Molecular, morphological, and cytological analysis of diverse *Brachypodium distachyon* inbred lines

E. Filiz, B.S. Ozdemir, F. Budak, J.P. Vogel, M. Tuna, and H. Budak

Abstract: *Brachypodium distachyon* (brachypodium) is a small grass with the biological and genomic attributes necessary to serve as a model system for all grasses including small grains and grasses being developed as energy crops (e.g., switchgrass and *Miscanthus*). To add natural variation to the toolkit available to plant biologists using brachypodium as a model system, it is imperative to establish extensive, well-characterized germplasm collections. The objectives of this study were to collect brachypodium accessions from throughout Turkey and then characterize the molecular (nuclear and organelle genome), morphological, and cytological variation within the collection. We collected 164 lines from 45 diverse geographic regions of Turkey and created 146 inbred lines. The majority of this material (116 of 146 inbred lines) was diploid. The similarity matrix for the diploid lines based on AFLP analysis indicated extensive diversity, with genetic distances ranging from 0.05 to 0.78. Organelle genome diversity, on the other hand, was low both among and within the lines used in this study. The geographic distribution of genotypes was not significantly correlated with either nuclear or organelle genome variation for the genotypes studied. Phenotypic characterization of the lines showed extensive variation in flowering time (7–22 weeks), seed production (4–193 seeds/plant), and biomass (15–77 g). Chromosome morphology of the collected brachypodium accessions varied from submetacentric to metacentric, except for chromosome 5, which was acrocentric. The diverse brachypodium lines developed in this study will allow experimental approaches dependent upon natural variation to be applied to this new model grass. These results will also help efforts to have a better understanding of complex large genomes (i.e., wheat, barley, and switchgrass).

Key words: AFLP analysis, karyotyping, mtDNA, cpDNA.

Résumé : Le *Brachypodium distachyon* (brachypodium) est une petite graminée dont les caractéristiques biologiques et génomiques en font un bon modèle pour toutes les graminées, incluant les petites céréales et les graminées envisagées pour la production de biocarburants (e.g. le panic érigé et le *Miscanthus*). Afin d'ajouter la variation naturelle à l'ensemble des outils à la portée des chercheurs utilisant le brachypodium comme modèle, il serait impératif d'établir des collections de ressources génétiques caractérisées de manière exhaustive. Les objectifs de ce travail étaient de constituer une collection d'accessions du brachypodium provenant de toute la Turquie et de caractériser la variation moléculaire (génomes nucléaire et des organites), morphologique et cytologique au sein de cette collection. Les auteurs ont collecté 164 lignées provenant de 45 régions géographiques diverses en Turquie et en ont dérivé 146 lignées fixées. La majorité (116 de 146) de ce matériel était diploïde. La matrice de similarité basée sur des analyses AFLP des lignées diploïdes a révélé une grande diversité puisque les distances génétiques variaient entre 0,05 et 0,78. La diversité au sein des génomes des organites était par contre faible, tant au sein que parmi les lignées employées dans ce travail. L'origine géographique des génotypes n'était pas corrélée significativement avec la variation des génomes nucléaire ou des organites. Une caractérisation phénotypique des lignées a révélé une grande variation pour ce qui est de la date de floraison (7–22 semaines), de la production de graines (4–193 graines/plante) et de la biomasse (15–77 g). La morphologie chromosomique chez ces brachypodiums variait de submétacentrique à métacentrique, à l'exception du chromosome 5, lequel était acrocentrique. Les diverses lignées de brachypodium développées au cours de ce travail rendront possibles des approches expérimentales reposant sur l'existence de variation génétique chez cette nouvelle graminée modèle. Ces résultats contribueront également aux efforts visant l'acquisition d'une meilleure connaissance des grands génomes complexes (i.e. le blé, l'orge et le panic érigé).

Mots-clés : analyse AFLP, caryotypage, ADNmt, ADNcp.

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Introduction

Brachypodium distachyon (L.) Beauv. (hereafter, brachypodium) has emerged as a robust model system to answer questions unique to the grasses. Brachypodium possesses several desirable attributes such as a small diploid genome (~300 Mbp), small physical stature, short life cycle, self fertility, and lack of seed shattering (Draper et al. 2001; Ozdemir et al. 2008; Garvin et al. 2008). In addition to these inherent attributes, a number of resources have been developed to facilitate the use of brachypodium as a model system, including highly efficient *Agrobacterium*-mediated transformation methods (Păcurar et al. 2008; Vain et al. 2008; Vogel and Hill 2008), EST libraries and sequences (Vogel et al. 2006*a*), BAC libraries and BAC end sequences (Huo et al. 2006, 2008), mutagenesis protocols, physical maps, and a high-density genetic map. Other resources that are currently being developed include sequence-indexed T-DNA populations, microarrays, conserved miRNAs and their targets (Unver and Budak 2009) and, most importantly, the complete genome sequence. A sequenced genome has become a requirement for a modern model system so it is important to note that a 4× draft assembly of the brachypodium genome is currently available (www.brachypodium.org) and a final 8× version will be available in 2009 (unpublished).

The need to develop brachypodium as a model grass has arisen because neither arabidopsis nor rice is suitable to easily address many questions unique to the grasses. Arabidopsis is an extremely powerful model system and the development of brachypodium has been greatly accelerated by the knowledge of what "worked" for arabidopsis researchers. However, there are many areas where arabidopsis, a dicot, is not a suitable model for the grasses. Although rice might seem a natural alternative, its large genome, long generation time, and specialized growth conditions limit its utility, especially for researchers in temperate areas (Ozdemir et al. 2008). For all these reasons, brachypodium has been proposed as an alternative model for the grasses.

Brachypodium $2n$ chromosome numbers of 10, 20, and 30 have been reported (Draper et al. 2001; Hasterok et al. 2004, 2006). However, it does not appear to be a simple polyploid series as initially thought. Rather, the $2n = 10$ and $2n = 20$ cytotypes both appear to be diploids and the $2n = 30$ cytotype seems to be an allotetraploid with genomes similar to those of the $2n = 10$ and $2n = 20$ cytotypes (Hasterok et al. 2004, 2006). It is the $2n = 10$ diploid that is primarily being used as a model system, and the genome size of this diploid is approximately 300 Mbp (Vogel and Hill 2008; Vogel et al. 2006*a*; Bennett and Leitch 2005). This genome size lies between those of arabidopsis (157 Mbp) and rice (490 Mbp) and is one of the smallest known among the grasses (Bennett 2007). Genomic in situ hybridization (GISH) analysis revealed a preponderance of repetitive DNA in the pericentromeric regions, reflecting the compactness of this genome (Sharma and Gill 1983).

The natural diversity of brachypodium is centered around the Mediterranean region, extending north into Europe and south into the Indian subcontinent. The distribution of the $2n = 10$ diploid may be somewhat restricted to the center of this larger distribution, but more sampling is necessary to verify this (Garvin et al. 2008). Within this region, Turkey is expected to be a rich source of brachypodium diversity, since the 3 major phytogeographical regions of Turkey (Euro-Siberian, Irano-Turanian, and Mediterranean), covering hot interior regions, cooler coastal areas, and colder mountainous regions, represent most of the environmental diversity found within the larger geographic range (Ozdemir et al. 2008). Unlocking and exploiting the genetic diversity found in brachypodium will facilitate cereal genome studies and improve breeding programs.

The aim of this study was to assemble and characterize a diverse collection of inbred lines from many locations in Turkey. The cytological, morphological, and molecular diversity of this collection was explored using amplified fragment length polymorphism (AFLP), organelle genome (mtDNA and cpDNA) variation, karyotyping, and phenotypic characterization.

Materials and methods

Plant materials and growth conditions

A total of 146 inbred lines were created from 1101 brachypodium individuals representing diverse geographic regions of Turkey (Table 1). Each line was put through 5 generations of single-seed descent and plants were covered during anthesis to prevent cross-pollination. In addition to the inbred lines we created, we used 3 previously developed brachypodium inbred lines: Bd2-3, Bd3-1, and Bd21 (Vogel et al. 2006*b*).

Seeds were stratified at 4 °C for 7–10 days in the dark between moist filter papers in Petri plates. After cold treatment, they were put under light at room temperature. After germination they were transferred to a peat–soil mixture in small pots. After the seedlings were established, they were transplanted into 15 cm diameter plastic pots containing a mixture of 35% peat, 32% vermiculite, 9% soil, and 24% sand (*v*/*v*) and grown under a 16 h light : 8 h dark photoperiod in a greenhouse (Filiz et al. 2009). For basal fertilization, the growth medium was treated with 200 mg/kg N, 100 mg/kg P, 50 mg/kg K, and 20 mg/kg S.

Morphological evaluation

Phenotypic characterization was conducted in the greenhouse during spring–summer seasons from 2005 to 2007 and in the field. We determined anthesis date, seed size and yield, plant height, leaf characteristics, plant stature, dry mass, and vernalization requirement. The genotypes were then planted in single-row plots of 3 m length with a spacing of 5 cm between the plants and 1 m between the rows. Data were taken from each sampled plant from each accession. For the biomass calculation, the dry mass of each plant was recorded following drying of the green parts at 70 °C for 2–3 days.

DNA extraction

Leaves from 2-week-old seedlings were ground and placed in 1 mL of DNA extraction buffer (50 mmol/L Tris–HCl, 25 mmol/L EDTA, 1 mol/L NaCl, 1% CTAB, 1 mmol/L 1,10-phenanthroline, and 0.15% 2-mercaptoethanol) and incubated at 60 °C for 1 h and then mixed with an equal volume of chloroform : isoamyl alcohol (24:1). After centri-

Table 1. Characterization of inbred lines.

Genotype	Latitude	Longitude	Elevation (m)	Average height (cm)*	Leaf color[†]	Leaf hairiness[‡]	Plant stature	Average seed size (cm)*		Seed production (weeks)[§]	Average biomass (g)*	Seed yield[‖]	Germination percentage	DNA content (pg/2C)
								Width	Length					
BdTR1A	41°25′17.86″N	27°28′36.81″E	124	30.0–41.0	2	2	Fairly erect	0.123	0.644	8	0.89	64	94	0.69
BdTR1B	41°5′7.15″N	26°55′53.29″E	141	29.5–40.5	2	2	Erect	0.123	0.665	8	0.88	63	94	0.69
BdTR1C	39°44′17.39″N	28°2′24.71″E	363	28.7–39.8	2	2	Fairly erect	0.120	0.661	8	0.89	63	95	0.69
BdTR1D	38°45′50.79″N	28°35′6.42″E	612	29.0–39.5	2	2	Fairly erect	0.118	0.622	8	0.9	65	95	0.69
BdTR1E	38°25′0.42″N	28°1′52.75″E	986	28.2–41.0	2	2	Fairly erect	0.124	0.666	8	0.9	63	95	0.69
BdTR1F	41°25′17.86″N	27°28′36.81″E	124	29.5–40.8	2	2	Fairly erect	0.124	0.629	8	0.9	64	95	0.69
BdTR1G	41°5′7.15″N	26°55′53.29″E	141	29.0–39.9	2	2	Fairly erect	0.121	0.616	9	0.87	64	95	0.69
BdTR1H	39°44′17.39″N	28°2′24.71″E	363	28.9–40.0	2	2	Fairly erect	0.124	0.636	8	0.89	64	95	0.69
BdTR1I	38°5′35.03″N	28°34′59.02″E	841	29.1–41.0	2	2	Fairly erect	0.121	0.606	8	0.89	64	95	0.69
BdTR1J	37°25′38.24″N	28°35′6.75″E	513	29.9–40.7	2	2	Fairly erect	0.114	0.654	8	0.89	63	95	0.69
BdTR1K	39°45′34.18″N	29°40′40.96″E	1007	28.9–40.5	2	2	Erect	0.127	0.652	8	0.89	62	95	0.695
BdTR1M	39°5′12.20″N	30°15′8.93″E	1076	29.0–40.0	2	2	Fairly erect	0.121	0.673	8	0.89	63	95	0.695
BdTR1N	38°5′59.53″N	30°14′44.11″E	1034	29.1–39.9	2	2	Fairly erect	0.119	0.641	8	0.89	63	95	0.696
BdTR2A	39°45′10.62″N	30°47′19.07″E	932	29.6–40.1	2	2	Fairly erect	0.112	0.593	10	0.79	183	100	0.696
BdTR2B	40°4′55.55″N	31°19′52.01″E	667	30.0–41.0	2	2	Fairly erect	0.112	0.585	10	0.78	183	100	0.696
BdTR2C	39°5′8.89″N	31°53′29.40″E	864	29.7–41.8	2	2	Fairly erect	0.103	0.591	10	0.78	183	100	0.696
BdTR2D	38°25′46.78″N	31°18′33.71″E	1301	29.2–40.2	2	2	Erect	0.114	0.586	10	79.8	186	100	0.696
BdTR2E	38°25′40.97″N	32°24′16.47″E	1012	28.5–39.0	2	2	Fairly erect	0.122	0.611	10	0.8	184	100	0.696
BdTR2F	37°46′41.64″N	31°53′5.68″E	1288	28.0–40.8	2	2	Fairly erect	0.108	0.589	9	0.78	184	100	0.696
BdTR2G	40°23′37.13″N	32°59′7.32″E	1596	30.9–40.0	2	2	Fairly erect	0.115	0.609	10	0.78	184	100	0.696
BdTR2H	39°45′23.35″N	32°25′56.46″E	787	30.0–41.7	2	2	Erect	0.119	0.611	10	0.78	184	100	0.696
BdTR2I	39°45′16.02″N	33°32′16.37″E	872	29.4–40.1	2	2	Fairly erect	0.119	0.617	10	0.79	184	100	0.696
BdTR2J	39°24′46.28″N	32°59′17.24″E	1192	29.0–39.2	2	2	Fairly erect	0.112	0.586	10	0.79	184	100	0.696
BdTR2K	38°45′28.75″N	34°4′18.34″E	1142	29.9–42.2	2	2	Fairly erect	0.111	0.597	10	0.8	184	100	0.696
BdTR2M	38°5′52.78″N	34°5′40.38″E	1406	30.0–41.2	2	2	Fairly erect	0.113	0.572	10	0.8	184	100	0.696
BdTR2N	37°46′4.28″N	33°31′10.58″E	1013	30.0–42.4	2	2	Erect	0.117	0.610	10	0.8	184	100	0.696
BdTR2O	39°45′23.35″N	32°25′56.46″E	787	31.1–39.5	2	2	Fairly erect	0.119	0.580	10	0.8	184	100	0.696
BdTR2P	39°45′16.02″N	33°32′16.37″E	872	30.0–39.0	2	2	Fairly erect	0.110	0.607	10	0.9	184	100	0.696
BdTR2R	39°24′46.28″N	32°59′17.24″E	1192	30.7–41.1	2	2	Fairly erect	0.115	0.611	10	0.79	184	100	0.696
BdTR2S	38°45′28.75″N	34°4′18.34″E	1142	29.9–40.0	2	2	Fairly erect	0.116	0.561	11	0.8	184	100	0.696
BdTR3A	37°46′4.28″N	33°31′10.58″E	1013	32.0–40.0	2	1	Erect	0.114	0.692	12	2	38	100	0.68
BdTR3B	37°6′31.87″N	34°4′17.06″E	2385	29.8–39.2	2	1	Erect	0.108	0.697	11	2	36	100	0.68
BdTR3C	36°46′58.92″N	32°57′46.71″E	1957	29.0–38.5	2	1	Erect	0.114	0.681	12	2	36	100	0.68
BdTR3D	40°24′19.91″N	34°38′10.10″E	1088	29.8–37.9	2	1	Erect	0.112	0.696	12	2.1	38	100	0.68
BdTR3E	39°44′53.45″N	34°39′1.15″E	1035	30.2–38.0	2	1	Erect	0.113	0.690	12	2.1	38	100	0.68
BdTR3F	39°5′31.64″N	35°11′18.62″E	1249	30.0–40.2	2	1	Erect	0.100	0.603	12	2.1	36	100	0.68
BdTR3G	38°24′16.86″N	35°9′34.32″E	1086	30.0–39.8	2	1	Fairly erect	0.106	0.650	11	2	34	100	0.68
BdTR3H	37°46′43.23″N	35°12′9.55″E	3196	29.7–38.5	2	1	Erect	0.100	0.638	11	2	34	100	0.68
BdTR3I	39°44′51.33″N	36°48′56.85″E	1574	29.5–38.5	2	1	Erect	0.100	0.669	11	2	34	100	0.68
BdTR3J	38°45′25.33″N	36°16′56.90″E	1849	30.2–38.0	2	1	Erect	0.094	0.600	12	2	34	100	0.68
BdTR3K	38°25′12.10″N	37°23′26.99″E	1709	30.0–38.0	2	1	Erect	0.094	0.615	12	2	34	100	0.68
BdTR3M	37°45′58.01″N	37°53′23.56″E	614	28.0–39.2	2	1	Erect	0.104	0.633	12	2	34	100	0.68

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Table 1 (*continued*).

Genotype	Latitude	Longitude	Elevation (m)	Average height (cm)*	Leaf color†	Leaf hairiness‡	Plant stature	Average seed size (cm)*		Seed production (weeks)§	Average biomass (g)*	Seed yield‖	Germination percentage	DNA content (pg/2C)
								Width	Length					
BdTR3N	37°26′4.39″N	36°49′9.55″E	787	29.9–38.2	2	1	Erect	0.108	0.684	11	1.98	34	100	0.68
BdTR3O	37°7′2.52″N	39°1′49.56″E	448	30.0–38.4	2	1	Erect	0.102	0.654	11	2	34	100	0.68
BdTR3P	37°45′41.57″N	39°34′56.72″E	1156	29.6–38.0	2	1	Erect	0.102	0.650	12	2	36	100	0.69
BdTR3R	37°26′13.88″N	40°7′50.51″E	710	29.5–39.0	2	1	Erect	0.104	0.650	12	2	36	100	0.69
BdTR3S	38°5′43.50″N	40°39′30.84″E	688	29.0–39.1	2	1	Erect	0.105	0.660	12	2.1	36	100	0.69
BdTR3T	37°27′31.68″N	41°14′39.03″E	975	29.7–39.0	2	1	Erect	0.108	0.656	11	2	36	100	0.69
BdTR4A	37°27′31.68″N	41°14′39.03″E	975	39.0–50.2	2	2	Erect	0.128	0.698	7	0.94	801	65	1.36
BdTR4B	37°47′51.69″N	41°46′25.09″E	612	38.5–49.0	2	2	Erect	0.123	0.663	7	0.95	801	65	1.36
BdTR4C	38°5′48.94″N	42°19′18.79″E	1713	38.4–49.2	2	2	Erect	0.128	0.669	7	0.95	798	65	1.36
BdTR4D	38°5′43.50″N	40°39′30.84″E	688	38.7–50.0	2	2	Erect	0.128	0.688	7	0.99	798	65	1.36
BdTR4E	37°27′31.68″N	41°14′39.03″E	975	38.5–50.1	2	2	Erect	0.122	0.666	7	0.94	790	65	1.36
BdTR4F	37°47′51.69″N	41°46′25.09″E	612	39.0–49.5	2	2	Fairly erect	0.127	0.665	7	0.95	790	65	1.36
BdTR4G	38°5′48.94″N	42°19′18.79″E	1713	38.9–49.0	2	2	Erect	0.128	0.690	7	0.95	791	65	1.36
BdTR4H	38°5′48.94″N	42°19′18.79″E	1713	38.0–49.0	2	2	Erect	0.131	0.693	7	0.98	791	65	1.36
BdTR4I	37°28′28.23″N	43°26′45.08″E	1957	38.3–48.5	2	2	Fairly erect	0.130	0.690	7	0.96	792	65	1.36
BdTR4J	36°45′59.24″N	44°32′6.90″E	886	38.0–51.0	2	2	Erect	0.128	0.672	7	0.95	792	65	1.36
BdTR4K	38°5′48.94″N	42°19′18.79″E	1713	38.1–49.3	2	2	Erect	0.125	0.680	7	0.98	792	65	1.36
BdTR5A	36°45′59.24″N	44°32′6.90″E	886	29.3–37.0	3	3	Fairly erect	0.109	0.701	14	1.7	24	100	0.69
BdTR5B	37°46′43.23″N	35°12′9.55″E	3196	29.2–37.0	3	3	Fairly erect	0.104	0.670	14	1.5	24	100	0.69
BdTR5C	39°44′51.33″N	36°48′56.85″E	1574	29.3–37.5	3	3	Fairly erect	0.109	0.673	14	1.5	24	100	0.69
BdTR5D	38°25′40.97″N	32°24′16.47″E	1012	29.0–37.2	3	3	Erect	0.107	0.691	14	1.6	24	100	0.69
BdTR5E	37°46′41.64″N	31°53′5.68″E	1288	29.0–37.1	3	3	Fairly erect	0.109	0.683	14	1.6	24	100	0.69
BdTR5F	40°23′37.13″N	32°59′7.32″E	1596	30.0–39.0	3	3	Fairly erect	0.107	0.700	14	1.7	24	100	0.69
BdTR5G	39°45′23.35″N	32°25′56.46″E	787	30.0–37.8	3	3	Erect	0.104	0.692	14	1.5	24	100	0.69
BdTR5H	39°45′16.02″N	33°32′16.37″E	872	29.2–36.9	3	3	Fairly erect	0.107	0.685	14	1.5	24	100	0.69
BdTR5I	40°23′37.13″N	32°59′7.32″E	1596	28.3–37.0	3	3	Fairly erect	0.112	0.684	14	1.5	24	100	0.69
BdTR5J	40°23′37.13″N	32°59′7.32″E	1596	28.9–37.2	3	3	Erect	0.114	0.688	14	1.6	24	100	0.69
BdTR5K	40°23′37.13″N	32°59′7.32″E	1596	29.1–36.8	3	3	Fairly erect	0.110	0.699	14	1.5	24	100	0.69
BdTR5M	40°23′37.13″N	32°59′7.32″E	1596	28.9–37.0	3	3	Fairly erect	0.102	0.679	14	1.7	24	100	0.69
BdTR5N	40°23′37.13″N	32°59′7.32″E	1596	28.9–36.8	3	3	Fairly erect	0.122	0.683	14	1.7	24	100	0.69
BdTR5O	39°45′16.02″N	33°32′16.37″E	872	29.0–38.0	3	3	Fairly erect	0.102	0.683	14	1.7	24	100	0.69
BdTR6A	38°5′48.94″N	42°19′18.79″E	1713	36.0–51.2	3	5	Erect	0.144	0.793	10	0.5	440	100	1.34
BdTR6B	37°28′28.23″N	43°26′45.08″E	1957	35.7–50.8	3	5	Fairly erect	0.141	0.799	10	0.6	390	100	1.34
BdTR6C	38°25′40.97″N	32°24′16.47″E	1012	35.2–51.0	3	5	Erect	0.143	0.788	11	0.5	379	100	1.34
BdTR6D	37°46′41.64″N	31°53′5.68″E	1288	35.3–51.4	3	5	Erect	0.144	0.782	11	0.5	398	100	1.34
BdTR6E	40°23′37.13″N	32°59′7.32″E	1596	35.0–51.2	3	5	Erect	0.144	0.794	11	0.5	438	100	1.34
BdTR6F	39°45′23.35″N	32°25′56.46″E	787	34.7–50.8	3	5	Erect	0.139	0.780	11	0.5	437	100	1.34
BdTR6G	39°45′16.02″N	33°32′16.37″E	872	34.9–51.0	3	5	Erect	0.138	0.763	10	0.5	437	100	1.34
BdTR6H	39°24′46.28″N	32°59′17.24″E	1192	35.1–52.0	3	5	Fairly erect	0.143	0.776	10	0.5	437	100	1.34
BdTR6I	38°45′28.75″N	34°4′18.34″E	1142	34.3–50.7	3	5	Erect	0.142	0.780	10	0.5	437	100	1.34
BdTR6J	38°5′52.78″N	34°5′40.38″E	1406	35.0–51.1	3	5	Erect	0.140	0.781	10	0.5	437	100	1.34
BdTR6K	37°46′4.28″N	33°31′10.58″E	1013	34.0–50.6	3	5	Fairly erect	0.140	0.800	10	0.5	437	100	1.34

Table 1 (*continued*).

Genotype	Latitude	Longitude	Elevation (m)	Average height (cm)*	Leaf color[†]	Leaf hairiness[‡]	Plant stature	Average seed size (cm)*		Seed production (weeks)[§]	Average biomass (g)*	Seed yield[‖]	Germination percentage	DNA content (pg/2C)
								Width	Length					
BdTR6M	37°6′31.87″N	34°4′17.06″E	2385	35.6–50.3	3	5	Erect	0.138	0.785	10	0.5	437	100	1.34
BdTR6N	36°46′58.92″N	32°57′46.71″E	1957	34.8–52.6	3	5	Erect	0.142	0.790	10	0.5	437	100	1.34
BdTR7A	39°44′53.45″N	34°39′1.15″E	1035	26.2–35.9	1	2	Fairly erect	0.113	0.638	22	15.77	54	100	0.69
BdTR7B	39°5′31.64″N	35°11′18.62″E	1249	25.8–36.1	1	2	Erect	0.112	0.630	22	15.87	54	100	0.69
BdTR8A	39°45′16.02″N	33°32′16.37″E	872	24.8–35.3	1	2	Erect	0.124	0.638	22	8.8	68	100	1.27
BdTR8C	39°24′46.28″N	32°59′17.24″E	1192	25.0–35.0	1	2	Erect	0.125	0.652	22	8.5	69	100	1.27
BdTR8F	37°46′4.28″N	33°31′10.58″E	1013	25.0–34.2	1	2	Erect	0.125	0.640	22	8.5	69	100	1.27
BdTR8I	37°6′31.87″N	34°4′17.06″E	2385	24.5–35.2	1	2	Fairly erect	0.126	0.650	22	8.9	69	100	1.27
BdTR8M	39°5′31.64″N	35°11′18.62″E	1249	24.9–34.8	1	2	Erect	0.128	0.641	22	8.9	68	100	1.27
BdTR8N	38°24′16.86″N	35°9′34.32″E	1086	25.2–35.0	1	2	Erect	0.129	0.650	22	8.7	68	100	1.27
BdTR9A	39°44′51.33″N	36°48′56.85″E	1574	29.4–45.2	1	3	Branchy	0.122	0.599	9	2.67	26	88	0.69
BdTR9B	39°44′17.39″N	28°2′24.71″E	363	29.7–45.0	1	3	Branchy	0.128	0.618	9	2.59	31	88	0.69
BdTR9C	38°45′50.79″N	28°35′6.42″E	612	29.9–44.8	1	3	Branchy	0.130	0.602	9	2.7	27	86	0.69
BdTR9D	38°25′0.42″N	28°1′52.75″E	986	30.2–45.2	1	3	Branchy	0.126	0.621	9	2.8	19	86	0.69
BdTR9E	38°5′35.03″N	28°34′59.02″E	841	30.0–46.0	1	3	Branchy	0.133	0.622	9	2.8	21	86	0.69
BdTR9F	37°25′38.24″N	28°35′6.75″E	513	28.9–45.0	1	3	Branchy	0.122	0.622	9	2.8	23	86	0.69
BdTR9G	39°45′34.18″N	29°40′40.96″E	1007	30.4–44.9	1	3	Branchy	0.122	0.610	9	2.78	24	86	0.69
BdTR9H	39°5′12.20″N	30°15′8.93″E	1076	30.8–45.9	1	3	Branchy	0.118	0.618	9	2.75	23	86	0.69
BdTR9I	38°5′59.53″N	30°14′44.11″E	1034	29.9–44.5	1	3	Branchy	0.119	0.611	9	2.68	23	86	0.69
BdTR9J	37°7′38.06″N	30°13′59.22″E	1073	30.3–44.7	1	3	Branchy	0.122	0.625	9	2.9	23	86	0.69
BdTR9K	39°45′10.62″N	30°47′19.07″E	932	30.2–45.3	1	3	Branchy	0.125	0.625	9	2.87	26	86	0.69
BdTR9M	40°4′55.55″N	31°19′52.01″E	667	29.7–45.0	1	3	Branchy	0.119	0.619	9	2.67	26	86	0.69
BdTR10A	38°25′46.78″N	31°18′33.71″E	1301	30.2–41.0	1	3	Erect	0.121	0.633	20	2.33	18	92	0.7
BdTR10B	38°25′40.97″N	32°24′16.47″E	1012	29.0–39.0	1	3	Erect	0.126	0.626	20	2.34	18	92	0.7
BdTR10C	37°46′41.64″N	31°53′5.68″E	1288	29.8–40.0	1	3	Erect	0.121	0.613	20	2.35	19	92	0.7
BdTR10D	40°23′37.13″N	32°59′7.32″E	1596	30.3–39.8	1	3	Fairly erect	0.122	0.624	20	2.34	19	92	0.7
BdTR10E	37°7′2.52″N	39°1′49.56″E	448	29.5–41.0	1	3	Erect	0.128	0.620	20	2.34	20	92	0.7
BdTR10F	37°45′41.57″N	39°34′56.72″E	1156	30.5–41.7	1	3	Fairly erect	0.123	0.620	20	2.37	21	92	0.7
BdTR10G	37°26′13.88″N	40°7′50.51″E	710	30.2–40.3	1	3	Erect	0.122	0.613	20	2.18	21	92	0.7
BdTR10H	38°5′43.50″N	40°39′30.84″E	688	29.7–40.0	1	3	Erect	0.133	0.621	20	2.15	21	92	0.7
BdTR10I	37°27′31.68″N	41°14′39.03″E	975	29.2–40.1	1	3	Fairly erect	0.124	0.610	20	2.18	21	92	0.7
BdTR10J	37°47′51.69″N	41°46′25.09″E	612	30.0–40.8	1	3	Erect	0.125	0.634	20	2.15	23	92	0.7
BdTR10K	38°5′48.94″N	42°19′18.79″E	1713	30.2–40.4	1	3	Erect	0.118	0.630	20	2.14	23	92	0.7
BdTR10M	41°25′17.86″N	27°28′36.81″E	124	29.8–39.6	1	3	Fairly erect	0.123	0.619	20	2.15	24	92	0.7
BdTR10N	41°5′7.15″N	26°55′53.29″E	141	30.3–39.9	1	3	Erect	0.125	0.622	20	2.17	24	92	0.7
BdTR10O	39°44′17.39″N	28°2′24.71″E	363	29.6–40.9	1	3	Erect	0.123	0.620	20	2.16	27	92	0.7
BdTR11A	38°25′0.42″N	28°1′52.75″E	986	30.0–39.5	1	3	Fairly erect	0.120	0.618	17	2.1	112	95	0.69
BdTR11B	41°25′17.86″N	27°28′36.81″E	124	28.3–39.0	1	3	Erect	0.125	0.613	17	2	112	95	0.69
BdTR11C	41°5′7.15″N	26°55′53.29″E	141	28.1–38.9	1	3	Erect	0.119	0.621	17	2.2	118	95	0.69
BdTR11D	37°27′31.68″N	41°14′39.03″E	975	29.6–39.1	1	3	Erect	0.123	0.610	18	2.1	119	95	0.69
BdTR11E	37°47′51.69″N	41°46′25.09″E	612	27.6–38.4	1	3	Erect	0.124	0.627	18	2.1	118	95	0.69
BdTR11F	38°5′48.94″N	42°19′18.79″E	1713	29.0–37.6	1	3	Fairly erect	0.122	0.612	18	2.3	118	95	0.69

Table 1 (*concluded*).

Genotype	Latitude	Longitude	Elevation (m)	Average height (cm)*	Leaf color[†]	Leaf hairiness[‡]	Plant stature	Average seed size (cm)*		Seed production (weeks)[§]	Average biomass (g)*	Seed yield[‖]	Germination percentage	DNA content (pg/2C)
								Width	Length					
BdTR11G	41°25′17.86″N	27°28′36.81″E	124	28.4–37.7	1	3	Erect	0.127	0.621	18	2	119	95	0.69
BdTR11H	41°5′7.15″N	26°55′53.29″E	141	29.0–39.0	1	3	Erect	0.123	0.618	18	2	119	95	0.69
BdTR11I	39°44′17.39″N	28°2′24.71″E	363	28.5–38.0	1	3	Erect	0.126	0.615	18	2	120	95	0.69
BdTR12A	36°46′58.92″N	32°57′46.71″E	1957	25.1–32.0	1	3	Erect	0.114	0.568	20	4.71	19	67	0.69
BdTR12B	40°24′19.91″N	34°38′10.10″E	1088	25.0–32.4	1	3	Fairly erect	0.111	0.588	20	4.72	16	67	0.68
BdTR12D	38°45′50.79″N	28°35′6.42″E	612	25.0–32.3	1	3	Erect	0.112	0.578	20	4.72	4	66.7	0.7
BdTR13A	39°45′23.35″N	32°25′56.46″E	787	30.2–41.9	3	4	Branchy	0.102	0.654	19	0.5	200	95	0.7
BdTR13B	39°45′16.02″N	33°32′16.37″E	872	31.2–42.0	3	4	Branchy	0.100	0.652	19	0.6	218	95	0.7
BdTR13C	39°24′46.28″N	32°59′17.24″E	1192	30.3–42.5	3	4	Branchy	0.100	0.650	19	0.4	219	95	0.7
BdTR13D	37°46′4.28″N	33°31′10.58″E	1013	29.5–41.9	3	4	Branchy	0.100	0.672	19	0.4	219	95	0.7
BdTR13E	37°6′31.87″N	34°4′17.06″E	2385	29.7–42.5	3	4	Branchy	0.110	0.649	19	0.6	210	95	0.7
BdTR13F	39°5′31.64″N	35°11′18.62″E	1249	30.0–41.4	3	4	Branchy	0.102	0.661	19	0.6	211	95	0.7
BdTR13G	38°24′16.86″N	35°9′34.32″E	1086	31.0–40.8	3	4	Branchy	0.100	0.664	19	0.6	214	95	0.7
BdTR13H	37°27′31.68″N	41°14′39.03″E	975	31.5–41.3	3	4	Branchy	0.109	0.675	19	0.7	216	95	0.7
BdTR13I	37°47′51.69″N	41°46′25.09″E	612	30.6–42.1	3	4	Branchy	0.104	0.650	19	0.5	216	95	0.7
BdTR13J	38°5′48.94″N	42°19′18.79″E	1713	31.0–41.5	3	4	Branchy	0.112	0.675	19	0.5	218	95	0.7
BdTR13K	38°5′48.94″N	42°19′18.79″E	1713	29.4–43.0	3	4	Branchy	0.110	0.673	19	0.5	219	95	0.7
BdTR13M	37°28′28.23″N	43°26′45.08″E	1957	29.9–42.6	3	4	Branchy	0.109	0.670	19	0.5	219	95	0.72
BdTR13N	36°45′59.24″N	44°32′6.90″E	886	31.0–41.5	3	4	Branchy	0.109	0.679	19	0.5	217	95	0.71
BdTR13O	38°5′48.94″N	42°19′18.79″E	1713	31.2–41.2	3	4	Branchy	0.100	0.673	19	0.5	216	95	0.71

*Values are the means of 15 replicates (individual plants).

[†]The degree of greenish leaf color from 1 to 3 in ascending order.

[‡]The degree of feathery leaf structure from 1 to 5 in ascending order.

[§]Number of weeks from planting to first seed production.

[‖]Values are the means of 7 replicates (individual plants).

fuging at 12 000 rpm (24 149*g*), the supernatant was transferred to a new tube, isopropanol was added, and the mixture was incubated for 30 min at room temperature to precipitate the DNA. The pellet was dried, resuspended in 200 μL of TE buffer (10 mmol/L Tris–HCl, 0.1 mmol/L EDTA, pH 8.0) plus 20 μg of RNase, and then incubated at room temperature overnight. The DNA concentration was quantified by spectrophotometry (TKO 100 fluorometer, Hoefer Scientific Instruments, San Francisco, California).

AFLP analysis

AFLP analysis was carried out using a LI-COR 4300 DNA Analysis System (LI-COR Biosciences, Germany). Restriction digestion of genomic DNA (100 ng) with an *Eco*RI–*Mse*I enzyme mix was performed at 37 °C for 2 h. Double-stranded *Mse*I and *Eco*RI adapters were ligated to the digested fragments with T4 DNA ligase at 20 °C for 2 h. Preamplification reactions were carried out with diluted DNA (1/10) from the ligation reaction. The reactions were placed in a thermocycler programmed to start 20 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min and then incubate at 4 °C. Selective amplifications were carried out using diluted DNA (1/40) from the preamplification reaction and the primer combinations E-AAC and M-CTT, E-AGG and M-CTT, E-AAG and M-CAA, E-ACG and M-CAA, E-ACG and M-CAC, and E-AAG and M-CAC (LI-COR, USA). The thermocycler was programmed to start 1 cycle of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, 2 more cycles with the annealing temperature (65 °C) lowered by 0.7 °C per cycle, and 23 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; the temperature was then held at 4 °C. The final PCR products were run on a 6.5% denaturing KB^{Plus} gel (LI-COR, USA).

Organelle genome analysis

Mitochondrial and chloroplast genomic DNA fragments were amplified using the primer pairs outlined by Budak et al. (2005*a*) and Isik et al. (2007). The restriction enzymes used were *Eco*RI, *Eco*RV, *Mbo*I, *Dra*I, *Taq*I, *Rsa*I, *Mse*I, and *Msp*I. The PCR was carried out as outlined by Budak et al. (2004*a*, 2004*b*, 2005*a*) using an MJ Research PTC-100 thermocycler programmed for 1 cycle of 2 min at 94 °C and 35 cycles of 1 min at 94 °C, 1 min at 54 °C, and 2 min at 72 °C. Based on the expected numbers and sizes of restriction fragments, 2.5% agarose gels were used to separate 15 μL aliquots of PCR products. Gels were stained with ethidium bromide. Amplified fragment bands were photographed using a Gel Doc 2000 (Bio-Rad, Hercules, California).

Scoring of gels and data analysis

Each nuclear and organelle genome marker was coded as "1" or "0", where "1" indicated the presence of a specific allele and "0" indicated its absence. Average genetic diversity (D) as a measure of genetic variation was calculated using $D = 1 - (1/L)\Sigma_l\Sigma_i p_{li}^2$, where p_{li} is the frequency of the ith allele at locus l and L is the number of loci (Weir 1996). The genetic similarity coefficients (GS) or the Dice coefficients (Sneath and Sokal 1973) were measured between genotypes to obtain a genetic similarity matrix based on nuclear and cytoplasmic banding patterns. Genetic similarity between 2 genotypes within 1 locus was calculated using the formula $GS_{ij} = 2N_{ij}/(N_i + N_j)$, where N_i and N_j represent the total number of bands present in cultivars i and j, respectively, and N_{ij} refers to the total number of common bands between the same cultivars (Nei and Li 1979). The NTSYS-pc version 2.1 software package (Rohlf 2000) was used for principal component analysis (PCA).

SAS PROC CLUSTER (SAS Institute Inc., Cary, North Carolina) was performed with distance matrices to generate composite groups based on a combination of intersite geographic distance and assemblage dissimilarity (Budak et al. 2005*a*). Regression analysis using SAS PROC REG was performed to determine associations between pair-wise genetic distances from nuclear and organelle DNA data sets and pair-wise geographic distances between populations. Additionally, analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed to estimate the amount of variation due to differences within and among genotypes.

PCA was performed following EIGEN and PROJECTION modules based on the similarity matrix and illustrated by the 3D PLOT module to exhibit associations among genotypes based on nuclear and organelle data sets. Bootstrap analysis was performed using the bootstrap module in PowerMarker and consensus trees were created using the consense module in the PHYLIP software package version 3.68 (Felsenstein 2008).

Flow cytometry analysis

Flow cytometry analysis was performed as described by Arumuganathan and Earle (1991) to identify the ploidy levels of the accessions used in this study. Briefly, mean DNA content was based on analysis of 1000 nuclei. Each genotype was analyzed by 4 separate extractions and flow cytometric runs (Budak et al. 2004*b*; Filiz et al. 2009). Base pair composition calculations were done as outlined by Godelle et al. (1993).

Cytological analysis

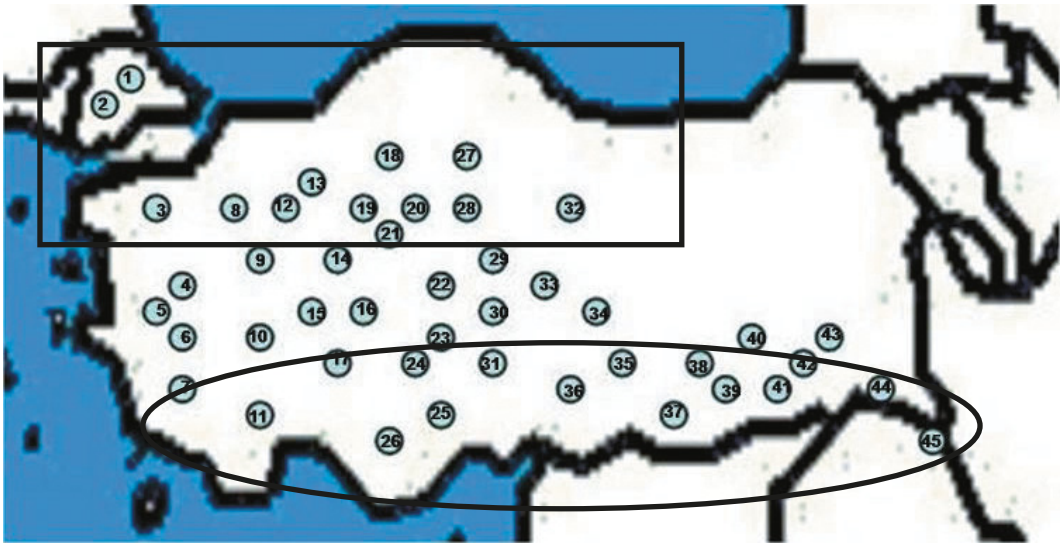
Seeds were germinated between 2 moistened filter papers. The seeds were kept at 4 °C for the first 10 days to break dormancy. Then, they were incubated at 23 °C in the dark until germination. Root tips were collected when roots were 1–1.5 cm long and then pretreated with cold water. The water was discarded after 16 h and replaced by Farmer's solution (3:1 (*v*/*v*) ethanol : acetic acid). Root tips were then stained in 2% acetocarmine dye for 3 h. Preparations were made by squashing root tips onto microslides.

Results

Morphological evaluation

For our analysis we used 1101 brachypodium individuals sampled from 45 locations in Turkey (Fig. 1). From these accessions we created 146 inbred lines. These lines were first grown under greenhouse conditions for morphological analysis. There was considerable phenotypic variation within this collection (Table 1). The inbred lines were placed into 13 groups (BdTR1 to BdTR13) based on their morphological characteristics. Plant height ranged from 21 to 52 cm and averaged 34 cm. Leaf color was rated as 1 (light green), 2 (green), or 3 (dark green) (Fig. 2A). The degree of leaf

Fig. 1. Geographic distribution of 45 collection sites throughout Turkey. The rectangle indicates the northern region, the ellipse indicates the southern region, and the remaining area is the central region.



hairiness was classified from 1 to 5, with 1 being almost hairless and 5 being very hairy (Fig. 2B). Plant stature varied from fairly erect to branchy (Fig. 2C). The time from planting to first seed production ranged from 7 weeks to 22 weeks and averaged 12 weeks. Like all other characteristics, the number of seeds produced per plant varied greatly. Among polyploid lines, BdTR4A and BdTR4B had the highest seed yield (average of 793 seeds/plant), and the diploid line BdTR12D exhibited the lowest seed yield (average of 4 seeds/plant). Since there was a great deal of variation in seed production, we vernalized all the lines for 6 weeks at 4 °C to determine whether vernalization affected seed production. There was no significant correlation between vernalization and seed production under these conditions. Viability of the harvested seeds (as measured by germination) ranged from 60% to 100% and averaged 90%. Like all other morphological features examined, average aboveground biomass showed considerable variation and ranged from 0.40 to 15.77 g/plant. The variable genotypes were significantly different, as revealed by a *t* test (LSD).

Flow cytometry analysis

Mean nuclear DNA content of the 1101 native brachypodium genotypes ranged from 0.70 pg/2C (diploid) to 1.35 pg/2C (polyploid) (Table 1). Based on their nuclear DNA content, lines were classified as either diploid (116 lines from 45 locations) or polyploid (30 lines from 4 locations) (Figs. 3A, 3B). At 8 locations both diploid and polyploid forms were found growing together. All of the polyploid lines fell into 2 phenotypic groups, BdTR4 and BdTR6. The remaining 11 BdTR groups were diploid. Thus, the most prevalent ploidy level of Turkish brachypodium was diploid. Since we are primarily interested in utilizing diploid accessions as a model, the 116 diploid lines were analyzed further.

Karyotyping brachypodium lines

A karyotype was produced for plants of 2 groups (Bd-TR1 and Bd-TR13) to confirm that it was similar to previously described karyotypes for the $2n = 10$ diploid form (Draper et al. 2001; Jenkins et al. 2003, 2005; Hasterok et al. 2004, 2006). Similar to previous reports, we observed 10 chromosomes, with 4 pairs of BdTR1 appearing submetacentric to metacentric and 1 pair appearing acrocentric (Fig. 4).

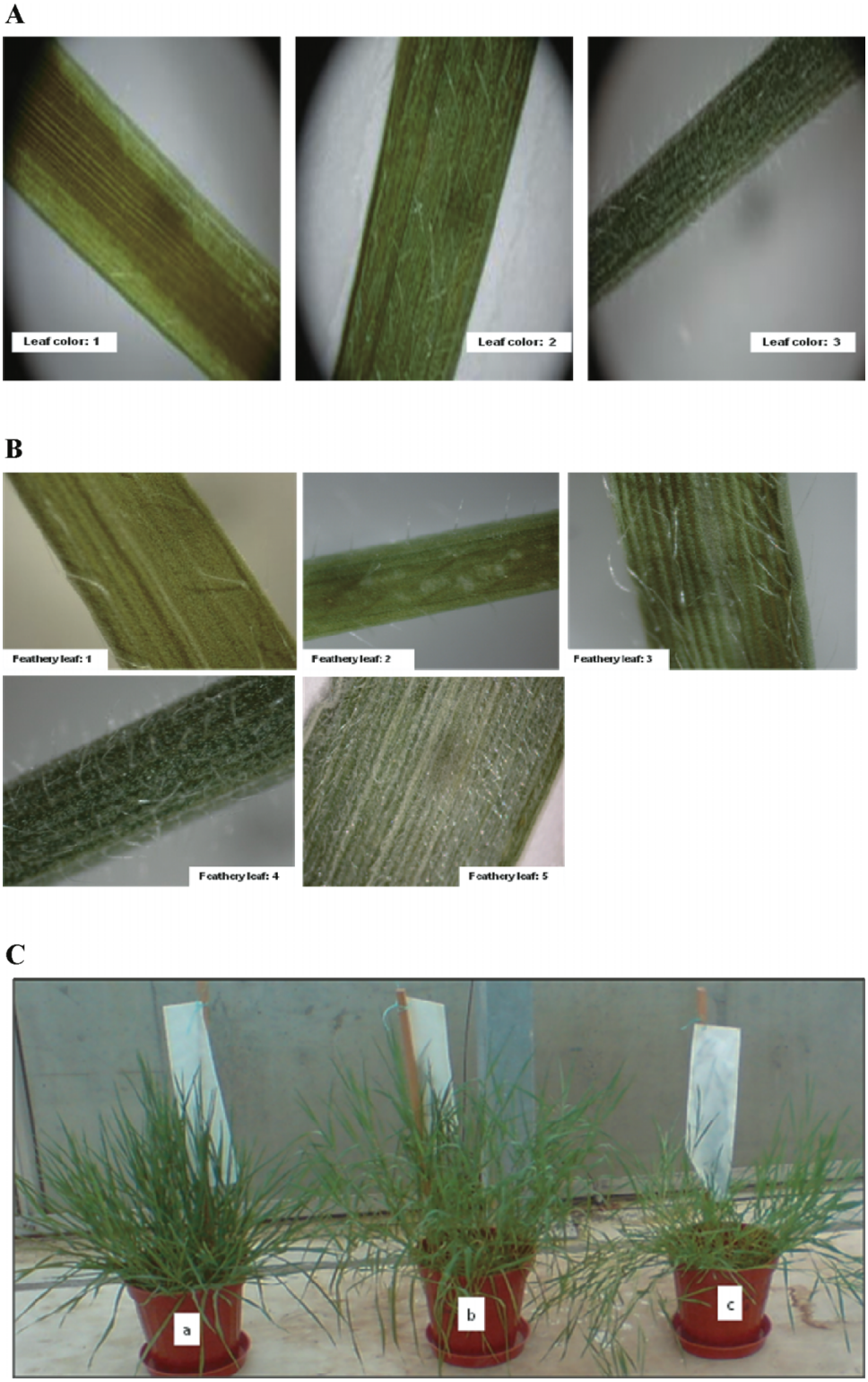
Molecular analyses

AFLP analysis

Analysis of genomic sequences by AFLP was used to genotypically characterize 116 diploid inbred lines. Five primer combinations produced 373 scorable bands, of which 266 (approximately 70%) were polymorphic. Genetic distance, determined by Nei's distance coefficient (Nei 1972), varied from 0.05 to 0.78 and averaged 0.67. A UPGMA (unweighted pair group method with arithmetic mean) dendrogram based on Nei's genetic distance separated the genotypes into 5 major clusters and several smaller clusters (Fig. 5). Group I consisted of inbred lines from BdTR7 and BdTR8, which have morphological similarities such as plant stature (erect type), seed production (22 weeks after planting), and plant height (~25 cm) (Table 1). BdTR13 lines and BdTR3 lines closely clustered together and also grouped with Bd21 in group V. The largest group in this study was group II, which consisted of BdTR5, BdTR9, BdTR10, and BdTR12. This largest group fell into the same cluster with Bd3-1 and Bd2-3. BdTR1 and BdTR2 clustered together, whereas group IV consisted of only BdTR11. A consensus neighbor-joining tree was also constructed and examination of the tree revealed groupings that were consistent with the UPGMA tree and the phenotypic groupings when compared with geographic origin.

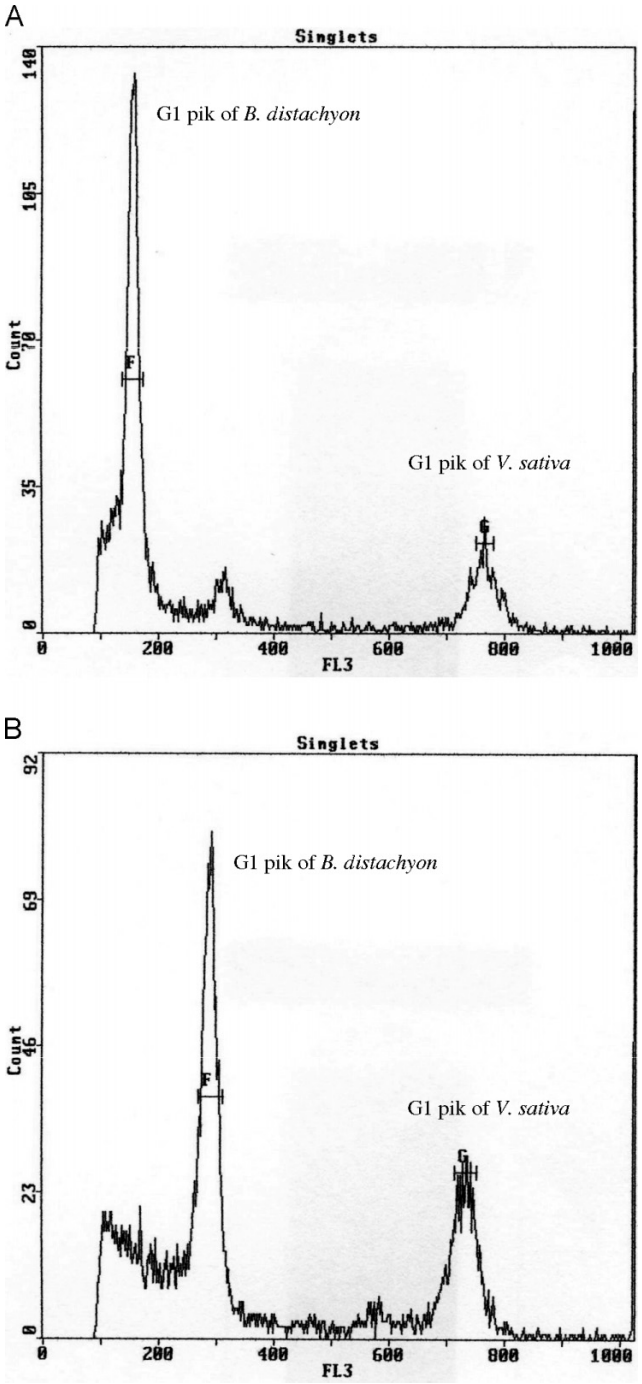
Most of the AFLP differentiation (coefficient of genetic differentiation, G_{ST}) was distributed among the populations. The hierarchical AMOVA showed there was high variation both among and within the 3 broad geographic regions (Table 2). The lines used in this study grouped together based on their geographic origin.

Fig. 2. Illustration of leaf color, leaf hairiness, and plant stature. (A) The leaf color scale of brachypodium, from 1 on the left to 3 on the right. (B) Leaves of brachypodium ecotypes showing five different degrees of hairiness, from 1 at the upper left to 5 at the lower right. Note the wide variation in trichome density. (C) Plant stature in brachypodium: (*a*) erect, (*b*) fairly erect, and (*c*) branchy.



The PCA of the AFLP matrix had Eigenvalues bigger than 1 that explained 90% of the variation observed. Principal component 1 (PC1) (*x*-axis) and PC2 (*y*-axis) explained 29.4% and 38.5% of the data set variation. Interestingly, lines originating from diverse locations grouped together. Adaptation patterns among the remaining inbred lines were not clear but some lines grouped with the ones that came from the southern region.

Fig. 3. Flow histograms of brachypodium. (A) Diploid brachypodium and (B) polyploid brachypodium.



Chloroplast and mitochondrial genome analysis

Chloroplast DNA (cpDNA) analysis indicated that the similarity among the diploid lines ranged from 0.09 to 0.98, with a mean similarity of 0.59. All scorable polymorphic and non-polymorphic bands were taken into account for the genetic distance calculations, which showed a low level of cpDNA variation among all genotypes. This is also valid for polyploid genotypes.

Mitochondrial DNA (mtDNA) analysis indicated that the similarity among all diploid lines ranged from 0.03 to 0.99, with a mean similarity of 0.32. As in the cpDNA analysis, we used all scorable polymorphic and non-polymorphic bands for the genetic distance calculations to be able to detect true mtDNA variation among the brachypodium genotypes studied. The mtDNA PCR-RFLPs also indicated that germplasm from different geographical regions might have a common genetic background because brachypodium genotypes representing various geographic regions did not differ. Additionally, geographically close genotypes were genetically highly distant. This result might be due to ecotype selection (Budak et al. 2005*a*; Isik et al. 2007). The mtDNA did not separate genotypes according to geographical location.

Both cpDNA and mtDNA analyses indicated that there was no significant correlation between genotypes and the number of bands detected (r = 0.10 and 0.12, $P < 0.05$) based on the primer pairs used, and these primers were not as polymorphic as the AFLP markers. cpDNA similarities among brachypodium genotypes were considerably higher than those within genotypes. Most of the cpDNA and mtDNA differentiation (G_{ST}) was distributed among the populations. The hierarchical AMOVA using both cpDNA and mtDNA showed there was high variation both among and within geographic regions. The amount of variation was higher among and within geographic regions (southern region, 14.7%; central region, 24.6%; and northern region, 23.1%) than within populations. These results are similar to findings in other plant species (Budak et al. 2005*a*; Isik et al. 2007).

PCA on the mtDNA matrix had Eigenvalues > 1 that explained 94.2% of the variation observed. PC1 (x-axis) and PC2 (y-axis) explained 30.1% and 38.2% of the variation, respectively. The cpDNA data matrix had Eigenvalues bigger than 1 which explained 94% of the variation. PC1 and PC2 explained most of the variation (30% and 28%, respectively). Brachypodium lines originating from diverse regions were not always separated using either mtDNA or cpDNA. Many lines originating from diverse locations in Turkey grouped together based on nuclear and cytoplasmic (mtDNA and cpDNA) analyses.

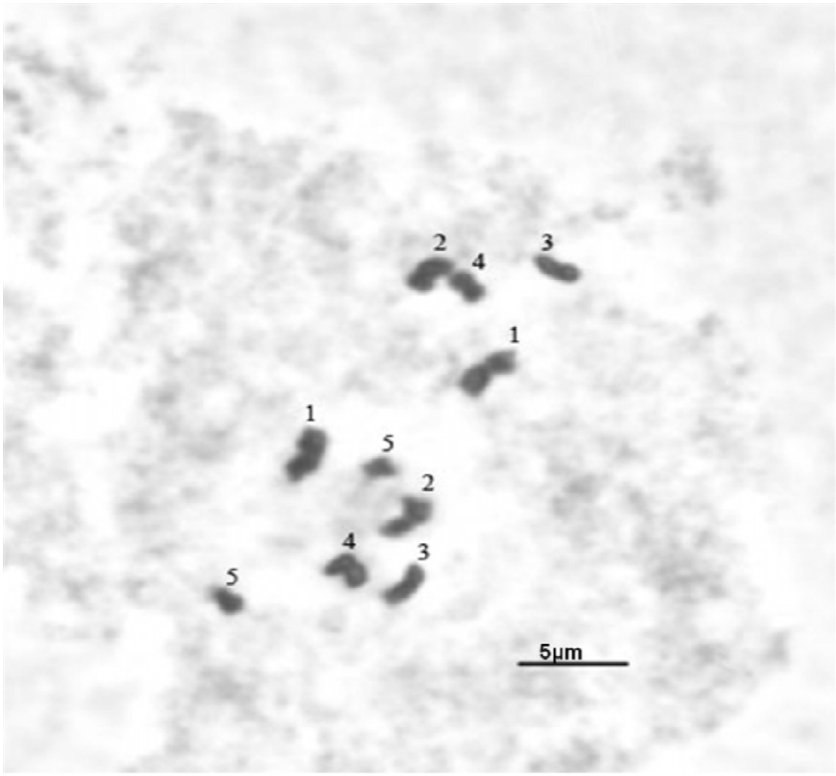
Population structure and geographic distributions

The F_{ST} values were significantly different from zero for all pair-wise comparisons between different regions. There was a significant differentiation among populations within regions but the F_{ST} values for 8 of the pair-wise comparisons within Eskisehir (39°50′N, 30°30′E, elevation 801 m) were not significantly different from zero.

Comparison of the geographical and genetic distances between populations within geographic regions did not show positive correlations. There were 2 populations (BdTR2C and BdTR1I) sampled from around Ankara (39°58′N, 32°53′E, elevation 864 m) and Konya (37°27′N, 32°35′E, elevation 975 m) (middle part of Turkey) that allowed this analysis. All of the interpopulational geographical distances were small. Ankara and Kirikkale (39°51′N, 33°32′E, elevation 748 m) were close to each other, but the F_{ST} value comparing these 2 populations was 0.11. Differentiation on the Ankara was more variable than on the Eskisehir, which was nearby. These findings showed significant population structure over a small geographical range.

To further explore the relationship between the pheno-

Fig. 4. Mitotic chromosomes of diploid brachypodium.



typic and genotypic classes, we mapped the collection locations of the 13 BdTR groups (Fig. 6). Interestingly, most of the BdTR classes were distributed over large but distinct regions. This suggests that long-distance seed dispersal plays a significant role in the ecology of brachypodium and that environmental factors influence the geographic distribution of certain classes.

The natural diversity of brachypodium is centered around the Mediterranean region, extending north into Europe and south into the Indian subcontinent (Garvin et al. 2008; Ozdemir et al. 2008). A better understanding of the geographic distribution of brachypodium is crucial to get a more precise picture of its migration history. Both nuclear and organelle markers are necessary to give a clear picture of the migration history of a plant species (Budak et al. 2005*a*). Organelle DNA markers showed that brachypodium genotypes from diverse geographical regions were closely related and genotypes from different geographic regions tended to cluster together. For instance, genotype BdTR13C, with the geographic location of 39°24′46.28″N, clustered with BdTR1M (39°5′12.20″N) at 90% similarity. Such findings have also been reported in other grass species (Budak et al. 2004*a*, 2005*a*; Isik et al. 2007). Like organelle genome variation, nuclear genome variation is geographically structured in brachypodium genotypes, which may be due to both environmental and genetic factors. It might also be because of sample size, sampling strategies, or a combination of these factors (Budak et al. 2005*a*). Regression analysis was used to assess whether genetic distances could be explained by geographic distance. Among the populations sampled in this study, genetic distance was not explained by geographic distance (correlation coefficient $r = 0.24$, $P < 0.05$). This finding clearly indicated that genetic relationships among lines could not be estimated based on geographical proximity alone and it also agreed with our previous findings (Budak et al. 2005*a*). The correlation coefficient between populational genetic distance and geographic distance computed by the Mantel test was $r = 0.19$, which explained the absence of association.

Discussion

In this study, we investigated a large native collection of brachypodium using nuclear (373 AFLP loci) and organelle genome (cpDNA and mtDNA RFLP) variation coupled with morphological and cytological analyses. We observed a high degree of variation in this collection. There was a great deal of variation in spikelet and grain morphology. This phenotypic data can be used to select parents for developing mapping populations to map genes that control vernalization, yield, and other important traits.

The amplified fragment length polymorphisms detected in this study can be used to genetically classify brachypodium genotypes. The AFLP genotyping results showed a strong correlation between the phenotypic classes and genotype, as has also been seen with SSR markers (Vogel et al. 2009). The AFLP genotyping results also indicated that genetic distance cannot be solely explained by geographic proximity. These results are similar to previous findings with buffalograss (*Buchloe dactyloides*) (Budak et al. 2004*a*, 2004*b*, 2005*a*). The sequence related amplified polymorphism analysis of 1500 individual brachypodium plants done by Filiz et al. (2009) indicated that genotypes from different geographic regions grouped together. In the present study, we observed that most classes of closely related genotypes originated

Fig. 5. UPGMA dendrogram of Turkish brachypodium inbred lines based on AFLP analysis.

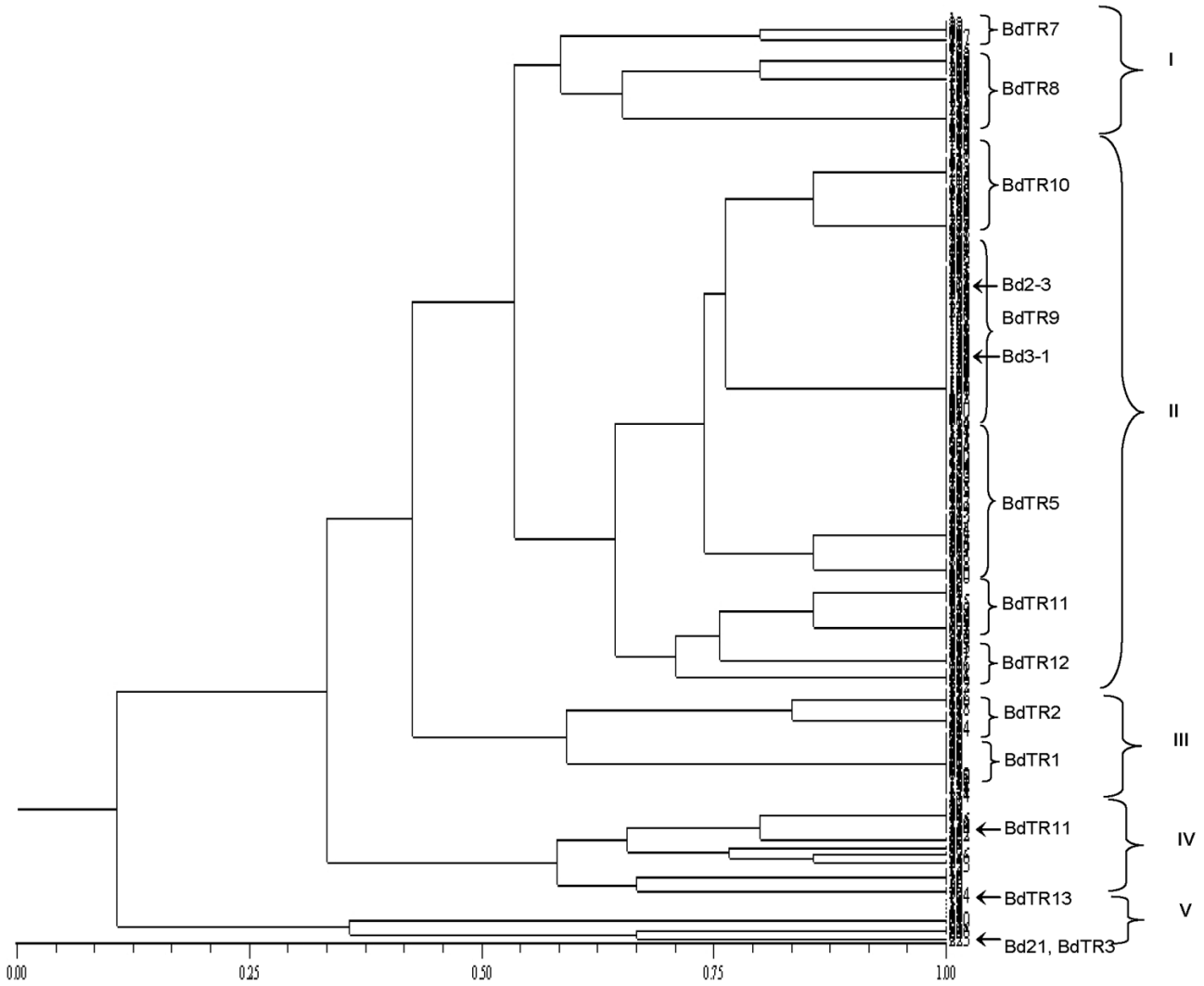


Table 2. Analysis of molecular variance within and among geographic regions using AFLP markers.

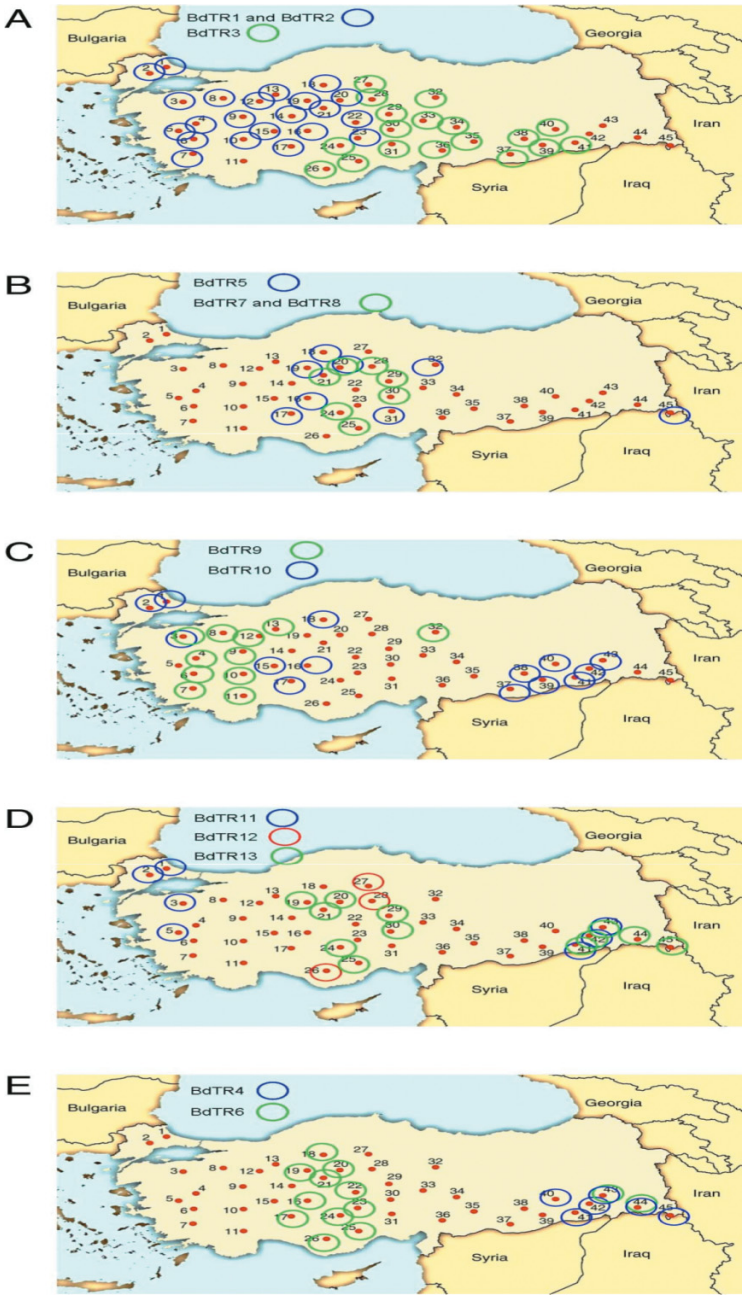
Source of variation	df	Sum of squares	Variation (%)
Among regions	44	231.16	24.32**
Among populations within southern region	89	187.16	32.76**
Among populations within central region	134	97.15	29.81*
Among populations within northern region	59	117.13	22.64
Total	1101	632.60	

Note: *, significant at 5% level ($P < 0.05$); **, significant at 1% level ($P < 0.01$).

from large but distinct geographic areas. This suggests long-distance seed dispersal via animals. Genetic distances for brachypodium lines from the 3 broad geographic regions sampled were correlated with geographic distances but the correlation coefficient was very low for each site. Our results agree with Huff et al. (1998), who found no significant association between geographic distance and genetic similarities in little bluestem (*Schizachyrium scoparium*) using RAPD markers. These results also parallel findings by Budak et al. (2005*a*), who did not find any significant relationship between geographic distance and genetic similarities in buffalograss, a C4 grass species. However, mapping the collection locations of the inbred lines revealed distribution patterns masked by the arbitrary division of the collection locations into 3 broad areas. This suggests extensive movement of seeds as well.

Genotypes representing various geographic regions did not differ for cpDNA PCR-RFLPs, indicating that germplasm from different geographical regions grouped together. Hence, these genotypes might have common genetic backgrounds. These results parallel findings in rye (Isik et al. 2007) and buffalograss (Budak et al. 2005*a*). Parsimony analysis conducted in the present study also indicated a close relationship between genotypes from different geo-

Fig. 6. Geographic distribution of the collection sites of all BdTR lines. (A–D) Collection sites of diploid lines. BdTR1 and BdTR2 classes and BdTR7 and BdTR8 classes grouped together because they were genotypically very similar. (E) Collection sites of polyploid lines. Note that many of the classes come from broad but distinct geographic areas.



graphic regions. The mtDNA and cpDNA polymorphism, however, might be used to genetically classify brachypodium genotypes according to cytoplasm type. Organelle marker variation was insignificant within (8.3%) and among genotypes (14.4%) as well. The reduced organelle diversity could be due to the uniparental inheritance of these markers (Budak et al. 2005*a*). Similar results have been found by other researchers studying organelle genome diversity (Budak et al. 2005*a*, 2005*b*). This analysis coupled with the morphological characteristics will help a better understanding of the ecotype classification and evolution of brachypodium genotypes.

In this study, we have demonstrated that a large amount of phenotypic and genotypic variation exists within and between Turkish brachypodium populations. To enable this diversity to be used by the scientific community, we created 116 diploid lines out of 146 inbred lines. All lines created in this study are freely available to the research community. This resource, in conjunction with the other tools created for brachypodium, will facilitate experimental approaches based on natural diversity. These studies will ultimately aid efforts toward improving crops with large complex genomes (e.g., wheat, barley, switchgrass, and *Miscanthus*).

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