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MOLECULAR ANALYSES OF THE GENERA *EREMOPYRUM* (LEDEB.) JAUB. & SPACH AND *AGROPYRON* GAERTNER (POACEAE) BY PCR METHODS

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Abstract

RAPD-PCR (Random Amplified Polymorphic DNA Polymerase Chain Reaction) and Post PCR (Polymerase Chain Reaction) Melting Curve Analysis (MCA) have been used to investigate the pattern of genetic variation among some species in the genera *Eremopyrum* (Ledeb.) Jaub. & Spach and *Agropyron* Gaertner (Poaceae). Thirteen primers have been used in the study based on the RAPD-PCR and MCA analyses. Each species produced a distinct pattern of DNA fragments which have been used as a measure of the degree of relationship between species by means of using the RAPD-PCR results with three primers selected for identifying the genetic similarities. Polymorphic melting profiles have been obtained with Post PCR MCA method using three primers. Genetic similarities are calculated for all the species studied with RAPD-PCR and MCA methods, the dendrograms are obtained with the MVSP (Multi Variate Statistical Package) software using UPGMA (Unweighted Pair Group Method with Arithmetic Averages) and Jaccard's Coefficient. Polymorphism between 18 populations of *Eremopyrum* and 6 *Agropyron* populations and within the species are determined by using RAPD-PCR and Post PCR melting curve analysis (MCA) respectively.

Introduction

Agropyron Gaertner s.l. is one of the largest genus encompassed more than 100 species in the tribe Triticeae (Dewey, 1983). Whereas Nevski (1934) treated as *Agropyron* s.str. a small genus consisting only those species which had keeled glumes. The other remaining taxa were placed in *Elytrigia* Desv*, Roegneria* C. Koch and *Elymus* L. It was agreed that *Agropyon* covering the species with P genome composed of three ploidy levels (Dewey, 1984). This narrow generic concept of *Agropyron* has been accepted by many authorities. The major Eurasian floras have followed the generic concept of Nevski (Tzvelev, 1976; Melderis *et. al*., 1980; Melderis, 1985).

Taxonomy of the Crested Wheatgrasses is especially complicated because many species and subspecies may freely hybridize with each other, and many of the hybrids are rather fertile (Knowles 1955; Asay & Dewey 1979). When several taxa grow together in breeding farms or experimental nurseries, considerable hybridization may occur between them and the identity of each collection may become obscure in future generations (Dewey, 1983).

In the Flora of Turkey, Melderis (1985) recognized only one species in the genus *Agropyron* viz. as *A. cristatum s.l.* which was further divided into two subspecies, namely subsp. *incanum* and subsp. *pectinatum.* According to this account, the first subspecies was confined to the high mountain steppes of East Anatolia and the latter was found throughout Turkey. On the other hand subsp. *pectinatum* represented by two varieties; var. *imbricatum* and var. *pectinatum*. While the former variety has the pilose spikelets and the latter has glabrous spikelets (Melderis, 1985). Löve (1984) recognized another species, *A. deweyi* from East Anatolia in Turkey. The seeds of this taxon were collected by J. R. Harlan in 1948 and cultivated in Evans Farm, Utah, U.S.A. He noted that this species might be a variant of *A. cristatum* arisen as a result of farming conditions far from its native habitat. Baum et al.(2008), designed a neotype of this species.

Eremopyrum (Ledeb.) Jaub. & Spach has been a well defined genus but there have been controversies over the concept of species in taxonomic treatments. In the past, a large number of the taxa were published in this genus, but some of them were placed in different genera in the past. *Eremopyrum* looked morphologically similar to *Agropyron*, because of this, they were often treated together under the same genus, *Agropyron* by many grass taxonomists (Grisebach, 1853). Therefore, the nomenclature of the genus has been rather confusing.

Eremopyrum consists of the species with F genome (Löve, 1984). Intergeneric hybridizations including *Eremopyrum* showed that there seemed to be a strong sterility barriers existing between *Eremopyrum* and the species included in the crossing efforts (Sakamoto 1967, 1968, 1972, 1974; Frederiksen & Bothmer 1989; Frederiksen 1993, 1994). This genus consists of diploid and tetraploid taxa. The diploid species, *E. triticeum* and *E. distans* have been well defined species and more distinct than the remaining ones. *E. orientale* is a tetraploid, thought to be originated from the diploid parents' *E. triticeum* and *E. distans* via allopolyploidization (Sakamoto, 1979). *E. boneapartis,* a species complex, includes both diploid and tetraploid taxa. Tetraploid taxa were also proposed as an allopolyploid, originated from the diploid parents *E. boneapartis* and *E. distans* (Sakamoto, 1979).

In the Flora of Turkey, Melderis (1985) recognized four species and two subspecies, namely, *E. distans* (K.Koch) Nevski, *E. orientale* (L.) Jaub. and Spach and *E. triticeum* (Gaertner) Nevski. *E. boneapartis* subsp. *boneapartis* (Spreng) Nevski, *E. boneapartis* subsp. *hirsutum* (Bertol.) Melderis.

RAPD-PCR is used in molecular systematics and constitution of plant genome successfully. Polymorphisms are detected by gel electrophoresis and thus RAPD markers are identified due to the sequence differences in the primer binding sites. Therefore, RAPDs are dominant markers. RAPDs are based on using only a single primer of about 8-10 nucleotides for DNA amplification (Babaoğlu *et al*., 2004).

Post-PCR MCA has become a robust and well-established method to characterize amplicons, for applications that include identification or the detection of polymorphisms. This latter approach is especially useful as a screening technique to reduce the number of sequencing reactions required to detect polymorphisms (Hoffmann *et al.,* 2007).

The aim of the study is to detect the genetic relationships and the polymorphism between the populations of the species and the intraspesific taxa of *Eremopyrum* (Ledeb.) Jaub. & Spach and *Agropyron* Gaertner (Poaceae) found in Anatolia by means of using RAPD-PCR and Post PCR melting curve analysis with arbitrary primers.

Material and Methods

Plant materials used: Dry specimens of 18 *Eremopyrum* (Ledeb.) Jaub. & Spach populations and 6 *Agropyron* Gaertner (Poaceae) populations used in this study were collected by the authors from different localities of Anatolia during the field surveys carried out as a part of a research project sponsored by the Turkish Scientific and Technical Reseach Council (TUBITAK) for a three year period starting from 2006. Species names of the populations and their locations are given in Table 1.

Primers used: The oligonucleotide primers have been obtained from Research Genetics Inc. (Huntsuille Al USA) and are listed Table 2. Potential RAPD markers as single arbitrary oligonucleotide primers (10-mers) for population studies in plants were choosen from literature (Massawe *et al*., 2003; Gomez *et al*., 2011).

Table 1. The species names and their locations used in this study.

Sample ID	Species name	Location	Altitude (m.)	Collector number
1.	*E. orientale*	Ankara; 30 km to Şereflikoçhisar.	906	E. Cabi 2247
2.	*E. orientale*	Aksaray; Aksaray to Şereflikoçhisar, around Tuz lake		G. Akaydın 7764c
3.	*E. orientale*	Konya; Cihanbeyli, Tuzla, around Karatepe village.	916	E. Cabi 008
4.	*E. orientale*	Ankara; 8 km to Sereflikochisar.	917	E. Cabi 2248
5.	*E. orientale*	Erzurum; Horasan town centre, roadside and wall side	1715	E. Cabi 2493
6.	*E.boneapartis* subsp. *boneapartis*	Urfa; Ceylanpınar, Cevri mainroad	478	E. Cabi 2252
7.	*E.boneapartis* subsp. *boneapartis*	Urfa; Ceylanpınar, edge of Gümüşsu and Beyazkule, Şıhanlı valley	401	E. Cabi 2253
8.	*E.boneapartis* subsp. *hirsutum*	Konya; Kulu to Konya 7-8 km, edge of mainroads, degraded areas	1136	E. Cabi 2242
9.	*E.boneapartis* subsp. *hirsutum*	Sivas; Boğazlıdere to Kutlukaya	1471	E. Cabi 1306
10.	*E.boneapartis* subsp. *hirsutum*	Konya; Cihanbeyli Tuz enterprise; edge of Tuz lake	909	E. Cabi 2245
11.	*E.boneapartis* subsp. *hirsutum*	Van; Van to Erciş,10-15 km to Erciş		G. Akaydın 7690
12.	*E.boneapartis* subsp. *hirsutum*	Kayseri; Sultanhanı to Bünyan 5 km from Sultanhanı	1203	E. Cabi 2249
13.	*E.boneapartis* subsp. *hirsutum*	Konya; Kulu to Konya 7-8 km, roadside, disturbed areas	1136	E. Cabi 2242
14.	*E. triticeum*	Ankara; Polatlı to Sivrihisar, 26 km from Polatli	814	E. Cabi 339
15.	*E. triticeum*	Konya; Yavşan Memlehanesi near Salt lake	908	E. Cabi 1706
16.	*E. triticeum*	Erzurum; Horasan town centre, roadsides	1715	E. Cabi 2492
17.	*E.distans*	Ağri; 9, 5 km from Doğubeyazıt to Iğdir	1545	E. Cabi 2256
18.	*E.distans*	Ağrı; Ağrı to Doğubeyazit	1730	E. Cabi 2506
19.	*A. cristatum* subsp. *pectinatum* var. *pectinatum*	Kars; Kuyucak village, Kuyucuk lake environs, dry pastures	1642	E. Cabi 2258
20.	*A. cristatum* subsp. *pectinatum* var. *pectinatum*	Sivas; Sivas Cumhuriyet University Campus, roadside and under the forest	1275	E. Cabi 2244
21.	*A. cristatum* subsp. *pectinatum* var. *pectinatum*	Ankara; Çayırhan Bird Sanctualy		G. Akaydın 10319
22.	*Agropyron cristatum* subsp. *pectinatum* var. *imbricatum*	Kars; Kuyucak village, Kuyucuk lake environs, dry pastures	1642	E. Cabi 2526
23.	*Agropyron cristatum* subsp. *incanum*	Van; Artos Mountain, edge between Gevaş and Çatak		
24.	*Agropyron cristatum* subsp. *incanum*	Erzurum; Aşkale to Bayburt, Kop mount, Kop pass, calcareous slopes	2401	E. Cabi 2545

Table 2. Primers used for RAPD-PCR and post PCR MCA.

Primer	Sequence (5´.....3´)
M1	GCTGCGGGAA
M2	GGTTCGCTCC
M3	*GTAGACCCGT*
M4	AAGAGCCCGT
M5	AACGCGCAAC
M6	CCCGTCAGCA
M13	GAGGGTGGCGGTTCT
B18	CCACAGCAGT
O3	GTGACGTAGG
O4	AATCGGGCTG
O11	TTATGAAACGACGGCCAGT
OPI 18	TGCCCAGCCT
OPB 08	GTCCACACGG

DNA extraction made for molecular analysis: DNA was extracted from dry leaves by using the DNA isolation kit (Roche Diagnostics, Manheim, Germany) for raw material of plant origin according to the manufacturer's instructions. Extracted DNA concentrations were measured with UV Visible Spectrophotometer (Model Carry 100, Varian, Netherlands). DNA concentrations in all samples were adjusted to 5 ng/µL. DNA preparations were stored at -20°C.

Random amplified polymorphic DNA polymerase chain reaction analysis: RAPD-PCR amplifications were performed using LightCycler 1.5 (Roche) in a total volume of 20 µL containing DNA Master SYBR Green I (Roche, 12 015 099 001) and DNA. DNA amplification program is as follows for all primers: an initial denaturation (5 min 95^{o}C), 45 cycles consisting of 20 s at 95^{o}C, 10 s at 40^{o}C, and 30 s at 72^{o}C segments and then cooling at 40^{o}C for 30 s. PCR products were electrophoresed in 1.5% agarose gels and photographed with a gel documentation system (Vilber Lourmat Image Analysis System, France).

Post polymerase chain reaction melting curve analysis: After initial polymerase activation and denaturation step at 95°C for 5 min, the samples underwent 45 amplification cycles, each comprising denaturation (95°C for 20 s), annealing (40°C for 10 s), and extension (72°C for 30 s) in the LightCycler instrument. The temperature transition rates were programmed at 20°C/s; and the fluorimeter gains were set with F1 equal to 1 (at 530 nm, measured in channel 1). Fluorescence was measured at the end of the annealing period of each cycle to monitor the progress of amplification. After completion, a melting curve was plotted by slow heating at 0.1°C/s until 65°C followed by cooling to 40°C at 20°C/s and finally holding at 40°C for 30 s. Fluorescence was measured continuously during the slow temperature rise.

Data analysis: Gel results of each amplicon were scored (present 1, absent 0) according to the presence and absence of fragments. Likewise, MCA results were also scored according to the presence or absence (present 1, absent 0) melting curve peaks. Scored data were fed into MVSP (Multi Variate Statistical Package) software (Kovach, 1999) using UPGMA (Unweighted Pair Group Method with Arithmetic Averages) and Jaccard's (1901) Coefficient to construct dendograms.

Results and Discussion

In this study, RAPD-PCR profiles of *Eremopyrum boneapartis* subsp. *hirsutum* (6 locations), *E. orientale* (5 locations), *E. triticeum* (3 locations), *E. boneapartis* subsp. *boneapartis* (2 locations), *E. distans* (2 locations); *Agropyron cristatum* subsp. *pectinatum var. pectinatum* (3 locations), *A. cristatum* subsp. *incanum* (2 locations), *A. cristatum* subsp. *pectinatum* var. *imbricatum* (1 location) have been obtained. Thirteen random primers are tested. Polymorphic bands are obtained with three primers, M3; M13 and OPI18 which are selected on the basis of the reproducibility, distribution, number and intensity of the bands (data is not shown here). All results are obtained in duplicate.

Dendrogram obtained from RAPD-PCR results, shows that two main groups to be distinguished on the basis of the populations used as the representative of the genera *Agropyron* and *Eremopyrum* (Fig. 1). Genetic diversity value between these two groups is determined as 0.20 to 0.50 using Jaccard's coefficient. *Agropyron* group contains subgroups of 2, *A. cristatum* subsp. *incanum* (ID 24 and ID 23), 1 *A. cristatum* subsp. *pectinatum* var. *imbricatum* (ID 22), and 3, *A. cristatum* subsp. *pectinatum var. pectinatum* (ID 20 and ID 21, ID19) respectively. *A. cristatum* subsp. *incanum*, ID 23 (Van), and ID 24 (Erzurum); *A. cristatum* subsp. *pectinatum* var. *pectinatum* ID 19 (Kars) and ID 21 (Ankara) are included in the same sub cluster with 0.0 using Jaccard's coefficient.

The second group covers all of the species of *Eremopyrum* with significant genetic similarity between *E. boneapartis* subsp. *hirsutum*, *E. orientale*, *E. boneapartis* subsp. *boneapartis, E. triticeum* and *E. distans* species. Interestingly, *E. distans* ID 17 (Ağrı) and ID 18 (Ağrı); *E. boneapartis* subsp. *hirsutum* ID 8 (Konya), ID 9 (Sivas) and ID 12 (Kayseri) are included in the same sub cluster with 0.0 using Jaccard's coefficient. Molecular data are found in most cases congruent with species on morphologically based data except one, *E. orientale* (ID 5).

Generally, melting curve analysis (MCA) is a fast and the post-PCR high throughput the method to scan for sequence variations in a targeted sequence. This study demonstrates for the first time, determination of genetic variations using the MCA with arbitrary primers in *Eremopyrum* and *Agropyron*. Result of this study revealed that genetic variation between *Eremopyrum* and *Agropyron* melting profile data reported in Tables 3, 4 and 5 have been obtained in triplicate by pooling DNA from multiple individuals prior to PCR amplification. This approach is also well suited for determination of the intra and the interspecies polymorphism in *Eremopyrum* (Ledeb.) Jaub. & Spach and *Agropyron* Gaertner (Poaceae).

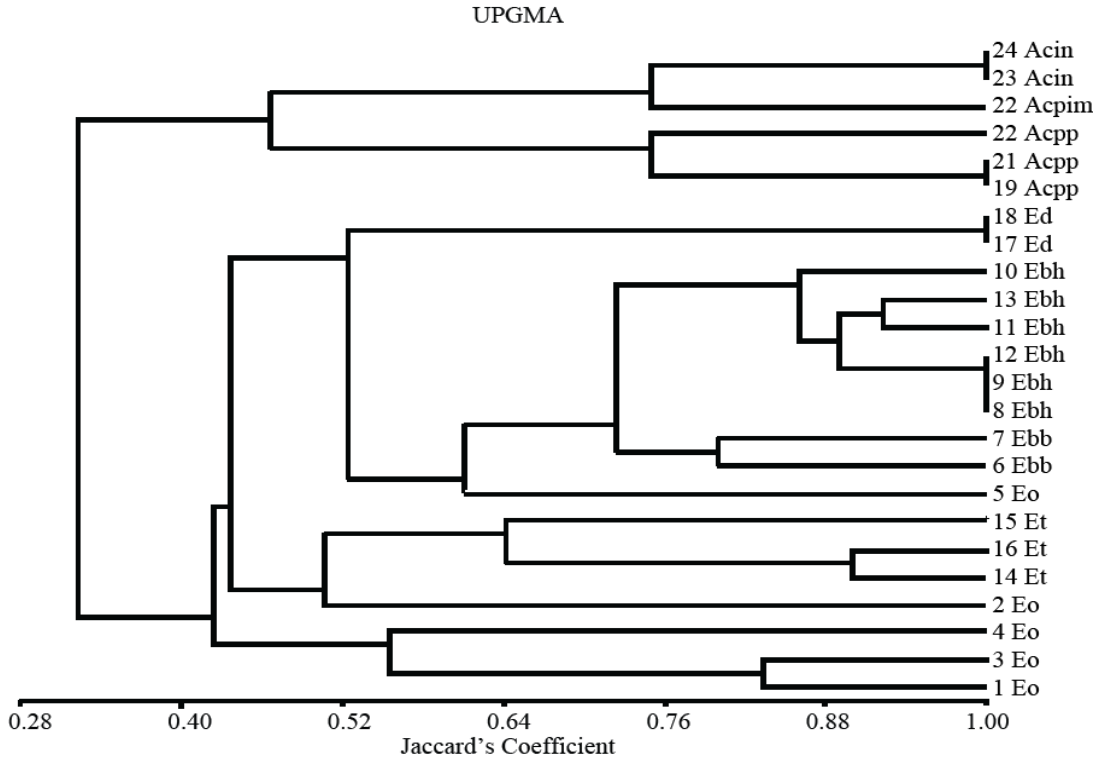

Fig. 1. Dendogram of *Eremopyrum* (Ledeb.) Jaub. & Spach species and *Agropyron* Gaertner species constructed by using RAPD-PCR results. Acin: *A. cristatum* subsp. *incanum*; Acpim: *A. cristatum* subsp. *pectinatum* var. *imbricatum*; Acpp: *A. cristatum* subsp. *pectinatum* var. *pectinatum*; Ed: *E. distans*; Ebh: *E. boneapartis* subsp. *hirsutum*; Ebb:*E. boneapartis* subsp. *boneapartis*; Eo: *E. orientale*; Et: *E. triticeum*

Table 3. Melting curve peaks results of *Eremopyrum* (Ledeb.) Jaub. & Spach species and *Agropyron* Gaertner species constructed by using M3 primer.

Species	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9
Eo	-	76.33	-	-	85.59	-	-	88.58	-
Ebb	-	76.79	-	82.57	-	-	-	88.22	-
Ebh	-	-	-	-	-	-	87.10	-	90.33
Et	-	75.36	-	80.76	-	-	87.18	-	90.46
Ed	-	-	-	-	-	86.54	-	-	90.05
Acpp	69.8	77.61	-	-	-	-	-	-	90.10
Acpim	70.4	-	-	83.29	-	-	87.95	-	-
Acin	70.3	-	79.21	-	-	86.25	-	-	-

Table 4. Melting curve peaks results of *Eremopyrum* (Ledeb.) Jaub. & Spach species and *Agropyron* Gaertner species constructed by using M13 primer.

Species	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Eo	-	77.97	-	-	87.00	-
Ebb	73.82	77.42	80.42	-	88.00	91.82
Ebh	74.48	77.81	80.42	-	86.50	-
Et	74.4	77.80	81.20	-	87.89	91.41
Ed	-	76.42	-	-	88.19	-
Acpp	-	-	-	84.27	-	-
Acpim	-	-	-	83.46	-	90.38
Acin	-	-	-	84.20	-	89.47

Table 5. Melting curve peaks results of *Eremopyrum* (Ledeb.) Jaub. & Spach species and *Agropyron* Gaertner species constructed by using OPI18 primer.

Species	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
Eo	74.65	-	-	-	-	85.94	90.03
Ebb	73.82	-	-	-	-	87.32	-
Ebh	74.77	-	-	-	-	87.05	-
Et	74.62	80.44	-	84.82	-	-	88.89
Ed	74.74	-	-	-	85.45	-	89.21
Acpp	74.63	79.41	-	-	-	87.45	-
Acpim	74.76	79.72	-	-	-	87.13	-
Acin	75.23	-	82.61	-	-	87.97	-

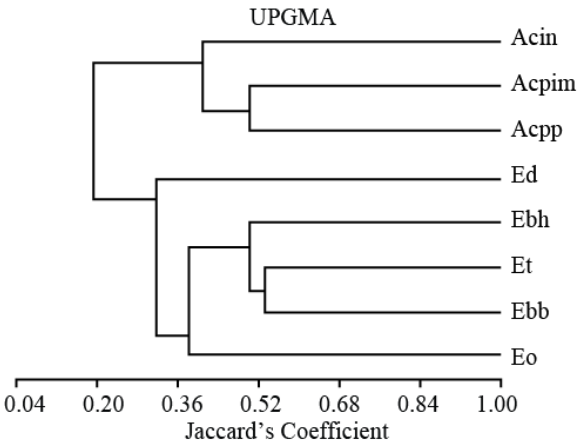

Fig. 2. Dendogram of *Eremopyrum* (Ledeb.) Jaub. & Spach species and *Agropyron* Gaertner species constructed by using Post PCR MCA results. Acin: *A. cristatum* subsp. *incanum*; Acpim: *A. cristatum* subsp. *pectinatum* var. *imbricatum*; Acpp: *A. cristatum* subsp. *pectinatum* var. *pectinatum*; Ed: *E. distans*; Ebh: *E. boneapartis* subsp. *hirsutum*; Ebb: *E. boneapartis* subsp. *boneapartis*; Eo: *E. orientale*; Et: *E. triticeum*

The taxa of *Eremopyrum* and the taxa of *Agropyron* are grouped into two major clusters. The first cluster is consisted of *A. cristatum* subsp. *incanum, A. cristatum* subsp. *pectinatum* var. *imbricatum* and *A. cristatum* subs. *pectinatum* var. *pectinatum* (Fig. 2). The second cluster is further divided into three more sub-clusters; *E. distans*; *E. boneapartis* subsp. *hirsutum, E. triticeum, E. boneapartis* subsp. *boneapartis* and *E. orientale*. The similarity matrix of the Post PCR MCA data is indicated that the species of *Eremopyrum* and the species of *Agropyron* are genetic similarity as they have showed the similarity values of 0.19 and 0.53 using the Jaccard's coefficient. Almost identical similarity values are obtained with the RAPD-PCR experiment (0.20 to 0.50).

Discussion

Molecular analyses used in this research study have indicated the intra and the interspecies polymorphism between 18 *Eremopyrum* populations and 6 *Agropyron* populations. RAPD-PCR and Post PCR MCA results with arbitrary primers have been shown to be useful methods to compliment morphological method results. We have named this new approach the Post PCR MCA with arbitrary primers to emphasize its rapid and high throughout its nature. We will continue our efforts to prove interlaboratory reproducibility, sensitivity, specificity, and predictive value of this approach in other molecular taxonomic studies.

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