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Molecular Characterization of Genetic Diversity Among *T. dicoccoides* and *T. dicoccon* Populations by RAPD-PCR Technique

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Abstract

It is necessary to characterize genetic diversity of the plant resources for their effective usage and protection. There are various well known marker systems to analyze and define the plant genomes. Randomly Amplified Polymorphic DNA (RAPD) is one of the efficiently used techniques for distinguishing the genetic variation among the wheat species as well as the other plants.

The objective of this study is to characterize the genetic diversity of 11 wild emmer (*T. dicoccoides*) and 8 emmer (*T. dicoccon*) populations each of which is found in Turkey. For this purpose wheat samples were analyzed with 25 RAPD markers of which 20 were found to be informative. Of the total 178 amplification products, 85 were polymorphic. An average percentage of polymorphism was detected as 47.75%. Dendrograms were constructed using an unweighted pair-group method with arithmetical averages (UPGMA). The UPGMA analysis revealed that the lowest similarity was between emmer wheat recorded as TUR 02456 and wild emmer wheat recorded as TUR 03399, whereas, the genetic distance between two emmer wheat which are recorded as TUR 03562 and TUR 03564 was the highest. Consequently, RAPD could clearly assessed the genetic diversity at inter and intraspecific levels and these species can be considered as valuable gene resources for future breeding and conservation programs.

INTRODUCTION

Transition of the mankind from hunter-gatherer to sedentary, agriculture-based life style began about 12.000 years ago [1]. The archeobotanical studies revealed that the first signs of domestication of various “founder crops” (emmer wheat, einkorn, barley, lentil, chickpea, and common flax) were found in farming villages those developed in Fertile Crescent (Figure 1), a region that encompasses a large area including modern-time Turkey, Iran, Iraq, Syria, Israel, Jordan and Lebanon [2].

Besides its worldwide usage, wheat has a unique place among the other crops as an agricultural and dietary material. According to the Food and Agricultural Organization (FAO), wheat is cultivated throughout 9.5 million hectares [3] with an annual production of 20 million tons in Turkey. In addition, annual worldwide wheat production is 620 million tons [4]. Although this amount seems to be quite

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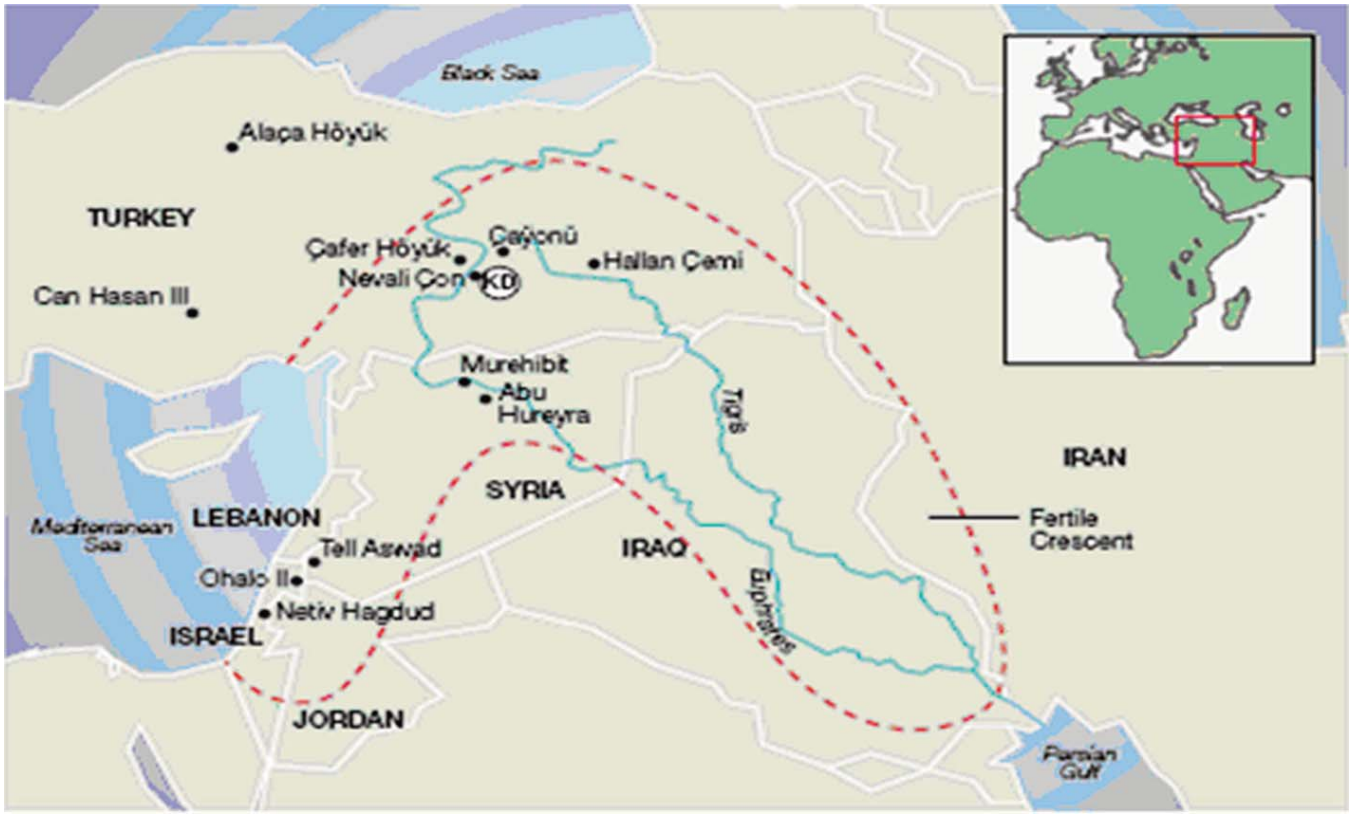

Figure 1. Fertile Crescent [1].

high, it's not sufficient to meet the world's growing wheat demand. In order to increase the wheat yield, various national and international breeding programs have been held. As a result, while making possible to get desirable field and harvest characters, these breeding programs also increased the vulnerability of crops to pests and diseases [5] consequently, these attempts have led to genetic bases of cultivars to become narrow [6,7].

From this point of view, progenitors of cultivated species can be considered as invaluable genetic resources that can be used to develop better cultivars with desired characteristics since wild relatives have precious genes in disease resistance, high protein content, ability to adapt stringent environmental conditions and other agronomically and economically important traits [8]. In this sense, it's vital to analyze the genetic diversity present both within cultivars and their wild relatives. Wild relatives of wheat are quite widespread in Turkey, and many researches indicated that the region of Karacadag located in southeastern Turkey, called as "core area", is the centre of origin [7-13]. Being one of them, wild emmer, *Triticum turgidum* L. ssp. *dicoccoides* referred as *Triticum dicoccoides,* is considered to be the wild progenitor of cultivated tetraploid (2n = 4X = 28, genome BBAA) and hexaploid (3n = 6X = 48, genome AABBDD) wheat and has been used for the improvement of wheat varieties [14]. Number of studies have been utilized for estimating its genetic diversity by various molecular techniques such as allozyme [15], rDNA [16], RAPD [17,18], microsatellites [19-21], and AFLP [12].

Like its wild progenitor *T. dicoccoides*, *T. dicoccon* is also considered to be a resource for durum and bread wheat [22,23]. However, there are not many researches on their agro-morphological and molecular characters. As a result of mankind's influence on wheat's evolutionary history, today *T. dicoccon* is neglected or underutilization. In the Old World, although *T. dicoccon* was used in diet and beer making as one of the main crops, it was replaced with free-treshing tetraploid and hexaploid

wheat varieties favoured by humans because of their better harvesting and agronomical properties. In this sense, even if *T. dicoccon* is one of the earliest domesticated wheat varieties, during 1960s its cultivation has reduced dramatically because of its low yield [24]. By the way, while it grows wild in the Middle East and Mediterranean basin, it is under cultivation in marginal farming areas of Turkey, Oman, Slovakia, Yemen, Italy, the Balkan Peninsula, Ethiopian highlands, Morocco, India, in some parts of Spain, etc. [25-27]. Recently, various findings about its agronomic and nutritive values have made this species popular again. Agriculturally, it has a capacity to grow at low temperatures, infertile soils and it also doesn't require elaborate techniques [28]; whereas its nutritive values are due to its high protein [29], high resistant starch, fibre and antioxidant contents [30]. Thus, there has been an increase in its cultivation areas in some countries like Italy [31,32]. In addition to above mentioned characters, *T. dicoccon*'s one of the most important traits is its resistance to rust and powdery mildew [24,33]. For these reasons, *T. dicoccon* has become of interest in breeding programs as a valuable genetic resource.

RAPD is one of the most popular among various molecular techniques which have been utilized to asses the genetic diversity present within cultivated species and its wild relatives. It has many advantages such as rapidity, low cost, requirement of small quantities of primers and genomic DNA, direct scoring data from agarose gels, clear and reliable detection of polymorphisms among different specimens [7].

The objective of this study was to estimate the genetic diversity of wild and domesticated emmer samples collected from different localities in Turkey by using RAPD-PCR technique. It's expected that the provided information would be supportive for future breeding and conservation programs.

MATERIALS AND METHODS

Wheat Samples

Wheat seeds belong to 8 populations of *T. dicoccon* and 11 populations of *T. dicoccoides* were obtained from collections of Field Crops Research Institute, Ankara, Turkey. Related information about the wheat samples are listed in Table 1.

DNA Extraction

Wheat seeds were planted in a nursery and DNAs were extracted from 14-days-old wheat seedlings. About 500 mg green leaf tissue from 10 individuals in each nursery was used for extraction. The extraction method was modified from Hulbert and Benetzen [34].

Genomic DNA samples were quantified on 1% agarose gel in 1x TBE buffer and 0.75 mg ml^{-1}

Table 1. List of *T. dicoccon* and *T. dicoccoides* samples analyzed.

No	Population Number	Species	Origin	Altitude (m)
1	TUR 02443	*T. dicoccon*	Sinop	1150
2	TUR 02453	*T. dicoccon*	Sinop	1200
3	TUR 0256	*T. dicoccon*	Sinop	1000
4	TUR 02637	*T.dicoccoides*	Şanlıurfa	480
5	TUR 03346	*T.dicoccoides*	Diyarbakır	850
6	TUR 03358	*T.dicoccoides*	Karacadağ	750
7	TUR 03362	*T.dicoccoides*	Karacadağ	1140
8	TUR 03369	*T.dicoccoides*	Karacadağ	1080
9	TUR 03371	*T.dicoccoides*	Karacadağ	1050
10	TUR 03376	*T.dicoccoides*	Karacadağ	1080
11	TUR 03388	*T.dicoccoides*	Karacadağ	810
12	TUR 03391	*T.dicoccoides*	Karacadağ	930
13	TUR 03399	*T.dicoccoides*	Karacadağ	1500
14	TUR 03402	*T.dicoccoides*	Karacadağ	990
15	TUR 03558	*T. dicoccon*	Sinop	1100
16	TUR 03560	*T. dicoccon*	Kastamonu	800
17	TUR 03562	*T. dicoccon*	Karabük	1020
18	TUR 03564	*T. dicoccon*	Kastamonu	1050
19	TUR 03565	*T. dicoccon*	Sinop	1050

HindIII digested I DNA (Boehringer-Manheim) was used as molecular weight marker. The final DNA concentrations were adjusted to 25 mg ml^{-1}, and the samples were stored at -20°C.

RAPD Amplification

Totally 25 RAPD primers which were selected from Operon Technologies (Alameda, Ca, USA) and University of British Columbia (Vancouver, Canada) were used in RAPD-PCR amplifications. The sequences, annealing temperatures, and GC% contents of the primers are listed in Table 2.

RAPD-PCR mixtures included 25 ng μl^{-1} of genomic DNA, 50 ng μl^{-1} of random primer, 25 mM μl^{-1} of $MgCl_2$, 2.5 mM μl^{-1} of dNTP mix, 5 µl of 10x PCR reaction buffer and 1.5 units of Taq DNA polymerase in a total volume of 50 µl.

The amplification reactions were performed as follow: (1) 94°C, 3 min. (2) 94°C, 30 s; (3) 32-52°C, 1 min; (4) 72°C, 2 min. Steps 2-4 were repeated for 40 times, and a last extension step was performed at 72°C for 10 minutes.

15 µl of RAPD-PCR amplification products were analyzed on 1.5% agarose gel in 1x TBE along with a DNA molecular marker. To check any possible DNA contamination a negative control containing all the ingredients except template DNA was always included. Electrophoresis carried out at 90V for 4 hours. Following electrophoresis, gels were visualized with ethidium-bromide staining and photographed by Sygene Gene Genius Bio Imaging System.

In order to ensure the reproducibility of the technique, previously optimized PCR conditions were used; the RAPD-PCR reactions were repeated twice; and only the reproducible bands were scored.

Table 2. List of sequences, annealing temperatures and GC% contents of primers.

Primer Sequences (5'−3')	Tm	%GC
OPA 01-CAG GCC CTT C	34°C	70
OPA 12-TCG GCG ATA G	32 °C	60
OPA 14-TCT GTG CTG G	32°C	60
OPF 01-ACG GAT CCT G	32°C	60
OPF 02-GAG GAT CCC T	32°C	60
OPF 03-CCT GAT CAC C	32°C	60
OPF 04-GGT GAT CAG G	32°C	60
OPF 05-CCG AAT TCC C	32°C	60
OPF 07-CCG ATA TCC C	32°C	60
OPF 09-CCA AGC TTC C	32°C	60
OPF 10-ACG GTA CCA G	32°C	60
OPF 14-TGC TGC AGG T	32°C	60
OPK 04-CCG CCC AAA C	34°C	70
OPK 16-GAG CGT CGA	32°C	60
OPO 02-ACG TAG CGT C	32°C	60
OPP 01-GTA GCA CTC C	32°C	60
OPP 09-GTG GTC CGC A	34°C	70
OPP 33-GTA AAA CGA CGG CCA GT	52°C	53
OPP 123-GGG ATT CGA C	32°C	60
OPP 232-CCG CTT GTT G	32°C	60
OPP 394-CGA CTC CAA C	32°C	60
OPP 437-CGG ATC GAC A	32°C	60
UBC 285-GGG CGC CTA G	36°C	80
UBC 493-CCG AAT CAC T	30°C	50
UBC 546-CCC GCA GAG T	34°C	70

Statistical Analysis

The fragments were scored as "1" in their presence and scored as "0" in their absence. The band matrices were analyzed following SIMQUAL program of the NTSYS-pc version 2.10 [35]. The similarity matrices were estimated using Dice coefficient and these matrices were used to construct the dendrograms with unweighted pair-group method with arithmetic averages (UPGMA).

RESULTS

Of 25 randomly selected primers, 20 (80%) were polymorphic for 19 wild and cultivated emmer genotypes. These 20 primers yielded total 178 amplification products and 85 of them (47.75%) were found to be polymorphic (Table 3; Figures 2, 3). The average number of amplified polymorphic bands per polymorphic primer was 8.9 and size of the fragments varied between 200-1600bp. Where the primers OPP 123 and OPO 02 gave the highest number of polymorphic RAPD markers, UBC 285 primer gave the lowest number (9%). The polymorphism percentages detected with 9 primers (OPA 12, OPP 437, OPP 09, OPF 03, OPK 04, OPP 123, UBC 546, OPO 02 and UBC 493) were 60 or above, and with the other 11 primers they were ranged from 9 to 43.

When *T. dicoccoides* and *T. dicoccon* samples were analyzed as separate groups, polymorphism percentages were calculated as 41.5% for *T. dicoccoides* and 32.9% for *T. dicoccon* samples. While OPP 09 and UBC 546 (7 polymorphic fragments) were the most informative primers for *T. dicoccoides* samples, it was OPF 02 primer (8 polymorphic fragments) for *T. dicoccon* samples.

Cluster Analysis Results

According to dendrogram based on Dice coefficient, which is ranged between 0.4680 (samples 3-12) and 0.9423 (samples 17-18), *T. dicoccoides* and *T. dicoccon* samples fell in two major clusters (Figures 4 and 5) . Group A includes *T. dicoccoides* sample recorded as TUR 03399 (sample 13). Group B is divided in two subclusters; while subcluster B1 includes *T. dicoccon* samples numbered as 1-3 and 15-19, subcluster B2 consists of *T. dicoccoides* samples numbered as 4-14 (except number 13). The closest similarity was found between *T. dicoccon* samples numbered as 17 and 18.

No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	8	19
1	1.0000																		
2	0.8089	1.0000																	
3	0.8131	0.6097	1.0000																
4	0.7450	0.7526	0.6315	1.0000															
5	0.6382	0.6352	0.6206	0.7142	1.0000														
6	0.6938	0.6966	0.5714	0.7450	0.8085	1.0000													
7	0.7291	0.7356	0.6067	0.7800	0.8043	0.8541	1.0000												
8	0.6363	0.6329	0.5185	0.7173	0.7619	0.7272	0.7674	1.0000											
9	0.6451	0.6666	0.5116	0.7628	0.6516	0.7311	0.7692	0.7951	1.0000										
10	0.6736	0.6976	0.6136	0.7676	0.6813	0.6947	0.7741	0.7058	0.7777	1.0000									
11	0.6732	0.6304	0.5531	0.7428	0.7010	0.8118	0.7676	0.6593	0.7291	0.6938	1.0000								
12	0.6336	0.6304	**0.4680**	0.7238	0.6391	0.7524	0.7272	0.6153	0.6666	0.6938	0.7500	1.0000							
13	0.5050	0.5333	0.4782	0.6601	0.6526	0.6666	0.6391	0.5617	0.6170	0.6458	0.6666	0.6862	1.0000						
14	0.6464	0.6222	0.5434	0.7572	0.7578	0.7474	0.7628	0.6516	0.7021	0.7083	0.7647	0.7450	0.7400	1.0000					
15	0.7924	0.7010	0.7070	0.7454	0.6666	0.7169	0.7692	0.6458	0.6732	0.7378	0.7522	0.7522	0.6542	0.7663	1.0000				
16	0.8910	0.7391	0.7659	0.7428	0.6391	0.6732	0.7272	0.6373	0.6875	0.6938	0.6538	0.6538	0.6078	0.6862	0.8256	1.0000			
17	0.7920	0.6739	0.7446	0.7238	0.7216	0.6930	0.7676	0.6373	0.6666	0.7142	0.6538	0.6153	0.5882	0.7254	0.8073	0.8461	1.0000		
18	0.7524	0.6304	0.7446	0.7047	0.6804	0.6534	0.7272	0.6373	0.6458	0.7346	0.6538	0.6153	0.5882	0.7058	0.8073	0.8269	**0.9423**	1.0000	
19	0.7959	0.6966	0.7472	0.7058	0.6382	0.6326	0.6666	0.5909	0.5806	0.6736	0.6336	0.5940	0.5858	0.6464	0.7547	0.8118	0.8514	0.8118	1.0000

Figure 2. Genetic similarity indexes of *T. dicoccides* and *T. dicoccon* samples.

Table 3. List of RAPD primers and polymorphism percentages obtained in wild and cultivated emmer species.

No	Primer	Total Number of Bands	Number of Polymorphic Bands	Polymorphism%
1	OPP 232	6	0	0
2	OPA 12	8	5	63
3	OPF 01	1	0	0
4	OPF 10	3	0	0
5	OPP 437	5	3	60
6	OPF 02	9	3	33
7	OPP 394	6	1	17
8	OPP 09	8	7	88
9	OPP 33	2	0	0
10	OPF 03	7	5	71
11	OPF 04	7	3	43
12	OPF 05	6	2	33
13	OPK 04	5	3	60
14	OPA 01	14	0	0
15	OPP 123	9	9	100
16	OPP 01	11	3	27
17	OPF 07	10	3	30
18	OPF 09	10	3	30
19	OPK 16	14	5	36
20	UBC 285	11	1	9
21	UBC 546	10	7	70
22	OPF 14	9	2	22
23	OPA 14	14	6	43
24	OPO 02	10	8	80
25	UBC 493	9	6	67
Total		204	85	48
Total number of bands obtained from polymorphic primers				178

DISCUSSION

RAPD is one of the most widely used molecular markers utilized in various molecular approaches. However, there have been some controversial discussions related to RAPD's reproducibility. When PCR conditions are strictly-optimized and only the reproducible amplification bands are scored, this problem can be eliminated. In the present study, as a result of amplification with totally 25 random primers of which 20 were found to be polymorphic and 47.75% polymorphism was obtained. Considering the dendrogram shown in Figure 3, RAPD could clearly assessed the genetic diversity at inter and intraspecific levels. In this respect, as reported in many researches [7,17,36-38] RAPD-PCR can be considered as a reliable and suitable technique for genetic diversity studies and germplasm evaluations among even close relative species. The obtained RAPD data are consistent with the data obtained from other marker systems [38,39].

In Figure 3, *T. dicoccoides* sample number 13 represents alone one of the two main clusters. It could be suggested that this sample might have different polymorphisms from the others. As mentioned by the authorities of Field Crops Research Institute, *T. dicoccoides* sample number 13 was collected from 1500 m (above sea level) which was the highest collection locality for *T. dicoccoides* in Turkey up to date (Table 1). Either

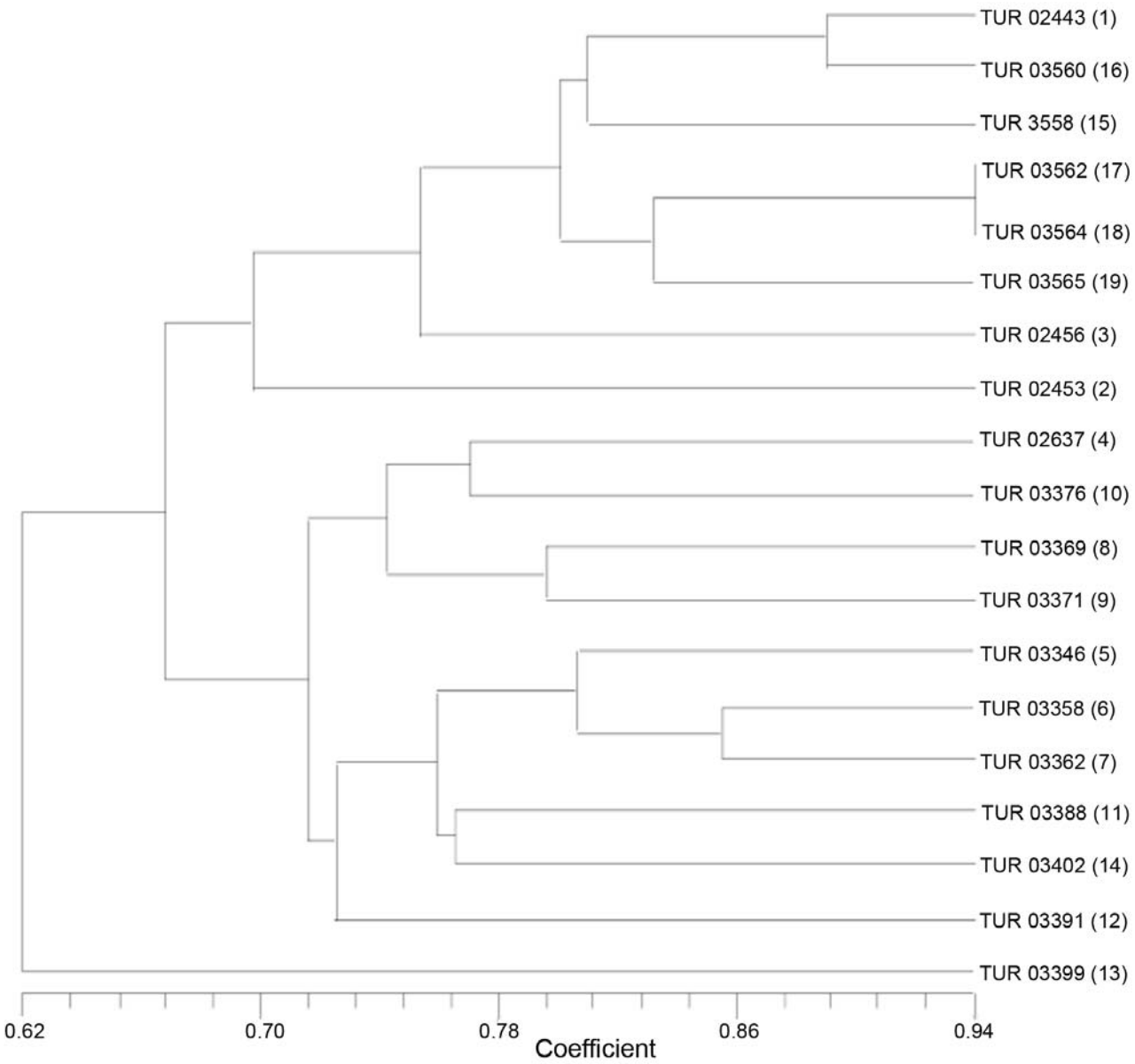

Figure 3. Dendrogram of *T. dicoccoides* and *T. dicoccon* species constructed using RAPD data.

adaptations to the changing environmental conditions those vary with altitude or other polymorphisms that emerge due to other independent causes might be responsible for these polymorphisms. According to some researchers, the ecological environment has been the major evolutionary force in wild emmer and other wild and cultivated species [40,41]. As well as longitude, latitude, climate, soil type and water availability, altitude is also one of the key triggering factors for polymorphisms to occur [17].

When the second main cluster (B) is taken into account, it seems that this cluster is divided into two subclusters originating from a common ancestor. Total of 8 *T. dicoccon* and 11 *T. dicoccoides* samples showed polymorphisms 32.9% and 41.5% within each subcluster respectively. This difference in the polymorphism percentages is consistent with the fact that cultivar forms are genetically more homogenous compared to landraces [42].

Although the above mentioned polymorphism percentages can be considered relatively lower than some other findings, they still reflect the degree of genetic diversity between these two groups. It is possible to observe different degrees of polymorphisms by using various numbers of primers

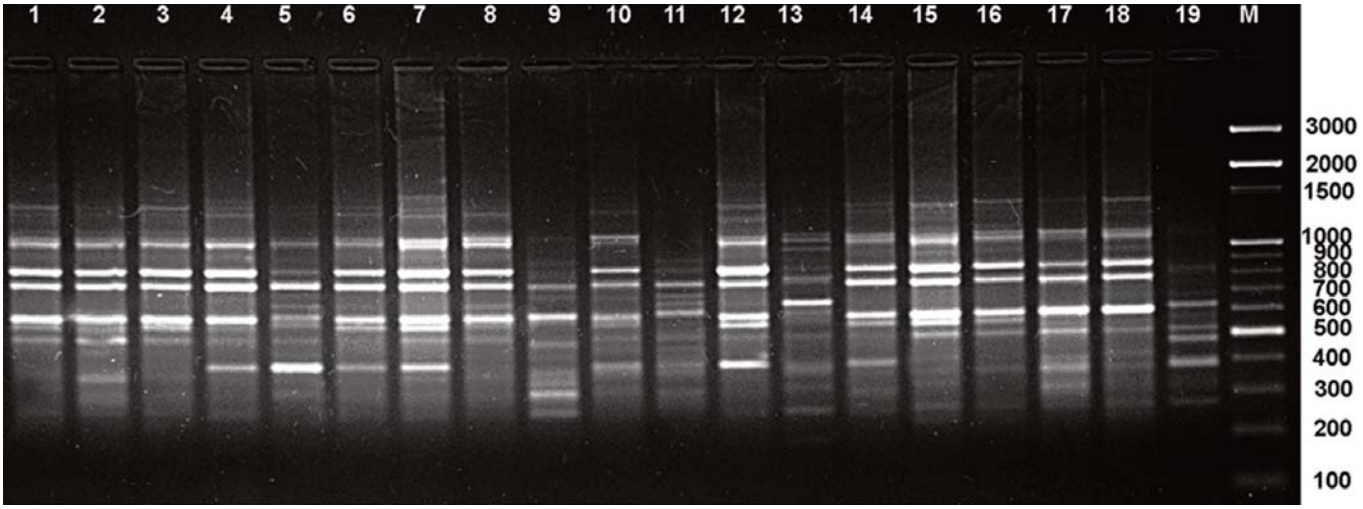

Figure 4. RAPD markers of *T. dicoccon* and *T. dicoccoides* populations using UBC 546 primer. Lane numbers correspond to numbers in Table 1. M: Molecular size marker (bp).

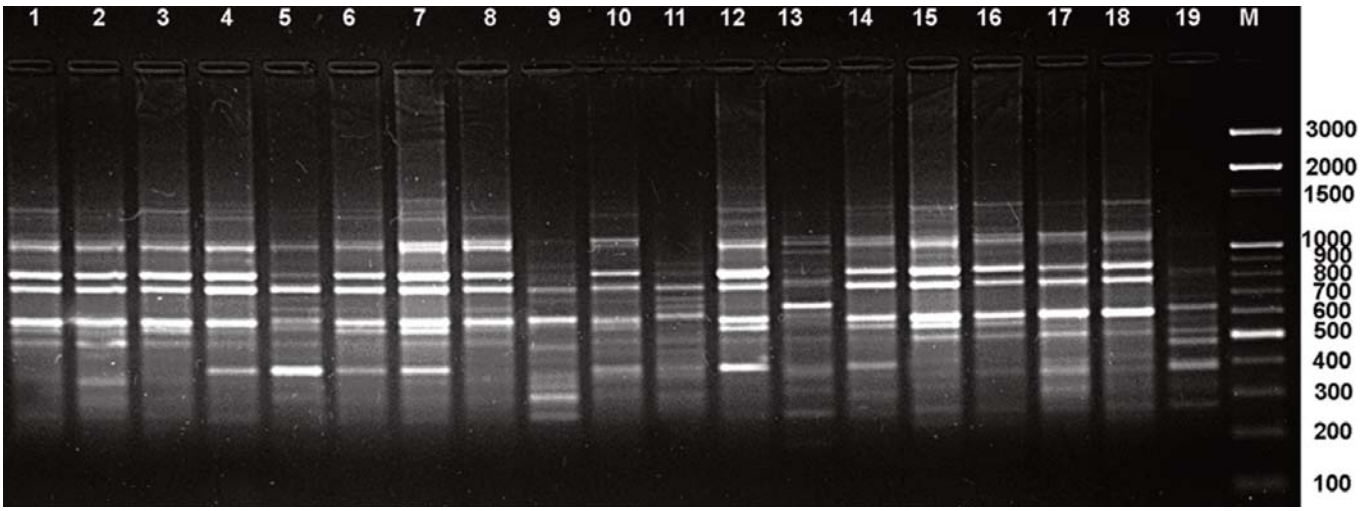

Figure 5. RAPD markers of *T. dicoccon* and *T. dicoccoides* populations using OPA 12 primer. Lane numbers correspond to numbers in Table 1. M: Molecular size marker (bp).

and different size of samples. In this sense, Aliyev et al. [43] suggest that the number of primers used in RAPD method should be neither too small nor too high. Moreover, some researchers indicate that it's only possible to assess the accurate polymorphism in *Triticum* and *Aegilops* when sufficient sample size were utilized [7,44,45]. Sun et al. [45] calculated 62.5% polymorphism among 46 genotypes of *T. aestivum* and *T. spelta* with 26 primers. In another study Fahima et al. [17] detected 81.4% polymorphism among 110 genotypes of *Triticum dicoccoides* by using 10 primers. The primers used in our study were chosen among the ones those previously revealed to be informative in *Triticum* species [7,46]. As a result, it could be suggested that lower polymorphism percentages obtained in this study might be the result of using smaller group size of *T. dicoccon* (8 samples) and *T. dicoccoides* (11 samples) to evaluate these two groups separately.

There are not many agro-morphological and molecular data available on *T. dicoccon*. Moreover, integrated approaches should be applied to reveal whether molecular data directly related to the agronomic traits or not. In this respect, Karagoz et al. [47] evaluated agro-morphologic characters such as plant height, number of days to heading, growth habit, number of stems per plant and spike length of *T. dicoccon* and *T. dicoccoides* samples. The samples of Karagoz et al.'s and ours were the same, except one. Our RAPD results and agro-morphologic data of Karagoz et al.'s were

consistently revealed the variation at two different levels among these species. In another recent study, Serpen et al. [30] quantified the phytochemicals and determined total antioxidant capacity of ancient wheat (emmer and einkorn). Five of our *T. dicoccon* populations were also among the samples analyzed by Serpen et al. Their results showed that there were significantly higher total antioxidant activity, total phenolics, ferulic acid and flavonoids in emmer samples.

In traditional breeding programmes, whereas strategies have been based on mostly agronomic, economic, and dietary criteria such as crop yield, disease resistance, protein and carbohydrate content, functionality, etc. less attention has been given on nutritional and health related properties. Recently increased evidence on nutritional and health beneficial properties of some wheat varieties including *T. dicoccon* revealed that these varieties have a potential capacity to treat diseases such as high blood cholesterol, colitis, allergies, celiac disease [48], coronary heart disease [49], type 2 diabetes [50,51], age-related eye diseases, and certain types of cancer [52-54]. This capacity could be due to the high resistant starch content and rich fibre [28], a different type of non-specific transfer proteins (nsLTPs) [55], and natural antioxidant ingredients of these varieties [30].

In addition to all these properties, as mentioned before its disease resistance, ability to grow in infertile soils even in cold climates and no requirement for elaborate techniques, *T. dicoccon* has taken renewed interest of many researchers. Moreover, since the founder genotypes of durum wheat populations were believed to be developed from emmer wheat, the rich polymorphisms that emmer encloses could be used to broaden durum and bread wheat's genetic bases [22,23]. Therefore, it's important to encourage the conservation and use of this species. Likewise, as being the immediate progenitor of all cultivated tetraploid and hexaploid wheat, wild emmer also can be used to transfer its invaluable genes to these species. Consequently, in the company of other approaches, it's crucial to analyze the genetic variation within and between these species in order to predict a way to use them efficiently in breeding and conservation programs. It's suggested that the further analysis should be done with bigger size of sample groups and via different molecular, agro-morphologic, etc. approaches.

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