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GENOME SIZE AND MORPHOLOGICAL VARIATIONS IN *BRACHYPODIUM DISTACHYON* L. ALONG ALTITUDINAL LEVELS

SIGNEM ONEY-BIROL[1*] AND SELMA TABUR[2]

[1]*Mehmet Akif Ersoy University, Faculty of Arts and Sciences, Department of Molecular Biology & Genetics, 15030, Burdur, Turkey*
[2] *Suleyman Demirel University, Faculty of Arts and Sciences, Department of Biology, 32260, Isparta, Turkey*
[*]*Corresponding author's: sobirol@mehmetakif.edu.tr*

Abstract

Brachypodium distachyon is an attractive model species for biological, physical, genomic and functional studies of the Triticeae. Altitude is an abiotic factor such as latitude/longitude, temperature, humidity and water conditions affecting the plant life. Many researchers have been working on changes in genome size and morphology to highlight the relation of elevational gradients. In this study, genome size and morphological variations was determined in *Brachypodium distachyon* L. (Poaceae) accessions collected from Turkey. Flow cytometric analysis was performed with 547 individuals representing 89 accessions of *B. distachyon* from different altitudinal habitats (from 0 to 1219 meters). 2C nuclear DNA content (± SD) of *B. distachyon* was estimated to be 0.736 ± 0.02 pg. In cytogenetical analysis, all the individuals from every accession were found to have diploid chromosome numbers (2n = 10). To determine the association with genome size (GS), morphologic traits and altitude obtained data were statistically analysed for ANOVA (p<0.005), pearson correlations (p<0.005), principal component analysis, factor analysis, discriminant analysis in Minitab 17 and SPSS 22 versions. The correlation analysis shows that there is no correlation between genome size and altitude. To see the changes of the morphological variations, 5 morphological features such as plant height (13.26± 5.39 cm), plant stature (mostly erect), seed height (7.34± 0.89 mm) and awn length (10.57± 2.24 mm), and 1000- seed weight (4.21± 1.00 mg) was used. We have not found any correlation with changing altitudinal gradients and morphology. However, when we grouped the altitudes from 0 to 600 meters in a collected gradient which represented first group and from 601 to 1219 meters which was the second collected group, we have found a positive correlation between genome size, seed height, awn length, 1000-seed weight and altitude. A negative correlation was only found in plant height along increasing altitudinal levels.

Key words: Altitude, *Brachypodium distachyon* L., Flow cytometry, Genome size variation, Morphology.

Introduction

The genome size (GS) of various plant species have shown significant variations from ~64 Mb to ~150.000 Mb (Leitch & Leitch, 2013) due to several genetic factors, one of which are the transposable elements] (Leitch & Bennett, 2007, Šmarda *et al.*, 2014). Recent studies on high-throughput next generation sequencing techniques show that variation in genome size has been based on junk or repetitive DNA that is generated by transposable elements of the transposable elements, the retrotransposons are playing a major role in genome expansion and plasticity due to their larger sizes and high copy numbers followed by DNA transposons (Levin 2002, Knight *et al.*, 2005; Nouroz *et al.*, 2015). Cytogenetic studies also highlight the chromosomal changes such as polyploidy or aneuploidy, which is significantly associated with genome size variation. Although many studies have detected the genome size variation on chromosomes, it is still unclear the existence of its rate (Schmuths *et al.*, 2004, Achigan-Dako *et al.*, 2008, Šmarda & Bureš, 2010). Several studies have reported the occurrence of B-chromosomes (Kellogg & Bennetzen, 2004, Sharbel *et al.*, 2005) and retrotransposon activity and distribution in various genomes (Kalendar *et al.*, 2000, Vitte & Bennetzen, 2006; Nouroz *et al.*, 2017). Moreover, it could be an adaptation to different climates, habitats or geographical patterns (Bennett, 1976; Chung *et al.*, 1998; Knight & Ackerly, 2002; Knight *et al.*, 2005; Doležel *et al.*, 2007).

Altitudinal gradients are excellent models for ecological and evolutionary studies to test the responses of climatic factors such as temperature, precipitation, wind, and sunshine on plant growth and development (Körner, 2007). Still *et al.*, (1999), implies that temperature decreases by 1°C for every 100m increase in altitude under dry air. The altitude above the sea level influences plant growth and development primarily through temperature effect. Temperature is an abiotic stress that can cause accumulation of nucleic acids, proteins and carbohydrates (Hirayama & Shinozaki, 2010). It has been shown that climate is significantly correlated with changes in GS content and has the strongest relationship into genome size (Šmarda *et al.*, 2014). Over many years there have been challenging questions in plant evolutionary biology that study correlations between genome size and changing ecological parameters or climatic parameters such as latitude, longitude, or altitude (Leitch & Bennett, 2007; Greilhuber & Leitch, 2013). It is still a contradictory question to give an idea about correlation between genome size and altitude above sea level or geographical latitude (Bennett & Leitch, 1995; Greilhuber, 1998; Bennett *et al.*, 2000; Knight *et al.*, 2005; Greilhuber *et al.*, 2005; Benor *et al.*, 2011). Few studies have reported a positive correlation between genome size and altitude (Laurie & Bennett, 1985; Rayburn & Auger, 1990; Caceres *et al.*, 1998; Kalendar *et al.*, 2000; Benor *et al.*, 2011). Instead many researchers found a negative relationship (Creber *et al.*, 1994; Rayburn *et al.*, 1994; Reeves *et al.*, 1998; Vinogradov, 2003; Greilhuber *et al.*, 2007) whereas did not find any correlation (Ceccarelli *et al.*, 1992; Lysák *et al.*, 2000; Knight *et al.*, 2005) between GS and altitude.

Brachypodium distachyon is a model crop for temperate grasses. It belongs to the Poaceae family and is related to the cereals such as wheat and barley (Catalan & Olmstead, 2000). It is used to study functional and structural genomics (Vogel *et al.,* 2006), biological systems in temperate grasses, dedicated biofuel crops and cool-season cereals (Draper *et al.,* 2001; Onda *et al.,* 2015). It has small size, simple growth requirements, self-fertile, a short life cycle, and a small diploid genome size (~355 Mbp). These features make it ideal for use in large-scale molecular studies. Many studies have been conducted on developing genomic knowledge (Zhang *et al.,* 2009; Vogel *et al.,* 2010; Mochida & Shinozaki 2013). Including much research on grain development (Guillon *et al.,* 2011), drought tolerance (Verelst *et al.,* 2013), and cell wall synthesis (Valdivia *et al.,* 2013) of *B. distachyon* as a model for temperate grasses. In this context, we presented a new set of data on this important species, concerning the mutual relations between the genome size, phenotypic variability and the altitude. The overall goal of this work was to identify the relationship among genome size, morphological variations and altitudinal gradients. We used 547 *B. distachyon* individuals, which belonged to 89 accessions distributed throughout Turkey from different altitudes ranging from sea level up to 1219 m to answer the questions as :(I) What is the variation of genome size and morphologic traits? (II) How much correlation is there in between genome size and morphology under various altitudinal habitats?

Material and Methods

Plant materials: The study species "*Brachypodium distachyon*" is a model plant. This study includes 547 individuals of *B. distachyon* collected from 89 different locations (accession) in Turkey representing different altitudes (0 m to 1219 meters) (Table 1). Seeds were collected for a region, taking care that there was a certain distance between areas of about 5.000 m^2 (accession). In addition, seeds were collected from a single plant. Due to the locational differences seeds were collected from that distance changing 2 and 17 different individual, details were shown in the Table 1 for each accession. The seeds were collected for both genome size and morphological variation analysis.

Vernalization and growth conditions: Collected, dry seeds were sown on filter paper in plastic petri dishes. We combined distilled water with Captan Solution (Captan WP 50%, 250gr/100lt) to inhibit the growth of microorganisms. Then, seeds were stratified at 4°C in the dark for 10 days to synchronize germination period for all individuals. For vernalization period, the germinated seeds were planted to a mixture of soil, turf, and sand (1:1:1). The pots were placed in the cold (approximately 5°C) for 8-14 weeks under fluorescent light in a greenhouse. After the process was completed, plants were transferred at 25°C under a 16 h day length at approximately 150 µEm-2s-1 and 18°C under a night length of 8 h at dark photoperiod. The humidity was maintained at 70%. Plants were controlled on daily basis for their water, light, and humidity requirements.

Nuclear genome size estimation: Nuclear genome size (DNA content: *pg*) analysis was performed for each individual collected from variable locations, the data for which is given in the Table 1. After completion of the vernalization (about 12 weeks), plants were transferred to the laboratory of genome size analysis for the individuals of each accession. The flow cytometry protocol described by Arumuganathan & Earle (1991) was used to obtain the DNA content. The protocol consists of preparing suspensions of intact nuclei by chopping plant tissues and lysing protoplasts in an MgSO4 buffer mixed with DNA standards and staining with propidium iodide (PI) in a solution containing DNase-free RNase. Specifically, 25 mg fresh/green leaf tissue and standard plant leaf was placed in a 10mm plastic petri dish on ice. We used rice (*Oryza sativa* L.) as an internal standard which has 0.99 pg 2C mean DNA content. The tissues were chopped into small pieces in 1 mL of solution A (24 mL MgSO4 buffer), 25 mg dithiothreitol, 500 µL propidiumiodide stock (5.0 mg propidium iodide in 1.0 mL ddH_2O), 625 µL Triton X100 stock (1.0 g Triton X100 in 10 mL ddH2O). The solution was filtered through a 40 µm nylon mesh into a micro-centrifuge tube and centrifuged at 14000 RPM for 14 seconds. The supernatant was discarded, the pellet was resuspended in 400 µL of solution B [7.5 mL solution A and17.5 µL RNase (DNase free)] and it was incubated for 15 min at 37°C before flow cytometric analysis [46-47]. Fluorescence intensities of the stained nuclei were measured by a flow cytometer FACSCaliburTM (Beckman Coulter, Inc., Fullerton, CA, USA). The values for nuclear DNA content were estimated by comparing fluorescence intensities of the nuclei of the test population with those of an appropriate internal DNA standard that is included with the tissue being tested. Mean DNA content per sample was based on analysis of 10000 nuclei per sample. The analysis was repeated if the coefficients of variation (CVs) of G0/G1 peaks of the sample was >2.0%. Nuclear genome size was calculated as a linear relationship between the ratio of 2C peaks of sample and standard. The nuclear DNA amount of the studied samples was calculated on the basis of the values of the G1/G2 peak means (Doležel & Bartoš, 2005).

Morphological measurements: Morphological data were taken from each individual for each location (accession). We measured total plant height (cm), plant stature (erect, fairly erect, branchy), seed height (mm), awn length (the longest within the spikelet) (mm) and 1000- seed weight (mg) under greenhouse conditions to estimate the variation in different altitude/latitude and longitudes belonging to 2-17 individuals for every accession. Plant height was measured from the soil level to the highest point of the spikelet length. Seed height represents total length of a seed on the spikelet without awn. Awn length was measured using the longest awn of a spikelet. To calculate 1000-seed weight for an individual 10 seeds of a spikelet was weighted and multiplied by the amount of the value to get grams of the seeds 10 1000 kernels. Duncan test was used to calculate the mean ratio for every accession to understand the morphological variation.

Table 1. Mean genome size and morphological features performed with latitude, longitude and altitude (m) per accession in *B. distachyon* species representing 89 different locations (N: the number of analyzed individuals for each accession).

Accession	Number of individuals	Latitude	Longitude	Altitude (m)	2C DNA content (pg)	Plant height (cm)	Plant stature	Seed height (mm)	Awn length (mm)	1000-seed weight (gr)
Bh1	6	N 41° 37.897'	E 026° 41.330'	100	0,74±0,008	10,37±1,32	Erect	6,98±0,41	10,23±1,63	4,52±0,75
Bh2	9	N 41° 40.695'	E 026° 20.271'	130	0,73±0,01	9,77±1,50	Erect	6,81±0,83	11,00±2,02	5,14±0,90
Bh3	6	N 41° 23.438'	E 026° 39.637'	86	0,74±0,005	10,29±1,20	Erect	7,89±1,03	11,04±1,75	4,13±0,32
Bh4	4	N 41° 15.686'	E 026° 37.298'	34	0,73±0,008	14,09±2,30	Fairly erect	7,72±0,57	11,06±2,48	3,18±0,39
Bh5	4	N 41° 12.275'	E 026° 28.639'	102	0,75±0,033	8,46±1,40	Erect	7,08±1,50	10,06±3,86	4,63±0,69
Bh6	8	N 41° 14.062'	E 026° 32.021'	85	0,74±0,007	11,93±1,82	Erect	7,53±0,97	10,02±0,88	3,45±0,88
Bh7	4	N 41° 30.528'	E 026° 53.279'	90	0,74±0,002	9,25±1,22	Erect	7,22±0,91	11,26±1,35	3,80±0,43
Bh8	5	N 41° 02.028'	E 027° 22.162'	147	0,73±0,005	10,53±0,88	Erect	7,49±0,33	11,59±1,25	5,68±0,77
Bh9	6	N 41° 00.891'	E 027° 25.351'	262	0,74±0,012	11,15±0,62	Erect	6,94±0,45	10,12±1,20	3,65±0,58
Bh10	6	N 41° 06.846'	E 027° 14.503'	104	0,74±0,024	13,27±3,30	Erect	7,42±0,67	11,19±2,07	3,62±0,52
Bh11	7	N 41° 12.029'	E 027° 11.192'	84	0,74±0,017	13,73±2,09	Erect	6,97±0,52	9,42±1,51	2,97±0,40
Bh12	3	N 41° 15.349'	E 027° 08.400'	54	0,74±0,013	12,33±1,85	Erect	7,39±0,38	11,49±1,24	4,97±1,23
Bh13	9	N 41° 17.392'	E 027° 32.824'	79	0,74±0,010	13,41±1,46	Erect	7,23±0,73	12,14±1,48	4,39±0,46
Bh14	6	N 41° 02.537'	E 027° 30.374'	220	0,74±0,008	15,16±1,96	Erect	7,06±0,37	10,48±0,96	3,95±0,26
Bh15	2	N 41° 65.837'	E 027° 28.290'	114	0,73±0,005	11,68±0,52	Fairly erect	7,00±0,55	9,64±1,37	4,45±0,64
Bh16	2	N 41° 05.786'	E 027° 55.796'	109	0,73±0,010	14,70±0,00	Erect	7,35±0,00	6,82±0,01	3,60±0,20
Bh17	5	N 41° 32.076'	E 027° 50.014'	136	0,75±0,015	14,42±2,57	Erect	7,42±0,42	9,83±1,40	3,76±0,37
Bh18	6	N 40° 47.703'	E 027° 21.792'	95	0,73±0,009	9,70±1,91	Erect	7,35±0,43	10,53±1,42	3,85±0,80
Bh19	4	N 40° 42.537'	E 027° 05.872'	203	0,75±0,003	10,92±1,57	Erect	7,64±0,26	10,26±1,09	3,43±0,13
Bh20	6	N 40° 49.633'	E 027° 04.405'	164	0,74±0,001	7,61±1,88	Erect	7,16±0,37	11,03±1,37	3,67±0,36
Bh21	6	N 40° 54.824'	E 027° 08.742'	177	0,75±0,000	12,25±2,22	Erect	7,17±0,70	10,70±1,48	4,87±0,88
Bh22	2	N 40° 56.578'	E 027° 18.347'	196	0,74±0,035	9,70±0,00	Erect	7,74±0,00	10,61±0,02	4,30±0,00
Bh23	7	N 40° 56.692'	E 026° 34.106'	115	0,74±0,009	12,06±2,33	Erect	6,90±0,49	10,72±1,60	3,70±0,63
Bh24	2	N 36° 16.609'	E 036° 13.662'	113	0,75±0,005	13,55±1,41	Erect	6,48±0,085	9,46±0,64	3,15±0,07
Bh25	2	N 36° 28.568'	E 036° 16.977'	133	0,74±0,009	6,65±1,20	Erect	6,53±1,32	8,38±1,85	3,55±0,64
Bh26	7	N 41° 06.690'	E 029° 25.759'	77	0,73±0,005	8,21±1,43	Erect	6,78±0,73	10,46±1,52	3,66±0,46
Bh27	7	N 41° 09.394'	E 029° 34.811'	9	0,73±0,006	12,29±1,47	Erect	7,05±0,68	9,52±0,66	3,80±0,57
Bh28	4	N 41° 07.090'	E 029° 39.577'	110	0,73±0,006	10,93±1,11	Erect	6,67±0,89	9,42±0,75	4,40±0,85
Bh29	6	N 40° 43.187'	E 026° 25.906'	18	0,73±0,01	13,23±6,39	Erect	6,71±0,53	8,50±0,37	5,23±1,27
Bh30	2	N 40° 36.007'	E 026° 24.876'	18	0,75±0,005	9,85±1,009	Fairly erect	6,61±0,85	11,33±0,33	2,85±0,50
Bh31	3	N 40° 43.009'	E 026° 34.751'	66	0,72±0,01	9,67±4,20	Erect	6,89±0,89	8,85±0,73	5,53±0,35
Bh32	5	N 40° 48.349'	E 026° 39.615'	100	0,70±0,09	6,87±2,03	Fairly erect	7,13±0,54	11,16±1,52	4,48±0,51
Bh33	2	N 40° 38.717'	E 026° 16.342'	38	0,74±0,002	8,28±1,35	Erect	6,44±0,099	10,31±1,03	3,65±0,35
Bh34	3	N 37° 28.679'	E 030° 33.541'	862	0,73±0,01	9,73±0,46	Erect	6,91±0,69	8,21±0,61	2,97±1,15
Bh35	3	N 37° 28.672'	E 030° 33.631'	854	0,74±0,003	8,27±0,34	Erect	6,96±0,35	8,20±0,13	4,37±1,03
Bh36	6	N 38° 50.750'	E 027° 01.257'	52	0,75±0,000	9,07±1,50	Erect	7,35±0,75	10,87±0,73	3,80±0,48
Bh37	7	N 39° 57.891'	E 027° 11.662'	305	0,75±0,006	11,93±1,45	Fairly erect	6,75±0,47	10,13±1,18	4,41±0,57
Bh38	16	N 39° 48.006'	E 027° 22.948'	357	0,74±0,009	13,38±2,74	Erect	6,94±0,54	9,55±1,53	3,44±0,87
Bh39	8	N 40° 12.070'	E 026° 16.389'	9	0,72±0,016	12,91±4,87	Erect	7,04±0,61	8,63±2,16	4,14±0,57
Bh40	13	N 39° 41.131'	E 027° 58.782'	196	0,74±0,009	12,20±2,43	Erect	7,03±0,55	10,41±1,19	3,99±0,60
Bh41	2	N 40° 14.653'	E 026° 17.708'	184	0,75±0,001	9,55±0,00	Fairly erect	7,30±0,00	9,78±0,03	3,30±0,01
Bh42	3	N 39° 40.773'	E 029° 08.846'	682	0,76±0,012	11,27±1,21	Erect	6,20±0,29	8,49±1,25	4,60±0,69
Bh43	6	N 40° 15.889'	E 026° 28.859'	18	0,76±0,018	14,32±1,81	Erect	6,73±0,73	8,75±1,26	4,65±1,04
Bh44	9	N 40° 03.470'	E 026° 35.758'	110	0,73±0,014	12,22±2,42	Fairly erect	6,99±0,55	8,93±1,56	3,36±1,09
Bh45	10	N 39° 36.650'	E 028° 58.074'	385	0,73±0,000	9,27±0,90	Erect	6,91±1,54	8,84±3,46	3,33±0,86

Table 1. (Cont'd.)

Accession	Number of individuals	Latitude	Longitude	Altitude (m)	2C DNA content (pg)	Plant height (cm)	Plant stature	Seed height (mm)	Awn length (mm)	1000-seed weight (gr)
Bh46	3	N 36° 40.512'	E 029° 07.864'	36	0,73±0,012	21,67±3,99	Fairly erect	6,92±0,75	9,92±2,61	3,90±0,78
Bh47	8	N 36° 05.815'	E 032° 56.128'	17	0,72±0,013	22,38±3,89	Fairly erect	7,36±0,97	9,69±2,25	5,10±1,04
Bh48	3	N 39° 37.738'	E 032° 42.293'	1085	0,74±0,014	11,87±3,26	Erect	6,72±0,43	9,83±0,30	4,27±0,70
Bh49	2	N 37° 53.990'	E 027° 16.727'	43	0,74±0,032	23,57±2,45	Branchy	7,94±0,92	13,46±0,01	4,85±0,92
Bh50	11	N 39° 34.620'	E 026° 51.108'	32	0,71±0,024	25,86±2,03	Erect	7,37±0,47	10,22±2,08	4,84±0,50
Bh51	2	N 40° 15.339'	E 026° 18.774'	265	0,76±0,013	11,68±1,16	Erect	7,84±0,55	10,16±0,21	4,55±0,35
Bh52	2	N 41° 05.835'	E 029° 45.760'	72	0,74±0,002	10,79±0,80	Erect	6,11±0,79	8,63±0,22	3,05±0,07
Bh53	8	N 40° 47.189'	E 029° 27.601'	181	0,73±0,020	13,77±6,32	Erect	7,75±0,67	10,92±2,19	2,95±0,54
Bh54	6	N 41° 05.347'	E 029° 45.249'	135	0,74±0,018	14,56±2,99	Erect	6,82±0,47	9,75±0,86	3,58±0,57
Bh55	5	N 38° 50.440'	E 034° 33.266'	1193	0,73±0,016	11,47±2,91	Fairly erect	7,43±0,55	11,30±1,96	3,70±1,03
Bh56	4	N 38° 44.470'	E 034° 50.725'	1110	0,74±0,006	12,23±3,74	Fairly erect	6,59±0,35	9,90±2,12	4,30±0,29
Bh57	2	N 38° 44.937'	E 034° 50.756'	1219	0,73±0,002	13,44±1,42	Erect	6,60±1,08	9,81±0,52	4,00±0,28
Bh58	4	N 38° 44.536'	E 034° 50.289'	1157	0,72±0,002	11,85±1,96	Fairly erect	6,90±0,47	9,25±1,53	3,75±0,66
Bh59	14	N 38° 43.864'	E 034° 49.910'	983	0,74±0,009	13,96±1,87	Fairly erect	7,20±0,75	9,60±1,25	3,09±0,68
Bh60	2	N 40° 06.910'	E 026° 25.482'	127	0,73±0,005	11,68±1,17	Erect	7,60±1,61	9,50±2,83	3,15±0,21
Bh61	9	N 38° 43.864'	E 034° 49.910'	47	0,74±0,016	14,40±3,17	Erect	6,89±0,71	10,37±2,09	4,11±0,86
Bh62	7	N 39° 46.888'	E 027° 24.375'	530	0,74±0,013	12,23±5,63	Erect	7,40±0,46	11,80±2,00	3,80±0,40
Bh63	12	N 39° 38.741'	E 027° 46.100'	252	0,73±0,019	15,12±3,13	Erect	7,40±1,19	11,21±1,75	4,23±0,79
Bh64	10	N 39° 42.073'	E 027° 33.289'	363	0,74±0,011	14,14±5,14	Fairly erect	6,90±0,68	9,52±2,59	4,46±0,56
Bh65	9	N 39° 44.669'	E 028° 21.423'	506	0,74±0,009	13,42±7,37	Erect	7,16±0,72	11,52±1,51	3,12±1,05
Bh66	3	N 39° 39.306'	E 029° 01.933'	616	0,73±0,002	13,43±3,91	Fairly erect	7,86±0,29	9,54±0,39	4,37±1,19
Bh67	6	N 37° 07.545'	E 028° 22.724'	634	0,81±0,090	10,16±1,23	Erect	7,19±0,47	9,74±2,25	3,47±0,88
Bh68	18	N 39° 34.643'	E 030° 07.208'	942	0,75±0,019	15,91±5,44	Erect	8,41±0,90	11,40±3,14	5,20±1,01
Bh69	2	N 36° 15.694'	E 033° 48.224'	214	0,74±0,005	26,35±1,84	Erect	9,58±0,73	16,38±1,20	3,70±0,06
Bh70	10	N 39° 32.222'	E 029° 38.014'	1027	0,75±0,031	8,91±1,52	Erect	7,82±0,50	11,89±2,08	4,99±0,75
Bh71	6	N 39° 43.058'	E 030° 40.601'	916	0,74±0,011	14,06±6,38	Erect	6,97±0,62	10,77±1,14	4,97±0,69
Bh72	12	N 39° 32.597'	E 032° 13.909'	995	0,72±0,001	9,94±0,93	Erect	8,54±0,62	13,13±1,90	5,62±0,84
Bh73	12	N 36° 57.506'	E 030° 35.570'	305	0,71±0,018	22,61±2,83	Fairly erect	7,43±0,49	9,49±0,98	3,87±0,63
Bh74	11	N 39° 29.645'	E 032° 26.810'	989	0,73±0,009	11,29±2,41	Erect	6,76±0,66	9,81±2,02	3,85±0,55
Bh75	8	N 39° 29.650'	E 031° 14.473'	1033	0,75±0,008	13,38±2,47	Erect	8,21±0,41	12,08±2,34	5,51±1,16
Bh76	2	N 36° 59.520'	E 028° 39.291'	34	0,71±0,001	28,78±1,96	Erect	8,41±0,23	14,06±0,02	4,40±0,09
Bh77	2	N 37° 03.270'	E 027° 22.703'	92	0,71±0,001	35,24±3,87	Fairly erect	8,47±0,12	11,75±0,00	4,20±0,16
Bh78	7	N 39° 30.126'	E 029° 52.618'	1052	0,73±0,011	11,53±2,04	Erect	8,45±0,70	13,49±1,95	5,60±0,33
Bh79	11	N 37° 29.540'	E 027° 20.380'	71	0,73±0,010	15,45±6,01	Erect	6,70±0,86	9,55±2,81	3,77±0,40
Bh80	15	N 37° 46.004'	E 027° 25.150'	69	0,74±0,022	21,12±2,24	Erect	7,87±0,60	10,24±2,91	5,17±0,95
Bh81	2	N 37° 47.955'	E 027° 18.279'	136	0,74±0,009	29,37±2,89	Erect	10,13±0,24	8,93±0,57	4,05±0,35
Bh82	2	N 36° 07.925'	E 033° 16.702'	70	0,60±0,002	24,00±2,21	Erect	7,35±0,45	9,19±0,01	5,10±0,17
Bh83	7	N 36° 18.237'	E 032° 15.957'	23	0,74±0,034	23,72±4,37	Fairly erect	8,37±0,76	14,52±1,60	4,76±0,49
Bh84	2	N 37° 09.827'	E 027° 35.402'	16	0,77±0,004	23,75±3,25	Erect	5,75±0,16	10,62±0,60	6,20±0,16
Bh85	2	N 37° 05.348'	E 027° 28.915'	31	0,73±0,002	33,30±4,43	Erect	10,56±0,18	16,71±0,09	3,60±0,34
Bh86	10	N 37°28.1805'	E030°56.588'	857	0,74±0,000	8,24±1,50	Erect	8,53±1,41	14,41±3,07	5,68±0,50
Bh87	7	N 38°19.1594'	E026°47.1620'	85	0,72±0,000	8,55±1,21	Fairly erect	8,49±0,82	13,13±2,53	4,94±0,49
Bh88	8	N 38°10.1494'	E 026°47.1945'	2	0,74±0,000	5,97±0,56	Erect	7,22±0,71	10,98±1,64	3,68±0,31
Bh89	10	N 41°02.0339'	E 031°03.4872'	251	0,72±0,022	8,25±1,83	Erect	7,29±0,68	11,10±1,70	3,75±0,55

* Values with insignificant difference ($p<0.01$) for each column are indicated with same letters (means ± SD)

[a]Accessions representing different letters are significantly different from each other according to Duncan test at $p<0.05$.

Statistical analysis: We used descriptive statistics to correlate the quantitative data obtained from genome size and morphological traits. A one-way analysis of variance was applied to test the correlation between genome size and morphological characters. We performed a Kolmogorow–Smirnow test to determine whether or not the associations of dependent and independent variables had a normal distribution. Pearson's correlation analyses were performed to investigate the correlation of genome size with altitude and morphology. A regression scatter plot was then drawn to determine the form of relationship between variables. Principal coordinate analysis was carried out to display the pattern of genome size of each accession with respective geographical distributions. Statistical procedures for ANOVA ($p<0.05$), pearson correlation, principal component analysis, factor analysis ($p<0.01$), discriminant analysis ($p<0.05$) were performed using Minitab 17 and SPSS 22.

To test the relationship among altitude and genome size and morphology, we first grouped the altitude levels per 100 meters to understand that there was a threshold for all the features studied. Then, we grouped the levels of altitudes per 100, 200, 300, 400, 500, 600 meters and tested whether there was a threshold for altitude.

Results

All data for genome size and 5 morphological characters (plant height, plant stature, seed height and awn length, and 1000- seed weight) data was tested for homogeneity of variances (O'Brien test, $p>0.05$) using MiniTab 17 and SPSS 22.

Genome size variation along altitudinal levels: We measured the genome size [pictogram (pg)] of 547 individuals representing 89 accessions of *B. distachyon* from different altitudinal levels (0 – 1219 m) of Turkey. GS values varied widely among individuals, from 0.54 (BD32-4) to 0.92 (BD-71-5, BD-71-6) pg (Table 1). The mean 2C DNA content of *B. distachyon* was determined to be 0.736 ± 0.02 pg ($p<0.001$, Kruskal–Wallis test). The scatter plot of all individuals shows that there was no correlation between altitude and genome size (Fig. 1a). To see how much individual variation between genome size and altitudinal gradients existed the statistical analysis was tested for every 100 meters. No correlation was observed from 0 to 600 meters tested with each 100 meters. However, altitudinal gradients have been grouped from 0 to 600 (group 1) meters as a same gradient and 601 to 1219 meters (group 2) as another gradient to determine level of altitude differences in genome size. So, a positive correlation between altitude and genome size was found with these two group altitudinal gradients. In the first altitudinal gradient (from 0 to 600m) having 409 individuals, the mean 2C DNA content was 0.734 ± 0.001 pg, ($p<0.005$) and in the second altitudinal gradient (from 601 to1219m) with 132 individuals, the 2C mean DNA content had 0,742 ± 0.002 pg genome size (Fig. 1b).

Morphological variation along altitudinal levels: We have used five traits (plant height, plant stature, seed height, awn length and 1000-seed weight) to test the morphological differences in changing altitude. A summary of descriptive statistics and significance of the analysis of variance for morphological characters is shown in Table 1. Studied individuals were morphologically variable, and statistically significant ($p<0.05$). The mean plant height was 13.26 ± 0.23 cm ranging from 5 to 35.24 cm for all studied habitats. Plants were mostly erect., where few accessions were fairly erect and only one accession (Bd49) showed branched stature. Seed height ranged from 4.59 to 10.74 mm and the mean was 7.34 ± 0.04 mm. The mean awn length was found to be 10.57 ± 0.1 and the mean 1000 – seed weight was 4.21 ± 0.04 mm. When we tested the correlation of morphological features with different levels of altitude, no correlation was found between altitudinal gradients (Fig. 2a). However, the two grouped altitudes showed a positive correlation between seed height, awn length and 1000-seed weight although a negative correlation between plant height and increased altitude was found in all datasets ($p<0.01$) (Fig. 2b).

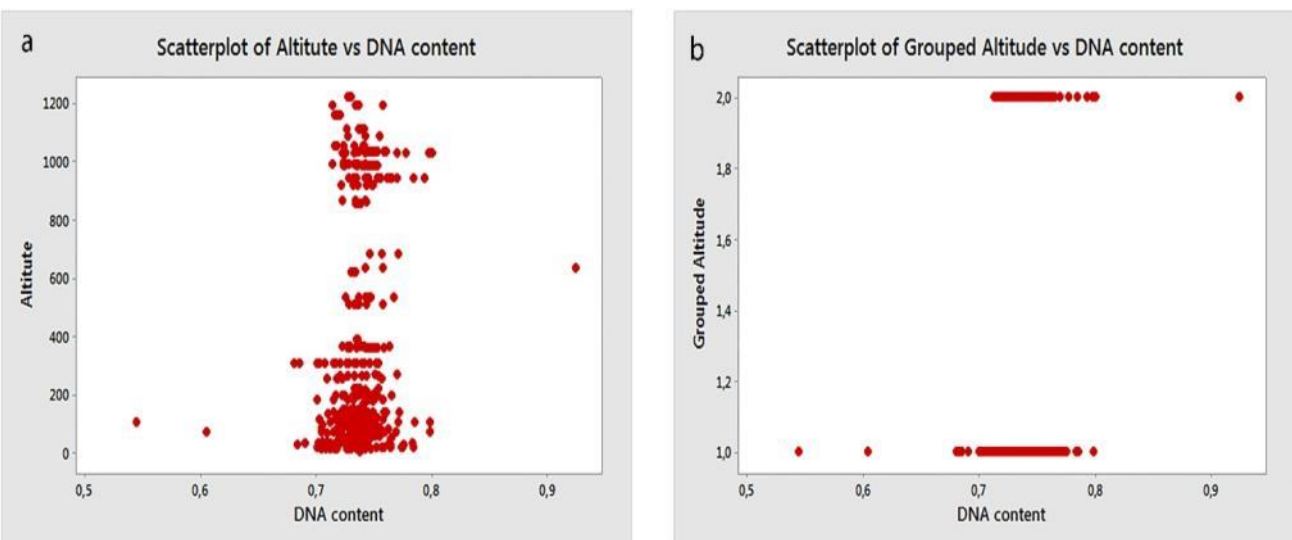

Fig. 1. Scatter plot shows the relationship between genome size (DNA content) and altitude (0m to 1219m) for 547 *B. distachyon* individuals from 89 accessions a) Genome size changes every 100m altitudinal levels b) Genome size changes grouped altitudinal levels (I group: 0-600m, II group 601-1219m).

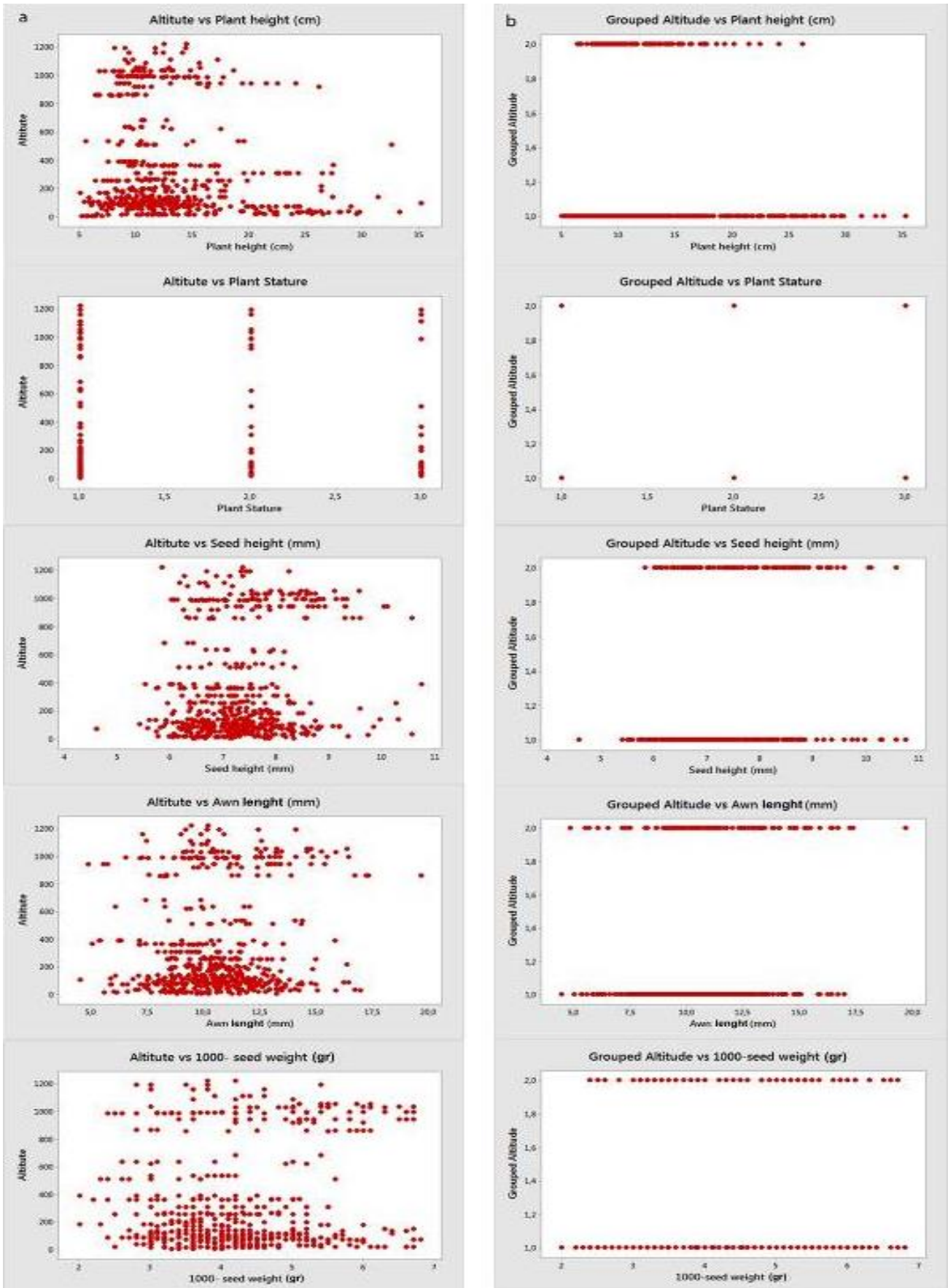

Fig. 2. Scatter plot shows the relationship between morphological features and altitudes (0m to 1219m) for 547 *B. distachyon* individuals from 89 accessions a) Morphological features every 100m altitudinal levels b) Morphological features for grouped altitudinal levels (I group: 0-600m, II group 601-1219m).

Table 2. Pearson's correlation matrix shows coefficient (r) and p value for each pair of variables.

	Altitude	Plant height (mm)	Seed height (mm)	Awn height (mm)	1000 seed weight	DNA content (pg)
Plant height (mm)	-0,157					
	0,000					
Seed height (mm)	0,175	0,178				
	0,000	0,000				
Awn height (mm)	0,136	-0,015	0,455			
	0,001	0,736	0,000			
1000 seed weight	0,150	0,117	0,251	0,273		
	0,000	0,007	0,000	0,000		
DNA content (pg)	0,106	-0,093	0,005	0,013	-0,030	
	0,014	0,030	0,901	0,760	0,492	

Six variables were used: Altitude, Plant height (cm), Seed height (mm), Awn length, 1000-seed weight, and DNA content (pg). ($p<0.05$)
Cell contents: Pearson correlation. p-value

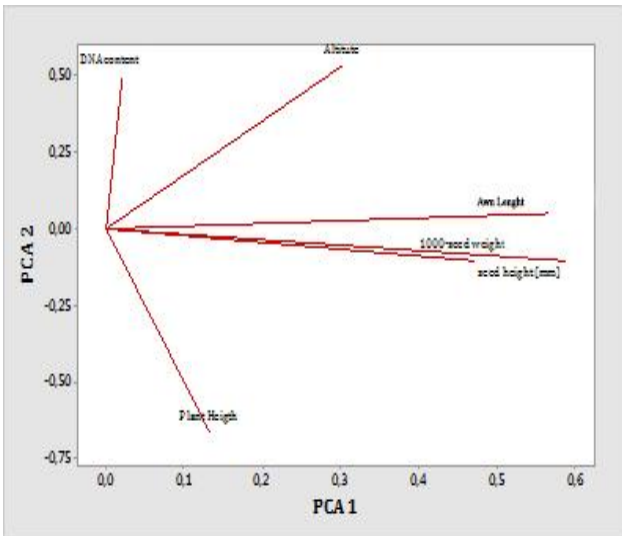

Fig. 3. Principal component analysis plot for first two component [PCA1 and PCA2] ($p<0.05$).

Table 3. Principal component analysis (PCA) loadings and the eingen (1, 2 and 3) values of each component.

Variable	Principal components (PC)		
	1	2	3
Altitute (m)	0,302	**0,533**	-0,215
Plant heigth (cm)	0,133	**-0,665**	0,454
seed height (mm)	**0,589**	-0,106	0,123
awn lenght (mm)	**0,567**	0,05	-0,072
1000-seed weight (mg)	0,472	-0,105	-0,095
DNA content (pg)	0,021	0,499	**0,847**
Eigenvalue	1,77	1,24	0,93
Proportion	0,30	0,21	0,15
Cumulative (%)	30	50	66

Statistical analysis of genome size and morphological traits with altitudinal gradients: We performed pearson correlation (Table 2), principal coordinate analysis (Table 3, Fig. 3), factor (Table 4) and discriminant (Table 5) to understand the relationship of altitude, genome size and morphology. We analyzed the species of *B. distachyon* individuals collected from 89 different locations in Turkey to estimate whether all characters are adequate to discriminate altitudinal levels. The pearson correlation analysis showed very low correlation values which is smaller than 0.02 ($p<0.01$) between all the traits and altitude (Fig. 3). The pearson correlation test indicates that the power of association between the altitude and genome size is very low (R = 0.106), and that the correlation coefficient is highly significantly different from zero ($p<0.01$). Also, we can conclude that 1.12% (0.106^2) of the variation in changing altitudes is explained by genome size. For morphological traits, the highest correlation was shown (R=0.175) between altitude and seed height that can explain about 3% of the association (Table 2). As shown in Table 3 and Fig. 3, the first principal component (PCA1) is strongly correlated with two of the morphological traits. PCA1 increases with increasing seed and awn length (mm). This suggests that these two features vary together. If seed height increases, then awn length tends to increase as well. The second principal component (PCA2) increases with two of the traits, decreasing plant height (cm) and increasing altitude (m). The third principal component (PCA3) increases with increasing DNA content (genome size). This suggests that genome size of all accessions is independent of any other features. We have shown that six different features have three different factors, which means that seed height, awn length and 1000-seed weight changes depending on each other. Plant height correlates with altitude. DNA content is not depended on any other feature. So, genome size is important to separate and understand the correlation between altitude and morphological variation (Table 4). In order to discriminate analysis (Table 5), genome size and morphological features of *B. distachyon* accessions from groups of altitudes were tested by canonical discriminant analysis (CDA). It was performed on 547 individuals, and the classificatory discriminant analysis was used in order to obtain the percentage of correctly classified individuals, based on genome size and morphological characters respectively. Using these characters to classify the individuals with the grouped altitudes shows that the individuals can be estimated by 82.1% correctly. The Wilk's Lambda of 0,86 has a highly significant value (Sig. = 0,000), thus, the group means appear to differ (Table 5).

Table 4. Rotated components with a factor correlation matrix for the three components (1, 2 and 3) (p<0.05).

Variable	Factors			Communalities
	1	2	3	
Altitute (m)		0,646		0,557
Plant Height (cm)		-0,845		0,772
seed height (mm)	0,790			0,642
awn lenght (mm)	0,746			0,577
1000-seed weight (mg)	0,636			0,416
DNA content (pg)			0,985	0,976
% Variance	**0,294**	**0,194**	**0,168**	

Extraction method: Principal component analysis
Rotation Method: Varimax rotation
Values ≤ 0,40 were eliminated from the table

Table 5. Morphological features and genome size selected by linear discriminant analysis to predict grouped altitudes.

Grouped altitude		Mean	Fisher's Linear Discriminant Function Coefficients	Function (1)	Wilks' Lambda	Canonical correlation	Chi-square	Eigen value	Classification results* (Count/ %)	
									Predicted group	
									1	2
Group 1	Plant height	13,60±5,73	0,55							
	Seed height	7,22±0,83	8,51							
	Awn lenght	10,35±2,08	0,42	-0,22					406 / 96,4	15 / 3,6
	1000-seed weight	4,09±0,91	4,26							
	DNA content	0,734±0,001	1516,17		0,86	0,38	81,54	0,164		
Group 2	Plant height	12,09±3,84	0,47							
	Seed height	7,75±1,00	9,17							
	Awn lenght	11,36±2,62	0,46	0,76					82 / 68,3	38 / 31,7
	1000-seed weight	4,66±1,18	4,80							
	DNA content	0,742±0,002	1531,76							

*82,1% of cross-validated grouped cases correctly classified.
≠ Significance value 0.00

Discussion

Genome size variation along altitudinal levels: The genome size and altitude relation is a controversial question for all researchers who focus on evolutionary biology. Some researchers found a negative correlation (Mangelsdorf & Cameron 1942; Wellhausen *et al.,* 1952; Longley & Kato, 1965; Bennett, 1976; Rayburn, 1990; Creber *et al.,* 1994; Singh *et al.,* 1996; Poggio *et al.,* 1998; Reeves *et al.,* 1998; Rosata *et al.,* 1998; Bottini *et al.,* 2000; Temsch & Greilhuber, 2001; Suda *et al.,* 2003; Knight *et al.,* 2005; Duskova *et al.,* 2010; Díez *et al.,* 2013; Talebi *et al.,* 2015; Realini *et al.,* 2015), and some found a positive relationship between genome size and altitude (Bennett, 1976; Smith *et al.,* 1976; Laurie & Bennett 1985; Rayburn & Auger, 1990; Godelle *et al.,* 1993; Caceres *et al.,* 1998; Cerbah *et al.,* 1999; Suda *et al.,* 2003; Benor *et al.,* 2011; Chalup *et al.,* 2014; Chumová *et al.,* 2015). However, another group of researchers could not find any correlation for genome size in different altitudinal levels (Rayburn, 1990; Palomino, 1993; Palomino & Sousa, 2000; Lysak *et al.,* 2000; Torrel & Valles, 2001; Suda *et al.,* 2005; Mráz, 2009; Wang, 2011; Kolano *et al.,* 2012), Whereas, we have found no correlation between individuals by changing altitudinal levels. The scatter plot of all individuals shows that there is no correlation between altitude (Fig. 1a)/grouped altitude (Fig. 1b) and genome size. With these two group altitudinal levels (group 1 and 2) we found a positive correlation between altitude and genome size correlation. In the first altitudinal gradient (from 0 to 600m) belonging to 409 individuals, the mean 2C DNA content is 0.734 ± 0.001 pg, (p< 0.005) and the second altitudinal gradient (from 601 to1219m) which has 132 individuals, the 2C mean DNA content was 0.742 ± 0.002 pg genome size (Fig. 1b, Table 5).

Morphological variation along altitudinal levels: In this study, morphologic features such as plant height, plant stature, seed and awn length and 1000-seed weight variation were determined using correlation analysis by changing altitudinal gradients in *B. distachyon.* In different plant groups, many researchers have shown that morphologic structure differentiates under changing altitudes (Vera, 1997; Cordell *et al.,* 1998; Kofidis *et al.,* 2003; Semagn *et al.,* 2004; Maliníková *et al.,* 2013; Wang *et al.,* 2014). Vera (1997) investigated the relationship between altitude and seed biomass distributed from 100 to 2090 meter heights in *Calluna vulgaris*, *Erica cinerea* and *Erica vagans* seeds and it was determined that *Calluna vulgaris* seeds collected from high altitudes showed the highest germination rates. Semagn *et al.,* (2004) determined the relationship between elevation and morphologic features for 16 characters in *Phytolacca dodecandra*, which was distributed from 1600 to 3000m, and the researchers have not found any correlation between elevation and morphological characters.

In the present work, when every altitudinal level was tested separately, no correlation was found between altitude and morphologic traits (Fig. 2a). Otherwise, grouped altitudes have positive correlation between altitude and seed height, awn length and 1000-seed weight and a negative correlation was determined between plant height and high altitude (p<0.01) (Fig. 2b). Similarly, Lavorel & Grigulis (2012) and Paniagua-Ibáñez *et al.,* (2015), examined a negative correlation with plant height and high altitude. On the other hand, Wang *et al.,* (2014) defined a positive correlation between plant height and altitude and a negative correlation between seed height, seed size, seed surface and altitude. The researchers proposed a variation on seed morphology in parallel to increasing altitude. Moles & Westoby (2003), Bu *et al.,* (2007) and Guo *et al.,* (2010) also reported a negative correlation between biomass, seed production and high altitude. Otherwise, Pluess *et al.,* (2005) emphasized that seed yield increased under high altitudinal levels within and among species. Some researchers found no correlation between high elevation and reproductive yield (Guo *et al.,* 2010). Results obtained by different researchers are likely to be explained with the different levels of adaptation for each species. This is not caused by genomic and morphological differences but between different individuals of the same species without sharp locational changes. The result of this study showed that the variation in altitude for genetic and morphological variation is 600 meters for *B. distachyon*. So, we may imply that 600 meters is a threshold for all the features studied. Each individial of the species showed morphological and genetical differences in each 600 m and the results are statistically significant (p<0.01).

Conclusion

Organisms may disappear by increasing temperature, extreme climate conditions, and different longitude/latitude and altitude gradients during adaptation to local conditions. Adaptive features of evolution in plants require genetic variation for local adaptation under selection in specific environments. Individuals can respond to environmental changes against the same genotype of the expression of different phenotypes. As the same with this study, although changes in separated localities with gradually difference between the individuals of the species depending on altitudinal gradient (every 100 meters) have not caused a correlation between genome size and morphology, in parallel to increased altitude (0 to 600m and 601 to 1219m). To discover the genetic basis to high elevation adaptation among individuals, genome-wide associated studies need to be done.

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