Pak. J. Bot., 41(5): 2477-2486, 2009.

BIOSYSTEMATICS STUDIES AMONG *EBENUS* L. SPECIES BASED ON MORPHOLOGICAL, RAPD-PCR AND SEED PROTEIN ANALYSES IN TURKEY

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Abstract

Taxonomic status of 14 Turkish endemic species and one variety of the genus *Ebenus* L. was analyzed using seed proteins and RAPD markers. These profiles were evaluated as characters to clarify the taxonomic relationships in the genus. A total number of 136 protein bands from all species, with molecular weight ranging between 116 kDa and 21 kDa were detected in SDS-PAGE electrophoregrams of the species studied. A total of 99 RAPD markers were generated by the 42 primers. The relative similarity between various taxa estimated by Jaccard's similarity index and clustered in UPGMA, is generally in accordance with taxonomic position. However, there are slightly different arrangements of these species from Flora of Turkey. *Ebenus argentea* Siehe ex Bornm. and *Ebenus longipes* Boiss. & Bal., are two different species in Flora of Turkey. However, this study revealed a close relationship between *E. argentea* and *E. longipes*. These taxa are clustered together and show genetic distance of 0.4% by SDS-PAGE and 33% by RAPD-PCR analysis. *Ebenus plumosa* Boiss. & Bal. var. *plumosa* has a seed protein banding profile very similar to *Ebenus plumosa* var. *speciosa* Hub.-Mor., genetic distance of 0.4%. In case of RAPD data, the genetic distance between two taxa is 44%.

Introduction

Ebenus L., (subfamily *Papilionoideae*) is a member of the Leguminosae and all the species in the genus in Turkey are endemic (Davis, 1988). The genus *Ebenus* is represented by a total of 15 taxa, including 14 species and one variety belonging to the *Euebenus* Boiss. Section (Huber-Morath, 1965) in Turkey. Of these taxa, 8 were Irano-Turanian elements and 7 East Mediterranean. The genus has 6 species occurring outside Turkey. The species in Turkey are found principally in the Mediterranean and inner Anatolian regions, as well as in the Aegean, east Anatolia and to a lesser extent the Marmara region (Aytaç, 2000). Although in terms of appearance, the genus *Ebenus* resembles *Astragalus* L., (milk-vetches), they can easily be distinguished from *Astragalus* by the calyx teeth in the flower structure which in *Ebenus* are at least twice as long as the tube, filaments joined in tube form (monadelphous stamens) and wings small enough to fit inside the calyx tube. Whilst there is no Turkish common name for the genus *Ebenus*, the plants are often called "morgeven" or "sarıgeven" by the locals because of their resemblance to milk-vetch (*Astragalus*) species. Since the flowers are generally purple in Turkey some species of the genus *Ebenus* could be utilized be horticulturally because for their colourful flowers. Secondly, as members of the genus often grow on poor soils, they could be used for erosion (Aytaç, 2000).

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Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based classification (Cnengyin *et al.,* 1992; Awasthi *et al.*, 2004). Electrophoretic patterns of total seed proteins as revealed by polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulphate (SDS) have been successfully used to resolve the taxonomic and evolutionary problems of some plant species (Ladizinsky & Hymowitz, 1979; Potokina *et al.*, 2000, Ghafoor & Arshad, 2008). PCR based techniques developed in recent years such as Random Amplified Polymorphic DNA (RAPD) (Willams *et al.*, 1990), are used to resolve taxonomical problems (Welsh *et al.,* 1991; Ranade & Farooki, 2002; Abbas *et al.,* 2009; Bibi *et al.,* 2009a, 2009b) on the basis of the discrete profiles produced. With respect to the family Leguminosae, some recent examples of PCR studies are done by Taylor-Grant & Soliman (1999), Potokina *et al.,* (1999) and Shehata (2004). The objectives of this study were to determine variations among the 14 Turkish endemic species and one variety of the genus *Ebenus* L., (Leguminosae) by using seed protein profiles (SDS-PAGE) and RAPD-PCR techniques. Accordingly, combined the molecular data with morphological data in order to obtain a better taxonomic classification of *Ebenus.*

Material and Methods

Plant material: The seed and dry leaves belonging to species of the genus *Ebenus* studied in this paper were obtained from collection of Zeki AYTAÇ at the herbarium of GAZI.

SDS-PAGE analysis: The total protein samples were extracted as described by Saraswati *et al.,* (1993). Total protein analysis was carried out by using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method described by Laemmli (1970). The gels were stained overnight with Coomassie Brilliant Blue G-250 according to Demiralp *et al.,* (2000).

DNA extraction and amplification: The CTAB (hexadecyltri-methylammonium bromide) method of Clark (1997) was used to extract total DNA. Amplification of genomic DNA was made on a Techne (UK) Progene thermocycler, using the arbitrary decamers. The 42 primers (Table 1) were selected from Operon Technologies (Alameda CA, USA). RAPD assays were performed according to the process recommended by Williams *et al.*, (1990). Amplification was carried out 45 cycles of 40 s at 96°C, 40 s at 30°C, 40 s at 72°C. PCR products generated by amplification were separated on 2% (w/v) agarose gel in TAE buffer at 40-90 V, stained with ethidium bromide. DNA fragments were visualized on UV light then photographed with Polaroid camera (DS34 Polaroid Direct Screen Instant Camera 100x81) using Polaroid Studio B/W film.

Data analysis: Protein patterns and amplification products generated by RAPD were scored for the presence (1) or absence (0) and compared with each other. The relative similarity between various taxa was estimated by Jaccard's similarity index and clustered in UPGMA (Nei, 1972).

Table 1. Primers used in the investigation.

Primer No.	Primer sequence (5'→3')	Primer no.	Primer sequence (5'→3')
A1	CAGGCCCTTC	UBC587	GCTACTAACC
A2	TGCCGAGCTG	UBC589	GACGGAGGTC
A7	GAAACGGGTG	OPB05	TGCGCCCTTC
B4	GGACTGGAGT	OPB08	GTCCACACGG
B6	TGCTCTGCCC	OPB10	CTGCTGGGAC
B7	GGTGACGCAG	OPA07	GAAACGGGTG
B15	GGAGGGTGTT	OPC 02	GGTCTACACC
B18	CCACAGCAGT	OPO02	TACCTTCCGT
SC1023	GGCTCGTACC	OPO04	AAGTCCGCTC
SCI038	GACCCCGGCA	OPO06	CCACGGGAAG
SCI053	CAGGGGACGA	OPR03	ACACAGAGGG
SC1079	CGCCACGTTC	OPU16	CTGCGCTGGA
SCI091	CTCGACTAGG	OPI18	TGCCCAGCCT
UBC372	CCCACTGACG	OPW 06	AGGCCCGATG
UBC373	CTGAGGAGTG	OPW10	TCGCATCCCT
UBC378	GACAACAGGA	LAI	GTGATCGCAC
UBC379	GGGCTAGGGT	LAII	GTTGCGATCC
UBC435	CTAGTAGGGG	LA12	ACGACCCACG
UBC440	CTGTCGAACC	LA13	CACCACGCCT
UBC441	CTGCGTTCTT	UTE	AGGCATCGTTGG
UBC586	CCGGTTCCAG	M13	GAGGGTGGCGGTTCT

Results and Discussion

The SDS-PAGE profile of the genus *Ebenus* seed proteins studied showed a range of bands varying from 21 kDa to116 kDa (Fermentas SM0431) (Fig. 1). A total of 136 protein bands from all taxa were considered for the taxonomic study. A similarity analysis based on SDS-PAGE profile of seed proteins was carried out. The software (Dendrogram Based Nei's (1972), Genetic distance: Method = UPGMA, Modified from NEIGHBOR procedure of PHYLIP Version 3.5) used displayed a dendrogram. Two major clusters were obtained (Fig. 2). Among these group, *E. plumosa* var. *plumosa* differs from *E. plumosa* var. *speciosa* by a genetic distance of 0.4%, *E. cappadocica* Hausskn. & Siehe ex Bornm differs from *E. plumosa* var. *plumosa* and *E. plumosa* var. *speciosa* by a genetic distance of 12% and 8% respectively, *E. macrophylla* Jaub. & Spach differs from *E. plumosa* var. *plumosa*, *E. plumosa* var. *speciosa* and *E. macrophylla* by a genetic distance of 12%, 17% and 27% respectively, *E. barbigera* Boiss., differs from *E. reesei* Hub.-Mor. by a genetic distance of 17% (Table 2). *E. boissieri* Barbey differs from *E. longipes* by a genetic distance of 8%; *E. bourgaei* Boiss., differs from *E. boissieri* and *E. longipes* by a genetic distance of 22% and 12% respectively. *E. argentea* differs from *E. pisidica* and *E. laguroides* by a genetic distance of 4%. *E. haussknechtii* Bornm., ex Hub.-Mor. showed the maximum distance to these two groups.

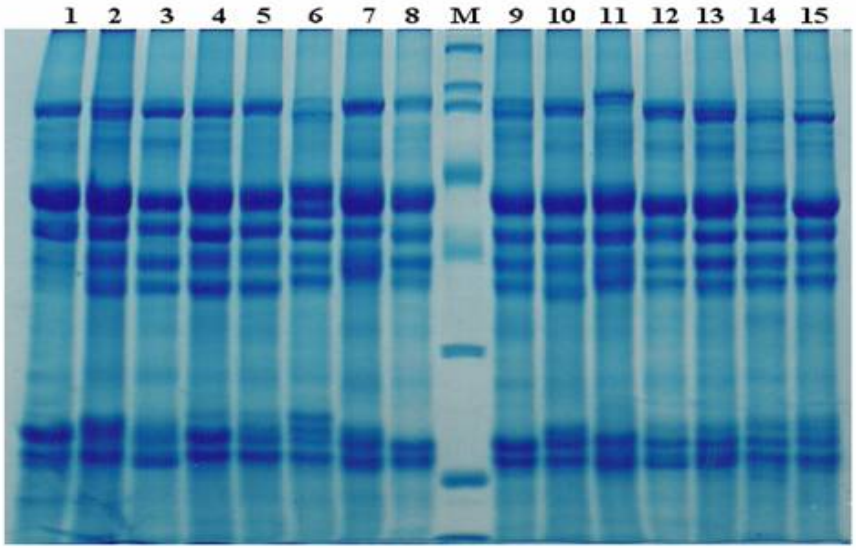

Fig. 1. Electrophoretic pattern produced by SDS-PAGE analysis of seed proteins of *Ebenus* species. (1) *E. macrophylla,* (2) *E. plumosa* var. *plumosa,* (3) *E. plumosa* var. *speciosa,* (4) *E. barbigera,* (5) *E. reesei,* (6) *E. haussknechtii,* (7) *E. depressa,* (8) *E. bourgaei,* (M) Marker; (Myosin (200 kDa), β-galaktosidaz (116 kDa), fosforilaz b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), karbonik anhidraz (31 kDa), tiripsin inhibitör (21 kDa), lizozim (14 kDa), (9) *E. cappadocica,* (10) *E boissieri,* (11) *E. hirsuta,* 12- *E. longipes,* (13) *E. argentea,* (14) *E. laguroides,* (15) *E. pisidica.*

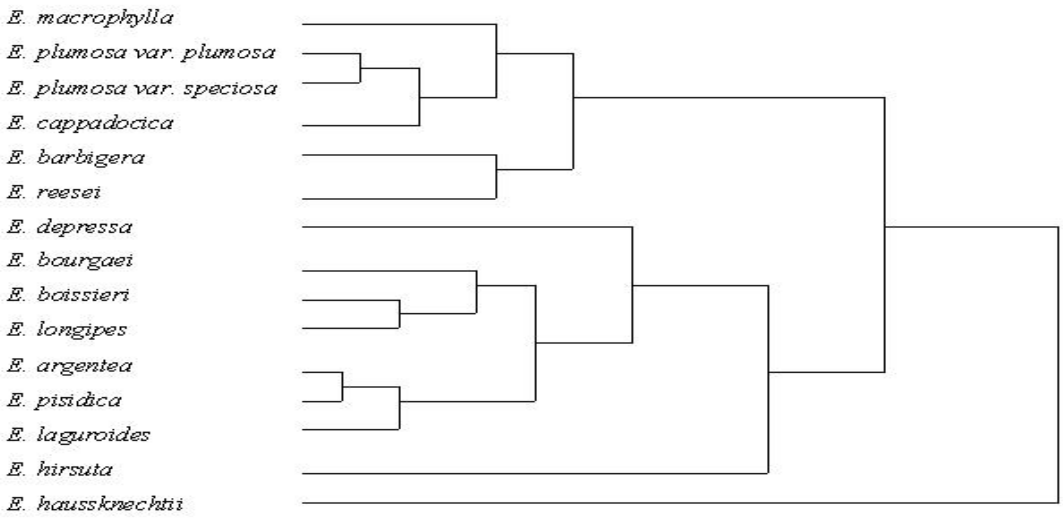

Fig. 2. UPGMA phenogram based on similarity matrix of seed storage proteins of *Ebenus* species.

A total of 99 RAPD markers were generated by the 42 primers, at a rate of 2.35 markers per primer. The size of the amplified products ranged from 150 – 1200 bp, with 1–5 bands per primer. Three primers (M13, OPI 18 and UTE) were finally selected for data matrix building and cluster analysis. Fig. 3 (Fermentas, Gene Ruler^{TM} DNA Ladder Mix, SMO331) shows the amplification products with primer UTE. Distance matrix analysis of the RAPD data was calculated using the computer program POPGENE (Data not shown). Genetic distance among studied species ranged from 0.11 (between *E. macrophylla* and *E. boissieri*) to 0.76 (between *E. plumosa* var. *plumosa* and *E. reesei*, and *E. cappadocica* and *E. haussknechtii*). The distance matrix based on RAPD data sets is graphically represented as a dendrogram using the UPGMA method. The dendrogram was divided into two main clusters (Fig. 4).

Table 2. Genetic distance values (%) based on seed storage proteins of *Ebenus* species.

	E. macrophylla	*E. plum.* var.*plum.*	*E. plum.* var.spec	*E. barbigera*	*E. reesei*	*E. haussknechtii*	*E. depressa*	*E. bourgaei*	*E. cappadocica*	*E. boissieri*	*E. hirsuta*	*E. longipes*	*E. argentea*	*E. lagurcides*	*E. pisidica*
E. macrophylla	**														
E.plum.var.plum.	12	**													
E.plum.var.spec	17	04	**												
E.barbigera	32	17	22	**											
E.reesei	32	27	22	17	**										
E.haussknechtii	65	57	51	44	32	**									
E.depressa	38	44	38	73	44	82	**								
E.bourgaei	38	44	38	73	44	65	27	**							
E.cappadocica	27	12	08	32	32	65	27	27	**						
E.boissieri	32	27	22	38	27	73	22	22	12	**					
E.hirsuta	44	38	32	65	51	91	44	32	22	27	**				
E.longipes	32	38	32	51	38	57	22	12	22	08	38	**			
E.argentea	51	44	38	57	57	65	38	17	27	22	32	12	**		
E.lagurcides	57	51	44	65	65	57	44	22	32	27	38	17	04	**	
E.pisidica	51	44	38	57	57	65	38	17	27	22	32	12	04	04	**

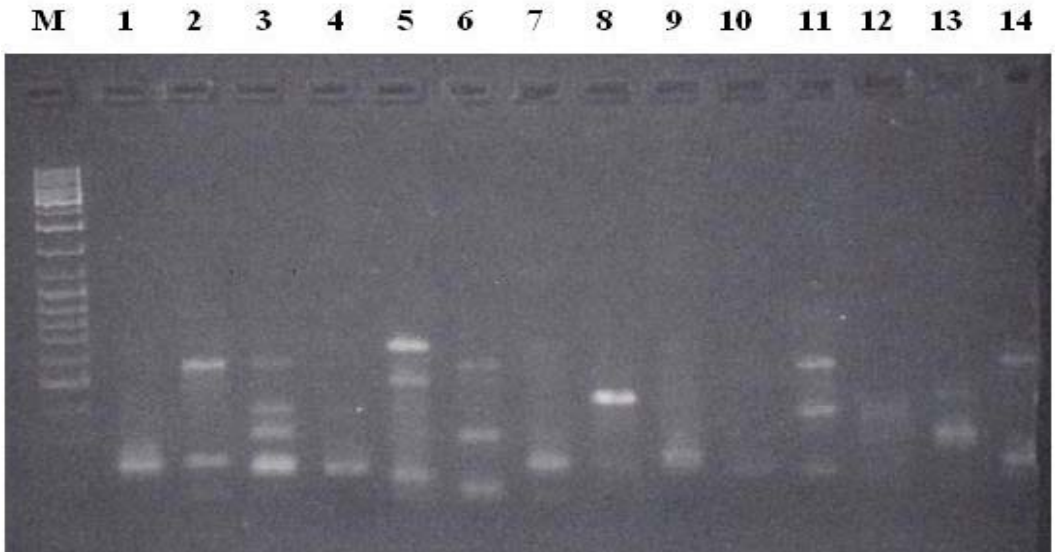

Fig. 3. RAPD profiles generated with the primer UTE. M: Marker (Fermentas, Gene Ruler^{TM} DNA Ladder Mix, SMO331; 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp) 1-*E. macrophylla* 2-*E. plumosa* var. *plumosa* 3-*E. plumosa* var. *speciosa* 4-*E. barbigera* 5-*E. reesei* 6-*E. haussknechtii* 7-*E. depressa* 8-*E. bourgaei* 9-*E. cappadocica* 10-*E boissieri* 11- *E. hirsuta* 12- *E. longipes* 13- *E. argentea* 14- *E. laguroides.*

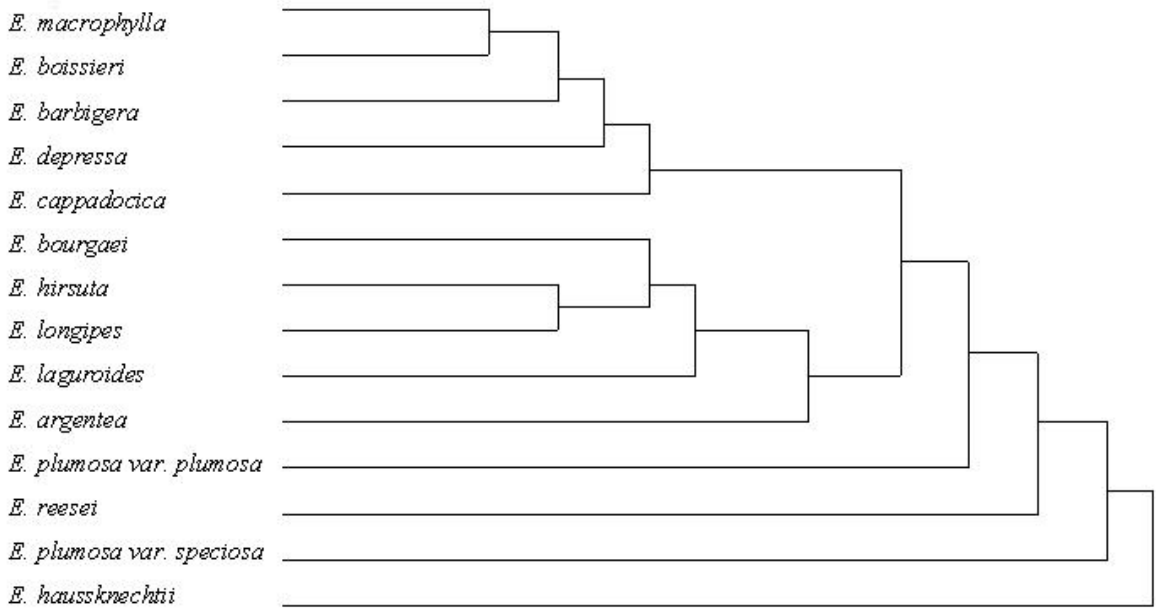

Fig. 4. UPGMA cluster analysis of RAPD data generated by M13, OPI 18 and UTE primers for *Ebenus* species.

Some of the RAPD markers used in this study were able to amplify one or two species. Thus, showed the utility of RAPD marker in the identification of species. One specific band was observed in species *E. macrophylla* (primer P2), whereas two such bands were observed in *E. reesei* and *E. laguroides* (primer OPA 01), *E. plumosa* var. *speciosa* and *E. reesei* (primer P3), *E. macrophylla* and *E. plumosa* var. *plumosa* (primer OPR 03). These bands could be potential species-specific markers after checking that every individual from that species shows the marker in question. These bands could be

potential species-specific markers after checking that every individual from that species shows the marker in question (Roman *et al*., 2003).

The present study is the first report of the use of a protein and DNA-based polymorphism assay to assess the variability on the Turkish endemic genus *Ebenus*. In the previous studies, only a few chemical works (Mitrocosta *et al.*, 1999), palynological and cytotaxonomical studies (Aytaç *et al.,* 2000, Aksoy *et al.,* 2001), fatty acid composition of *Ebenus* species seeds (Azcan *et al.,* 2001), and fractionating the storage protein of *Ebenus cretica* L., seeds (Syros *et al*., 2003) have been recorded on the *Ebenus* species but there are no report dealing with molecular studies.

Morphological, palynological and cytotaxonomical studies based on two varieties. *E. plumosa* var. *plumosa* and *E. plumosa* var. *speciosa,* the species revealed no structural differences (Aytaç, 2000; Pınar *et al.*, 2000). SDS-PAGE results revealed a close relationship between *E. plumosa* var. *plumosa* and *E. plumosa* var. *speciosa* at the genetic genetic distance of 0.4%. Aksoy *et al.,* (2001) reported that although morphologically the two varieties can be distinguished and the morphological identification based on size and number of flowers and length of the spike, which sometimes is not enough to separate them, however, there are cytological differences which exist between the two varieties. Despite all these, in case of RAPD, these two taxa seem not very close, 44% of genetic distance. The other two taxa, *E. longipes* and *E. argentea* were found identical using morphological characters such as number of pairs of leaflets on the basal leaves and structure of the bracts which used in the species identification key of Flora of Turkey. Morphological quantitative characteristics such as height and width may vary according to ecological factors. However, distribution areas of these two species do also overlap and they grow in calcareous steppe. Detailed morphological, palynological and cytogenetical comparisons could not distinguish *E. longipes* from *E. argentea* and they reduced *E. argentea* as synonym of *E. longipes* (Aytaç *et al.,* 2000). In the present study based on the analysis of the seed proteins obtained using SDS-PAGE and RAPD, these two species showed a genetic distance of 12% for SDS-PAGE, whereas genetic similarity of the two species were 33% for RAPD. We cannot build our hypothesis of species identification only on RAPD because it is not areliable method of identification alone. It can work in combination with other strong co-dominant methods, therefore, the distance revealed by protein profile results and RAPD-PCR cannot equally be observed with morphological analysis. For example, *E. boissieri* and *E. longipes*, morphologically different, but, they show a similar proteins profile and RAPD results, indicating a closer relationship. In addition, *E. argentea*, *E. pisidica* and *E. laguroides* are in the same group in SDS-PAGE result, although they are not very close from a morphological point of view. The other taxa, *E. haussknechtii* as the most dissimilar taxon of this group is a prostrate so, this result may not be suprising. *E. barbigera* and *E. reesei* are grouped together for SDS-PAGE, and their geographic distribution are similar and they are morphologically similar as well. By contrast, our results showed that genetic distances of *E. cappadocica* to *E. depresa* and *E. bourgaei* were 27% for both taxa. In addition, *E. boissieri* and *E. hirsuta* have yellow corolla and *E. boissieri* is shorter than *E. hirsuta*. They are morphologically similar, according to seed proteins, genetic distance is 22%. For all other species studied with SDS-PAGE, are in broad agreement with Flora of Turkey. Our results are in agreement with Sammour (1991) and Juan *et al.*, (2007), seed proteins are useful characters to discriminate between species. In addition these characters show low level of environmental and evolutionary variability.

Despites the dendrogram obtained from RAPD profile (Fig. 4) discriminates between *Ebenus* species, it is not completely compatible with systematic similarity in Flora of Turkey and SDS-PAGE results. Thus, the relationships established by electrophoretic profile of RAPD do not match the established relationships based on morphological or traditional characters. RAPD seems to show more variation than morphological analysis. It can be explained for the reason that the protein and RAPD profile has the advantage of representing full genome of a species unlike morphological differences which are more dependent to environmental conditions (Sing, 1994). RAPD-PCR method provides a direct analysis of the genome not possible with morphological methods and therefore, serves as a powerful tool for biosystematics studies (Taylor-Grant, 1999). Since the RAPD technique does not require any previous knowledge of the target genome and is relatively simple and rapid to carry out, RAPD markers have been extensively used in population genetics, analyses of biodiversity and studies of relationships among species at different levels (Thorman & Osborn, 1992; Williams *et al.*, 1993; Ma *et al.*, 2004). However, mismatching resulting in non-specific amplification products may occur due to RAPD assay employs short nucleotide sequences of unknown location and/or functions and the technique sometimes may be limited to obtain a result because of representing only one segment of DNA (Taylor-Grant, 1999). Therefore, it could be estimated when the more primers amplified only all of the species are examined and specific makers are obtained to compare the species with each other.

In conclusion, taxonomic classification of plant species is basically depend on the morphological and anatomic characters, these features are changeable and sometimes difficult to observe, so it is necessary to be supported by molecular techniques. This study represents a first approach in using molecular markers as a tool to study molecular systematic in *Ebenus*. In the future studies, the analysis of additional population and species, additional primers to RAPD-PCR studies and the use of different types of molecular markers such as AFLP (Amplified Fragment Length Polymorphism), SSR (Self Sustained Sequence Replication), ITS (international transcribed spacers) will improve the accuracy of resolution of accurate classification of the genus *Ebenus* and for other plant species.

Acknowledgements

We are grateful for financial support of Gazi University BAP Scientific Research Project.

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(Received for publication 24 July 2008)