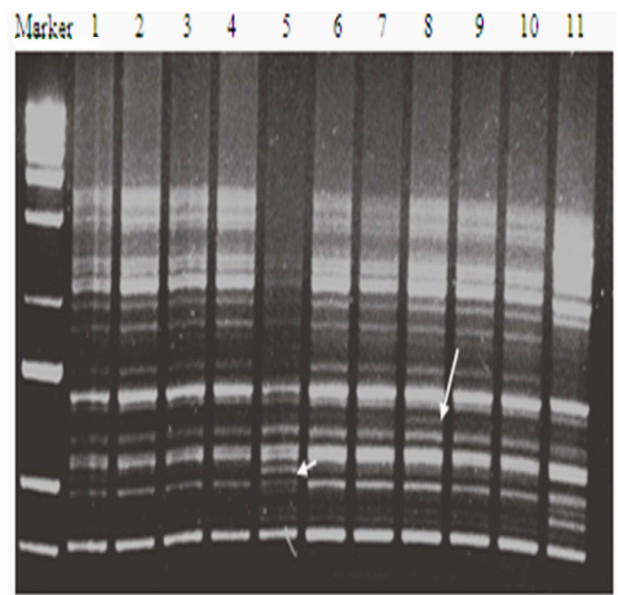


S 151

Fig. 2. RAPD profiles of 10 accessions of 'Amasya' generated with primers S248 and S151. Lane 1, 'Amasya 20' lane 2, 'Amasya 21'; lane 3, 'Amasya Uludag'; lane 4, 'Amasya 22'; lane 5, 'Amasya 41'; lane 6, 'Amasya 9'; lane 7, 'Amasya 38', lane 8, 'Amasya'; lane 9, 'Amasya 40'; lane 10, 'Amasya 50'; lane 11, 'Granny Smith'



S 35



S 125

Fig. 3. RAPD patterns resulting from amplification with primer S35 and S125. The arrows indicate polymorphic RAPD markers, which discriminate between the 10 'Amasya' accessions. Lane 1, 'Amasya 20' lane 2, 'Amasya 21'; lane 3, 'Amasya Uludag'; lane 4, 'Amasya 22'; lane 5, 'Amasya 41'; lane 6, 'Amasya 9'; lane 7, 'Amasya 38', lane 8, Amasya; lane 9, 'Amasya 40'; lane 10, 'Amasya 50'; lane 11, 'Granny Smith'

Code	'Amasya 20'	'Amasya 21'	'Amasya Uludag'	'Amasya 22'	'Amasya 41'	'Amasya 9'	'Amasya 38'	'Amasya'	'Amasya 40'	'Amasya 50'
S21-850										
S32-1750										
S32-1000										
S32-900										
S35-1750										
S35-1250										
S38-2000										
S98-3750										
S101-2150										
S101-2100										
S101-900										
S120-1750										
S125-1300										
S125-1100										
S126-1750										
S128-1950										
S139-2000										
S139-1700										
S161-1750										
S165-1250										
S188-2000										
S188-850										
S280-2000										
S443-1250										
S443-850										
S461-1600										

Fig. 4. Polymorphic bands identified by RAPD with 17 of the selected primers on several accessions. A dark-grey box represents the presence of the marker; the white box represents the absence of marker

Conclusions

The results obtained by RAPD analysis of the ten accessions of 'Amasya' clearly show that morphological traits alone could lead to misidentification of cultivars. The RAPD technique allowed the identification of polymorphic markers, though few (6.46%), which discriminate between accessions of the same cultivar. The results of this study show that it can also be used to distinguish accessions of the same cultivar.

Acknowledgments

This work received financial support from the Uludag University Scientific Research Projects' Commission for the research Project 2004/48. We gratefully acknowledge Zeynel Dalkilic for his helpful comments on the manuscript.

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